DRUG SUSCEPTIBILITY TESTING OF SECOND AND THIRD LINE ANTI-TUBERCULOSIS DRUGS USED IN THE MANAGEMENT OF EXTENSIVELY DRUG RESISTANT TUBERCULOSIS

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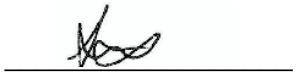
ABSTRACT

Drug resistant tuberculosis is a major contributor to South Africa's quadruple burden of disease. Management of this infection in a highly HIV endemic area is a constant challenge. There is a paucity of new anti-tuberculosis agents in the developmental and clinical trial phases to address the problem of extensively-drug resistant tuberculosis (XDR-TB). In an attempt to affect a cure in patients with XDR-TB, it has become necessary to re-introduce previously used anti-tuberculosis drugs, as well as antimicrobial agents designed for treatment of non-tuberculosis infections. Whilst these drugs may have previously been tested and shown efficacy in drug susceptible tuberculosis, their activity in XDR TB strains was not tested before introduction for management of XDR-TB in KwaZulu-Natal, South Africa. Drug susceptibility testing (DST) plays an integral role in the diagnosis and treatment options for tuberculosis. It is able to decrease the burden and spread of resistant tuberculosis. However DSTs methods for second line anti –TB drugs (SLDs) and third line anti-TB drugs (TLDs) have not been standardised. Critical concentrations of these anti-TB drugs remain unknown or vary within and between settings thus further hampering the control of TB.

PREFACE

This study represents original work by the candidate and has not been submitted in any other form to another University. Assistance where received, has been duly acknowledged.

All routine and experimental work (growth of isolates from storage, into liquid and solid media, phenotypic and genotypic methods of drug susceptibility testing and time kill experiments) described in this dissertation was performed by the candidate in the Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Professor P. Moodley.



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DECLARATION

Plagiarism

DECLARATION

I ……SALONA MOODLEY……, declare that

(i) The research reported in this dissertation, except where otherwise indicated, is my original work.

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PRESENTATIONS

This work has been presented by the candidate at the following conferences and symposiums:

- The 2nd International TB Conference of South Africa. KwaZulu-Natal, Durban, International Convention Centre, 1-4 June 2010. Oral Presentation
- 4th Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) Congress. South Africa, KwaZulu-Natal, Durban, The Elangeni Hotel, 8-11 September 2011. Poster presentation
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LIST OF ABBREVIATIONS

ACP – Acyl Carrier Protein

AECOM – Albert Einstein College of Medicine

AFB – Acid Fast Bacilli

AMIK – Amikacin

ARV – Antiretroviral

CAP – Capreomycin

CDC – Centers for Disease Control and Prevention

cfu/ml – Colony forming units per millilitre

CLO – Clofazimine

CLSI – Clinical and Laboratory Standard Institute

CTAB-NACL – Cetyltrimethylammonium bromide-sodium chloride

CTAB – N-cetyl-N,N,N-trimethyl ammonium bromide

DFC – Drug Free Control

DMF – Dimethyl Formamide

DNA – Deoxyribonucleic Acid

DOTS – Directly Observed Therapy

DOTS – Directly Observed Therapy Short Course

DR-TB – Drug Resistant Tuberculosis

DST – Drug Susceptibility Tests

EDTA – Ethylenediamine Tetra-Acetic Acid

EMB – Ethambutol

et al – and others

ETH – Ethionamide

FDA – Food and Drug Administration

FLDs – First Line Drugs

g – Grams

HCL – Hydrochloric Acid

HIV – Human Immunodeficiency Virus

i.e. – that is

INH – Isoniazid

IMI – Imipenam

IU – International units

KAN – kanamycin

KZN – KwaZulu–Natal

LAM – Lipoarabinomannan

LIN – Linezolid

LJ – Lowenstein Jensen

L – Litres

MDR – TB-Multi Drug Resistant Tuberculosis

M&C – Meropenem and Clavulanic acid

MGIT – Mycobacteria Growth Indicator Tube

mg – milligrams

MIC – Minimum Inhibitory Concentration

ml – Millilitres

mM – milli-Molar

MODS – Microscopic Observation Drug Susceptibility Assay

MOXI – Moxifloxacin

MTB – *Mycobacterium tuberculosis*

MTT salt – [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]

NADH – Nicotinamide Adenine Dinucleotide Dehydrogenase

NAD – Nicotinamide Adenine Dinucleotide

NCCLS – National Committee for Clinical Laboratory Standards

OADC – Oleic Albumin Dextrose Catalase

OD – Optical density

PACT – Polymyxin B, Amphotericin B, Carbenicillin, Trimethoprim

PAS – Para aminosalicylic acid

PBP – Penicillin binding proteins

PBS – Phosphate buffered saline

PCR – Polymerase Chain Reaction

PD – Pharmacodynamics

PK – Pharmacokinetic

pmols – picomols

PZA – Pyrazinamide

RNA – Ribonucleic acid

rpm – revolutions per minute

R – Rifampicin

SA – South Africa

SANDOH – South African National Department of Health

SDS – Sodium dodecyl sulphate

SLD – Second Line Drugs

SM – Streptomycin

SOP – Standard Operating Procedure

TB – Tuberculosis

TBE – Tris-Borate-EDTA

TE – Tris-EDTA

TKE – Time Kill Experiments

TLD – Third Line Drugs

µg – microgram

µl – microliter

USA – United States of America

UV – Ultra violet

V – Volts

viz – namely

WHO – World Health Organisation

XDR – TB-Extensively Drug Resistant Tuberculosis

ZN – Ziehl Neelsen

CHAPTER 1

INTRODUCTION

Previously effective therapeutic strategies to manage tuberculosis (TB) have unfortunately not succeeded in eradicating the causative agent, *Mycobacterium tuberculosis* (MTB). The escalating incidence of TB in Africa, in particular amongst the human immunodeficiency virus (HIV) type 1 (HIV-1) infected population, coupled with increasing antimicrobial resistance, underscores the urgency to address public health interventions and optimise anti-mycobacterial drug use. HIV is responsible for the highest number of deaths caused by any single infectious disease (WHO, 2011a). TB follows closely, and in 2010, the World Health Organisation (WHO) revealed that there were 9 million cases of TB globally, which caused 1.3 million deaths (WHO, 2011a).

Multi drug resistant TB (MDR-TB) is defined as an isolate that is resistant to the two most potent first line anti-TB agents, isoniazid (INH) and rifampicin (RIF) (WHO, 2008a). In the 1990's, cases of MDR-TB was reported in New York, Florida and London, (Breathnach *et al*., 1998; Coronado *et al.*, 1993; Edlin *et al.*, 1992; Frieden *et al.*, 1996;) and was attributed to delayed diagnosis, inappropriate treatment regimens, non-compliance to therapy and high rates of nosocomial transmission (Dooley *et al.*, 1992). At the turn of the century, the extent of the spread of MDR-TB was highlighted through reports of its presence in almost 90 countries and regions worldwide (Shah, *et al*., 2007). In 2008, 440 000 cases of MDR-TB were reported worldwide (WHO, 2010). This however represents the tip of the iceberg with large numbers of cases being unreported. The under reporting has been attributed to a significant number of cases being either not diagnosed, or incorrectly diagnosed; or if diagnosed are not correctly treated. It was also reported that only 20% of cases that were diagnosed, were treated according to the accepted international guidelines (Nathanson *et al.*, 2010). India and China have the highest burden of MDR-TB, and account for almost 50 % of the world's cases (Nathanson *et al.*, 2010).

In 2010, the number of globally reported MDR-TB cases increased to 650 000. This was a third more than the figure that was reported in 2008 (WHO, 2011b). In South Africa (SA), the first case of MDR-TB was detected in the Western Cape in 1985 (Weyer *et al.*, 1995). In

KwaZulu-Natal (KZN), MDR-TB strains were traceable as far back as 1994 (Pillay and Sturm, 2007). In 2006/2007, the incidence of MDR-TB in KZN was 780 per 100 000 population, with only 145 per 100 000 being culture confirmed positive, thus indicating that over 80 % of reported cases were not culture confirmed and additionally did not have drug susceptibility tests (DSTs) performed on them (Moodley *et al*., 2011).

The MDR form of MTB further evolved into a more resistant form termed extensively drug resistant (XDR-TB). XDR-TB is MDR-TB (resistance to RIF, INH) with additional resistance to the fluoroquinolones, plus one of the injectables viz: capreomycin (CAP) and/or kanamycin (KAN) and/or amikacin (AMIK) (CDC, 2006; Shah *et al*., 2005; WHO, 2006a).

XDR-TB was identified as far back as the late 1980's and early 1990's in the United States, Latvia and Russia (Koening, 2008). By the end of 2004, XDR-TB had spread to17 countries in a period of 4 years (Banerjee *et al.*, 2008). In 2008 XDR-TB was reported to be present in at least 45 countries (WHO, 2008a). By 2011 XDR-TB was present in 69 countries (WHO, 2011b).

In 2006, 53 cases of XDR-TB were identified in the Tugela Ferry region of KZN, SA (Gandhi *et al*., 2006). Fifty two (98%) patients died approximately 2 weeks after diagnostic sputum collection for detection of TB. Forty- four (83%) were co-infected with HIV (Gandhi *et al*., 2006). Whilst the first clinical case of XDR-TB was reported in 2006 in KZN, an analysis of the TB isolate bank in KZN revealed that the XDR form of the microbe was present in KZN from as early as 2001 (Pillay and Sturm, 2007). India, China, Russia and South Africa, have the largest number of cases of drug resistant TB (DR-TB), and these countries are home to more than half the world's cases of DR-TB (WHO, 2012b).

In 2007, the first report of totally drug resistant strains of TB (TDR-TB) was reported in Italy (Migliori *et al.*, 2007). Further reports of TDR-TB then followed in Iran (Velayati *et al*., 2009) and India (Udwadia *et al*., 2011). TDR-TB refers to MTB that is resistant to all first line anti-TB drugs (FLD) and all second line anti-TB drugs (SLD). Infection with these strains makes successful treatment of TB almost impossible (Velayati *et al.*, 2009).

Various factors aid in the development of drug resistance. These include poor treatment compliance, HIV co-infection and poverty (Thaver and Ogunbanjo, 2007). *In vivo* drug resistance involves complex interactions between the organism, site of infection and the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the drug (Kim, 2005). De-novo resistance was observed initially among TB patients that received sub-optimal drug treatment (Pillay and Sturm, 2007). Inadequate and inappropriate treatment has been attributed as being primarily responsible for *de novo* drug resistance (Kim, 2005 ; Pillay and Sturm, 2007).

The observation that already resistant strains of TB (MDR including XDR and TDR) are now being isolated from newly diagnosed TB patients supports transmission of already resistant strains within the community (Pillay and Sturm, 2007 ; Ramtahal *et al*., 2012) .

The treatment of DR-TB remains a challenge. WHO classifies the SLDs used for treatment of MDR-TB into 5 classes (WHO, 2008b). In KZN, MDR-TB patients were treated with SLDs viz: AMIK or KAN and ofloxacin. The emergence of XDR-TB prompted health officials to look at alternatives. In 2007, CAP and para-aminosalicylic acid (PAS) were re-introduced in SA as SLD for the management of patients with XDR-TB (Dheda *et al*., 2010; Master, 2011). In addition, antimicrobial agents viz: linezolid (LIN) and imipenam (IMI) that were already being used for the management of non-mycobacterial infections were reported as showing *in vitro* activity against DR-MTB (Anger *et al.*, 2010; Migliori *et al.*, 2009). Meropenam (in the same carbapenam class as imipenam) in combination with clavulanic acid (M&C) has shown *in vitro* activity against DR-TB (Hugonnet *et al*., 2009) and there are currently early bactericidal activity studies underway to determine its effect on MTB *in vivo* (Niehaus, 2012). CAP, PAS, LIN and M&C are now being used in various combinations with other FLDs and SLDs for the management of DR-TB in KZN (Master, 2011). However, standardised DST data linked to clinical outcomes are lacking.

In SA, the high prevalence of HIV-1 co-infection compounds the problem of management of TB. Patients may already be on antiretroviral (ARV) treatment, or are often commenced on ARV treatment together with anti-TB drugs (Abdool Karim *et al*., 2009; Abdool Karim *et al*., 2010). This results in poly-pharmacy with resultant serious drug interactions and toxicities

(Burman, 2005). Anti-TB drugs in TB and HIV co-infected patients have to therefore be carefully selected. In the era of DR-TB this is not an easy decision as the choices are limited. The prescriber has to ensure that all drugs being administered are effective against the strain of TB targeted. Whilst empirical treatment was previously an option, this is no longer possible given the diversity of drug resistant mutants that are currently circulating in our setting.

It has therefore become mandatory that initial empirical treatment for DR-TB that is commenced on diagnosis of cases, be modified as soon as possible by reliable DSTs for SLDs. The tailoring of empirical anti-TB drug therapy by DSTs as close to the time of initial diagnosis as possible will assist in guiding treatment and prevent the use of drugs that are not effective (Kim, 2005) .

Many factors influence the results and the reproducibility of a DST. A major challenge in the interpretation of DSTs is that there are no standardised tests for the SLDs used in the management of DR-TB in KZN (Shah *et al*., 2007; Van Ingen *et al*., 2010). DSTs are also influenced by physico-chemical factors in the test environment and are therefore difficult to standardise and reproduce (Canetti, *et al.*, 1969; Krüüner, *et al*., 2003).

Routine DSTs, measure the efficacy of a drug at a given time point. The interaction of the drug and microbe over time is not measured. Time kill experiments (TKEs) are the closest simulation to measure the latter, and may be used as an indicator of clinical outcome. However, the labour intensive nature of these assays does not allow it to be performed routinely. A proxy for TKEs is to use DSTs. Ideally, in the absence of clinical outcome data, DSTs employed routinely must be interpreted by extrapolating the performance of drugs against organisms in TKEs.

The gold standard DST for the FLDs is the agar dilution method (NCCLS, 2003). Liquid based assays such as the [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay have also been standardised for FLDs and proved to be reliable when tested against the gold standard DST (Martin *et al.*, 2005). For INH and RIF DSTs have been aligned to the activity of the drugs measured over time by TKEs (Jayaram *et al*., 2003; Jayaram *et al*., 2004; Steenwinkel *et al*., 2010; Vilchèze *et al*, 2011). However, the parameters

for phenotypic DSTs for SLDs have not been fully defined. In KZN, SA, DSTs for the more recently introduced SLDs and TLDs are not routinely performed. In addition, TKEs for these drugs have not been conducted and the DSTs when performed are not interpreted in conjunction with the former. We therefore sought to determine the reliability and reproducibility of DSTs for SLDs and TLDs in KZN.

The objectives of this study were to therefore:

1. Validate the reproducibility of the agar dilution DST (gold standard) for SLDs and TLDs viz: CAP, PAS, LIN, and M&C.
2. Validate the reproducibility of a liquid based DST (viz: MTT) for SLDs and TLDs viz: CAP, PAS, LIN, M&C.
3. Compare results obtained using the agar based DST with that of the MTT assay.
4. Perform TKEs for CAP, PAS, LIN and M&C.
5. Interpret DST results in conjunction with TKEs.
6. Attempt to resolve discrepancies by sequencing the known resistance genes.

CHAPTER 2

BACKGROUND

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis (TB). This organism is spread via the airborne or droplet routes of infection (Knechel, 2009; Mathema *et al*., 2006). It currently ranks second, only to HIV as the most frequent cause of death attributable to a single infectious agent (WHO, 2012b).

SA ranks fifth on a list of high TB burden countries, behind India, China, Indonesia and Nigeria (Donald *et al.*, 2009). Additionally, HIV infection is highest in these regions (WHO, 2012b). HIV infection increases the risk of TB reactivation as well as new infections with MTB (Getahun *et al*., 2010). In 2006, it was estimated that 1 in 4 young adults in SA are infected with HIV (Dorrington *et al.*, 2006), and 2/3 of them might also be infected with TB.

In the pre-antibiotic era, 65% to 75% of patients' survived pneumonia caused by *S.pneumoniae* (Bordow *et al.*, 2005*).*This was due to the ability of the host immune system to counteract the infection. The emergence and spread of drug resistant MTB strains places us in a situation akin to the pre-antibiotic era. However, a major difference in KZN is the fact that the population is largely HIV immune-compromised. KZN is likely to be the province with the highest incidence of DR-TB, with 4170 cases of MDR-TB, including 443 XDR-TB, were identified in 58 independent sites in KZN between the 1 January 2006 and 30 June 2007 (Moodley *et al.*, 2011). Studies have shown that the increase in the number of TB cases in KZN over the last 25 years, correlates with the exponential increase in HIV infected people in this province (Abdool Karim *et al*., 2009; Naidoo *et al*, 2007). DR-TB in a largely HIV induced immune-compromised population alters the survival odds when compared to the pre-antibiotic era, when the population at large were immune-competent.

2.1 The causative agent

Mycobacterium tuberculosis (MTB) is the causative agent of tuberculosis. This organism belongs to the order Actinomycetales, family Mycobacteriaceae and genus, Mycobacterium

(Rastogi *et al*., 2001). It is a rod shaped obligate aerobe, and is classified as a Gram positive bacterium due to the lack of a classical outer membrane. MTB is between 0.2-0.5µm in width and between 2-4µm in length (Knechel, 2009). The family Mycobacteriaceae differs from most other bacteria by being acid fast. This refers to the ability of the organism to withstand exposure to extreme acidic and alkaline conditions and is not decolorized by acidic solutions as applied in the Ziehl-Neelsen (ZN) stain. This acid fast property is attributed to the structure of the cell wall. The cell wall has a thick and virtually impermeable outer membrane, which is largely due to it being composed of numerous long chains of mycolic and fatty acids (Langford *et al*., 2011). This composition protects the organism and contributes to the virulence of the disease by enhancing resistance to various anti-TB drugs (Brennan, 2003). Other integral components of the cell wall include lipids and glycolipids, which are uniquely arranged and are parallel to the peptidoglycan layer (Knechel, 2009). There are various metabolic pathways required for the production of these mycobacterial cell wall lipids and these pathways serve as the main targets for the anti-TB drugs.

2.2 Resistance in *Mycobacterium tuberculosis*

The control of TB has been declared a national emergency in SA (Abdool Karim *et al*., 2009). DR-TB has been on the rise worldwide, with SA being listed as one of the high burden countries. Since 2007, SA has reported increasing numbers of patients with XDR-TB (Olson *et al*., 2011; WHO, 2010). The management of patients with DR-TB is far from optimal. A large proportion of these patients are either not diagnosed, and therefore remain untreated, or are diagnosed, but are treated with ineffective drugs or are lost to follow up. Moodley *et al* (2011) revealed that over 65% of the culture confirmed MDR-TB patients in KZN, including XDR-TB, were not treated at the MDR-TB referral center. The suboptimal management of patients promotes the continued transmission of DR-TB in the communities.

Resistance can be divided into 3 categories based on the patient's history of previous TB treatment:

a. Primary resistance: a patient is infected with an already resistant strain of MTB and was not on any treatment for TB prior to this (Cohn *et al*., 1997; Mathema *et al.*, 2006; Zhang and Yew, 2009).

b. *De Novo*/Acquired resistance: A patient is on anti-TB treatment, or has had one or more previous TB treatment episodes which add up to more than one month. It is perceived that the resistant isolate has become resistant due to mutations and had selective advantage due to the treatment episodes (Cohn *et al*., 1997; Mathema *et al.*, 2006; Zhang and Yew, 2009).

c. Initial resistance: the patient's history of previous anti-TB treatment is unknown; therefore, it may be a combination of primary resistance, and undisclosed acquired resistance (Cohn *et al*., 1997; Zhang and Yew, 2009).

MTB resistance may be classified based on drug susceptibility patterns.

a. Mono-resistant TB: TB caused by MTB resistant to one of the FLDs (Table 1) (WHO, 2013)

b. Multi-drug resistant TB (MDR-TB): TB caused by MTB resistant to the two most potent FLDs viz. INH and RIF (WHO, 2008a; WHO, 2013).

c. Pre-extensively drug resistant TB (pre-XDR-TB): TB caused by an MDR MTB strain with additional resistance to either fluoroquinolones or a second line injectable drug (i.e. CAP, KAN or AMIK), but not both (SANDOH, 2011).

d. Extensively-drug resistant TB (XDR-TB): TB caused by an MDR MTB strain with additional resistance to one of the fluoroquinolones and any of the second line injectable drugs (CAP, KAN, AMIK) (WHO, 2008a; WHO, 2013).

e. Totally drug resistant TB (TDR-TB): TB caused by an XDR-TB strain with additional resistance to 5 other drugs. TDR-TB strains are therefore resistant to a total of 9 different drugs (Migliori *et al*., 2007; Velayati *et al*., 2009).

Reported XDR-TB cases have escalated since the outbreak in 2006 (Gandhi *et al.,* 2010; Moll *et al.,* 2007; Olson *et al.,* 2011). Pillay and Sturm (2007) reported that this outbreak and many of the previous MDR and XDR-TB cases were caused by the F15/LAM4/KZN strain. XDR-TB has since been documented in more than 60 different clinics and hospital settings in KZN (Moodley *et al.*, 2011). It has also been reported in all of SA's provinces (SAPA, 2007).

Higher morbidity and mortality rates are present in patients with DR-TB, due to the treatment options being limited and less successful as opposed to that of the susceptible form of infection (Gandhi *et al*., 2010). These morbidity and mortality rates are even higher when DR-TB patients are co-infected with HIV (Reid and Shah, 2009). The management of patients with DR-TB is often prolonged and complex. This may result in poor patient compliance, thereby promoting transmission to others as well as *de novo* resistance development in the individual.

The cost to treat a patient with drug susceptible TB in the United States of America (USA) is $ 10, whereas to retreat a patient it costs $ 5000-10000 i.e. the cost of 1 retreatment is equivalent to initially treat 500-1000 patients (Caminero, 2005). According to a survey conducted by Dr. I.H. Master in 2010, it costs R67 per month for treating a patient for standard TB in the intensive phase (first phase of treatment) and R42 per month for the continuation phase (second phase of treatment). For MDR-TB treatment in the intensive phase and the continuation phase, it costs R1207 and R968 per month respectively. XDR-TB treatment in the intensive and continuation phase costs a markedly higher price of R6654 and R4263 per month respectively (Master, 2011).

2.3 Factors contributing to drug resistance

It is generally accepted that acquired bacterial resistance is a man-made problem Lalloo (2010), reported on the factors that result in the selection of resistance in MTB in patients on anti-TB treatment. These factors include administration of ineffective drugs as well as incorrect doses, poor absorption of the drug, drug antagonism, shortages of drugs at hospitals and clinics etc. Clinicians further intensify the problem, if they continue treatment with a failing regimen, or if only one anti-TB drug is added to the treatment regimen, when treatment failure occurs (Lalloo, 2010).

A factor contributing to the spread of DR-TB is delayed diagnosis of TB. Until 2007, culture and susceptibility testing was restricted to patients that had interrupted treatment, second episodes of TB and treatment failures (SANDOH, 2006; Singh *et al*., 2007). With the emergence of XDR-TB, the policy changed in that now a culture is mandatory in each patient suspected of TB (Moodley *et al*., 2011). All positive cultures for MTB should be subject to DST. However, the laboratory capacity to support the new recommendation is insufficient and needs to be extended urgently. Another problem that needs to be resolved is the poor communication between laboratory staff and clinicians and between clinicians and patients resulting in a significant loss to follow up. Poor management practices at clinics and flaws in the health system also contribute to resistance development (Lawn *et al.*, 1998; Lienhardt *et al*., 2001; Steen and Mazonder, 1998; Wondimu *et al.,* 2007). This includes poor record keeping, lack of directly observed treatment (DOTS), lack of follow up and poor contact tracing. This is fuelled by frequent changes of hospital staff, poor education in the management of TB patients and poor infection prevention measures (Lalloo, 2010).

2.4 Treatment of Tuberculosis

Treatment of patients suffering from TB is of medical as well as public health importance. It aims at curing the patient, preventing relapses and, in doing so, preventing death from the disease (Harries and Maher, 1996).

In SA, TB treatment regimens are based on the WHO recommendations for the management of patients with TB disease. Therefore the management of TB is largely dictated by guidelines

as opposed to individualized treatment based on susceptibility patterns. A number of lessons have been learnt from the management of drug susceptible infection in the past. These have been used to shape the approach to the management of DR-TB (Di Perri and Bonora, 2004) and include the following: a single anti-TB agent should not be added to a failing regimen, antibiotic mono-therapy should not be considered and at least 3 anti-TB drugs which the patient has never taken should be incorporated into the regimen. Overall, the clinician should aim to treat the patient with at least 5 anti-TB agents to which the organism is not resistant.

Combined drug therapy is thought to be more effective than mono-therapy since it decreases the selection for resistance. (Rieder, 2002; Schluger *et al.*, 1996) .According to the South African National Department of Health (SANDOH) policy guidelines for the treatment of DR-TB, patients with MDR-TB and XDR-TB must be on their appropriate regimen of drugs for a minimum of 6 months during the intensive phase and at least 18 months during the continuation phase. The duration of both of these phases must also be guided by the TB culture conversion of each patient (SANDOH, 2011). If severe side effects occur, then treatment must be stopped. Treatment must also be stopped, if only one drug can be tolerated by the patient, to prevent selection for resistance. It is permissible for one anti-TB drug to be exchanged for another because of side effects, not because of treatment failure (SANDOH, 2011). If, however alternative treatment is not available, then the number of drugs can be reduced as long as a minimum of three drugs with proven *in vitro* activity remain. Culture results and DST results must be assessed prior to this change.

There is currently no established regimen for the treatment of XDR-TB. There is also no guarantee of a cure, as the MTB isolate infecting the patients may be resistant to the anti- TB drugs that are being used for treatment (Banerjee *et al*., 2008).

In the era of DR-TB, effective management has to be based on reliable DSTs as opposed to guidelines. The diversity of susceptibility profiles currently seen amongst TB cases in SA underscores the importance of directed therapy.

2.5 Anti-Tuberculosis Agents

Novel anti-TB drugs are still a while away from field testing. In the interim, scientists and policy makers are exploring the possibility of using antibacterial agents not traditionally used for TB treatment as possible anti-tuberculosis agents. The exact positioning of these drugs in the TB treatment algorithms has not been fully elucidated, but may form part of the second or third line treatment regimen (Banerjee *et al.*, 2008). The classes of drugs were referred to as first line drugs (FLD), second line drugs (SLD) and third line drugs (TLD) (table 1) (Banerjee *et al.*, 2008). However, due to increasing resistance, this classification is no longer applicable since SLDs and TLDs are used interchangeably.WHO has recently released a new classification of anti-TB drugs (table 2) (SANDOH, 2011; WHO, 2008b)

Table 1: Classes of Anti -TB Drugs

First Line Drugs	Second Line Drugs	Third Line Drugs
Isoniazid	Fluoroquinolones	Linezolid
Rifampicin	Injectables:	Clarithromycin
Streptomycin	Amikacin and Kanamycin	Imipenem
Pyrazinamide	Capreomycin	Amoxacillin and Clavulanate
Ethambutol	Ethionamide	Clofazimine
	Para-aminosalicylic acid	
	Cycloserine	

Table 2: World Health Oganisation grouping of Anti-TB Drugs

Group	Drugs
Group 1: First line oral drugs	Isoniazid
	Rifampicin
	Ethambutol
	Pyrazinamide
Group 2: Injectable drugs	Kanamycin
	Amikacin
	Capreomycin
	Streptomycin
Group 3: Flouriquinolones	Levofloxacin
	Moxifloxacin
	Gatifloxacin
Group 4: Oral bacteriostatic second line drugs	Ethionamide
	Prothionamide
	Cycloserine
	Terizidone
	Para-amino salicylic acid
Group 5: Drugs of unclear efficacy (Not recommended for use in MDR-TB patients)	Clofazamine
	Amoxicillin/Clavulanate
	Clarithromycin
	Azithromycin
	Linezolid
	Thioacetazone
	Imipenem
	High dose of isoniazid

2.5.1 First line drugs (FLDs)

Streptomycin (SM)

In 1944 SM was discovered by Selman Waksman and was the first record of a drug effective against TB (Schatz and Waksman, 1944). It binds to the 30S ribosomal unit, and interferes with the decoding of aminoacyltRNA and thus inhibits mRNA translation, or causes insufficient translation (Da Silva and Palomino, 2011). By interfering with ribosomal function, it prevents protein and polypeptide synthesis (Kolyva and Karakousis, 2012). SM shows strong bacteriostatic activity against TB (Schatz and Waksman, 1944) and bactericidal activity against bacilli in the exponential phase (Di Peri and Bonora, 2004). However a few months after being introduced in the treatment of tuberculosis meningitis in children, relapse was seen and was attributed to the development of resistance to SM (Kolyva and Karakousis, 2012). This provided initial evidence that mono-therapy for the treatment of MTB rapidly led to resistance development. Patients have poor oral absorption of SM (Kolyva and Karakousis; 2012). It therefore needs to be administered as an intra-muscular injection, which has been described as very painful (Gundersen and Bietti 1949). There is also several safety concerns associated with treatment using SM. One of the most severe is the development of ototoxicity with resultant deafness.

Isoniazid (INH)

INH was first synthesized in 1912 in Prague (Meyer and Mally, 1912). It was only in the early 1950's that it was discovered to be effective against TB (McDermott, 1969; Robitzek and Selikoff, 1952). INH is a small water soluble molecule, which is a hydrazide of isonicotinic acid. It is able to enter the host cells easily (Mackaness and Smith, 1952), and is then able to pass through the MTB membrane (Bardou *et al*., 1998). The active form of INH inhibits synthesis of phospholipids (Brennan, *et al*., 1970; Kolykova and Karakousis, 2012) and nucleic acid (Gangadharam *et al*., 1963). It was also shown to prevent the metabolism of nicotinamide adenine dinucleotide (NAD) (Bekierkunst, 1966). The main mode of action of INH against MTB is the inhibition of the mycolic acid synthesis and elongation of fatty acids of MTB (Quémard *et al*., 1995). This is achieved by supressing the activity of the InhA enzyme (Dessen *et al*., 1995; Rozwarski *et al*., 1998; Zhang and Guo, 2012). INH displayed bacteriostatic activity against mature resting bacilli, but bactericidal activity for rapidly

dividing bacteria both intracellularly and extracellularly (Di Perri and Bonora, 2004; Snell, 1998). Due to its early bactericidal activity it has an increased capacity to render patients non-infectious more rapidly than some of the other anti-TB agents (Di Perri and Bonora, 2004).

Pyrazinamide (PZA)

PZA was first discovered as an anti-TB agent in 1954 (Zhang and Mitchison, 2003).This compound is an analogue of nicotinamide, which is a Vitamin B_3 precursor that has bactericidal activity against MTB (Di Perri and Bonora, 2004). It is thought to act as a sterilizing drug, and kills bacteria within an acidic environment i.e. a pH of 5.5 (McDermott and Tompsett, 1954; Mitchison, 1985). It also plays a unique role in that it is able to shorten the duration of therapy (Steele and Des Preze, 1988; Zhang and Mitchison, 2003), from 9-12 months, to a period of 6 month in combination therapy with INH and RIF (Iseman, 2002). This is due to its ability to successfully eradicate bacilli that are in a semi-dormant state within acidic environments during active inflammation whilst all other anti-TB drugs are unable to do this (Heifets and Lindholm-Levy, 1992). PZA given in the 2 months of intensive therapy is thought to rapidly reduce bacterial load and the 4 months of maintenance therapy will then eradicate the remaining organisms (Heifets and Lindholm-Levy, 1992). It was also shown to have a strong synergistic relationship with R (Grosset, 1978). PZA has no or very little effect on other mycobacteria, including *M. bovis* (Fuursted *et al*., 1993; Zhang and Mitchison, 2003). It is believed that this is due to the lack of the enzyme pyrazinamidase (Pzase) in *M. bovis* (Zhang and Mitchison, 2003). This enzyme is involved in the drugs activity against MTB, as it hydrolyses the PZA to pyrazinoic acid (Scorpio and Zhang, 1996), which is the active form of PZA (Zhang *et al*., 2003). Pyrazinoic acid has been reported to interfere with the energetics and proton motive force of the membrane as well as the membrane transport function of MTB (Zhang *et al.*, 2003).

Ethambutol (EMB)

EMB was discovered in 1961 (Thomas *et al*., 1961). It is commonly referred to as a "companion drug" since it is frequently used in combination with other drugs for the treatment of TB (WHO, 2006b). Although it was not very effective in killing bacteria, it prevented resistance when used in combination with other drugs (Hoek *et al*., 2009) and displays bacteriostatic activity (Di Perri and Bonora, 2004). Its mode of action is linked to the inhibition of 3 arabinosyltransferase enzymes (*EmbA, EmbB* and *EmbC*) (Goude *et al*., 2009). The first two are involved in converting arabinose into arabinan, and lastly arabinogalactan. This is an important constituent of the MTB cell wall (Goude *et al*., 2009). EmbC facilitates the formation and production of lipoarabinomannan (LAM), which is a cell wall glycolipid (Goude *et al*, 2009). EMB inhibits many cellular pathways. It inhibits ribonucleic acid (RNA) metabolism (Forbes *et al*., 1962; Forbes *et al*., 1965) and in turn prevents MTB growth (Zhang and Guo, 2012). EMB was shown to prevent phospholipid synthesis (Cheema and Khuller, 1985a; Cheema and Khuller, 1985b) and to inhibit the transfer of mycolic acids into the cell wall (Takayama *et al*., 1979).

Rifampicin (RIF)

RIF was discovered in the early 1960's and this, in combination with PZA and INH, allowed for a reduction in treatment duration, from 18-24 months to 6 months (Kabra and Srivastava, 2011).It is described as a potent 'sterilising' drug and its added benefit in combination with INH is the ability to kill very slow dividing and non-replicating bacilli (Blumberg *et al*., 2003; Jindani *et al*., 1980; Rieder *et al*., 2002). It also kills persistent bacilli, throughout the duration of TB treatment (Grosset *et al.*, 1998; Mitchison, 1985). To date, INH and RIF are the most effective antimycobacterial agents known to rapidly decrease the bacterial load caused by susceptible strains (Harries and Maher, 1996, WHO, 2008a). RIF is able to pass through the MTB membrane, as it is lipophilic in nature (Wade and Zhang, 2004). Its mode of action involves inhibiting transcription. This occurs when it binds to the bacterial dependent enzyme, RNA polymerase, and inhibits activity of the β subunit of this enzyme. This unit is responsible for transcribing the bacterial RNA (Buurman *et al*., 2001).

2.5.2 Second line drugs (SLDs)

The treatment of DR-TB involves using toxic drugs that are less potent than FLDs. These have to be used for a longer duration. In SA, there are numerous anti-TB drugs that are used for treating MDR-TB and XDR-TB. Not all of these drugs have been placed into set treatment regimens. Table 2 categorizes SLDs into 5 different classes, based on their efficacy and experience of use. Some of these drugs present in the SLD category are placed in the TLD category of other treatment regimens (SANDOH, 2011; WHO, 2008b)

Cycloserine

Cycloserine is recommended by the WHO as a SLD for the treatment of MDR-TB (Hwang *et al*., 2013). It is useful as an additional drug to the MDR-TB treatment regime, as it may prevent the development of drug resistance, since it has not shown cross resistance with other active TB drugs (SANDOH, 2011; Wolinsky, 1993). Cycloserine displays bacteriostatic activity with MTB, however due to its many toxic effects at its effective dose, it is seldom included in national treatment programmes (David, 2001; Hwang *et al.*, 2013). Its mode of action involves inhibiting peptidoglycan synthesis (Kolyva and Karakousis, 2012).

Ethionamide (ETH)

ETH belongs to a group of drugs called thioamides (Di Perri and Bonora, 2004). Its mode of action is similar to that of INH, in preventing the synthesis of mycolic acids which are found on the cell wall of MTB (Di Perri and Bonora, 2004; Quemard *et al*., 1992). It does this via inhibition of the *inhA* gene product enoyl-ACP reductase (Johnsson *et al*., 1995), when it binds to the reductase InhA enzyme (Kolyva and Karakousis, 2012). It exhibits bactericidal activity *in vitro* (Steenken and Montabine, 1960). Side effects of ETH are dose related, and it is thought to be responsible for causing nausea, vomiting, dizziness, fatigue and depression. It has also been reported to cause gastro-intestinal disturbances, hepatotoxicity and hyperthyroidism (Banerjee *et al*, 2008; Di Perri and Bonora, 2004). Patients generally have poor tolerance against ETH (Mahmoudi and Iseman, 1993).

Aminoglycosides

Amikacin (AMIK) and kanamycin (KAN) belong to the aminoglycoside family. They have very strong concentration dependent bactericidal properties (Freeman *et al*., 1997; Gilbert, 2000). Their bactericidal activity is only effective against rapidly dividing mycobacteria (Heifets and Lindholm-Levy, 1989). They are thus not effective when bacilli are not replicating, which may be for long periods, during the stationary phase of growth (Heifets and Lindholm-Levy, 1989; Jana and Deb, 2006). Aminoglycosides have to penetrate the mycobacterium, in order for them to reach their molecular target (Jana and Deb, 2006). They are responsible for preventing protein synthesis in MTB, by binding to the 30S subunit proteins (Kotra *et al*., 2000) and 4 nucleotides of the 16S rRNA and a single amino acid of the S12 protein. This action interferes with the decoding of site 1400 in the 16S rRNA of the 30S subunit (Jana and Deb, 2006; Melancon *et al*., 1992). This results in the incorrect amino acid being inserted into the polypeptide (Heifets and Lindholm-levy, 1989; Perri and Bonora, 2004). Thus these polypeptides are non-functional. When the aminoglycosides bind to the ribosomal unit this makes these molecular targets unavailable for translation resulting in cell death (Kotra *et al.,* 2000). The mode of action of AMIK and KAN are thought to be similar, but it is thought that their binding sites on the molecular targets are different. The prolonged use of aminoglycosides leads to toxicity related nephrotoxicity and ototoxicity (Peloquin *et al.*, 2004). The side effects of these agents are duration and dose related (Smith *et al.*, 1986). It is thought that AMIK is generally active against SM resistant strains of MTB (Hoffner and Kallenius, 1988; Perri and Bonora, 2004). However, AMIK resistant strains are generally resistant to SM. Cross resistance with KAN is well described (McClatchy *et al.*, 1977).

Fluoroquinolones

Ofloxacin , ciprofloxacin, sparfloxacin, levofloxacin, gatifloxacin and moxifloxacin all belong to the fluoroquinolone class of drugs (Perri and Bonora, 2004).Ofloxacin and ciprofloxacin have both been approved as SLDs for the treatment of MDR-TB, by the WHO, Centers for Disease Control and Prevention (CDC) and the American Thoracic Society (Blumberg *et al*., 2003 ; Crofton *et al*., 1997). The safety and tolerability of gatifloxacin and moxifloxacin, is still being investigated, as they are both fairly new fluoroquinolones. They have been described as being the most potent of the fluorquinolone group (Lalloo and Ambaram, 2010).

It is believed that all fluoroquinolones have similar modes of action. They are capable of penetrating macrophages and are responsible for bactericidal activity (Auwera *et al*., 1988; Rastogi *et al.*, 1996). Fluoroquinolones interfere with DNA (deoxyribonucleic acid) gyrase, and prevents DNA replication and transcription (Kolyva and Karakousis, 2012; Zhang and Guo, 2012). It affects the function of this enzyme, which is to prevent excessive supercoiling of the DNA during replication or transcription. Thus flouoroquinolones prevent the correct number of unwinding to occur for 1 DNA helix to divide into two. They are thereby able to block off bacterial DNA replication and inhibit normal cell division (Drlica *et al*., 1996).

Capreomycin (CAP)

CAP is a complex cyclic polypeptide isolated from *Streptomyces capreolus* (Donomae, 1966). It is bacteriostatic and its mode of action involves binding to the 16S rRNA ribosomal subunit and altering its structures (Wade and Zhang, 2004), thus inhibiting the translocation reaction. It is responsible for the inhibition of protein synthesis through interaction with ribosomes (Maus *et al*., 2005a). It displays strong activity on persistent non-replicating forms of MTB (Filippini *et al*., 2010; Heifets *et al*., 2005). CAP cannot be administered orally, but rather intramuscularly, or intravenously. It is a fairly expensive anti-TB drug with numerous side effects. Some of the most serious side effects include nephrotoxicity, ototoxicity, electrolyte imbalances, anaemia, leukopenia, hypersensitivity, and hypokalaemia (Donomae, 1966; Garfield *et al*., 1966; Obrien, 1993). Over the past few years, there has been an increase in DST research on CAP. CAP has been tested on both solid and liquid based DST methods in an effort to determine a critical concentration, breakpoint concentrations or minimum inhibitory concentration (MIC) for MTB. In 2003 a study compared the proportion method to the colorimetric resazurin microtitre assay **(**REMA) (Martin *et al*., 2003). This study used 150 susceptible and MDR-TB clinical isoaltes. These isolates were collected from Bolivia, Peru and other eastern European countries. The proposed breakpoint value for CAP was 10µg/ml when using the proportion method and 2.5µg/ml when using REMA (Martin *et al*., 2003). In 2010 a comparative study by Van Ingen *et al*, compared the agar dilution method to the MGIT 960 system using 28 MDR and XDR-TB isolates (Van Ingen *et al.*, 2010). These two DST methods revealed MICs that were fully concordant.The breakpoint concentrations were the same as the above mentioned study. Similar breakpoint concentrations were also expressed in

a study by Kam *et al*, in 2010. This study made use of the absolute concentration method, the 1% proportion method and the radiometric BACTEC 460 method. The breakpoint concentrations were 1.5-2 mg/l in the BACTEC method, 8mg/l in the 7H10 agar method and 40mg/l in the Lowenstein Jensen medium (LJs) used for the absolute concentration method (Kam *et al*., 2010). This still follows the trend that values are higher in the solid media DST methods.

Para-amino salicylic acid (PAS)

PAS was discovered to have anti-TB activity in 1943 by Lehmann (Lehmann, 1946). It is classified as bacteriostatic and prevents conversion of para–amino benzoic acid into folic acid, and in so doing, it prevents the biosynthesis of folic acid (Peloquin *et al*., 1999). Bacteria are often unable to use external sources of folic acid, thus PAS prevents access to folic acid which is required by the bacterium for cell growth and multiplication. PAS also interferes with the uptake of iron by bacteria (Wade and Zhang, 2004). It is administered orally, and the current granular formulation of PAS, is more tolerable, and less toxic than the previous tablet formulation (Akhtar *et al*., 1968). Side effects include hypersensitivity reactions, hypothyroidism, thrombocytopenia, jaundice, lymphadenopathy, leucocytosis, malabsorption, nausea and other gastrointestinal problems (Akhtar *et al*., 1968). It also affects the uptake of vitamin B12, and thus a supplement of this should be given to patients on PAS treatment (Paaby and Norvin, 1966). Research on DST methods using PAS on MTB isolates aims to determine the MIC, breakpoint and critical concentrations. This will aid in determining concentrations of PAS for treatment regimens. The study by Kam *et al*, in 2010 (mentioned above) proposed that the critical concentrations for PAS in LJs are 1mg/l and in 7H10 agar and the BACTEC 460 system is 0.5-1mg/l. According to Martin *et al*, (2003) the critical concentration for PAS when using LJs or 7H11 medium, is 0.5µg/ml and when using the liquid 7H9 broth in the REMA method it is 2µg/ml.

With resistance to the above SLDs emerging, newer options of chemotherapy are needed to manage XDR-TB.

2.5.3 Third Line Drugs (TLDs)

Linezolid (LIN)

LIN is the first oxazolidinone that was developed and approved for clinical use (Bozdogan and Appelbaum, 2004). It exhibits good antimycobacterial activity (Cynamon *et al*., 1999) as well as good antimicrobial activity against a wide variety of Gram positive pathogens (Stevens *et al.,* 2004). LIN inhibits synthesis of bacterial ribosomal proteins (Shinabarger, 1999). This agent is able to bind on the 50S ribosomal subunit close to the 30S unit, and thereby prevents the formation of a 70S initiation complex (Rodriguez Diaz *et al*., 2003). This mode of action is unique, and is believed to preclude cross resistance to other available anti-TB drugs. LIN possesses a unique structure that is completely synthetic, and therefore there is no pre-existing resistance (Moellering, 2003). It displays excellent activity on MTB cultures in both the logarithmic and stationary phase of growth. This is a characteristic that has only been demonstrated with INH and RIF. Recent studies have shown that LIN is effective in the treatment of MDR-TB (Huang *et al*., 2008), however it must always be used in combination with other SLDs and TLDs. Several studies also show synergistic activity between LIN and fluoroquinolones which has been proven to enhance linezolid's effect in the latent phase of infection (Rodriguez Diaz *et al*., 2003). LIN also has synergistic activity with RIF and can therefore be used to treat patients infected with MTB strains that are resistant to INH but not to RIF (Rodriguez Diaz *et al*., 2003). Treatment with LIN over long durations is associated with many side effects e.g. neurological toxicity like peripheral neuropathy as well as pancreatitis and anaemia (Fortún *et al*., 2005). LIN was tested against MDR and XDR-TB clinical isolates and MTB complex strains using the agar dilution and automated MGIT method (Van Ingen *et al*., 2010). This was done in an effort to determine the MIC of LIN against MTB isolates of varying susceptibility profiles. This will aid in determining the best concentrations at which LIN is most efficient against MTB. The DST results revealed a breakpoint concentration of 1 µg/ml for LIN when using both these methods. This breakpoint concentration was also shown by Alcalá *et al* in 2003 when using the agar proportion method. A study in 2007 by Richter *et al* was the first to report a LIN resistant clinical isolate, which had an MIC of 8µg/ml.

Meropenem combined with Clavulanic Acid (M&C)

Meropenem is a ß- lactam drug, which belongs to the class of carbapenems. These have no effect on MTB as it is inactivated by the enzyme ß –lactamase produced by the bacteria (Cole *et al*., 1998). When meropenem was combined with a ß-lactamase inhibitor, such as clavulanic acid, it was shown to inhibit the growth of XDR-TB (Hugonnet *et al.*, 2009). A similar bactericidal activity was also displayed when amoxicillin and clavulanic acid were combined and administered to TB patients in 1998 (Chambers *et al*., 1998). Strong inhibitory activity of this combination was also shown on anaerobically grown MTB cultures (Hugonnet *et al*., 2009). M&C was also active against the growth of rapidly multiplying bacteria as well as persistent bacteria (Hugonnet *et al*., 2009). This *in vitro* study which tested 13 XDR-TB isolates revealed that most M&C MIC values for these isolates were less than 1µg/ml. These MIC values were also similar to that of the susceptible strain (H37Rv) that was tested. These two anti- TB drugs are both food and drug administration (FDA) approved and are non-toxic, with very few side effects, thus rendering them appropriate for TB treatment in adults and children. Clavulanic acid is also the only FDA approved ß-lactamase inhibitor that irreversibly inhibits *blaC* (Hugonnet *et al*., 2009).

2.6. Resistance mechanisms

Bacterial resistance to anti-TB agents can be due to drug inactivation, alteration of the structure of the drugs target, a decrease in the quantity of the drug within the host or bacterial cell, which can be caused by either decrease in permeability, and/or an increase in activity of efflux pumps (Li and Nikaido, 2004). Resistance can also occur when the pro-drug is not converted to its active form. This is necessary for INH, ETH and PZA (Kolyva and Karakousis, 2012; Raynaud *et al.*, 1999; Scior *et al.*, 2002). There are also various forms of bacterial resistance. Intrinsic resistance refers to resistance that is due to a natural feature of the organism. In MTB, an example of intrinsic resistance would be its use of the low permeable cell wall that it possesses (De Rossi *et al*., 2006). This wall is made up of layers of lipids and glycolipids that are arranged in a unique manner, which helps control the type of molecules that enter the cell. It can thus prevent the entry of various drug molecules (Brennan 2003; Niederweis, 2003). Acquired resistance occurs when an exogenous resistant gene is acquired or when a spontaneous mutation develops (Hogan and Kolter, 2002; Normark and

Normark, 2002). MTB has a unique ability to remain in a latent state within a host who shows no signs or symptoms of possessing the MTB strain. It can then convert to its active form, which is then infectious (Stewart *et al*., 2003; Zhang, 2004). Resistance of MTB to various anti-TB agents is often a result of mutations in chromosomal genes. The sites and positions of these mutations vary in each gene, but are specific for the various anti-TB agents. These mutations are often found on the gene that is the drug target, or that controls the activation of the actual anti-TB agent (Wade and Zhang, 2004; Zhang and Telenti, 2000). Another mechanism of resistance that MTB makes use of are efflux pump systems (Levy, 2002; Nikaido, 1994).This system controls the entry of various drug molecules, and is also able to pump out drug molecules from within the cell, thus protecting MTB from the mode of action of the anti-TB agent, and ensuring its survival (Lomovskaya and Watkins, 2001). Efflux pump systems exhibit specificity, and thus can mediate the transport of specific molecules into and out of the cell (Neyfakh, 2002).

Table 3 shown below (Banerjee *et al.*, 2008) summarizes the specific genes that develop mutations, which in turn results in resistance for the listed anti-TB agents.

Table 3 : Anti -TB drugs and Drug Resistance conferring Gene Mutations

Anti-TB drug	**Mutated genes conferring resistance**
First Line Drugs	
Isoniazid	*katG,inhA,ahpC,oxyR,kasA,furA,ndh*
Rifampacin	*rpoB*
Streptomycin	*rrs,rpsl,gidB,*
Pyrazinamide	*pncA*
Ethambutol	*embCAB*
Second Line Drugs	
Fluoroquinolones	*gyrA, gyrB*
Injectables:	
Amikacin and Kanamycin	*rrs*
Capreomycin	*rrs, tlyA*
Ethionamide	*inhA, etaA/ethA*
Para-aminosalicylic acid	*thyA*
Cycloserine	*alrA , ddl* (shown only in *M.smegmatis)*
Third Line Drugs	
Linezolid	*rrl1 and rrl2*
Clarithromycin	-
Imipenem	-
Amoxacillin and Clavulanate	bpb coding genes
Clofazimine	-

2.6.1 Resistance mechanisms of the drugs used in this study

Resistance to INH

Resistance to INH can be due to mutations in many genes. These include *ndh, kasA, inhA, oxyR, furA, ahpC* (Banerjee *et al*., 2008; Riska *et al.*, 2004). Mutations in the *katG* gene are most common, and account for over 50 % of resistant cases (Banerjee *et al*., 2008; Riska *et al.*, 2004). Mutations in the *katG* gene interfere with the function of the catalase-peroxidase, which is to activate the INH pro-drug (Kolyva and Karakousis, 2012). Mutations at this gene can result in insertions, deletions, missense, nonsense mutations and even entire gene deletions, although the latter is uncommon (Kolyva and Karakousis, 2012). Single point mutations also occur in the *katG* gene, which results in a substitution of threonine for serine at the 315 site. This is the most common mutation that accounts for resistance to INH (Abate *et al.*, 2001; Marttila *et al.*, 1998).This mutation results in a decrease in the activity of catalase and peroxidase (Rousse *et al*., 1996; Saint-Joanis *et al*., 1999). Low level resistance to INH was also revealed when mutations were present in the *inhA* gene. This was also evident at the promoter region of the operon (*mabA inhA*) (Musser *et al*., 1996). Mutations in the *inhA* gene, decreases the affinity of the enzyme to nicotinamide adenine dinucleotide dehydrogenase (NADH), which it is dependent on (Basso *et al*., 1998). Mutations at the promoter region of the operon results in over expression of the wild-type enzyme. Mutations were present in the *ndh* gene, in INH resistant MTB isolates. These isolates did not have mutations in the *katG* and *inhA* genes (Lee *et al*., 2001). The *ndh* gene is responsible for encoding NADH dehydrogenase, therefore mutations at this site, affects the peroxidation of INH (Miesel *et al*., 1998).

Resistance to INH was also found in isolates that lack mutations in all of the above mentioned genes. This forms a quarter of the INH resistant isolates (Karakousis, 2009), thus implying that there are alternative mechanisms of resistance to INH. The efflux pump system was suggested to be one of these alternate mechanisms. A study by de Steenwinkel *et al* (2010) showed that when reserpine, which is the efflux pump inhibitor, was added to the INH containing agar, there was a decrease in the minimum inhibitory concentration (MIC).

Resistance to CAP

Resistance to CAP is caused by mutations in the *rrs* gene (Jugheli *et al.*, 2009; Maus *et al*., 2005b) and *tlyA* gene (Kashuba *et al*., 1999; Maus *et al*., 2005a). Mutations that occur in the *rrs* gene encoding for the 16S rRNA is responsible for resistance to CAP and AMIK (Alangaden *et al*., 1998; Taniguchi *et al*., 1997). Resistance to CAP and viomycin is due to mutations in the *tlyA* gene, which encodes for 2'-o-methyltransferase of the 16S rRNA and the 23S r RNA (Johansen *et al*., 2006) and is responsible for the addition of methyl groups to the rRNA (Sander *et al*., 1996). Cross resistance between KAN and AMIK is common but not with SM (Jureen *et al.*, 2010). Cross resistance has been reported between CAP and viomycin, however there is very little information available on this mechanism (Mclatchy *et al*., 1977).

Resistance to PAS

The mechanisms of resistance to PAS are still unclear. It is thoughout to be associated with mutations in the *thyA* gene (Rengarajan *et al*., 2004) and in Thr202Ala region (Kolyva and Karakousis, 2012). Mutations at the *thy A* gene, codes for the enzyme thymidylate synthase A (Rengarajan *et al.*, 2004). This enzyme is associated with the biosynthesis of thymine in the folate pathway, and it is also the enzyme which is responsible for activating PAS. PAS is a pro-drug, similar to INH, PZA and ETH, and it thus requires a fully functional activating enzyme, to ensure that it is converted to its active state (Rengarajan *et al*., 2004). A mutation at the *thyA* gene, will therefore interfere with thymidylate synthaseA ability to activate PAS, this will in turn prevent PAS from its mode of action against the MTB isolates.

Mutations at these sites however are not present in all PAS resistant MTB strains (Mathys *et al.*, 2009) and are even present in some PAS susceptible MTB strains (Leung *et al*., 2010) thus implying that there are additional mechanisms of resistance to PAS, that need to be investigated. Mathys *et al* (2009) attempted searching for mutations in 8 other genes, to determine if this could be associated with resistance to PAS. These 8 genes included 5 other enzymes involved in the folate pathway and thymine biosynthesis as well as in 3 N-acetyl transferase genes. However no mutations were found in any of these sites. Additional work is required to gain more insight into other mechanisms of resistance.

Resistance to LIN

The synthetic nature of LIN proves to be advantageous, since no prior mechanisms of resistance are evident among gram positive bacteria (Moellering, 2003). However, resistance has recently been reported. To identify these mechanisms of resistance, various genes have been analysed and sequenced. These include the gene that codes for the 23S rRNA methyltransferase (Richter *et al*., 2007), which is the *erm37* gene. Other genes that have been investigated include the entire 23S rRNA gene and the *rplV* and the *rplD* genes. The two latter genes code for the L4 and L22 ribosomal proteins respectively. Sequences however revealed that there were no differences between susceptible and resistant strains or between these genes in the wild type H37Rv strain and the resistant isolates (Richter *et al.*, 2007). Isolates displaying high level resistance to LIN , where MICs were between 16-32mg/L revealed mutations at the G2061T and G2576T region of the 23S rRNA (Hilleman *et al.,* 2008). These mutations however, were not present in the isolates that showed low level resistance to LIN (with MICs of 4-8mg/L). Efflux pumps as a possible mechanism of resistance was also investigated (Richter *et al.*, 2007). Other mechanisms of resistance that require further research, is the role of the ribosomes of the LIN resistant MTB strains, as well as the rate at which the drug is being imported into the MTB (Richter *et al*., 2007).

Resistance to M&C:

All ß-lactams, meropenem inhibits penicillin binding proteins (PBPs) and therefore prevents the production of peptidoglycan. MTB prevents that by producing a ß-lactamase coded for by the *blaC* gene. Mutations in the PBP coding region of the genome lead to alterations in the drug target (Lister *et al*., 2009). Mutations in the *blaC* gene result in inactivation of the β-lactamase enzyme (Lister *et al.*, 2009). Such mechanisms of resistance also exist in other bacterial species (Lister *et al.*, 2009).

2.7 Drug Susceptibility Tests (DSTs)

2.7.1 Importance of DSTs

A vital factor in TB control is the development of a DST that is rapid, reliable and affordable. This would ensure that the appropriate treatment can be administered to patients, and thus

prevent the spread of TB, as well as the spread of resistance strains. DSTs can be used as tools to evaluate treatment regimens that are currently being used, and develop means to cope with the emergence of drug resistance. It is able to offer insight to various concentrations and combinations of anti-TB drugs that may be successful yet are not being administered to patients. These tests however are difficult to develop due to the slow growth of MTB, the dangers of working with such an infectious organism, the presence of many non-tuberculosis strains that are present in the patients' samples, which often give false positives and the poor laboratory infrastructure that is evident in countries with high rates of TB.

DSTs must be evaluated for routine use, against not only test isolates but also clinical isolates to determine if there is a difference in sensitivity and specificity of results. This should be done, to ensure that the *in vitro* results obtained can also be successfully compared to patients' clinical outcome. The relationship between DST results and how they can be used to guide treatment options can be optimised by ensuring that laboratory staff interpret results correctly, and that these reliable results are then sent to clinicians on time (Lobue *et al*., 2009). Although laboratory staff may be trained sufficiently to read results, great care should also be taken to ensure that they are trained appropriately to conduct the DSTs correctly. This is due to the sensitive nature of DSTs. They are easily affected by numerous external and internal factors. Great measures must be taken to ensure that conditions are appropriate for the different tests and kept constant during the duration of the DST. These factors include, the type of growth medium that is used, the pH of this medium, the temperature at which incubation occurs, the type of solvent that the anti-TB drug is dissolved in, as well as errors that must be taken into account for such as loss of drug that may occur during filter sterilization, the inoculum of the TB culture must be standardised for all DSTs and care must be taken to ensure that dilutions of the culture or the anti-TB drugs are carried out correctly and carefully (Kim, 2005). By training laboratory staff appropriately, these factors can be controlled and thus results read from DSTs have a greater chance of being reliable and reproducible.

A major concern is that DSTs are not conducted routinely. Instead they are only conducted when a case seems suspicious or if there is treatment failure. This was evident in countries where resources were limited and which also lacked laboratory infrastructure, staff and equipment. The WHO report in 2010, highlighted that only one third of the countries

understudy conducted routine DSTs on suspected cases of TB (WHO, 2010). Additionally, a study in 2008 revealed that a common problem that was present in many countries, was the lack of DSTs being conducted on new cases of TB (Young *et al.*, 2008). In the countries that were investigated in this study, less than 5 % of new TB cases had access to DSTs (Young *et al.*, 2008). The lack of DSTs being conducted has more serious implications. This is largely due to the fact that standard treatment regimens are based on data that is obtained from population-level-drug resistance, because of the ever changing epidemiology of DR-TB. Gaps in data collection imply that the standard treatment regimens may be inaccurate (Shah *et al.*, 2011).

In SA, DSTs on various SLD are not being conducted routinely e.g. oflaoxacin and kanamycin. This means that patients are still being administered these two drugs, despite a lack of resistance data for them (Shah *et al*., 2011).The study by Shah *et al*, (2011), showed that 68% of the patients at the Tugela Ferry hospital in KZN had XDR-TB, which showed patterns of being resistant to 8 drugs i.e. INH, RIF, EMB, SM, Ofloxacin, KAN, CAP, and ETH. These patients however, had been receiving treatment of which they were resistant to. This in turn increases and spreads resistance as well as increases the risk for treatment failure (Shah *et al.*, 2011). DSTs for SLD are therefore critical, in determining a patient's susceptibility profile, and ensuring that the correct treatment is administered, this will aid in preventing patients from being subjected to anti -TB drugs that they are already resistant to.

When MDR-TB patients are treated properly it can result in at least half of the TB patients converting to a negative sputum culture, within 3 months and with improved outcomes (Telzak *et al.*, 1995). DSTs for SLDs are not standardized throughout the world and do not have good reproducibility, even among national reference laboratories (Kim *et al.*, 2004). Despite numerous published recommendations (WHO, 2001), and validation of DSTs on SLDs in various studies (Krüüner *et al.*, 2006 ; Rüsch-Gerdes *et al.*, 2006; Sanders *et al.*, 2004) there are still many variations of the critical concentrations of these SLDs, during DSTs in the different laboratories (Kim *et al.*, 2004; Pfyffer *et al.*, 1999). Another problem faced when performing DSTs on SLDs is the lack of quality control (Fattorini *et al.*, 2008; Laszlo *et al.*, 2002). Further research is required to determine *in vitro* efficacy of results obtained during DSTs on SLDs, as there is limited information published on the link between, *in vitro* efficacy

results of drugs and the actual effect of the anti-TB agents on patients i.e. the clinical outcome (Banerjee *et al*., 2008 ;Krüüner *et al.*, 2006; WHO , 2001).

Discrepancies were evident when comparing DST results using two different methods i.e. a solid medium based DST and a liquid medium based DST for cycloserine, ETH, CAP, and to a lesser extent, clofazimine and rifabutin (Pfyffer *et al.*, 1999). These differences could be due to a variety of possibilities i.e. due to the two different DST methods, due to the isolate/ strain that is tested containing a mixed population or due to the isolate/strain being border line resistant to the drug tested.

It is presumed that DSTs on TLDs will experience the same problems as SLD. In order for a DST to be successful, it must be optimised for a control TB isolate (H37Rv). The procedure must be properly standardised and be simple so as to avoid errors, and obtain reproducible results. A DST for MTB can be determined by either observing growth of the TB isolate in the presence of antimicrobials, or by observing the metabolic inhibition of it. It can be detected by macroscopic observation of the growth in drug free controls (DFC) and drug containing wells. It can also be detected by measuring the metabolic activity or products, by lysis with mycobacteriophages, and lastly it can be done using molecular based techniques to identifying genetic mutations which confer drug resistance (Kim, 2005).

2.8 Types of DSTs

2.8.1 Phenotypic methods

DSTs on solid media are accepted as the gold standard for phenotypic DSTs of FLDs (Canetti *et al*., 1963; Canetti *et al.*, 1969; NCCLS, 2003; Piersimoni *et al*., 2008). This is largely due to it being easily standardised on solid media, as well as it is more reproducible, as it is a less sensitive method than a liquid based DST. The use of LJs and Middlebrook media has been standardized for FLDs, yet are still unreliable and not reproducible for SLDs and TLDs (Shah *et al*., 2007; Van Ingen *et al.*, 2010).

Direct DST

This DST has been well established on solid media such as Middlebrook 7H10 agar or LJs (CLSI, 2007; Kent *et al*., 1985; Kim, 2005). It involves incorporating a known concentration of the drug, mainly the breakpoint concentration, into the media and thereafter inoculating the decontaminated clinical sample (Siddiqi *et al.*, 2012). An advantage of this method over the indirect DST method is that it provides results faster and it offers a representation the patient's original bacterial population (Ramachandran and Parmasivan, 2003). The microscopic observation drug susceptibility assay (MODS) and the nitrate reductase assay (NRA) are examples of non-commercial direct DSTs.

Indirect DST

This method differs to the direct DST method, since it involves inoculating with a culture that is isolated from a clinical specimen (Siddiqi *et al.*, 2012). This means that the test can only be performed 3-6 weeks after the specimen was received, so that a culture could be isolated from the specimen (Siddiqi *et al.*, 2012). This culture is then standardized and inoculated onto the growth media (egg based LJs, or Middlebrook agar) which contains the anti-TB drugs (Ramachandran and Parmasivan, 2003). A control is also included and it contains the drug free media which is then inoculated with the same standardised MTB culture.

1 % Proportion method

This method is often used as a reference or gold standard method for DST (NCCLS, 2003). It can be performed on agar based or egg based media and is inexpensive, simple, and does not require any equipment, except an incubator. The proportion method uses the principle of observing "growth or no growth" of the MTB culture when exposed to the concentration of the drug being tested (Richter *et al.*, 2009). It makes use of just one concentration of the drug that is being tested. This concentration is the critical concentration, which is the lowest concentration that shows resistance, if growth of the bacilli is present (Heifets, 1991). The wild type strain does not grow when exposed to the critical concentration (Heiftes, 1991). Various dilutions of the MTB culture is prepared and inoculated onto the media containing

the drug at its critical concentration. One of the dilutions should reveal colonies that can be counted (between 20-200 colonies). A control must also be included, which will follow the same procedure described above, but on drug free media. This is the DFC. The resistant proportion of MTB can then be interpreted as a percentage of the total proportion (obtained from the DFC).Resistance is defined if over 1 % of the bacterial population is able to grow when exposed to the critical concentration (Richter *et al*., 2009).The proportion method is time consuming and requires an incubation period of 3-6 weeks before results can be obtained (Ramachandran and Parmasivan, 2003).

Absolute concentration method

This method is also used frequently for DST, and is usually performed on LJs (Richter *et al*., 2009). Reading of results and preparation of the culture inoculum are easy. It is based on inoculating a standardized inoculum of the test culture on drug free media and media that has various concentrations of the drug under test (Ramachandran and Paramasivan, 2003). Several graded concentrations of each test drug are used, and resistance is recorded as the lowest concentration of the anti-TB agent that is able to prevent the bacilli from producing colonies, which means inhibiting growth of the organism i.e. the minimum inhibitory concentration (MIC) (Ramachandran and Paramasivan, 2003). This method of testing depends on the viability of the organism (Ramachandran and Paramasivan, 2003), and is affected when using an inoculum of MTB that has many smaller sub-populations within it. This may result in incorrect results recorded. In order to prevent this, single colonies of the test organism should be prepared before running this DST, this would ensure presence of only that population of the MTB during the test.

Agar Dilution Minimum Inhibitory Concentrations

Agar dilution MICs are done in a similar manner. A serial dilution of the drug is made and different drug concentrations are incorporated into the Middlebrook agar plates. Then a standardised inoculum of the culture is added to this plate, and thereafter incubated for 3 weeks. MIC determination offers more information than the DST, as it will not only reveal resistance, or susceptibility, but also the exact and smallest concentration at which no growth of the isolate is observed.

Resistance ratio method

This DST method compares the growth of an unknown strain of MTB, to the growth of a known control strain such as H37Rv (Ramachandran and Paramasivan, 2003). Both of these strains are inoculated on media containing the same concentration of the drug that is being tested. After 3-6 weeks of incubation, results are read. Resistance is recorded as the ratio of the MIC of the unknown test strain to the MIC of the reference strain, specific for each set of drug dilutions (Ramachandran and Paramasivan, 2003). This method, like the absolute concentration method, is also affected by the viability and the inoculum size of the strain. Prior testing of the standard laboratory strain is important, to ensure that this strain exhibits its usual and known susceptibility pattern, and does not show any unexpected variation, as this will affect interpretation of results during the actual DST.

E-test

This test is a fairly new phenotypic DST. It makes use of plastic strips containing various concentration of the drug that is being tested. When this strip is placed on the surface of the culture media, one is able to determine the MIC, by noting the area of the strip that is able to inhibit the growth of the microorganism. The MIC is interpreted as the point where the ellipse intersects the e-test strip (Varma *et al.*, 2002). This test can be performed on 7H10 Middlebrook agar, and can usually yield results within 5-15 days (Richter *et al.*, 2009). This method however is not very reliable and often has a high rate of displaying false resistance, when compared to BACTEC or the conventional proportion method (Ramachandran and Paramasivan, 2003).

Mycobacteria Growth Indicator Tube (MGIT)

MGIT is an example of a liquid based DST. There are two different types of MGIT tests, the manual BBL MGIT and the automated non-radiometric BACTEC MGIT 960 system. Both of these systems monitor the growth indicator tubes every hour, and are based on the same principle of detecting growth of the MTB in the tubes containing drugs compared to the drug free tubes (Richter *et al.*, 2009). The system uses fluorometric detection based on oxygen

consumption in the tubes which occurs when there is bacterial growth in the tubes (Richter *et al*., 2009). Acid fast bacilli (AFB) such as MTBn metabolize and use up oxygen, this in turn leads to the intensification of the oxygen quenched fluorescent dye which is present within the tubes (Springer *et al.*, 2009). This indicator fluoresces under the 365 nanometer UV lamp (Adikaram *et al.*, 2012; Piersimoni *et al.*, 2006). The automated system will display a notification once a positive result is detected however the manual system requires that results are read manually. Results can be obtained between 7-10 days after incubation in the system. Both systems detect positives at the same rate, however the manual system can only be used for INH, RIF, EMB and SM, whereas the automated system can be used for these drugs as well as PZA. The latter also has software (Richter *et al*., 2009) for performing DST on SLD and new anti-TB drugs (Krüüner *et al.*, 2006; Martin *et al.*, 2008, Rodrigues *et al*., 2008; Rüsch-Gerdes *et al*., 2006). It has been used to determine the critical concentration of ofloxacin, moxifoxacin, prothionamide, LIN, CAP, AMIK, KAN and ETH. The sensitivity and specificity of the manual and automated system are very similar, and thus the manual system serves as a cheaper alternative if FLD are being tested. However the specificity of this DST is questioned. A number of factors may affect results obtained i.e. purity and homogeneity of the inoculum, the inoculum size and the environmental contamination that may occur during the inoculation process (Piersimoni *et al*., 2006).

Microscopic Observation Drug Susceptibility (MODS) Assay

This liquid based DST is a rapid, reliable and affordable tissue culture based assay that is well established for detecting resistance to INH and RIF (Shah *et al*., 2011). It can be performed using either sputum samples which are decontaminated or MTB culture (Richter *et al*., 2009). These are inoculated into a 24 well flat bottomed tissue culture plate containing 7H9 Middlebrook broth and various concentrations of the anti-TB drug being tested. Thereafter plates are sealed and placed in plastic heat sealable bags, and incubated at 37°C. The plates have to be viewed daily from day 4 till day 21 for the detection of MTB, which displays a cording morphology in liquid media (Moore *et al*., 2004). This is done using an inverted light microscope. The plates are placed in plastic bags to reduce the risk of spills, as well as to contain the spill if it does occur within the bag. Since plates are moved in and out of the incubator daily from day 4 to day 21 (or until a positive result is detected) the use of the

plastic bags prevents the spread of aerosols in the air, which is hazardous to laboratory staff (Singh *et al*., 2012). Results are obtained much faster with the MODS assay, compared to the conventional gold standard of DST on solid media, which takes 3 weeks to detect growth (Ejigu *et al.*, 2008; Richter *et al.*, 2009). This assay is also very sensitive in detecting both smear negative (Arias *et al.*, 2007) and positive respiratory samples (Banerjee *et al*., 2008; Moore *et al.*, 2006; Shiferaw *et al*., 2007). Another advantage of this assay is that it does not require a culture source to detect MTB. Instead it is capable of identifying MTB directly from sputum samples, as well as pleural fluids or the pleural biopsy (Tovar *et al.*, 2008). MODS also detected MDR-TB successfully (Moore *et al.*, 2006; Shiferaw *et al*, 2007). A downfall of the MODS assay however, is that it is less sensitive for detecting MTB in cerebro-spinal fluid (Caws *et al.*, 2007), and it is unable to distinguish between MTB and non-tuberculosis mycobacteria. Thus it has low specificity for MTB. The latter is a major concern, as it will yield false positive results, and thus the MODS assay has to still be used in conjunction with a confirmation test such as microscopy. It also requires appropriate training for viewing of the plate.

Colorimetric DST assays

There are various types of colorimetric methods, some of the earlier types include the use of MTT salt as far back as 1983(Abate *et al*., 1998; Mosmann, 1983), and more recently, in 1995 Yajko *et al,* (1995) used Alamar blue as an oxidation reduction indicator to detect MTB. These along with other colorimetric assays such as Malachite green and Resazurin microtitre assay (REMA) are easy to perform and interpret and results are reliable (Richter *et al*., 2009). They make use of consumables that are generally already present in the laboratory, thus making them inexpensive. They are based on colour changes which occur as a result of the metabolic activity of the MTB, and they do not require the use of specialised equipment, as these colour changes can be detected visually.

Resazurin Microtitre Assay (REMA)

REMA is a liquid based colorimetric DST that is used for the detection of MDR-TB strains (Palomino *et al.,* 2002). It can be performed using decontaminated sputum samples or MTB culture. This assay is done in a sterile flat bottomed, 96 well plate. Broth is added to wells, and thereafter, a dilution of the anti-TB drug that is being tested is done within the wells to

achieve various concentrations of the drug. The MTB in a standardised liquid form is then added to the wells. A DFC and a control MTB strain with known susceptibility are included to ensure the test is performed correctly. The plates are incubated for 7 days at 37°C, and thereafter the resazurin is added to each well. A colour change from blue to pink indicates the growth of bacteria and also indicates the conversion of the oxidised state of resazurin to its reduced state (Palomino *et al*., 2002). The MIC is recorded as the lowest concentration of the drug that inhibits this colour change (Martin *et al*., 2003).This test shows good correlation with the results obtained from the proportion method. It has also been used successfully for DST using SLD (Martin *et al*., 2003). In an effort to reduce the production of aerosols this test can also be performed within screw cap tubes, however this then increases the cost of the test and makes it more labour intensive compared to working within a 96 well plate (Abate *et al*., 1998; Foongladda *et al*., 2002; Martin *et al*., 2003).

Nitrate reductase assay

This assay was first described in 1989 by Emil Kalfin, and is based on the principle of MTB reducing nitrate to nitrite (Kalfin *et al*., 1989; Panaiotov and Kandtardjiev, 2002). It is used routinely for biochemical identification of mycobacterial species. The nitrate reductase agent has been recently changed, from the Griess reagent to a crystalline reagent, which is less toxic and has a longer shelf life (Lampe 1981; Panaiotov and Kandtardjiev, 2002; Warren *et al*., 1983). This test is conducted in LJ tubes, which contains the drug at various concentrations, the MTB culture and the 0, 1 % potassium nitrate (KNO_3). These tubes are incubated at 37° C for 7 days. From day 8 to day 10, the KNO_3 is reduced when the crystalline reagent is added to the DFC tube, if a colour change occurs, then the crystalline reagent is also added to the tubes containing drugs, and the result are read. An isolate is considered to be resistant if a colour change from pink to red-purple occurs , and if this colour intensity is more than that in the 1: 10 diluted growth control (Ängeby *et al.*, 2002).

Malachite green

This colorimetric assay is based on the ability of malachite green to show viability of MTB (Farnia *et al.*, 2008). It has been used as a means of DST for SLD and TLD against MDR and XDR-TB strains (Farnia *et al*., 2008). Malachite green is a triphenylmethane dye. It is cheaper than the other dyes used in the colorimetric methods. This assay can be performed using

decontaminated sputum samples directly or MTB culture. In an effort to make this assay safer, and reduce the spread of aerosols, it can be conducted in microtubes with caps (Farnia *et al.*, 2008) instead of 96 well plates. The microtubes which contain broth, and various concentrations of the drug being tested, are inoculated with the MTB culture and incubated for 7 days at 37°C. The DFC tubes are tested first by the addition of the malachite green, at day 7, 14, or 21 or until a colour change takes place. The dye is originally a dark green colour in its oxidised form, which then converts to a colourless solution when in a reduced state. This only occurs when there is metabolism of MTB. The various time points were included as different strains of MTB metabolise at different rates. Once a colour change occurs in the DFC and indicates that sufficient metabolic activity has occurred, the malachite green can then be added to the tubes containing drugs and results can be read.

The [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

This colorimetric method is similar to the ones described above, in that it is inexpensive, reliable, easy to perform and does not require sophisticated equipment to read results. It has been widely tested against various FLD (Abate *et al*., 1998; Mshana *et al*., 1998). This assay can be performed directly on decontaminated sputum samples or MTB cultures. It is based on the principle that live bacteria are able to use dehydrogenase to reduce the indicator (MTT salt) which is yellow in its oxidised state, to formazan, which is purple (Mosmann, 1983).The amount of formazan produced, is directly proportional to the number of live cells present (Mosmann, 1983). Results can be obtained after 10 days of incubation, when the MTT salt ([(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is added to the wells .A disadvantage of this assay, is that it is not specific for MTB and thus a colour change will still occur even if a contaminant is present in the well. This is due to the fact that any bacteria will be able to reduce the MTT salt.

2.8.2 Genotypic methods

Genotypic methods of DST, makes use of molecular techniques to detect mutations in specific target genes that are associated with conferring resistance to specific anti -TB drugs (Banerjee *et al.*, 2008). Currently there are numerous genotypic methods that can be used i.e. polymerase chain reaction (PCR) single strand conformation polymorphism (PCR SSCP),

hybridisation assays, PCR restriction fragment length polymorphism analysis (PRA), multiplex allele specific (MAS) PCR heteroduplex formation (Richter *et al*., 2009).
These genotypic methods of DST are faster and are safer in that they do not require constant work with MTB culture, but rather with the MTB DNA. They are also able to determine resistance even if the sample has lost its viability. Most of them also involve reactions in an automated closed system thus reducing the introduction of contaminants (Richter *et al*., 2009). The disadvantages however, are that these methods are expensive, require specialised equipment as well as extensive training for laboratory staff (Banerjee *et al*., 2008). A further limitation is that only a portion of the genes associated with resistance for different anti -TB drugs and their sites of mutations have been revealed. These do not always confer resistance, as they are often absent in some resistant strains, or even present in susceptible ones. This implies the possibility of additional target genes being involved, but they currently remain undiscovered. False results may also occur due to contaminating amplicons or chromosomal DNA, especially when the same samples are used routinely or over long periods of time.

Currently there are also two commercial DNA line probe assays that can be used for the detection of DR-TB. These are the strip assay INNO LiPA Rif.TB (Innogenetics, Belgium) and the MTB drug resistance genotyping test (GenoType MTBDR) assay (Hain Life Science, Germany) (Brossier *et al*., 2006). Either MTB cultures or direct clinical specimens can be used for both these methods. The ability for these assays to be performed using direct clinical specimens means that, the turn around time for results will also be faster than conventional methods which rely on cultures.The INNO LiPA Rif.TB has higher sensitivity and specificity when MTB cultures are used. This method targets the *rpoB* gene, and is thus able to detect resistance to RIF. The GenoType MTBDR method, which was introduced in 2004, is able to detect mutations in the *rpoB* and in addition the *katG* gene. It is thus able to detect resistance to RIF as well as high level INH resistance (Ling *et al*., 2008).

There are two other variations of the Geno Type MTBDR method. This includes the MTBDRplus which is the second generation assay and the MTBDRsl assay. The former assay is able to detect mutations in the *inhA* gene, which confers to low-levels of INH resistance (Ling *et al*., 2008). Both of these Geno Type methods reveal excellent sensitivity and specificity for RIF however it only displays high specificity for INH. The sensitivity of this

test to INH is low and varies (Ling *et al.*, 2008). The GenoType MTBDR assay makes use of the following steps, DNA extraction, multiplex PCR, solid phase reverse hybridisation and detection of the resistant gene mutations (Hillemann *et al.*, 2006; and Hillemann *et al.*, 2007).This method is used in SA for routine diagnosis on selected clinical specimens and positive TB cultures (Ling et al.,2008). It has yielded ated results between periods of 6 hours to two days (Barnard *et al*., 2008). This is remarkably faster than the conventional method of DST's, therefore making it a rapid screening tool, which aids in early iniatiation of treatment options.

The MTBDRsl assay is a fairly new method which is capable of simultaneously detecting resistance to fluoroquinolones, ethambutol, aminoglycosides. It does this by recognising mutations in the *gyrA*, *embB* and *rrs* genes respectively. This assay is based on the same principle as the MTBDR assay, except it is now able to also detect resistance to SLD, and thus identify XDR-TB isolates. Studies have shown that this assay is specific for the above mentioned anti-TB agents, and that it is accurate in that manner. The sensitivity of this assay varies for the various anti-TB agents (Kiet *et al.*, 2010).This method should not be exclusively used as a DST method.

In 2010, WHO recommended the use of GeneXpert MTB/RIF (Xpert) as a first diagnostic test for patients suspected to have MDR-TB (WHO, 2010 Replaced with WHO, 2011c). This system is able to diagnose TB as well as detect resistance to RIF (Boehme *et al.*, 2010; Boehme *et al.*, 2011).It is the latest breakthrough in molecular genotypic methods and can be performed directly on sputum. Results are obtained within 2 hours (Piatek *et al*., 2013; WHO, 2011c). This means that diagnosis of TB, as well as detection of resistance to RIF, can be made quickly and thus results in an overall decrease in time for treatment initiation. This method displays higher sensitivity than sputum microscopy in detecting MTB and is also able to detect smear negative MTB. It is similar in accuracy at detecting MTB to culture methods (Piatek *et al*., 2013; WHO, 2011c) Overall there have been many studies which have shown the high specificity and sensitivity of the GeneXpert for MTB (Scott *et al.*, 2011). A disadvantage of this technique, as with all molecular based techniques, is that it is based on detecting DNA, and thus does not distinguish between live or dead mycobacteria (Meyer-Rath *et al.*, 2012). In addition, DST using this method is only available for testing against RIF. It is

also expensive, and the cost of the actual machine is approximately USD17 000 (Piatek *et al*., 2013). It then requires a cartridge for each test to be performed which costs USD 9. 98. The machine requires an uninterrupted supply of electricity and proper waste disposal is needed for the cartridges. As with most molecular techniques, technical support is required, and the laboratory staff needs to be appropriately trained.

2.9 Time Kill Experiments (TKEs)

TKEs are not methods of DSTs. Instead TKEs offer valuable information on the various anti - TB drugs and their effect on MTB over a period of time (Budha *et al*., 2009). They can be used to understand how drugs behave in respect to its pharmocodynamics (PD) which is the drugs effect on the body (Budha *et al*., 2008). If TKEs were performed in vivo, using an animal model, one could also observe the pharmacokinetics (PK), which is how the body reacts to the drug (Budha *et al*., 2008). Past studies have conducted *in vitro* and *in vivo* TKEs using FLDs and some SLDs. TKEs are time consuming and laborious to perform, and require large quantities of consumables for the various time points. It is for these reasons that they are not performed routinely.

TKEs can be performed in two manners, using various concentrations of each drug, and observing the PD at different time points, or by using a constant concentration of the drug, and still observing the PD properties at different time points. This study made use of the latter method, of a constant concentration. Although this may seem similar to the normal agar dilution method and MTT method, which also makes use of a static/constant concentration of the anti-TB drug, it differs in that it is not read at the end of the experiment, like the agar dilution and MTT methods. Instead of this "once off " end point determined MIC, TKEs requires colony counts of the colony forming units (CFUs) to be counted at each time point. Since different dilutions were performed, the dilution which yields 20-200 colonies is chosen for each time point, for each of the drugs and for each of the isolates tested. These colony counts enable one to observe the bactericidal activity of the various drugs over time, as well as to determine the degree at which the drug is able to kill (concentration dependant) (Budha *et al*., 2008; Budha *et al*., 2009; Jayaram *et al*., 2003; Jayaram *et al*., 2004). Additionally TKEs also gives insight to the rate at which this killing is done (time dependant).

CHAPTER 3

MATERIALS AND METHODS

This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal as per ethics approval number 274/09.

3.1 Antimicrobial Drugs

The antimicrobial drugs that were selected for testing in this study included capreomycin (CAP), para-aminosalicylic acid (PAS), linezolid (LIN), meropenem (MER), meropenem in combination with clavulanic acid (M&C).Whilst very little is known about the efficacy of these drugs against MDR and XDR-TB (WHO, 2008b), they are currently incorporated into the treatment regimens for the treatment of MDR and XDR-TB in SA. Thus DST and TKE information obtained from this study will assist in guiding the appropriate placement of these agents in the management of TB. Isoniazid (INH) was included as a control. All anti-TB agents used were purchased from Sigma Aldrich, USA, except MER and LIN which were donated for research use by AstraZeneca, South Africa and Pfizer, South Africa

3.2 *Mycobacterium tuberculosis* isolates

Based on susceptibility profiles, 12 MTB isolates were selected from the collection available in the TB laboratory of the Deapratment of Infection Prevention and Control at the University of KwaZulu-Natal. The isolates included:

- 2 fully susceptible
- 1 INH mono resistant
- 3 MDR
- 1 pre-XDR
- 5 XDR

The isolates had previously been identified to species level by standard methodology and the susceptibility profile was determined by the 1% proportion method. H37Rv was used as a susceptible MTB control for all DSTs and for the time kill experiment (TKE).

Table 4 provides the susceptibility patterns to the various anti-TB drugs that are used for classification in the different categories.

Table 4 : Susceptibility Profile of MTB Isolates

Isolate Name	**Susceptibility Profile**					
	INH	RIF	EMB	STRE	KANA	OFLO
Susceptible Isolates						
H37Rv	S	S	S	S	S	S
V4207	S	S	S	S	S	S
Mono resistant Isolates						
V666	R	S	S	S	S	S
MDR Isolates						
V1435	R	R	S	R	S	S
V2475	R	R	S	S	S	S
V4258	R	R	S	R	S	S
Pre XDR Isolates						
R 413	R	R	S	S	R	S
XDR Isolates						
R 26	R	R	S	S	R	R
R 252	R	R	S	R	R	R
R 502	R	R	S	S	R	R
R 506	R	R	S	R	R	R
KZN605	R	R	R	R	R	R

KEY:					
INH	-	Isoniazid	STRE	-	Streptomycin
RIF	-	Rifampicin	KANA	-	Kanamycin
EMB	-	Ethambutol	OFLO	-	Ofloxacin

3.3 Retrieval of Isolates from Storage

The selected isolates were retrieved from storage in the -70 degrees Celsius (^{o}C) freezer, and thawed to room temperature. These isolates were then revived, by inoculating 500 microlitres (µl) of the thawed stock into 5 millilitres (ml) of sterile Middlebrook 7H9 broth (Becton Dickinson and Company, Difco Laboratories, USA) (Appendix 1.1), using a sterile glass pasteur pipette. The broth contained the following antibiotics: polymyxin B (200.000 IU/L) (Sigma-Aldrich, USA), amphotericin B (20mg/L) (Sigma-Aldrich, USA), carbenicillin (100mg/L) (Sigma-Aldrich, USA) and trimethoprim (20mg/L) (Sigma-Aldrich, USA). These antibiotics are collectively referred to as PACT, and are incorporated into the broth to make it selective and reduce risk of contamination by non-mycobacterial organisms. The broth was also supplemented with oleic-albumin-dextrose-catalase (OADC) (100ml/L) (Becton Dickinson and company, USA). After inoculation, the tubes were incubated at 37 ° C for 3-6 weeks.

3.4 Culture onto Middlebrook 7H11 agar plates

Once growth of the culture was observed in the Middlebrook 7H9 broth (Becton Dickinson and Company, Difco Laboratories, USA), a plastic sterile quadloop was used to transfer the broth and to perform a four way streak onto Middlebrook 7H11 agar (BD, Difco Laboratories, USA) (Appendix 1.2) plates. This was done to ensure that a single colony is obtained. This therefore ensures that a pure culture is used for the drug susceptibility tests, rather than a mixed culture. These plates were placed in a gas permeable plastic bag and heat sealed. It was thereafter incubated at 37°C for 3 weeks. Growth was observed after the 3 weeks, and thereafter a single colony was chosen, and inoculated into 5ml of sterile Middlebrook 7H9 broth (Becton Dickinson and Company, Difco Laboratories, USA) (Appendix1.1) which contained PACT and OADC (Section 3.3). This was performed for all 11 isolates and H37Rv. The tubes of broth were then incubated at 37°C for 3 weeks. Once growth was observed in the tubes, after the incubation of 3 weeks, 200µl of this broth culture was aspirated using a sterile glass pasteur pipette, and inoculated onto Middlebrook 7H11 agar (BD, Difco Laboratories, USA) (Appendix1.2)plates. A sterile plastic quadloop was used to spread the broth evenly across the agar plate. For each of the 11 isolates and H37Rv, 5 Middlebrook 7H11 agar (BD, Difco Laboratories, USA) plates were prepared. These plates were then placed in a gas

permeable plastic bag and heat sealed and incubated at 37°C for 3 weeks. After 3 weeks, the culture present on each plate were transferred into separate cryovials containing a storage media for TB culture (Appendix 1.3).These cryovials were stored at -70°C and were used as the stock culture for all isolates and H37Rv for all DSTs, TKE and genotypic methods that followed.

3.5. Agar Dilution Method

The agar dilution experiment was performed following the procedure outlined by Isenburg, (2004), with slight modifications. Results were read by observing if the agar plates had growth of MTB or no growth, and colony counts on the drug free control quadrants were performed.

3.5.1 Preparation of Drug Solutions

All anti-TB drugs were in their chemically pure powder form, and were stored at temperatures recommended by the manufacturer, until use. Various stock concentrations of each drug were prepared. These were prepared at double the required concentration (Appendix1.4.1-1.4.6), and to only reach the required concentration in 100ml of Middlebrook 7H10 agar (BD, Difco Laboratories, USA) (Appendix 1.6). Stock solutions of the anti-TB drugs were measured accordingly and dissolved in 10 ml of their appropriate solvent or deionised water. All anti-TB drugs used were dissolved in deionised water except clavulanic acid, which was dissolved in 0.1M phosphate buffer at a pH of 6. (Appendix 1.4.1-1.4.6) The solutions were sterilized by membrane filtration using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA). From the stock solutions, 7 double dilutions were performed i.e. 10 ml of the first stock solution of the anti-TB drug (double the required concentration) was added to 10 ml of sterile appropriate solvent, this was mixed well and repeated 6 times for the next 6 dilutions to obtain the remaining concentrations .This was done for each anti-TB drug to achieve the 7 required concentration of each drug (Table 5). It is for this reason that a concentration higher than what is required, is made up at the starting point. Each dilution was then aliquoted into 1.1ml volumes in sterile cryovials tubes, and stored at -80 °C until use. For each drug, stock solutions and serial dilutions were prepared in triplicate. Once a frozen drug solution was

thawed, it was used immediately and any remainder was discarded and was not re-frozen or re-used. Working solutions of the anti-TB drugs for the MTT assay and the time kill experiment (TKE) were prepared freshly from the stock solution, and the necessary dilutions were performed to obtain the concentration for these 2 assays. This was also made possible by originally preparing a higher stock concentration i.e. each concentration of drug per 100 ml of media.

Table 5: Concentrations of Anti -TB Drugs used for Agar and MTT Assay

Anti-TB Drug	Concentration (µg/ml)
Isoniazid	0.062;0.125 ;0.25 ;0.5 ;1;2 ;4
Capreomycin	1 ;2 ;4 ;8; 16; 32; 64
Para-aminosalicylic acid	0.25 ;0.5 ;1; 2; 4; 8; 16
Linezolid	0.125; 0.25; 0.5; 1; 2; 4; 8
Meropenem	0.125; 0.25; 0.5; 1; 2; 4; 8
Meropenem:Clavulanic Acid	(0.25:8);(0.5:8);(1:8);(2:8);(4:8);(8:8);(16:8)

3.5.2 Preparation of Agar Dilutions

Middlebrook 7H10 agar (Becton Dickinson and Company, Difco Laboratories, USA) plates were prepared (Appendix1.6) and used for the agar dilution method. Briefly, appropriate amounts of Middlebrook 7H10 agar powder (19g/L), Bacto Casitone (Becton Dickinson and Company, Difco Laboratories, USA) (1g/L) were incorporated into 90ml of deionised water. This was then dissolved using a magnetic stirrer and placed on a magnetic plate. Thereafter, glycerol (5 ml/L) (Merck, SA) was added and this suspension was autoclaved for 15 minutes at 121^{o}C using the media setting. This was left to cool in a water bath at a temperature of 55 °C. When this media reached the water bath temperature, OADC (Becton Dickinson and company, USA) was added (100ml/L). M&C were not tested with the agar dilution method since the temperature of the agar at the time that the antimicrobial agents were added would

have inactivated the ß-lactamase inhibitor. Since MER was used as a control for M&C, this drug was also not tested using the agar dilution method. These two drugs were investigated using the MTT assay method.

The remaining 4 pre-aliquoted anti-TB drugs were then thawed, and 1 ml of each antibiotic was added to the 100ml of agar, to obtain the desired test concentration. The mixture was swirled gently, and a repeater pipette (Eppendorf), was used to transfer 5ml of the agar into the quadrants of quadrant Petri dishes. This step was performed 7 times for each anti-TB drug. Each time, a different concentration of the anti-TB drug was added to the media, till all 7 concentrations were completed. A drug free control (DFC) was also prepared. The agar plates were allowed to solidify before use. Remaining plates were stored at 4°C, but were used with a week of preparation.

3.5.3 Standardisation of the Inoculum

Colonies from a 3 week old 7H11 Middlebrook agar (Appendix 1.2) plate of culture was used for the standardization step for each of the 11 isolates and H37Rv. Briefly, confluent growth covering an entire 7H11Middlebrook agar plate (diameter 65cm) was harvested and suspended into a tube containing 0,5ml of sterile phosphate buffer saline (PBS) (Sigma-Aldrich, USA) (Appendix 1.7) with 0.05 % Tween 80 (Merck, SA) (Appendix 1.8) and 4 glass beads (approximately 5mm in diameter). The tubes were agitated for 2 minutes using a vortex and remaining clumps were allowed to settle for 15 minutes. Therafter, 3ml of PBS solution containing 0.05 % Tween 80 (Merck, SA) was then added to the tubes, which were agitated for a further 5 minutes. The remaining clumps were allowed to settle for 45 minutes. The top layer of culture was removed using a sterile glass pipette and discarded.The remaining contents within the tube was mixed with sterile triple distilled water to match an optical density of McFarland $BaSo_4$ suspension No 1(Appendix 1.10). Three 10-fold serial dilutions (0.5ml of the suspension into 4.5 ml of sterile distilled water) were made to prepare the required inoculum concentration of 10^{-4} colony forming units per ml (cfu/ml).

3.5.4 Inoculation of plates

The Middlebrook 7H10 agar (Becton Dickinson and Company, Difco Laboratories, USA) plates containing the 7 different concentrations of the anti-TB drugs in separate quadrants, were then seeded with 100µl per quadrant of the standardised MTB cultures (Section 3.5.3). The same volume of a 10-fold dilution of the inoculum (10^{-3}cfu/mL) was used for the drug free controls. This further dilution was used for the DFC, to ensure that a countable number of colonies (20-200) were achieved. All agar plates were swirled gently to evenly distribute the inoculum over the agar. Plates were allowed to stand for 30 minutes, before they were incubated at 37° C for 3 weeks, in heat sealed gas permeable plastic bags.

3.5.5 Interpretation of results:

At day 21 the plates were removed from incubation and results were read. Growth was recorded as either being present or not. The lowest concentration of the 4 anti-TB drugs that inhibited growth (MIC) was noted. Colony counts of the DFC quadrants were performed and recorded.

3.6 MTT Reduction Assay

This assay was chosen in an effort to determine how accurate it is to the gold standard, agar dilution method. Although there is a lack of guidelines and standardized protocols when testing SLDs and TLDs, the information gained from using the MTT assay and comparing it to the agar dilution method can be used to aid in its validation. In regions of high TB and HIV co-infection, the WHO recommends the use of validated liquid DST's when testing SLDs and TLDs (WHO, 2008b). For this assay to be validated, it is required to be easy to perform, rapid, safe, reliable and cost effective. The MTT assay meets all of these requirements, however the sensitivity and specificity of this method when using the anti-TB agents that were chosen for this study, remains unknown. Thus a comparison between the MTT assay and the agar dilution assay needs to be made, to determine if these two methods provide accurate and reproducible results that are sensitive and similar to eachother.

3.6.1 Preparation of the test plate

The MTT assay was performed in a sterile Greiner CELLSTAR 96-well, flat bottomed microtitre plate (Sigma-Alrdich, USA). Since this assay requires incubation for a period of 9 days at 37 °C, 100 µl of sterile distilled water was added to the peripheral wells around the plate to prevent drying out. The assay was performed following the methodology illustrated in Abate *et al*, (1998), with slight modifications.

Briefly 100µl sterile Middlebrook 7H9 broth (Becton Dickinson and Company, Difco Laboratories, USA) (Appendix 1.1) was inoculated into the inner wells from left to right of each row (100µl/well). The initial stock concentration for each of the anti-TB drugs were prepared to reach the desired concentration in 100ml of media for the agar dilution method (Section 3.5.1) (Appendix 1.4.1-1.4.6),therefore these were now too concentrated for the small volumes of broth used in the MTT assay. Appropriate dilutions were performed to reach double the desired highest concentration of each drug in 200µl (total final volume in each well of the 96 well plates). Once this concentration of double the highest concentration was made, 100 µl of it was added to the first well, which already contained 100µl of 7H9 Middlebrook broth. Therefore a double dilution was performed within the first well of the 96 well plate to achieve the highest required concentration, thereafter six two-fold serial dilutions of the antimicrobial agents in 7H9 Middlebrook broth were performed (across the row, from left to right) (final volume 100 µl/well). Once all 7 concentrations were achieved, the last 100µl of the broth and drug solution is discarded. This step (6 dilutions down the row) was not carried out for clavulanic acid, as it required a constant concentration of 8µg/ml when incorporated with meropenem (Table 5), therefore this concentration was added to each of the M&C wells directly. The inoculum for each isolate was standardised as described above (Section 3.5.3) and 100 µl containing 10^{-4} cfu/ml were added to each well (which gives the total final volume of 200µl in each well). Each row included a drug free control and each plate included a well with broth only. All broth used during drug susceptibility testing did not contain PACT or PANTA, as this would affect the results obtained, as they may play a role in inhibiting growth of MTB, or may even interact with the MTT salt. The plates were held together securely using elastic bands and they were then heat sealed in a gas permeable plastic bag and incubated at 37° C for 7 days. On day 8, the plates were removed from the incubator and, if the broth control showed no contamination, then 15 µl of MTT salt (3-[4, 5-

dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide (5mg/ml) (Sigma-Aldrich, USA) (Appendix 2.1) was added to each well. The plates were then sealed once again, as described above and re-incubated overnight at 37^{o}C. On day 9, 50µl of the formazan solubilisation buffer which is a SDS-DMF solution (Appendix 2.4) was added to each well and the plate was sealed and re-incubated overnight. This was a 1:1 volume of the 20 % Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA) solution (Appendix 2.2) and a 50% aqueous N,N Dimethylformamide solution (Sigma-Aldrich, USA) (Appendix 2.3). On day 10, if no colour change was observed in the un-inoculated broth, the test was ready for reading. Each isolate was tested in triplicate.

3.6.2 Interpretation of MTT assay results

A yellow colour indicated growth inhibition, and a colour change to purple indicated actively metabolizing cells. The lowest concentration of the antimicrobial agent that showed growth inhibition (yellow) was recorded as the MIC.

3.7 Time Kill Experiments (TKEs)

Due to the labour intensive nature of time kill experiments (TKEs), only 6 isolates were used. The isolates selected were chosen according to their susceptibility profile, and included: 1 susceptible, 2 MDR-TB and 2 XDR-TB isolates, with H37Rv as a control strain. These isolates were V4207, V1435, V4258, KZN605 and R252 (susceptibility profile available in Table 4). This experiment was conducted following the methodology outlined by a protocol provide by Dr Catherine Vilchèze of Albert Einstein College of Medicine, USA.

TKEs were performed for each drug at a concentration twice that of the MIC as determined by the agar dilution methodology (Section 3.5). Bacterial suspensions with a 1 McFarland (Appendix 1.10), density were prepared for each isolate. This was done following the standardisation method explained in section 3.5.3. Different volumes (0.5 ml; 1ml; 1.5ml and 2ml) of this suspension for each of the 5 isolates and H37Rv were added to sterile Nalgene plastic inkwell square bottom bottles (Thermo Fisher Scientific, USA) containing 15 ml of Middlebrook 7H9 broth (Becton Dickinson and Company, Difco Laboratories, USA) (Appendix 1.1) containing 20 % tyloxapol (Sigma-Aldrich, USA) (Appendix3.1). Tyloxapol

(Sigma-Aldrich, USA) was added to the media, to prevent the MTB cultures from clumping as they grew. The bottles were incubated at 37 ° C, in an Innova 44 Orbital shaker incubator (New Brunswick Scientific,USA) at a speed of 150 rpm and the optical density (OD) of each culture was read daily from day 3 onwards using a spectrophotometer, at a wavelength of 600nm. Once an OD of 0.7 was obtained, the suspension was diluted 15 times i.e. by adding 3 ml of the suspension to 42 ml of sterile Middlebrook 7H9 broth. This new suspension was then agitated, and further divided into 10 ml aliquots in inkwell bottles. Anti-microbial drugs were added at the required concentration for each drug (at double the MIC obtained during the agar dilution and the MTT assay). This concentration was obtained after further diluting from the stock solution prepared in Section 3.5.1. It was calculated to be twice that of the MIC obtained (for each isolate with the corresponding anti-TB drug) in a total volume of 10ml Middlebrook 7H9 broth (in the inkwell bottles). Each isolate was tested in triplicate for each drug. For each of the triplicate runs, a fresh culture was grown up and used for each isolate, and new dilutions of the anti-TB drugs were prepared from the concentration of the working solution (Appendix 1.4.1-1.4.6).

On days 0, 3, 7, 14, 21 and 28 growth was quantified. After continuous agitation at 150 rpm within a shaker incubator (New Brunswick Scientific, USA), 10-fold serial dilutions were made with the culture suspension. This was done by adding 100µl of the culture suspension of each isolate to 900 µl PBS with 20% (v/v) tyloxapol (Appendix 3.2). This was done 5 times to achieve the following dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. From each of the 5 dilutions above, 50 µl was removed and plated out in duplicate on separate Middlebrook 7H11 agar plates. The plates were heat sealed in gas permeable plastic bags and incubated for 3 weeks at 37° C. This procedure was done at each of the 6 time points for each of the selected isolates and their 5 anti-TB drugs.

Plates containing a countable number of colonies (between 20 and 200) were used to perform colony counts. Raw data of these colony counts are present in Appendix 7. Once the colony counts were performed, the data was used to construct the time-kill curves (Section 4.2.1). The number of colony forming units per ml (cfu/ml) was calculated by multiplying the counted number of colonies by 20 X the dilution factor. The exact formula used is described in detail in Appendix 5. The results of the duplicate plates were averaged and this number was

used to construct the time kill curves. The raw data of the average log values for the various time points are provided in Appendix 6

3.8 Detection of mutations in genes coding for drug targets

3.8.1 Culture prior to DNA Extraction:

The 11 clinical isolates (Table 4) and H37Rv were sub-cultured onto Middlebrook 7H11 agar (BD, Difco Laboratories, USA) plates (65mm). These plates were then sealed in plastic heat sealable bags and incubated at 37°C for 3 weeks.

3.8.2 DNA Extraction:

DNA extraction was performed using the N-acetyl-N, N, N-trimethylammonium bromide(CTAB) method. The procedure was carried out as previously described by Van Soolingen *et al*, (1991), with slight modifications.

The growth of each of the 11 isolates and H37Rv was harvested from the 65mm agar plates (Section 3.8.1), using a sterile loop, and suspended in a 2ml microfuge tube (Eppendorf) containing 50 µl of lysozyme (Sigma-Aldrich, USA) of concentration 10mg/ml (Appendix 4.2). The suspensions were placed in a water bath at 37°C for 1 hour. The tubes were then removed, and 75 µl of a proteinase K (Roche Diagnostics, Mannheim, Gemany) of concentration 10mg/ml (Appendix 4.3) and 10 % Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA) (Appendix 4.4) solution was added to each of the tubes. The tubes were then thoroughly mixed with the reagent by vortexing and the tubes were incubated in a water bath at 65 °C for 10 minutes. Following this, 100 µl of 5M Sodium chloride (NaCl) (Sigma-Aldrich, USA) (Appendix 4.5) was added to each tube and vortexed for a few seconds. Thereafter, 100µl of pre-warmed (65°C) CTAB-NaCl (Sigma-Aldrich, USA) (Appendix 4.6) solution was added to the tubes. These suspensions were agitated using a vortex until the fluid turned milky. This was followed by incubation at 65˚C for 10 minutes. Next, 750µl of a chloroform (Sigma-Aldrich, USA): isoamyl alcohol (Sigma-Aldrich, USA) (24:1) mixture (Appendix 4.7) was added to the tubes. The fluid was agitated for 10 seconds, and then centrifuged for 20 minutes at 12 000rpm. The supernatant was transferred to a sterile 1.5 ml microfuge tube (Eppendorf), and 500 µl of isopropanol (Sigma-Aldrich, USA) was added.

After gently mixing by inverting three times, the contents of each tube were then observed for the formation of a thin thread. If this was present, the mixture was centrifuged for 30 minutes at 12 000rpm. Thereafter, the isopropanol was discarded. The pellet was washed in 1 ml 70 % cold ethanol (Merck, SA) (Appendix 4.8). Tubes were inverted and then centrifuged for 5 minutes at 12 000rpm. The ethanol was discarded, and the pellet was washed again, but now with only 500 µl of 70 % ethanol (Merck, SA). After centrifugation and removal of the ethanol (as described above), the pellet was allowed to dry by evaporation for about 30 minutes at room temperature. Depending on the size of the pellet, 20 to 50 µl of 1x TE buffer (Tris-ethylenediamine tetra acetic acid or EDTA) was added (Appendix 4.1). The DNA was mixed in the buffer by inversion. The DNA was then stored in the fridge at 4°C until use.

3.8.3 Gel Electrophoresis

The concentration and quality of the DNA was determined by means of gel electrophoresis. A 1% agarose gel (Lonza, USA) (Appendix 4.9) was prepared by mixing agarose with 1 x Tris-borate-EDTA buffer (TBE buffer) (Appendix 4.10). To dissolve the agarose, the mixture was heated in a microwave oven. The solution was allowed to cool to 40-45^{o}C, after which ethidium bromide (Sigma-Aldrich, USA) (Appendix 4.11) was added. This was then poured into a gel casting tray, which was secured with masking tape on each end, to prevent the gel from leaking out of the tray. Attached within the casting tray, was a 20 well plastic comb. Once the gel was poured into the casting tray with the comb securely attached, it was left to solidify for 30-40 minutes at room temperature after which the combs and tape were removed. The gel was placed in an electrophoresis tank (Hoefer) filled with 1 X TBE buffer (Appendix 4.10). On a sheet of parafilm, 5 µl of DNA of each isolate was mixed with 3 µl of gel loading dye (Appendix 4.12). This was then pipetted into the corresponding well of the gel. One well was used for the 6XMassRuler Low range DNA ladder (Fermentas, Thermoscientific, USA).This was included to serve as a molecular weight marker, so that the quantification and sizing of the DNA fragments on the agarose gel could be determined. An electrical current of 100 volts (V) was applied to the gel for 30-45 minutes. Thereafter, the gel was removed from the tank, and an image of the bands was captured by using the Syngene gel imaging system and GeneSnap software.

The size and brightness of the bands were compared to that of the molecular weight marker. This was used as a crude measurement for the quality and concentration of the DNA which was then diluted with nuclease free water to achieve optimal concentrations. The dilutions were stored in the fridge at 4˚C till further processing.

3.8.4 Polymerase Chain Reaction (PCR)

Genomic DNA that was isolated from the 11 MTB isolates and H37Rv (Section 3.8.2) was used as the target DNA, to determine possible mutations at specific genes that confer resistance to the related anti-TB drugs. PCR was performed using the Expand High Fidelity PCR System kit (Roche Diagnostics, Germany). All reagents for the PCR reaction were provided by this kit. However, the optimal concentrations of the various constituents had to be determined, i.e. the optimal concentration of the enzyme or the $MgCl_2$. These factors together with optimising the actual PCR conditions, such as temperature at which the reaction is run and the duration of the cycles etc. are very important. They will impact on the final PCR product that was amplified and obtained.

Details of primers, PCR conditions and mastermix used are shown in tables 6, 7 and 8 respectively. The mastermix components (Table 8) were aliquoted into PCR tubes (placed on ice) and the specific DNA for each of the isolates and H37Rv were added to these tubes as well. The primers that were used for each reaction are displayed in Table 6. The PCR reaction took place within a thermocycler (Applied Biosystems, USA) and the cycling conditions used for the various runs are displayed in Table 7.

After each PCR experiment, gel electrophoresis was performed, following the same procedure as described above (Section 3.8.3). This was done to detect the presence of a band, within the molecular marker range, which indicated that the PCR was successful. These PCR products were stored in the fridge at 4˚C until the purification experiment

Table 6 : Primer Details for target genes

Gene	Primer Details
rrs Region 2 1400rrs	Fwd:KMSA [AAGTACCCCGCCTGGGGAGTACGG] Rev:KMRA [GGTGGGACAACACCTGGAACAAGTC] Jugheli *et al.,* 2009.
tlyA: Region 1	Fwd: tlyA1[AAGGCATCGCACGTCGTCTTTCC] Rev: tlyA2 [TGTCGCCCAATACTTTTTCTACGC] Designed using PRIMER3
thyA: Region 1	Fwd: thyA [GCCTCCGTTGTACTCCTGTG] Rev: thyA [ACACGCGTCACTCCTTGATT] Designed using PRIMER 3
rrl: Region 3	Fwd: rrl5 [GGTTGAAGACTGAGGGGATGAG] Rev: rrl 6 [GCCTTAGGTCCCGACTCACCCT] Richter *et al.*, 2007
rrl: Region 5	Fwd: rrl 9[CCCAAACCGACACAGGTGGTCA] Rev: rrl10[AAACTACCCGCCAGGCACTGTC] Richter *et al.* 2007.

Table 7 : PCR Conditions for genes of interest

Gene	PCR Cycling Conditions	
rrs Region 2 1400rrs	Initial Denaturation:	5mins @94ºC
	The next 3 steps had 40 cycles:	
	Denaturation:	45s @ 94 ºC
	Annealing:	45s @ 66ºC
	Extension:	45s@72ºC
	Final Extension:	10mins @ 72 ºC
	Amplicon Size:	831bp
tlyA	Initial Denaturation:	2 mins @ 94ºC
	The next 3 steps had 40 cycles	
	Denaturation:	45s @ 94 ºC
	Annealing:	45s @ 60 ºC
	Extension:	45s @ 72ºC
	Final Extension:	10mins @ 72 ºC
	Amplicon Size:	981 bp
thyA	Initial Denaturation:	5mins @ 94ºC
	The next 3 steps had 40 cycles	
	Denaturation:	45s @ 94 ºC
	Annealing:	45s @62 ºC
	Extension:	45s @ 72ºC
	Final Extension:	10mins @ 72 ºC
	Amplicon Size:	981bp

Rrl :Region 3	Initial Denaturation:	5mins @ 94ºC
	The next 3 steps had 40 cycles.	
	Denaturation:	45s @ 94 ºC
	Annealing:	45s @ 59 ºC
	Extension:	45s @ 72ºC
	Final Extension:	10mins @ 72 ºC
	Amplicon Size:	594bp
Rrl: Region 5	Initial Denaturation:	5mins @ 94ºC
	The next 3 steps had 40 cycles:	
	Denaturation:	45s @ 94 ºC
	Annealing:	45s @ 59ºC
	Extension:	45s @ 72ºC
	Final Extension:	10mins @ 72 ºC
	Amplicon Size:	623bp

Table 8: PCR Mastermix for Genes of Interest

Gene	**Mastermix (1 reaction)**	
rrs: Region 2 -1400rrs	1.Buffer (10X):	5 µl
tlyA: Region 1	2.$MgCl_2$(25nM):	3 µl
rrl: Region 3	3.dNTPs(10mM each):	1 µl
rrl: Region 5	4.Primer Fwd (10pmol):	1 µl
	Primer Rev (10pmol):	1 µl
	5.Enzyme(2.5 U):	0.75 µl
	6.Water:	35.95µl
	Total Volume:	48 µl
		+ 2 µl DNA
thyA: Region 1	1. Buffer (10X):	5µl
	2.$MgCl_2$(25nM):	3,3 µl
	3.dNTPs(10mM each):	1 µl
	4. Primer Fwd (10pmol):	1 µl
	Primer Rev (10 pmol):	1µl
	5.Enzyme(2.5 U):	0.45 µl
	6.Water:	36.25 µl
	Total Volume:	48 µl
		+ 2 µl DNA

3.8.5 PCR Product Purification

The PCR products were purified using the Invitrogen PureLink PCR purification kit (Version A) (Life Technologies, USA).The manufacturer's instructions were followed. Briefly, 3 main steps were conducted: DNA binding, DNA washing and DNA eluting. The binding step involved making a 4:1 solution of PureLink binding buffer with isopropanol and the PCR product. The combination was thoroughly mixed and then added to the PureLink spin columns that were provided. The columns were centrifuged for 1 minute at 10 000 X g or 9092rpm. The flow through was then discarded and the spin column was placed into a collection tube. The spin column remained in the collection tube, awaiting the DNA washing step. Wash buffer was combined with 100 % ethanol. Six hundred and fifty microlitres of the mixture was added to the column. The column was then centrifuged again for 1 minute at 10000 X g or 9092 rpm. The flow through was discarded and the spin column was placed in the collection tube. The column was then centrifuged for 2-3 minutes at 12 000X g or 9960 rpm. This was done to remove any residual wash buffer. The collection tube was discarded, but the spin column was kept, awaiting DNA elution. For DNA elution, the column is placed in a clean 1.7 ml PureLink elution tube. Thereafter, 50µl sterile distilled water was passed through the centre of the column. The column was incubated at room temperature for a minute, and thereafter centrifuged for 2 minutes at 12 000Xg or 9960 rpm. The spin column was then discarded, as the purified PCR product was moved into the elution tube during centrifugation. This purified PCR product within the elution tube is the final product and was stored in a freezer at -20˚C, until further use.

3.8.6 Preparation before Sequencing:

A reaction was performed on the PCR products (Section 3.8.5) before sequencing occurs. It was performed to purify the sequencing template and removes unwanted primers and dNTPs which may destroy the sequencing reaction. This was performed using the ABI Prism BigDye Terminator v3.0 Cycle sequencing ready reaction kit (Applied Biosystems, USA).This step requires 2.5µl of DNA which must be a final concentration of 20ng/µl of the DNA to be used for each reaction. A mastermix comprising of the reagents provided by the kit (5 X sequencing buffer, BigDye reaction mix) was added to the DNA, forward and reverse primers

(specific for each of the target genes mentioned earlier) and water. These were added to a MicroAmp96 well reaction plate (Applied Biosystems, USA), and was placed in a thermocycler, under the following conditions: 1 cycle at 96˚C for 1 minute.
25 cycles of the following 3 steps:

96˚C for 10 seconds

50˚C for 5 seconds

60˚C for 4 minutes

The last cycle is a hold cycle at 4˚C.

Once this step was completed, the plate with the sequencing reaction product was cleaned.

3.8.7 Sequencing Plate Clean-up

A 3 M sodium acetate (Appendix 4.13) (Applied Biosystems, USA) of pH 5.2 (5µl) and 100% ethanol (50µl) (Merck, SA) solution was constituted and added to each well containing the sequencing reaction products. The sodium acetate is responsible for precipitating the DNA from the ethanol. The plate was then sealed with an adhesive foil cover (Thermowell sealing tape) (Corning, USA) and agitated using a vortex. Thereafter, the plate was centrifuged for 20 minutes at 3000Xg or 4980 rpm. The foil cover was then removed and the plate inverted in a quick, smooth motion onto a folded pad of paper towels. This step was done with great care, as handling the plate to roughly would have resulted in the pellet being dislodged. The inverted plate on the paper towel was placed into the centrifuge for 1 minute at 150Xg. The plate was immediately removed and 150 µl of cold 70 % ethanol (Merck, SA) (Appendix 4.8) was added to each well. The plate was re- sealed with an adhesive foil cover, and agitated using a vortex. Thereafter it was centrifuged for 5 minutes at 3000Xg or 4980 rpm. The plate was then inverted on a folded paper towel again and centrifuged at 150Xg for 1 minute. The plate was removed from the centrifuge, and placed on a thermocycler (Applied Biosystems, USA), where it was left to dry for 5 minutes at 50˚C. After drying the plate, it was sealed with an adhesive foil cover, and then the entire plate was wrapped in foil, to protect the dyes from exposure to light. Thereafter it was stored at -20 ˚C awaiting sequencing.

3.8.8 Sequencing using the 3130 XL Genetic Analyser.

The plate was removed from the freezer, and samples were re-suspended in 10µl highly-deionized (Hi-Di) formamide (Applied Biosystems, USA) and thereafter agitated thoroughly using a vortex. The PCR products were then denatured, by placing in a thermocycler (Applied Biosystems, USA) at 95˚C for 3 minutes and at 4˚C for a minimum of 3 minutes. The plate was then sequenced in the 3130 XL Genetic analyser (Applied Biosystems, USA) and followed the Sanger sequencing method.

CHAPTER 4

RESULTS

This section describes the findings of the agar dilution experiment and the MTT assay (Table 9). It then describes results of the time kill experiments (Figures 1-11) for 5 specific isolates and H37Rv and lastly this section describes the sequencing results for the 4 different target genes encoding the drug targets for the specific anti-TB agents used (Table 11).

4.1 MIC Interpretation (Refer to Table 9)

H37Rv displayed an average INH MIC of 0.125 mg/L with both methods of DST. The susceptible isolate V4207 showed an MIC in the agar dilution test of 0.25 mg/L and in the MTT test of 0.125 mg/L. This is within the acceptable range of one dilution difference. According to WHOs breakpoint concentrations (Table 10), (WHO, 2009) for INH in agar (0,2mg/L) and in broth (0,1mg/L) both H37Rv and V4207 would be considered susceptible to INH. The mono-resistant, MDR-TB, Pre-XDR-TB and XDR-TB isolates all have the same MIC value for INH in both test methods, of 8mg/L. Thus based on the WHO breakpoint concentration (2009) (Table 10), they would be resistant to INH.

When exposed to CAP, H37Rv has a MIC value of 4 mg/L for both test methods. V4207 (susceptible isolate) has a MIC value of 8 mg/L in agar and 4 mg/L in the MTT assay. Based on the WHO breakpoint concentrations (Table 10) for CAP of 10 mg/L in agar, and 2,5 mg/L in broth, both H37Rv and V4207 would be considered susceptible to CAP with the agar dilution method but resistant with the MTT assay. The MIC for the INH mono resistant isolate (V666) and some of the MDR-TB isolates (V1435 and V2475) are 8 mg/L with agar dilution and 4 mg/L with MTT. V4258 (MDR-TB) has an MIC of 4 mg/L in agar, and according to the breakpoint concentrations, this would make it together with V666 (INH mono resistant), V1435 (MDR-TB) and V2475 (MDR-TB) susceptible to CAP. With the MTT assay V4258 (MDR-TB) has an MIC of 2 mg/L and is thus susceptible with both methods, whereas the rest are resistant with the MTT assay. The pre-XDR-TB isolate, R413, has a MIC of 8 mg/L for both agar and MTT. These MIC values thus make it susceptible to CAP in the agar method, but resistant in broth. All XDR-TB isolates (R26, R502, R506 and

KZN605) except R252 have the same MIC for agar (32 mg/L) and broth (8 mg/L). R252 has a lower MIC value for both methods i.e.8 mg/L in agar and 4 mg/L in broth. Despite this difference in MIC value, all XDR-TB isolates are considered resistant to CAP with both broth and agar methods.

The MIC values obtained for PAS using the MTT assay were markedly lower than the MIC values obtained by the agar dilution method. Experiments using the MTT assay method were repeated up to 5 times, and always obtaining inconsistent and lower values, that were far from those obtained by the agar method. Both H37Rv and V4207 (susceptible) have an MIC of 2 mg/L, which are the same as the breakpoint concentration as described in the WHO Guidelines. The INH mono resistant isolate V666, had a MIC for PAS of 0.5 mg/L, and according to WHO, can be considered susceptible. Two of the MDR-TB isolates (V1435 and V2475) have an MIC value of 2 mg/L, which is also the same as the breakpoint concentration. The remaining MDR-TB isolate, V4258, has a lower MIC of 1 mg/L, thus making it susceptible to PAS. Pre-XDR-TB isolate, R413 and XDR-TB isolates (R26 and R502) have MIC values of 4 mg/L, thus making it resistant to PAS. The remaining XDR-TB isolates (R252, R506 and KZN 605) have MIC values of 2 mg/L, which is the breakpoint concentration of PAS in an agar medium (WHO, 2009) (Table 10).

Linezolid MICs varied between 0.5mg/L and 0.25mg/L for all isolates with the lowest MICs obtained in the MTT assay. According to the WHO criteria (WHO, 2009), all isolates are deemed susceptible (Table 10).

Meropenem on its own has no activity against MTB. It was still tested as a control for M&C and all MIC values were 16mg/L, which was the highest concentration that was tested. It can thus be dedused that MTB would still grow at higher concentrations of meropenem on its own, if higher concentrations were tested. When meropenem is combined with clavulanic acid (M&C), however, it shows great anti-mycobacterial activity against MTB when using the MTT assay. The MICs in the MTT assay for all but one isolate varied between 0.5 and 1 mg/L. This value is at the breakpoint concentration as suggested by WHO (Table 10). V1435 is the only isolate which had an MIC value of 2

mg/L, thus suggesting that it is resistant to meropenem in combination with clavulanic acidThe MICs that were obtained using the agar dilution assay, for M&C were not used, as clavulanic acid is sensitive to heat, and thus its activity against MTB is affected when it is exposed to the high temperature of the 7H10 agar during plate preparation.

Table 9 displays the MICs that were obtained using the MTT assay and the agar dilution method, when the 5 anti-TB agents were tested against the 11 TB isolates and H37Rv.

Raw data of all MIC values obtained during the agar dilution and MTT assay are shown in Appendix 8

Table 9: MIC values of Agar Dilution and MTT Assay

MIC of Isolates/Strains µg/ml

MIC Method	Isolate/Strain	INH	CAP	PAS	LIN	MER	M&C
Agar	H37Rv	0.125	4	2	0.5	16	32
MTT	H37Rv	0.125	4	0.5	0.5	16	1
Agar	V4207	0.25	8	2	0.5	16	32
MTT	V4207	0.125	4	0.5	0.5	16	1
Agar	V666	8	8	0.5	0.5	16	32
MTT	V666	8	4	0.25	0.5	8	1
Agar	V1435	8	8	2	0.5	16	32
MTT	V1435	8	4	0.5	0.25	16	2
Agar	V2475	8	8	2	0.5	16	32
MTT	V2475	8	4	0.5	0.5	16	1
Agar	V4258	8	4	1	0.5	16	32
MTT	V4258	8	2	0.25	0.5	16	1
Agar	R 413	8	8	4	0.5	16	32
MTT	R 413	8	8	0.5	0.25	16	0.5
Agar	R 26	8	32	4	0.5	16	32
MTT	R 26	8	8	0.25	0.5	16	1
Agar	R 252	8	8	2	0.5	16	32
MTT	R 252	8	4	0.25	1	16	1
Agar	R 502	8	32	4	0.5	16	32
MTT	R 502	8	8	0.25	0.25	16	0.5
Agar	R 506	8	32	2	0.5	16	32
MTT	R 506	8	8	0.25	0.5	16	1
Agar	KZN605	8	32	2	0.5	16	32
MTT	KZN605	8	8	0.25	0.25	16	0.5

Table 10: WHO proposed critcial concentration for Anti-TB Drugs

Drug group[a]	Drug	DST category	DST method available	DST crtitical concentration (µg/ml)				
				Löwenstein-Jensen[b]	Middlebrook 7H10[b]	Middlebrook 7H11[b]	BACTEC460	MGIT960
Group 1 First-line oral anti-TB agents	Isoniazid	I	Solid, liquid	0.2	0.2	0.2	0.1	0.1
	Rifampicin	I	Solid, liquid	40.0	1.0	1.0	2.0	1.0
	Ethambutol	II	Solid, liquid	2.0	5.0	7.5	2.5	5.0
	Pyrazinamide	II	Liquid	–	–	–	100.0	100.0
Group 2 Injectable anti-TB agents	Streptomycin	II	Solid, liquid	4.0	2.0	2.0	2.0	1.0
	Kanamycin	II	Solid, liquid	30.0	5.0	6.0	4.0	–
	Amikacin	II	Liquid	–	–	–	1.0	1.0
	Capreomycin	II	Solid, liquid	40.0	10.0	10.0	1.25	2.5
	Viomycin	V	None	–	–	–	–	–
Group 3 Fluoroquinolones	Ciprofloxacin[d]	III	Solid, liquid	2.0	2.0	2.0	2.0	1.0
	Ofloxacin	III	Solid, liquid	2.0	2.0	2.0	2.0	2.0
	Levofloxacin	IV	Solid, liquid	–	2.0	–	–	2.0
	Moxifloxacin	IV	Liquid	–	–	–	0.5	0.25
	Gatifloxacin[e]	IV	Solid	–	1.0	–	–	–
Group 4[c] Oral bacteriostatic second-line anti-TB agents	Ethionamide	IV	Solid, liquid	40.0	5.0	10.0	2.5	5.0
	Prothionamide	IV	Solid, liquid	40.0	–	–	1.25	2.5
	Cycloserine	IV	Solid	40.0	–	–	–	–
	Terizidone	IV	None	–	–	–	–	–
	P-aminosalicylic acid	IV	Solid, liquid	1.0	2.0	8.0	2.0	–
	Thioacetazone	V	None	–	–	–	–	–
Group 5[c] Antituberculosis agents with unclear efficacy (not recommended by WHO for routine use in MDR-TB patients)	Clofazimine	V	Liquid	–	–	–	4.0	–
	Amoxicillin/clavulanate	V	None	–	–	–	–	–
	Clarithromycin	V	None	–	–	–	–	–
	Linezolid	V	Liquid	–	–	–	1.0	1.0

[a] WHO *Guidelines for the programmatic management of drug-resistant tuberculosis* (*25*).

[b] Indirect proportion method recommended. Other solid media methods (resistance ratio, absolute concentration) have not been adequately validated for second-line drugs.

[c] Routine DST for group 4 and 5 drugs is not recommended.

[d] Ciprofloxacin is no longer recommended to treat drug-susceptible or drug-resistant TB (*25*).

[e] Gatifloxacin only to be used in exceptional circumstances (*25*).

4.2 Time Kill Experiments

The isolates used were V4207 (Susceptible), V1435 and V4258 (both MDR-TB), R252 and KZN605 (both XDR-TB). H37Rv was used as reference susceptible strain. The time kill curves (TKCs) (Figures 1-12) were constructed based on the average numbers of colony forming units (cfus) obtained in 6 experiments.The range of values and raw data used to construct the TKC for each of the time-points for each of the 6 experiments are given in Appendix 6 and Appendix 7. The time kill experiments will be illustrated in two manners.

4.2.1 Time Kill Curves of tested isolates and H37Rv by Anti-TB Drugs

The results for H37Rv are shown in Figure 1. This strain was killed when exposed to CAP, LIN and M&C respectively. This total kill was achieved by CAP on day 14, by M&C on day 21 and by LIN by day 28. This effect was expected as H37Rv is a susceptible strain. Exposure to PAS, resulted in H37Rv steadily decreasing in number of colony forming units (cfu), from 5.06 Log cfu/mL to 2.15 Log cfu/mL, however it did not kill H37Rv completely during the exposure time of 28 days. INH caused a decrease in number of cfus of H37Rv, over the first 3 days, where a decrease of almost 1.5 log cfu/ml occurred. A small increase in number of cfus (0. 43 log cfu/ml) then occurred between day 3 and day 7, this was followed by yet another decrease in cfus (0.13 log cfu/ml). From day 14 till day 28, there is a steady increase in the number of cfus of H37Rv. H37Rv continued to grow, when it was not exposed to any anti-TB agent.

V4207 (Figure 2) is a susceptible clinical isolate, which shows very similar behaviour to that of H37Rv, in that exposure to CAP, LIN and M&C resulted in complete kill, at day 7 and day 14 respectively. However there were 2 differences in its behaviour compared to H37Rv. V4207 was eradicated completely by PAS at day 14 and although INH did not kill it completely, no regrowth occurred.

V1435 (Figure 3) is a MDR-TB clinical isolate, which was killed by CAP and M&C completely at day 21, while exposure to LIN, resulted in complete eradication by day 28. Exposure to INH has no effect as shown by an almost identical curve as the drug free control. Exposure to PAS shows an initial decrease by 3.45 log cfu/ml from day 0 to day 3. However

it recovers thereafter and increases by 4.58 log cfu/ml from day 3 till day 28, thus indicates resistance to PAS.

V4258 (Figure 4) which is also a MDR-TB isolate behaves very similarly to V1435, in that it is also killed when exposed to M&C, CAP, and LIN, at day 7, 14 and 28 respectively and INH has no effect. The only difference with V1435 is that it shows a marked decrease of 3.56 log cfu/ml when exposed to PAS. However it was still not killed completely in the presence of PAS over the 28 day observation period.

XDR-TB strain R252 (Figure 5) is only eradicated completely in the presence of CAP and this is achieved at day 21.This isolate shows resistance to INH and PAS, where it increases in number of cfu by a 2.38 log cfu/ml and 2.16 log cfu/ml respectively from day 0 to day 28. When exposed to LIN, it increases in number of cfu (0.52 log cfu/ml) from day 0 to day 3. Thereafter it decreases by 0.98 log cfu/ml till day 14. From then till day 28 it shows resistance to LIN, and increases by 1.95 log cfu/ml. Exposure to M&C results in a decrease of 1.04 log cfu/ml. Although M&C displayed an inhibitory effect to R252, it was not killed completely within 28 days.

KZN 605 (Figure 6) is also an XDR-TB strain and showed continuous growth in the presence of INH. PAS and LIN are the only anti-TB drugs that kill off KZN 605 completely, at day 21 and 28 respectively. M&C, also displays inhibition with a decrease in number of cfus from day 0 till day 28 (2.13 log cfu/ml). Like with R252, M&C does not kill KZN605 completely within the 28 day period. Exposure to CAP, results in an initial decrease in number of cfu (1.33 log cfu/ml) from day 0 till day 14, but thereafter this XDR-TB isolate shows resistance, when it increases in number of cfu (1.59 log cfu/ml) from day 14 till day 28.

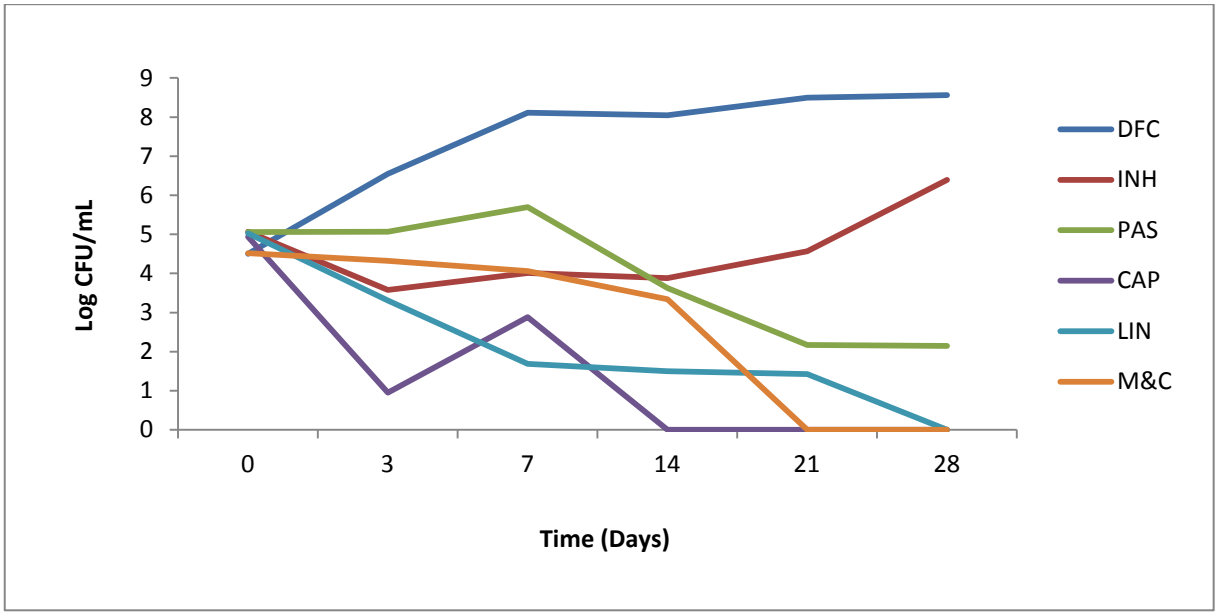

Figure 1: Time kill curve of H37Rv under drug free conditions and when exposed to the 5 anti-TB drugs.

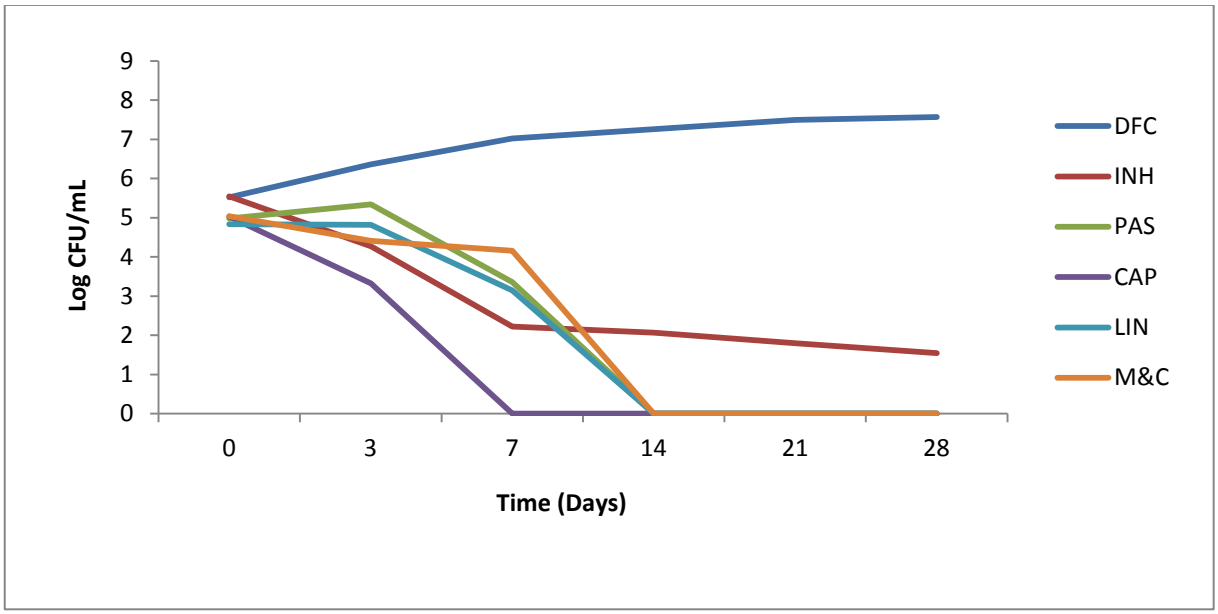

Figure 2: Time kill curve of V4207 under drug free conditions and when exposed to the 5 anti-TB drugs.

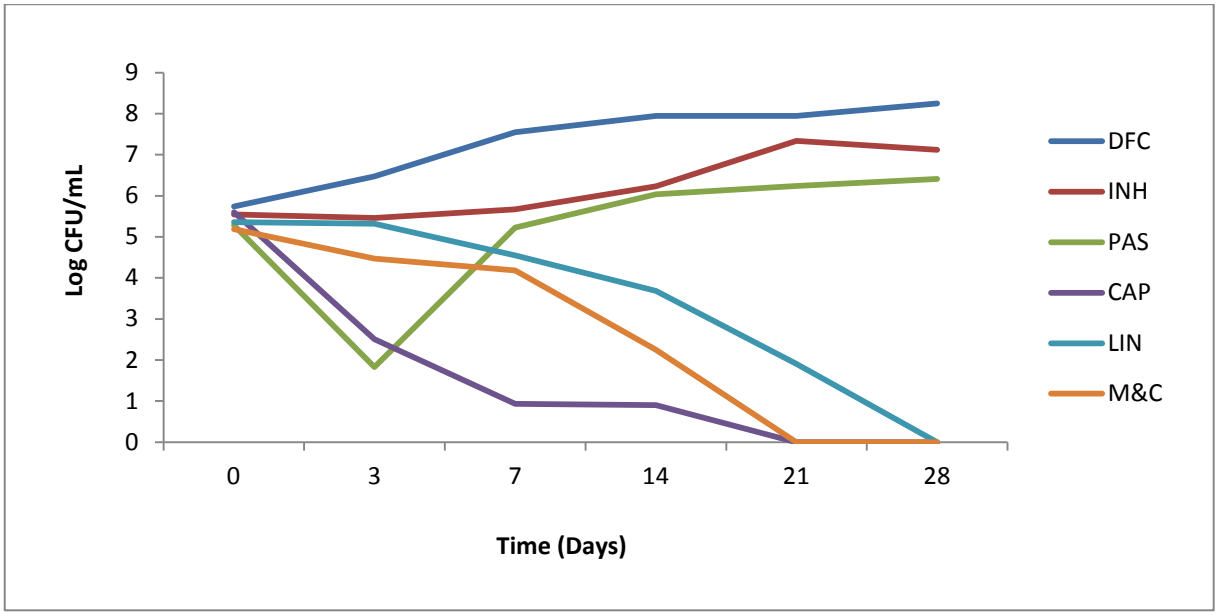

Figure 3: Time kill curve of V1435 under drug free conditions and when exposed to the 5 anti-TB drugs

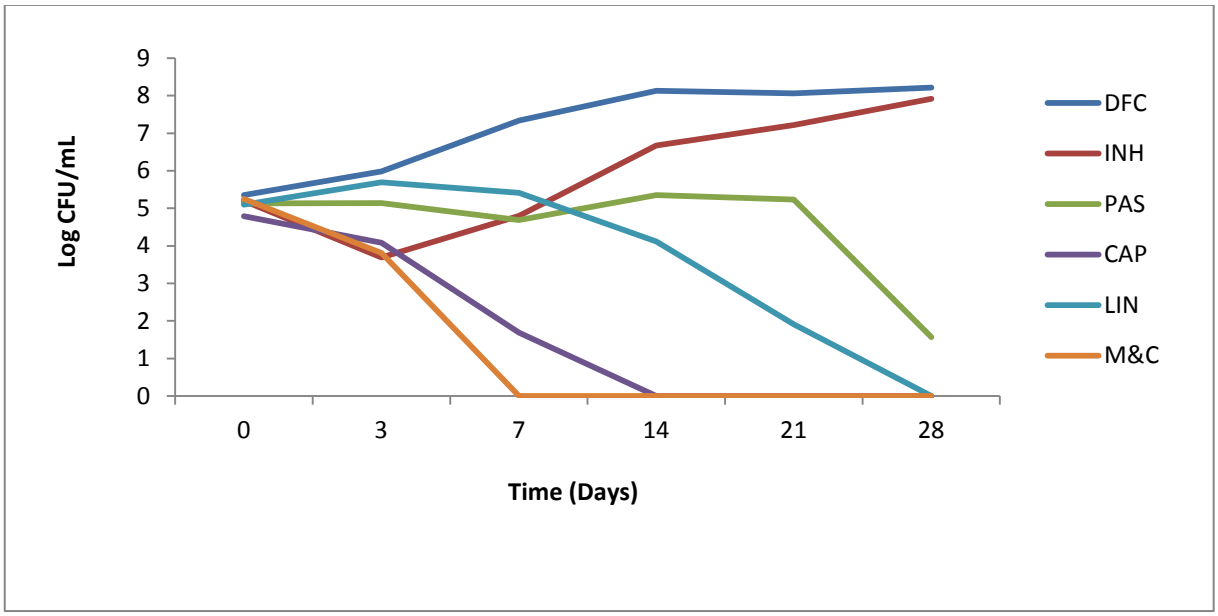

Figure 4: Time kill curve of V4258 under drug free conditions and when exposed to the 5 anti-TB drugs

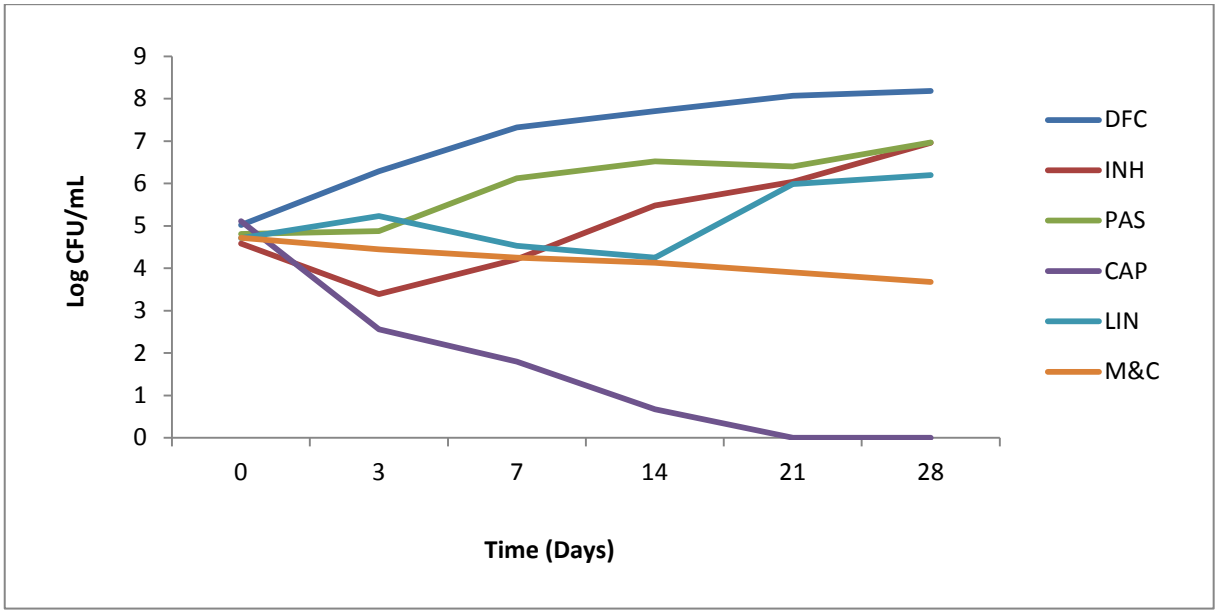

Figure 5: Time kill curve of R252 under drug free conditions and when exposed to the 5 anti-TB drugs

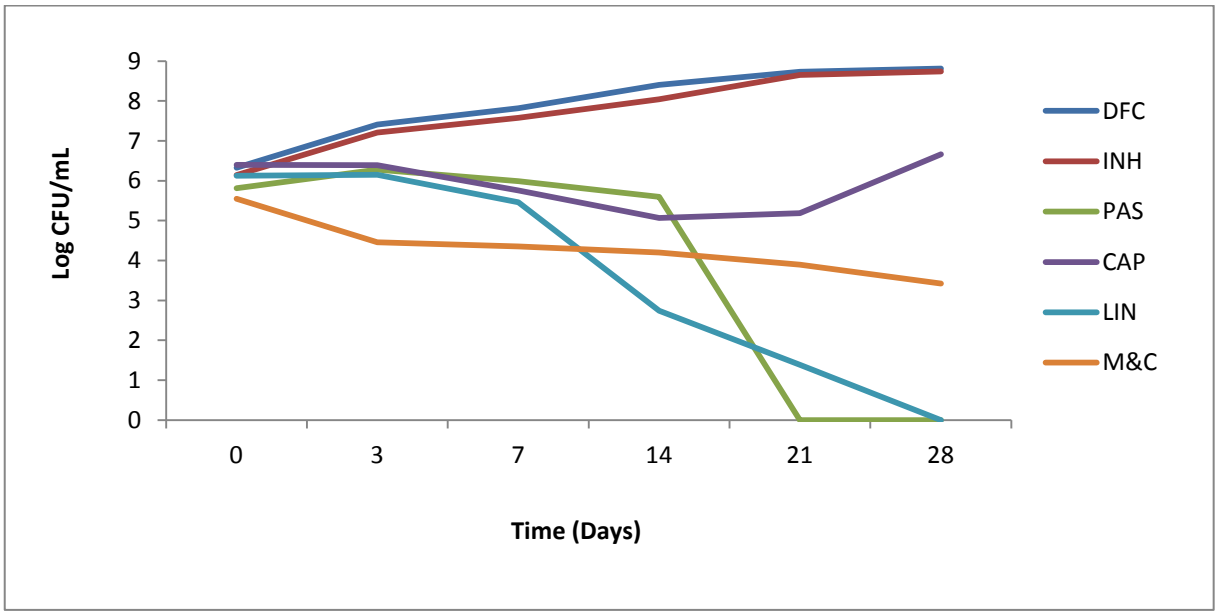

Figure 6: Time kill curve of KZN 605 under drug free conditions and when exposed to the 5 anti-TB drugs

Fig. 7-11 shows the same results as Fig. 1-6 but displayed in a different manner. In summary, INH had a killing effect on only one isolate. CAP killed all except one isolate. PAS inhibited all and showed late killing of 4 isolates. One isolate (XDR-TB) was resistant to LIN while all others were killed at different rates. Two isolates (both XDR-TB) showed tolerance to M&C while the other four were eliminated.

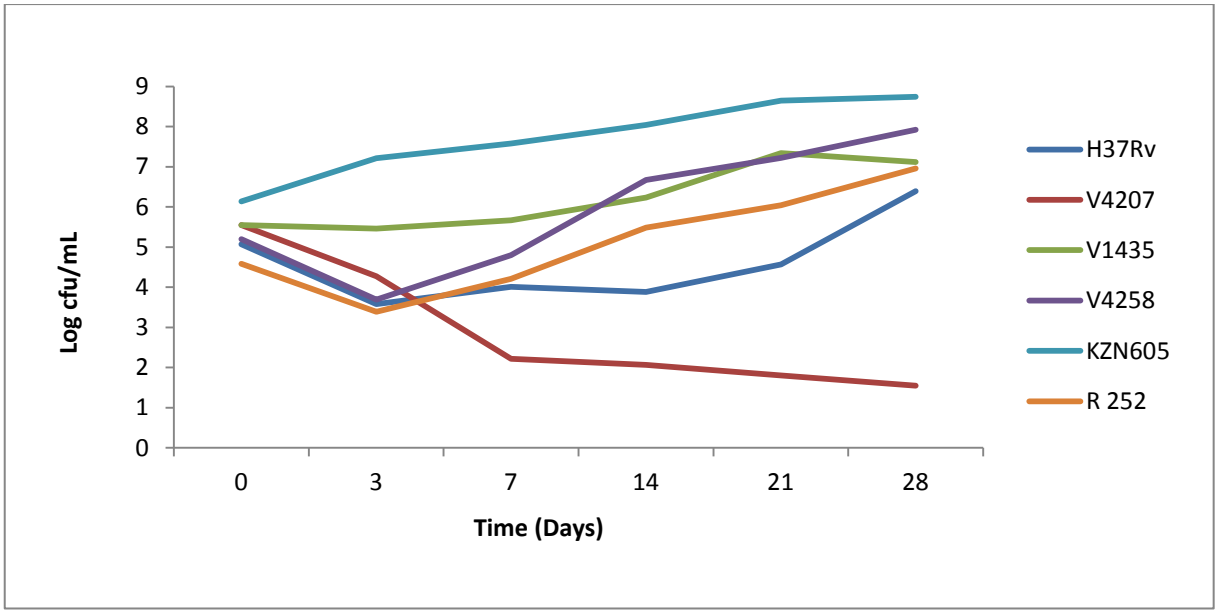

Figure 7: Time kill curve of the 5 isolates and H37Rv when exposed to isoniazid

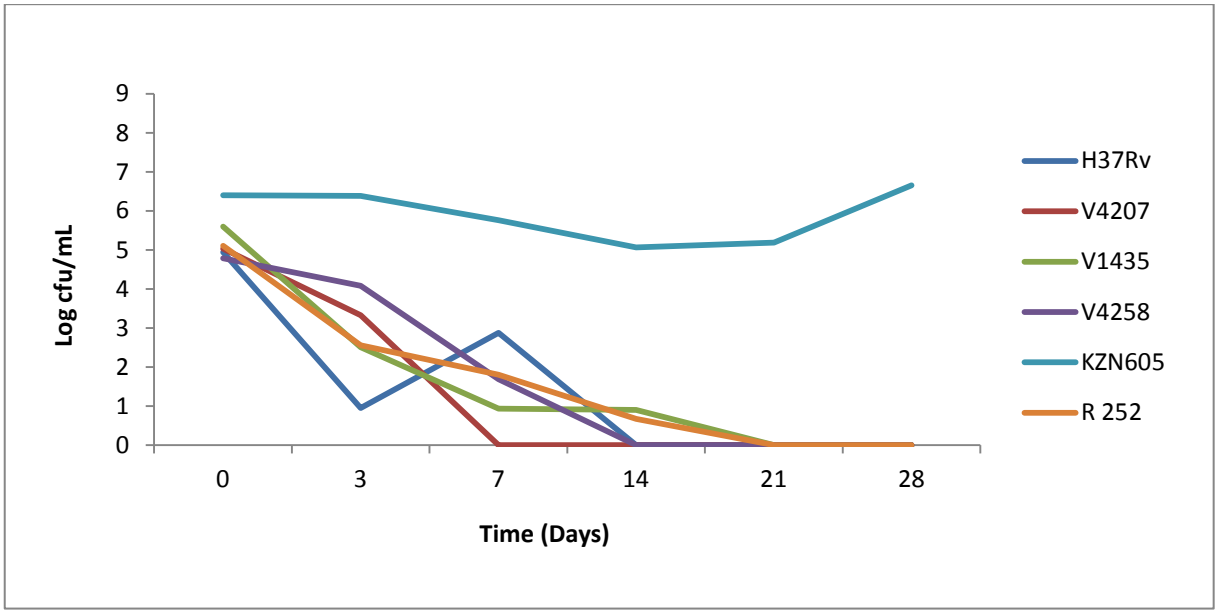

Figure 8: Time kill curve of the 5 Isolates and H37Rv when exposed to capreomycin

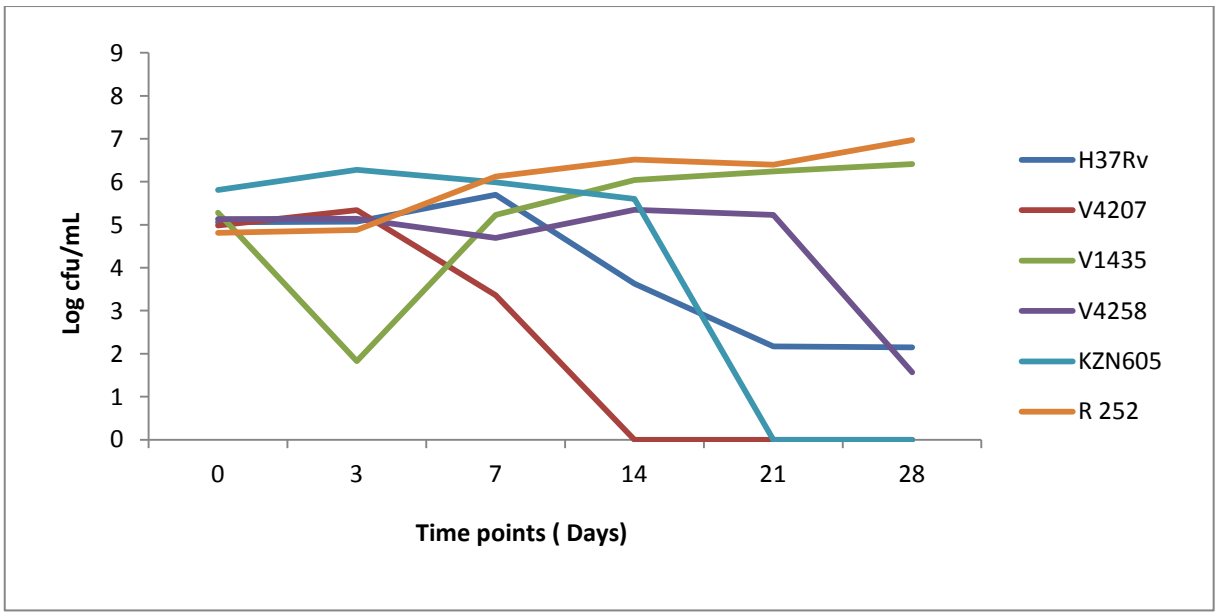

Figure 9: Time kill curve of the 5 isolates and H37Rv when exposed to para-aminosalicylic acid

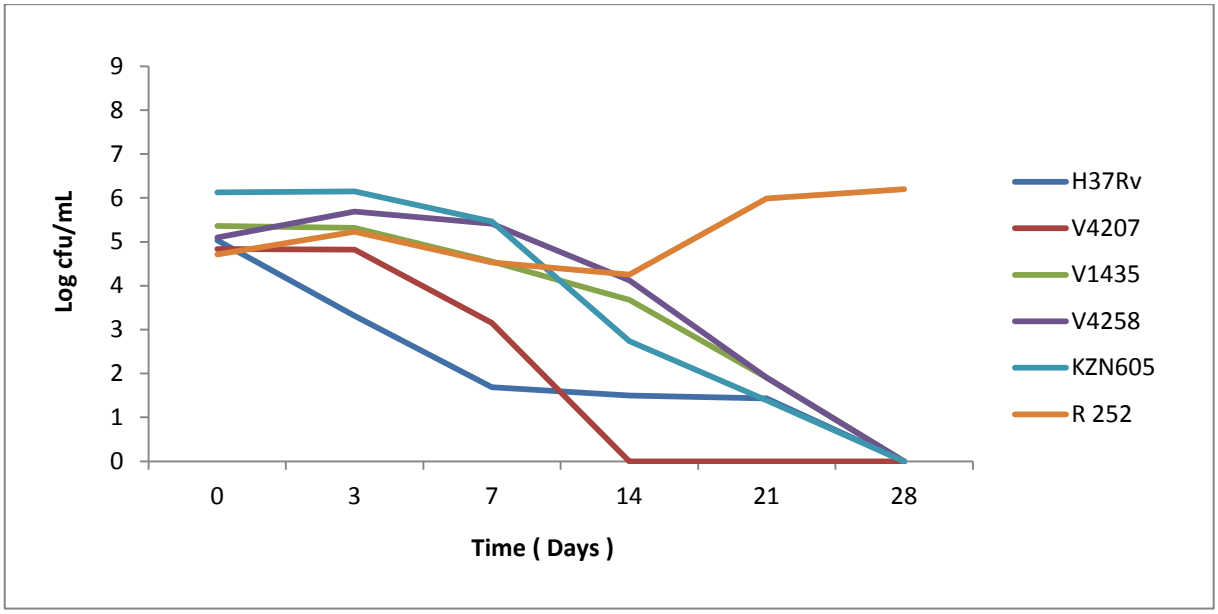

Figure 10 Time kill curve of the 5 Isolates and H37Rv when exposed to linezolid

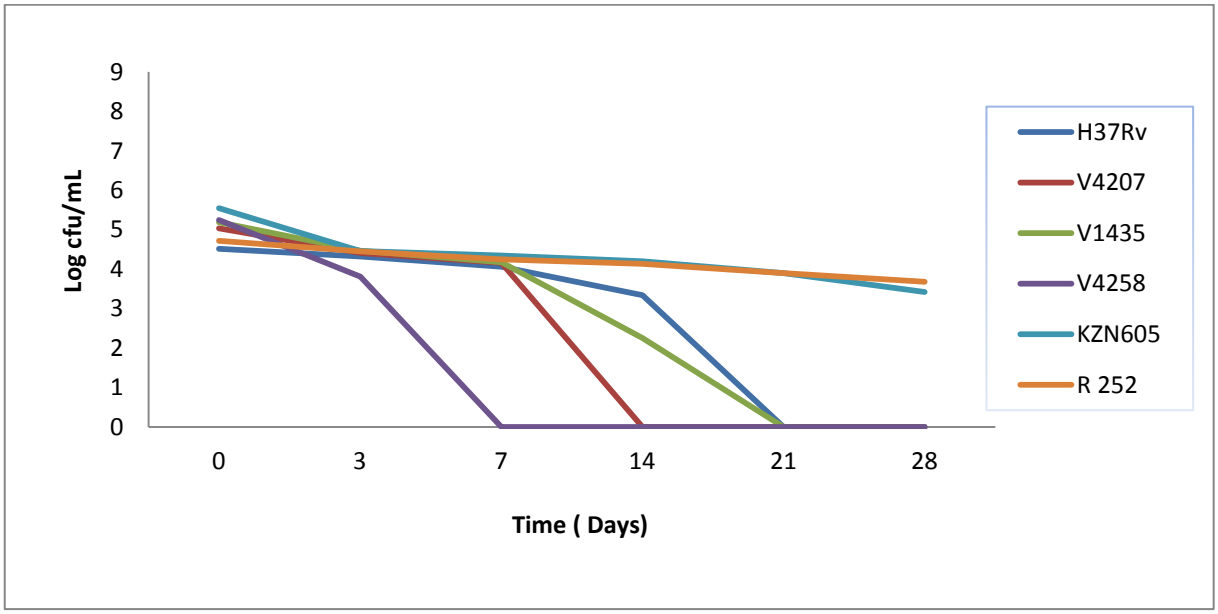

Figure 11: Time kill curve of the 5 isolates and H37Rv when exposed to meropenem in combination with clavulanic acid.

4.3 Sequencing Results

This section describes the mutations that were detected by means of the sequencing experiments. The results are shown below in table 11.The sequencing results showed that H37Rv and R252 have no mutations in any of the genes investigated. R26, R506 and KZN605, are all XDR-TB isolates, and were shown to have the following mutations A1401G, A74G, and G2399A in the *rrs, tlyA* and *rrl*2 genes respectively. R413, V666, V1435, V2475, V4207, V4258, all have mutations A74G and G2399A in the *tlyA* and *rrl2* genes respectively. R502 only had mutations A1401G and G2399A in the *rrs* and *rrl2* genes respectively. It was not possible to detect mutations for certain genes in specific isolates. This was due to poor quality of the DNA that was isolated and sequenced. This is a strange phenomenon, as even though the DNA was deemed poor quality, for a specific isolate it was able to detect mutations in other genes, coding resistance to other anti-TB drugs i.e V4207, V666, V1435, V4258 and R502

Table 11: Mutations detected in specified Genes of Interest for 11 Isolates and H37Rv

Isolate	Profile	*rrs*	*tlyA*	*thyA*	*rrl1*	*rrl2*
H37Rv	Susceptible	None	None	None	None	None
V4207	Susceptible	None	A74G	PQ	None	G2399A
V666	Mono resistant	PQ	A74G	None	None	G2399A
V1435	MDR	PQ	A74G	None	None	G2399A
V2475	MDR	None	A74G	None	None	G2399A
V4258	MDR	None	A74G	PQ	None	G2399A
R 413	Pre XDR	None	A74G	None	None	G2399A
R 26	XDR	A1401G	A74G	None	None	G2399A
R 252	XDR	None	None	None	None	None
R 502	XDR	A1401G	PQ	None	None	G2399A
R 506	XDR	A1401G	A74G	None	None	G2399A
KZN 605	XDR	A1401G	A74G	None	None	G2399A

PQ-Poor Quality of DNA

CHAPTER 5

DISCUSSION AND CONCLUSION

The global rise of MDR and XDR-TB has forced attention to be focused on the search for additional drugs to treat patients infected with these drug resistant strains. This search not only includes development of novel drugs but also to assess existing drugs that have so far not been considered for the treatment of TB, and older anti-TB drugs that were removed from use when the current FLDs with high effectivity and low toxicity came into the market. Such drugs have to be evaluated by means of *in vitro* and *in vivo* testing. *In vitro* testing includes different forms of susceptibility testing while *in vivo* testing involves testing in animals as well as in patients. The work presented here involves the *in vitro* evaluation of two "old" anti-TB drugs, PAS and CAP, and two drugs developed for the treatment of infections other than TB, LIN and the combination of M&C. Two different DSTs, one agar based and one broth based, were used. The time-kill experiment (TKE) was also performed to better understand the activity of the drug on the various isolates over a period of time. The results of these tests were compared with each other and with the presence of mutations in the genes coding for the targets of the drugs tested.

Eleven isolates were used. They were chosen based on their susceptibility profiles established by means of the 1% proportion method on Middlebrook 7H10 agar which is routinely used in our setting. These isolates included susceptible, INH mono-resistant, MDR-TB, pre XDR-TB and XDR-TB isolates (Table 4). This allowed validation of the routine susceptibility test method for the drugs investigated. MICs were determined using solid and liquid based DSTs. The results were compared with each other and the isolates were then classified as susceptible or resistant using the WHO proposed breakpoints for DST on solid and in liquid media, shown in Table 10 (WHO, 2009). These results were then correlated with kill over time as well as with the presence of known resistance conferring mutations in the genes coding for the respective drug targets, to gain an overall understanding of the cause of resistance or susceptibility of MTB to the various anti-TB agents.

When comparing MICs obtained with the agar dilution method with those obtained in Middlebrook 7H9 broth with MTT as a growth detector, we found that if there were

differences in MIC, the broth MIC was invariably lower than the MIC on the agar medium. This confirms observations of others which were reflected in the lower proposed breakpoints for resistance in liquid medium (WHO, 2009). A possible explanation for the higher MICs obtained in solid as compared to liquid media is that the liquid based media are read after a shorter incubation period. The longer incubation time may partially inactivate the drugs, resulting in growth at higher concentrations.

Despite the proposed breakpoints for INH (Table 10) (WHO, 2009) being one dilution lower in liquid as compared to solid media, the values obtained with both were identical for all isolates.The MICs obtained for CAP and LIN revealed that the broth method always yielded a lower MIC than the agar dilution. The MICs were generally only one or two dilution factors lower than that of the agar method. This is in keeping with the difference in proposed breakpoints for CAP. Breakpoints for LIN on solid media have not been proposed (Table 10) (WHO, 2009).

Based on the proposed breakpoints, MICs for PAS were expected to be identical with both methods. This however, was not the case in the results obtained. Except for one, all isolates had significantly higher MIC values with the agar dilution method. Different from the opinion of the WHO committee (WHO, 2009), it seems that PAS follows the trend of most anti-TB drugs.

As mentioned before, no breakpoints have been proposed for agar MICs for LIN (Table 10). However, both tests produced very similar and reproducible results. Most were identical while some different by one dilution. Differences in MIC values of one dilution are interpreted as being the same. The MIC for all isolates tested against LIN using the agar dilution method is 0.5µg/ml. This MIC for isolates of varying susceptibilities was also shown in a study by Tato *et al*, 2006. This study, together with our findings implies that the level of resistance of various isolates tested, did not influence the MIC obtained for LIN.

The use of meropenem alone displayed results as expected, which was resistance at high concentration (8-16ug/ml). These high MIC's were expected, as meropenem falls under the class of antibiotics of ß-lactams. MTB is resistant to ß-lactams, as it produces a broad

spectrum ß-lactamase (BlaC) which catalyzes the hydrolysis of the ß-lactam ring, thus inactivating meropenem (Hugonnet and Blanchard, 2007). The addition of clavulanic acid decreased the MICs to 1-2 mg/l. This is the result of inactivation of the ß-lactamase by means of an irreversible bond between the enzyme and clavulanic acid. A study by Hugonnet *et al.*, in 2009, also compared the effect of M&C on MTB. This study illustrated M&C's strong activity against XDR-TB (Hugonnet *et al*., 2009). Our study had similar findings and similar MIC values to theirs, of not being higher than 1µg/ml.

All experiments were done in triplicate, and sometimes, even more than that. Both methods are sensitive to the size of the inoculum and the actual standardisation procedure. As a means to reduce errors in this study, the standardisation procedures for both these DSTs methods were kept constant. A concern with respect to MIC determination is that TB isolates can be composed of various mycobacterial sub-populations, which may have different susceptibility patterns. This will therefore affect susceptibility testing, by producing discrepant results. In an effort to avoid this, single colonies were grown up for each isolate. Its progeny was then used for all the tests in this study.

The agar dilution method only yields results after 3 weeks. The MTT assay however, was able to yield results after just 9 days, as at that point there is sufficient growth to allow the reduction of the MTT salt to formazan. This resulted in the reagent to change colour. However, colour changes from yellow to purple are not always distinct. Therefore, the MTT assay was repeated several times. Recent studies made use of a spectrophotometer or a colorimeter to circumvent this problem (Raut *et al.*, 2008). The MTT assay is cheaper than the agar dilution method and easy to perform and interpret. It must be performed using a young culture, which is between 2 to 4 weeks old, as older cultures affect the results adversely (Canetti *et al.*, 1969; Raut *et al.*, 2008). This is because the assay requires cells that are metabolically active, as these are the only ones which will be able to produce the dehydrogenase enzyme, responsible for reducing the tetrazolium dye. Raut *et al* (2008) claimed that when the MTT assay showed an isolate to be resistant to a drug, that is usually correct, but if this assay reveals an isolate to be susceptible, then this may not be accurate, and should be confirmed with another laboratory test. Our study does not support this. Although there were discrepancies between the two methods with lower MICs in the MTT, none of

these isolates changed from susceptible to resistant (Table 9). However, we have to keep in mind that only 12 isolates were tested. Great care was taken to avoid cross contamination, since the close proximity of the wells easily leads to such an event. One of the measures to avoid cross contamination was to cover wells that were already inoculated with the lid. Testing of MDR-TB and XDR-TB isolates in a microtitre plate is a laboratory hazard, as it is able to generate aerosols easily, and the risks of spills seem higher as it is a liquid based test. As a precautionary measure, the lids of the plates were held securely to the plate with elastic bands on each side and thereafter heat sealed in a gas permeable bag. As with all handling of MTB isolates, the safety rules should be strictly adhered to, in an effort to minimise the hazards.

Results from this study suggest that the MTT assay can be used as a rapid screening test to confirm DST results. This assay should always be used in conjunction with another conventional method, rather than being the sole DST. It proves useful in that it is able to offer information on efficacy of various anti-TB agents within 9 days, as opposed to 3-6 weeks with the conventional method. Thus this assay would be useful in resource limited settings, with high rates of DR-TB, where clinicians require DST information promptly. Results from this assay will undoubtly assist with treatment options. As mentioned previously (Section 3.6.1), since the MTT assay is a DST, the presence of other antibiotics in the media, can alter DST results. Thus one must ensure that the TB isolates are always tranferrend into broth that is free of PACT or PANTA, when conducting the MTT assay.
In a routine laboratory, initial culture of MTB isolates is performed on PACT/PANTA containing media. This is done to prevent growth of contaminants that may have escaped the decontamination process. For DSTs, sub-cuture of the isolates onto PACT/PANTA-free broth will be required. However, this is labour intensive and may lead to further delays in the generation of DST results.

Since we expected isolates with the same susceptibility pattern to behave similarly, the TKEs were performed for two susceptible (including H37Rv), two MDR-TB and two XDR-TB isolates. The isolates were inoculated into the broth to which tyloxapol was added to prevent clumping. Unlike Tween 80, this is a detergent which has been shown not to influence TKEs with antimicrobials. When Tween 80 is present in the growth medium, it releases free fatty

acids lowering the pH and thus facilitating the killing of MTB (Vandal *et al*., 2009). Ortalo-Magne *et al* in 1996, suggest that Tween 80 may also be involved in stripping the glycoprotein capsule of MTB, thus making the bacterium susceptible to acidic conditions. Tyloxapol was therefore used in the TKEs in an effort to avoid biased results, where the killing of MTB may be enhanced by the actual media properties, rather than the anti-TB agents that were being tested.

TheTKE were performed using twice that of the MIC that was obtained when using the agar dilution method and MTT assay. Whilst MICs are available for these drugs, it is not known how these drugs perform over time in vitro and in vivo against MTB. Twice the MIC was chosen as an arbitrary starting point since not much is currently known on this topic, and it is preferable to start at a higher MIC for TKE studies than what is needed to kill or inhibit growth of an organism.

TKEs can be performed in two manners. In the first method the concentration of the drug is decreased over time, mimicking the decrease in concentration during the dose-time interval *in vivo*. The second method uses a constant concentration of the drug. Both measure the decrease in number of viable bacteria at different time points during the experiment. Since we did not have access to the equipment needed for the first, this study made use of the second method. MIC methodology measures growth inhibition irrespective of whether the organisms are killed in the process. Minimal bactericidal concentration (MBC) determination following MIC provides information on differences in the concentration that inhibits and that which kills. TKEs provide information different from MBCs in that it shows kill over time. This requires counts of viable bacteria at various time points. The method applied here involves subculturing and thereafter peforming counts of the colony forming units (CFUs). This method involves sampling of the broth in which the experiment is performed at 6 time points. Ten-fold serial dilutions are made and plated out to obtain a subculture with a number of colonies that can be counted reliably. The dilution which yields 20-200 colonies is used. Trying to count more than 200 colonies results in counting errors while less than 20 colonies results from unreliable dilution.

Time kill curves (TKCs) of INH and the 5 isolates and H37Rv show that INH as expected was unable to kill the two MDR-TB and two XDR-TB isolates. It was also unable to kill all cells of the susceptible isolate. As described previously, that part of the bacterial population survives when exposed to INH resulting in regrowth at a later time point (Colangeli *et al.*, 2005; Viveiros *et al.*, 2002). This exposure dependant kill pattern for mycobacteria under INH therapy has also been reported in *in vivo* animal studies (Jayaram *et al.*, 2004). The initial rapid kill rate followed by a slow kill rate along with the re-growth of bacteria observed in this study is remarkably similar to that observed in an *in vitro* study by Gumbo *et al.*, (2007) using *M.tuberculosis* H37Ra. Similar results were also found in several other *in vitro* drug susceptibility assays. It was hypothesized that the development of drug induced tolerance and transient resistance to INH is responsible for the cessation of INH bactericidal activity (Viveiros *et al.*, 2002; Wallis *et al.*, 1999).

CAP was able to kill the full population of all isolates except one of the two XDR-TB isolates. The kill of one XDR-TB isolate is an unexpected result, as this isolate shares the same MIC with the other XDR-TB isolates. This phenomenon needs confirmation using a larger sample size. Drug tolerance or latency could also be the cause of the one XDR-TB isolate (KZN 605)tocontinue to grow in the presence of CAP.The MTB strain could be in a state of reduced metabolic activity, that can involve either replicating sporadically, very slowly or not at all (Primm and Franzblau, 2007).The two XDR-TB isolates could have behaved differently during the TKE, despite their identical MIC values due to specific needs by the specific MTB isolate i.e. nutrition, space, aeration, and the production of toxic metabolites (Vadaddy *et al*., 2010). *In vivo* studies have shown that MTB is able to shift into a non-replicating drug tolerant state, when nutrients are depleted, thus ensureing that the organism is able to remain in a dormant state within the host for long periods of time, till conditions are favourable for replication (Vadaddy *et al*., 2010). Perhaps this could have occurred with this XDR-TB isolate (KZN 605) in the presence of CAP.

Within the timeframe, PAS killed the full population of both susceptible isolates. It was also able to partially kill two other isolates, one being an MDR-TB isolate, and the other an XDR-TB isolate. No killing was observed with the remaining two of which one was MDR-TB (V1435) and one XDR-TB (R252). All isolates had an MIC in the susceptibility range. PAS

has formerly been described as bacteriostatic. This was only confirmed for some of the isolates. The unexpected behaviour of the various isolates, with differing susceptibility profiles, suggest that their classification of being MDR or XDR isolates, was unable to completely and correctly predict their performance over the 28 day period when exposed to PAS. Previous research by Odenholt-Tornqvist *et al*, in 1991, revealed that when MTB is exposed to sub-MICs of β-lactams, this results in various adaptive modifications by the organism. These modifications include changes in the cell wall structure, altering the ribosomal density as well as the formation of filaments. Since the TKE in this study is an *in vitro* one, one is unable to observe if these modifications occurred. Although this unexpected behaviour was observed in the presence of PAS, and not the class of β-lactams as described in literature, it still remains a possibile theory as to why the remaining MDR and XDR-TB isolates behaved so differently to the other isolates of their respective profiles.

LIN killed the full population of 4of the 5 isolates and H37Rv within 28 days. These isolates all had MICs in the susceptible range. One XDR-TB isolate (R252), which was also susceptible based on the MIC, was not killed. This phenomenon is similar to that which was observed when the isolates were exposed to CAP, and can possibly be due to the above mentioned reasons as well. An *in vitro* study by Cremades *et al*, in 2011, revealed that repeated exposure of LIN to MTB, at subinhibitory concentrations, does not result in MTB generating resistant mutants.The study also showed that LIN exhibited bactericidal activity against susceptible isolates at high concentrations (Cremades *et al*., 2011). This was also shown in our study, as V4207 and H37Rv were both killed at day 14 and day 28 respectively. Our study also shows that LIN was also able to exhibit this property to both MDR-TB and one XDR-TB isolates, except R252. Another finding by Cremades et al., 2011, is that the bactericidal activity of LIN is low against bacteria in a nonreplicating phase, even if these strains are susceptible.

The combination of M&C, killed the full population of both susceptible and both MDR-TB isolates within 1 to 3 weeks. The two XDR-TB isolates, despite being susceptible based on the MICs, were not killed within the time period, again indicating drug tolerance.

TKEs are useful in elucidating information regarding the effect of a specific concentration of the anti-TB drugs tested on each isolate over a period of 28 days to a certain degree. However, it may not be a complete reflection of what happens *in vivo.* There are numerous additional factors that contribute to the effect of the drug on the MTB. These include host factors, i.e. the additional effect of the immune system on the MTB infection. There are also physiological factors that can influence the effect of the anti-TB agent on MTB, such as pH (Vadaddy *et al*., 2010). Various interaction such as plasma-protein binding of the anti-TB agents are also absent in *in vitro*TKEs (Vadaddy *et al*., 2010). An additional factor is that in an *in vivo* setting one is able observe the effect of other drugs on the anti-TB agents that are being tested. This drug interaction often occurs since there are many patients who are co-infected with TB and HIV, and thus receive treatment for both of these infections.

Sequencing of the genes coding for the targets of CAP, PAS and LIN showed three mutations, two of which (one for CAP and one for LIN) were also found in susceptible isolates. The *thyA* gene, which was thought to code for resistance to PAS (Rengarajan *et al.*, 2004), showed no mutations in our study.This was a concerning factor, since the MDR and XDR-TB isolates V1435 and R252 respectively, showed resistance to PAS during the TKE, yet showed no mutations in this gene. These two isolates continued to increase in CFU, despite them having the same MIC as H37Rv and V4207 which were both killed in the presence of PAS. A similar finding was made by Mathys *et al* in 2009 when phenotypic MIC values obtained by the BACTEC /Alert and dilution assay were compared to the genotypic sequencing results. They had alsoobserved that mutations were not present in the *thyA* gene, despite the clinical isolates displaying resisitance. They then investigated 8 other genes that could confer resistance to PAS, but discovered that none of these sites had any mutations. This thus implies that mutations at the *thyA* gene are not necessary to reflect resistance to PAS. This supports our findings. Mathys *et al*, (2009) suggested that this resistance could be dependant on the PAS concentration. Perhaps the next step to determine if this is true is to test various concentrations of PAS in a TKE, in an effort to determine which concentration is most efficient at inhibiting growth of MTB.

Findings of previous studies suggest that mutations in both the *tlyA* and the *rrs* genes confer resistance to CAP (Jugheli *et al*., 2009; Maus *et al.*, 2005a and Maus *et al.*, 2005b). Our genotypic results however, are not supported by these findings. Our results indicate that there

were mutations in the *tlyA* gene at the A74G region, for all MDR-TB, pre-XDR-TB, mono-resistant-TB, one susceptible isolate and 3 out of the 5 XDR-TB isolates that were tested. This mutation was not present in the 2 remaining XDR-TB isolate as well as the susceptible control strain, H37Rv. This result is conflicting to the MICs that were obtained with both the agar dilution and the MTT assay, which implies that all isolates except KZN 605 are susceptible to CAP, based on the break point concentration proposed by WHO in 2009 (Table 10). According to the TKE, only KZN 605 continued to grow in the presence of CAP. This is conflicting, since V4207, V1435 and V4258, which were also tested in the TKE, also contained mutations in the *tlyA* gene, yet they were killed off in the presence of CAP. A similar finding was made by Engstrom *et al*, in 2011. Their study had a much larger sample size and also compared MIC values and genotypic sequencing information. It was discovered that not all of the mutations in the *tlyA* gene leads to resistance to capreomycin, and thus *tlyA* can not be used as a sensitive genetic marker (Engstrom *et al*., 2011). This was due to the fact that frameshift or missense mutations at this region of the *tlyA* gene do not lead to resistance to CAP. Perhaps the mutations that were displayed in the *tlyA* gene of the susceptible and MDR-TB isolates, in our study,which were still susceptible to CAP according to the break point concentrations by WHO, in 2009 (Table 10), were also these missense mutations. Thus this mutation was not a true reflection of resistance to CAP. Our study also sequenced the *rrs* gene. According to our sequencing results, all the XDR-TB isolates had mutations in A1401G region, except R252. This would imply that all isolates are resistant to CAP. This is supported by the MICs that were obtained in our study, where all isolates except R252 had an MIC of 32 µg/ml, which classifies it as resistant to CAP according to the WHO breakpoints (2009) (Table 10). The one XDR-TB isolate (R252), which did not have the mutation at the *rrs* gene, also had a lower MIC, making it susceptible to CAP. Thus mutations in the *rrs* gene, seem to be a better and more sensitive marker to CAP resistance than *tlyA*. This finding is also supported by the work of Engstrom *et a*l, (2011).

Sequencing results for LIN revealed no mutations in the *rrl1* region. The *rrl2* region, however, had mutations at the G2399A site in all the isolates tested except H37Rv and one (R252) of the 5 XDR-TB isolates. Surprisingly, all isolates had the same MIC value of 0.5µg/ml, and thus were all susceptible to LIN at this concentration. During the TKE, R252

was the only isolate which continued to grow in the presence of LIN, for the entire 28 day period. It is unlikey that this mutation at the *rrl2* region conferred this resistance to LIN, since it was also present in H37Rv, which was killed in the presence of LIN, during the same TKE, under the same conditions. It is possible that this mutation, could be a silent mutation, and does not affect the isolates resistance to LIN.

The results indicate drug tolerance of MTB for PAS, LIN and M&C. This differs from that with INH since INH tolerance is only seen in a subpopulation of the bacterial cells. The tolerance that is observed against the non-first line drugs involves the whole population of organisms of each tolerant isolate. This is a novel observation. Its clinical importance needs to be further investigated.

The tolerance phenomenon was only observed in MDR-TB isolates irrespective of whether these had evolved to XDR-TB or not. Due to the small number of isolates tested, this could be a chance observation. If this observation is confirmed when the number of isolates are increased, then this may reflect an additional mechanism of resistance. The mechanism on which this tolerance is based needs to be elucidated. Since the tolerance for the different drugs were not observed in the same isolate, more than one mechanism might be involved.
Perhaps the next step of this TKE is to test different concentrations of each drug at different time points, and to test various combinations of drugs, for e.g. it was reported that LIN has a synergistic relationship with fluoroquinolones (Diaz *et al.*, 2002). One could perform TKEs using various concentrations of two or more drugs to gain better insight of how these agents behave synergistically over time. The additional resistance to CAP which was illustrated in the results indicates that the XDR-TB strain that circulates in KZN can be classified as a TDR-TB strain. It is resistant to INH, RIF, PZA, EMB, ETH, SM, KAN, AMIK and the quinolones (Dookie *et al.*, 2012).

According to Olson *et al*, (2011) at least 3 to 4 new classes of anti-TB drugs are required to treat TDR-TB. The ability to develop these new drugs however, is hampered by various financial and technical factors. There is a 90 % failure rate from target identification to the regulatory approval of the new drug, and this often has a 50% failure rate even in Phase III

trials. This entire process will take at least 10 -14 years and often longer, when dealing with drugs for TB. The cost to develop just one drug is US $ 1.5 billion, from the discovery to the approval step. This cost however does not include the cost for follow up studies that are necessary to detect adverse events and side effects of the drug, after it has been launched, nor does it cover the manufacturing compliance and drug delivery costs. Although many prospective drugs are in their developmental stages, they may only be ready for use in a few years' time.

CONCLUSION

The agar dilution assay was reproducible for all SLD and TLD anti-TB drugs tested. The MTT assay provided MIC results that were reproducible to the agar dilution method for all anti-TB drugs except PAS. This illustrates that the MTT assay can be used as an alternate method of DST. An additional advantage of this assay is that results can be obtained within 9 days. This is important to speed up diagnosis and treatment options.TKEs provided insight to the rate of kill of the anti-TB drugs tested. It highlighted the phenomenon of drug tolerance for some of the MDR and XDR-TB isolates. This was observed when PAS, M&C, LIN were unable to kill off the population of these isolates over the 28 day testing period. This was unexpected as these isolates fell within the susceptible category based on the MIC results. Sequencing was used to resolve discrepancies of the susceptibility patterns that were observed using the phenotypic methods of DST. The sequencing results however did not fully resolve these discrepancies as mutations that were detected for CAP and LIN, were also present in the susceptible isolates. Additionally, one of the XDR-TB isolates resistant to CAP did not have any mutations present in the *rrs* or *tlyA* gene. Thus indicating other means of DR. This work shows that three of the four alternative drugs for the treatment of patients in KZN that are infected with the TDR-TB strain are active *in vitro*. However, LIN and PAS have such serious side effects that most patients will not be able to complete a full 2 year course with these drugs. In addition, further investigations are needed to establish the role in clinical practice of the tolerance phenomenon that was observed.

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix 1

Solutions used for retrieval of isolate and agar dilution experiment.

1.1 Middlebrook 7H9 Broth (For DST use)

Middlebrook 7H9 broth (Becton Dickinson & Company, Difco Laboratories, USA)	4.7g
Bacto Casitone (Becton Dickinson & Company, Difco Laboratories, USA)	1g
Glycerol (Merck, SA)	2ml
Distilled water	900ml
OADC (Becton Dickinson and company, USA)	100ml

The broth powder was weighed out and added to a flask containing the water.
Bacto casitone and glycerol were added. The contents in the flask was dissolved using a magnetic stirrer bar on a magnetic plate. The top of the flask was then covered with foil and autoclaved for 15 minutes at 121 °C. The broth was then cooled to room temperature and the OADC was added. PACT was not added to the broth when it was used for DST use, as these antibiotics may have influenced the results by affecting growth of MTB.

For growth use: PACT was added to the 1 L of broth at the following volumes:

Polymyxin B (Sigma-Aldrich, USA)	200.000 IU/L
Amphotericin B (Sigma-Aldrich, USA),	20mg/L
Carbenicillin (Sigma-Aldrich, USA)	100mg/L
Trimethoprim (Sigma-Aldrich, USA)	20mg/L

1.2 Middlebrook 7H11 agar

Middlebrook 7H11 agar (Becton Dickinson & Company, Difco Laboratories, USA)	21g
Bacto Casitone (Becton Dickinson & Company, Difco Laboratories,	

USA)	1g
Glycerol (Merck, SA)	5ml
Distilled water	900ml
L-asparagine (Sigma- Aldrich, USA)	5g
OADC (Becton Dickinson and company, USA)	100ml

Middlebrook powder was weighed out and added to a flask containing the water. The Bacto casitone and L asparagine were then weighed out and also added. The glycerol was added to the flask. The solution was dissolved using a magnetic stirrer bar. The top of the flask was then covered with foil and autoclaved at 121 °C for 15 minutes.

The flask was taken out and allowed to cool in a water bath before OADC was added. The flask was swirled gently and thereafter plates were poured aseptically.

1.3 MTB storage media

Protease peptone (Becton Dickinson & Company, Difco Laboratories, USA)	4g
glycerol (Merck, SA)	32ml
Distilled water	400ml

All reagents were added to the 400 ml of water in the flask. This was dissolved with the aid of a magnetic stirrer.

The contents was then autoclaved at 121°C for 15 minutes

1.4 Anti-TB drugs

1.4.1 Isoniazid (Sigma-Aldrich, USA):

A stock solution of 800µg/ml was made in sterile deionised water. This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and 7 double dilutions were performed. This was done to obtain 7 working concentrations: (400µg/ml; 200µg/ml; 100µg/ml; 50µg/ml; 25µg/ml; 12.5µg/ml; 6.25µg/ml). Therefore when 1 ml of the 7 above mentioned concentrations is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentrations of (4µg/ml; 2µg/ml; 1µg/ml; 0.5µg/ml; 0.25µg/ml; 0.125µg/ml; 0.0625µg/ml) was achieved.

1.4.2 Capreomycin in the form of capreomycin sulphate from *Streptomyces capreolus* (Sigma-Aldrich, USA):

A stock solution of 12800µg/ml was made in sterile deionised water. This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and 7 double dilutions were performed. This was done to obtain 7 working concentrations: (6400µg/ml; 3200µg/ml; 1600µg/ml; 800µg/ml; 400µg/ml; 200µg/ml; 100µg/ml). Therefore when 1 ml of the 7 above mentioned concentrations is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentrations of (64µg/ml; 32µg/ml; 16µg/ml; 8µg/ml; 4µg/ml; 2µg/ml; 1µg/ml) was achieved.

1.4.3 Para-aminosalicylic acid in the form of 4-Aminosalicylic acid (Sigma-Aldrich, USA):

A stock solution of 3200µg/ml was made in sterile deionised water. This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and 7 double dilutions were performed. This was done to obtain 7 working concentrations: (1600µg/ml; 800µg/ml; 400µg/ml; 200µg/ml; 100µg/ml; 50µg/ml; 25µg/ml). Therefore when 1 ml of the 7 above mentioned concentrations is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentrations of (16µg/ml; 8µg/ml; 4µg/ml; 2µg/ml; 1µg/ml; 0.5µg/ml; 0.25µg/ml) was achieved.

1.4.4 Linezolid in the form of zyvox (Pfizer, SA):

A stock solution of 1600µg/ml was made in sterile deionised water. This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and 7 double dilutions were performed. This was done to obtain 7 working concentrations: (800µg/ml; 400µg/ml; 200µg/ml; 100µg/ml; 50µg/ml; 25µg/ml; 12.5µg/ml). Therefore when 1 ml of the 7 above mentioned concentrations is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentrations of (8µg/ml; 4µg/ml; 2µg/ml; 1µg/ml; 0.5µg/ml; 0.25µg/ml; 0.125ug/ml) was achieved.

1.4.5 Meropenem in the form of meronem (AstraZeneca, SA):

A stock solution of 1600µg/ml was made in sterile deionised water. This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and 7 double dilutions were performed. This was done to obtain 7 working concentrations: (800µg/ml; 400µg/ml; 200µg/ml; 100µg/ml; 50µg/ml; 25µg/ml; 12.5µg/ml). Therefore when 1 ml of the 7 above mentioned concentrations is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentrations of (8µg/ml; 4µg/ml; 2µg/ml; 1µg/ml; 0.5µg/ml; 0.25µg/ml; 0.125ug/ml) was achieved.

1.4.6 Clavulanic acid in the form of potassium clavulanate from clavuligerus (Sigma-Aldrich, USA):

A stock solution of 1600µg/ml was made in sterile 0.1M phosphate buffer at a pH of 6 (Appendix 1.5). This was filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and only 1 double dilutions was performed. This was done to obtain 1 working concentration: (800µg/ml). Therefore when 1 ml of the above mentioned concentration is added to 100ml of Middlebrook 7H10 media to make the agar dilutions, then the required concentration of (8µg/ml) was achieved.

1.5 0.1M Phosphate buffer at pH 6

Potassium dihydrogen orthophosphate (Merck, SA) KH_2PO_4 1.36g

di-Sodium hydrogen orthophosphate (Merck, SA) Na_2HPO_4 1.42 g

KH_2PO_4 (molar mass 136.09) and Na_2HPO_4 (molar mass 141.96) were weighed out and added to 80 ml of distilled water. This solution was adjusted to a pH of 6, and the volume increased to 100ml, with water. Aliquots of 10ml were made and were autoclaved at 121 for 15 minutes.

1.6 Middlebrook 7H10agar plates

Middlebrook 7H10 agar (Becton Dickinson & Company, Difco Laboratories, USA) 19g

Bacto Casitone (Becton Dickinson & Company, Difco Laboratories, USA) 1g

Glycerol (Merck, SA)	5ml
Distilled water	900ml
OADC (Becton Dickinson and company, USA)	100ml

Middlebrook powder was weighed out and added to a flask containing the water. The Bacto casitone was then weighed out and also added. The glycerol was added to the flask. The solution was dissolved using a magnetic stirrer bar. The top of the flask was then covered with foil and autoclaved at 121 °C for 15 minutes.

The flask was taken out and allowed to cool in a water bath before OADC and the anti-TB drug (at the required concentration) was added. The flask was swirled gently and thereafter plates were poured aseptically.

1.7 Phosphate Buffered Saline (PBS) (Sigma-Aldrich, USA)

1 Tablet was dissolved in 200 ml of sterile distilled water.

The solution was autoclaved at 121° C for 15 minutes.

1.8 0.05 % Tween 80 (Sigma-Aldrich, USA)

0.05ml Tween 80 was added to 80 ml of sterile distilled water. This was stirred and dissolved. If Tween still did not dissolve, the solution could be heated up to 56°C. The entire solution was then filter-sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA). This solution can be stored for up to 2 months at room temperature.

1.9 20% Tween 80 (Sigma-Aldrich, USA)

20 ml of Tween 80 was added to 80 ml sterile distilled H_2O. This was stirred and dissolved. If Tween still did not dissolve, the solution could be heated up to 56°C. The entire solution was then filter-sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA). This solution can be stored for up to 2 months at room temperature.

1.10 McFarland No. 1 Standard

a) 0.5 ml of concentrated H_2SO_4 (Merck, SA) was added to 49.5 ml of triple distilled water, to make a 1 % H_2SO_4 solution.

b) 0.175g of $BaCl_2.2H_2O$ (Sigma-Aldrich, USA) was added to 10 ml of triple distilled water to make a 1 % $BaCl_2.2H_2O$ solution.

Thereafter 4.950 ml of 1 % H_2SO_4 solution was combined with 50 µl of 1 % $BaCl_2.2H_2O$ solution. The tube containing this McFarland 1 suspension was then stored in foil or away from direct sunlight.

Appendix 2

MTT Assay solutions

2.1 MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5- diphenyltetrazolium bromide (5mg/ml) (Sigma Aldrich, USA)

0,1g of the MTT salt was weighed out and added to 20ml PBS of pH 6.8 (Appendix 1.7)

Three separate tubes of this solution were made. The tubes were vortexed, however if there was difficulty with dissolving, then tubes were placed in a water bath of 25°C for about 15-30 minutes.

Using a water bath at a higher temperature causes the MTT solution to change colour, and thus the tubes have to be monitored regularly. The MTT solution was then filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA) and tubes were covered in foil and stored at 4 °C.

2.2 20 % Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA)

2g of SDS was weighed and added to 10ml of distilled water. This solution was not vortexed, but instead it was inverted and place in a water bath at 30-35°C to help dissolve all the SDS. This was then filter sterilised using a Millipore 0.22 µm pore size filter unit (Merck Millipore, SA)

SDS is hazardous and the fine particles are able to disperse easily. This should be weighed out and made in a fume hood or while wearing a safety face mask.

2.3 50 % aqueous N, N Dimethylformamide (DMF) (Sigma-Aldrich, USA)

Equal volumes of DMF and distilled water were combined to make a 50 % solution i.e. 20 ml of DMF and 20 ml of distilled water.

This was stored in an amber bottle or in a bottle covered with foil and stored away from direct light.

2.4 1: 1 vol/vol SDS-DMF solution (of the 20% SDS and 50 % DMF solution)

Equal volumes of each of the solutions were combined i.e. 30 ml of the 20 % SDS solution to 30 ml of the 50 % DMF.

SDS was added to the DMF, instead of vice versa, as this prevents a white precipitate from forming. This SDS-DMF solution was stored in a amber bottle or bottle covered in foil and kept away from light.

Appendix 3

Time kill experiment solutions

3.1 20 % Tyloxapol (Sigma-Aldrich, USA)

2ml of tyloxapol was added to 8 ml of sterile distilled water.

This was then sterilised by filtering through Millipore 0.22 µm pore size filter unit (Merck Millipore, SA).

3.2 PBS and 20 % Tyloxapol (Both : Sigma-Aldrich, USA)

625µl of this solution was added to 250 ml of PBS (Appendix 1.7) and was used to make 900 µl aliquots for the dilution blanks for the TKE.

The remaining 20 % tyloxapol solution was stored away at 4°C.

Appendix 4

DNA isolation and sequencing solutions

4.1 10x TE Buffer

Trizma base (Sigma-Aldrich, USA) 1.21g

EDTA (Sigma-Aldrich, USA) 0.37g

The trizma base was weighed out and dissolved in 80ml of distilled water. It was the adjusted to a pH of 8 using concentrated HCl (Merck).The EDTA was then added and dissolved. The final pH of the entire suspension was checked and adjusted to a final volume of 100ml. This was then autoclaved at 121°C for 15 minutes.

This was then used to make a 1x TE Buffer.

1x TE Buffer

10ml of 10x TE Buffer was diluted into 90ml of triple distilled water. This solution was stored at room temperature.

4.2 Lysozyme (10mg/ml) (Sigma-Aldrich, USA)

1ml of distilled water was added to 10mg of lysozyme powder. This solution was stored at 4°C until use.

4.3 Proteinase K (10mg/ml) (Roche Diagnostics)

10ml of distilled water was added to 100mg of proteinase K powder. This solution was store at 4°C until use.

4.4 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA)

10g of SDS powder weighed and dissolve in 100ml of distilled water. Refer to Appendix 2.2 for more details on how to work with SDS.

4.5 5M Sodium Chloride (NaCl) (Sigma-Aldrich, USA)

14.6 g of NaCl powder was weighed out and dissolved in 50ml of distilled water. This solution was then autoclaved at 121°C for 15 minutes.

4.6 CTAB-NaCl Solution

NaCl (Sigma-Aldrich, USA) 4.1g

CTAB (Sigma-Aldrich, USA) 10g

The above powders were weighed out and dissolved in 100ml of distilled water. The solution was heated to 65°C until powders are completely dissolved.

4.7 Chloroform: Isoamyl alcohol (24:1) (Sigma-Aldrich, USA)

1ml of isoamyl alcohol (Sigma-Aldrich, USA) was added to 24ml of chloroform (Sigma-Aldrich, USA).

4.8 70% ethanol

70ml of absolute ethanol (Merck, SA) was added to 30ml of distilled water. Store at -20°C.

4.9 1% agarose gel (140ml)

Agarose powder (Lonza, USA)	1.4g
1x TBE buffer	140ml
Ethidium bromide	140µl

The agarose powder was weighed and added to a flask containing 1x TBE buffer (Appendix 4.10). The mixture was heated in a microwave to dissolve the powder. It was then allowed to cool and ethidium bromide was added.

4.10 10xTBE Buffer

Trizma base (Sigma-Aldrich, USA)	108g
Boric acid (Sigma-Aldrich, USA)	55g
EDTA (Sigma-Aldrich, USA)	9.3g

The above reagents were weighed out and dissolved in 1000ml of distilled water.

1x TBE

100ml of 10x TBE was added to 900ml of distilled water.

4.11 Ethidium Bromide (5µg/ml)

10mg of ethidium bromide powder was weighed out and dissolved in 20ml of distilled water. The bottle was then wrapped in foil and stored at 4°C. Great care must be taken when working with ethidium bromide and nitrile gloves should be used.

4.12 Sample loading dye

1% Double dye

Bromphenol Blue (Sigma-Aldrich, USA)	1g
Xylene cyanole (Sigma-Aldrich, USA)	1g

The above powders were weighed out and dissolved in 100ml of distilled water.

50 ml of loading dye was then prepared from the above 1% Double Dye (DD) stock solution

10x TBE	5 ml
Glycerol (Merck, SA)	25 ml
1% Double dye	5 ml

The required amounts of the above mentioned reagents were added to 15ml of distilled water. This was then dissolved properly and stored at 4°C until it was used for gel electrophoresis.

4.13 3M Sodium Acetate NaOAc (Sigma Aldrich, USA) at a pH of 5.2

40.8 g of NaOAc was weighed out and dissolved in 70 ml of deionised water.

Glacial acetic acid was used to obtain a pH of 5.2. The volume was increased to a final volume of 100ml.

Appendix 5

Formula for calculations performed to construct a TKC from raw data

Time kill curves were constructed by adding the colony counts of each triplicate set (A, B, C). These sets were also done in duplicate (A1, A2, B1, B2, C1, C2)

Therefore for each of the 6 time points (Day 0, Day 3, Day 7, Day 14, Day 21, and Day 28) an average of the total colony counts were calculated. This was divided by 20 (as only 50µl out of the total 1000µl was inoculated to do the colony counts) and multiplied by the dilution factor that gave a countable number of colonies (10, 100, 1000, 10000, 100000). This value was then converted to a log value.

A final average log value for the Log A, Log B and Log C was used for each isolate for the specific drugs

$$\text{Log}\left\{\frac{\text{(Sum of colony count A1*+A2*)}}{20}\ \text{X Dilution Factor}\right\}$$

Note * : the A1* and A2* values in the above formula were substituted by B1 and B2, C1 and C2 values, to get a total average log value of the entire colony count data for the triplicate experiment at each time point.

This final average value was used to construct the time kill curves (Figure 1-Figure 11).

Refer to the example (Below) of how the raw data was used to calculate the log values which were in turn used to construct the time kill curves for each isolate.

Appendix 6

Raw data used to calculate log values for the time kill curves

H37Rv A1 and A2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	34100	2130000	146000000	174000000	192000000	219000000
INH	69000	0	6300	2170000	2310000	1170000
PAS	191000	126000	3270000	2950000	3310000	2750000
CAP	118000	0	1100	0	0	0
LIN	73000	32100	0	0	0	0

Log Values of H37Rv A1 and A2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.53	6.33	8.16	8.24	8.28	8.34
INH	4.84	0	3.8	6.34	6.36	6.07
PAS	5.28	5.1	6.51	6.47	6.52	6.44
CAP	5.07	0	3.04	0	0	0
LIN	4.86	4.51	0	0	0	0

H37Rv B1 and B2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	51000	1630000	114000000	31100000	212000000	295000000
INH	131000	1480000	12800	0	0	3490000
PAS	146000	84000	145000	0	0	0
CAP	56000	700	800	0	0	0
LIN	100000	0	0	0	0	0

Log Values of H37Rv B1 and B2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.71	6.21	8.06	7.5	8.33	8.47
INH	5.12	6.17	4.11	0	0	6.54
PAS	5.16	4.92	5.16	0	0	0
CAP	4.75	2.85	2.9	0	0	0
LIN	5	0	0	0	0	0

H37Rv C1 and C2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	32400	2900000	139000000	13900000	188000000	261000000
INH	56000	1200	17400	1790000	55000000	2900000
PAS	56000	150000	265000	25600	0	0
CAP	99000	0	500	0	0	0
LIN	174000	258000	121000	31000	19900	0

Log Values of H37Rv C1 and C2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.51	6.46	8.14	7.14	8.27	8.42
INH	4.75	3.08	4.24	6.25	7.74	6.46
PAS	4.75	5.18	5.42	4.41	0	0
CAP	5	0	2.7	0	0	0
LIN	5.24	5.41	5.08	4.49	4.3	0

Average of H37Rv log values for A1,A2, B1, B2, C1 and C2 for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.58	6.33	8.12	7.63	8.29	8.41
INH	4.9	3.08	4.05	4.2	4.7	6.36
PAS	5.06	5.07	5.7	3.63	2.17	2.15
CAP	4.94	0.95	2.88	0	0	0
LIN	5.03	3.31	1.69	1.5	1.43	0

H37Rv A1 and A2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	24000	3230000	126000000	306000000	500000000	500000000
INH	158000	16400	13100	4800	30500	3010000
M&C	29400	20300	11700	2700	0	0

Log Values of H37Rv A1 and A2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.38	6.51	8.1	8.49	8.7	8.7
INH	5.2	4.21	4.12	3.68	4.48	6.48
M&C	4.47	4.31	4.07	3.43	0	0

H37Rv B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	24600	2690000	99000000	269000000	500000000	500000000
INH	146000	9600	6500	2600	25700	2070000
M&C	37000	19900	10000	1700	0	0

Log Values of H37Rv B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.39	6.43	8	8.43	8.7	8.7
INH	5.16	3.98	3.81	3.41	4.41	6.32
M&C	4.57	4.3	4	3.23	0	0

H37Rv C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	28700	23400000	149000000	332000000	500000000	500000000
INH	221000	11400	9700	3900	28300	3120000
M&C	32400	22700	12700	2300	0	0

Log Values of H37Rv C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.46	7.37	8.17	8.52	8.7	8.7
INH	5.34	4.06	3.99	3.59	4.45	6.49
M&C	4.51	4.36	4.1	3.36	0	0

Average of H37Rv log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.41	6.77	8.09	8.48	8.7	8.7
INH	5.23	4.08	3.97	3.56	4.45	6.43
M&C	4.52	4.32	4.06	3.34	0	0

Final log values used to construct the time kill curves for H37Rv

Total Average of Log values for H37Rv A1, A2, B1, B2, C1, C2 for TKE 1 & TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.5	6.55	8.11	8.05	8.5	8.56
INH	5.07	3.58	4.01	3.88	4.57	6.39
PAS	5.06	5.07	5.7	3.63	2.17	2.15
CAP	4.94	0.95	2.88	0	0	0
LIN	5.03	3.31	1.69	1.5	1.43	0
M&C	4.52	4.32	4.06	3.34	0	0

Raw data used to calculate log values for the V4207 time kill curves

V4207 A1 and A2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	85000	258000	640000	1110000	1550000	1990000
INH	104000	0	0	0	0	0
PAS	112000	143000	14700	0	0	0
CAP	88000	5000	0	0	0	0
LIN	56000	143000	0	0	0	0

Log Values of V4207 A1 and A2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.93	5.41	5.81	6.05	6.19	6.3
INH	5.02	0	0	0	0	0
PAS	5.05	5.16	4.17	0	0	0
CAP	4.94	3.7	0	0	0	0
LIN	4.75	5.16	0	0	0	0

V4207 B1 and B2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	57000	277000	1010000	1620000	2110000	2760000
INH	147000	225000	0	0	0	0
PAS	134000	245000	900	0	0	0
CAP	103000	500	0	0	0	0
LIN	89000	253000	11500	0	0	0

Log Values of V4207 B1 and B2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.76	5.44	6	6.21	6.32	6.44
INH	5.17	5.35	0	0	0	0
PAS	5.13	5.39	2.95	0	0	0
CAP	5.01	2.7	0	0	0	0
LIN	4.95	5.4	4.06	0	0	0

V4207 C1 and C2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	50000	153000	380000	540000	920000	1260000
INH	318000	52000	0	0	0	0
PAS	57000	299000	900	0	0	0
CAP	134000	4000	0	0	0	0
LIN	68000	8200	239000	0	0	0

Log Values of V4207 C1 and C2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.7	5.18	5.58	5.73	5.96	6.1
INH	5.5	4.72	0	0	0	0
PAS	4.76	5.48	2.95	0	0	0
CAP	5.13	3.6	0	0	0	0
LIN	4.83	3.91	5.38	0	0	0

Average of V4207 log values for A1,A2, B1, B2, C1 and C2 for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.8	5.34	5.8	6	6.16	6.28
INH	5.23	3.36	0	0	0	0
PAS	4.98	5.34	3.36	0	0	0
CAP	5.03	3.33	0	0	0	0
LIN	4.84	4.82	3.15	0	0	0

V4207 A1 and A2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	1730000	24300000	178000000	353000000	700000000	700000000
INH	382000	315000	28000	14900	5300	1900
M&C	78000	24100	11400	0	0	0

Log Values of V4207 A1 and A2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.24	7.39	8.25	8.55	8.85	8.85
INH	5.58	5.5	4.45	4.17	3.72	3.28
M&C	4.89	4.38	4.06	0	0	0

V4207 B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	1700000	20500000	160000000	357000000	700000000	700000000
INH	336000	32800	25600	10800	2100	400
M&C	116000	27500	17200	0	0	0

Log Values of V4207 B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.23	7.31	8.2	8.55	8.85	8.85
INH	5.53	4.52	4.41	4.03	3.32	2.6
M&C	5.06	4.44	4.24	0	0	0

V4207 C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	1730000	25900000	180000000	308000000	700000000	700000000
INH	3180000	326000	29700	15700	5400	2500
M&C	146000	25300	15600	0	0	0

Log Values of V4207 C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.24	7.41	8.26	8.49	8.85	8.85
INH	6.5	5.51	4.47	4.2	3.73	3.4
M&C	5.16	4.4	4.19	0	0	0

Average of V4207 log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.24	7.37	8.24	8.53	8.85	8.85
INH	5.87	5.18	4.44	4.13	3.59	3.09
M&C	5.04	4.41	4.16	0	0	0

Final log values used to construct Time kill curves for V4207

Total Average of Log values for V4207 A1, A2, B1, B2, C1, C2 for TKE 1 and TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.52	6.36	7.02	7.26	7.5	7.57
INH	5.55	4.27	2.22	2.07	1.8	1.55
PAS	4.98	5.34	3.36	0	0	0
CAP	5.03	3.33	0	0	0	0
LIN	4.84	4.82	3.15	0	0	0
M&C	5.04	4.41	4.16	0	0	0

Raw data used to calculate log values for the KZN 605 time kill curves

KZN 605 A1 and A2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	1810000	16800000	19900000	11700000	400000000	400000000
INH	2470000	400000000	400000000	400000000	400000000	600000000
PAS	370000	2760000	419000	400000	0	0
CAP	7100000	2130000	510000	302000	192000	2760000
LIN	880000	300000	940000	0	0	0

Log Values of KZN 605 A1 and A2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.26	7.23	7.3	7.07	8.6	8.6
INH	6.39	8.6	8.6	8.6	8.6	8.78
PAS	5.57	6.44	5.62	5.6	0	0
CAP	6.85	6.33	5.71	5.48	5.28	6.44
LIN	5.94	5.48	5.97	0	0	0

KZN 605 B1 and B2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	2370000	18800000	13500000	400000000	400000000	600000000
INH	1240000	400000000	400000000	400000000	400000000	600000000
PAS	332000	1710000	1480000	400000	0	0
CAP	1730000	2410000	640000	293000	236000	12500000
LIN	1450000	3100000	82000	4300	0	0

Log Values of KZN 605 B1 and B2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.37	7.27	7.13	8.6	8.6	8.78
INH	6.09	8.6	8.6	8.6	8.6	8.78
PAS	5.52	6.23	6.17	5.6	0	0
CAP	6.24	6.38	5.81	5.47	5.37	7.1
LIN	6.16	6.49	4.91	3.63	0	0

KZN 605 C1 and C2 Mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	1740000	288000000	29800000	400000000	400000000	800000000
INH	1850000	400000000	400000000	400000000	400000000	600000000
PAS	2130000	1510000	1480000	400000	0	0
CAP	1330000	2960000	590000	18100	82000	2720000
LIN	2000000	2920000	322000	37700	14900	0

Log Values of KZN 605 C1 and C2 mean colony count values for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.24	8.46	7.47	8.6	8.6	8.9
INH	6.27	8.6	8.6	8.6	8.6	8.78
PAS	6.33	6.18	6.17	5.6	0	0
CAP	6.1	6.47	5.77	4.26	4.91	6.43
LIN	6.3	6.47	5.51	4.58	4.17	0

Average of KZN 605 log values for A1,A2, B1, B2, C1 and C2 for TKE 1						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.29	7.65	7.3	8.09	8.6	8.76
INH	6.25	8.6	8.6	8.6	8.6	8.78
PAS	5.81	6.28	5.99	5.6	0	0
CAP	6.4	6.39	5.76	5.07	5.19	6.66
LIN	6.13	6.15	5.46	2.74	1.39	0

KZN 605 A1 and A2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	2380000	16100000	226000000	500000000	700000000	700000000
INH	1790000	374000	3590000	28800000	500000000	500000000
M&C	364000	25100	22000	16600	9200	3600

Log Values of KZN 605 A1 and A2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.38	7.21	8.35	8.7	8.85	8.85
INH	6.25	5.57	6.56	7.46	8.7	8.7
M&C	5.56	4.4	4.34	4.22	3.96	3.56

KZN 605 B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	2020000	14200000	190000000	500000000	700000000	700000000
INH	1830000	343000	3710000	31700000	500000000	500000000
M&C	359000	29700	22900	14900	6300	2600

Log Values of KZN 605 B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.31	7.15	8.28	8.7	8.85	8.85
INH	6.26	5.54	6.57	7.5	8.7	8.7
M&C	5.55	4.47	4.36	4.17	3.8	3.41

KZN 605 C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	2450000	13500000	232000000	500000000	700000000	700000000
INH	360000	2110000	3500000	31500000	500000000	500000000
M&C	335000	31300	22300	15700	8600	2000

Log Values of KZN 605 C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.39	7.13	8.37	8.7	8.85	8.85
INH	5.56	6.32	6.54	7.5	8.7	8.7
M&C	5.53	4.5	4.35	4.2	3.93	3.3

Average of KZN 605 log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.36	7.16	8.33	8.7	8.85	8.85
INH	6.02	5.81	6.56	7.49	8.7	8.7
M&C	5.55	4.46	4.35	4.2	3.9	3.42

Final log values used to construct time kill curves for KZN 605

Total Average of Log values for KZN605 A1, A2, B1, B2, C1, C2 for TKE 1 &TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.33	7.41	7.82	8.4	8.73	8.81
INH	6.14	7.21	7.58	8.04	8.65	8.74
PAS	5.81	6.28	5.99	5.6	0	0
CAP	6.4	6.39	5.76	5.07	5.19	6.66
LIN	6.13	6.15	5.46	2.74	1.39	0
M&C	5.55	4.46	4.35	4.2	3.9	3.42

Raw data used to calculate log values for the R252 time kill curves

R 252 A1 and A2 Mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	59000	1390000	2170000	4500000	28100000	35500000
INH	21100	0	3400	460000	31700	240000
PAS	36300	40000	1670000	3010000	2890000	11600000
CAP	86000	0	0	0	0	0
LIN	49000	246000	50000	16800	352000	1160000

Log Values of R 252 A1 and A2 mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	4.77	6.14	6.34	6.65	7.45	7.55
INH	4.32	0	3.53	5.66	4.5	5.38
PAS	4.56	4.6	6.22	6.48	6.46	7.06
CAP	4.93	0	0	0	0	0
LIN	4.69	5.39	4.7	4.23	5.55	6.06

R 252 B1 and B2 Mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	46000	3250000	3520000	19300000	31000000	35600000
INH	28700	2600	2400	153000	15500	99000
PAS	37000	40000	1350000	3090000	2110000	9300000
CAP	154000	5300	500	0	0	0
LIN	54000	228000	32700	19600	1520000	3370000

Log Values of R 252 B1 and B2 mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	4.66	6.51	6.55	7.29	7.49	7.55
INH	4.46	3.41	3.38	5.18	4.19	5
PAS	4.57	4.6	6.13	6.49	6.32	6.97
CAP	5.19	3.72	2.7	0	0	0
LIN	4.73	5.36	4.51	4.29	6.18	6.53

R 252 C1 and C2 Mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	50000	760000	860000	2370000	23800000	27900000
INH	33700	4700	100	253000	1880000	940000
PAS	205000	274000	1010000	3760000	2620000	7400000
CAP	162000	8900	500	100	0	0
LIN	52000	86000	23200	17000	1700000	1010000

Log Values of R 252 C1 and C2 mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	4.7	5.88	5.93	6.37	7.38	7.45
INH	4.53	3.67	2	5.4	6.27	5.97
PAS	5.31	5.44	6	6.58	6.42	6.87
CAP	5.21	3.95	2.7	2	0	0
LIN	4.72	4.93	4.37	4.23	6.23	6

Average of R252 log values for A1,A2, B1, B2, C1 and C2 for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	4.71	6.18	6.27	6.77	7.44	7.52
INH	4.44	2.36	2.97	5.41	4.99	5.45
PAS	4.81	4.88	6.12	6.52	6.4	6.97
CAP	5.11	2.56	1.8	0.67	0	0
LIN	4.71	5.23	4.53	4.25	5.99	6.2

R 252 A1 and A2 Mean colony count values for TKE 3						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	169000	2400000	212000000	421000000	500000000	700000000
INH	33300	29800	279000	355000	22100000	365000000
M&C	112000	30000	15300	12600	7000	4400

Log Values of R 252 A1 and A2 mean colony count values for TKE 3						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	5.23	6.38	8.33	8.62	8.7	8.85
INH	4.52	4.47	5.45	5.55	7.34	8.56
M&C	5.05	4.48	4.18	4.1	3.85	3.64

R 252 B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	237000	2700000	240000000	433000000	500000000	700000000
INH	146000	26100	262000	376000	22100000	270000000
M&C	35100	30400	17000	12300	7000	4000

Log Values of R 252 B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.37	6.43	8.38	8.64	8.7	8.85
INH	5.16	4.42	5.42	5.58	7.34	8.43
M&C	4.54	4.48	4.23	4.09	3.85	3.6

R 252 C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	234000	2460000	242000000	500000000	500000000	700000000
INH	32200	22900	311000	315000	3770000	254000000
M&C	37700	24700	21700	16200	10100	6500

Log Values of R 252 C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.37	6.39	8.38	8.7	8.7	8.85
INH	4.51	4.36	5.49	5.5	6.58	8.4
M&C	4.58	4.39	4.34	4.21	4	3.81

Average of R252 log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.32	6.4	8.36	8.65	8.7	8.85
INH	4.73	4.42	5.45	5.54	7.09	8.46
M&C	4.73	4.45	4.25	4.13	3.9	3.68

Final log values used to construct time kill curves for R 252

Total Average of Log values for R 252 A1, A2, B1, B2, C1, C2 for TKE 2 &TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.02	6.29	7.32	7.71	8.07	8.18
INH	4.58	3.39	4.21	5.48	6.04	6.96
PAS	4.81	4.88	6.12	6.52	6.4	6.97
CAP	5.11	2.56	1.8	0.67	0	0
LIN	4.71	5.23	4.53	4.25	5.99	6.2
M&C	4.72	4.45	4.25	4.133	3.9	3.68

Raw data used to calculate log values for the V4258 time kill curves

V4258 A1 and A2 Mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	195000	382000	20300000	11800000	21400000	76000000
INH	147000	0	3800	215000	1850000	14400000
PAS	206000	137000	144000	330000	219000	0
CAP	75000	10600	200	0	0	0
LIN	73000	255000	298000	11500	600	0

Log Values of V4258 A1 and A2 mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	5.29	5.58	7.31	7.07	7.33	7.88
INH	5.17	0	3.58	5.33	6.27	7.16
PAS	5.31	5.14	5.16	5.52	5.34	0
CAP	4.88	4.03	2.3	0	0	0
LIN	4.86	5.41	5.47	4.06	2.78	0

V4258 B1 and B2 Mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	149000	220000	15100000	102000000	24000000	58000000
INH	59000	1200	800	157000	97000	12300000
PAS	137000	134000	40000	180000	250000	0
CAP	78000	12700	0	0	0	0
LIN	302000	650000	238000	10300	900	0

Log Values of V4258 B1 and B2 mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	5.17	5.34	7.18	8.01	7.38	7.76
INH	4.77	3.08	2.9	5.2	4.99	7.09
PAS	5.12	5.13	4.6	5.26	5.4	0
CAP	4.89	4.1	0	0	0	0
LIN	5.48	5.81	5.38	4.01	2.95	0

V4258 C1 and C2 Mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	240000	810000	21100000	164000000	33800000	31200000
INH	217000	1200	700	293000	3310000	14000000
PAS	92000	144000	21000	185000	87000	50000
CAP	41000	12700	600	0	0	0
LIN	89000	700000	237000	19500	0	0

Log Values of V4258 C1 and C2 mean colony count values for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	5.38	5.91	7.32	8.21	7.53	7.49
INH	5.34	3.08	2.85	5.47	6.52	7.15
PAS	4.96	5.16	4.32	5.27	4.94	4.7
CAP	4.61	4.1	2.78	0	0	0
LIN	4.95	5.85	5.37	4.29	0	0

Average of V4258 log values for A1,A2, B1, B2, C1 and C2 for TKE 2						
	Day 0	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
DFC	5.28	5.61	7.27	7.76	7.41	7.71
INH	5.09	2.05	3.11	5.33	5.93	7.13
PAS	5.13	5.14	4.69	5.35	5.23	1.57
CAP	4.79	4.08	1.69	0	0	0
LIN	5.1	5.69	5.41	4.12	1.91	0

V4258 A1 and A2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	248000	2120000	26500000	336000000	500000000	500000000
INH	199000	305000	2990000	114000000	351000000	500000000
M&C	162000	6700	0	0	0	0

Log Values of V4258 A1 and A2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.39	6.33	7.42	8.53	8.7	8.7
INH	5.3	5.48	6.48	8.1	8.55	8.7
M&C	5.21	3.83	0	0	0	0

V4258 B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	237000	1880000	21800000	270000000	500000000	500000000
INH	182000	172000	3200000	130000000	430000000	500000000
M&C	238000	5700	0	0	0	0

Log Values of V4258 B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.37	6.27	7.34	8.43	8.7	8.7
INH	5.26	5.24	6.51	8.11	8.63	8.7
M&C	5.38	3.76	0	0	0	0

V4258 C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	324000	2660000	29000000	332000000	500000000	500000000
INH	224000	175000	3030000	62000000	222000000	500000000
M&C	141000	6700	0	0	0	0

Log Values of V4258 C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.51	6.42	7.46	8.52	8.7	8.7
INH	5.35	5.24	6.48	7.8	8.35	8.7
M&C	5.15	3.83	0	0	0	0

Average of V4258 log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.42	6.34	7.41	8.49	8.7	8.7
INH	5.3	5.32	6.49	8	8.51	8.7
M&C	5.25	3.81	0	0	0	0

Final log values used to construct time kill curves for V4258

Total Average of Log values for V4258 A1, A2, B1, B2, C1, C2 for TKE 2 &TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.35	5.98	7.34	8.13	8.06	8.21
INH	5.2	3.69	4.8	6.67	7.22	7.92
PAS	5.13	5.14	4.69	5.35	5.23	1.57
CAP	4.79	4.08	1.69	0	0	0
LIN	5.1	5.69	5.41	4.12	1.91	0
M&C	5.25	3.81	0	0	0	0

Raw data used to calculate log values for the V1435 time kill curves

V 1435 A1 and A2 Mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	660000	2600000	3420000	17700000	26000000	16200000
INH	280000	322000	94000	295000	3010000	36000
PAS	145000	0	282000	278000	2030000	3220000
CAP	570000	4400	600	500	0	0
LIN	227000	40000	20700	15500	1800	0

Log Values of V 1435 A1 and A2 mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.82	6.41	6.53	7.25	7.41	7.21
INH	5.45	5.51	4.97	5.47	6.48	4.56
PAS	5.16	0	5.45	5.44	6.31	6.51
CAP	5.76	3.64	2.78	2.7	0	0
LIN	5.36	4.6	4.32	4.19	3.26	0

V 1435 B1 and B2 Mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	750000	338000	9400000	17800000	14300000	152000000
INH	347000	21000	34300	171000	1920000	1920000
PAS	182000	0	162000	2090000	1430000	3350000
CAP	181000	0	0	0	0	0
LIN	96000	262000	37100	4500	300	0

Log Values of V 1435 B1 and B2 mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.88	5.53	6.97	7.25	7.16	8.18
INH	5.54	4.32	4.53	5.23	6.28	6.28
PAS	5.26	0	5.21	6.32	6.16	6.53
CAP	5.26	0	0	0	0	0
LIN	4.98	5.42	4.57	3.65	2.48	0

V 1435 C1 and C2 Mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	311000	2100000	3610000	14400000	3550000	35200000
INH	100000	3120000	224000	13600000	14300000	1910000
PAS	255000	309000	107000	2250000	1830000	1560000
CAP	600000	7700	0	0	0	0
LIN	540000	860000	59000	1600	0	0

Log Values of V 1435 C1 and C2 mean colony count values for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.49	6.32	6.56	7.16	6.55	7.55
INH	5	6.49	5.35	7.13	7.16	6.28
PAS	5.41	5.49	5.03	6.35	6.26	6.19
CAP	5.78	3.89	0	0	0	0
LIN	5.73	5.93	4.77	3.2	0	0

Average of V1435 log values for A1,A2, B1, B2, C1 and C2 for TKE 2						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.73	6.09	6.69	7.22	7.04	7.65
INH	5.33	5.44	4.95	5.94	6.64	5.71
PAS	5.28	1.83	5.23	6.04	6.24	6.41
CAP	5.6	2.51	0.93	0.9	0	0
LIN	5.36	5.32	4.55	3.68	1.91	0

V1435 A1 and A2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	311000	3050000	230000000	416000000	700000000	700000000
INH	363000	276000	2570000	3440000	190000000	341000000
M&C	142000	23600	16200	2200	0	0

Log Values of V1435 A1 and A2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.49	6.48	8.36	8.7	8.85	8.85
INH	5.56	5.44	6.41	6.54	8.28	8.53
M&C	5.15	4.37	4.21	3.34	0	0

V1435 B1 and B2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	2030000	35900000	317000000	500000000	700000000	700000000
INH	1520000	322000	2860000	3560000	28600000	247000000
M&C	155000	33500	11900	0	0	0

Log Values of V1435 B1 and B2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	6.31	7.56	8.5	8.7	8.85	8.85
INH	6.18	5.51	6.46	6.55	7.46	8.39
M&C	5.19	4.53	4.08	0	0	0

V1435 C1 and C2 Mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	289000	3500000	253000000	433000000	700000000	700000000
INH	373000	301000	2080000	2720000	243000000	500000000
M&C	171000	33000	17500	2600	0	0

Log Values of V1435 C1 and C2 mean colony count values for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.46	6.54	8.4	8.64	8.85	8.85
INH	5.57	5.48	6.32	6.43	8.39	8.7
M&C	5.23	4.52	4.24	3.42	0	0

Average of V1435 log values for A1,A2, B1, B2, C1 and C2 for TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.75	6.86	8.42	8.68	8.85	8.85
INH	5.77	5.48	6.4	6.51	8.04	8.54
M&C	5.19	4.47	4.18	2.25	0	0

Final log values used to construct time kill curves for V1435

Total Average of Log values for V 1435 A1, A2, B1, B2, C1, C2 for TKE 2 &TKE 3						
	Day 0	Day 3	Day 7	Day 14	Day 21	Day 28
DFC	5.74	6.47	7.55	7.95	7.95	8.25
INH	5.55	5.46	5.67	6.23	7.34	7.12
PAS	5.28	1.83	5.23	6.04	6.24	6.41
CAP	5.6	2.51	0.93	0.9	0	0
LIN	5.36	5.32	4.55	3.68	1.91	0
M&C	5.19	4.47	4.18	2.25	0	0

Appendix 7: Raw data of colony counts for Time kill experiment

Colony Counts of Time Kill Experiment 1

	H37Rv																													
1	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	175	23	3	0	0	>300	>200	103	2	0	>500	>400	>350	>200	57	>500	>400	>350	>200	100	>500	>400	>350	>200	124	>500	>400	>350	>200	130
B1	220	21	7	0	0	>300	>200	84	19	0	>500	>400	>350	197	45	>500	>400	>350	188	107	>500	>400	>350	>200	119	>500	>400	>350	>200	157
C1	173	75	17	0	0	>300	>200	>200	16	12	>500	>400	>300	128	67	>500	>400	>350	181	131	>500	>400	>350	>200	121	>500	>400	>350	>200	162
A2	166	67	9	0	0	>300	>200	110	0	0	>500	>400	>350	>200	89	>500	>400	>350	>200	74	>500	>400	>350	>200	68	>500	>400	>350	>200	89
B2	>200	30	3	0	0	>300	>200	79	11	0	>500	>400	>350	126	69	>500	>400	>350	123	58	>500	>400	>350	>200	93	>500	>400	>350	>200	138
C2	151	35	9	0	0	>300	>200	206	13	4	>500	>400	>300	143	72	>500	>400	>350	158	147	>500	>400	>350	>200	67	>500	>400	>350	>200	99

	H37Rv																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	198	33	6	0	2	0	0	0	0	0	20	1	0	0	0	>250	>200	98	32	5	>250	>200	103	68	11	>250	>200	60	20	0
B1	>200	71	3	0	0	0	0	84	0	0	62	2	0	0	0	0	0	0	0	0	0	0	0	0	0	>250	>200	187	47	0
C1	224	29	4	0	0	12	0	0	0	0	65	1	4	3	0	>250	>200	123	16	0	>250	>200	107	69	30	>250	>200	175	54	10
A2	>200	36	5	0	0	0	0	0	0	0	43	4	0	0	0	>250	>200	119	58	1	>250	>200	128	85	12	>250	>200	57	6	0
B2	>200	60	4	2	0	0	0	64	0	0	66	5	0	0	0	0	0	0	0	0	0	0	0	0	0	>250	>200	162	38	0
C2	>200	27	6	1	0	0	0	0	0	0	109	4	3	4	0	>250	148	56	8	0	>250	>200	54	36	25	>250	>200	115	30	0

	H37Rv																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	125	6	0	0	>200	89	0	0	0	>200	197	158	81	3	>250	>200	162	11	0	>250	>200	189	38	8	>250	>200	161	45	0
B1	>200	93	12	0	0	>200	39	0	0	0	>200	78	39	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	40	0	0	0	>200	56	13	3	0	>200	96	68	7	0	121	13	8	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	66	15	0	0	>200	37	0	0	0	>200	>200	169	61	5	>250	>200	133	8	0	>250	>200	142	19	30	>250	>200	114	3	0
B2	>200	53	5	0	0	>200	45	0	0	0	>200	67	36	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	16	4	0	0	>200	94	23	11	0	>200	169	65	8	0	135	45	10	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 1

	H37Rv																													
2	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	72	14	4	0	0	0	0	0	0	5	3	6	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	38	2	2	0	5	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	68	25	2	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	198	46	2	1	0	0	0	0	0	0	6	5	2	5	3	0	0	0	0	0	0	0	0	0	0	0	0		0	0
B2	>200	18	4	2	0	2	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	31	0	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	H37Rv																													
LIN	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	41	3	0	0	177	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	23	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	61	0	0	0	>200	97	13	1	0	>200	54	25	1	0	147	0	0	0	0	121	15	0	0	0	0	0	0	0	0
A2	>200	32	8	0	0	144	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	77	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	113	6	0	0	>200	161	42	1	0	>200	67	16	3	0	163	0	0	0	0	78	8	0	0	0	0	0	0	0	0

	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	40	9	0	0	>200	134	19	0	0	>250	>200	37	0	0	>250	>200	68	5	0	>250	>200	99	20	0	>250	>200	121	46	0
B1	>200	28	1	2	2	>200	159	25	6	0	>250	>200	62	7	0	>250	>200	95	11	0	>250	>200	134	26	0	>250	>200	150	69	12
C1	>250	24	1	1	0	233	36	0	0	0	>250	>200	12	0	0	>250	>200	19	0	0	>250	>200	38	0	0	>250	>200	59	32	0
A2	>250	45	4	0	0	>200	124	14	0	0	>250	>200	27	0	0	>250	>200	43	0	0	>250	>200	56	0	0	>250	>200	78	49	0
B2	>200	29	5	0	0	>200	118	18	2	0	>250	>200	39	4	0	>250	>200	67	3	0	>250	>200	77	4	0	>250	>200	126	52	9
C2	>250	26	9	2	0	>200	117	12	0	0	>250	>200	26	0	0	>250	>200	35	0	0	>250	>200	54	0	0	>250	>200	67	37	0

Colony Counts of Time Kill Experiment 1

	V4207																													
3	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	63	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	53	7	0		298	58	8	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	165	2	0	0	182	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	41	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	94	4	0		246	167	9	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	153	15	0	0	216	29	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	41	15	3	0		65	12	0	0	76	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	70	8	3	0	>200	126	18	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	32	0	0	0	>200	148	20	3	0	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	71	18	1	5		78	16	1	0	71	7	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	64	9	0	0	>200	119	17	2	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	25	6	0	0	>200	151	22	1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	46	0	5	3	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>400	38	3	7	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>400	64	7	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>400	42	0	10	1	25	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>400	65	4	3	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>400	70	5	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 1

4	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
LIN	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	22	3	1	0	>200	46	13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	43	11	3	0	>200	158	17	2	0	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	28	21	0	0	82	0	0	0	0	>200	157	25	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	34	5	0	0	>200	97	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	46	14	2	0	>200	95	8	5	0	65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	40	28	0	0	0	0	0	0	0	>200	82	22	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	>300	96	16	5	>400	>300	269	90	53	>400	>300	>200	88	25	>400	>300	>200	83	8	>400	>300	>200	>200	>200	>400	>300	>250	>200	>200
B1	>400	>300	150	19	3	>400	>300	121	156	13	>400	>300	>200	79	25	>400	>300	>200	>200	>200	>400	>300	>200	>200	>200	>400	>300	>250	>200	>200
C1	>400	>300	120	22	6	>400	>300	>300	318	156	>400	>300	>200	106	18	>400	>300	>200	>200	>200	>400	>300	>200	>200	>200	>400	>400	>400	>400	>400
A2	>400	>300	85	28	9	>400	>300	264	78	36	>400	>300	>200	111	44	>400	>300	>200	34	5	>400	>300	>200	>200	>200	>400	>300	>250	>300	>200
B2	>400	>300	87	68	10	>400	>300	>200	32	9	>400	>300	>200	56	0	>400	>300	>200	>200	>200	>400	>300	>200	>200	>200	>400	>300	>250	>200	>200
C2	>400	>300	54	20	7	>400	>300	>300	291	132	>400	>300	>200	192	21	>400	>300	>200	>200	>200	>400	>300	>200	>200	>200	>400	>400	>400	>400	>400
	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	316	134	4	1	>400	>300	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300
B1	>400	228	68	20	1	>250	136	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300
C1	>400	315	128	11	4	>400	>300	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300
A2	>400	294	113	2	5	>400	>300	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300
B2	>400	204	56	50	5	>250	>300	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300
C2	>400	305	57	12	0	>400	>300	>200	>200	>200	>400	>400	>300	>300	>200	>400	>400	>300	>300	>200	>400	>400	>400	>300	>200	>400	>400	>400	>300	>300

Colony Counts of Time Kill Experiment 1

5	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	195	38	13	0	>350	>250	160	21	2	>350	>200	0	0	0	>350	>200	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>400	189	27	8	0	>350	>250	98	12	3	>350	72	70	4	4	>200	>200	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>400	183	136	13	6	>350	212	52	11	2	>350	>250	57	17	2	>300	>300	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>400	175	56	6	0	>350	>250	116	6	4	>350	169	0	0	0	>350	>200	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>400	143	47	9	0	>350	>250	73	45	1	>350	>250	78	15	5	>200	>200	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>400	>20	77	8	3	>350	198	99	21	1	>350	>250	91	6	6	>300	>200	0	0	0	0	0	0	0	0	0	0	0	0	0

	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	>30	>20	32	20	>250	>250	107	3	1	>250	251	27	3	1	>200	150	0	0	0	>250	118	11	0	0	>300	>200	137	6	0
B1	>400	297	119	21	3	>250	>250	147	5	0	>250	196	36	6	0	>200	164	0	0	0	>250	151	73	0	0	>300	>200	>200	21	4
C1	>400	>30	67	48	11	>250	>250	151	16	8	>250	154	30	11	0	93	9	8	0	0	>250	46	0	0	0	>300	>200	163	49	0
A2	>400	>30	>20	39	24	>250	>250	106	4	0	>250	131	24	1	2	>200	152	0	0	0	>250	74	14	0	0	>300	>200	139	25	2
B2	>400	228	54	11	5	>250	>250	94	9	4	>250	163	28	4	0	>200	129	0	0	0	>250	85	3	0	0	>300	>200	>200	104	7
C2	>400	>30	66	27	6	>250	>250	145	26	6	>250	146	29	6	0	88	13	3	0	0	>250	36	0	0	0	>300	>200	109	14	0

	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
LIN	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>400	293	63	8	1	>350	>250	17	9	0	>350	212	48	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>400	>30	89	10	0	0	0	0	8	0	>350	31	3	2	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>400	>30	105	28	3	>350	>250	129	21	13	>350	189	30	5	1	178	10	0	0	0	64	0	0	0	0	0	0	0	0	0
A2	>400	288	25	34	3	>350	>250	13	0	0	>350	158	46	8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>400	222	56	31	0	0	0	0	23	0	>350	51	6	3	0	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>400	>30	95	8	2	>350	>250	163	60	11	>350	133	39	6	1	199	22	0	0	0	85	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 2

R 252

1	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	26	4	0	0	>300	>200	51	20	1	>300	>200	128	52	11	>400	>300	>200	16	0	>400	>300	>200	139	4	>400	>300	>200	189	6
B1	136	22	0	0	0	>300	>200	189	23	2	>300	>200	183	31	1	>400	>300	>200	137	28	>400	>300	>200	138	7	>400	>300	>200	180	16
C1	>200	22	1	0	0	>300	>200	35	10	2	>300	>200	47	39	26	>300	>200	109	53	27	>400	>300	>200	120	0	>400	>300	>200	132	0
A2	>200	33	6	0	0	>300	>200	88	30	2	>300	>200	89	67	6	>400	>300	>200	29	0	>400	>300	>200	142	6	>400	>300	>200	166	36
B2	157	24	0	0	0	>300	>200	136	21	0	>300	>200	169	27	18	>400	>300	>200	56	16	>400	>300	>200	172	20	>400	>300	>200	176	53
C2	>200	28	7	1	0	>300	>200	41	12	2	>300	>200	39	15	6	>300	>200	128	83	32	>400	>300	>200	118	0	>400	>300	>200	147	12

R 252

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	113	18	6	0	0	0	0	0	0	0	26	0	0	0	0	>300	>200	29	3	1	157	0	0	0	0	>200	>200	19	6	0
B1	136	11	11	0	1	11	0	0	0	0	18	0	0	0	0	>300	81	14	6	2	85	4	0	0	0	>200	29	2	0	0
C1	178	112	13	8	3	26	6	0	0	0	1	0	0	0	0	>300	106	26	0	0	>300	>200	117	14	2	>200	>200	43	5	0
A2	98	31	9	1	0	0	0	0	0	0	8	0	0	0	0	>300	>200	17	2	1	160	0	0	0	0	>200	>200	5	4	0
B2	151	26	14	0	0	15	3	0	0	0	6	2	0	0	0	>300	72	16	8	0	70	3	0	0	0	>200	70	0	0	0
C2	159	118	17	0	0	21	0	0	0	0	0	0	0	0	0	>300	147	31	2	0	>200	>200	71	16	5	>200	>200	51	1	0

R 252

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	179	28	3	3	1	>200	0	0	0	0	>250	>200	88	8	0	>300	>200	139	14	5	>300	>200	153	31	8	>300	>200	>200	62	4
B1	221	27	2	0	0	>200	0	0	0	0	>250	>200	83	11	0	>300	>200	163	4	0	>300	>200	116	16	0	>300	>200	>200	20	0
C1	>200	77	34	0	0	>200	151	82	0	0	>250	>200	63	32	2	>300	>200	179	23	4	>300	>200	149	30	0	>300	>200	>200	26	11
A2	184	36	4	0	0	>200	0	0	0	0	>250	>200	79	15	6	>300	>200	162	36	1	>300	>200	136	23	0	>300	>200	>200	54	0
B2	167	10	7	1	0	>200	0	0	0	0	>250	>200	52	10	0	>300	>200	146	12	0	>300	>200	95	0	0	>300	>200	>200	73	0
C2	>200	128	33	5	0	>200	123	63	0	0	>250	>200	38	5	0	>300	>200	197	27	6	>300	>200	113	22	0	>300	>200	>200	48	12

Colony Counts of Time Kill Experiment 2

	R 252																													
2	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	41	5	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	61	5	3	0	32	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	83	18	0	0	36	0	0	0	0	3	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	45	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	93	6	1	0	21	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	194	79	16	2	0	53	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	R 252																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
LIN	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	37	0	3	0	>200	89	19	0	0	>200	28	3	1	0	104	5	0	0	0	>200	188	20	0	0	>200	>200	69	9	0
B1	198	31	8	1	0	>200	87	18	3	0	169	22	2	0	0	87	1	0	0	0	>200	>200	95	32	6	>200	>200	148	30	4
C1	>200	28	5	1	0	>200	56	7	0	0	126	23	3	0	0	>200	1	1	0	0	>200	189	124	9	1	>200	>200	39	6	0
A2	>200	12	1	0	0	>200	157	27	0	0	>200	22	2	0	0	64	0	0	0	0	>200	164	24	0	0	>200	>200	47	6	0
B2	125	23	4	1	0	>200	141	20	6	0	158	13	0	0	0	109	11	0	0	0	>200	>200	57	16	5	>200	>200	189	58	6
C2	>200	24	2	2	0	>200	30	5	0	0	106	17	0	0	0	>200	16	3	0	0	>200	>200	46	12	1	>200	>200	62	22	0

	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	116	49	30	1	>250	194	23	2	0	>400	>300	>200	106	5	>400	>300	>200	62	11	>400	>300	>200	138	49	>400	>300	>200	>200	36
B1	>200	63	28	3	0	>250	>200	22	2	0	>400	>300	>200	83	26	>400	>300	>200	184	54	>400	>300	>200	132	51	>400	>300	>200	>200	25
C1	>200	123	29	2	0	>250	>200	43	5	0	>400	>300	>200	88	25	>400	>300	>200	>200	95	>400	>300	>200	165	77	>400	>300	>200	159	28
A2	>200	79	18	5	1	>250	188	43	3	1	>400	>300	>200	97	8	>400	>300	>200	56	8	>400	>300	>200	76	37	>400	>300	>200	>200	40
B2	>200	86	8	0	0	>250	>200	0	0	0	>400	>300	>200	68	27	>400	>300	>200	>200	48	>400	>300	>200	108	20	>400	>300	>200	>200	33
C2	>200	117	30	5	0	>250	>200	38	3	0	>400	>300	194	123	8	>400	>300	>200	>200	69	>400	>300	>200	173	76	>400	>300	>200	153	34

Colony Counts of Time Kill Experiment 2

3	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	58	12	3	0	0	0	0	0	0	38	7	5	0	0	>200	137	42	15	0	>200	>200	83	10	0	>200	>200	>200	63	6
B1	>200	40	2	1	0	6	6	1	0	0	3	0	0	0	0	>200	77	9	0	0	>200	30	0	0	0	>200	>200	>200	50	12
C1	>200	107	8	0	0	8	2	0	0	0	5	0	0	0	0	>200	154	30	1	0	>200	>200	185	17	2	>200	>200	>200	67	11
A2	>200	89	0	0	0	0	0	0	0	0	0	0	0	0	0	>200	78	37	1	0	>200	>200	102	21	0	>200	>200	>200	81	10
B2	>200	19	3	0	0	6	0	0	0	0	5	0	0	0	0	177	80	0	0	0	>200	67	52	50	13	>200	>200	>200	73	0
C2	>200	110	3	0	0	4	1	0	0	0	2	0	0	0	0	>200	139	23	0	0	>200	>200	146	25	3	>200	>200	>200	73	3

	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	94	0	0	1	>200	49	13	0	0	>200	105	8	3	0	>200	97	12	1	0	>200	116	4	3	0	0	0	0	0	0
B1	>200	98	12	0	0	>200	57	10	0	0	>200	7	2	0	0	>200	70	10	0	0	>200	110	6	3	0	0	0	0	0	0
C1	>200	48	6	0	0	>200	66	1	0	0	>200	9	0	0	0	>200	88	13	0	0	>200	44	0	0	0	>200	>200	3	0	0
A2	>200	112	14	9	4	>200	88	15	0	0	>200	39	9	2	0	>200	>200	21	0	0	>200	103	6	3	0	0	0	0	0	0
B2	>200	39	4	0	0	>200	77	14	0	0	>200	33	4	0	0	>200	110	20	0	0	>200	140	10	0	0	0	0	0	0	0
C2	>200	44	1	0	0	>200	78	7	0	0	>200	12	3	1	0	>200	97	14	3	2	152	43	0	0	0	>200	>200	2	0	0

	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	33	4	1	0	76	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	48	19	0	0	68	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	12	8	1	0	71	6	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	42	17	0	0	30	5	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	30	19	0	0	59	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	29	7	2	0	56	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 2

V4258

4	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
LIN	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	31	7	4	0	>200	120	16	0	0	>200	163	5	0	0	61	18	2	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	137	44	0	0	>200	185	36	3	0	>200	122	1	0	0	50	5	0	0	0	9	0	0	0	0	0	0	0	0	0
C1	>200	53	9	0	0	>200	243	49	0	0	>200	117	1	0	0	84	8	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	42	3	2	0	>200	135	16	0	0	>200	135	13	1	0	54	13	1	0	0	6	0	0	0	0	0	0	0	0	0
B2	>200	165	48	0	0	>200	>200	29	3	0	>200	116	5	0	0	53	7	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	36	4	0	0	>200	206	21	0	0	>200	120	3	0	0	111	9	0	0	0	0	0	0	0	0	0	0	0	0	0

V1435

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	>200	61	6	0	>500	>300	>250	25	4	>500	>300	178	37	3	>300	>200	>200	99	23	>400	>300	>200	124	17	>400	>300	>200	86	20
B1	>250	>200	40	4	0	>500	138	0	0	0	>500	>300	>200	36	8	>300	>200	>200	44	11	>400	>300	>200	80	20	>400	>300	>200	152	97
C1	>250	158	69	18	0	>500	>200	119	14	3	>500	>300	175	33	0	>300	>200	>200	79	25	>400	>300	182	94	18	>400	>300	>200	169	76
A2	>250	>200	5	0	0	>500	>300	>250	1	0	>500	>300	164	9	0	>300	>200	>200	78	48	>400	>300	>200	136	10	>400	>300	>200	76	13
B2	>250	>200	35	5	0	>500	>200	0	0	0	>500	>300	>200	58	3	>300	>200	>200	134	29	>400	>300	>200	63	10	>400	>300	>200	75	55
C2	>250	153	16	8	0	>500	>200	91	28	3	>500	>300	186	27	0	>300	>200	>200	65	16	>400	>300	173	69	16	>400	>300	>200	183	30

V1435

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	>200	12	2	0	>300	177	19	2	0	>200	43	0	0	0	>200	158	43	2	1	>300	>200	140	0	0	181	112	23	0	0
B1	>250	168	17	0	0	121	3	0	0	0	179	27	0	0	0	>200	91	20	0	0	>300	>200	109	5	2	>300	>200	118	28	0
C1	>250	>200	1	0	0	>350	>200	143	14	6	>200	139	19	0	0	>300	>200	112	62	13	>300	>200	>200	81	10	>300	>200	79	25	3
A2	>250	>200	16	3	0	>300	145	14	2	0	>200	51	0	0	0	>200	137	14	0	0	>300	>200	161	0	0	179	113	32	0	0
B2	>250	179	18	0	0	89	4	4	0	0	164	30	2	0	0	>200	80	18	0	0	>300	>200	83	3	0	>300	>200	74	19	0
C2	>250	>200	9	3	0	>350	>200	169	80	3	>200	85	11	0	0	>300	>200	179	74	20	>300	>200	>200	62	18	>300	>200	112	36	1

Colony Counts of Time Kill Experiment 2

	V1435																													
5	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
PAS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	80	10	0	0	0	0	0	0	0	>300	147	5	0	0	>300	156	139	10	1	>300	>200	93	0	0	>400	>300	184	49	0
B1	>250	94	22	0	0	0	0	0	0	0	>300	93	15	1	0	>300	>200	91	17	1	>300	>200	87	4	0	>400	>300	190	21	0
C1	>250	89	9	0	0	>300	156	38	6	0	>300	64	0	0	0	>300	>200	138	13	8	>300	>200	85	20	0	>400	>300	82	11	2
A2	>250	65	0	0	0	0	0	0	0	0	>300	135	9	0	0	>300	122	76	28	5	>300	>200	110	0	0	>400	>300	138	13	6
B2	>250	88	14	0	0	0	0	0	0	0	>300	69	3	1	1	>300	>200	118	15	3	>300	>200	56	2	0	>400	>300	145	13	0
C2	>250	166	17	2	0	>300	153	11	8	0	>300	43	0	0	0	>300	>200	87	22	10	>300	>200	98	11	1	>400	>300	74	21	0

	V1435																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
CAP	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	>200	20	1	0	16	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>250	93	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>250	>200	37	18	3	43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>250	>200	34	7	0	28	0	0	0	0	3	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>250	88	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>250	>200	26	5	0	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	V1435																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
LIN	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	121	10	0	0	>300	0	0	0	0	118	10	0	0	0	86	2	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>250	56	7	0	0	>200	156	41	0	0	175	29	0	0	0	30	3	0	0	0	3	0	0	0	0	0	0	0	0	0
C1	>250	91	37	0	0	>300	>200	37	2	1	>200	24	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>250	106	4	0	0	>300	0	0	0	0	89	15	0	0	0	69	7	0	0	0	18	0	0	0	0	0	0	0	0	0
B2	>250	40	2	0	0	>200	106	43	0	0	196	38	1	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>250	125	17	0	0	>300	>200	49	2	0	>200	35	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 3

H37Rv

1	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	125	47	8	0	0	>200	>200	159	87	28	>200	>200	>200	>200	75	>200	>200	>200	>200	167	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
B1	117	33	7	0	0	>200	>200	137	72	17	>200	>200	>200	>200	49	>200	>200	>200	>200	128	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
C1	126	38	3	0	0	>200	>200	>200	109	23	>200	>200	>200	>200	66	>200	>200	>200	>200	160	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
A2	115	27	5	2	0	>200	>200	164	98	16	>200	>200	>200	>200	51	>200	>200	>200	>200	139	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
B2	129	42	9	1	0	>200	>200	132	76	12	>200	>200	>200	>200	50	>200	>200	>200	>200	141	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200
C2	161	56	12	8	3	>200	>200	>200	125	29	>200	>200	>200	>200	83	>200	>200	>200	>200	172	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

H37Rv

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	81	59	23	1	91	29	0	0	0	67	17	0	0	0	28	9	2	0	0	163	83	22	5	0	>200	>200	164	58	18
B1	>200	75	13	0	0	51	7	0	0	0	27	5	0	0	0	12	2	0	0	0	119	51	18	3	0	>200	>200	98	31	16
C1	>200	122	26	3	0	55	12	0	0	0	54	0	0	0	0	22	11	4	0	0	135	82	16	6	0	>200	>200	155	62	22
A2	>200	77	40	20	1	73	22	0	0	0	64	13	0	0	0	20	8	1	0	0	142	68	14	6	0	>200	>200	137	42	12
B2	>200	71	11	0	0	45	12	0	0	0	38	0	0	0	0	14	5	0	0	0	138	70	11	4	0	>200	>200	109	29	9
C2	>200	99	29	4	0	59	15	0	0	0	43	2	0	0	0	17	8	0	0	0	148	87	29	7	1	>200	>200	157	46	18

H37Rv

	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	145	98	63	2	0	94	43	4	3	0	48	12	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	192	110	52	18	0	110	63	2	1	0	53	7	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	164	81	30	0	0	115	75	6	0	0	55	14	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	149	99	61	1	0	109	63	12	9	0	69	22	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	178	122	45	14	0	89	41	1	0	0	47	28	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	160	91	70	0	0	112	76	7	2	0	72	32	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 3

	R 252																													
2	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	77	29	10	4	>200	>200	143	56	14	>300	>300	>300	>200	117	>300	>300	>300	>300	>200	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300
B1	>200	128	45	18	7	>200	>200	158	61	20	>300	>300	>300	>200	139	>300	>300	>300	>300	>200	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300
C1	>200	116	37	9	0	>200	>200	120	44	18	>300	>300	>300	>200	115	>300	>300	>300	>300	>200	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300
A2	>200	92	31	12	0	>200	>200	97	25	11	>300	>300	>300	158	95	>300	>300	>300	>300	171	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300
B2	>200	109	30	8	1	>200	>200	112	47	17	>301	>300	>300	182	101	>300	>300	>300	>300	183	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300
C2	>200	118	24	6	0	>200	>200	126	68	22	>302	>300	>300	>200	127	>300	>300	>300	>300	>200	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300

	R 252																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	179	63	31	12	1	142	76	32	20	6	>200	138	80	25	0	>200	182	98	24	10	>300	>200	>200	119	76	>300	>300	>300	>200	>200
B1	>200	61	33	10	0	136	90	46	22	6	>200	127	72	13	0	>200	178	105	38	11	>300	>200	177	98	59	>300	>300	>300	173	128
C1	184	63	37	9	6	113	67	31	11	5	>200	147	86	11	0	>200	139	71	27	8	>300	>200	193	113	52	>300	>300	>300	>200	136
A2	154	81	27	13	1	156	84	44	18	3	>200	141	89	36	0	>200	173	64	11	2	>300	>200	152	102	49	>300	>300	>300	192	115
B2	172	85	17	12	0	125	87	30	18	8	>200	135	75	16	0	>200	198	113	45	19	>300	>200	>200	123	61	>300	>300	>300	>200	142
C2	138	49	32	21	6	116	64	23	9	0	>200	164	91	17	0	>200	176	108	39	17	>300	>200	184	105	47	>300	>300	>300	199	118

	R 252																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	53	12	9	0	152	76	26	9	0	65	34	11	7	0	67	27	5	0	0	42	19	7	0	0	29	6	1	0	0
B1	172	64	34	16	1	140	73	22	7	0	89	19	6	0	0	62	9	0	0	0	39	12	4	0	0	13	4	0	0	0
C1	188	84	46	12	0	125	51	12	1	0	101	25	9	0	0	73	18	4	0	0	45	26	9	1	0	26	11	4	0	0
A2	>200	59	22	12	0	148	65	17	1	0	88	39	2	0	0	59	20	7	0	0	28	9	3	0	0	15	7	1	0	0
B2	179	83	42	12	1	164	78	29	5	0	81	28	12	3	0	61	13	0	0	0	31	11	0	0	0	27	8	1	0	0
C2	189	89	45	10	1	122	54	15	7	0	116	37	11	2	0	89	34	12	0	0	56	27	8	1	0	39	15	0	0	0

Colony Counts of Time Kill Experiment 3

3	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>300	>200	116	41	17	>300	>300	>200	82	47	>300	>300	>300	>300	106	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B1	>300	>200	97	36	9	>300	>300	>200	75	22	>300	>300	>300	>300	98	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C1	>300	>200	126	44	16	>300	>300	>200	63	26	>300	>300	>300	>300	118	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
A2	>300	>200	122	31	13	>300	>300	>200	79	33	>300	>300	>300	>300	120	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B2	>300	>200	105	25	17	>300	>300	>200	67	18	>300	>300	>300	>300	92	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C2	>300	>200	119	37	14	>300	>300	>200	72	25	>300	>300	>300	>300	114	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300

	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	>200	94	17	0	>200	188	95	27	9	>200	>200	180	70	27	>200	>200	>200	127	54	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
B1	>200	>200	108	13	10	>200	178	100	33	11	>200	>200	172	83	39	>200	>200	>200	130	71	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
C1	>200	174	77	11	0	>200	162	91	22	7	>200	>200	186	85	33	>200	>200	>200	148	66	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
A2	>200	>200	85	13	0	>200	186	97	20	8	>200	>200	179	62	24	>200	>200	>200	161	76	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
B2	>200	>200	75	18	0	>200	165	87	24	15	>200	>200	199	108	44	>200	>200	>200	187	99	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
C2	>200	186	76	14	0	>200	>200	120	54	20	>200	>200	164	76	27	>200	>200	>200	167	79	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200

	KZN605																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	185	112	29	21	138	90	40	17	0	108	55	17	0	0	83	24	8	0	0	58	27	9	0	0	21	8	0	0	0
B1	>200	173	89	29	16	165	94	46	15	6	119	23	7	0	0	79	19	6	0	0	25	12	7	0	0	16	3	1	0	0
C1	>200	148	63	20	10	179	91	35	12	4	124	37	14	0	0	92	39	15	1	0	47	26	5	0	0	13	0	0	0	0
A2	>200	179	113	35	15	113	69	24	8	0	112	26	6	0	0	83	28	9	0	0	34	19	6	0	0	15	1	0	0	0
B2	>200	186	87	33	18	132	78	37	9	2	110	28	9	0	0	70	19	11	1	0	38	11	1	0	0	10	0	0	0	0
C2	>200	187	78	16	13	134	79	22	9	2	99	21	5	0	0	65	16	2	0	0	39	21	13	4	0	7	3	0	0	0

Colony Counts of Time Kill Experiment 3

4	V1435																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	162	71	37	12	>300	>300	140	99	30	>300	>300	>300	>300	91	>300	>300	>300	>300	166	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B1	>200	>200	94	62	28	>300	>300	>200	177	92	>300	>300	>300	>300	175	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C1	>200	151	78	29	15	>300	>300	168	106	63	>300	>300	>300	>300	147	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
A2	>200	149	59	17	9	>300	>300	165	115	66	>300	>300	>300	>300	139	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B2	>200	>200	109	78	35	>300	>300	>200	182	101	>300	>300	>300	>300	142	>300	>300	>300	>300	>200	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C2	>200	138	99	45	33	>300	>300	182	159	51	>300	>300	>300	>300	106	>300	>300	>300	>300	183	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300

	V1435																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	188	62	12	0	>200	148	77	17	0	>200	>200	145	65	27	>200	>200	163	86	33	>300	>300	>200	>200	101	>300	>300	>300	>200	179
B1	>200	>200	69	29	10	>200	170	89	20	9	>200	>200	146	62	23	>200	>200	172	75	27	>300	>300	>200	137	77	>300	>300	>300	>200	135
C1	>200	184	62	16	2	>200	144	67	13	5	>200	>200	98	46	15	>200	>200	97	69	31	>300	>300	>200	>200	118	>300	>300	>300	>200	>200
A2	>200	175	75	16	2	>200	128	53	13	0	>200	>200	112	42	12	>200	>200	181	67	29	>300	>300	>200	>200	89	>300	>300	>300	>200	162
B2	>200	>200	83	12	9	>200	152	89	23	8	>200	>200	140	79	27	>200	>200	184	66	19	>300	>300	>200	149	53	>300	>300	>300	>200	112
C2	>200	189	73	14	1	>200	157	73	16	6	>200	>200	110	48	13	>200	>200	175	62	24	>300	>300	>200	>200	125	>300	>300	>300	>200	>200

	V1435																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	70	30	15	0	109	57	14	5	0	78	24	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	77	30	13	0	167	89	19	9	1	62	17	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	84	25	17	0	172	71	16	5	0	98	39	13	0	0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	72	29	18	0	127	67	12	7	0	84	28	0	0	0	22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	78	29	11	0	168	92	23	10	2	57	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	87	27	18	0	158	88	18	8	2	77	26	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 3

	V4207																													
5	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	>200	98	23	7	>300	>300	>200	129	61	>300	>300	>300	>200	86	>300	>300	>300	>300	191	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B1	>200	>200	73	17	3	>300	>300	>200	95	43	>300	>300	>300	>200	63	>300	>300	>300	>300	182	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C1	>200	>200	95	32	18	>300	>300	>200	126	72	>300	>300	>300	>200	92	>300	>300	>300	>300	149	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
A2	>200	>200	75	29	12	>300	>300	>200	114	82	>300	>300	>300	>200	92	>300	>300	>300	>300	162	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
B2	>200	>200	97	30	10	>300	>300	>200	110	68	>300	>300	>300	>200	97	>300	>300	>300	>300	175	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300
C2	>200	>200	78	33	16	>300	>300	>200	133	59	>300	>300	>300	>200	88	>300	>300	>300	>300	159	>300	>300	>300	>300	>300	>300	>300	>300	>300	>300

	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>250	193	119	52	21	>200	166	93	11	3	138	91	34	11	6	78	32	7	1	0	28	6	0	0	0	13	0	0	0	0
B1	>250	164	97	39	25	193	85	41	9	0	127	67	26	7	1	59	12	6	1	0	12	0	0	0	0	2	1	0	0	0
C1	>250	>200	194	77	39	>200	168	76	9	0	162	97	40	15	4	92	26	10	3	0	37	15	6	0	0	15	6	0	0	0
A2	>250	189	95	50	28	>200	149	72	16	1	142	75	48	17	9	71	14	12	2	0	25	9	1	0	0	6	0	0	0	0
B2	>250	172	110	95	52	135	74	30	10	0	129	62	35	12	6	49	11	4	0	0	9	0	0	0	0	2	0	0	0	0
C2	>250	187	124	92	46	>200	158	74	12	0	135	88	39	26	10	65	18	13	7	0	17	11	3	0	0	10	3	0	0	0

	V4207																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	38	13	1	0	124	22	6	0	0	71	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	52	17	6	0	143	54	12	0	0	81	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	70	16	6	1	127	31	6	0	0	94	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	40	14	2	0	117	26	4	0	0	43	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	64	13	0	0	132	43	9	0	0	91	39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	76	18	6	1	126	27	8	0	0	62	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Colony Counts of Time Kill Experiment 3

6	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
DFC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	132	65	21	5	>200	>200	119	52	16	>200	>200	>200	137	69	>300	>300	>300	>200	182	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200
B1	>200	128	58	14	1	>200	>200	87	58	23	>200	>200	>200	102	55	>300	>300	>300	>200	141	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200
C1	>200	155	71	29	7	>200	>200	104	47	28	>200	>200	>200	159	92	>300	>300	>300	>200	162	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200
A2	>200	116	52	26	0	>200	>200	93	39	12	>200	>200	>200	128	73	>300	>300	>300	>200	154	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200
B2	>200	109	30	11	2	>200	>200	101	61	20	>200	>200	>200	116	45	>300	>300	>300	>200	129	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200
C2	>200	169	91	36	9	>200	>200	162	89	36	>200	>200	>200	131	61	>300	>300	>300	>200	170	>300	>300	>200	>200	>200	>300	>300	>300	>200	>200

	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
INH	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	96	31	13	2	>200	164	54	26	10	>200	>200	168	78	12	>300	>300	>200	>200	62	>300	>300	>300	>200	179	>300	>300	>300	>300	>200
B1	>200	99	37	12	0	>200	94	47	22	9	>200	>200	173	82	17	>300	>300	>200	>200	59	>300	>300	>300	>200	180	>300	>300	>300	>300	>200
C1	>200	120	36	11	0	>200	89	39	17	6	>200	>200	148	57	9	>300	>300	>200	>200	24	>300	>300	>300	>200	103	>300	>300	>300	>300	>200
A2	>200	103	28	15	3	>200	141	87	35	11	>200	>200	131	63	5	>300	>300	>200	>200	52	>300	>300	>300	>200	172	>300	>300	>300	>300	>200
B2	>200	83	28	10	0	>200	78	27	14	5	>200	>200	147	71	14	>300	>300	>200	>200	71	>300	>300	>300	>200	>200	>300	>300	>300	>300	>200
C2	>200	104	26	10	0	>200	86	36	18	8	>200	>200	155	69	12	>300	>300	>200	>200	38	>300	>300	>300	>200	119	>300	>300	>300	>300	>200

	V4258																													
	Day 0					Day 3					Day 7					Day 14					Day 21					Day 28				
M&C	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
A1	>200	86	14	8	0	39	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B1	>200	133	59	7	0	20	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C1	>200	74	19	4	0	35	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A2	>200	76	12	9	0	28	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B2	>200	105	25	7	0	37	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C2	>200	67	16	2	0	32	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 8: Raw data of MIC values from the agar dilution and MTT assay

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**INH A**												
	1	8	0.125	8	8	8	8	8	8	8	0.125	8	0.125
	2	8	0.125	8	8	8	0.062	8	8	8	0.25	8	0.25
	3	0.062	0.125	8	8	8	8	8	8	8	0.5	8	0.062
MTT	**INH A**												
	1	8	0.125	8	8	8	8	8	8	8	0.125	8	0.125
	2	8	0.125	8	8	8	8	8	8	8	0.125	8	0.125
	3	8	0.125	8	8	8	8	8	8	8	0.125	8	0.25
AGAR	**INH B**												
	1	8	0.25	8	8	8	8	8	8	8	0.25	8	0.125
	2	8	0.062	1	8	8	8	8	8	8	0.125	8	0.062
	3	8	0.25	4	8	8	8	8	8	8	0.25	8	0.125
MTT	**INH B**												
	1	8	8	8	8	8	8	8	8	8	0.062	8	0.062
	2	8	8	8	8	8	8	8	8	8	0.062	8	0.25
	3	8	8	8	8	8	8	8	8	8	0.062	8	0.062
AGAR	**INH C**												
	1	8	8	8	8	8	8	8	8	8	0.25	8	0.125
	2	8	8	8	8	8	8	8	8	8	0.25	8	0.125
	3	8	8	4	8	8	8	8	8	8	0.125	8	0.125
MTT	**INH C**												
	1	8	8	8	8	8	8	8	8	8	0.125	8	0.25
	2	16	8	8	8	8	8	8	8	8	0.125	8	0.125
	3	8	8	4	8	8	8	8	8	8	0.125	8	0.125
MTT	**INH D**												
	1	8	0.125	8	8	8	8	8	8	8	0.125	8	0.062
	2	8	0.25	8	8	8	8	8	8	8	0.125	8	0.125
	3	8	0.125	8	8	8	8	8	8	8	0.062	8	0.125
MTT	**INH E**												
	1	8	8	4	8	8	8	16	8	8	0.125	8	0.25
	2	8	8	4	8	8	8	8	8	8	0.062	8	0.25
	3	8	8	8	8	8	8	8	8	8	0.125	8	0.125

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**PAS A**												
	1	4	2	4	4	2	1	2	2	1	1	1	2
	2	4	2	4	4	2	1	1	8	2	1	1	2
	3	4	2	4	4	2	0.5	1	4	1	2	1	2
MTT	**PAS A**												
	1	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.5	0.125	0.25	0.125
	2	0.125	0.25	0.125	0.125	0.125	0.25	0.125	0.5	0.25	0.125	0.5	0.125
	3	0.125	0.125	0.125	0.125	0.125	0.25	0.125	0.5	0.5	0.125	0.5	0.125
AGAR	**PAS B**												
	1	2	2	4	4	4	2	4	2	2	2	2	4
	2	2	4	2	8	2	2	0.5	2	2	4	2	2
	3	2	4	2	2	2	2	4	2	2	4	1	2
MTT	**PAS B**												
	1	0.25	0.5	0.5	0.25	0.25	0.25	0.25	0.5	0.25	0.25	0.25	0.25
	2	0.25	0.5	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.25	0.5
	3	0.125	0.125	0.25	0.25	0.125	1	0.125	0.5	0.5	0.5	0.25	0.5
AGAR	**PAS C**												
	1	4	2	4	4	2	1	0.5	2	2	2	1	2
	2	4	8	4	8	2	1	0.5	2	2	2	1	2
	3	2	8	2	4	2	0.25	0.5	2	2	2	2	4
MTT	**PAS C**												
	1	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.25	0.5
	2	0.125	0.25	0.25	0.25	0.25	0.5	0.25	0.5	0.5	0.5	0.5	0.5
	3	0.25	0.25	0.5	0.25	0.25	1	0.25	2	0.5	0.5	0.25	0.25
AGAR	**PAS D**												
	1	4	2	2	4	2	2	0.5	2	2	1	1	2
	2	2	2	4	4	2	2	0.5	4	2	2	2	2
	3	4	2	4	4	4	2	0.5	2	2	2	1	2
MTT	**PAS D**												
	1	0.25	0.25	0.25	0.25	0.25	0.25	0.125	0.5	0.125	0.5	0.25	0.125
	2	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.5	0.5	0.125	0.25	0.125
	3	0.25	0.5	0.25	0.25	0.125	0.25	0.25	0.5	0.125	0.125	0.25	0.25

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**CAP A**												
	1	16	8	8	32	32	32	16	4	4	4	4	4
	2	32	8	8	32	32	32	4	4	8	8	4	4
	3	32	8	4	32	32	16	8	4	4	8	4	2
MTT	**CAP A**												
	1	8	4	8	16	16	16	4	4	4	4	4	4
	2	8	4	8	16	16	16	4	4	4	4	4	4
	3	8	4	8	16	8	16	4	64	4	4	4	4
AGAR	**CAP B**												
	1	32	8	8	32	32	32	8	8	8	8	8	4
	2	32	8	8	32	32	32	8	8	8	8	4	4
	3	32	8	8	32	32	32	8	8	8	8	4	4
MTT	**CAP B**												
	1	8	4	4	4	4	8	4	2	2	4	2	2
	2	8	8	4	4	8	8	4	2	2	2	1	2
	3	4	4	2	2	4	16	4	2	2	2	1	1
AGAR	**CAP C**												
	1	32	8	8	32	32	16	8	8	4	8	4	4
	2	32	8	4	32	32	32	8	8	8	4	4	4
	3	16	8	8	32	32	32	8	4	8	8	8	4
MTT	**CAP C**												
	1	8	4	8	8	8	8	4	4	4	2	2	4
	2	16	4	8	8	8	32	4	4	4	4	2	2
	3	8	8	2	16	16	8	4	4	4	4	2	4
AGAR	**LIN A**												
	1	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	1	0.5	1
	2	0.5	0.5	0.5	0.5	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3	0.5	0.25	0.5	0.5	2	0.5	0.5	0.5	1	0.5	0.5	0.5
MTT	**LIN A**												
	1	0.25	2	2	0.25	0.5	1	0.5	1	0.25	4	0.5	1
	2	0.5	1	2	1	0.5	0.5	0.5	0.5	0.25	2	0.5	0.5
	3	0.5	1	2	1	0.5	0.25	0.5	0.5	0.5	2	0.5	0.5

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**LIN B**												
	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MTT	**LIN B**												
	1	0.5	1	0.25	0.25	0.25	0.125	1	0.25	0.5	0.5	0.25	0.25
	2	0.25	1	0.5	0.25	0.25	0.25	1	0.25	0.5	0.5	0.25	0.5
	3	0.25	1	0.25	0.25	0.5	0.125	0.5	0.25	0.5	1	0.25	0.5
AGAR	**LIN C**												
	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	2	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5	1	0.5	0.5	0.5
	3	0.5	2	0.5	0.5	1	0.5	0.5	0.5	0.5	1	0.5	0.5
MTT	**LIN C**												
	1	0.5	1	0.25	1	0.5	0.25	0.5	0.25	0.25	0.5	0.5	0.5
	2	0.5	1	0.25	0.25	0.5	0.25	0.5	0.25	0.5	0.5	0.5	0.5
	3	0.25	0.5	0.5	1	0.25	0.5	0.5	0.5	0.5	0.5	0.5	0.25
AGAR	**LIN D**												
	1	0.5	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
	2	0.5	0.5	0.5	0.5	1	1	0.5	0.5	1	0.5	0.5	1
	3	0.5	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	1	0.5	0.5
MTT	**LIN D**												
	1	0.25	0.5	0.25	0.25	0.5	0.25	0.5	0.25	0.5	0.5	0.25	0.5
	2	0.5	1	0.5	0.5	0.5	0.25	0.5	0.25	0.5	0.5	0.5	0.5
	3	0.5	1	0.25	0.25	0.5	0.25	0.5	0.25	0.5	0.5	0.5	0.25
AGAR	**MER A**												
	1	16	16	16	0.5	16	16	16	16	16	16	16	16
	2	16	16	16	16	16	16	16	16	16	16	16	16
	3	16	16	16	0.5	16	16	16	16	16	16	16	16
MTT	**MER A**												
	1	16	16	32	8	8	8	16	16	16	16	16	16
	2	16	16	32	8	8	16	16	16	16	16	16	16
	3	16	16	32	8	8	8	16	16	16	16	16	16

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**MER B**												
	1	16	16	16	16	16	16	16	16	16	16	16	16
	2	16	16	16	16	16	16	16	16	16	16	16	16
	3	16	16	16	16	16	16	16	16	16	16	16	16
MTT	**MER B**												
	1	8	16	4	4	4	4	8	8	16	16	16	16
	2	16	16	8	4	4	8	8	8	16	16	16	16
	3	16	16	8	8	4	4	8	8	16	16	16	16
AGAR	**MER C**												
	1	16	16	16	16	16	16	16	16	16	16	16	16
	2	16	16	16	16	16	16	16	16	16	16	16	16
	3	16	16	16	16	16	16	16	16	16	16	16	16
MTT	**MER C**												
	1	16	16	16	16	16	16	8	16	16	16	16	16
	2	16	16	16	16	16	16	8	16	16	16	16	16
	3	16	16	16	16	16	16	8	16	16	16	16	16
AGAR	**M&C A**												
	1	16	32	32	32	32	16	32	32	32	32	32	32
	2	16	32	32	8	32	16	16	32	32	32	32	32
	3	32	32	32	32	32	16	32	32	32	32	32	32
MTT	**1**	32	2	4	1	1	0.5	1	2	1	0.5	1	1
	2	32	4	4	0.5	1	0.5	2	2	1	1	1	1
	3	32	4	4	0.5	1	0.5	1	2	1	1	1	2
AGAR	**M&C B**												
	1	32	32	32	32	32	32	32	32	32	32	32	32
	2	32	32	32	32	32	32	32	32	32	32	32	32
	3	32	32	32	32	32	32	32	32	32	32	32	32
MTT	**1**	1	16	32	0.5	1	0.5	2	2	1	1	1	1
	2	1	16	32	0.5	1	0.5	1	2	1	0.5	1	1
	3	1	16	32	0.5	1	0.5	1	2	1	1	1	2

MIC Values (µg/ml) from Agar Dilution Method and MTT Assay													
METHOD	**DRUG**	**R 26**	**R 252**	**R 413**	**R 502**	**R 506**	**KZN 605**	**V666**	**V1435**	**V2475**	**V4207**	**V4258**	**H37Rv**
AGAR	**M&C C**												
	1	16	16	32	32	32	32	32	32	32	32	32	32
	2	32	32	32	32	32	32	32	32	32	32	32	32
	3	32	32	32	32	32	16	32	32	32	32	32	32
MTT	**1**	1	1	0.5	0.5	1	0.5	1	2	1	1	1	1
	2	1	2	0.5	0.5	1	0.5	1	2	1	1	1	1
	3	0.5	1	1	0.5	1	1	1	2	1	0.5	1	1
MTT	**M&C D**												
	1	0.5	1	0.5	0.5	1	0.5	1	2	1	1	1	1
	2	0.5	1	0.5	0.5	1	2	1	2	1	1	1	2
	3	0.25	4	1	0.5	1	1	1	2	1	2	1	1
MTT	**M&C E**												
	1	1	4	4	0.5	1	0.5	1	2	1	1	1	1
	2	1	1	4	0.5	1	1	1	2	1	1	1	1
	3	0.5	2	2	0.5	1	1	1	2	1	0.5	1	1
MTT	**M&C F**												
	1	1	1	1	0.5	1	0.5	1	2	1	1	1	1
	2	1	1	0.5	0.5	1	0.5	1	2	1	1	1	1
	3	0.5	1	1	1	1	1	1	2	1	1	1	2