Reinfection dynamics of *Mycobacterium tuberculosis*

by

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DECLARATION

I, Joni Mitchell declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Reinfection is an important mechanism leading to recurrent tuberculosis. Recently, molecular epidemiological studies have shown that in high incidence settings, recurrent tuberculosis may occur through reinfection. Animal model experiments have shown that a reinfecting mycobacterial strain is specifically targeted to existing granulomas and that these structures are more dynamic than was previously thought. In this study we hypothesised that primary infection with *M. tuberculosis* may reprogramme human macrophages thereby preventing or facilitating reinfection with a secondary mycobacterial strain.

Two antibiotic-resistant *M. tuberculosis* H37Rv variants were generated by electrotransformation of marked plasmids, designated Kan^R and Hyg^R. A THP1 human macrophage cell line was infected and reinfected with different combinations of these marked strains as well as a hypervirulent *M. tuberculosis* Beijing strain. Mycobacterial growth has been assessed by colony forming unit enumeration and confirmed with polymerase chain reaction (PCR) analysis.

In vitro growth curves of wild-type and differentially marked *M. tuberculosis* H37Rv Kan^R and Hyg^R strains were compared in the BACTEC™ mycobacterial growth indicator tube (MGIT™) system in parallel with conventional liquid culturing. *In vitro* liquid culture growth curves of hypervirulent clinical Beijing strain isolates were also compared to *M. tuberculosis* H37Rv growth curves. Through this it was established that there was no fitness cost as result of plasmid integration and that these strains of varying virulence had similar growth curves. Competitive dynamics within THP1 human macrophage cells were then assessed and have shown that there were no significant differences in growth patterns between primary and secondary infecting strains during THP1 cell reinfection.

The findings of this study answered fundamental questions regarding reinfection of mycobacterial strains. It was established here that human macrophages can indeed be reinfected with a second virulent mycobacterial strain.

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DEDICATION

for my parents

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
BA	blood agar
bp	base pair
CFU	colony forming unit
DC	dendritic cell
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
D-PBS	Dulbecco's phosphate buffered saline
FBS	foetal bovine serum
G	gauge
IL-12	interleukin 12
INF-γ	interferon-gamma
IS*6110*	Insertion sequence *6110*
kV	kilovolt
LJ	Lowenstein-Jensen
MCS	multiple cloning site
µF	microfarad
µg	microgram
ml	millilitre
mM	millimolar
MOI	Multiplicity of infection
nM	nanomolar
nm	nanometre
NO	nitric oxide
NOS	nitric oxide synthase
OADC	oleic acid albumin dextrose complex
OD_{600}	optical density at 600nm
PANTA™	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
RFLP	restriction fragment length polymorphism
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
Th1	T helper cell type 1

Th2	T helper cell type 2
THP1	human acute monocytic leukaemia cell line
TNF-α	tumour necrosis factor-alpha
UV	ultraviolet
WHO	World Health Organisation
ZN	Ziehl Neelsen

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Studies have shown that a large proportion of recurrent cases of tuberculosis are caused by a mycobacterial strain other than the one involved in the initial infection. This happens more frequently than was previously believed in high incidence areas, such as the Western Cape, South Africa (Warren *et al*., 2004; Van Rie *et al*., 1999). This phenomenon justifies further investigation, as it directly impacts on patient treatment and future vaccine strategies. It has been shown that some mycobacterial strains are far more virulent than others, which is postulated to influence the outcome of a reinfection in a host (Warren *et al*., 2004), but has not yet been clarified. It is not known whether some mycobacterial strains may actually confer protection against reinfection with a secondary strain, or whether the presence of a specific strain, such as highly virulent members of the Beijing family, makes a host more susceptible to reinfection.

1.2 Literature review

1.2.1 The worldwide tuberculosis epidemic

Tuberculosis kills more people than any other single micro-organism (Dolin *et al*., 1994). Approximately 1.6 million people died of tuberculosis infection in 2005 according to the latest World Health Organisation (WHO) Report (2007). However, this is a relatively small number considering that an estimated one-third of the world population is infected with *Mycobacterium tuberculosis* in a latent or

clinically silent persistent state (Bloom and Murray, 1992; Sudre *et al*., 1992). Persistence refers to the resilience of *M. tuberculosis* bacilli to survive in spite of the host immune response and chemotherapy (Gomez and McKinney, 2004). Latency can be defined as an undetectable tuberculosis infection, with the threat of reactivation later in life, which may occur after the initial infection has been controlled by the immune response (Parrish *et al.*, 1998). When tuberculosis disease is not active it can be described as dormant, which may refer to the unculturable metabolic state of the *M. tuberculosis* bacilli (Gomez and McKinney, 2004). Whether from an initial infection or reactivation, there are 8 million active tuberculosis cases each year, where Africa constitutes the largest numbers at an incidence rate of 343 per 100 000 people per year, and South-Africa at 600 per 100 000 people, illustrated in **Figure 1.1** (WHO, 2007).

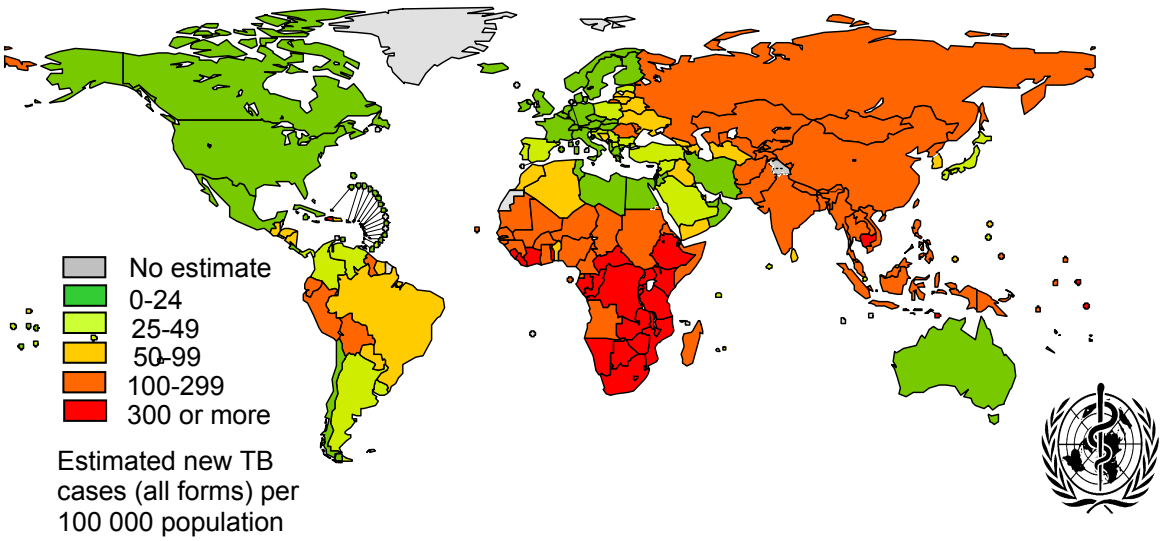

Figure 1.1. World map showing the estimated incidence rates of active tuberculosis during 2005. It is evident from the map that the highest incidence of tuberculosis infection occurs in sub-Saharan African countries (WHO, 2007).

1.2.2 Immunology of tuberculosis

Tuberculosis is an airborne disease. Airborne tuberculosis bacilli are inhaled into the lung where alveolar macrophages phagocytose the bacilli. Some ingested tuberculosis bacilli are killed by phagocytes but most survive, depending on their virulence and the ability of *M. tuberculosis* to manipulate and evade the macrophage antimycobacterial immune response (Dannenberg and Rook, 1994; Flynn and Chan, 2003). The ingested mycobacteria activate macrophages and dendritic cells (DC) to produce tumour necrosis factor-alpha (TNF-α) and interleukin 12 (IL-12) (Young and Robertson, 2002), and IL-12 in turn induces interferon-gamma (INF-γ) production (Fenton and Vermeulen, 1996). INF-γ can be produced by either the innate or acquired immune response and directs the immune response to initiate the development a of T-helper cell 1 (Th1) response (Barnes *et al.*, 1994; Flynn and Chan, 2001). Innate immunity is provided by natural killer cells and the acquired immune compartment is constituted by $CD4^+$ and $CD8^+$ T cells (Young and Robertson, 2002). Surviving mycobacteria multiply within the macrophage and are released when cytotoxic $CD8^+$ T cells lyse the phagocyte for uptake by activated macrophages (Kaufmann and Flesch, 1988). **Figure 1.2** illustrates the anti-inflammatory response of a macrophage or a DC to *M. tuberculosis* infection.

IFN-γ and TNF-α alone are insufficient to kill *M. tuberculosis,* but together they initiate antimycobacterial functions within murine macrophages (Flesch and Kaufmann, 1990). Phagosome-lysosome fusion produces an oxidative burst, which is the generation of reactive oxygen intermediates (ROI) (reviewed by Chan and Kaufmann, 1994). Additionally the L-arginine-dependent cytotoxic pathway induces the production of powerful antimycobacterial toxins, nitric oxide (NO) and reactive nitrogen intermediates (RNI), by the action of inducible nitric oxide synthase (NOS), using L-arginine as substrate, (Chan *et al.*, 1995).

Another process of direct killing of mycobacteria is through a T cell granule protein, granulysin, produced by cytotoxic $CD8^+$ T cells, which are assisted by a pore drilling protein known as perforin (Flynn and Ernst, 2000).

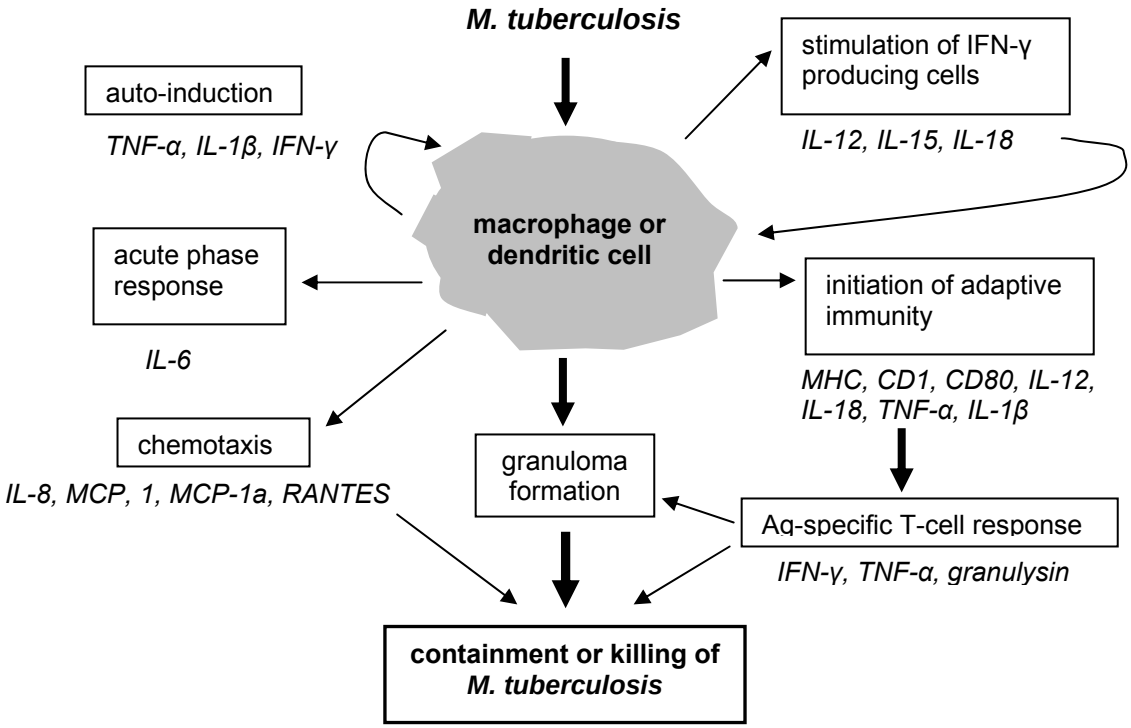

Figure 1.2. Inflammatory cytokine response of an activated phagocyte (macrophage or dendritic cell) to *M. tuberculosis*, directing early events of the immune response (Van Crevel *et al.*, 2002).

These events at the primary site of infection are the start of a granuloma, a central area of necrotic tissue and extracellular bacilli, surrounded by macrophages containing intracellular bacilli which are in turn surrounded by CD^+4 and CD^+8 T cells, B cells and fibroblasts (Flynn and Chan, 2001). In 90% of infected individuals the infection is contained at this point and remains clinically latent, but 10% may present with clinical tuberculosis at some later stage (Parrish *et al*., 1998). This implies that the immune response cannot eliminate an initial tuberculosis infection but is protective enough to prevent disease progression (Chan and Kaufmann, 1994).

Exposure to most infectious pathogens usually results in an asymptomatic period of incubation or latency, which may progress to clinical disease. With *M. tuberculosis*, the latency period is extremely variable, resulting in either rapid progression to clinical disease within a few weeks or the infection may remain latent with the threat of reactivation many years later (Parrish *et al*., 1998; Fine and Small, 1999). It has been debated for decades whether the majority of active tuberculosis cases results from endogenous reactivation or whether they arise as a result of exogenous reinfection (Chiang and Riley, 2005).

1.2.3 Tuberculosis reinfection

Recurrent cases of tuberculosis have always been assumed to be caused by endogenous reactivation of a previous primary infection and reinfection was considered to occur rarely due to the immunity conferred by the initial infection (Small *et al.*, 1993). It was in fact suggested that the idea of exogenous reinfection should be abandoned (Stead, 1967). However after re-evaluation of the same data it was concluded that reinfection may play a part in tuberculosis development, but only in areas of relatively high incidence (Romeyn, 1970; Chiang and Riley, 2005).

In contrast to the dogma that a successfully treated tuberculosis patient is being protected against later tuberculosis infections (Stead, 1967), a study done in the United Kingdom by Vynnycky and Fine (1997) found that primary tuberculosis infection confers little protection against exogenous reinfection, with only 16% in adolescents and 41% in adults. However, the risk associated with disease after reinfection is still lower than that of developing disease after primary exposure (Vynnycky and Fine, 1997).

More recently, it was concluded that successfully treated tuberculosis patients are at higher risk for tuberculosis reinfection than the general population (Verver *et al.*, 2005). Furthermore, it was found that the immunity that develops as a result of active disease would confer the same protection against exogenous reinfection as the immunity that develops after a period of latency following the first episode of active disease (Van Rie *et al*., 1999). It was suggested that reinfection is unpredictable and may cause reinfection disease at any stage of a tuberculosis infection or may even cause reactivation of a latent infection (Warren *et al.*, 2004), the various scenarios being described in **Figure 1.3**.

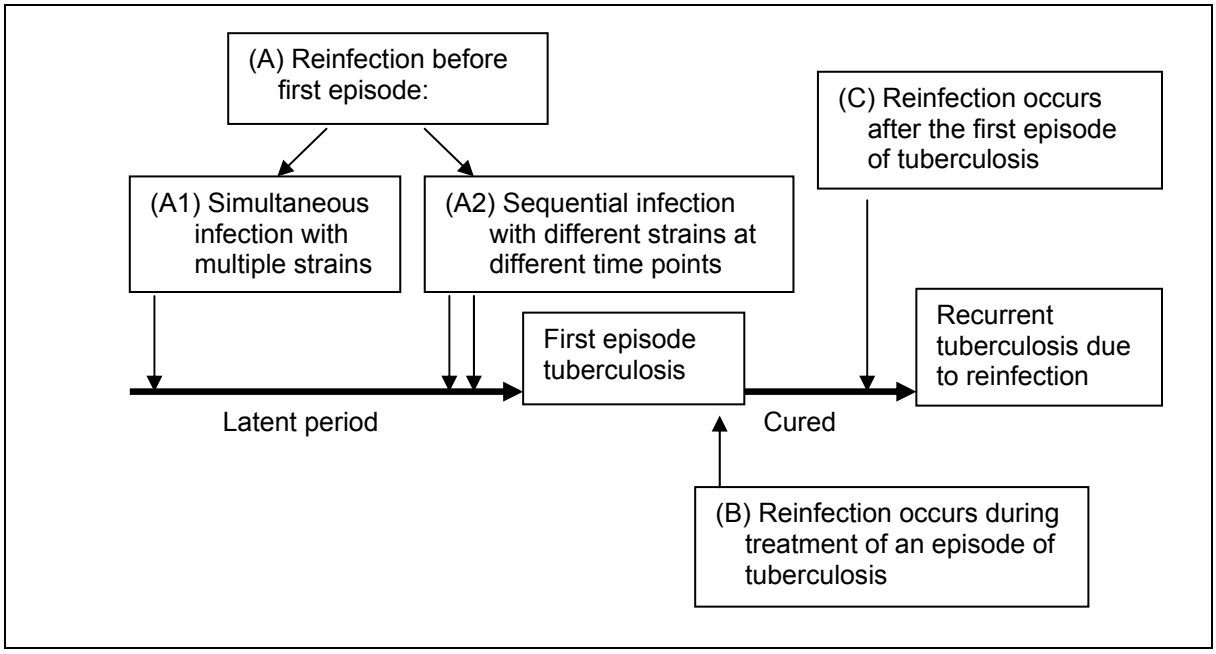

Figure 1.3. Reinfection may occur at different stages of tuberculosis infection: (**A**) Before the first episode, either (**A1**) simultaneously or (**A2**) sequentially, (**B**) during treatment of an episode and (**C**) after curative treatment (Chiang and Riley, 2005).

Deoxyribonucleic acid (DNA) fingerprinting and polymerase chain reaction (PCR) based strain classification of *M. tuberculosis* showed that reinfection with a secondary tuberculosis strain in patients in high risk areas actually occurs at a relatively high rate (Warren *et al*., 2004; Van Rie *et al*., 1999). In contrast to this,

different studies reported that reinfection does not only occur in high risk areas but also in areas with low incidence of tuberculosis (Bandera *et al.*, 2001; Garcia de Viedma *et al.*, 2002). However, it seems clear that the greater the prevalence of *M. tuberculosis* the greater the risk of tuberculosis reinfection (Fine and Small, 1999; Romeyn, 1970; Van Rie *et al.*, 1999).

1.2.4 The *M. tuberculosis* Beijing strain family

The *M. tuberculosis* Beijing strains are widely known for their frequent appearance in outbreaks and association with drug resistance, suggesting that Beijing strains have enhanced fitness and the potential to spread. Multidrug resistance (MDR) have been increasing world-wide and most MDR *M. tuberculosis* strains have been found to be Beijing strains (Kruuner *et al.*, 2001). In the Western Cape of South Africa 28% of drug resistant *M. tuberculosis*-infected patients are infected with drug-resistant Beijing-like isolates (Streicher *et al.,* 2004), the prevalence of which is illustrated in **Figure 1.4**.

All Beijing-genotype strains share a number of independent genetic markers (Bifani *et al.*, 2002). One of these constitutes an insertion sequence *6110* (IS*6110*) restriction fragment length polymorphism pattern (RFLP), which is highly similar, but not entirely identical in all Beijing strains. IS*6110* is an insertion sequence-like element specific to a group of mycobacterial strains, designated *M. tuberculosis* complex, and identification of strains are based on the chromosomal position of IS*6110* and different amounts of copy numbers. The spoligotype pattern for the Beijing family involves hybridisation to a 9 spacer region between spacers 35 and 43 (Kremer *et al.*, 2004) and the IS*6110* insertion element namely A1 in the origin of replication between the genomic dnaA-dnaN locus (Kurepina *et al.*,1998). The Beijing spoligotype is characterised by the deletion of spacers 1 to 34 in the direct

repeat region (Kremer *et al.*, 2004) and has been found to be identical for all strains of the Beijing family (Van Soolingen *et al*., 1995).

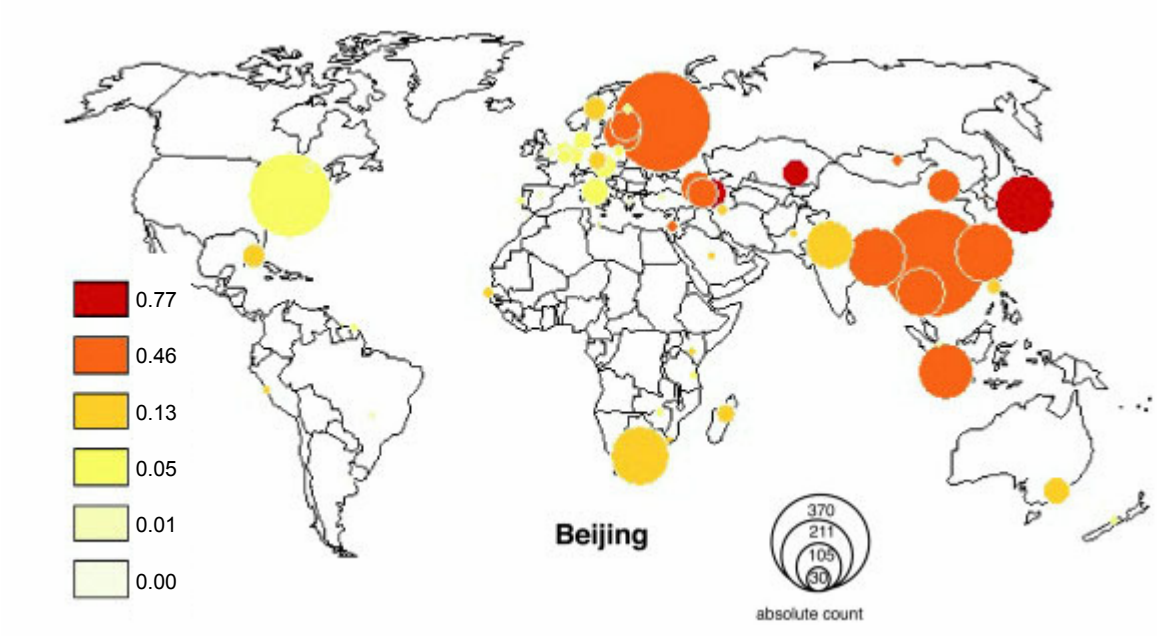

Figure 1.4. World map showing absolute (diameter) and percentage (colour) numbers of Beijing strains within each country. Beijing comprising 24.5% of the 50 most frequently found strain families in the world (Brudey *et al*., 2006).

The Beijing genotype may represent a higher level of evolutionary development of *M. tuberculosis*, indicative of a more recent common ancestor (Kremer *et al.*, 2004). Beijing strains display a number of genetic differences compared to other *M. tuberculosis* strains, which may have advanced their ability to overcome the protective effects of BCG vaccination and resistance to treatment with anti-tuberculosis drugs (Glynn *et al.*, 2002; Van Soolingen *et al.*, 1995; Van Soolingen, 2001). The members of the Beijing family have a faster growth rate within monocytes than other strains – this hypervirulence may contribute to their advantage over other genotypes to acquire resistance (Li *et al.*, 2002).

In this study, Beijing strains from a hypervirulent cluster were used. These hypervirulent strains have been observed, through molecular epidemiology, to spread more readily than any other strain. This observation was supported by *in vivo* studies in which mice succumbed to a Beijing strain infection within 4 weeks after inoculation, as opposed to *M. tuberculosis* H37Rv, which killed mice within 6 weeks (personal communication, Hernandez-Pando). Furthermore Theus *et al.* (2005) found that persistent cluster strains such as the Beijing family may induce a rapid anti-inflammatory response from infected macrophages, by blocking mechanisms or switching mechanisms on, to secure an increased chance of survival. By altering cell function, Beijing strains could influence the cells' anti-inflammatory responses (Theus *et al*., 2005). This raises the question whether a primary infecting Beijing strain could alter the cell in such a way to prevent the cell from being reinfected with another strain of *M. tuberculosis* or alternatively, encourage reinfection.

1.2.5 Models for mycobacterial-host interaction

Little is understood of the underlying immune interaction between the host and tuberculosis bacillus and its evasion of host immunity during persistent infection, reactivation and reinfection. Moreover, the ability to investigate all aspects of tuberculosis infection is difficult and sometimes impossible to achieve in humans, therefore vast efforts in tuberculosis research have been invested in the development and refinement of various animal and cell culture models.

1.2.5.1 Animal models of tuberculosis

Various animal models have been used to study the pathogenesis of *M. tuberculosis* infection. Mouse models are well established and most commonly

used, considering the cost and availability to genetically altered mutants. This has contributed much to the study of immunology, resistance and reinfection of tuberculosis (Gupta and Katoch, 2005). However, the inability of mice to develop tuberculosis that closely resembles human tuberculosis disease is a major disadvantage of the mouse model (Gupta and Katoch, 2005).

Tuberculosis in guinea pigs and rabbits closely resemble the pathological features seen in human tuberculosis, but unlike humans, guinea pigs are innately susceptible and therefore succumb quickly to pulmonary tuberculosis infection, which is a drawback of this model (Cosma *et al.*, 2003; Gupta and Katoch, 2005).

Non-human primate models have been investigated for the study of tuberculosis infection because they develop natural human-like tuberculosis. Unfortunately, only small numbers of subjects could be studied at one time because of the high cost and difficulty in handling, as well as ethical issues (McMurray, 2000; Walsh *et al.*, 1996), compromising the accuracy of a study conducted in this model.

Disadvantages of animal models for the study of tuberculosis are that they are expensive and time-consuming. Even though mouse, guinea pig, rabbit and non-human primate models have helped in the understanding of immunology, assessment of virulence and pathogenesis of tuberculosis disease, no single animal model has been found to reproduce human disease mechanisms and clarify the underlying interactions between host and bacillus that leads to disease (Gupta and Katoch, 2005; Silver *et al*., 1998a).

1.2.5.2 Animal models for tuberculosis reinfection studies

Reinfection studies have been performed in various animal models with a wide variety of outcomes. For instance, Ziegler *et al.* (1985) found a lower bacterial

load and a lesser tendency of *M. tuberculosis* to disseminate after reinfection, therefore showing some protection. In their study, different clinical *M. tuberculosis* strains of variable virulence were used for the initial infection and reinfection in guinea pigs (Ziegler *et al.*, 1985). A murine model using environmental mycobacteria as the primary infecting strain followed by reinfection with *M. tuberculosis* showed a large difference in outcome and pathogenesis (Hernandez-Pando *et al.*, 1997). A low mycobacterial dose induced a Th1 response, which was protective, but a high dose induced a combined Th1 and Th2 response that lead to increased susceptibility to reinfection (Hernandez-Pando *et al.*, 1997). Therefore, the presence of the Th2 response accompanied by the damage caused by TNF-α, accelerated pathogenesis (Hernandez-Pando *et al.*, 1997). From these animal studies it may be deduced that the immune response could be primed during an initial infection with *M. tuberculosis* or other mycobacteria, to either be protective or enhance susceptibility, depending on the intensity of the initial infecting dose.

During a vaccine study in mice, Repique *et al*. (2002) showed that a chronic tuberculosis infection did not completely protect against reinfection. Similarly, studies performed to investigate the dynamics of a granuloma in zebra fish using *M. marinum* found that the original infecting strain attracted macrophages containing phagocytosed mycobacteria from a secondary infection. It seemed that the newly infected macrophages quickly migrated to granulomas as an adaptive immune response, even though the original infecting mycobacterium could not be eliminated (Cosma *et al.*, 2004). These reinfection animal studies in turn support the hypothesis that a current or previous tuberculosis infection does not protect against reinfection.

It is therefore clear that there are two contrasting hypotheses regarding tuberculosis reinfection dynamics, as ascertained from animal models, which may

in fact not be applicable to human immune responses, as no animal model can mimic human disease exactly. The use of human cell culture as a model for reinfection may thus provide a more accurate view of human tuberculosis reinfection.

1.2.5.3 Cell culture models for the study of mycobacterial-macrophage interaction

Cell culture models are rapid, cost-effective and more manageable than animal models and provide good models for early stage infection (Theus *et al*., 2006). Cell culture techniques have been used to investigate many aspects of tuberculosis, including the immunology of tuberculosis (Crowle and May 1981; Fenton *et al.*, 1997; Silver *et al*., 1998a), mechanisms of apoptosis (Durrbaum-Landmann *et al.*, 1996; Riendeau and Kornfeld, 2003) and phagocytosis (Shattock *et al.*, 1994; Aston *et al.*, 1998; Rajavelu and Das, 2007), intracellular growth (Li *et al.*, 2002; Stokes and Doxsee, 1999; Theus *et al.*, 2004) and virulence (Silver *et al*., 1998b; Zhang *et al.*, 1998; Manca *et al.*, 1999).

Various human cell culture systems have been used as a model to study early stage infection with *M. tuberculosis*, each with its own advantages and disadvantages. There are two basic types of cell culture systems: primary cell isolates and *in vitro* differentiated cell lines. An example of primary cell isolates is human alveolar macrophages (Fenton *et al.*, 1997; Aston *et al.*, 1998; Borelli *et al.*, 2002). Primary cell isolates are terminally differentiated and may be expected to show some donor variability (Durrbaum-Landmann *et al.*, 1996) and are difficult to obtain in large numbers, but cells from human origin may be more representative of a clinical situation than differentiated cells (Stokes and Doxsee, 1999).

Some examples of *in vitro* differentiated cells are human monocyte-derived macrophages (Crowle and May, 1981; Stokes and Doxsee, 1999) and human macrophage-like cell lines such as the human acute monocytic leukaemia cell line, THP1 (Riendeau and Kornfeld, 2003; Rajavelu and Das, 2007; Theus *et al.*, 2006). Using these immortalised continuously cycling cells may decrease the variability between results and large amounts of cells can be produced (Stokes and Doxsee, 1999).

The human macrophage-like cell line, THP1, can be differentiated using phorbol 12-myristate 13-acetate (PMA). PMA stimulates Protein Kinase C, mimicking the effect of diacylglycerol involved in natural macrophage differentiation. This triggers the THP1 cells to differentiate into a macrophage-like morphology causing the cells to become adherent, phagocytic and to stop reproducing (Riendeau and Kornfeld, 2003).

Little is known of the dynamic interaction of reinfection with a secondary infecting tuberculosis strain following a primary infection. The THP1 model presents an ideal opportunity to elucidate mycobacterial responses and factors of the host immune system involved in these processes.

1.3 Problem statement

Limited information is available on the dynamic interaction between multiple virulent *M. tuberculosis* strains during reinfection of disease. Reinfection may occur in a treated patient following cured primary tuberculosis infection, during persistent infection, or as a superinfection.

1.4 Hypothesis

We hypothesise that infection with a mycobacterial strain will influence reinfection with a secondary mycobacterial strain. A primary tuberculosis infection may either have a protective effect or the presence of a specific strain, such as a highly virulent member of the *M. tuberculosis* Beijing family, may facilitate or even enhance reinfection by a secondary mycobacterial strain.

1.5 Aims of this study

The aims of this study was to investigate the likelihood of reinfection within a human macrophage-like cell line and to establish how primary infection with a hypervirulent Beijing strain of *M. tuberculosis* will influence a secondary infection with *M. tuberculosis* H37Rv.

1.6 Experimental approach

In this study it was envisaged to use a THP1 macrophage-like cell line to evaluate the intracellular dynamics between a secondary infecting marked *M. tuberculosis* H37Rv strain and a primary differentially marked *M. tuberculosis* H37Rv strain. In order to assess how strains of varying virulence affect the outcome of a reinfection, it was proposed to use a hypervirulent *M. tuberculosis* Beijing strain as the primary infecting strain and marked *M. tuberculosis* H37Rv as the secondary infecting strain. Growth was assessed by colony forming unit (CFU) enumeration and confirmed by real-time PCR.

CHAPTER 2

MATERIALS AND METHODS

2.1 Safety consideration and location of study

Due to the biohazardous nature of the work involved in this study, all experimental work was done in the biohazard level 3 laboratories of the Department of Biomedical Sciences, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg Campus, South Africa.

2.2 Mycobacterial strains and culture conditions

M. tuberculosis H37Rv (ATCC 27294) and two hypervirulent clinical isolates of the Beijing family, strains 208 and 209, were propagated in Middlebrook 7H9 broth (Becton Dickinson, Microbiology systems, Sparks, MD, USA), supplemented with 10% Middlebrook oleic acid albumin dextrose complex (OADC) (Becton Dickinson, Microbiology systems, Sparks, MD, USA) and 0.05% Tween 80 (Sigma, St Louis, MO, USA). Cultures were grown in filtered cap cell culture flasks (Cellstar®, Greiner Bio-One, Frickenhausen, Germany), stationary, at 37ºC for a period of 28 days or as individual experiments required.

For CFU enumeration *M. tuberculosis* cultures were plated on Middlebrook 7H10 solid media (Becton Dickinson, Microbiology systems, Sparks, MD, USA), supplemented with 10% OADC and 0.5% glycerol (Merck, Gauteng, South Africa). Ten-fold serial dilutions were made using 0.9% saline (Merck, Gauteng, South Africa) containing 0.01% Tween 80 and plates were incubated at 37ºC for 21 days.

2.3 Generation of marked *M. tuberculosis* H37Rv strain variants

In order to study the dynamics of reinfection of tuberculosis, two marked *M. tuberculosis* H37Rv variants were generated, namely *M. tuberculosis* Kan^R, containing the plasmid pIVK1 integrated into its genome, and *M. tuberculosis* Hyg^R, containing the integrated plasmid pIVH1. Plasmids were kindly provided by Dr J. Gomez, Laboratory of Infection Biology, Rockefeller University, New York, United States of America. Plasmids were constructed from a pUC19 backbone, containing an ampicillin resistance gene (*bla*) (**Figure 2.1**), with an additional complete L5 integrase gene (*int*) and a phage attachment site (*att*P), which ensured that the plasmid would efficiently integrate into the *M. tuberculosis* chromosomal *att*B site. From this backbone, the plasmid pIVK1 (**Figure 2.2**), which in addition contains the insert aminoglycoside (3') phosphotransferase (*aph*), conferring kanamycin resistance (Kan^R), was constructed. The plasmid pIVH1 (**Figure 2.3**), which contains a Hygromycin B phosphotransferase (*hph*) insert conferring hygromycin resistance (Hyg^R), was also constructed. Detailed sequence maps of pIVH1 and pIVK1 can be seen in Appendix A.

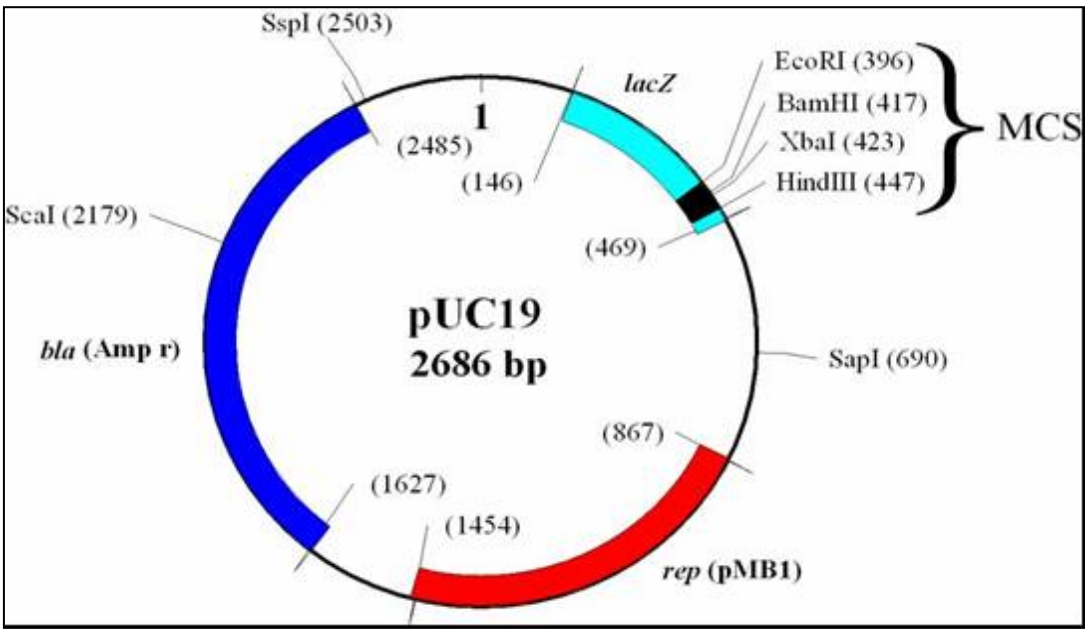

Figure 2.1. pUC19 plasmid restriction map. pUC19 (2686 bp) is represented with the genes, *lacZ* with a multiple cloning site (MSC), *rep* (pMB1), the origin of replication and *bla,* conferring ampicillin resistance, as well as a few restriction enzyme sites.

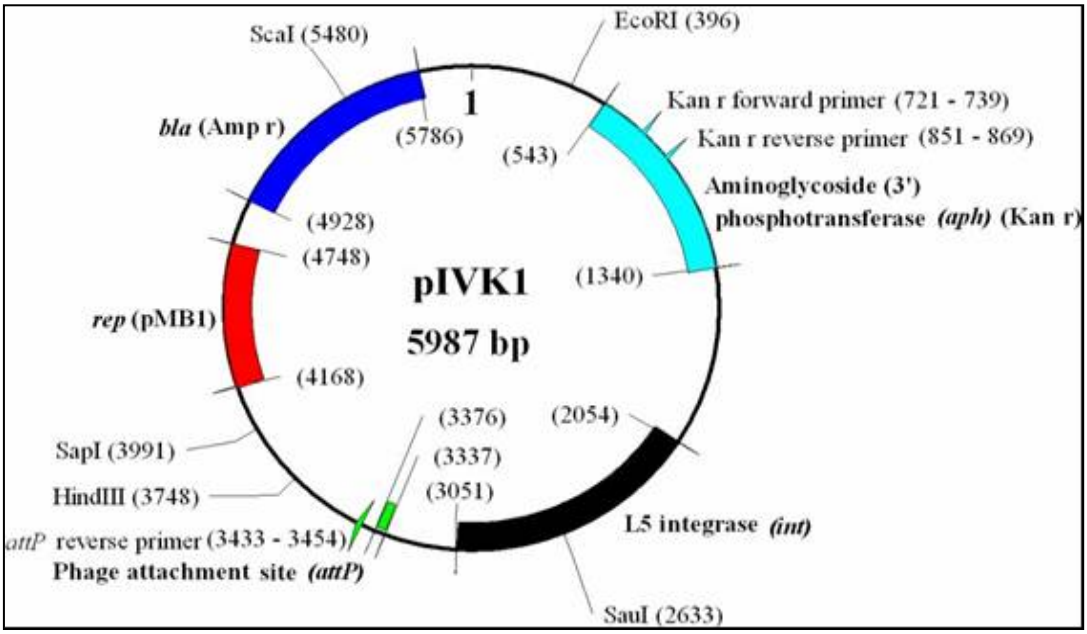

Figure 2.2. pIVK1 plasmid restriction map. pIVK1 (5987 bp) is represented with the genes, *aph*, Aminoglycoside (3') phosphotransferase, conferring kanamycin resistance; *int*, a L5 integrase site; *att*P, a phage attachment site; *rep* (pMB1), the origin of replication and *bla,* conferring ampicillin resistance. The Kanr forward and reverse primer set (section 2.5) and *att*P reverse primer (section 2.6) are shown as well as some restriction enzymes sites.

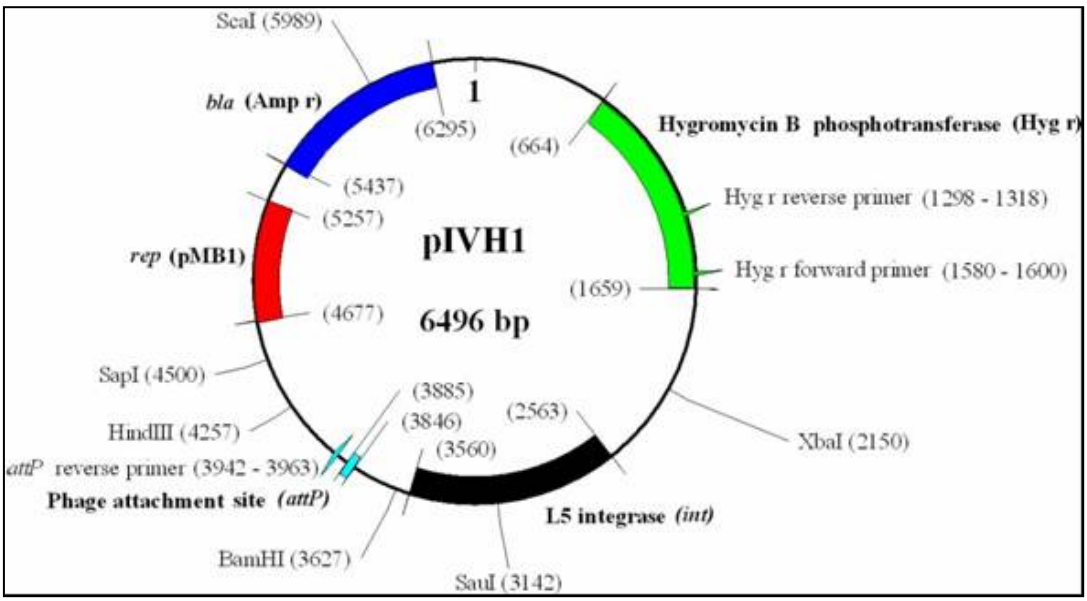

Figure 2.3. pIVH1 plasmid restriction map. pIVH1 (6496 bp) is represented with the genes, *hph* Hygromycin B phosphotransferase, conferring hygromycin resistance; *int*, a L5 integrase site; *att*P, a phage attachment site; *rep* (pMB1), the origin of replication and *bla*, conferring ampicillin resistance. The Hygr forward and reverse primer set (section 2.5) and *att*P reverse primer (section 2.6) are shown as well as some restriction enzyme sites.

2.4 Electrotransformation of *M. tuberculosis* H37Rv strain variants

Liquid cultures of *M. tuberculosis* H37Rv were propagated in Middlebrook 7H9 broth, as described in section 2.2, for approximately 2 weeks until log phase was reached. The plasmids were prepared for integration in 1.5% glycine (Merck, Gauteng, South Africa) for 24 hours at 37ºC prior to making electrocompetent cells. The *M. tuberculosis* H37Rv cultures were harvested by low-speed centrifugation, the centrifugation step was repeated in 10% glycerol, after which the cells were finally resuspended in 10% glycerol. The pIVK1 and pIVH1 plasmid DNA was cleaned by ethanol precipitation on the day of electroporation, after which the DNA was added to the electrocompetent cells in a pre-warmed cuvette and pulsed. A Bio-Rad Gene Pulser® (Hercules, CA, USA) was used for the transformation by electroporation (Voltage = 2.5 kV; Resistance = 1000 Ohms, Capacitance = 25µF, extended to 125µF). The electroporated mycobacterial cells

were incubated in 7H9 liquid media at 37ºC overnight and then pelleted by low speed centrifugation. Thereafter, electrotransformed cells were plated on 7H11 solid media (Becton Dickinson, Microbiology systems, Sparks, MD, USA) plates, supplemented with 10% OADC and 0.5% glycerol as well as selective antibiotics, either 50 µg/ml kanamycin (Roche, Indianapolis, IN, USA) or 100µg/ml hygromycin (Roche, Indianapolis, IN, USA). Following incubaction of plates for 3 to 4 weeks at 37ºC, single colonies were picked and streaked on Lowenstein-Jensen (LJ) slopes (National Health and Laboratory services (NHLS), Cape Town, South Africa) for long term storage of pure cultures.

2.5 PCR confirmation of marked *M. tuberculosis* H37Rv variants

In order to confirm that *M. tuberculosis* Kan^R contains the pIVK1 plasmid integrated in its genome, a PCR for the amplification of a 148 bp product was done, using the primer set designated Kanr r, reverse primer, and Kanr f, the forward primer. Similarly, a PCR for the amplification of a 302 bp product was done to confirm that *M. tuberculosis* Hyg^R contains pIVH1, using the primer set designated Hygr r as reverse primer and Hygr f as forward primer **(Table 2.1)**. The DNA was obtained by boiling colonies from the respective strains, grown on 7H11 agar, in 0.2ml 0.9% Saline with 0.001% Tween 80 in a heating block for 20 minutes. Hot-start PCR was done, using the HotStar Taq kit (Qiagen, Southern Cross Biotechnology, Germany) using the reaction mixture as prescribed in the product sheet (Appendix B).

Table 2.1. PCR primers used to detect the presence of pIVK1 or pIVH1

Primer name	Orientation	Primer sequence	T_m	Product size
Kan^R amplification				
Kanr f	**Forward**	**5'- CGT TGC CAA TGA TGT TAC - 3'**	**52ºC**	**148 bp**
Kanr r	**Reverse**	**5'- TAC CTG GAA TGC TGT TTT - 3'**	**50ºC**	
Hyg^R amplification				
Hygr f	**Forward**	**5'- CTG CGG AAC GAC CAG GAA TT - 3'**	**62ºC**	**302bp**
Hygr r	**Reverse**	**5'- CGG CTC ATC ACC AGG TAG GG - 3'**	**66ºC**	

The PCR conditions for the Kan^R reaction were programmed on an Eppendorf thermocycler PCR machine (Hamburg, Germany), with an initial denaturation at 95ºC for 15 minutes, followed by 35 cycles of:

Denaturation: 94ºC for 30 seconds,

Annealing: 50ºC for 30 seconds and

Extension: 72ºC for 30 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the sample was held at 4ºC until use.

The PCR conditions for Hyg^R reaction were programmed on the PCR machine with an initial denaturation at 95ºC for 15 minutes, followed by 35 cycles of:

Denaturation: 94ºC for 30 seconds,

Annealing: 60ºC for 30 seconds and

Extension: 72ºC for 30 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the sample was held at 4ºC until use.

PCR products were separated on 2% agarose gels (Appendix B) (Whitehead Scientific, Cape Town, South Africa) prepared with Tris-acetate-EDTA (TAE) buffer pH 8.3 (Appendix B) and visualised under ultraviolet (UV) illumination.

2.6 PCR confirmation of plasmid integration

In order to prove that the pIVK1 and pIVH1 plasmids were integrated into the *M. tuberculosis* H37Rv genome a section of DNA at the L5 *att*P integration site was amplified, using the primer set designated *att*P f, forward primer, and *att*P r, the reverse primer **(Table 2.2)**. This primer set yielded a 184 bp product on amplification, which contains a section of DNA spanning both the *M. tuberculosis* and plasmid sides of the *att*P integration site **(Figure 2.4)**. For this hot-start PCR reaction the template DNA prepared in section 2.5 was also used.

Table 2.2. PCR primers for the analysis of plasmid integration into *att*P integration site

Primer name	Orientation	Primer sequence	T_m	Product size
***att*P amplification**				
***att*P f**	**Forward**	**5'- TCT GCT ACC ACG TAC AGT CG -3'**	**62ºC**	**184 bp**
***att*P r**	**Reverse**	**5'- CAG AAG AGT CGC ACA AGA GTT -3'**	**62ºC**	

The PCR conditions for the *att*P reaction were programmed on an Eppendorf thermocycler with an initial denaturation at 95ºC for 15 minutes, followed by 35 cycles of:

Denaturation: 94ºC for 30 seconds

Annealing: 62ºC for 30 seconds

Extension: 72ºC for 30 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the samples were held at 4ºC until use.

PCR products were separated and visualised on 1% agarose gels (Appendix B), made with TAE buffer (Appendix B), and visualised under UV illumination.

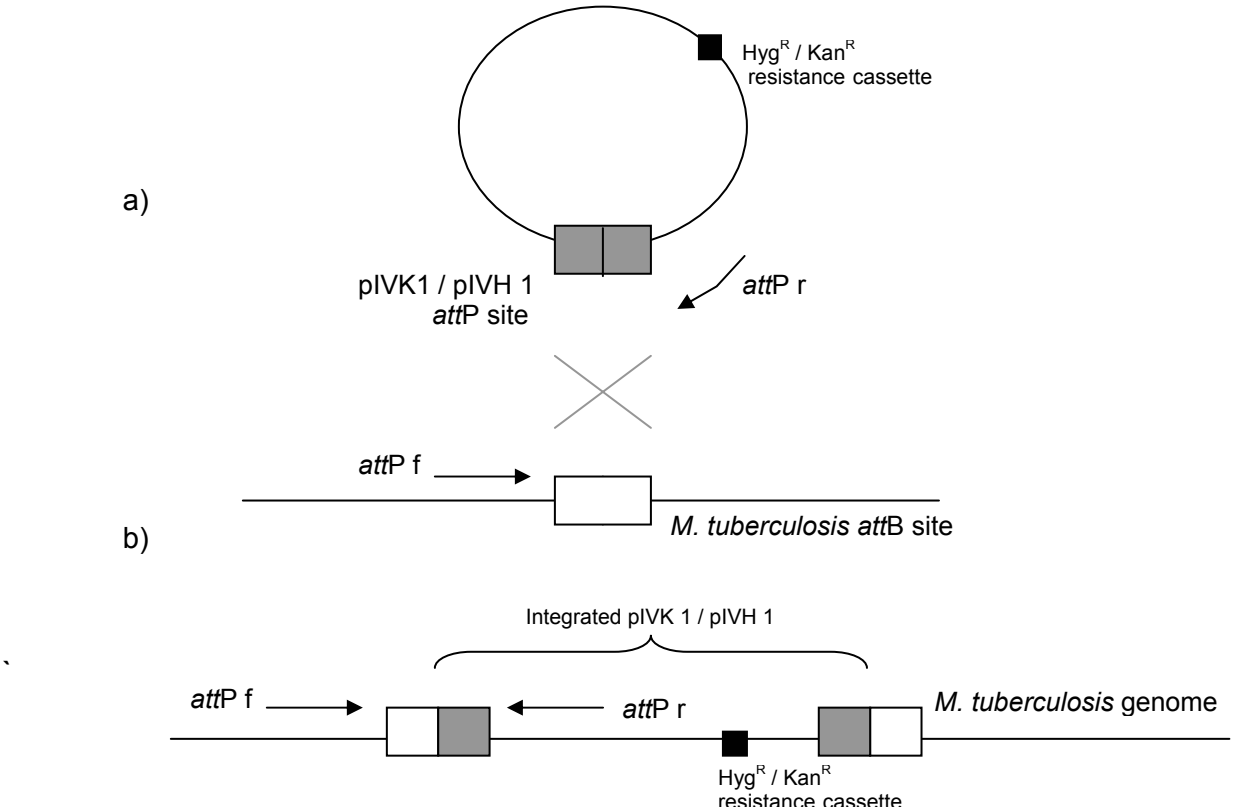

Figure 2.4. Schematic representation of the integration of plasmids, pIVK1 and pIVH1 into the *M. tuberculosis* genome. a) Plasmid *att*P (grey box) and *M. tuberculosis att*B (white box) DNA segments, showing the alignment of the integration sites. The black box indicates the Hyg^R or Kan^R resistance cassettes within the respective plasmids. b) The product of integration in the *M. tuberculosis* genome is shown. Arrows indicate the location and direction of the forward, *att*P f, and reverse, *att*P r, primers in both schemes.

2.7 *In vitro* growth curves of mycobacterial strains

2.7.1 The MGIT^{TM} system

The BACTEC^{TM} mycobacteria growth indicator tube (MGIT^{TM}) system (Becton Dickinson, Diagnostic systems, Sparks, MD, USA) was used for *in vitro* growth curve experiments. Each MGIT^{TM} tube contains 7ml modified Middlebrook 7H9 broth base, which was supplemented with 0.8ml Growth Supplement/BBL^{TM} MGIT^{TM} PANTA^{TM} (Polymyxin B (6,000 units), Amphotericin B (600 µg), Nalidixic acid (2,400 µg), Trimethoprim (600 µg), Azlocillin (600 µg)), containing OADC enrichment and a mixture of antibiotics immediately prior to inoculation with 0.5ml mycobacterial culture suspension. The MGIT^{TM} system works on the principle of

measuring oxygen consumption by mycobacteria using an orange fluorescent compound, sensitive to oxygen levels, embedded into silicone at the bottom of each MGIT^{TM} tube.

2.7.2 Establishing the optimal CFU titre for growth curve inoculation

In vitro MGIT^{TM} culturing of marked *M. tuberculosis* Kan^{R} and Hyg^{R} strains involved initial inoculation of a single colony from 7H11 plates, suspended in 0.5ml 0.9% Saline containing 0.001% Tween 80, into a MGIT^{TM} tube, supplemented with 0.8ml growth supplement, added immediately prior to inoculation. The wild-type *M. tuberculosis* H37Rv strain, on the other hand, was inoculated with 0.5ml H37Rv frozen stock into a MGIT^{TM} tube. Cultures were incubated at 37ºC in the MGIT^{TM} system until it reached a reading of between 200 and 400 MGIT^{TM} units. The cultures were tested for purity by Ziehl Neelsen (ZN) staining (Appendix B) and inoculation on a blood agar (BA) (NHLS, Cape Town, South Africa) plates at 37ºC for 2 days.

For pilot growth curve experiments, 0.5ml of the initial MGIT^{TM} cultures was added to a fresh MGIT^{TM} tube supplemented with 0.8ml growth supplement, added immediately prior to inoculation. The inoculum dilution was replicated by suspending 0.5ml of the initial MGIT^{TM} culture in 7.8ml 0.9% Saline containing 0.04% Tween 80 and was plated on 7H10 agar plates as described in section 2.2, plating dilutions from 10^{0} to 10^{-4}. This was done in order to determine the inoculum titre CFU. MGIT^{TM} readings were taken every second day during log phase, for a period of 2 weeks, until stationary phase was reached. Following that, readings were taken twice a week for another 2 weeks to establish a 4 week growth curve.

2.7.3 *In vitro* MGIT™ growth curve

After establishing that between 10^2 and 10^3 bacteria was an optimal inoculum for a growth curve, MGIT™ tubes were inoculated with each of *M. tuberculosis* Kan^R, Hyg^R and H37Rv cultures, at 5 different concentrations, 1 x 10^3, 5 x 10^3, 1 x 10^4, 5 x 10^4 and 1 x 10^5 for each strain. All of these inoculations were done in duplicate and the experiment was repeated. Each 7ml MGIT™ tube was supplemented with 0.8ml growth enrichment medium and MGIT™ readings were taken every second day for 20 days. In addition to this, the inoculum titre was determined by replicating the inoculum as described in section 2.7.2. At the end of each growth curve experiment, all cultures were tested for purity as described in section 2.7.2.

2.7.4 Antibiotic supplementation to select for *M. tuberculosis* Kan^R and Hyg^R strain growth

M. tuberculosis Kan^R and Hyg^R strain cultures were plated in ten-fold serial dilutions, (10^1 to 10^6) in duplicate. These dilutions were plated on both 7H10 and 7H10 supplemented with the recommended concentrations of kanamycin (50µg/ml) and hygromycin (100µg/ml).

2.7.5 Liquid 7H9 culture growth curve

An initial culture was set up using approximately 10^5 bacteria from frozen stock or a single colony from 7H10 plates, which was inoculated into 10ml 7H9 broth. In order to determine that cultures were pure, testing was performed by ZN staining and inoculating a BA plate for 2 days on each initial culture as described before in section 2.7.2. Optical density (OD) readings were taken, using a Novaspec II (Biochrom Ltd, Cambridge, England) at 600nm on day 7, and again

at 24 to 48 hours later, depending on the result. When an OD_{600} of ± 0.8 was reached, the culture was scaled up by adding 500µl of initial culture to 50ml of 7H9. On the day of inoculation, CFU assessment of the initial culture was determined by plating on 7H10 agar, in dilutions from 10^{-2} to 10^{-7}, as well as from the scaled-up liquid culture, in dilutions from neat to 10^{-4}. OD_{600} readings were taken on days 0, 3, 7, 14, 21 and 28. On day 7 serial dilutions from 10^{-1} to 10^{-5} were plated and on days 14, 21 and 28 cultures were diluted from 10^{-3} to 10^{-9} and incubated as described in section 2.2.

2.7.6 Preparation of *M. tuberculosis* stock cultures

Liquid cultures of *M. tuberculosis* H37Rv, Kan^R, Hyg^R and Beijing strains, 208 and 209, were grown in 7H9 Middlebrook broth and log phase stocks were frozen at -80ºC, supplemented with 15% glycerol for long term storage.

2.8 Cell culture experiments

2.8.1 THP1 cells and culture conditions

The THP1 (ATCC TIB-202) cell line was kindly provided by Hanne Veenstra, Department of Biomedical Sciences, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg Campus. A THP1 macrophage-like cell line was cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMax™ (Invitrogen, Paisley, UK), containing 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen, Paisley, UK) and 50µg/ml gentamicin (Roche, Indianapolis, IN, USA) (Appendix B), at 37°C with 5% CO_2 and 95% humidity. Initially, THP1 cells were cultured using 1% pen/strep (5000 units/ml penicillin and 5000µg/ml streptomycin) (Invitrogen, Paisley, UK) as

antibiotic, which was replaced by gentamicin after contamination problems were encountered. Since gentamicin has a bacteriostatic effect on *M. tuberculosis* (Ho *et al*., 1997), the cells were washed before any *M. tuberculosis* infection experiments were done as described in section 2.8.4. Cell growth was monitored on a daily basis and media was changed according to the general appearance and amount of the cells, approximately every third day.

2.8.2 Preparation of THP1 cells for storage

A healthy culture of cells was pelleted by low speed centrifugation, at 1000rpm for 5 minutes, resuspended in FBS and cooled on ice for 5 minutes. A 20% DMSO dilution was made in unsupplemented RPMI and cooled on ice for 5 minutes. The cells were frozen at a final concentration of 10% DMSO by combining the cell mixture and 20% DMSO solution in an equal volume. Aliquots were wrapped in paper towel to allow cells to cool slowly when put in the -80°C freezer (to store cells for up to a month) or liquid nitrogen tank (for long term storage).

2.8.3 Preparation of THP1 cells from frozen stock

Frozen THP1 cell stocks were thawed at 37°C as quickly as possible and diluted in approximately 10ml complete RPMI medium, to dilute the dimethyl sulphoxide (DMSO) (Sigma, St Louis, MO, USA) in which the cells were frozen. The cells were pelleted by low speed centrifugation, at 1000rpm for 5 minutes in a bench-top centrifuge (Eppendorf 5810 R, Hamburg, Germany), and then resuspended in 10ml complete RPMI medium and aliquoted into cell culture flasks to be incubated as described in 2.8.1.

2.8.4 Treatment of THP1 cells to differentiate them into macrophage-like cells

THP1 cells were differentiated into macrophages by the addition of 100nM PMA (Sigma, St Louis, MO, USA) to 5 x 10^5 cells per well. The cells were incubated overnight at 37ºC in complete RPMI medium containing no antibiotics and washed 3 times with Dulbecco's phosphate buffered saline (D-PBS) (Invitrogen, Paisley, UK) to remove any non-adherent cells. Following that, the cells were resuspended in fresh complete medium and allowed to rest for 3 days to fully develop into macrophages (Riendeau and Kornfeld, 2003), after which they were ready for infection as depicted in **Figure 2.5**. The PMA used was prepared by dissolving it in absolute ethanol (50µg/ml) and diluted 123:1000 in medium and then again 1:1000 into the cell suspension in order to render the residual ethanol concentration negligible.

The optimal PMA treatment was predetermined by first treating the cells with different concentrations of PMA (5nM, 8.1nM and 100nM) for different time periods, then assessing their differentiation progress after 16 hours, 48 hours and 72 hours.

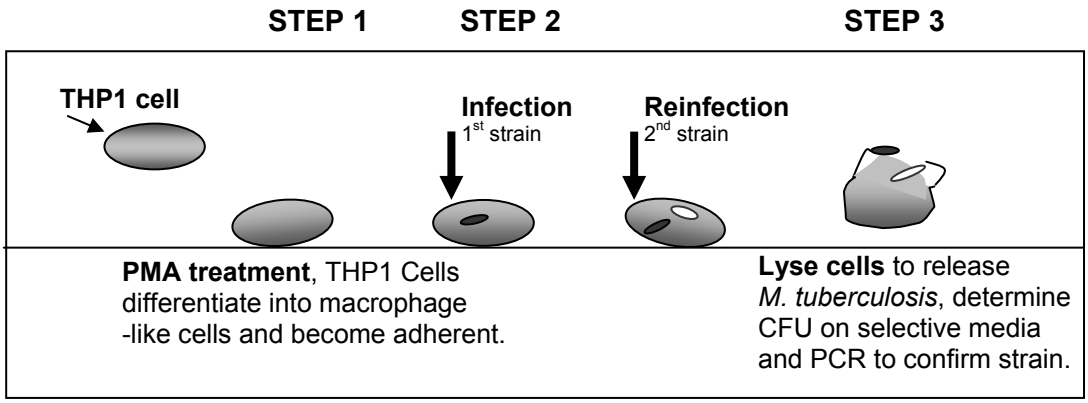

Figure 2.5. Schematic representation of the THP1 cell reinfection. THP1 cells were treated with PMA in order to promote differentiation into macrophage-like cells, making them adherent, phagocytic and to halt cell division. Cells were infected with a primary *M. tuberculosis* strain, followed by a secondary strain, and finally lysed to release the phagocytosed mycobacteria. Cell lysates were plated on selective media to assess CFU, and PCR was performed in parallel to identify the strains.

2.8.5 Preparation of mycobacterial cultures for infection

Frozen cultures were thawed and passaged 30 times up and down through a 1ml 25G needle. Thereafter, the desired amount of culture was pelleted by low speed centrifugation at 3000rpm for 20 minutes in a bench-top microfuge (Heraeus, Biofuge Pico, Kendro laboratory supplies, Osterode, Germany). The pellet of each strain was washed once with complete RPMI medium, to dilute the glycerol and Tween 80 used to culture and freeze the mycobacteria in, after which it was centrifuged again at 3000rpm for 20 minutes. The resulting pellet was resuspended in complete medium and further passaged through a 25G syringe 10 times.

2.8.6 *M. tuberculosis* H37Rv Kan^R, Hyg^R and Beijing growth in THP1 cells

THP1 cells were infected at a multiplicity of infection (MOI) of approximately 5:1 (mycobacteria:THP1 cell), in triplicate for each strain in wells of Costar® 24 well

plates (Corning Incorporated, NY, USA), for each time point, Day 0 (4 hours post infection), Day 1, Day 3 and Day 7. The cells were incubated for 16 hours at 37ºC to allow phagocytosis to occur, after which non-phagocytosed mycobacteria were removed by washing cells 3 times with D-PBS, followed by resuspension of cells in fresh complete medium, refer to **Table 2.3** for the experimental design and **Figure 2.6** for the plate setup and distribution of strains.

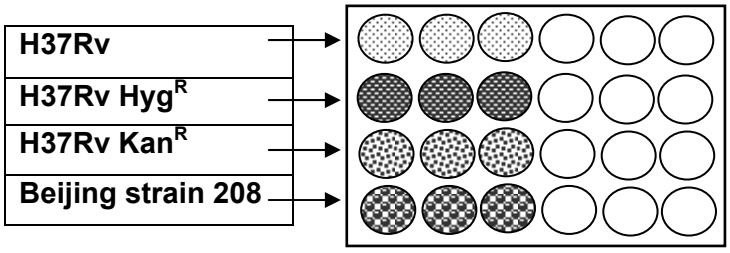

Figure 2.6. Schematic diagram of THP1 cell growth curve. This diagram depicts a 24 well cell culture plate with wells containing 5 x 10^5 THP1 cells per well and 2.5 x 10^6 mycobacteria per well, as listed on the left, in triplicate. Four plates were used per growth curve experiment, to be harvested at different time points, Day 0 (4hrs), Day 1 (16hrs), Day 3 and Day 7.

Mycobacteria were harvested at each time point as stated above, by lysing non-adherent macrophages from each well with 100µl 0.25% Sodium Dodecyl Sulphate (SDS) (Merck, Gauteng, South Africa) into each well containing 1ml medium, and then adherent macrophages by washing wells 3 times with 0.025% SDS. The mycobacteria were then pelleted by low speed centrifugation at 3000rpm for 20 minutes and resuspended in 7H9 Middlebrook medium. Mycobacterial uptake by THP1 cells were subsequently enumerated by CFU.

Table 2.3. THP1 cell growth curve experimental design

Timeline	Action Taken
± 7 days	Thawing THP1 cells and growing until adequate numbers are reached
16 hours	PMA treatment to differentiate THP1 cells into macrophage-like cells, 5×10^5 cells / well; 3 wells per strain (12 wells / plate); 4 plates Remove PMA by washing 3 x with D-PBS
3 days	Rest cells to fully become macrophage-like
Day 0 **(4 hrs)**	Infect all wells with MOI of 5:1 bacilli of each mycobacterial strain Harvest Day 0 plate, 4 hours post infection
Day 1 **(16 hrs)**	Wash 3 x with D-PBS to remove non-phagocytosed bacilli and replace media of remaining 3 plates Harvest Day 1 plate, 16 hours post infection
Day 3	Harvest Day 3 plate and replace media of last plate
Day 7	Harvest Day 7 plate

2.8.7 Reinfection experiment in THP1 cells

The THP1 cells were used to evaluate the intracellular interaction between a secondary infecting marked *M. tuberculosis* H37Rv and a primary infecting marked H37Rv strain. The experiment was repeated using a *M. tuberculosis* Beijing strain as the primary infecting strain and a marked *M. tuberculosis* H37Rv as secondary infecting strain as depicted in **Figure 2.7**. Growth and strain differentiation was determined by CFU enumeration on selective media and confirmation of strains was done by PCR analysis.

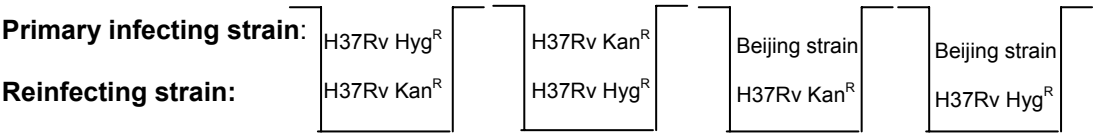

Figure 2.7. Schematic layout of the mycobacterial reinfection experiment. Each box represents a well of a cell culture plate containing THP1 cells – the top row displays the primary infecting strain whereas the bottom row indicates the reinfecting strain. The experiment was carried out in triplicate to provide statistically significant results.

Cells were infected as described in section 2.8.6, at a MOI of approximately 5:1 (mycobacteria:THP1 cell) in triplicate for each strain, in wells of a 24 well plate. For each time point, Day 0_1 (4 hours post primary infection), Day 0_2 (4 hours post reinfection), Day 1, Day 3 and Day 7, cells were incubated for 16 hours to allow phagocytosis to occur, and non-phagocytosed mycobacteria were removed by washing cells as described in section 2.8.6. Cells were then reinfected with a secondary mycobacterial strain at the same MOI of approximately 5:1 in triplicate in wells containing primary infected THP1 cells. The cells were again incubated for 16 hours at 37ºC to allow uptake of the reinfecting strain to occur, and the non-phagocytosed mycobacteria were removed by washing cells as described before (section 2.8.6). The sequence of primary and secondary infecting strains is indicated in **Figure 2.8**. See **Table 2.4** for experimental design and **Figure 2.9** for timeline of reinfection experiments.

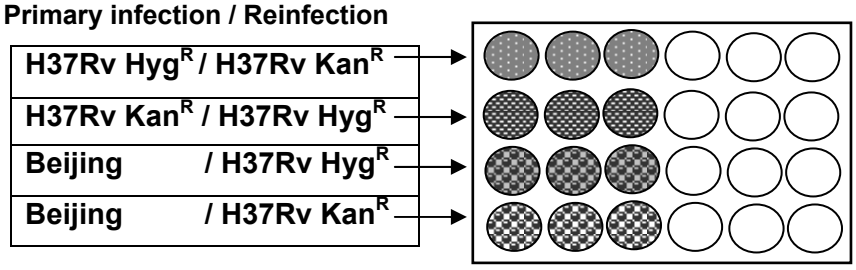

Figure 2.8. Schematic diagram of THP1 cell reinfection experiment. This diagram depicts a 24 well cell culture plate with each well containing 5 x 10^5 THP1 cells per well and 2.5 x 10^6 mycobacteria per well, for each strain, with the sequence of primary and reinfecting strains listed on the left, in triplicate. Five plates were used per growth curve experiment, to be harvested at different time points, Day 0_1 (4hrs post primary infection), Day 0_2 (4hrs post reinfection), Day 1 (16hrs post reinfection), Day 3 and Day 7.

Mycobacteria were harvested at each time point, as described in section 2.8.6. Mycobacterial uptake of cells was subsequently enumerated by CFU and PCR was performed on denatured DNA to identify the respective strains.

Table 2.4. Experimental design for THP1 cell reinfection

Timeline	Action Taken
± 7 days	Thawing THP1 cells and growing until adequate numbers are reached
16 hours	PMA treatment to differentiate THP1 cells into macrophage like cells, 5×10^5 cells / well; 3 wells per reinfection experiment; 5 plates Remove PMA by washing 3 X with D-PBS
3 days	Rest cells to fully become macrophage-like
Day 0_1 **(4 hrs post primary infection)**	Infect all wells with MOI 5:1 bacilli of each primary infecting mycobacterial strain Harvest Day 0_1 plate, 4 hours post infection
Day 0_2 **(4 hrs post reinfection)**	Wash 3 X with D-PBS to remove non-phagocytosed bacilli and replace media of remaining 4 plates Reinfecting all wells with 5:1 bacilli of reinfecting mycobacterial strains Harvest Day 0_2 plate, 4 hours post infection
Day 1 **(16 hrs post reinfection)**	Wash 3 X with D-PBS to remove non-phagocytosed bacilli and replace media of remaining 3 plates Harvest Day 1 plate, 16 hours post infection
Day 3	Harvest Day 3 plate and replace media of last plate
Day 7	Harvest Day 7 plate

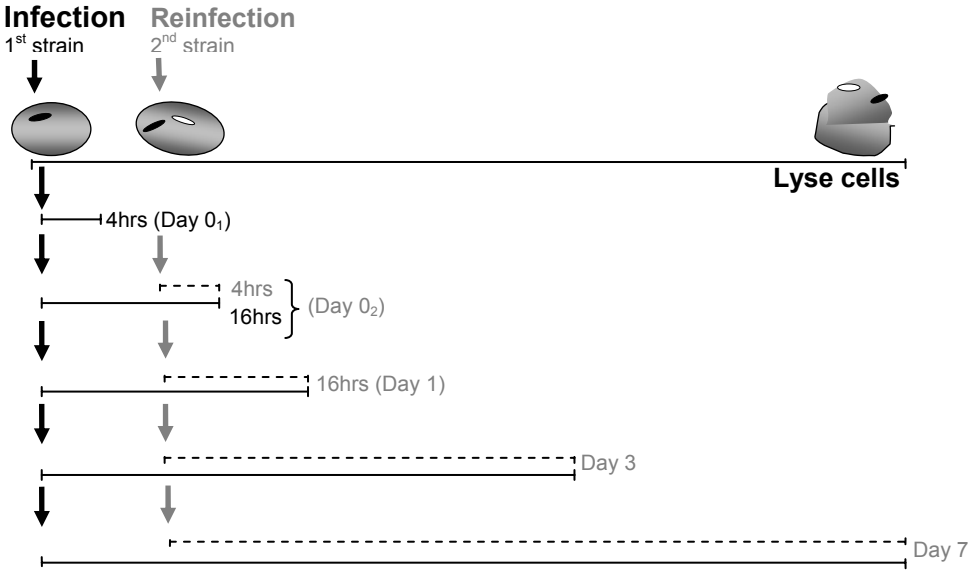

Figure 2.9. Schematic diagram of the timeline of reinfection experiments. Primary infection is indicated by the black arrows and reinfection is indicated by the gray arrows on each line. For the reinfection experiments, there were 5 time points where cells were harvested. The solid lines indicate the course of the primary infections whereas the dotted lines indicate reinfection.

During the various reinfection experiments a hypervirulent *M. tuberculosis* Beijing strain and two marked virulent strains, *M. tuberculosis* Kan^R and *M. tuberculosis* Hyg^R, respectively, have been used (**Figures 2.7** and **2.8**). The differentially marked *M. tuberculosis* strains were used to facilitate enumeration of individual strains on selective media following each time point during the THP1 cell reinfection experiment. In addition, differentiation was done by PCR using primers designed for specific areas within the individual antibiotic resistant cassettes for the marked *M. tuberculosis* H37Rv strains, in addition to primers designed to identify Beijing strains (**Figure 2.5**).

2.8.7.1 CFU analysis to enumerate mycobacterial strains from reinfected THP1 cell lysates

Following THP1 cell reinfection, the resulting mycobacterial content of each of the triplicate wells were resuspended in 7H9 liquid media in individual 2ml Eppendorf tubes, after which 100µl of each tube was used to plate ten-fold serial dilutions (10^{-1} to 10^{-5}) from. These aliquots were plated on both hygromycin-supplemented 7H10 plates and kanamycin-supplemented 7H10 plates in order to differentiate strains. The *M. tuberculosis* Beijing and marked H37Rv strain reinfection experiments' samples were plated onto 7H10 solid media, as well as on 7H10 supplemented with the respective antibiotics. Plates were incubated and CFU enumerated using standard mycobacterial procedures as described in section 2.2.

2.8.7.2 PCR analysis to identify mycobacterial strains of reinfected THP1 cells

The remaining portions of cell lysates, which were plated for CFU enumeration, were denatured by boiling at 100ºC for 20 minutes for PCR analysis. DNA

extraction was performed by phenol and ethanol precipitation as explained in Appendix B. Standard hot-start PCR reactions were done using the air dried DNA samples, incorporating Syto® 9 (Invitrogen, Paisley, UK), a green fluorescent agent used to detect PCR products using melting curves in the Rotor-gene 6000 (Corbett, Australia). The *M. tuberculosis* Hyg^R and Kan^R strain PCR was done using the primers described in **Table 2.1** and the Beijing strain PCR was done using the primers described in **Table 2.5**.

Standard hot-start PCR for *M. tuberculosis* Hyg^R amplification was done on all the samples of the marked reinfection experiment as well as the Beijing infected THP1 cells reinfected with *M. tuberculosis* Hyg^R. The PCR machine was programmed as follows with an initial denaturation at 95ºC for 15 minutes, followed by 45 cycles of:

Denaturation:	94ºC for 45 seconds,
Annealing:	62ºC for 45 seconds and
Extension:	72ºC for 45 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the samples were held at 4ºC until use.

The standard Hot-Start PCR conditions for the Kan^R reaction were performed on the marked reinfection experiment as well as the Beijing infected THP1 cells reinfected with H37Rv Kan^R. The PCR machine was programmed as follows with an initial denaturation at 95ºC for 15 minutes, followed by 45 cycles of:

Denaturation:	94ºC for 45 seconds,
Annealing:	50ºC for 45 seconds and
Extension:	72ºC for 45 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the sample was held at 4ºC until use.

Table 2.5. PCR primers used for the detection of Beijing strains

Primer name	Orientation	Primer sequence	T_m	Product size
Beijing strain identification				
F29/780F	**Forward**	**5'-AGATCGGCT TTCTTCCCGC-3'**	**62ºC**	**203 bp**
Xho1	**Reverse**	**5'-TTCAACCATCGCCGCCTCTAC-3'**	**62ºC**	

The standard hot-start PCR conditions for the Beijing reaction were programmed on all the Beijing infected THP1 cells reinfected with a marked H37Rv strain. The PCR machine was programmed as follows with an initial denaturation at 95ºC for 15 minutes, followed by 45 cycles of:

Denaturation: 94ºC for 45 seconds,

Annealing: 62ºC for 45 seconds and

Extension: 72ºC for 45 seconds

The final extension step was performed at 72ºC and continued for 15 minutes and the sample was held at 4ºC until use.

2.8.7.3 Melt analysis for the detection of PCR products

The PCR products were subjected to high resolution melt analysis in a Rotor-gene 6000. The thermal denaturation profile was measured over the temperature range 80°C to 95°C and fluorometric readings, detecting the Syto® 9 in PCR products, were taken every 0.1°C.

A plot representing the thermal denaturation point used to identify each strain was generated using Rotor-gene software, which calculated the derivative of the intensity of fluorescence at different temperatures. A temperature range was identified for each strain and values above a certain threshold implied that sufficient DNA was available for amplification.

2.8.8 Statistical analysis

Experiments were performed in triplicate and repeated to obtain statistically meaningful results. Data obtained from the different groups were compared statistically. The student's *t-test* was used and a $P < 0.05$ value was considered statistically significant.

CHAPTER 3

RESULTS

3.1 Generation of differentially marked *M. tuberculosis* H37Rv strains

Following electrotransformation of *M. tuberculosis* H37Rv with hygromycin and kanamycin marked plasmids, portions of single colonies from newly transformed strains were picked from 7H11 plates, on which the electroporated cultures were plated, and boiled for 20 minutes at 100ºC to release the DNA for PCR analysis. *M. tuberculosis* Hyg^R colonies had two different morphologies, large and small, of which only the large colonies were found to contain the pIVH1 plasmid, confirmed by yielding a 302 bp amplification product during the PCR for detection of the Hyg^R insert (**Figure 3.1**). *M. tuberculosis* Kan^R colonies displayed uniform colony morphology and these colonies were initially found not to contain the pIVK1 plasmid, ascertained during the PCR for detection of the Kan^R cassette (**Figure 3.1**). The PCR for the detection of the Kan^R vector was repeated using a further 10 randomly selected colonies from the electroporated culture and only 3 of the colonies yielded the 148 bp product, confirming the presence of PIVK1 (**Figure 3.2**).

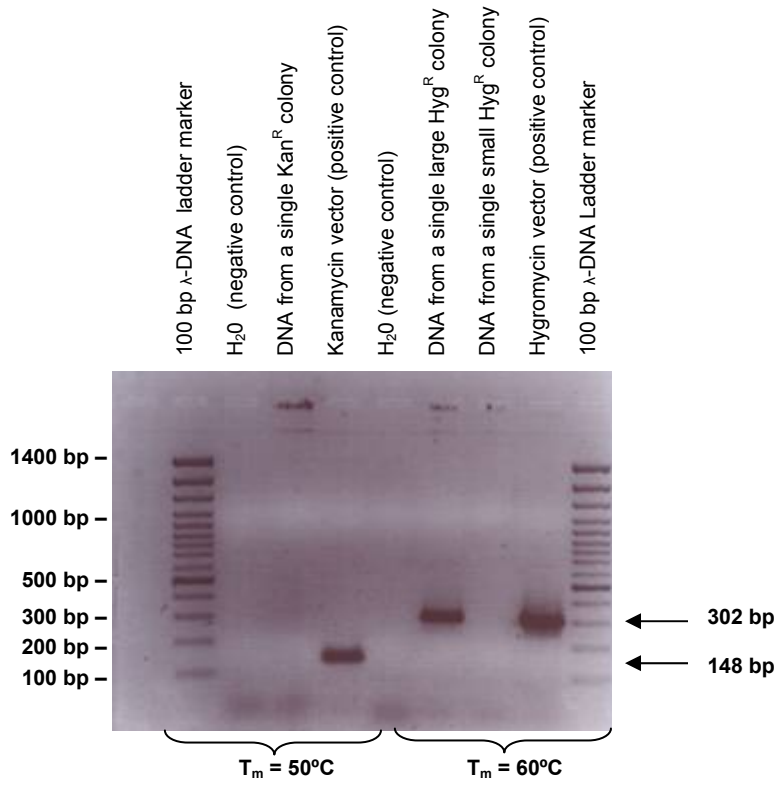

Figure 3.1. PCR to detect the presence of marked plasmids in the electrotransformed mycobacterial DNA samples. Lanes 1 and 9 represents the 100 bp λ-DNA Ladder marker, lanes 2 to 4 represent PCR of the kanamycin-resistant vector, the PCR was done at an annealing temperature of 50ºC: lane 2, H_2O (negative control); lane 3, bacterial DNA from a single Kan^R boiled colony; lane 4, known kanamycin-resistant vector, pIVK1 (positive control). Lanes 5 to 8 represent hygromycin-resistant vector PCR, the annealing temperature for this PCR was 60ºC: lane 5, H_2O (negative control); lanes 6 and 7, bacterial DNA from single large and small boiled Hyg^R colonies; lane 8, known hygromycin-resistant vector, pIVH1 (positive control).

The antibiotic selection plasmids, pIVH1 and pIVK1 are integrating plasmids. Therefore, it had to be ascertained whether the plasmids' *att*P integration site was integrated into the *M. tuberculosis* genomic *att*B site. DNA extracted from one large *M. tuberculosis* Hyg^R colony and the three positive *M. tuberculosis* Kan^R colonies were thereafter tested for integration into the *M. tuberculosis* genome. The *M. tuberculosis* Hyg^R clone was positive for the 184 bp product, which indicated integration into the *M. tuberculosis* genome. Of the three selected colonies of *M. tuberculosis* Kan^R, only one showed the presence of the 184 bp product (**Figure 3.3**), which indicated integration of the kanamycin-resistant marker into the correct position in the *M. tuberculosis* genome. The rest of the partial colonies, positive for both the respective resistant inserts and *att*P/*att*B integration, as determined by PCR were streaked on Lowenstein-Jensen slopes for long-term storage.

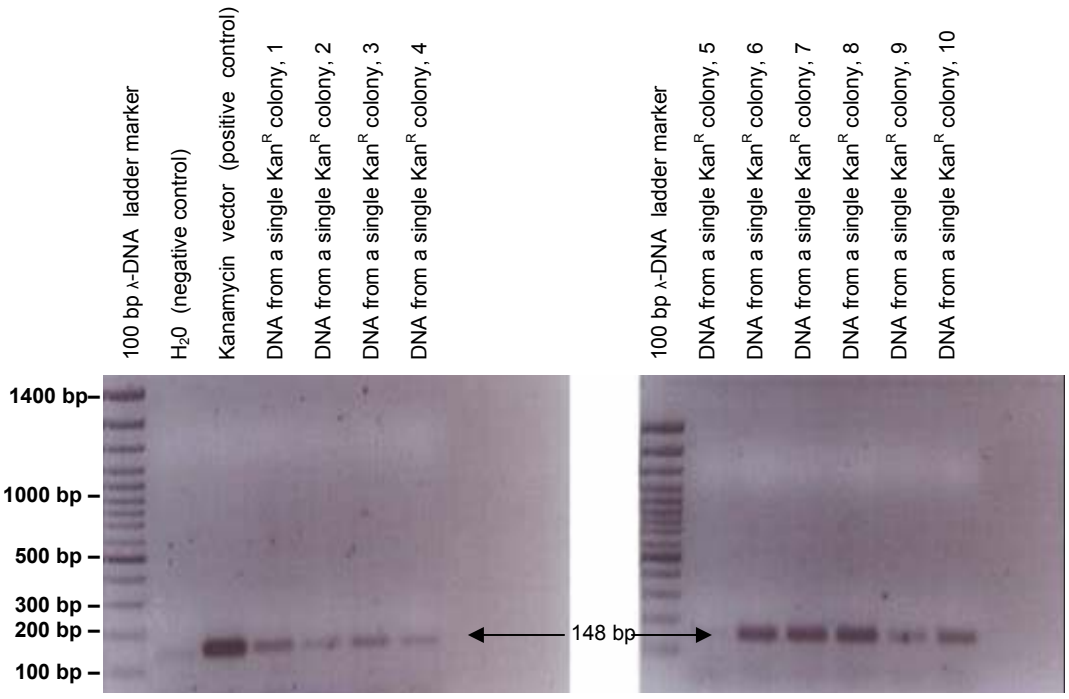

Figure 3.2. PCR to detect presence of marked plasmids in 10 *M. tuberculosis* Kan^R transformants. Lanes 1 and 8 represent the 100 bp λ-DNA Ladder marker; Lane 2, H_2O (negative control); Lane 3, Kan^R vector (positive control); Lanes 4 to 13, bacterial DNA from 10 Kan^R single colonies; (The T_m of the Kan^R primers was 50ºC)

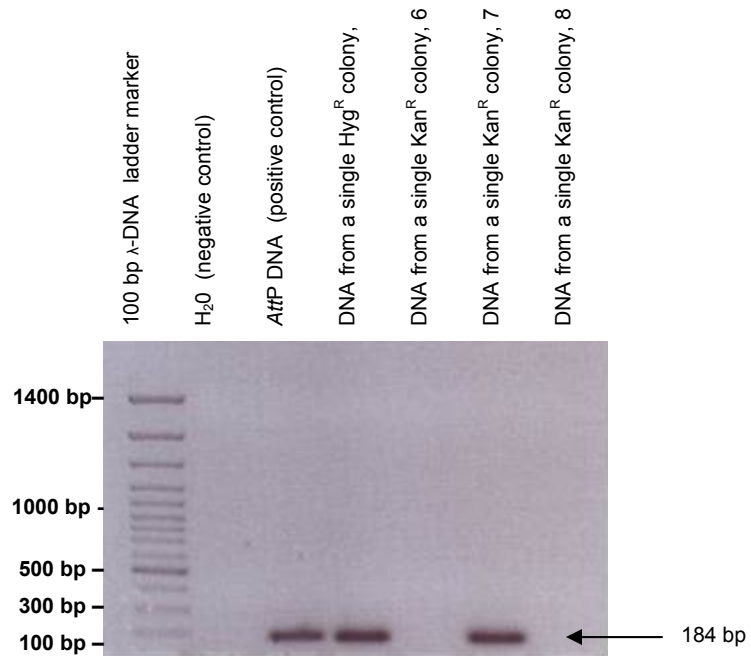

Figure 3.3. PCR for the detection of the *att*P plasmid integration site in the electrotransformed mycobacterial DNA. Lane 1 represents the 100 bp λ-DNA Ladder marker, lane 2, H_2O (negative control); lane 3, *att*P DNA – positive control; Lane 4, single Hyg^R colony; Lanes 5 to 7 , single Kan^R colonies (The *att*P primers' T_m was 62ºC).

3.2 *In vitro* growth curves

3.2.1 *In vitro* MGIT™ growth curves

Upon completion of optimisation experiments, it was found that an inoculum titre of 10^2 – 10^3 was optimal for starting a 3 week growth curve in the MGIT™ system. Five different starting concentrations were used for each culture – the intended concentrations were 1 x 10^3, 5 x 10^3, 1 x 10^4, 5 x 10^4 and 1 x 10^5. These inoculums were done in duplicate and repeated. The lowest concentration, closest to 1 x 10^3, gave the most comparable growth pattern for all cultures. MGIT™ readings, an indication of the growth rate of the mycobacteria, were taken every second day for 20 days. Both *M. tuberculosis* Hyg^R and *M. tuberculosis* Kan^R cultures displayed similar growth patterns in MGIT™ units as compared to wild-type H37Rv, although H37Rv displayed a slightly longer lag phase as seen in **Figure 3.4**.

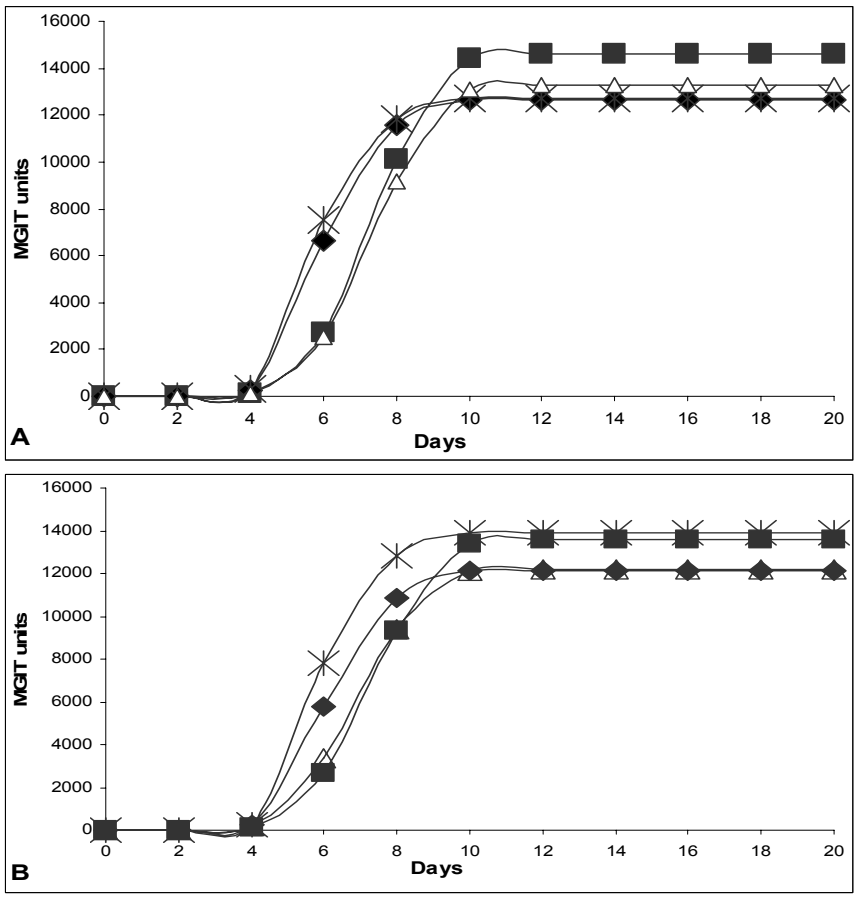

Figure 3.4. MGIT™ *in vitro* growth curves of *M. tuberculosis* H37Rv, Hyg^R and Kan^R. Hyg^R (♦) and Kan^R (Ж) cultures showed similar growth patterns to wild type *M. tuberculosis* H37Rv in MGIT units, H37Rv (■/Δ) displaying a slightly longer lag phase than Hyg^R and Kan^R. Inoculum titres of cultures were as follows, for A: H37Rv (■), 9.84 x 10^3; H37Rv (Δ), 1.095 x 10^3; Hyg^R (♦), 1.72 x 10^3; Kan^R (Ж), 1.01 x 10^3 and B: H37Rv (■), 5.95 x 10^2; H37Rv (Δ) 5.95 x 10^2; Hyg^R (♦), 1.72 x 10^3; Kan^R (Ж), 1.01 x 10^3. The graphs in B are repeat experiments of A.

3.2.2 Liquid culture growth curves of *M. tuberculosis* strains

3.2.2.1 Comparison of growth kinetics of *M. tuberculosis* Hyg^R and Kan^R to *M. tuberculosis* H37Rv

During 28 day *in vitro M. tuberculosis* culturing in Middlebrook 7H9 medium, starting with a 1:500 inoculum dilution, *M. tuberculosis* H37Rv and recombinant strain variants, *M. tuberculosis* Hyg^R and Kan^R, displayed similar growth patterns,

when comparing CFU (**Figure 3.5 A** and **B**) and OD_{600} readouts (**Figure 3.6 A** and **B**). These growth curves were performed twice and in duplicate.

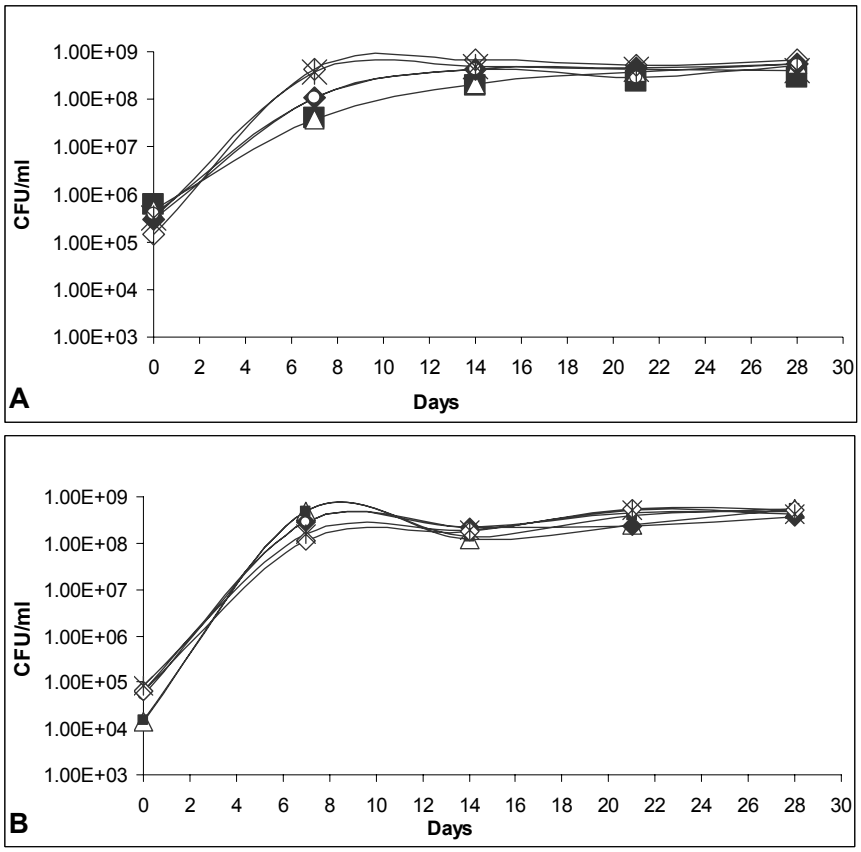

Figure 3.5. Comparison of CFU/ml during liquid culture growth curves A and B. B represents the repeated growth curve experiment of A. The growth curve of each strain *M. tuberculosis* H37Rv, Hyg^R and Kan^R were done in duplicate with an inoculum dilution of 1:500. Recombinant strains Hyg^R (○/♦) and Kan^R (◊/Ж) displayed similar growth curves to wild-type *M. tuberculosis* H37Rv (■/∆).

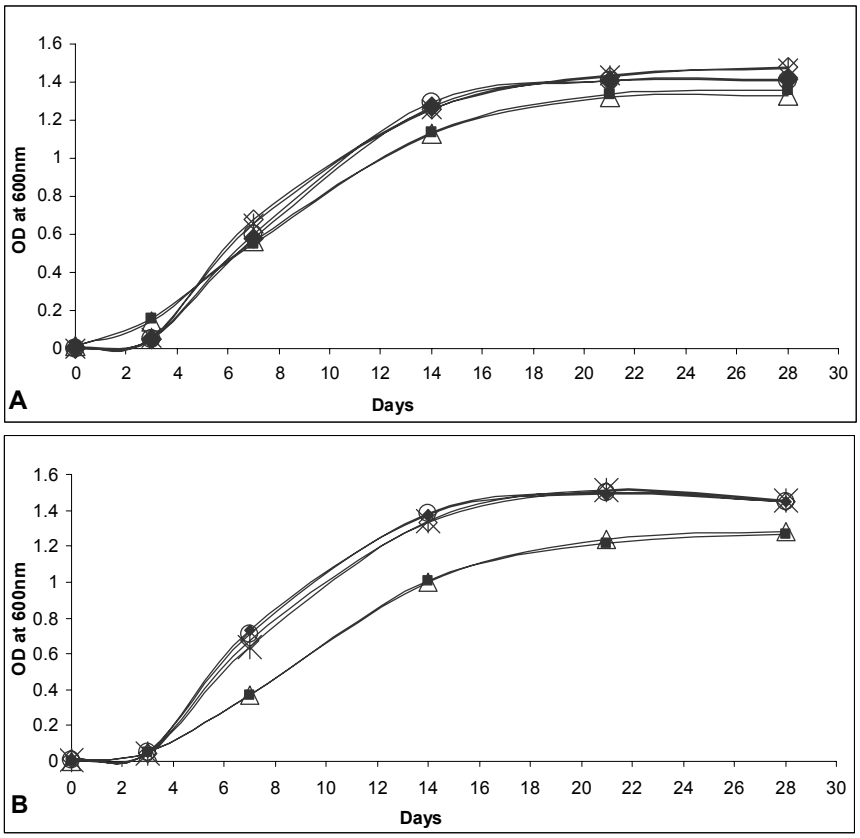

Figure 3.6. Comparison of OD_{600} during liquid culture growth curves A and B. B represents the repeated growth curve experiment of A. The growth curve of each strain *M. tuberculosis* H37Rv, Hyg^R and Kan^R were done in duplicate with an inoculum dilution of 1:500. Recombinant strains Hyg^R (○/♦) and Kan^R (◊/Ж) displayed similar growth curves to wild-type *M. tuberculosis* H37Rv (■/∆).

3.2.2.2 Comparison of growth kinetics of two hypervirulent Beijing strains to *M. tuberculosis* H37Rv

Two hypervirulent Beijing strains, 208 and 209, were cultured in Middlebrook 7H9 liquid media and displayed similar growth patterns during a 28 day *in vitro* growth curve, starting with a 1:500 inoculum dilution. Growth curves were done in duplicate and repeated to confirm that the mycobacterial strain growth was comparable during reinfection experiments, as determined by two different readouts, CFU (**Figure 3.7 A** and **B**) and OD_{600} (**Figure 3.8 A** and **B**). Only one Beijing strain was needed for the reinfection experiment, and taking into account

that both strains 208 and 209 grew comparable to *M. tuberculosis* H37Rv, strain 208 was arbitrarily chosen to use in subsequent reinfection experiments.

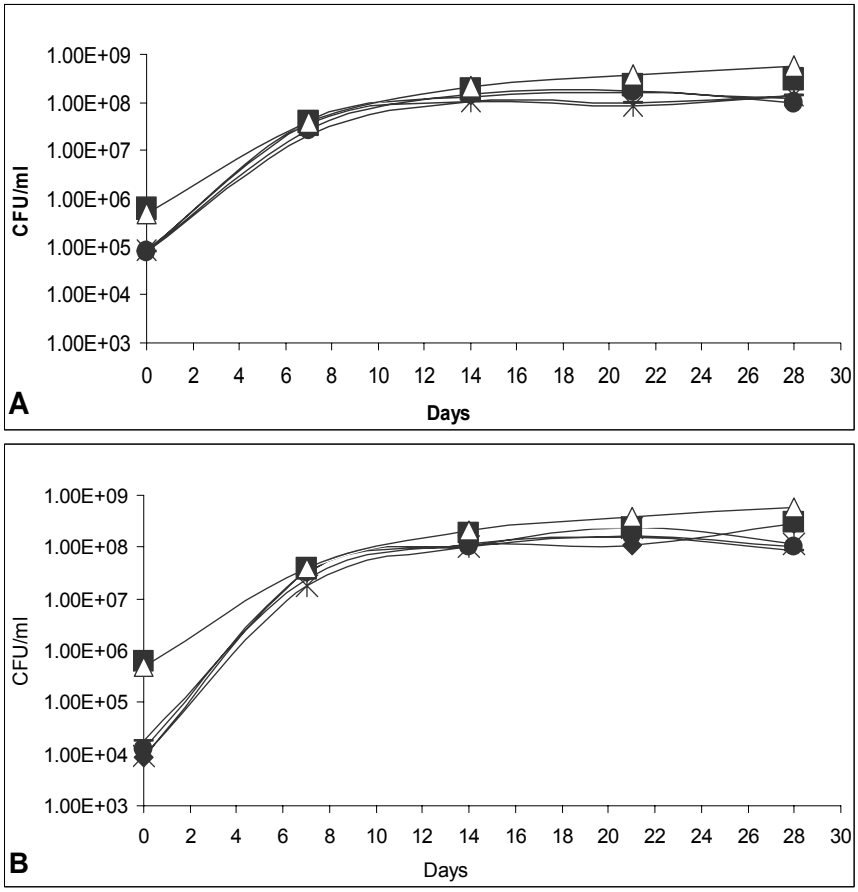

Figure 3.7. Comparison of CFU/ml of Beijing strains to *M. tuberculosis* H37Rv during liquid culture growth curves A and B. B represents the repeated growth curve experiment of A. The growth curves of each Beijing strain, 208 and 209, and *M. tuberculosis* H37Rv were done in duplicate with a starting inoculum of 1:500. Hypervirulent clinical Beijing strains 208 (♦/●) and 209 (Ж/**–**) grew similarly to wild-type *M. tuberculosis* H37Rv (■/∆).

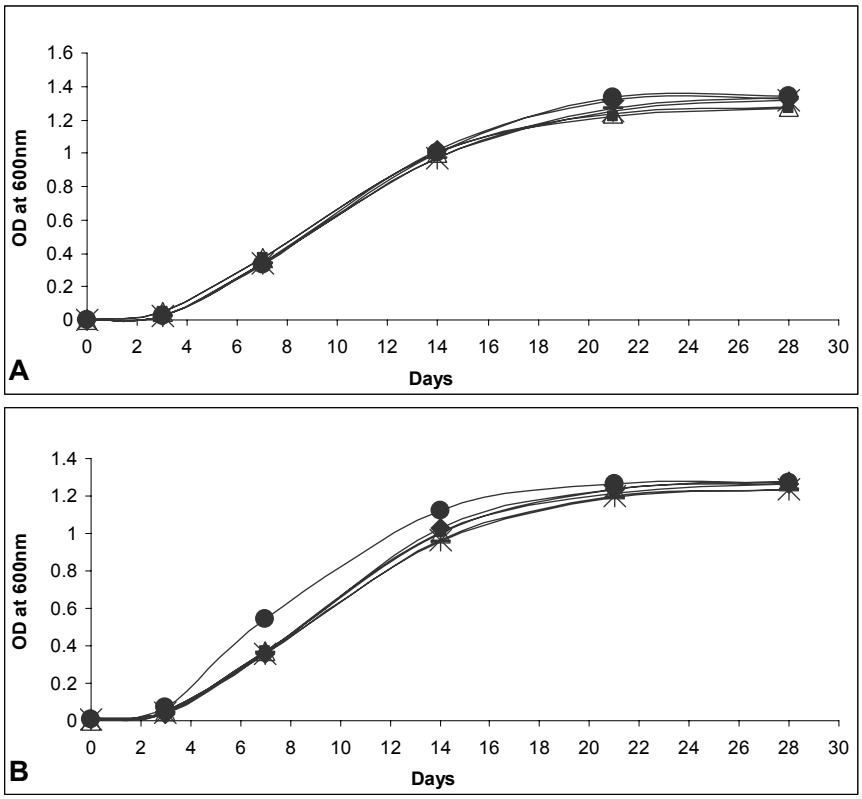

Figure 3.8. Comparison of OD_{600} of Beijing strains to *M. tuberculosis* H37Rv during liquid culture growth curves A and B. B represents the repeated growth curve experiment of A. The growth curves of each Beijing strain, 208 and 209, and *M. tuberculosis* H37Rv were done in duplicate. Hypervirulent clinical Beijing strains, 208 (♦/●) and 209 (Ж/–) displayed similar growth patterns to wild-type *M. tuberculosis* H37Rv (■/∆).

3.2.2.3 Purity testing of *M. tuberculosis* liquid cultures

Before growth curve experiments were performed, initial propagated liquid cultures for MGIT™ and Middlebrook 7H9 liquid cultures were subjected to purity testing to rule out contamination of any other bacterial or fungal origin. On ZN smears, only acid-fast stained bacilli were to be seen and if no growth was observed after 2 days of incubation at 37ºC on BA plates, it indicated the absence of bacterial or fungal contamination.

3.3 Assessment of optimal antibiotic concentration for *M. tuberculosis* Hyg^R and Kan^R strain growth

In order to assess if the selective antibiotics inhibited growth of the respective resistant strains, the recommended concentration of antibiotic was used in selective Middlebrook 7H10 plates and compared to growth on Middlebrook 7H10 plates with no selection. During the reinfection experiment only selective media was used, therefore it had to be ascertained that the results would be accurate. There was no significant difference between plating on unsupplemented Middlebrook 7H10 and Middlebrook 7H10 plates containing the recommended concentrations of hygromycin or kanamycin (**Figure 3.9**). It can be stated that the CFU counted during the reinfection experiments were the true values and not influenced by the addition of potentially inhibiting antibiotics.

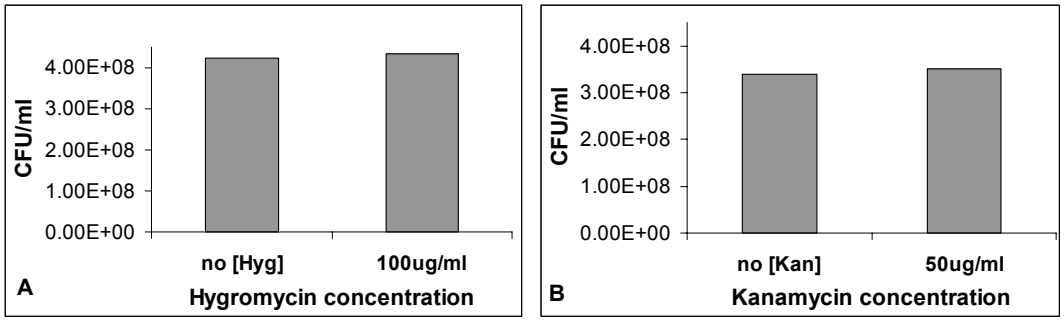

Figure 3.9. Optimisation of antibiotic concentrations for use in Middlebrook 7H10 plates. For hygromycin (**A**) the recommended concentration (100µg/ml) was tested against no selection and the recommended concentration of hygromycin had no significant inhibitory effect on CFU. For kanamycin (**B**) concentration, the recommended concentration (50µg/ml) was tested against no selection and the recommended concentration of kanamycin had no significant inhibitory effect on CFU either.

3.4 Cell culture experiments

3.4.1 *In vitro* culturing of THP1 cells

THP1 cells were initially cultured using complete RPMI medium supplemented with 1% pen/strep (5000 units/ml penicillin and 5000µg/ml streptomycin) as antibiotic in order to inhibit non-mycobacterial growth. Prior to PMA treatment of cells, as described in section 3.4.2, pen/strep was removed by washing cells once in complete RPMI, to eliminate any possibility that the antibiotic might influence mycobacterial growth. However, cells became contaminated within a few days following removal of the pen/strep. After repeated cell culture experiments became contaminated following the removal of pen/strep, fresh stocks of THP1 cells were cultured in the presence of 50µg/ml gentamicin, which prevented contamination once the antibiotic was removed.

3.4.2 Optimisation of PMA treatment

PMA was used to differentiate THP1 cells into macrophage-like adherent cells. The optimal concentration and length of treatment had to be determined so that the greatest number of healthy differentiated macrophage-like cells could be obtained. The following concentrations of PMA were assessed over a period of 3 days: 100nM, 8.1nM and 5nM, taking into consideration different published methods found in the literature (Riendeau and Kornfeld, 2003; Theus *et al.* 2005). Cells were viewed on a daily basis for morphological changes during treatment. The cells responded better to 100nM PMA treatment overnight, compared to lower concentrations for longer times. Cells started to differentiate overnight and continued to do so after PMA was washed off, after which cells were rested for 3 days to fully differentiate into macrophage-like cells. PMA-treated cells were

propagated for a total of 15 days, replacing media every third day, to ensure that cells survive for the duration of the reinfection experiment.

3.4.3 *M. tuberculosis* H37Rv, Kan^R, Hyg^R and Beijing growth in THP1 cells

Growth curves of the Hyg^R and Kan^R marked *M. tuberculosis* H37Rv strains, wild-type H37Rv and Beijing strain 208 were assessed and showed comparable growth patterns in THP1 cells during a 7 day growth curve experiment (**Figure 3.10**). The aim was to infect 5 x 10^5 THP1 cells per well with 2.5 x 10^6 mycobacteria (MOI, 5:1), however, the actual mycobacterial numbers could only be obtained after culturing an aliquot of the inoculums. For the actual inoculum titres, CFU/ml at 4 hours after infection and CFU/ml at 16 hours after infection, which are indicated as averages of triplicate wells, plated in duplicate, are displayed in **Table 3.1**. The initial infection of the THP1 cells was done for 4 hours (Day 0) and then the rest of the THP1 cells were left to be infected for a total of 16 hours (Day 1). Only an average of 1.35% of the mycobacterial inoculum was taken up by the cells after 4 hours of infection, whereas 6.71% was taken up after 16 hours of infection. The amount of mycobacteria increased in a log phase pattern following the 4 hour infection up to the overnight infection (Day 1). Following this the growth remained stationary for the rest of the experiment, at Day 3 and Day 7 (**Figure 3.10**).

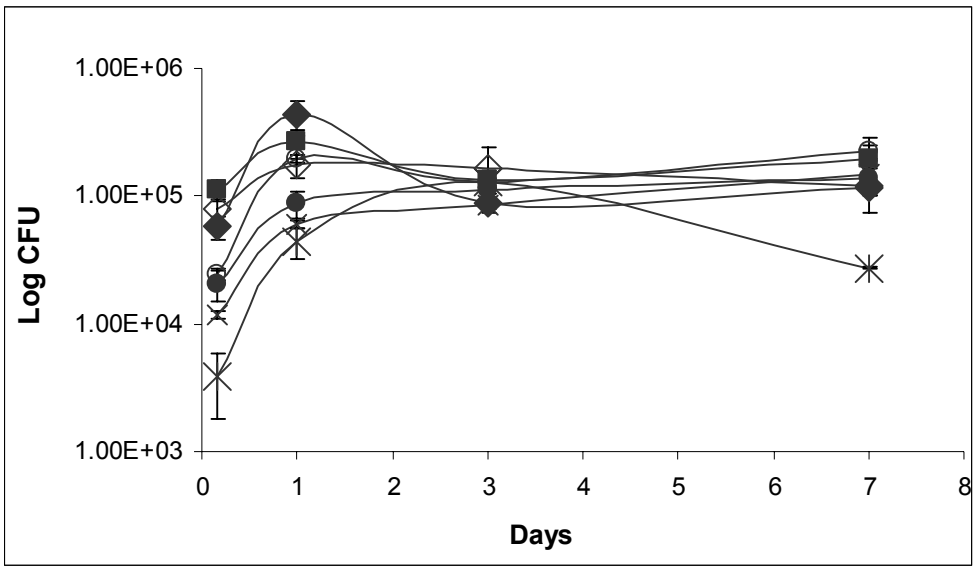

Figure 3.10. Growth of mycobacterial strains within THP1 cells. *M. tuberculosis* Hyg^R (x/Ж), *M. tuberculosis* Kan^R (●/○) and Beijing strain 208 (♦/◊) all replicated comparably to wild-type *M. tuberculosis* H37Rv (■) in THP1 cells. These growth curves were done in triplicate and repeated, with average values displayed on the graphs.

Table 3.1. Infection of THP1 cells, displaying percentage uptake of inoculum after 4hrs and after 16 hours of infection

Mycobacterial strain	Inoculum	Mycobacterial uptake 4hrs after infection		Mycobacterial uptake 16 hrs after infection	
	CFU/ml	Average CFU/ml	% of inoculum	Average CFU/ml	% of inoculum
208 1	3.60E+06	7.90E+04	2.19	1.75E+05	4.86
208 2 (repeat)	4.10E+06	5.73E+04	1.40	4.28E+05	10.44
Kan^R 1	2.90E+06	2.40E+04	0.83	1.97E+05	6.79
Kan^R 2 (repeat)	1.15E+06	1.18E+04	1.03	8.87E+04	7.71
Hyg^R 1	1.15E+06	3.83E+03	0.33	4.42E+04	3.84
Hyg^R 2 (repeat)	9.05E+05	2.08E+04	2.30	6.00E+04	6.63
	Average:		**1.35%**		**6.71%**

3.4.4 Reinfection of mycobacterial strains in THP1 cells

THP1 cell reinfection experiments were done using different primary and secondary infecting strains. Firstly, marked *M. tuberculosis* Hyg^R infected THP1 cells were reinfected with *M. tuberculosis* Kan^R, then this reinfection experiment was repeated using *M. tuberculosis* Kan^R as the primary infecting strain and

M. tuberculosis Hyg^R as reinfecting strain. Secondly, *M. tuberculosis* Beijing strain 208 infected THP1 cells were reinfected with either of the Hyg^R and Kan^R marked *M. tuberculosis* strains. Cell growth and morphology were documented at every time point, Day 0_1 (4 hours after primary infection), Day 0_2 (4 hours after reinfection), Day 1, Day 3 and Day 7, during reinfection experiments (**Figure 3.11**). During reinfection in both combinations of infecting and reinfecting strains, with *M. tuberculosis* Hyg^R and *M. tuberculosis* Kan^R, it was observed that the growth pattern of the primary and reinfecting strains looked similar (**Figures 3.12** and **3.13**). The average percentage uptake of the reinfecting strain (1.96%) following the primary infection was the same as that of the primary infecting strain average (1.61%), following the 4 hour infection (**Table 3.2**). The overnight (16 hours) uptake of the mycobacterial inoculum was slightly higher during reinfection (average, 11.33%) than during primary infection (average, 9.25%), however, this was not statistically significant (P value = 0.261) (**Table 3.3**).

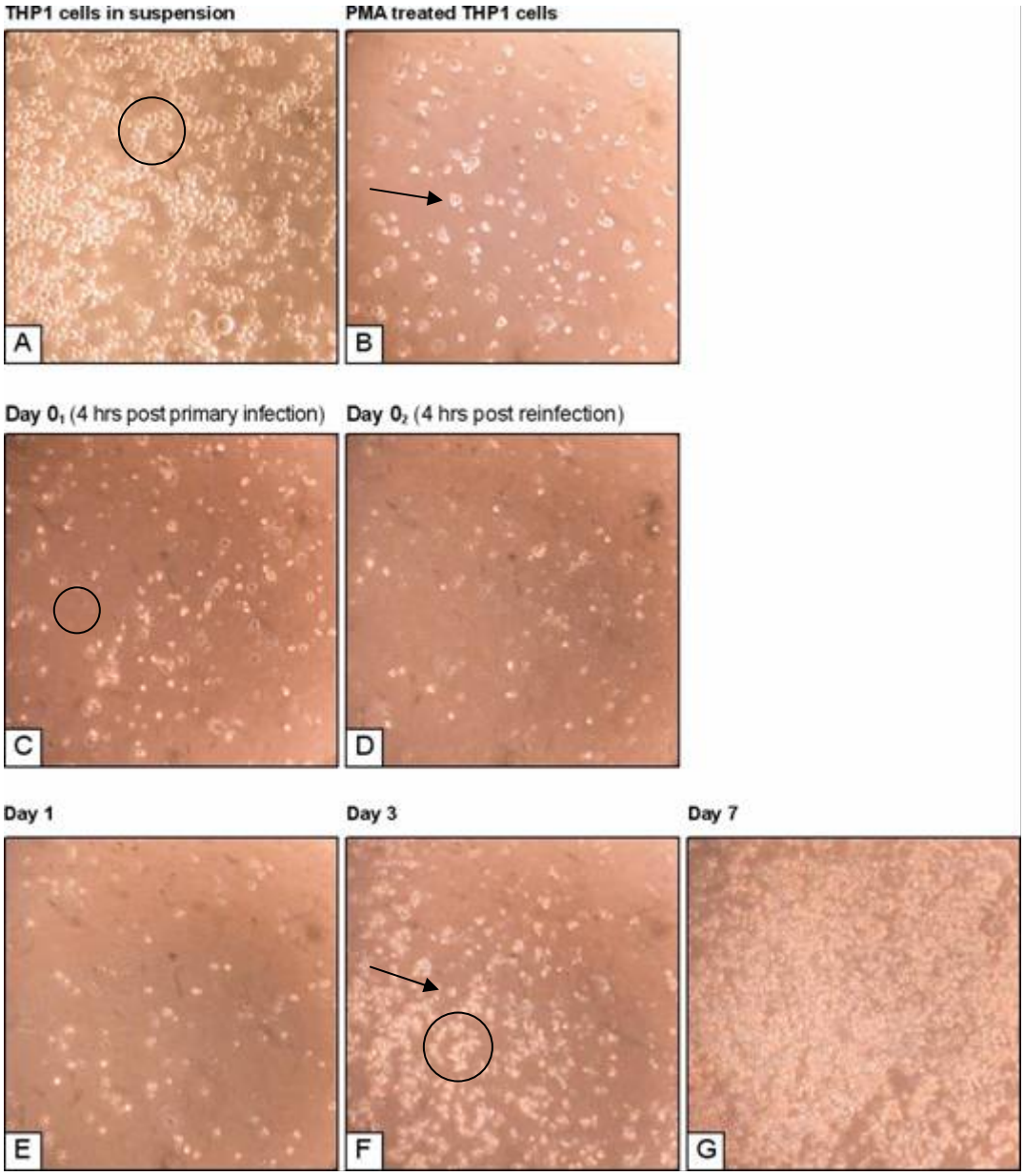

Figure 3.11. THP1 cells at different stages of the experiment. **A**, THP1 cells in suspension, where cells tend to clump, indicated by the circle; **B**, PMA-treated THP1 cells, cells starting to differentiate into macrophage-like morphology, with a well differentiated cell indicated by the arrow; **C**, Day 0_1 4 hours post infection, cells appeared alive and well differentiated, with mycobacteria visible in the background, indicated by the circle; **D**, Day 0_2, 4 hours post reinfection, cells appeared alive and well differentiated with more mycobacteria visible in the background; **E**, Day 1, cells appeared less but alive and well differentiated; **F**, Day 3, dead cell debris started to accumulate, indicated by the circle, some living cells still visible, indicated by the arrow; G, Day 7, dead cell debris obscured the whole field with no more living cells visible. Cells were photographed at 100 X magnification.

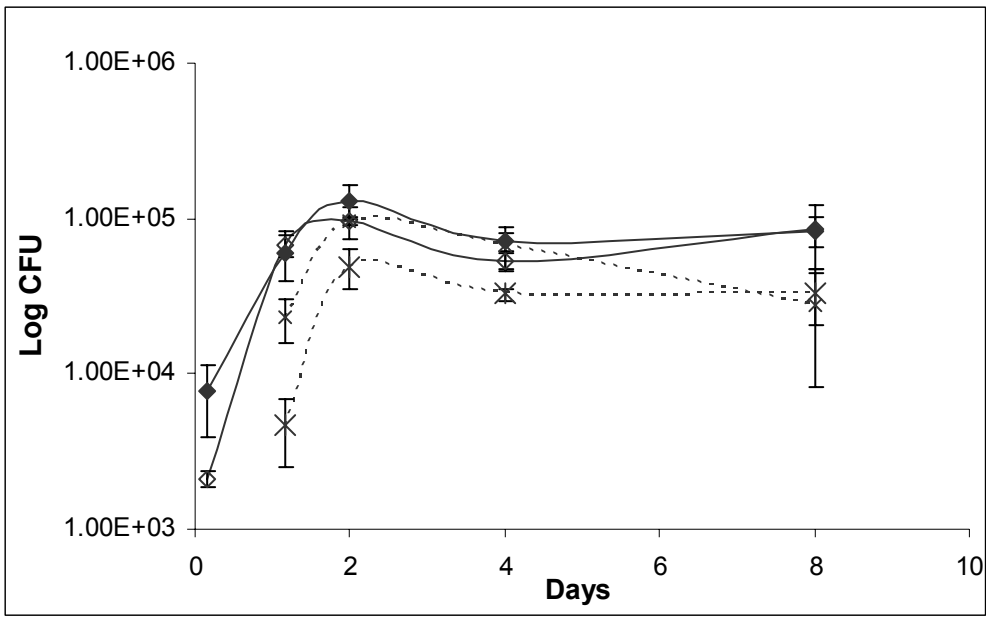

Figure 3.12. THP1 cells infected/reinfected with *M. tuberculosis* Hyg^R/Kan^R. *M. tuberculosis* Hyg^R (♦/◊) infected THP1 cells reinfected with *M. tuberculosis* Kan^R (x/Ж). No difference could be observed between primary infecting (solid line) and reinfecting (dotted line) strain growth patterns. This experiment was done in triplicate and repeated, with average values displayed on the graph.

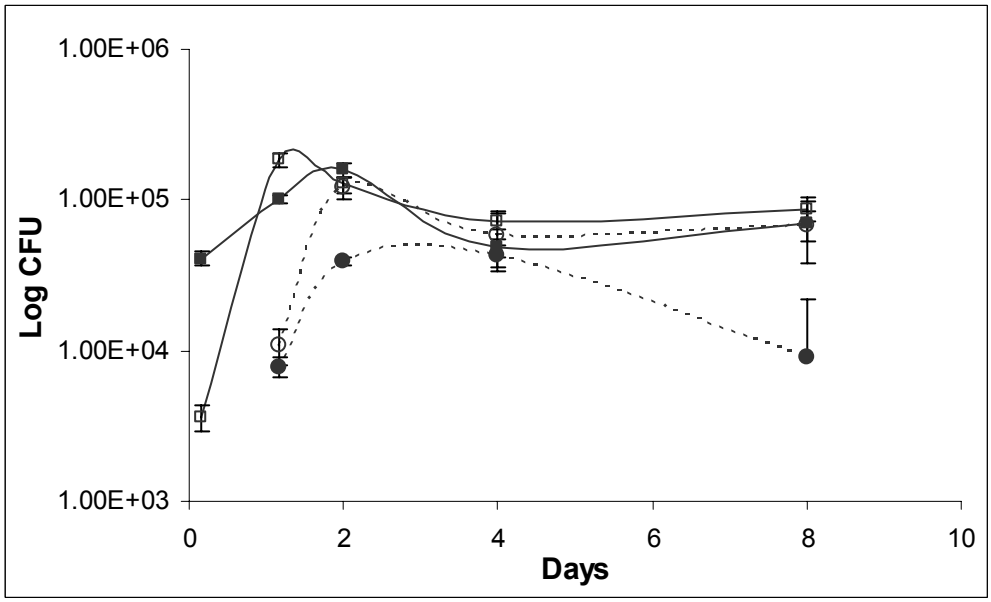

Figure 3.13. THP1 cells infected/reinfected with *M. tuberculosis* Kan^R/Hyg^R. *M. tuberculosis* Kan^R (■/□) infected THP1 cells reinfected with *M. tuberculosis* Hyg^R (●/○). No difference could be observed between primary infecting (solid line) and reinfecting (dotted line) strain growth patterns. This experiment was done in triplicate and repeated, with average values displayed on the graph.

Table 3.2. Reinfection of THP1 cells, displaying percentage uptake of inoculum after 4 hours of infection. (A, B and C, refers to each of the triplicate wells)

Mycobacterial strain	Inoculum (CFU/ml)	% of inoculum uptake after 4hrs infection (CFU/ml)					
Primary infecting strain		**A**	%	**B**	%	**C**	%
Hyg^R 1	1.19E+06	2.35E+03	0.20	2.20E+03	0.18	1.90E+03	0.16
Kan^R 1	2.70E+06	3.20E+03	0.12	4.50E+03	0.17	3.20E+03	0.17
Hyg^R 2	6.25E+05	1.20E+04	1.92	5.60E+03	0.90	5.45E+03	0.90
Kan^R 2	7.90E+05	4.30E+04	5.44	3.60E+04	4.56	4.40E+04	4.56
Reinfecting strain							
Hyg^R re 1	8.20E+05	8.35E+03	1.02	8.75E+03	1.07	6.40E+03	1.07
Kan^R re 1	6.25E+05	2.30E+04	3.68	3.05E+04	4.88	1.60E+04	4.88
Hyg^R re 2	1.03E+06	1.23E+04	1.19	1.32E+04	1.28	7.45E+03	1.28
Kan^R re 2	5.75E+05	3.75E+03	0.65	7.15E+03	1.24	3.15E+03	1.24

Average of primary infection: 1.61%
Average of reinfection: 1.96%

Table 3.3. Reinfection of THP1 cells, displaying percentage uptake of inoculum after 16 hours of infection. (A, B and C, refers to each of the triplicate wells)

Mycobacterial Strain	Inoculum (CFU/ml)	% of inoculum uptake after 16hrs infection (CFU/ml)					
Primary infecting strain		**A**	%	**B**	%	**C**	%
Hyg^R 1	1.19E+06	7.85E+04	6.60	6.25E+04	5.25	6.00E+04	5.04
Kan^R 1	2.70E+06	2.05E+05	7.59	1.80E+05	6.67	1.70E+05	6.67
Hyg^R 2	6.25E+05	5.90E+04	9.44	8.30E+04	13.28	4.00E+04	13.28
Kan^R 2	7.90E+05	9.75E+04	12.34	9.80E+04	12.41	1.09E+05	12.41
Reinfecting strain							
Hyg^R re 1	8.20E+05	4.10E+04	5.00	4.10E+04	5.00	3.70E+04	5.00
Kan^R re 1	6.25E+05	1.12E+05	17.92	1.04E+05	16.64	7.00E+04	16.64
Hyg^R re 2	1.03E+06	1.16E+05	11.26	1.44E+05	13.98	1.06E+05	13.98
Kan^R re 2	5.75E+05	4.85E+04	8.43	6.35E+04	11.04	3.50E+04	11.04

Average of primary infection: 9.25%
Average of reinfection: 11.33%

When the *M. tuberculosis* Beijing strain 208 infected THP1 cells were reinfected with respective Hyg^R and Kan^R marked *M. tuberculosis* H37Rv strains, little difference in growth of the reinfecting strains was observed. During the Beijing strain infected THP1 cells reinfected with *M. tuberculosis* Hyg^R and Kan^R, there were little difference between the growth rates of the Beijing and marked

M. tuberculosis strains. However, there was a slight decrease in CFU during the 16 hour *M. tuberculosis* Hyg^R reinfection, indicated by the arrow in **Figure 3.14**. Furthermore, when the Beijing strain infected THP1 cells were reinfected with *M. tuberculosis* Kan^R, the Beijing strain suffered a retardation of growth during the 16 hour reinfection with the *M. tuberculosis* Kan^R strain, indicated by the arrow in **Figure 3.15**. The 4 hour inoculum uptake was also the same for both primary infection (average 1.87%) and reinfection experiments (average 1.58%) (**Table 3.4**), and the overnight inoculum uptake of the reinfecting strains was statistically significantly higher (average 8.94%) than the primary inoculum uptake (average 1.96%) (P value = 0.004) (**Table 3.5**).

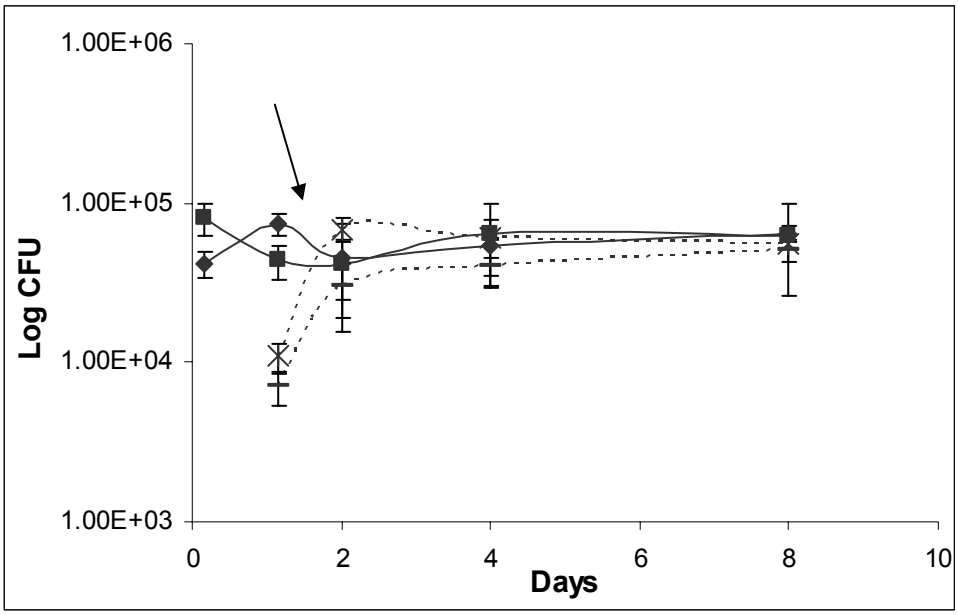

Figure 3.14. THP1 cells infected/reinfected with *M. tuberculosis* Beijing strain 208/Hyg^R. Beijing strain 208 (■/♦) infected THP1 cells reinfected with *M. tuberculosis* Hyg^R (Ж/-). The primary infecting Beijing strain (solid line) growth decreased during the 16 hour infection of the *M. tuberculosis* Hyg^R reinfecting (dotted line) strain, as indicated by the arrow. This experiment was done in triplicate and repeated, with average values displayed on the graph.

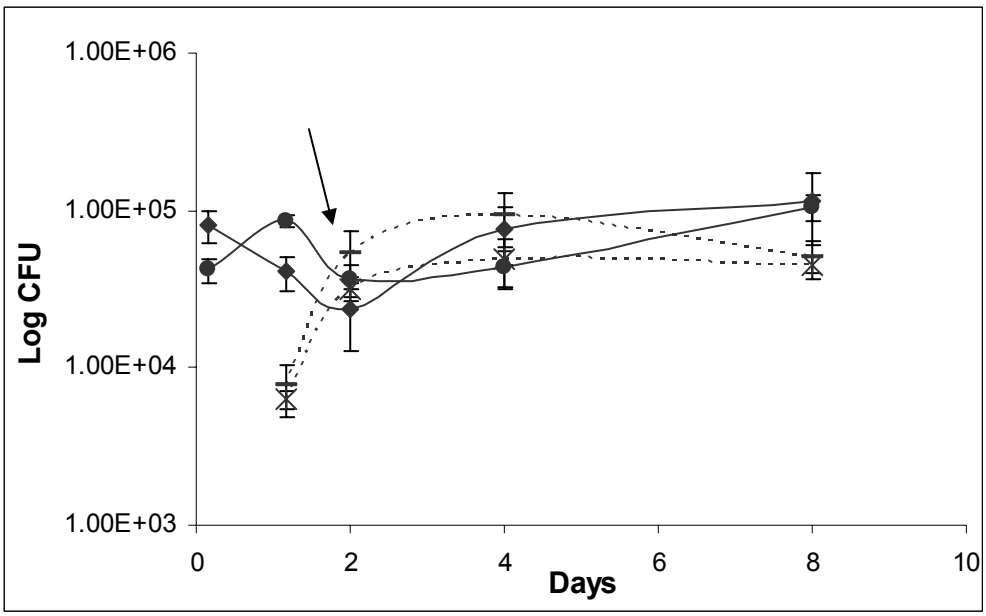

Figure 3.15. THP1 cells infected/reinfected with *M. tuberculosis* Beijing strain 208/Kan^R. Beijing strain 208 (●/♦) infected THP1 cells reinfected with *M. tuberculosis* Kan^R (Ж/-). The growth of the primary infecting Beijing strain (solid line) suffered a retardation of growth during the 16 hour infection of the *M. tuberculosis* reinfecting (dotted line) strain, as indicated by the arrow. This experiment was done in triplicate and repeated, with average values displayed on the graph.

Table 3.4. *M. tuberculosis* Beijing strain-infected and reinfected THP1 cells, displaying percentage uptake of inoculum after 4 hours of infection. (A, B and C, refers to each of the triplicate wells)

Mycobacterial Strain	**Inoculum** (CFU/ml)	**% of inoculum uptake after 4hrs infection (CFU/ml)**					
Primary infecting strain		**A**	**%**	**B**	**%**	**C**	**%**
208 1 (reinfected with Hyg^R)	2.95E+06	3.95E+04	1.34	3.55E+04	1.20	5.05E+04	1.71
208 1 (reinfected with Kan^R)	2.95E+06	3.95E+04	1.34	3.55E+04	1.20	5.05E+04	1.71
208 2 (reinfected with Hyg^R)	3.50E+06	1.02E+05	2.91	6.70E+04	1.91	7.45E+04	2.13
208 2 (reinfected with Kan^R)	3.50E+06	1.02E+05	2.91	6.70E+04	1.91	7.45E+04	2.13
Reinfecting strain							
Hyg^R re 1	4.90E+05	8.65E+03	1.77	1.08E+04	2.20	1.32E+04	2.69
Kan^R re 1	6.95E+05	4.80E+03	0.69	1.03E+04	1.48	7.85E+03	1.13
Hyg^R re 2	4.90E+05	5.80E+03	1.18	8.95E+03	1.83	6.40E+03	1.31
Kan^R re 2	4.05E+05	7.25E+03	1.79	5.75E+03	1.42	5.85E+03	1.44

Average of primary infection: 1.87%
Average of reinfection: 1.58%

Table 3.5. *M. tuberculosis* Beijing strain-infected and reinfected THP1 cells, displaying percentage uptake of inoculum after 16 hours of infection. (A, B and C, refers to each of the triplicate wells)

Mycobacterial Strain	Inoculum (CFU/ml)	% of inoculum uptake after 16hrs infection (CFU/ml)					
Primary infecting strain		**A**	**%**	**B**	**%**	**C**	**%**
208 1 (reinfected with Hyg^R)	2.95E+06	8.09E+04	2.74	8.12E+04	2.75	6.08E+04	2.06
208 1 (reinfected with Kan^R)	2.95E+06	9.42E+04	3.19	8.07E+04	2.74	8.32E+04	2.82
208 2 (reinfected with Hyg^R)	3.50E+06	4.72E+04	1.35	3.16E+04	0.90	5.21E+04	1.49
208 2 (reinfected with Kan^R)	3.50E+06	3.13E+04	0.89	5.13E+04	1.46	3.97E+04	1.13
Reinfecting strain							
Hyg^R re 1	4.90E+05	7.95E+04	16.22	5.80E+04	11.84	6.95E+04	14.18
Kan^R re 1	6.95E+05	4.10E+04	5.90	7.75E+04	11.15	4.00E+04	5.76
Hyg^R re 2	4.90E+05	4.30E+04	8.78	2.20E+04	4.49	2.60E+04	5.31
Kan^R re 2	4.05E+05	2.90E+04	7.16	3.80E+04	9.38	2.90E+04	7.16

Average of primary infection: 1.96%
Average of reinfection: 8.94%

In order to confirm the strain differentiation by selective CFU enumeration of the mycobacterial strains, following reinfection experiments, melt analysis was done on boiled cell lysates for identification of *M. tuberculosis* Hyg^R, Kan^R and Beijing strains. Using primer sets designed to identify each strain, identification was made by measuring the temperature ranges of melting curves, by detection of the fluorescence of Syto® 9 in PCR amplification products. Samples positive for the *M. tuberculosis* Hyg^R strain produced melting curves between the temperature range of 92ºC and 94ºC, using the Hygr r-Hygr f primer set. *M. tuberculosis* Kan^R strain positive samples produced melting curves between 80ºC and 82ºC, using the Kanr r-Kanr f primer set, and *M. tuberculosis* Beijing strain positive samples were identified by the primer set F29/780F-XhO 1, producing melting curves between temperatures 80ºC and 90ºC. Unfortunately, only a few samples produced melting curves, owing to the small amount of DNA extracted from each sample, as displayed in **Table 3.6**.

Table 3.6. Melt analysis for the detection of PCR products

PCR product	*M. tuberculosis* Hyg^R	*M. tuberculosis* Kan^R	*M. tuberculosis* Beijing strain
Temperature range	92ºC – 94ºC	80ºC – 82ºC	88ºC – 90ºC
Sample	**PCR amplification products detected in the following samples marked with an X:** (Samples on which PCR was done, to identify each respective strain, indicated by shaded areas)		
Hyg^R Day 0_1	**-**	**-**	
Hyg^R/Kan^R Day 0_2	**X**	**-**	
Hyg^R/Kan^R Day 1	**X**	**X**	
Hyg^R/Kan^R Day 3	**X**	**-**	
Hyg^R/Kan^R Day 7	**X**	**-**	
Kan^R Day 0_1	**-**	**-**	
Kan^R/Hyg^R Day 0_2	**-**	**-**	
Kan^R/Hyg^R Day 1	**-**	**-**	
Kan^R/Hyg^R Day 3	**-**	**X**	
Kan^R/Hyg^R Day 7	**X**	**-**	
208 Day 0_1	**-**	**X**	**-**
208/Hyg^R Day 0_2	**X**		**X**
208/Hyg^R Day 1	**X**		**-**
208/Hyg^R Day 3	**X**		**-**
208/Hyg^R Day 7	**-**		**-**
208/Kan^R Day 0_2		**-**	**X**
208/Kan^R Day 1		**-**	**-**
208/Kan^R Day 3		**X**	**X**
208/Kan^R Day 7		**-**	**-**

CHAPTER 4

DISCUSSION

4.1 Generation of differentially marked *M. tuberculosis* Hyg^R and Kan^R strains

In this study we successfully generated Hyg^R and Kan^R marked *M. tuberculosis* strains, following electroporation of the pIVK1 and pIVH1 plasmids into the *M. tuberculosis* genome. Selection of cultures was necessary, as the plasmid DNA may not have penetrated into every mycobacterial cell and may not have integrated into the correct position into the mycobacterial genome, which had to be established.

4.2.1 *In vitro* growth kinetics of marked *M. tuberculosis* Hyg^R and Kan^R variants

The MGIT^{TM} system measures oxygen consumption, which is a different principle, compared to traditional liquid culture that measures growth by OD or CFU. At different starting point inoculums the MGIT^{TM} growth curves were inconsistent at first, but using low concentrations of approximately 1 x 10^3 CFU/ml, results were comparable for all cultures. Both Hyg^R and Kan^R cultures displayed similar growth patterns in MGIT^{TM} units as compared to wild-type *M. tuberculosis* H37Rv. However, *M. tuberculosis* H37Rv displayed a slightly longer lag phase. *M. tuberculosis* Hyg^R and Kan^R cultures grew slightly faster than *M. tuberculosis* H37Rv, implying that the plasmid integration did not negatively affect the growth kinetics of the Hyg^R and Kan^R marked *M. tuberculosis* H37Rv variants. In fact, the plasmid integration seemed to have rather promoted growth slightly.

Conventional liquid culture methods were also investigated. A 28 day Middlebrook 7H9 liquid media broth culture with an initial inoculum dilution of 1:500 was used, measuring growth by both CFU and OD. The recombinant *M. tuberculosis* H37Rv strains displayed similar growth kinetics with no difference to wild-type *M. tuberculosis* H37Rv growth. This confirmed that neither of the marked strains' growth patterns were negatively affected as a result of the plasmid integration into their genomes.

4.2.2 *In vitro* growth kinetics of two hypervirulent *M. tuberculosis* Beijing strains

Hypervirulent clinical *M. tuberculosis* Beijing family strains, 208 and 209, were cultured and displayed comparable growth kinetics to wild-type *M. tuberculosis* H37Rv during a 28 day 7H9 liquid culture, starting with a 1:500 inoculum dilution. There was no observable difference in growth by either CFU or OD readings. It was important to verify that there were no significant differences in growth kinetics between the Hyg^R and Kan^R marked *M. tuberculosis* H37Rv variants and the Beijing strains to be able to compare the growth dynamics of primary and secondary infecting strains during reinfection experiments.

4.3 Growth kinetics of mycobacterial strains within a human THP1 macrophage-like cell line

Growth curves of Hyg^R and Kan^R marked *M. tuberculosis* H37Rv, wild-type H37Rv and strain 208 of the Beijing family showed comparable growth patterns during a 7 day THP1 cell growth curve, following infection of THP1 cells. There was no significant difference in growth kinetics between strain 208, a hypervirulent clinical Beijing strain and the laboratory strain *M. tuberculosis* H37Rv. This is in contrast to the findings of Theus *et al.* (2005), who found that members of the Beijing family displayed faster growth rates in a THP1 cell model. This may imply that the

M. tuberculosis Beijing strain has lost some virulence due to repeated culturing in other hands. However, the frozen Beijing strain specimen was thawed only once and cultured in 7H9 liquid media to log phase, and thereafter frozen in 15% glycerol for preparation of stock vials. It is known that sub-culturing of *M. tuberculosis* diminishes the organism's virulence significantly. On the other hand, the marked *M. tuberculosis* H37Rv variants were sub-cultured about 5 times, and together with their preparation for electroporation as well as the electroporation process itself, they may also have had decreased virulence, with an effect on the infection capability of the organisms.

During the THP1 cell growth curve there was one log increase in CFU between the 4 hour infection, Day 0, and the 16 hour reinfection, Day 1. However, between the time points following the overnight infection, Day 3 and Day 7, there was no significant increase in CFU counts. There could be several factors that may have influenced mycobacterial growth in THP1 cells. Only an average of 1.35% of the inoculum was taken up by the cells after 4 hours of infection in contrast to an average of 6.71% after overnight infection. The overnight inoculum uptake value might not have been a true reflection of uptake, but probably represented a combination of growth and mycobacterial uptake into the THP1 cells. During and after PMA treatment to differentiate the cells into macrophage-like morphology, the antibiotic gentamicin, used for contamination prevention, was removed from the complete medium added to the cells, because it was shown to have a bacteriostatic effect on *M. tuberculosis* (Ho *et al*., 1997). This was done to prevent the gentamicin from affecting the mycobacterial growth post-infection. When the mycobacteria were harvested from the cells, 0.025% SDS was used to lyse the THP1 cells, which at this low concentration had no apparent effect on mycobacterial growth. Therefore, it was assumed that growth kinetics of the different mycobacterial strains assessed was a true reflection of their growth under these conditions.

The doubling time of wild-type *M. tuberculosis* H37Rv is 20 to 24 hours in Middlebrook 7H9 liquid media and in macrophage culture approximately 28.6 hours (Zhang *et al*., 1998). When comparing CFU counts of liquid culture to CFU of THP1 cell culture, it was found that the liquid culture reached stationary phase by Day 7, while CFU counts of THP1 cell culture only increased (log phase) between 4 hours infection (Day 0_1) and 16 hours infection (Day 1). One explanation for this could be that the MOI or starting point concentration of mycobacteria was too high and that the THP1 cells became saturated with mycobacteria at an early stage of the infection. Another possible reason for the early stationary phase may be the fact that the mycobacterial cultures were used for infection directly after thawing. In this case the mycobacteria had no time to adjust to their environment and become metabolically active after being frozen at -80°C. For future experiments it is advisable to allow the frozen cultures to grow for 72 hours in Middlebrook 7H9 liquid media at 37ºC before use.

4.4 Reinfection dynamics of Hyg^R and Kan^R marked *M. tuberculosis* H37Rv variants and a hypervirulent Beijing strain in THP1 cells, using different primary and secondary infecting strains

During reinfection experiments the 4 hour inoculum uptake was consistent (average 2%) for all strains. However, 2% of the inoculum is a very low uptake percentage. An explanation for this could be that the amount of THP1 cells in the final inoculum might have been less than the initially calculated concentration, as a result of all the washing steps and media changes. There is also a possibility that not all the cells treated with PMA differentiated and adhered to the well surfaces of the plates.

The intended MOI was 5:1 (mycobacteria:THP1 cells), in other words 2.5 X 10^6 mycobacteria infecting 5 x 10^5 THP1 cells, for THP1 cell growth curves and reinfection experiments. The actual inoculum titre for the growth curve as well as

reinfection experiments was between 4 x 10^5 and 4 x 10^6. When plating out thawed liquid culture preparations, the inoculum titre results were reproducible. However, the reason for the varied inoculum results may be ascribed to the rigorous inoculum preparation, leading to a loss of mycobacteria.

It is also well known that mycobacteria tend to clump in liquid culture. In order to rid the cultures of clumps after thawing, suspensions were passed through a syringe 30 times using a 25G needle to achieve single cell suspensions. The suspensions were then centrifuged twice in RPMI medium to wash out the Tween 80 and glycerol, which is harmful to the THP1 cells, followed by another 10 times of passaging through a syringe. The preparation of the inoculum may thus account for the varied loss of mycobacteria.

An inoculum of between 4 x 10^5 and 4 x 10^6 gave a MOI of between 0.8 to 8 mycobacteria per THP1 cell, with an average of 4.4, which was lower than the intended MOI of 5:1. The amount of THP1 cells, however, may have been much less as the washing and media changes could also have depleted the cells as mentioned previously. A shortcoming of this study therefore was the resultant MOI of the secondary infection. It was the intention to infect each THP1 cell with exactly five mycobacteria with both the primary and reinfecting strain, respectively, to be able to compare the behaviour of the strains. The fact that only 2% of the primary infecting strain inoculum was taken up after 4 hours of infection, may suggest that there had been enough uninfected macrophages available for reinfection after the primary infection. Alternatively, as stated before, the amount of cells may have been depleted and most of the macrophages may have been infected by the primary mycobacterial inoculum.

Viewing of the cells under phase microscopy revealed that there had been more cells before PMA treatment than after. This loss was probably due to the many

PBS washes to remove the excess PMA. However, THP1 cells did not appear significantly depleted after media changes. Another indication for the early stationary phase during THP1 cell infection or reinfection may be the morphology of the *M. tuberculosis* infected THP1 cells, which confirmed the decreased life-span of infected cells. By Day 3 very little of the THP1 cells were still alive and dead cell debris started to accumulate. By Day 7 the dead cell debris obscured the whole field, indicating that most of the cells had died.

During CFU counting it was observed that the morphology of the *M. tuberculosis* colonies plated from the THP1 cell lysates after infection had changed. All four strains had exhibited decreased size colonies after passing through THP1 cells, when compared to the inoculums plated on the same day from the same frozen aliquot. The colony size of especially the *M. tuberculosis* H37Rv and Beijing strains had become very small, pinprick size, and this altered morphology was observed throughout the experiment. Contrary to this, colonies of the *M. tuberculosis* Hyg^R strain remained relatively normal throughout the experiment. The *M. tuberculosis* Kan^R colonies on the other hand were mixed in size, some very large, some normal but most were very small. This may indicate that there was possible phenotypic alteration to the mycobacteria inside the THP1 cell, which may have affected their metabolic state.

The PCR that was done to confirm the identification of mycobacterial strains, did not yield products in all the samples, suggesting that there was either too little DNA or that inhibitory agents were present.

The possibility that the individual strains could have been contaminated throughout the experiments could be ruled out by the fact that the marked *M. tuberculosis* strains were plated on selective antibiotic media, on which only the selected strain would grow. The PCR data, however, confirmed that the intended strains only

were present during reinfection experiments, specifically in the case of the Beijing strain, where non-selective media was used.

During reinfection with the different combinations of *M. tuberculosis* H37Rv Hyg^R and H37Rv Kan^R strains, the reinfecting strain displayed a similar replication rate to the primary infecting strain. There were no significant differences in growth kinetics between primary and secondary infecting strains in both combinations of infection. However, the 16 hours post-infection uptake of the mycobacterial inoculum was slightly higher following reinfection (average 11.33%) as compared to the primary infection (average 9.25%). On the contrary, there was no difference in the 4 hour inoculum uptake between the two strains. This suggests that either the reinfecting strain was taken up more readily or that its growth was promoted, or alternatively that the primary infecting strain was suppressed in some way. Either way, the primary infecting mycobacterial strain manipulated the cell in such a way as to promote the uptake or growth of the reinfecting strain. This finding correlates with that of Cosma *et al*. (2004), who showed that mycobacteria-infected macrophages are attracted to an established granuloma, showing that a controlled primary infection attracts reinfecting mycobacteria. The higher uptake of reinfecting mycobacteria suggests that the signalling to reinfecting mycobacteria is initiated at a cellular level rather than at a structural level. This may help to explain why successfully treated tuberculosis patients are at higher risk for developing recurrent tuberculosis disease than the general population (Verver *et al*., 2005).

Where the primary Beijing-infected cells were reinfected with the respective marked *M. tuberculosis* H37Rv Kan^R and Hyg^R strains, there were also no differences between the 4 hour inoculum uptake for both primary infection and reinfection. However, the 16 hour inoculum uptake of the reinfecting strain was significantly higher, with an average of 8.94% than the primary infection uptake (average 1.96%). This indicates that pre-infection with a Beijing strain enhances

the THP1 cell's susceptibility to reinfection. This corroborates the findings of Theus *et al.* (2005) who showed that *M. tuberculosis* Beijing strains could alter cell function and influence the cell's anti-inflammatory response. Furthermore, the *M. tuberculosis* Beijing strain suffered a retardation of growth following the 16 hours infection period of the reinfecting strain, suggesting that reinfection with a second mycobacterial strain suppresses the growth of the primary infecting strain. This supports the results from the Hyg^R and Kan^R marked *M. tuberculosis* H37Rv experiments, which showed an insignificant higher infection rate for the reinfection when compared to the primary infection. Therefore, it is plausible that *M. tuberculosis*-infected THP1 cells are reinfected more readily than naïve cells.

From this work a number of possibilities for further investigation have arisen. Further information would be gained by reinfection experiments in THP1 cells using either of the two marked *M. tuberculosis* H37Rv strains as primary infecting strain and *M. tuberculosis* Beijing 208 strain as the reinfecting strain. Valuable comparative data should be obtained by observing the difference in growth dynamics compared to the reinfection experiments where the Beijing strain was used as the primary infecting strain. Moreover, competitive dynamics of strains could also be evaluated by dual or super infection experiments in THP1 cell cultures. This could be done using different inoculum titres of different mycobacterial strains with variable virulence. Additionally, during reinfection or dual infection experiments in THP1 cell culture, cytokine analysis could be done as a measure of mycobacterial virulence and cellular immune response.

CHAPTER 5

CONCLUSION

The two antibiotic-resistant marked *M. tuberculosis* H37Rv strains, designated Hyg^R and Kan^R, replicated comparably during *in vitro* growth experiments, using different methods and read-outs, and was also comparable with wild-type H37Rv growth. Neither of the marked mycobacterial strains showed signs of a reduced fitness cost as result of the plasmid integration into their genome. Additionally, there were no significant differences in growth kinetics between primary and secondary infecting strains during THP1 cell reinfection of *M. tuberculosis* H37Rv Hyg^R and Kan^R strains. There were also no significant differences in growth kinetics following primary infection with a hypervirulent Beijing strain and reinfection with either of the marked *M. tuberculosis* H37Rv strains.

However, during both reinfection experiments with the combination of marked *M. tuberculosis* H37Rv strains and the *M. tuberculosis* Beijing strain reinfected with the respective marked *M. tuberculosis* H37Rv strains, the overnight uptake of the reinfecting strain was higher than the primary infecting strain, therefore it is possible that *M. tuberculosis*-infected THP1 cells are reinfected more readily than naïve cells.

Furthermore, pre-infecting cells with the *M. tuberculosis* Beijing strain resulted in slight growth retardation following the 16 hour infection of the reinfecting strain, both for *M. tuberculosis* Hyg^R and Kan^R. This indicated that the primary infecting strain was suppressed by the reinfecting strain.

These findings open the way to design further experiments using different strains of *M. tuberculosis* in order to better understand reinfection of tuberculosis and the underlying dynamics of multiple bacterial strain infections.

APPENDIX A

PLASMID AND PRIMER SEQUENCES

1. pIVK1 plasmid

1.1 Restriction enzyme analysis (Methylation: dam-No dcm-No

Enzymes with more than one site are not shown)

1.1.1 Screened with 84 enzymes, 44 enzyme restriction sites found

AatII 1	GACGT/C	5922	EheI 1	GGC/GCC	237
Acc65I 1	G/GTACC	408	**HindIII 1**	**A/AGCTT**	**3748**
AlwNI 1	CAGNNN/CTG	4523	KpnI 1	GGTAC/C	412
ApaBI 1	GCANNNNN/TGC	186	MstII 1	CC/TNAGG	2633
ApaI 1	GGGCC/C	2344	NaeI 1	GCC/GGC	2760
Asp718I 1	G/GTACC	408	NarI 1	GG/CGCC	236
AvrII 1	C/CTAGG	2138	NruI 1	TCG/CGA	631
BbeI 1	GGCGC/C	239	PflMI 1	CCANNNN/NTGG	1237
BbvII 1	GAAGACNN/	3131	SacI 1	GAGCT/C	406
BglII 1	A/GATCT	2605	**SapI 1**	**GCTCTTCN/**	**3991**
Bsc91I 1	GAAGACNN/	3131	**SauI 1**	**CC/TNAGG**	**2633**
Bsp1407I 1	T/GTACA	2881	**ScaI 1**	**AGT/ACT**	**5480**
BstEII 1	G/GTNACC	2321	SciI 1	CTC/GAG	574
Bsu36I 1	CC/TNAGG	2633	SpII 1	C/GTACG	2357
CvnI 1	CC/TNAGG	2633	SpoI 1	TCG/CGA	631
EagI 1	C/GGCCG	2243	SstI 1	GAGCT/C	406
Ecl136II 1	GAG/CTC	404	StuI 1	AGG/CCT	1598
Eco52I 1	C/GGCCG	2243	SunI 1	C/GTACG	2357
Eco56I 1	G/CCGGC	2758	Tth111I 1	GACN/NNGTC	2621
EcoICRI 1	GAG/CTC	404	XhoI 1	C/TCGAG	572
EcoNI 1	CCTNN/NNNAGG	886	XmaIII 1	C/GGCCG	2243
EcoRI 1	**G/AATTC**	**396**	XmnI 1	GAANN/NNTTC	5599

1.1.2 List by site order

186 -	ApaBI	412 -	KpnI	2243 -	XmaIII	2758 –	Eco56I
236 -	NarI	572 -	XhoI	2321 -	BstEII	2760 -	NaeI
237 -	EheI	574 -	SciI	2344 -	ApaI	2881 -	Bsp1407I
239 -	BbeI	631 -	SpoI	2357 -	SplI	3131 -	Bsc91I
396 -	**EcoRI**	631 -	NruI	2357 -	SunI	3131 -	BbvII
404 -	Ecl136II	886 -	EcoNI	2605 -	BglII	**3748 -**	**HindIII**
404 -	EcoICRI	1237 -	PflMI	2621 -	Tth111I	**3991 -**	**SapI**
406 -	SacI	1598 -	StuI	2633 -	Bsu36I	4523 -	AlwNI
406 -	SstI	2138 -	AvrII	2633 -	MstII	**5480 -**	**ScaI**
408 -	Acc65I	2243 -	Eco52I	2633 -	CvnI	5599 -	XmnI
408 -	Asp718I	2243 -	EagI	**2633 -**	**SauI**	5922 -	AatII

1.1.3 Non-cut enzymes

AccIII	AflII	AgeI	AscI	AsuII	BclI
Bpu1102I	BspMII	BssHII	ClaI	Csp45I	CspI
DraIII	Eco47III	Eco72I	EcoRV	EspI	FseI
HpaI	I-PpoI	MfeI	Mlu113I	MluI	NcoI
NheI	NotI	PacI	PinAI	PmaCI	PmeI
RleAI	SacII	SfiI	SgrAI	SnaBI	SpeI
SrfI	SstII	SwaI	XbaI		

1.2 Primer sequences

Kanr forward primer (Kanr f) – 5’-CGTTGCCAATGATGTTAC-‘3

Kanr reverse primer (Kanr r) – 5’-TACCTGGAATGCTGTTTT-‘3

(Kanr r reverse complementary) – 3’-AAAACAGCATTCCAGGTA-‘5

***att*P reverse (*att*P r) –** 5’-CAGAAGAGTCGCACAAGAGTT-‘3

(*att*P r reverse complementary) – 3-‘AACTCTTGTGCGACTCTTCTG-‘5

1.3 Restriction sites and primer locations on pIVK1 sequence

```
1           TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA
61          CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG
121         TTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGC
```

BbeI

EheI

ApaBI NarI

181 ACCATA/TGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGG/C/GC/C

241 ATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTAT

301 TACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGT

KpnI

Asp718I

Acc65I

SstI

SacI

EcoICRI

EcoRI Ecl136II

361 TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTG/AATTCGAG/CT/CG/GTAC/CCGGGGAT

421 CCTCTAGCCAACAAAGCGACGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATA

481 AAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTG

SciI

XhoI

541 TTATGAGCCATATTCAACGGGAAACGTCTTGC/TC/GAGGCCGCGATTAAATTCCAACATGG

NruI

SpoI

601 ATGCTGATTTATATGGGTATAAATGGGCTCG/CGATAATGTCGGGCAATCAGGTGCGACAA

661 TCTATCGCTTGTATGGGAAGCCCCATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTA

Kanr f

721 G**CGTTGCCAATGATGTTAC**AGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGC

781 CTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCACCACTG

Kanr r EcoNI

841 CGATCCCCGGG**AAAACAGCATTCCAGGTA**TTAGAAGAATATCCTGA/TTCAGGTGAAAATA

901 TTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTTGCATTCGATTCCTGTTTGTAATTGTC

961 CTTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATAACGGTT

1021 TGGTTGATGCGAGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGA

1081 AAGAAATGCATAATCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTGATTTCT

1141 CACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGTATTGATGTTGGACGAG

PflMI

1201 TCGGAATCGCAGACCGATACCAGGATCTTGCCATCCT/ATGGAACTGCCTCGGTGAGTTTT

1261 CTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTGATATGAATA

1321 AATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTAATCAGAATTGGTTAATTGGTTGT

1381 AACACTGGCAGAGCATTACGCTGACTTGACGGGACGGCGGCTTTGTTGAATAAATCGAAC

1441 TTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTTCCGTGGC

1501 AAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGG

StuI

1561 CTCCCTCACTTTCTGGCTGGATGATGGGGCGATTCAGG/CCTGGTATGAGTCAGCAACACC

1621 TTCTTCACGAGGCAGACCTCACTAGAGTCGACCAAGACGATCACCGGGCTCGTCGGAGCT

1681 GGAGGCGCAGCGGGAGCTGGCTCTGTATTCTCAGGCAAGGCCGGAGGCCCTGGAGGAAAC

1741 ACCACGGCGTCCGCTGTCGGATGGTCAGGTTTGACCGCAACCGGCGGTCCCGGAGGCTCT

1801 GTGATCGACATCCTCAGCGTCGCCGGAAAGTCGCCTGGAGATCGGACCTACAACGACCAG

1861 CTCTACATAGGCGGCGCACAACAGAACTCAGCTGGCGGGAACGGCAATGCTCCTGGCGGC

1921 GGCGGGGCTGGTGCCCAGGTCTCCGCACAGAGCGGCGGTGCTGGCGCTCGCGGCCAGGCG

1981 TGGTTCTTCGCGTACTGACAAGAAACCCCCCTCTTTAGGACTCAGTGTCCTTGGGAGGGG

2041 GGCTTTTTGCGTTTCAGGAGGTCTTGGCCAGCTTGGACATCGCCTCAGCGATAGCCTCGT

AvrII

2101 CGCGGGCCTCAGACGCCATCTGGTACTTCATCGCCATC/CTAGGAGTCGTGTGACCGAGAC

2161 GGGCCATCAGCTCCTTGGTCGTCGCACCTGCCTGAGCGGCGAACGTAGCGCCGACAGCGC

XmaIII

EagI

Eco52I

2221 GGAGGTCGTGGATGCGGAGTTCC/GGCCGACCGATCTTGGCGTAGCCACGCTTCAGCGACT

BstEII

2281 TGGTGAACGCGGACTTCGACAGCCGGTTGCCCTGCGTCGTG/GTCACCAGGAATGCCTCGG

```
                                  SunI
                ApaI              SplI
2341      GGCC/CTTGTTCATCTTC/GTACGGTCCTTCATGTGCGCTCGGATCATCTCCGCGACGTGAG
2401        GCGGAACCGTCACAGGACGCTTCGACCGGACGGTCTTGGCGTTGCCAACGACGATCTTGT
2461        TCCCCACGCGGGAAGCGCCACGGCGCACCCGGAGCTTCATCGTCATGCCGTCGTCCACGA
2521        TGTCCTTGCGGCGAAGCTCGATCAGCTCTCCGAACCGGAGGCTCGTCCACGCCAGGATGT

                                                                 SauI
                                                                 CvnI
                                                                 MstII
                                      BglII              Tth111I  Bsu36I
2581      ATGCCGCGATCCGGTAGTGCTCGAA/GATCTCAGCGGCGACG/ATGTCCAGCTCC/TCAGGCG
2641        TCAGCGCCTCTACGTCGCGCTCATCGGCTGCCTTCTGCTCGATCCGGCACGGGTTCTCTG

                                                                   NaeI
                                                                 Eco56I
2701      CGATCAGCTTGTCCTCGACCGCTGTGTTCATCACCGCCCGGAGGACGTTGTAGGCATGCC/
2761        GGCGGGCAGTCGGGTGCTTCCTACCCATCCCGGCCCACCACGCACGCACCAGAGCTGGCG
2821        TCATCTCTGTGACCGCCACTTCACCTAGCACCGGGTAGATGCGGCGCTCCGCGTGCCCGC

            Bsp1407I
2881      T/GTACAGATCCCTGGTGCCGTCTGCGAGGTCGCGCTCCACGAGCCACTTCCGGGTGTACT
2941        CCTCCAGCGTGATGGCGCTGGCGGCTGCCTTCTTCGCCCGGTCCTGTGGAGGGGTCCAGG
3001        TCTCCATCTCGATGAGCCGCTTCTCGCCCGCGAGCCAGGCTTCGGCGTCCATCTTGTTGT
3061        CGTAGGTCTGCAGCGCGTAGTACCTCACACCGTCCTGCGGGTTGACGTATGAGGCTTGGA
                  BbvII
                  Bsc91I
3121      TCCTCCCGCTG/CGCTGAGTCTTCAGCGATCCCCATCCGCGACGTGCCAACTAGGTCTCCT
3181        CTCGTCGTGAACAAGGCTACCGGGTTGCAACTCCTGTGCAACTCTCAGGCTTCAACGCGC
3241        TTCTACGACCTGCAATTTCTTTCCACTTAGAGGATGCAGCCGAGAGGGGGTAAAAACCTA
3301        TCTTGACCGGCCCATATGTGGTCGGCAGACACCCATTCTTCCAAACTAGCTACGCGGGTT
3361        CGATTCCCGTCGCCCGCTCCGCTGGTCAGAGGGTGTTTTCGCCCTCTGGCCATTTTTCTT

                        attP r
3421        TCCAGGGGTCTGCAACTCTTGTGCGACTCTTCTGACCTGGGCATACGCGGTTGCAACGCA
3481        TCCCTGATCTGGCTACTTTCGATGCTGACAAACGAATAGAGCCCCCCGCCTGCGCGAACA
3541        GACGAGGGGCATTCACACCAGATTGGAGCTGGTGCAGTGAAGAGAATAGACCGGGACAAG
3601        GTTGCACCGGGAGTTGCAGCGGTCGGAACCCTCGCCGTCGGCGGGCTGGCGTTCGCCCTG
3661        TCGTTCACGGCTCTCAGCGAGCTGGCTGCGGCCAACGGGGTGGCCCAAGCAGAGATGGTG

                                  HindIII
3721      CCCTTGGTGGTCGACCTGCAGGCATGCA/AGCTTGGCGTAATCATGGTCATAGCTGTTTCC
3781        TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTG
```

```
3841        TAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCC
3901        CGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGG

                                    SapI
3961      GAGAGGCGGTTTGCGTATTGGGCGCTCTTCC/GCTTCCTCGCTCACTGACTCGCTGCGCTC
4021        GGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCAC
4081        AGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAA
4141        CCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCA
4201        CAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGC
4261        GTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATA
4321        CCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTA
4381        TCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCA
4441        GCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA

                                    AlwNI
4501      CTTATCGCCACTGGCAGCAGCCA/CTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGG
4561        TGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGG
4621        TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGG
4681        CAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAG
4741        AAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAA
4801        CGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGAT
4861        CCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTC
4921        TGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTC
4981        ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATC
5041        TGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGC
5101        AATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTC
5161        CATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTT
5221        GCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGC
5281        TTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAA
5341        AAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTT
5401        ATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATG

                                    ScaI
5461      CTTTTCTGTGACTGGTGAGT/ACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACC
5521        GAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAA
                                    XmnI
5581      AGTGCTCATCATTGGAAAA/CGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTT
5641        GAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTT
5701        CACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAG
5761        GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTA
5821        TCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
```

AatII

```
5881        AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGT/CTAAGAAACCATTATTAT
5941                        CATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC
```

2. pIVH1 plasmid

2.1 Restriction enzyme analysis (Methylation: dam-No dcm-No

Enzymes with more than one site are not shown)

2.1.1 Screened with 59 enzymes, 28 enzyme restriction sites found

Acc65I 1	G/GTACC	408	KpnI 1	GGTAC/C	412
AlwNI 1	CAGNNN/CTG	5032	MstII 1	CC/TNAGG	3142
Asp718I 1	G/GTACC	408	NotI 1	GC/GGCCGC	620
AvrII 1	C/CTAGG	2647	NruI 1	TCG/CGA	1713
BamHI 1	**G/GATCC**	**3627**	PflMI 1	CCANNNN/NTGG	1850
BclI 1	T/GATCA	1462	**SapI 1**	**GCTCTTCN/**	**4500**
BglII 1	A/GATCT	3114	**SauI 1**	**CC/TNAGG**	**3142**
Bpu1102I 1	GC/TNAGC	511	**ScaI 1**	**AGT/ACT**	**5989**
Bsp1407I 1	T/GTACA	3390	SgrAI 1	CR/CCGGYG	1524
Bsu36I 1	CC/TNAGG	3142	SpoI 1	TCG/CGA	1713
CvnI 1	CC/TNAGG	3142	SspI 1	AAT/ATT	6313
DraIII 1	CACNNN/GTG	1195	Tth111I 1	GACN/NNGTC	3130
EspI 1	GC/TNAGC	511	**XbaI 1**	**T/CTAGA**	**2150**
HindIII 1	**A/AGCTT**	**4257**	XmnI 1	GAANN/NNTTC	6108

2.1.2 List by site order

408 -	Asp718I	1462 -	BclI	3114 -	BglII	**3627 -**	**BamHI**
408 -	cc65I	1524 -	SgrAI	3130 -	Tth111I	**4257 -**	**HindIII**
412 -	KpnI	1713 -	SpoI	3142 -	MstII	4500 -	SapI
511 -	EspI	1713 -	NruI	3142 -	Bsu36I	5032 -	AlwNI
511 -	Bpu1102I	1850 -	PflMI	3142 -	CvnI	**5989 -**	**ScaI**
620 -	NotI	**2150 -**	**XbaI**	**3142 -**	**SauI**	6108 -	XmnI
1195 -	DraIII	2647 -	AvrII	3390 -	Bsp1407I	6313 -	SspI

2.1.3 Non-cut enzymes

AflII	AgeI	AscI	AsuII	BssHII	ClaI
Csp45I	Eco47III	Eco72I	EcoNI	EcoRV	HpaI
I-PpoI	MfeI	MluI	NcoI	NheI	NsiI
PacI	PinAI	PmaCI	PmeI	RleAI	SciI
SfiI	SnaBI	SpeI	SrfI	StuI	SwaI
XhoI					

2.2 Primer sequences

Hygr forward (Hygr f) –	5’-CTGCGGAACGACCAGGAATT-‘3
(Hygr f reverse complementary) –	**3’-AATTCCTGGTCGTTCCGCAG-‘5**
Hygr reverse (Hygr r) –	**5’-CGGCTCATCACCAGGTAGGG-‘3**
*att*P reverse (*att*P r) –	5’-CAGAAGAGTCGCACAAGAGTT-‘3
(*att*P r reverse complementary) –	**3’-AACTCTTGTGCGACTCTTCTG’-5**

2.3 Restriction sites and primer locations on pIVH1 sequence

```
1           TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA
61          CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG
121         TTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGC
181         ACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCC
241         ATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTAT
301         TACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGT
```

KpnI

Acc65I
Asp718I

361 TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATTCGAGCTCG/GTAC/CCAAACAG
421 TCGAGATCCTTGCCGAGCTGGGATGGAAGCTCGGCCGACCACCCTGGAGGAGATGATCGA

Bpu1102I
EspI

481 GGATGCCAGGGCCTTTCACGCCCGCCGCTGC/TGAGCGTCCGCCGCCGGGCCCGCACCGCC
541 GTCGGCCGGCCCGCTCCGGGCTCGCAGCAGCGGGCTTCGGCGCGGGCCCGGGGCTCCCGA

NotI

601 GCGCGGGCGGGGCTCCGGGC/GGCCGCCGGGGGCCGGGGGCGGCGCCGGGCGGCCCGGGGC
661 GTCAGGCGCCGGGGGCGGTGTCCGGCGGCCCCCAGAGGAACTGCGCCAGTTCCTCCGGAT
721 CGGTGAAGCCGGAGAGATCCAGCGGGGTCTCCTCGAACACCTCGAAGTCGTGCAGGAAGG
781 TGAAGGCGAGCAGTTCGCGGGCGAAGTCCTCGGTCCGCTTCCACTGCGCCCCGTCGAGCA
841 GCGCGGCCAGGATCTCGCGGTCGCCCCGGAAGGCGTTGAGATGCAGTTGCACCAGGCTGT
901 AGCGGGAGTCTCCCGCATAGACGTCGGTGAAGTCGACGATCCCGGTGACCTCGGTCGCGG
961 CCAGGTCCACGAAGATGTTGGTCCCGTGCAGGTCGCCGTGGACGAACCGGGGTTCGCGGC
1021 CGGCCAGCAGCGTGTCCACGTCCGGCAGCCAGTCCTCCAGGCGGTCCAGCAGCCGGGGCG
1081 AGAGGTAGCCCCACCCGCGGTGGTCCTCGACGGTCGCCGCGCGGCGTTCCCGCAGCAGTT

DraIII

1141 CCGGGAAGACCTCGGAATGGGGGGTGAGCACGGTGTTCCCGGTCAGCGGCACCCT/GTGCA
1201 GCCGGCCGAGCACCCGGCCGAGTTCGCGGGCCAGGGCGAGCAGCGCGTTCCGGTCGGTCG

Hygr r

1261 TGCCGTCCATCGCGGACCGCCAGGTGGTGCCGGTCATC**CGGCTCATCACCAGGTAGGG**CC
1321 ACGGCCAGGCTCCGGTGCCGGGCCGCAGCTCGCCGCGGCCGAGGAGGCGGGGCACCGGCA
1381 CCGGGGCGTCCGCCAGGACCGCGTACGCCTCCGACTCCGACGCGAGGCTCTCCGGACCGC

BclI

1441 ACCAGTGCTCGCCGAACAGCTT/GATCACCGGGTCGGGCTCGCCGACCAGTACGGGGTTGG

SgrAI

1501 TGCTCTCGCCGGGCACCCGCAGCA/CCGGCGGCACCGGCAGCCCGAGCTCCTCCAGGGCTC

Hygr f

1561 GGCGGGCCAGCGGCTCCCAG**AATTCCTGGTCGTTCCGCAG**GCTCGCGTAGGAATCATCCG

1621 AATCAATACGGTCGAGAAGTAACAGGGATTCTTGTGTCACAGCGGACCTCTATTCACAGG

NruI

SpoI

1681 GTACGGGCCGGCTTAATTCCGCACGGCCGGTCG/CGACACGGCCTGTCCGCACCGCGGTCA

1741 GGCGTTGACGATGACGGGCTGGTCGGCCACGTCGGGGACGTTCTCGGTGGTGCTGCGGTC

PflMI

1801 GGGATCGCCAATCTCTACGGGCCGACCGAGGCGACGGTGTACGCCACCGC/CTGGTTCTGC

1861 GACGGCGAGGCGCCGCCAGGCCCCGCCGATCCCCGTCCCCCGCGTCGTCGAGCGCGGTGC

1921 CGACGACACCGCCGCGTGGCTCGTCACGGAGGCCGTCCCCGGCGTCGCGGCGGCCGAGGA

1981 GTGGCCCGAGCACCAGCGGTTCGCCGTGGTCGAGGCGATGGCGGAGCTGGCCCGCGCCCT

2041 CCACGAGCTGCCCGTGGAGGACTGCCCCTCCGACCGGCGCCTCGACGCGGCGGTCGCCGA

XbaI

2101 GGCCCGGCGGAACGTCGCCGAGGGCTTGGTGGACCTCGACGACCTCGACT/CTAGAGTCGA

2161 CCAAGACGATCACCGGGCTCGTCGGAGCTGGAGGCGCAGCGGGAGCTGGCTCTGTATTCT

2221 CAGGCAAGGCCGGAGGCCCTGGAGGAAACACCACGGCGTCCGCTGTCGGATGGTCAGGTT

2281 TGACCGCAACCGGCGGTCCCGGAGGCTCTGTGATCGACATCCTCAGCGTCGCCGGAAAGT

2341 CGCCTGGAGATCGGACCTACAACGACCAGCTCTACATAGGCGGCGCACAACAGAACTCAG

2401 CTGGCGGGAACGGCAATGCTCCTGGCGGCGGCGGGGCTGGTGCCCAGGTCTCCGCACAGA

2461 GCGGCGGTGCTGGCGCTCGCGGCCAGGCGTGGTTCTTCGCGTACTGACAAGAAACCCCCC

2521 TCTTTAGGACTCAGTGTCCTTGGGAGGGGGGCTTTTTGCGTTTCAGGAGGTCTTGGCCAG

2581 CTTGGACATCGCCTCAGCGATAGCCTCGTCGCGGGCCTCAGACGCCATCTGGTACTTCAT

AvrII

2641 CGCCATC/CTAGGAGTCGTGTGACCGAGACGGGCCATCAGCTCCTTGGTCGTCGCACCTGC

2701 CTGAGCGGCGAACGTAGCGCCGACAGCGCGGAGGTCGTGGATGCGGAGTTCCGGCCGACC

2761 GATCTTGGCGTAGCCACGCTTCAGCGACTTGGTGAACGCGGACTTCGACAGCCGGTTGCC

2821 CTGCGTCGTGGTCACCAGGAATGCCTCGGGGCCCTTGTTCATCTTCGTACGGTCCTTCAT

2881 GTGCGCTCGGATCATCTCCGCGACGTGAGGCGGAACCGTCACAGGACGCTTCGACCGGAC

2941 GGTCTTGGCGTTGCCAACGACGATCTTGTTCCCCACGCGGGAAGCGCCACGGCGCACCCG

3001 GAGCTTCATCGTCATGCCGTCGTCCACGATGTCCTTGCGGCGAAGCTCGATCAGCTCTCC

BglII

3061 GAACCGGAGGCTCGTCCACGCCAGGATGTATGCCGCGATCCGGTAGTGCTCGAA/GATCTC

SauI
CvnI
Bsu36I
Tth111I MstII

3121 AGCGGCGACG/ATGTCCAGCTCC/TCAGGCGTCAGCGCCTCTACGTCGCGCTCATCGGCTGC
3181 CTTCTGCTCGATCCGGCACGGGTTCTCTGCGATCAGCTTGTCCTCGACCGCTGTGTTCAT
3241 CACCGCCCGGAGGACGTTGTAGGCATGCCGGCGGGCAGTCGGGTGCTTCCTACCCATCCC
3301 GGCCCACCACGCACGCACCAGAGCTGGCGTCATCTCTGTGACCGCCACTTCACCTAGCAC
Bsp1407I
3361 CGGGTAGATGCGGCGCTCCGCGTGCCCGCT/GTACAGATCCCTGGTGCCGTCTGCGAGGTC
3421 GCGCTCCACGAGCCACTTCCGGGTGTACTCCTCCAGCGTGATGGCGCTGGCGGCTGCCTT
3481 CTTCGCCCGGTCCTGTGGAGGGGTCCAGGTCTCCATCTCGATGAGCCGCTTCTCGCCCGC
3541 GAGCCAGGCTTCGGCGTCCATCTTGTTGTCGTAGGTCTGCAGCGCGTAGTACCTCACACC

BamHI
3601 GTCCTGCGGGTTGACGTATGAGGCTTG/GATCCTCCCGCTGCGCTGAGTCTTCAGCGATCC
3661 CCATCCGCGACGTGCCAACTAGGTCTCCTCTCGTCGTGAACAAGGCTACCGGGTTGCAAC
3721 TCCTGTGCAACTCTCAGGCTTCAACGCGCTTCTACGACCTGCAATTTCTTTCCACTTAGA
3781 GGATGCAGCCGAGAGGGGGTAAAAACCTATCTTGACCGGCCCATATGTGGTCGGCAGACA
3841 CCCATTCTTCCAAACTAGCTACGCGGGTTCGATTCCCGTCGCCCGCTCCGCTGGTCAGAG

***att*P r**
3901 GGTGTTTTCGCCCTCTGGCCATTTTTCTTTCCAGGGGTCTGC**AACTCTTGTGCGACTCTT**
3961 **CTG**ACCTGGGCATACGCGGTTGCAACGCATCCCTGATCTGGCTACTTTCGATGCTGACAA
4021 ACGAATAGAGCCCCCCGCCTGCGCGAACAGACGAGGGGCATTCACACCAGATTGGAGCTG
4081 GTGCAGTGAAGAGAATAGACCGGGACAAGGTTGCACCGGGAGTTGCAGCGGTCGGAACCC
4141 TCGCCGTCGGCGGGCTGGCGTTCGCCCTGTCGTTCACGGCTCTCAGCGAGCTGGCTGCGG

HindIII
4201 CCAACGGGGTGGCCCAAGCAGAGATGGTGCCCTTGGTGGTCGACCTGCAGGCATGCA/AGC
4261 TTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCA
4321 CACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA
4381 CTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAG

SapI
4441 CTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC/
4501 GCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCT
4561 CACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATG
4621 TGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTC
4681 CATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGA
4741 AACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCT
4801 CCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTG
4861 GCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG

```
4921        CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTAT

                                                                  AlwNI
4981       CGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCA/CTGGTAAC
5041        AGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAAC
5101        TACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC
5161        GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTT
5221        TTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATC
5281        TTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATG
5341        AGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCA
5401        ATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCA
5461        CCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAG
5521        ATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC
5581        CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGC
5641        AGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCT
5701        AGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATC
5761        GTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGG
5821        CGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATC
5881        GTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAAT

                                                                  ScaI
5941       TCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGT/ACTCAACCAAG
6001        TCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGAT

                                                                 XmnI
6061       AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAA/CGTTCTTCGGGG
6121        CGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCA
6181        CCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGA
6241        AGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTC

                          SspI
6301       TTCCTTTTTCAAT/ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATA
6361        TTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTG
6421        CCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATC
6481                                                  ACGAGGCCCTTTCGTC
```

3. Beijing primer sequence and location

3.1 Primer sequence for Beijing strain identification

F29/780F – **5'-AGATCGGCTTTCTTCCCGC-3'**

Xho1 – 5'-TTCAACCATCGCCGCCTCTAC-3'

Xho1 (reverse complimentary) – **3'-GTAGAG GCGGCGATGG TTGAA-5'**

Beijing strain: F29/780F + xhoI = 62 + 141 = 203 bp

3.2 Restriction sites and primer locations on pIVH1 sequence

```
1     CCTTGCTGTCCCGCCAATACGGCGCCGACACAGAAACGTTTTACAGGAAGCCGAATGAGG
61    ACCTCGCCCATATCCGTCGGCTTTTCGGCGACACCGAGGAGTACATGACGGGCCGACTCG
121   TCTTCCGCGACACGAAGCTCACCAACAAAGACGACCTCGAAGCCCGCGGCGCTAAGACTC
181   TCACCGAGGTGAAATTCGAGAACGCCATCAACCGGGTGACCGCAAAGGCAAACCTTCGCC
241   AGATGGAACGCGTGATCCCCGGCAGCGGGTTCGCGTTCTCACTTGTCTACGAGGTCTCCT
301   TCGGCACCCCCGGCGAGGAACAGAAGGCGTCTCTGCCTTCCTCCGATGAGGTCATCGAGG
361   ACTTCAACGCCATCGCGCGCGGCCTGAAGTTGCTCGAACTCGACTACCTCGGCGGCAGCG
421   GAACCCGTGGCTACGGGCAGGTCAAGTTCAGCAACCTGAAAGCCCGCGCCGCAGTCGGCG
481   CCCTCGACGGTTCTCTGCTGGAGAAGCTAAACCATGAACTCGCGGCTGTTTAGGTTCGAC
541   TTCGACCGCACACACTTCGGCGACCACGGCCTCGAGTCGTCCACGATTAGCTGCCCCGCG
601   GACACCCTCTACTCTGCGCTTTGCGTTGAAGCGCTACGGATGGGTGGCCAGCAGCTGCTT
661   GGCGAACTCGTTGCGTGCTCGACGCTGCGGTTGACCGATCTGCTGCCCTATGTGGGGCCC
721   GATTACCTGGTTCCCAAGCCCCTGCACAGCGTTCGGTCCGACGGCTCAAGTATGCAGAAG

                    F29/780F
781   AAGCTGGCGAAGAAGATCGGCTTTCTTCCCGCTGCCCAGCTCGGCAGCTTCCTCGATGGC
841   ACGGCCGACCTGAA

                TGAACC GCCCCGGTGA GTCCGGAGAC TCTCTGATCT GAGACCTCAG
 CCGGCGGCTG GTCTCTGGCG TTGAGCGTAG TAGGCAGCCT CGAGTTCGAC CGGCGGGACG

                Xho1                                          IS6110
 TCGCCGCAGT ACTGGTAGAG GCGGCGATGG TTGAACCAGT CGACCCAGCG CGCGGTGGCC
 AACTCGACAT CCTCGATGGA CCGCCAGGGC TTGCCGGGTT TGATCAGCTC GGTCTTGTAT
```

APPENDIX B

SOLUTIONS AND REACTIONS

1. PCR Reagents

1.1 Tris-acetate-EDTA (TAE) buffer

0.04M Tris (Merck, South Africa)

0.2mM Na_2EDTA (Fluka, Netherlands)

0.02M NaOHAc (Merck, South Africa)

No need to sterilise, stored at room temperature

1.2 Agarose

1% agarose (Whitehead Scientific, South Africa)

1g agarose in 100ml TAE buffer

2% agarose

2g agarose in 100ml TAE buffer

1.3 HotStar Taq 1 X reaction mix (Qiagen, Southern Cross Biotechnology, Germany)

2.5µl 10 X PCR buffer (containing 15mM $MgCl_2$)

2µl 25mM $MgCl_2$

1µl 10mM dNTPs

0.5µl of each primer at a working concentration of 50pmol/µl

1µl 5 units/µl Taq polymerase

5µl Q solution

0.5µl template DNA

Nuclease-free H_2O up to a final volume of 25µl

1.4 HotStar Taq 1 X reaction mix with Syto9 (Invitrogen, UK) for melt analysis

2.5µl 10 X PCR buffer (containing 15mM $MgCl_2$)

2µl 25mM $MgCl_2$

1µl 10mM dNTPs

0.5µl of each primer at a working concentration of 50pmol/µl

1µl 5 units/µl Taq polymerase

5µl Q solution

1µl 0.5mM Syto® 9 working concentration

2µl template DNA

Nuclease-free H_2O up to a final volume of 25µl

2. Culture media

2.1 Middlebrook 7H9 liquid media (1L)

4.7g Middlebrook 7H9 powder (Becton Dickinson, Microbiology systems, USA)

0.5g Tween 80 (Merck, South Africa)

900ml distilled H_2O

Autoclave at 121ºC and cool before adding OADC

Add 100ml Middlebrook OADC (Becton Dickinson, Microbiology systems, USA)

after autoclaving and cooling to room temperature

2.2 Middlebrook 7H10 agar plates (1L)

19g Middlebrook 7H10 agar (Becton Dickinson, Microbiology systems, USA)

5ml glycerol (Merck, South Africa)

900 ml H_2O

Autoclave at 121ºC for 15 minutes and cool to 56ºC before adding OADC

Add 100ml Middlebrook OADC after autoclaving and cooling at 56ºC for 2 hours

2.3 Saline / 0.01% Tween

9g NaCl

0.1g Tween 80

1L H_2O

Autoclave to sterilise (121°C for 20 minutes)

3. Cell culture reagents

3.1 Complete RPMI medium (50ml)

45ml RPMI with GlutaMax™ (Invitrogen, UK)

5ml Heat-inactivated FBS (Invitrogen, UK)

50µl 50mg/ml Gentamicin (Roche, USA)

3.2 **PMA** (Phorbol Myristate Acetate)

50µg/ml PMA (Sigma, USA) diluted in absolute ethanol (Merck, South Africa)

123µl stock in 1ml complete RPMI medium

1µl in 1ml cell suspension

3.3.1 0.25% SDS

25g SDS (Merck, South Africa) powder in 1L distilled H_2O

No need to sterilise SDS, autoclaving will lead to precipitation and it cannot be filter sterilised

3.3.2 0.025% SDS

100ml 0.25% SDS in 1L H_2O

4. DNA extraction buffers

4.1 PK (proteinase K) buffer 10X pH7.8 (500ml)

6.05g Tris

9.3g EDTA

25g SDS

4.2 Phenol Chloroform Iso-amyl alcohol

24ml Chloroform (Merck, South Africa)

24ml Phenol (Merck, South Africa)

1ml Iso amyl alcohol (Merck, South Africa)

4.3 3M NaAc, pH 5.5 (100ml)

40.82g NaAc-3H_2O (Merck, South Africa)

Adjust pH with Glacial Acetic Acid (Merck, South Africa)

5. DNA extraction

5.1 Phenol extraction procedure

Add 100µl Proteinase K (Roche, USA) and 100µl PK buffer to 1ml crude denatured DNA sample

Incubate at 42ºC for at least 2 hours

Add 500µl Phenol Chloroform Iso amyl alcohol and centrifuge at high speed (1400rpm) for 5 minutes

Transfer the supernatant / top layer to a new tube

Add 500µl Chloroform and centrifuge at high speed (14000 rpm) for 5 minutes

Transfer the supernatant / top layer to another new tube

Add 100µl 3M NaAc and 1ml Isoproponol (Popan-2-ol) (Merck, South Africa)

Store at –20°C

5.2 Ethanol precipitation

Centrifuge frozen DNA aliquots at high speed (14000 rpm) for 30 minutes in cold room

Add 1ml 70% ethanol and centrifuge at high speed (14000 rpm) for 30 minutes in cold room

Remove supernatant and leave overnight to air dry

6. Ziehl-Neelsen staining

6.1 0.3% Ziehl-Carbol Fuchsin (200ml) (prepared according to standard WHO/UATLD method)

1g Basic Fuchsin (Sigma, USA)

10ml Absolute ethanol

5g Phenol

Up to 200ml distilled H_2O

6.2 3% Acid alcohol solution (100ml) (prepared according to the standard WHO/UATLD method)

3 HCl (Merck, South Africa)

Diluted in 100ml 95% absolute ethanol

6.3 Staining method:

Stain slide with Ziehl-Carbol Fuchsin for 7- 10 minutes, flaming intermittently until steam comes off

Rinse of with distilled H_2O.

Decolourise with 3% acid-alcohol for 2 minutes.

Rinse off with distilled H_2O

Counter stain with 3% Methylene Blue (NHLS, South Africa) for 5 minute.

Rinse with distilled H_2O

Air dry and view under microscope with oil-immersion lens

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