Investigation of the role of gene deletions in the virulence of an outbreak strain of *Mycobacterium tuberculosis*

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Abstract

In 2001, a large tuberculosis outbreak took place at Crown Hills School, Leicester, UK. This outbreak had unusual features including a high number of infected individuals with rapid development of symptoms. Genotypic investigation of the causative strain, *Mycobacterium tuberculosis* CH, identified unique deletions of five loci compared with the reference strain *M. tuberculosis* H37Rv genome. Among the five deleted loci, a locus involving two adjacent genes named *rv1995* and *rv1996* was found. There was an evolutionary clue that these two genes were the last deletions within the genome of CH. Phenotypically, CH grew less rapidly, was less resistant to low pH and H_2O_2, but stimulated more production of IL-10 and IL-6 from monocyte derived macrophages than H37Rv. The aim of this project was to determine the contribution of the deletion of *rv1995* and universal stress protein encoding gene, *rv1996* to the phenotype and hypervirulence of the CH strain.

The present study involved construction of shuttle expression vectors for *rv1995* and *rv1996*. Both genes were amplified from the H37Rv genome, ligated successfully with the digested pSMT3 plasmid, and then cloned giving two expression vectors, pAAO1 and pAAO3 for *rv1995* and *rv1996* respectively. Both vectors were sent to Imperial College and inserted into CH to enable expression of *rv1995* and *rv1996,* and investigated. Thereafter, knocking out of the target genes from candidate strains and phenotypic characterisation were planned. Suicide vectors for *rv1995* and *rv1996* were developed using insertional mutagenesis strategy and named as pANO1 and pANO2, respectively. Using PCR and bioinformatic analysis, it was shown that *rv1995* and *rv1996* are present in *M. bovis* BCG and absent from *M. smegmatis*. Thus, the constructed suicide vectors were transformed into the BCG and H37Rv strains to knock out the sequences of target genes from their genomes by homologous recombination. H37Rv recombinants were stored, whereas BCG recombinants were used as a model for investigation. The mutant successfully created was for *rv1996* and named as *M. bovis* BCG *rv1996*::hyg^R. Phenotypic *in vitro* experiments showed that there was no significant difference between the growth rates of the wildtype BCG and its mutant. Both strains showed similar levels of resistance to various concentrations of $NaNO_2$ and to H_2O_2. However, the mutant strain showed less acid resistance than the BCG wildtype strain.

It was expected that deletion of *rv1996* from CH would be the one responsible for microbiological attenuation of CH compared to H37Rv based on the prediction of the universal stress protein function in other bacteria. However, it can be concluded from this work that *rv1996* had a minor or no role on exposure of *M. tuberculosis* to H_2O_2 and NO stress under the experimental conditions followed in this work. Only a difference on exposure to acid stress was found on disruption of the gene in BCG. This was compatible with the results provided from the Imperial College team on complementation of CH by pAAO3 carrying *rv1996*, whereas complementation by pAAO1 carrying *rv1995* induced no change in any of the investigated phenotypic characters. This is the first study to implicate *rv1996* in a role on exposure of mycobacteria to acid stress. It could be also hypothesised that deletion of *rv1996* as a *dosR* regulated gene might be associated with failure of latency development, therefore, increasing the likelihood of active disease and propensity of CH to cause the outbreak. Lastly, the substantial role of *rv1995/96* locus deletion as a phylogenetic marker of the CH strain is evident. More work is needed to confirm these findings and to identify other factors that might be related to the CH phenotype.

Publications

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Dedication

For

My Older Brother

Mohammed

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Statement of Originality

The accompanying thesis submitted for the degree of Ph.D. entitled ‘‘An investigation of the role of *rv1995* and *rv1996* gene deletions on virulence of an outbreak strain of *Mycobacterium tuberculosis* CH’’ is based on work conducted by the author in the Department of Infection, Immunity and Inflammation during the period between September 2002 and May 2009 and in the Department of Genetics during the period between April 2015 and October 2017 of the University of Leicester.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed ---------- Date ----------

Abbreviations

ADC	Albumin-dextrose-catalase
AIDS	Acquired immune deficiency syndromes
Amp	Ampicillin
ASP	Acid shock proteins
ATCC	American Type Culture Collection
ATR	Acid tolerance response
BCG	Bacille Calmette-Guerin
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
BSA	Bovine serum albumin
CAS	Central Asian Strain
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonuclic Acid
CFP-10	Culture filtrate protein-10
CFU	Colony forming units
CFP-10	Culture filtrate protein-10
CH	Crown Hills
Ct	Cycle threshold
dATP	Deoxy adenosine tri phosphate
DCO	Double cross over
dCTP	Deoxy cytocine tri phosphate
dGTP	Deoxy guanosine tri phosphate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxy nucleotides tri phosphate
dosR	Dormancy survival regulator
dTTP	Deoxy thymedine tri tphosphate
EDTA	Ethylene diamine tetraacedic acid
ELISA	Enzyme linked immunosorbent assay

ELISPOT	Enzyme-linked immunospot
EMB	Ethambutol
ESAT-6	Early secretly antigen target-6
FAM	5-carboxyfluorescein
FDA	Food and Drug Administration
g	Gramme
GLIP	Genome level-informed PCR
GSE	Guanidium chloride sarkosyl EDTA
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HPA	Health Protection Agency
HR	homologous recombination
hr	hour
Hyg	Hygromycin
IFNγ	Interferon gamma
IL	Interlukin
INH	Isoniazid
IPTG	Isoprpyl-β-D-thiogalactosylpyranoside
IR	Illegitimate recombination
IS	Insertion sequence
Kan	Kanamycin
KatG	Catalase: peroxidase
Kb	Kilo base
LA	Luria Bertani agar
LB	Luria-Bertani broth
LJ	Lowenstein-Jensen medium
LSP	Large sequence polymorphisms
MAPC	Mycolic acids (MA), arabinogalactan (AG) and peptidoglycan (PG) complex
MCS	Multi cloning site
MDR-TB	Multiple drug resistance to tuberculosis

MDM	Monocyte-derived macrophage
µg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
µl	Microlitre
mM	Millimole
min	Minute
mRNA	Messenger ribonucleic acid
MTB	*Mycobacterium tuberculosis*
MTBC	Mtb complex
NCTC	National Collection of Type Cultures
ng	Nano gram
NHS	National Health Service
OADC	Albumin-oleic acid-dextrose-catalase
OD	Optical density
ORFs	Open reading frames
oriE	Origin of replication for *E. coli*
oriM	Origin of replication for *M. tuberculosis*
PCR	Polymerase chain reaction
PGL	Phenolic glycolipid
PhaB	Phage amplified biologically assay
pmol	Picomole
PPD	Purified protein derivative
PZA	Pyrazinamide
qRT-PCR	Quantitative real time PCR
RD	Region of difference
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RNAse	Ribonuclease
RNA-Seq	RNA Sequencing
RNI	Reactive nitrogen intermediate

ROI	Reactive oxygen intermediate
rpm	Revolution per minute
RT-PCR	Reverse transcriptase PCR
SCID	Sever compound immune deficiency
SCO	Single cross over
SDS	Sodium dodecyl sulphate
Sec	Second
SNP	Single-nucleotide polymorphism
STR	Streptomycin
Ta	Annealing temperature
TAE	Tris-acitate EDTA
TAMRA	N, N, N, N'-tetramethyl-6-carborhodamine
Taq	*Thermus aquaticus*
TB	Tuberculosis
TbD1	Mtb-specific deletion 1
TE	Tris-EDTA
Tm	Melting temperature
TNF	Tumour necrosis factor
TST	Tuberculin skin testing
USP	Universal stress protein
UV	Ultraviolet
V	Volts
v/v	Volume to volume
WGS	Whole genome sequence
WHO	World health organisation
w/v	Weight to volume
XDR-TB	Extensively drug resistant to tuberculosis
X-Gal	5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside
ZN	Ziehl–Neelsen acid-fast stain

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CHAPTER 1:

INTRODUCTION

1.1. Worldwide pandemic of tuberculosis.

Over a century ago, in 1882, Robert Koch, a German scientist, identified the tuberculosis bacillus (Koch's bacillus) as the causative agent of tuberculosis, when he was working to prove the germ theory of infectious disease (Koch, 1882; Sakula, 1982; Cambau and Drancourt, 2014).

Tuberculosis is a well-known infectious disease caused chiefly by *Mycobacterium tuberculosis*, mainly transmitted by inhalation of cough-propelled droplets. *M. tuberculosis* is an intracellular pathogen that can survive and multiply within host phagocytes. The disease is one of the oldest recorded human diseases which exists worldwide with an enormous impact on human morbidity and mortality (Madigan *et al*., 2003; Cano *et al*., 1988; Prescott *et al*., 2002; Smith, 2003; WHO Global Report, 2015; Mohajan, 2015). In all its forms (Section 1.7), the disease has always been found in the developing world, however along with the era of human immunodeficiency virus, HIV (Section 1.1.4) it involved the industrialized world as well (WHO Global Report, 2010; Glaziou *et al*., 2015; Al-Hajoj and Varghese, 2015).

In 1990, the global tuberculosis incidence rate was 143 cases per 100,000 (Dolin *et al*., 1994) and decreased slowly between 1999 and 2007 to reach 139 cases per 100,000 population in 2008 (WHO Global Report, 2009). WHO has set a goal to reduce the prevalence and mortality rates of tuberculosis by 2015 to be half their level in 1990 (WHO Global Report, 2009). It seems that achievement of this goal is out of reach as the global level recently reported in 2014 estimated 133 cases per 100,000 population (WHO Global Report, 2015; Glaziou *et al*., 2015; WHO, Global Plan to Stop TB 2011-2015).

1.1.1. Historical points of pandemic of tuberculosis.

Until the 1700s, tuberculosis cases were sporadic, but later, it became epidemic because of the increase of population density, the industrial revolution, and unfavourable living conditions in addition to human movement. Tuberculosis had become an endemic disease by the end of the 19th century (Godreuil *et al*., 2007; Smith *et al*., 2003). In the 20th

century, the incidence of this disease decreased as a result of improving living standards, sanitation, introduction of BCG vaccine in 1921 (Section 1.5), and eventually the use of anti-TB agents (Section 1.3). However, despite these developments, the prevalence of tuberculosis began to increase again by the start of the 1990s, because of the high incidence of HIV (Section 1.1.4) and the emergence of multi-drug-resistant strains (Section 1.4). Other factors were lower living standards in many poor parts of the world like Sub-Saharan Africa and South East Asia, especially the Sub-Indian continent. Immigration of populations from high tuberculosis prevalence regions to low prevalence countries is also an important factor for the prevalence of tuberculosis (Gleson *et al*., 2012; Borgdorff *et al*., 2010; Dasgupta *et al*., 2005; Al-Hajoj, 2010; WHO Global Report, 2010; WHO Global Report, 2015; WHO Global Plan to Stop TB 2011-2015). Consequently, tuberculosis at the end of the 20th century has again become one of the most significant causes of death (Kochi, 1994; Dye *et al*., 1999; Raviglione *et al*., 1995; Godreuil *et al*., 2007; WHO Global Report, 2015; WHO Global Plan to Stop TB 2011-2015).

In 1990, there were an estimated 7.5 million cases of tuberculosis and 2.5 million deaths worldwide (Raviglione *et al*., 1995). The highest prevalence of tuberculosis infection and estimated annual risk of the disease were in sub-Saharan Africa and South East Asia. However, tuberculosis has also failed to decline in many industrialized countries, and in Eastern European countries and the former Soviet Union, cases and deaths were increasing (Raviglione *et al*., 1995). In 1993, one third of the world's population was estimated to be infected with *M. tuberculosis* and more than 5 million of these were dually infected with HIV and *M. tuberculosis* (Kochi, 1994). A similar report issued by WHO estimated that about 2 billion of world population, carry TB bacilli, but around 10% of population develop the disease in their lifetime (WHO TB/HIV Policy, 2004).

In 1999, there was an estimated increase of new TB cases from 8.0 million in 1997 to 8.4 million (WHO Global Report, 2001). In 2007, the WHO Global report estimated that there were 9.27 million worldwide incident cases of TB (WHO Global Report, 2009). Most of the estimated cases were in Asia (55%), especially the sub-Indian continent (India only has 2.0 million cases), and Africa (31%), especially in sub-Saharan Africa, with a small proportion of cases reported in the Eastern Mediterranean Region, the European region and

the region of the Americas with percentages of 6%, 5% and 3% respectively. In 2007, out of the 9.27 million TB cases, an estimated 1.37 million were HIV-positive and 79% of these HIV-positive cases were in Africa, in particular Saharan African countries that have a high incidence of HIV, and 11% were in South-East Asia (WHO Global Report, 2009).

A steady slow decrease in the incidence per /100,000 population of notified TB cases was observed in the period from 2005 to 2014 (Figure 1.1) (WHO Global Report, 2015). Despite a decrease in global TB incidence and mortality, however, tuberculosis was responsible for 1.3 million deaths in 2012 (WHO Global Report, 2013).

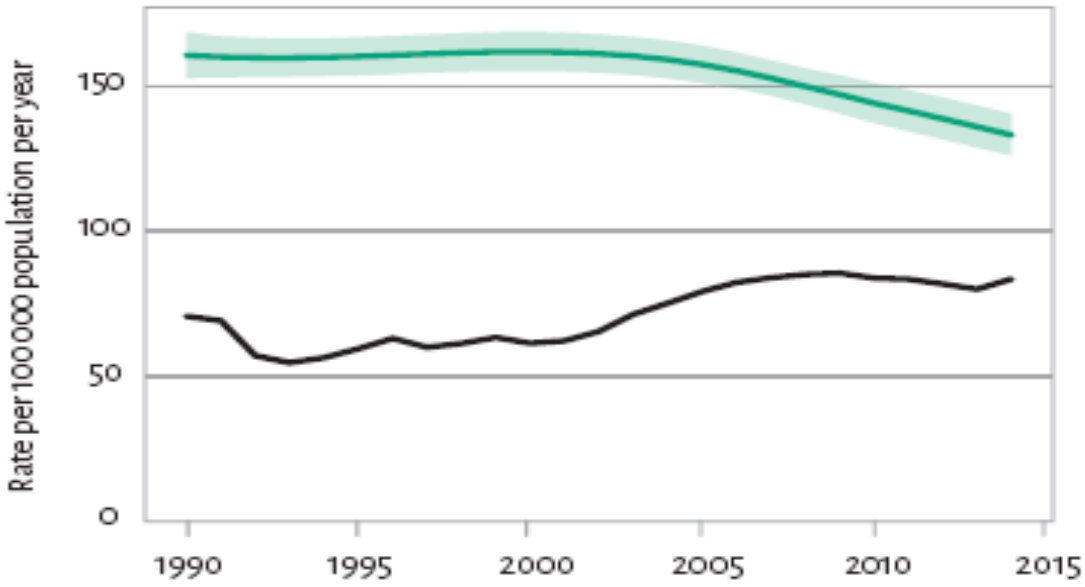


Figure 1.1: Absolute number of notified worldwide TB cases in (Black) and TB incidence in (Green) in the period from 1990-2014 (WHO Global Report, 2015).

As indicated in the WHO Global Tuberculosis Report 2015 (WHO, 2015), the incidence rate varies extensively among countries worldwide (Figure 1.2). The lowest rates were found mainly in high-income countries including Western Europe, Canada, USA, Australia and New Zealand. In these countries, the incidence rate was less than 10 cases per 100 000 population per year. The highest rates were found in poor countries including most sub-Saharan African and South-East Asian regions with an incidence rate more than

125 cases per 100 000 population per year. Most countries in the region of Latin America have rates below 50/100,000 population per year (WHO Global Report, 2015). The data obtained by WHO Global Report further indicated that tuberculosis is a socio-economic pandemic disease.

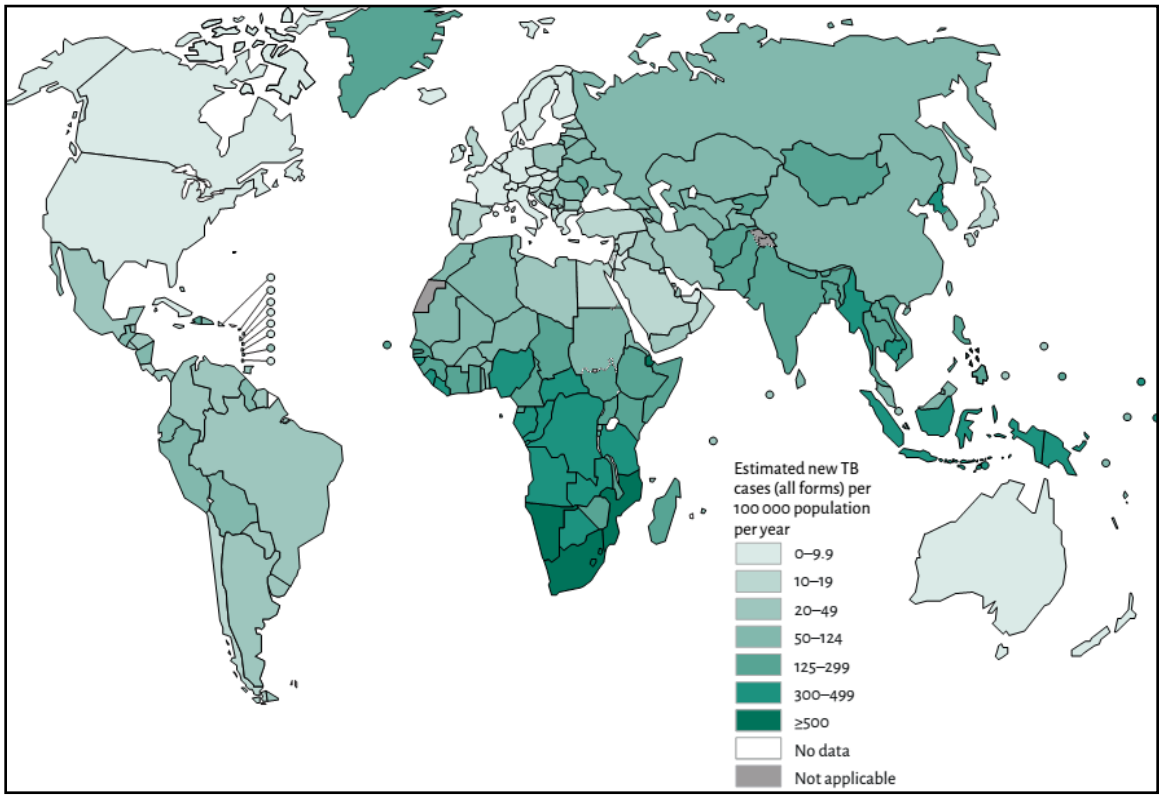


Figure 1.2: Estimated tuberculosis incidence rates, 2014 (WHO, Global Tuberculosis Report, 2015).

1.1.2. Tuberculosis in the United Kingdom.

In European countries, tuberculosis was the main attributable cause of death in the 18-19th centuries (Cole, 2002). The mortality was between 200 and 300 per 100000 at the beginning of the 19th century. In the late 19th and early 20th centuries, as a result of improvements in the standards of living in many Western Europe countries the incidence of tuberculosis markedly declined (Kato-Maeda *et al*., 2001; Cole, 2002; Euro tuberculosis, 2005; WHO Global Report, 2007). However, studies indicate that there is a wide variation

between the western and eastern European countries. For example, in 2004, the notified cases were less than 13/100000 population in Ireland and the UK compared to more than 100/100000 in Romania and the Russian Federation and a rate of 216/100000 in Kazakhstan (Walls and Shingadia, 2007). The overall European region contribution of world TB cases is only 5%. In Western Europe, although tuberculosis still causes disease among the old native -born population, high-risk groups such as HIV-infected patients, prisoners, immigrants and drug addicts notably contribute to the overall burden (Carvalho *et al*., 2010).

In the United Kingdom, the incidence of tuberculosis decreased steadily during the 20th century until the 1980s. In 1950, about 50,000 cases were reported every year in England and Wales, declining to around 5,000 to 6,000 cases in the mid 1980s. Since that time there has been a significant increase in the tuberculosis cases to 7000 cases reported each year (Ormerod *et al*., 1998; Rose *et al*., 2001; British Thoracic Society, 2000; Walls and Shingadia, 2007). In the UK, a total of 6,864 tuberculosis cases were reported in 2001, representing a rate of 11.6 per 100,000 of the population (HPA report, 2012). Tuberculosis continued to increase again in the UK regions to reach a total of 8,917 tuberculosis cases as reported in 2009, a rate of 14.4 per 100,000 (HPA, 2012). In 2013, a total of 7,892 cases were notified with a rate of 12.3 cases per 100,000 of population. The rate of tuberculosis in the UK has remained fairly stable over the past ten years (PHE report, 2014).

Geographically, most tuberculosis cases take place in big cities, predominantly in London, which accounted for 39% of total cases reported in 2007 with a rate of 43.2 per 100,000 (HPA report, 2008). According to the PHE reports (2014), London still accounts for the highest proportion of cases in the UK (37.8%), with a rate of 35.5 cases per 100,000. The West Midlands PHE Centre area comes after with a rate of 17.3 per 100,000 which accounts for 12.4% of the total cases reported in the UK (PHE report, 2014). Leicester has one of the highest tuberculosis rates in the UK. It had a tuberculosis rate that was four times the national average even before the Crown Hills Community College outbreak in 2001 (Watson and Moss, 2001). In 2012, Leicester had the uppermost incidence rate of tuberculosis in the East Midlands with a rate of 56 cases per 100,000, compared with the incidence rate in Leicestershire County and Rutland that was 5/100,000 (TB strategy

Board, 2013). The major burden of tuberculosis is still intense in large urban areas which pose a considerable risk of local outbreaks and spread TB among populations (PHE report, 2014; TB strategy Board, 2013; Crofts *et al*., 2008).

Ethnically, in the UK, the tuberculosis rate was highest among the Black African ethnic group with a rate of 309 per 100,000 followed by Indian/Bangladeshi/Pakistani ethnic groups (212 per 100,000). The rate among persons in the white ethnic group is 3 per 100,000 (HPA report, 2008). Recent reports in 2014 showed that the highest rates per /100,000 population were in the Indian, Black-African and Pakistani ethnic groups (132, 123 and 114 per 100,000 respectively) (PHE report, 2014).

Regarding country of birth, the tuberculosis rates remain very powerfully linked to place of birth and period of residence in the UK. It has been found that children born in the UK have very low rates of TB infection, whereas immigrant children born in countries with high incidence of tuberculosis (less than five years) and recently arrived in the UK have the highest rates (Walls and Shingadia, 2007; Walls and Shingadia, 2004; Rose *et al*., 2002). Among non-UK born, there was a decline in the prevalence from a peak of 98 per 100,000 in 2005 to 70 per 100,000 in 2013. The rate of tuberculosis among the non-UK born population was 18 times greater than the rate in the UK born (PHE report, 2014).

1.1.2.1. Tuberculosis outbreaks in the UK.

Outbreaks of tuberculosis in the UK are uncommon with one about every two years. Surveys indicate that it is unusual for there to be more than a dozen cases reported in these outbreaks (Davies, 2001). There are three places which are the most common settings; healthcare establishments, educational establishments and prisons (Ruddy *et al*., 2004; Rajakumar *et al*., 2004; Anderson *et al*., 2010). For example, in a London teaching hospital, an outbreak of MDR-TB was reported when a MDR-TB patient who is HIV-negative was admitted to an isolation room with HIV-positive patients. Seven HIV-positive contacts developed MDR-TB (Breathnach *et al*., 1998). Investigators suggested that patients suspected or diagnosed as TB-positive should not be admitted in the same sections with immuno-compromised patients, and they must be isolated in negative-pressure rooms,

particularly if they are proven to have MDR-TB (Breathnach *et al*., 1998). Studies indicated that healthcare workers were at increased risk of disease (Sepkowitz, 1994). For example, in a survey of healthcare personnel in England and Wales held in the period from 1988 to 1993, one hundred nineteen cases of TB were identified, including 42 doctors and 61 nurses (Meredith *et al*., 1996). Also, in another survey done in 2005, at least 105 incidents of tuberculosis in hospital-based healthcare workers were reported (Anderson *et al*., 2007). The risk may be decreased through utilization of respiratory isolation rooms, environmental precautions, and immediate management of all suspected TB cases, however the risk of infection is certainly not reducible to zero (Sepkowitz, 1994).

Prisons and other detention setting populations are particularly vulnerable to tuberculosis infection. TB forms that affect the lungs or larynx can be transmitted to other prisoners and exceptionally to staff (PHE report, 2013). The rate of TB among prisoners is found to be much higher than UK average rates. For example, a study done in London reported that TB rates in the prison population are 208 per 100,000 (Storey *et al*., 2007). The socio-epidemiologic and clinical data of 205 TB infected prisoner patients in the UK between 2004 and 2007 showed that, in contrast to non-prisoners, those who are infected in the prison were more likely to be UK-born (47 vs. 25%), to have the pulmonary form of disease (75 vs 56%) and to be white (33 vs. 22%), than other tuberculosis patients (Anderson *et al*., 2010).

1.1.2.1.1. Tuberculosis outbreaks in UK schools.

Outbreaks of tuberculosis in UK schools are infrequent. Few incidents of outbreaks were reported in the last 2 decades. The most recent tuberculosis outbreak in the UK took place in summer 2015. Almost 200 children were infected with tuberculosis after an outbreak at a school in Devon. 1,400 pupils and staff were tested during the summer term in 2015 and 200 of those tested showed a positive TB screening result. Fortunately, only 10 of the 200 pupils infected were diagnosed and treated for active TB, with the remaining individuals treated for a latent TB infection. It was believed that the infection was brought in by a pupil from overseas, who was ill for several months (British Broadcasting Corporation, report 2015; The Guardian, report 2015).

Another outbreak was in Birmingham at a Girls' School in the summer of 2008. All 200 pupils were tested after a pupil and one of their relatives had tested positive for tuberculosis. Investigations by health officials found that 30 pupils tested positive for the disease and they were treated with anti-TB drugs (British Broadcasting Corporation, report 2008; The Guardian, report 2008; Birmingham Post, report 2013).

In 2001, the largest tuberculosis outbreak ever in the United Kingdom was reported in Leicester city. This outbreak occurred in a school called Crown Hills Community College (Ewer *et al*., 2003; Shafi *et al*., 2002; Rajakumar *et al*., 2004). The outbreak was attributed to a single strain, named *Mycobacterium tuberculosis* CH, which was transmitted extensively among the students (section 1.9).

1.1.3. Tuberculosis in Saudi Arabia as a country with distinctive circumstances.

Saudi Arabia has a unique population dynamic, since more than two million pilgrims visit the country annually for the Hajj pilgrimage, in addition, more than six million expatriates (non-Saudi immigrant workers) living in the country come from endemic areas with high tuberculosis prevalence (Memish *et al*., 2003; Al-Hajoj *et al*., 2010). Tuberculosis still represents an important health problem although, living standards have been improved, anti-tuberculosis medications are freely donated and the mass BCG vaccination is implemented (Al-Kassimi *et al*., 1993; Al-Hajjaj, 2000). Different studies estimated variable incidence of TB among the Saudi population. The overall changes showed a decline in the incidence from 44/100000 in 1990 to about 14/100,000 in 2011. This proportion appeared to correlate with the course for TB elimination by 2050 set by the WHO (Abouzeid *et al*., 2012; Memish *et al*., 2014).

Some studies estimated higher incidence when Non Saudi residents were included. In a comparison with Saudis, non-Saudis had about a 2-fold higher TB incidence rate (Gleason *et al*., 2012; Memish *et al*., 2014; Abouzeid *et al*., 2012). Furthermore, latent tuberculosis infection (LTBI) (Section 1.7.2) is more common among immigrants from

high tuberculosis prevalence regions, and they have a greater risk of disease reactivation (Dasgupta and Menzies, 2005; Borgdorff *et al*., 2010; Al-Jahdali *et al*., 2010). It seems that Saudi Arabia has a TB profile similar to that in developed countries, however immigration and human population movements, HIV and drug resistance are reasons that facilitate the spread of tuberculosis in the country (Al-Bishri *et al*., 2014; Al-Hajoj *et al*., 2010; Al-Hajoj *et al*., 2013).

1.1.3.1. Tuberculosis in Hajj seasons in Saudi Arabia.

Saudi Arabia hosts the principal annual Islamic mass gathering in the world (the Hajj). Every year, millions of Muslims come into Saudi Arabia for pilgrimage. Mass gatherings pose extraordinary opportunities for dissemination of infectious diseases (Ahmed *et al*., 2006, Memish, 2002, Shafi *et al*., 2012). Several reports have indicated that the international travel, migration and severe overcrowding during pilgrimage leads to a great risk of transmission of airborne infectious diseases including TB (Memish and Ahmed, 2002; Wilder-Smith, *et al*., 2003; Al-Hajoj *et al*., 2013; Gleason, *et al*, 2012). There are several documented cases of tuberculosis having been acquired during air travel with one index case passenger on board. Fortunately, the risk of such transmission remains low (Ormerod, 2000; Memish and Ahmed, 2002; Al-Jahdali *et al*., 2003; Al-Hajoj *et al*., 2013). Tuberculosis transmission is enhanced by crowding (Al-Jahdali *et al*., 2003, Shafi *et al*., 2012) and is the most common reason for pneumonia hospitalization during Hajj (Alzeer *et al*., 1998, Alzeer, 2009). Furthermore, pilgrims coming to Hajj have been shown to have increased TB skin test conversion, upon their return (Wilder-Smith *et al*., 2005). In a previous study of community-acquired respiratory diseases during the 1994 Hajj season, bacteriological diagnosis confirmed that *M. tuberculosis* was the most common pathogen, since it was identified in 13 of 46 (20%) patients (Alzeer *et al*., 1998). Other studies reported that more than 50% of the pilgrims complain of cough (Wilder-Smith and Memish 2003; Wilder-Smith *et al*, 2005). Hence, transmission of *M. tuberculosis* infection is potentially likely to occur during the pilgrimage (Wilder-Smith, *et al*., 2003; Ahmed *et al*., 2006; Shafi *et al*., 2012).

Saudi Arabia has already established the national tuberculosis control programme (NTCP) since 1992 (Al-Hajjaj *et al*., 1996; Al-Hajjaj *et al*., 2000). Recommendation has been made to allow only visitors with a pre-visa chest x-ray for Hajj. Visitors with suspicious infection are not prevented from travelling to the Hajj, but they have to start treatment before arrival (Alrajhi *et al*., 2002). Screening of foreign workers for tuberculosis using the Mantoux test and chest x-ray in Saudi local hospitals and medical centres as soon as they arrive in the country is required. This is especially important for those who come from areas endemic for tuberculosis and drug-resistant *M. tuberculosis*, for example, the Indian sub-continent (Alrajhi *et al*., 2002; Gleason, *et al*, 2012). Recently, a nationalization policy with the WHO is in place with the aim of decreasing dependency on non-Saudi workers and increasing employment opportunities for nationals (WHO and Saudi Arabia strategy 2012-2016). Also, Saudi Arabia established the first guidelines for testing and identification of individuals with LTBI as a key constituent of TB control programmes. Treatment of those LTBI patients can reduce the risk of developing active tuberculosis in most of treated patients (Al Jahdali *et al*., 2010).

1.1.4. HIV/AIDS and TB co-infection.

Since the discovery of the HIV in 1981, the prevalence of tuberculosis and HIV infection has risen (Mohanty and Basheer, 1995). One of the most important reasons leading to death in TB patients is its concurrent infection with HIV (Zumla *et al*., 2000; Volpe *et al*., 2006; WHO TB/HIV Policy, 2004).

The HIV/AIDS pandemic has a major effect on the global epidemiology of tuberculosis, particularly in developing countries (Zumla *et al*., 2000). TB accelerates the course of HIV infection leading to the appearance of the acquired immune deficiency syndrome (AIDS) and HIV infection promotes the development of TB infection to active disease (Hopewell and Chaisson, 2000; Toossi *et al*., 2001; WHO TB/HIV Policy, 2004). TB has become the major attributable cause of death in HIV-infected people (Toossi *et al*., 2001; WHO TB/HIV Policy, 2004). According to WHO, it has been estimated that one third of forty million individuals with HIV worldwide are co-infected with MTB. HIV

positive individuals are about 50 times more susceptible to development of active TB than HIV-negative individuals (WHO TB/HIV Policy, 2004). Furthermore, approximately 90% of patients with HIV die within months of getting infected with TB if not properly treated (Kawai *et al*., 2006; WHO TB/HIV Policy, 2004). Most of patients who are co-infected with both diseases are living in sub-Saharan African countries where HIV infection rates are high and access to treatment is difficult. Additionally, anti-TB drugs are ineffective in many cases, due to increasing drug resistance.

In 2004, it has been estimated that about 8 million Africans were co-infected by HIV and *M. tuberculosis* (WHO TB/HIV Policy, 2004; Toossi *et al*., 2001). In South East Asia, the association between these two pathogens has also been reported (Toossi *et al*., 2001). More than 60,000 adults in South and South East Asia were estimated to have co-infection with HIV and MTB. The rapid spread of HIV in India and Thailand suggested that the TB/HIV epidemic in parts of South East Asia may reach the level of the TB-HIV epidemic in sub-Saharan Africa (Mohanty and Basheer, 1995). For example, in an Indian study, it was found that 55% of HIV positive patients are infected with *M. tuberculosis* compared to 25% of HIV negative patients (Devies *et al*., 2005). In the United Kingdom, the co-infection rate was estimated as 3.7% (Delpech *et al*., 2004). In the United States, it was reported that 26% of TB cases are attributed to co-infection with HIV (Corbett *et al*., 2003).

Overall, the co-infection rates of tuberculosis with HIV were highest in countries in the african region, with a percentage of 32% of TB cases have also HIV. This region accounted for 74% of tuberculosis cases among people living with HIV worldwide (Figure 1.3) (WHO Global Report, 2015). In Uganda, for example, it is still counted among 22 countries having the highest TB burden worldwide. An estimated tuberculosis /HIV co-infection rate of 54% was reported in the 2010 tuberculosis cohort (WHO Global Report, 2011).

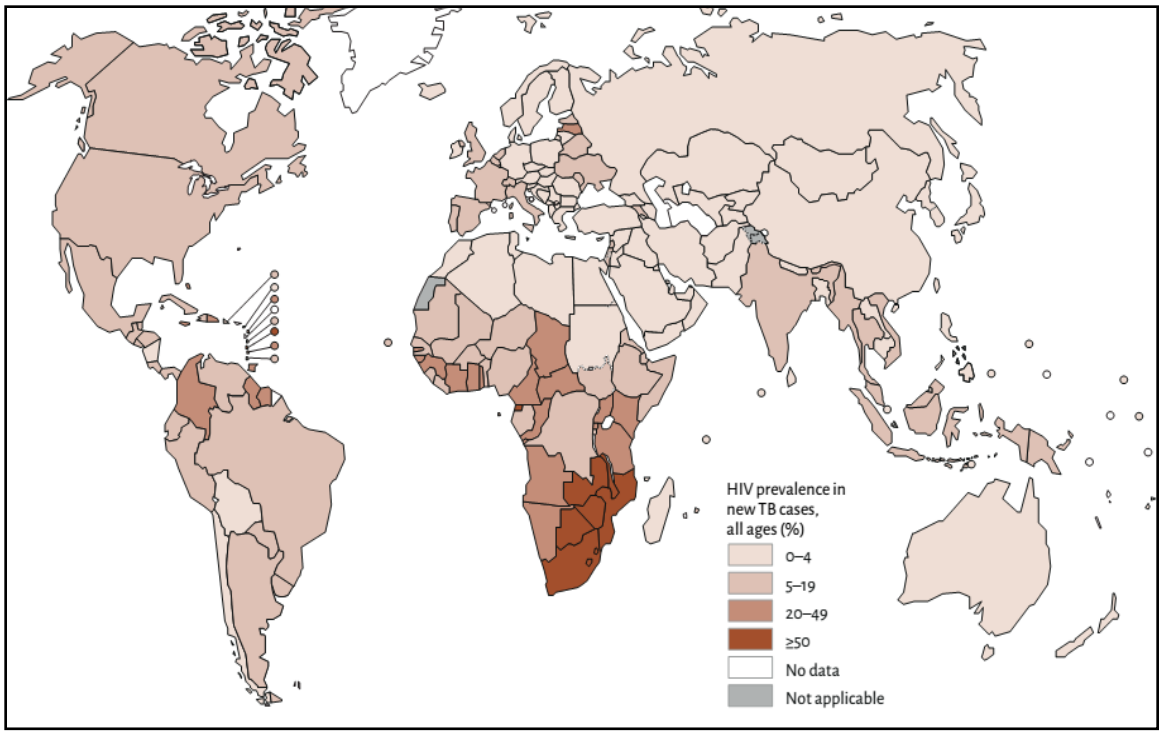


Figure 1.3: HIV prevalence in new tuberculosis cases, 2014 (WHO, Global Tuberculosis Report, 2015).

1.2. Diagnosis of Tuberculosis.

1.2.1. Examination for tuberculosis symptoms.

Dry or productive cough is the most common symptom associated with active pulmonary tuberculosis. Sometimes, if sputum is present, it is streaked with blood (Daley *et al*., 2003; American Thoracic Society, 2000). The cough may continue for weeks or months and sometimes it is associated with chest pain, and difficulties in breathing. Individuals with pulmonary tuberculosis often have a fever of low-grade and suffer from night-sweats. The patients who have acute disease often lose interest in food and consequently they lose weight (British Thoracic Society, 2000; American Thoracic Society, 2000; Daley *et al*., 2003).

Tuberculosis is not only a pulmonary disease. *M. tuberculosis* can spread to other tissues and organs and is called extra-pulmonary tuberculosis (EPTB) (Thwaites *et al*.,

2000; Memish *et al*., 2014; Lee, 2015). For example, lymph nodes, pleura, meninges, musculoskeletal system, joints and urinary tract and abdomen may be affected (Grange, 1988; Grange and Zumla, 2002; Lee, 2015; Purohit and Tehmina Mustafa, 2015). Tuberculous meningitis is the severest form of TB which may be fatal and usually complicated with severe neurologic deficits in more than 50% of cases. Young children are commonly affected. Typically, it begins 3 to 6 months after the primary TB infection (Thwaites *et al*., 2000; Galdwin and Trattler, 1997; Smith, 2003). Tuberculomas, another Central Nervous System (CNS) form, are structures developed by the enlargement of TB granulomas in the brain. They are mainly due to inflammatory responses and they may be accompanied with convulsions (Kumar *et al*., 2007). Tuberculosis also can be a disseminated lethal form, known as miliary tuberculosis (Galdwin and Trattler, 1997; Smith, 2003; Chan *et al*., 1993; Shingadia, 2004; Sharma and Mohan, 2004; Lee, 2015; Purohit and Tehmina Mustafa, 2015).

1.2.2. Radiography (Chest X-ray).

In active pulmonary disease, chest X-ray may reveal infiltrates, consolidations and/or cavities mostly in the upper lungs. This may be associated with hilar or mediastinal lymph nodes and/or pleural effusion. In HIV infected persons, any abnormality may indicate TB. Chest X-ray can be used for the diagnosis of active tuberculosis, but it has poor sensitivity in patients with latent, minimally active tuberculosis or childhood tuberculosis (Daley *et al*., 2003; Al Zahrani *et al*., 2000).

1.2.3. Tuberculin skin test for detecting latent tuberculosis.

The tuberculin skin test (TST) is the current test used widely for detecting tuberculosis in persons who do not have tuberculosis symptoms (Perkins *et al*., 2000; Drobniewski, 2003). In TST a purified protein derivative (PPD) containing a mixture of antigens obtained from *M. tuberculosis* is injected to test for hypersensitivity and to diagnose latent tuberculosis (Booth *et al*., 1996). The two major tuberculin skin tests used are the Mantoux and Heaf tests. Mantoux is given intra-dermal, whereas the Heaf test is given by multiple puncture of the skin (Booth *et al*., 1996**;** Andersen *et al*., 2000).

Individuals with a positive tuberculin test are not given BCG, because it is unnecessary and may cause aggressive immune reaction (Booth *et al*., 1996**;** American Thoracic Society, 2000). TST is poor in its specificity among persons who have been vaccinated with BCG or exposed to environmental mycobacteria (Edwards *et al*., 1972; Booth *et al*., 1996; Andersen *et al*., 2000; Singh *et al*., 2002), because of shared antigens (Andersen *et al*., 2000; Lalvani *et al*., 2001).

1.2.4. Laboratory diagnosis of tuberculosis.

The only definite diagnostic criterion for confirming tuberculosis is the demonstration of the existence of tubercle bacilli in clinical specimens (Marie *et al*., 2003). This is based on the basic routine diagnostic procedures; Ziehl-Neelsen (ZN) stain and culture on Lowenstein-Jensen (LJ) medium (Marie *et al*., 2003; Hale *et al*., 2001). However, many other new diagnostic tools have been introduced such as ELISA as an example of serologic tests (Lodam *et al*., 1998) or molecular methods, such as amplification of mycobacterial DNA by PCR (Sandin, 1996).

1.2.4.1. Examination of smears.

The most widely used laboratory test, at present, is the direct microscopic examination of a sputum smear for acid-fast bacilli (Ziehl-Neelsen stain). The sensitivity of the Ziehl–Neelsen (ZN) test is variable (Salfinger *et al*., 1994; Hale *et al*., 2001). In general, smear examination detects only 40-80% of pulmonary tuberculosis cases (Kim *et al*., 2001). The advantage of ZN is that it is easy to perform and cheap; but its low sensitivity is a major disadvantage (Salfinger *et al*., 1994; Hale *et al*., 2001; Marie *et al*., 2003).

1.2.4.2. Examination of mycobacterial cultures.

Culture is a sensitive technique that is central for the confirmation and identification of mycobacteria and for drug susceptibility testing (Marie *et al*., 2003; Al Zahrani *et al*., 2000). In spite of its high sensitivity, culture has two drawbacks: suitable facilities are not available in many poor countries and it is time consuming (Al Zahrani *et al*., 2000; Marie

et al., 2003). For example, culture on Lowenstein-Jensen (LJ) medium takes from 4 to 8 weeks (Al Zahrani *et al*., 2000; Marie *et al*., 2003).

1.2.4.3. Examination of immune response against tuberculosis.

Serologic tests using ELISA to detect specific antibodies against *M. tuberculosis* antigens have been proposed for the diagnosis of tuberculosis; for example, detection of ESAT-6 antibody (Lodam *et al*., 1998; Lyashchenko *et al*., 1998). Many commercial kits were offered with such an aim; however, none of them perform sufficiently well to replace smear microscopy. Thus, it was concluded that they have little or no role in pulmonary tuberculosis diagnosis (Steingart *et al*., 2007).

QuantiFERON-TB Test

QuantiFERON-TB assay has been described using ELISA technique to detect and quantify the cytokine IFN-γ released from T-lymphocytes stimulated with PPD, ESAT-6 or CFP-10 peptides (Desem and Jones, 1998; Mazurek and Villarino, 2003; Mazurek *et al*. 2005). It has been found that this test is more accurate than TST (Mazurek *et al*., 2001).

Enzyme-linked immunospot (ELISPOT) assay

ELISPOT assay is based on the detection of specific interferon-γ secreting T-cells in the presence of ESAT-6 and CFP-10 (Lalvani *et al*., 2001; Andersen *et al*., 2000; Ewer *et al*., 2003). ESAT-6 and CFP-10 have been identified as major antigens that induce interferon-γ production from T-cells in humans infected with *M. tuberculosis* or pathogenic *M. bovis* but not those immunized with BCG (Andersen *et al*., 2000; Ewer *et al*., 2003). It has been found that it was more accurate and sensitive than TST. In the CH outbreak, ELISPOT assay results were found not to be associated with BCG vaccination in BCG-vaccinated students (Ewer *et al*., 2003). In spite of the advantages of ELISPOT, it is sophisticated to apply and requires separation of mononuclear cells; a procedure that is not done in most clinical laboratories in poor countries. In contrast, TST is cheap and easy to apply (Lalvani *et al*., 2001; Ewer *et al*., 2003; Huebner *et al*., 1993).

1.2.5. Molecular diagnosis of tuberculosis.

Since *M. tuberculosis* is a slowly growing organism, isolation, identification, and drug sensitivity testing take more than 2 weeks. Many molecular techniques have been developed to decrease the time needed for diagnosis from weeks to days and to increase accuracy. Scientists have suggested that fast and accurate diagnosis of tuberculosis would be a very important element to control of tuberculosis (Andersen *et al*., 2000; Mustafa *et al*., 2003).

1.2.5.1. PCR-based sequencing for detecting *M. tuberculosis* DNA.

PCR-based sequencing has become a powerful tool for the rapid and specific diagnosis of many infectious agents (Kocagoz *et al*., 1993; Soini and Musser, 2001). The microorganism is identified by comparing the sequence of a PCR product against reference sequences. For example, PCR was used for a rapid detection of the gene coding for the 16S rRNA (Sonini and Musser, 2001; Ramaswamy and Musser, 1998). Furthermore, PCR-based sequencing was also used to determine genetic markers or mutations associated with *rpoB*, the gene associated with drug resistance to rifampin (Telenti *et al*., 1993; Sonini and Musser, 2001). This technique has been used extensively to investigate the CH strain outbreak (Rajkumar *et al*., 2004; Newton *et al*., 2006).

1.2.5.2. Amplicor *M. tuberculosis* assay.

The Amplicor *M. tuberculosis* test can be used for detection of *M. tuberculosis* DNA directly from clinical respiratory specimens (Soini and Musser, 2001). The basis of this assay is to amplify the *M. tuberculosis* DNA by PCR using specific primers designed to amplify a 584-bp region of the 16S rRNA. The PCR product is denatured to form a single strand and added for hybridization with a labelled specific probe for members of the *M. tuberculosis* complex (Huang *et al*., 1996; Lebrun *et al*., 1997). Commercially, it was approved by the FDA under the name AMPLICOR (r) MTB (Bergman *et al*., 1996).

1.2.5.3. GeneXpert MTB/RIF.

GeneXpert MTB/RIF (Cepheid, Sunnyvale, California, USA) was recently announced as a semi-quantitative nested real-time polymerase chain reaction (RT-PCR) assay for the coincident detection of *M. tuberculosis* complex and rifampin resistance (RIF) directly from clinical samples (Zeka *et al*., 2011; Alcaide and Coll, 2011; Bunsowa *et al*., 2014). In two multicentre studies, a single GeneXpert MTB/RIF test detected *M. tuberculosis* in almost all smear-positive specimens and in about 75% of the smear-negative samples (Boehme *et al*., 2010; Boehme *et al*., 2011). Other studies have reported high sensitivity and specificity for this test, especially in smear-positive pulmonary samples (Bunsowa *et al*., 2014).

1.2.5.4. Phage based assay of MTB (FASTPlaqueTB).

One of the new diagnostic tools for tuberculosis is diagnosis by mycobacteriophage-based method (Marei *et al*., 2003; Perkins, 2000). This method is based on the mycobacteriophage replication system that reflects the presence of live *M. tuberculosis* complex in clinical samples (Perkins, 2000). The specific mycobacteriophage is added into a sample. If tubercle bacilli exist, the phage will rapidly infect and replicate inside. Then, a virucidal solution is added which destroys all phage that have not infected the bacilli. The mixture of phages and sample is transferred into a lawn of sensor cells of a non-pathogenic rapid-growing mycobacterial host (*M. smegmatis*) allowing the intracellular phages to replicate and plaques will be produced. When the sample is negative, no plaques will be present (Wilson *et al*., 1997; Heifets *et al*., 1999). FASTPlaqueTB has been recommended to be used instead of the standard culture for *M. tuberculosis*, because it is sensitive, cheap and able to detect *M. tuberculosis* in clinical samples within 1 day from collection (Marei *et al*., 2003).

1.2.6. Diagnosis of extra-pulmonary tuberculosis.

Regarding diagnosis of extra-pulmonary tuberculosis (EPTB) cases, some patients with EPTB may develop constitutional symptoms such as fever, weight loss, anorexia, fatigue and malaise (Sharma and Mohan, 2004). Culture of TB-affected serosal fluids, measuring biochemical markers such as gamma interferon in the affected fluid, and molecular tools like PCR may be useful in the diagnosis of these cases (Purohit and Tehmina Mustafa, 2015; Lee, 2015).

Nucleic Acid Amplification techniques (NAAT) using a PCR assay targeted for various genes specific to *M. tuberculosis* strains are routinely used (Dinnes *et al*., 2007; Purohit and Tehmina Mustafa, 2015). Body fluids, such as pleural, peritoneal cerebrospinal (CSF), and pericardial fluids, often provide important diagnostic clues in EPTB patients (Lee, 2015). The advantages of NAAT over culture are higher sensitivity, as it can detect as few as 1-10 organisms in clinical specimens, at least under research conditions (Purohit and Tehmina Mustafa, 2015; Lee, 2015).

Because culture and smear microscopy are not attainable to monitor patients with EPTB, biopsy is the most efficient method for diagnosing EPTB for which, surgery is usually required. Clinical follow up is the usual way to evaluate the response to treatment (Lee, 2015). However, the diagnosis of EPTB can be obscure, requiring a high index of suspicion (Sharma and Mohan, 2004; Lee, 2015; Purohit and Tehmina Mustafa, 2015).

1.3. Treatment of tuberculosis.

Treatment of tuberculosis aims at curing the patient, reducing the spread of *M. tuberculosis* to other individuals and to prevent a relapse of this disease. The anti-tuberculosis chemotherapy programme differs from other anti-microbial treatment regimens since it needs use of multiple drugs simultaneously to prevent the emergence of drug-resistant organisms. In addition, prolonged chemotherapy is necessary for at least six months (Nahid *et al*., 2016; Zumla *et al*., 2015; American Thoracic Society report, 2003; Petrini *et al*., 1999; Ramaswamy and Musser, 1998; Tiexeira *et al*., 2001).

Chemotherapy for tuberculosis began in the late 1940s after the discovery of streptomycin in the early 1940s (Medical Research Council Investigation, 1948). Since then, treatment guidelines and recommendations for management of tuberculosis are updated regularly, including treatment regimens, approved anti-tuberculosis drugs and its dosage.

Anti-tuberculosis drugs currently used and approved by FDA for use in the treatment of tuberculosis are first-line drugs which include Isoniazid (INH), Rifampin, Rifabutin, Rifapentine, Pyrazinamide (PZA) and Ethambutol (EMB) and second-line drugs which include Cycloserine, Ethionamide, Streptomycin, Amikacin/kanamycin, Capreomycin, Para-amino salicylic acid, Levofloxacin and Moxifloxacin (Nahid *et al*., 2016). The first-line TB drugs are the most effective and tolerable among all TB drugs, and are now recommended in a four-drug combination to treat drug-susceptible TB cases. Due to its lower efficacy, tolerability and higher toxicity in comparison to first line drugs, the use of second-line TB drugs has been limited for patients having drug-resistant TB or intolerant to first-line drugs (Shin and Kwon, 2016).

For treatment of tuberculosis, present WHO recommendations (WHO Global Report, 2010; Zumla *et al*., 2015) make a clear distinction between new cases and retreatment cases. New tuberculosis cases (irrespective of HIV status) should be treated with a combination of rifampicin, isoniazid, pyrazinamide, and ethambutol for 2 months then by isoniazid and rifampicin only for the next 4 months. Treatment for more than 6 months is not recommended since it does not demonstrate clinical advantage (WHO Global Report, 2010; Zumla *et al*., 2015). However, in special situations some clinicians suggest treatment for 9-12 months in cases of TB meningitis because of the high risk of mortality and disability and 9 months for cases of TB of bones and/or joints because of the difficulties of treatment response evaluation (National Collaborating Centre for Chronic Conditions, 2006 and 2011; American Thoracic Society Report, 2003). Adjuvant corticosteroid treatment is recommended for TB meningitis and pericarditis, except in cases of suspected drug resistance. In tuberculosis meningitis, ethambutol should be replaced by streptomycin (Thwaites *et al*., 2004).

1.4. Multidrug resistant tuberculosis.

Multidrug resistant tuberculosis (MDR-TB) is defined as tuberculosis caused by bacteria which are resistant to at least isoniazid and rifampin, with or without resistance to other mycobacterial agents (American Thoracic Society, 1998; Johansen *et al*., 2003). Resistance may occur when a doctor does not prescribe a proper chemotherapy regimen or when a patient is unable to adhere to therapy (Johansen *et al*., 2003; American Thoracic Society, 2003; Soini, 2001). Resistance to rifampin alone is rare (Ridzon *et al*., 1998). Therefore, rifampin resistance is used as a marker for MDR-TB (Drobniewski and Pozniak 1996). Another term is the extensively drug-resistant TB (XDR-TB) which is defined as tuberculosis caused by a strain of *M. tuberculosis* resistant to isoniazid, rifampin, any fluoroquinolone and at least one of the following drugs: kanamycin, capreomycin, or amikacin among the second-line drugs (Holtz, 2007; Singh, 2007).

It has been estimated that more than 400,000 cases of new MDR-TB cases are reported every year as a result of poor control of anti-TB drug use and transmission of drug-resistant strains (WHO Global Report, 2007; WHO Stop TB Strategy, 2007). In 2013, 450,000 new MDR-tuberculosis cases were estimated by WHO and 170,000 died from MDR-tuberculosis (WHO Global Report, 2014). Among patients with pulmonary TB who were notified in 2013, an estimated 300,000 had MDR-tuberculosis. More than half of these patients were in China, India and the Russian Federation (Zumla *et al*., 2015). Out of these MDR-tuberculosis cases, eligible for treatment with MDR-tuberculosis regimens, 123,000 were notified to WHO in 2014. India, South Africa and the Russian Federation accounted for about half of the cases (Figure 1.4) (WHO Global Report, 2015).

MDR-TB and XDR-TB became a serious public health problem worldwide, because only a few effective drugs are available for treatment, consequently therapy became more costly and not easy to implement, especially in poor developing countries (De Beenhouwer *et al*., 1995; Walls and Shingadia, 2007; Singh, 2007; WHO Global Report, 2015; Zumla *et al*., 2015; Al-Hajoj *et al*., 2013; Abubakar *et al*., 2013).

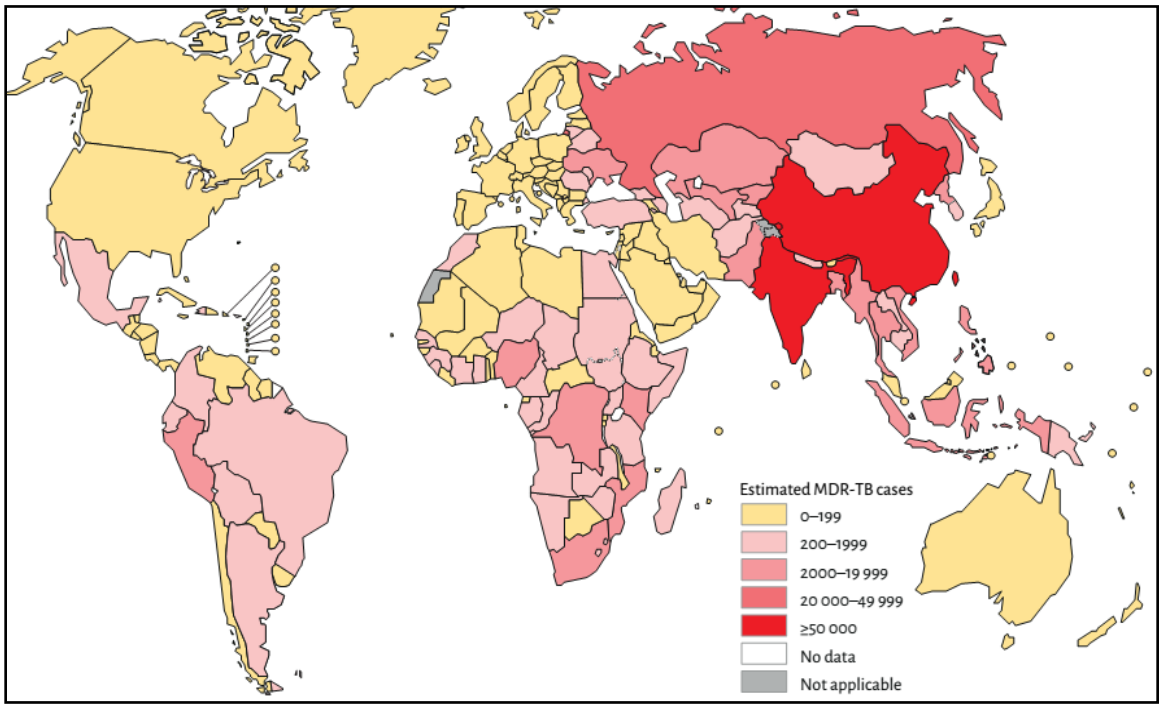


Figure 1.4: Distribution of MDR-tuberculosis cases among reported pulmonary TB cases, 2014 (WHO, Global Tuberculosis Report, 2015).

1.4.1. Genetic basis of drug resistance for tuberculosis.

The occurrence and spread of drug resistant TB hinder the success of TB treatment and control programs worldwide. Drug-resistant TB represents a major problem, necessitating new diagnostic tools, and therapeutics (Farhat *et al*., 2013). Resistance of *M. tuberculosis* to anti-mycobacterial drugs is due to mutations (Table 1.1) and no plasmids or transposable elements are involved (Ramaswamy and Musser, 1998; Somoskovi *et al*., 2001; Smith *et al*., 2003; Bushra *et al*., 2016; Veyrier *et al*., 2009; Becq *et al*., 2009). Individual point mutations (deletions or insertions) induce resistance to single drugs, and the gathering of these individual mutations leads to MDR-TB (Ramaswamy and Musser, 1998; Somoskovi *et al*., 2001; Petrini *et al*., 1999; Riska *et al*., 2000; Ormerod, 2005).

Drug name	Drug Target	Gene correlated with resistance	References
Isoniazid	Catalase-peroxidase enzyme Mycolic acid synthase	*katG* *inhA*	(Petrini *et al*., 1999; Phelan *et al*., 2016)
Rifampin	RNA polymerase	*rpoB*	(Somoskovi *et al*., 2001; Telenti *et al*., 1993; Phelan *et al*., 2016)
Pyrazinamide	pyrazinamidase (PZase)	*pncA*	(Ramaswamy and Musser, 1998; Scorpio *et al*., 1999)
Ethambutol	Arabinosyl transferase	*embB*	(Ramaswamy and Musser, 1998; Telenti *et al*, 1997; Phelan *et al*., 2016)
Streptomycin	Ribosomal S12 protein 16S rRNA	*rpsL* *rrs*	(Cooksey *et al*, 1996; Ramaswamy and Musser, 1998; Phelan *et al*., 2016)
Kanamycin	16S rRNA	*rrs*	Ramaswamy and Musser, 1998, Alangaden *et al*., 1998)
Quinolones	DNA gyrase	*gyrA*	(Reece and Maxwell 1991; Ramaswamy and Musser, 1998; Kocagoz, *et al*., 1996)

Table 1.1: Genetic sites for drug resistance in *M. tuberculosis*.

Beside the traditional drug-resistance genes (encoding a drug metabolizing enzyme or the target protein of the drug), three other categories of gene mutations may have a role. First, mutations that decrease the access of the drug to inside the cell by either decreasing cell wall permeability or activating drug efflux pumps that remove the drug outside the cell

which are expected to increase concentrations of drugs needed to inhibit bacterial growth, possibly providing a step toward complete drug resistance (Nikaido, 1994). Second, mutations which are needed to compensate for the fitness costs of other drug resistance mutations, may be developed (Schrag *et al*., 1997). Third, mutator phenotypes can raise the rate at which rare useful mutations arise and hence provide a selective advantage under therapy (Denamur *et al*., 2006).

Farhat *et al*. (2013) searched for convergent positive selection among drug resistant *M. tuberculosis* strains. They investigated the genomes of 123 recently and previously sequenced *M. tuberculosis* strains. They recovered all previously identified resistance markers. They further proved a positive selection in an extra 39 genomic loci among resistant isolates. These loci encoded pathways for DNA repair, cell wall synthesis and transcriptional regulation. Mutations in these loci could result in drug resistance or improve the fitness costs related to resistance. For evidence to support their hypothesis, they evaluated the functional influence of the detected mutations in the *ponA1* gene, one of the targets of independent mutation (TIM). They constructed two *ponA1* mutants in an H37Rv laboratory strain; one that lacked the *ponA1* gene and another had a *ponA1* G1095T mutation. Afterwards they compared the survival of the wildtype to the mutant strains under different concentrations of isoniazid, rifampicin, streptomycin and ofloxacin. In the presence of rifampicin, the mutant strain having the *ponA1* G1095T mutation showed an *in vitro* growth advantage more than other strains, indicating that the independent mutation had improved the tolerability of the strain to rifampicin (Farhat *et al*., 2013).

1.5. Tuberculosis vaccines - current and future.

1.5.1. Current tuberculosis vaccines.

Currently, the Bacille Calmette-Guerin (BCG) vaccine is used on a wide scale as a live attenuated vaccine against tuberculosis throughout the world. It was given to humans for the first time in 1921 (Takayama *et al*., 1972; Mahairas *et al*., 1996; Behr, 2002). *M. bovis* BCG is a live attenuated vaccine derived from a virulent, clinical isolate of *M. bovis*

by several subcultures (Bloom and Fine, 1994; Benevolo-de Andrade *et al*., 2005). More than three billion persons have been vaccinated with BCG with no major side effects (Bloom and Murray, 1992; Hanson *et al*., 1995; Mahairas *et al*., 1996). Interestingly, as *M. microti* (vole bacillus) is harmless for both human and cattle, it also has been tried as a live vaccine in clinical trials (Mahairas *et al*., 1996; Pyme *et al*., 2002; Pyme *et al*., 2003). Nearly one million individuals have received *M. microti* vaccine in European programmes and immunization with *M. microti* was found to induce protection against TB nearly equal to that conferred by BCG (Hart and Sutherland, 1977; Brosch *et al*., 2000). The attenuation of both *M. bovis* BCG and *M. microti* has been attributed to the loss of important virulence factors due to deletion of a DNA segment designated as region of difference-1 (RD1) (Section1.8.3), which is present in the virulent *M. tuberculosis* complex members (Pyme *et al*., 2002; Pyme *et al*., 2003).

1.5.1.1. BCG vaccine history.

The vaccine of BCG was developed by the French scientists, Albert Calmette and Camille Guerin in the Pasteur Institute in Paris, France. Initially the aim of their experiment was to get a homogenized bacterial growth with no clumping in the culture suspension of *M. bovis*. Over a 14 year period from 1908 to 1921, they observed changes in the colony morphology and gradual reduction of virulence during passage of *M. bovis in vitro* in potato extract medium that contains glycerin and ox bile salts (Calmette and Plotz, 1929; Bloom and Fine, 1994). Surprisingly, these cultures were not able to lose the clumped morphology (Bloom and Fine, 1994; Benevolo-de Andrade *et al*., 2005). However, after 230 passages *in vitro*, the sample was tested in animals (chimpanzees, guinea pigs, mice, and cattle) and has never been shown to revert to a virulent phenotype but it retained immunological features without disease (Takayama *et al*., 1972; Sakula, 1983; Benevolo-de Andrade *et al*., 2005). Subsequently, in 1921, BCG was given to humans for the first time using oral immunisation (the route initially used by Calmette and Guerin), in order to protect a newborn child who was born from a mother who died of tuberculosis a short time after the birth, and who lived with the grandmother, who also had tuberculosis. Soon after, in France BCG was introduced as an oral vaccine administered in milk (Calmette and Plotz, 1929; Fine *et al*., 1989; Benevolo-de Andrade *et al*., 2005).

In 1924, this vaccination method began and was used in France and Belgium and it had a wide acceptance in Scandinavian countries (Sakula, 1983; Benevolo-de Andrade *et al*., 2005). From 1921 to 1927 in France and Belgium, the BCG vaccine showed high efficiency in protecting children against tuberculosis (Calmette and Plotz, 1929; Benevolo-de Andrade *et al*., 2005). In 1928, the vaccine had become internationally accepted and large vaccination programmes were started in France and Scandinavia, but further dissemination of the vaccine was stopped in 1930 as a consequence of the Lubeck disaster. The Lubeck disaster involved 250 babies immunized orally with BCG; 73 of them passed away from TB infection within the first year, whereas another 135 developed disease symptoms. It was found that the administered BCG had been contaminated with virulent *M. tuberculosis*, which was kept in the same place. Consequently, a legal action was taken against the BCG vaccine manufacturers at that time (Benevolo-de Andrade *et al*., 2005).

During the period from 1924-1926, nurses from the Ulleval Hospital, in Oslo (Norway) noticed that oral BCG did not develop allergic response against skin test in many of the vaccinated, which was considered as one of the accepted criteria of developing immunity against TB. They tried to vaccinate two non-allergic individuals via the subcutaneous route. Fortunately, these two individuals became tuberculin test positive 6 weeks after vaccine injection. Later, it was proved that BCG parenteral administration had no harmful effect and this route results in the development of a positive reaction to the tuberculin skin test (TST) (Section 1.2.3) (Heimbeck, 1948). Subsequently, the oral vaccine was replaced in almost all countries by intra-dermal administration (Sakula, 1983; Benevolo-de Andrade *et al*., 2005). In 1961 the WHO recommended using BCG as a vaccine against tuberculosis. Consequently, BCG was sub-cultured by numerous laboratories worldwide (Marttin, 2005). Following the Second World War, BCG became widely used in Europe and in developing countries and is still used (Bloom and Fine, 1994; Fine, 1995; Benevolo-de Andrade *et al*., 2005; WHO Global Report, 2008).

1.5.1.2. Stability of BCG attenuation.

BCG has never been shown to revert to virulence in animals, indicating that the attenuating mutation in this strain is a stable deletion that led to stability of BCG attenuation

(Hozouri *et al*., 2014). This clarifies the reason of safety of BCG vaccine in an immunocompetent host throughout all these years (Takayama *et al*., 1972; Mahairas *et al*., 1996; Behr *et al*., 1999). This stability of BCG has been attributed to the loss of the protein secretion system, T-cell antigens ESAT-6 and CFP-10 encoded by the *rv3875* and *rv3874* genes respectively (Section 1.8.3) due to deletion of RD1 (Mahairas *et al*., 1996; Brodin *et al*., 2004; Berthet *et al*., 1998; Pym *et al*., 2003).

1.5.1.3. BCG vaccination.

BCG vaccination is given in more than 154 countries with 111 reporting coverage of more than 90%. It is recommended by WHO to be administered in areas of high tuberculosis incidence (WHO Global Report, 2017).

In the UK, the BCG vaccination programme was started in 1953 and since then, several changes have been introduced to match the changes in the epidemiology of TB. The programme initially targeted school-leaving age children because the peak incidence of the disease was in young adults. More recently, TB rates have become clearly higher in new immigrants, and the rates in the native population have continuously decreased. Therefore, the BCG programme was directed to protect these new entrants, especially UK born infants to parents from high-prevalence TB countries. In the 1990s, BCG coverage in 10-14 year old schoolchildren was approximately 70% and tuberculin-positive children were exempt from taking the vaccine. As a result of the continuing decrease in TB rates in the UK population, the schools programme was stopped in 2005 and the programme now is a neonatal one, targeting populations at risk for TB exposure. It divides the population at risk into highest, moderate and low priority groups. Generally, any infant born, or anyone who is tuberculin negative living or working in an area with a rate of TB $\geq$ 40 cases /100,000 population, is a candidate for BCG vaccination in the UK (Public Health England, 2017; Green Book, 2011).

In the United States, BCG vaccination is not recommended for routine use in children, nor is it used as a control strategy against tuberculosis for three major reasons: (1) Because the vaccine interferes with the tuberculin skin test producing a positive tuberculin test in recipients; (2) Because of the low incidence of new tuberculosis cases in

the United States; (3) Uncertainty about the efficiency of the BCG vaccine. These reasons have led the U.S. Public Health Service to recommend TST testing for diagnosis and drug therapy for infected individuals (American Thoracic Society, 1996). However, BCG may be given in the USA in critical areas; for example, to tuberculin-negative infants and children who are unable to receive izoniazid or are continuously exposed to active tuberculosis by a close household contact (Packe *et al*., 1988; Colditz *et al*., 1994; American Thoracic Society, 1996).

1.5.1.4. BCG efficacy.

BCG is a safe and inexpensive vaccine that has been used for more than 70 years (Fine, 1989). Although BCG remains the most broadly used vaccine worldwide which has been given to over 3 billion people since 1948 (Hanson *et al*., 1995; Colditz *et al*., 1994; Colditz *et al*., 1995; Marttin, 2005), the ongoing death toll of tuberculosis remains approximately 2 million deaths per year (Zumla *et al*., 1999; Mostowy *et al*., 2003). The efficacy of the vaccine is still debated, however this continued high mortality rate is better attributed to other non-vaccine efficacy related factors such as inaccessibility in remote areas and poor countries, drug resistance, the break-through of HIV, poverty, poor hygiene and special groups like prisoners and drug addicts (Soysal *et al*, 2005; Colditz *et al*., 1995; Godreuil *et al*., 2007; Banuls *et al*., 2015; Monteiro-Maia and Pinho, 2014).

The efficacy of BCG might have been affected by methods and routes of administration and by the environments and features of the populations in which BCG vaccines have been studied (American thoracic society CDC report, 1996; Monteiro-Maia and Pinho, 2014). In a meta-analysis study Roy and his colleagues concluded through numerous clinical trials that BCG has 60-80% protective efficiency against serious forms of tuberculosis in children (Roy *et al*., 2014; Monteiro-Maia and Pinho, 2014). For this reason, the WHO continues to recommend BCG vaccination for infants (Nelson and Wells, 2004). However, WHO does not recommend repeating BCG vaccination in the absence of evidence of whether or not it confers protection (WHO Global Tuberculosis Programme on Vaccines, 1995; Dantas *et al*., 2006).

1.5.2. Future tuberculosis vaccines.

BCG principally provides protection to young children from TB and its severe forms. However, protection against pulmonary TB in adults is limited (Singh *et al*., 2014; Fine, 1995). One third of the world is latently infected with MTB, and approximately 10% of this one third will develop active disease during their lifetime (Gupta *et al*., 2012). The relative inefficiency of BCG vaccine against latent TB, as well as drug resistance, non-compliance of drugs used in treatment and the rise in HIV infection, have increased the need for development of a new improved vaccine (Soysal *et al*, 2005; Monteiro-Maia and Pinho, 2014; Singh *et al*., 2014). Two broad approaches have been pursued for developing new vaccines against *M. tuberculosis* infection.

1.5.2.1. Live recombinant mycobacterial vaccines.

Improving efficacy of BCG vaccines

Recombinant BCG expressing or over expressing a specific gene(s) of *M. tuberculosis* immunodominant antigens could achieve a more comprehensive protection against *M. tuberculosis* infection (Liang *et al*., 2015; Marttin, 2005; Tang *et al*., 2008). For example, it has been found that animals vaccinated with recombinant BCG (BCG::RD1) expressing ESAT-6 demonstrated enhanced protection against challenge with *M. tuberculosis* (Pym *et al*., 2003). In another example, it was reported that rBCG strains overexpressing immunodominant antigens, Ag85A (rBCG::85A), Ag85B (rBCG::85B) or both (rBCG::AB) showed more protection against *M. tuberculosis* than BCG control. The better and longer-lasting protection was achieved by rBCG::AB in animal experiments indicating that rBCG::AB could be a very hopeful TB vaccine candidate (Wang *et al*., 2012). However, a recombinant BCG (rBCG) vaccine would share numerous advantages and disadvantages with the original BCG vaccine. On the favourable side, the vaccine most likely would be inexpensive and of excellent safety. On the negative side, such a vaccine would not be recommended for immunocompromised persons including HIV infected patients. Also, it will share with BCG its interference with tuberculin skin testing (Tang *et al*., 2008; Horwitz *et al*., 2000; Andersen and Dherty, 2005). In addition, it could have a lot of regulatory difficulties (Dixit *et al*., 2010; Graves and Hokey 2011).

Attenuated *M. tuberculosis* vaccines

Construction of attenuated *M. tuberculosis* vaccines depends on the knockout strategy of an effective gene or locus of genes of virulent *M. tuberculosis*. Usually the targeted gene or genes belong to the RD1 region (Collins, 2000). For example, ESAT-6 knockout mutant of *M. bovis* has been proved to cause reactive intradermal skin test without major pathological changes in guinea pigs proposing it to be a live candidate vaccine (Wards *et al*., 2000). Also, *M. tuberculosis* H37Rv:ΔRD1 has been suggested to be a better live vaccine because its ability to multiply *in vivo* is better than *M. bovis* BCG (Lewis *et al*., 2003; Lagranderie *et al*., 1996, Brandt *et al*., 2002). Furthermore, a *M. tuberculosis phoP* mutant, with a mutation in a transcriptional regulator, has been suggested to be a potential candidate live vaccine, because of its attenuation in animals (Perez et al., 2001; Marttin, 2005). Recently, Kaushal *et al*. (2015) demonstrated that aerosol immunization with a MTBΔ*sigH* mutant provided protection against a fatal TB challenge signifying that future TB vaccine candidates can be constructed on the basis of MTBΔ*sigH* (Kaushal *et al*., 2015). These vaccines are promising being derived from the MTB itself not a bovine strain, hence it is expected to have many immunologically important genes which are lost from *M. bovis* BCG (Ferrer *et al*., 2010) and in many of the clinical trials on animal models they were more effective than BCG. However, most of these vaccines need to be further evaluated, including whether the deletions or mutations in these strains result in adequate attenuation for wide scale use in humans. Also, it remains necessary to verify whether these attenuated MTB vaccines will be able to prevent infection in humans or not (Graves and Hokey, 2011; O'Shea and McShane, 2016).

1.5.2.2. Non-viable molecular vaccines (DNA and protein subunit vaccines).

Non-viable molecular vaccines are promising new generation of vaccines that can elicit an immune response. A DNA vaccine depends on the fact that when a recombinant plasmid or suitable virus carrying the gene encoding the antigen of interest is introduced into a host, the gene will be expressed directly under the control of a strong promoter. A DNA vaccine can be a substitute for exposing the antigen presenting cell to *M. tuberculosis* antigens to stimulate T-lymphocyte cells (Meerak *et al*., 2013; Orme, 2013; Sarhan, 2007;

Andersen and Dherty, 2005; Martin, 2005). Immunization in animal experiments with constructs of plasmid DNA that encode one of the *M. tuberculosis* secreted components, antigen 85A (Ag85A), antigen 85B or antigen ESAT-6, has shown an effective protection against *M. tuberculosis* challenge (Fan *et al*., 2009; Meerak *et al*., 2013; Orme, 2013; Baldwin *et al*., 1999; Orme *et al*., 2001). This technique is also useful for identifying the antigens of *M. tuberculosis* that lead to the development of a more efficient vaccine (Fan *et al*., 2009; Meerak *et al*., 2013; Orme, 2013).

As for protein subunit vaccines, these are based on using a single purified recombinant protein or mixtures of recombinant proteins, in an attempt to develop a substitute for BCG vaccine (Orme *et al*., 2013; Martin, 2005; Olsen *et al*., 2000). For example, the ESAT-6 antigen has been found to be highly immunogenic and induces cellular responses in mice (Brandt *et al*., 2000). This result was encouraging and demonstrates the potential of a protein subunit based vaccine to protect against tuberculosis (Olsen *et al*., 2000; Griffiths and Khader, 2014). DNA and protein subunit vaccines have important advantages over other vaccines; they are easy to prepare and store. In addition, they are safe to use especially for immunocompromised individuals such as HIV patients (Huygen, 2005; Wang *et al*., 2015).

1.6. The genus *Mycobacterium.*

The genus *Mycobacterium* comprises more than 70 species (Brosch *et al*., 2000). This genus belongs to the family Mycobacteriaceae which is one of the order Actinomycetales, Suborder Corynebacterineae. The majority of the species that belong to this genus are slow-growing, harmless saprophytes disseminated in soil and water, but a minority are human or animal pathogens or both (Brosch *et al*., 2000; Cole, 2002; Cole *et al*., 1998; Jacobs *et al*., 1991; Madigan *et al*., 2003). All mycobacteria are aerobic non-motile rods that possess an exceptionally lipid-rich cell wall. They do not form capsules or spores (Grange, 1988; Jacobs *et al*., 1991). However, it was recently claimed that *Mycobacterium marinum* and also *Mycobacterium bovis* BCG have the ability to form spores (Singh *et al*., 2010). This was contested by Traaq and colleagues who concluded that it is unlikely mycobacteria have this characteristic (Traaq *et al*., 2010).

1.6.1. Pathogenic species of *Mycobacterium* genus.

The genus *Mycobacterium* encompasses pathogenic species; either opportunistic or obligate pathogens. The most important pathogenic mycobacteria are those of the *M. tuberculosis* complex and *M. leprae,* which cause tuberculosis and leprosy, respectively (Muttucumaru *et al*., 2004; Brosch *et al*., 2000). The Human, Bovine and African types of mycobacteria are similar in their clinical features and many bacteriologists, in order to avoid confusion, reported them as *M. tuberculosis* complex (Grange, 1988). The *M. tuberculosis* complex (MTBC), include *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canettii* and *M. marinum*.

M. tuberculosis is the main causative agent in the majority of human tuberculosis cases, while *M. africanum* is a chief cause of human TB in sub-Saharan Africa. The agent of tuberculosis in voles is *M. microti,* but not for humans. *M. bovis* infects a broad range of mammalian species, for example cattle, goats and elephants, in addition to humans. *M. canettii* is very rarely encountered, but may cause human disease (Cole, 2002; Brosch *et al*., 2000), and *M. marinum* causes tuberculosis to fish and amphibia (Stinear *et al*., 2008). Many other mycobacteria are opportunistic human pathogens such as the *M. avium* complex (*M. avium* and *M. intracellulare*), *M. ulcerans*, *M. abcessus* and *M. fortuitum* (Muttucumaru *et al*., 2004).

1.7. Immunity and Pathogenicity of *M. tuberculosis*.

Tubercle bacilli enter the human body mainly through the respiratory route after inhalation of these tiny droplets expelled into the air. The progression of the pulmonary form of tuberculosis depends on four successive steps: phagocytosis of the bacilli, their intracellular multiplication, the latent contained phase of infection and finally the active lung infection. These steps can progress towards different clinical scenarios: spontaneous cure, disease, latent infection and re-activation, or re-infection (Figure 1.5). Immunosuppressed individuals are more at risk of developing active tuberculosis once infected, particularly patients with AIDS (Banuls *et al*., 2015; Ernest, 2012).

Pathogenicity and virulence of *M. tuberculosis* depends on microbial and host factors. The susceptibility and specificity of the host play a major role (Brosch *et al*., 2000; Madigan *et al*., 2003; Wei *et al*., 2000). The virulence of *M. tuberculosis* depends mainly on its capability to survive and multiply in human macrophages and other cells, although it does not involve known invasive enzymes or toxins (Smith, 2003; Finlay and Falkow, 1997; American Thoracic Society, 2000; Madigan *et al*., 2003; Philips and Ernst, 2012).

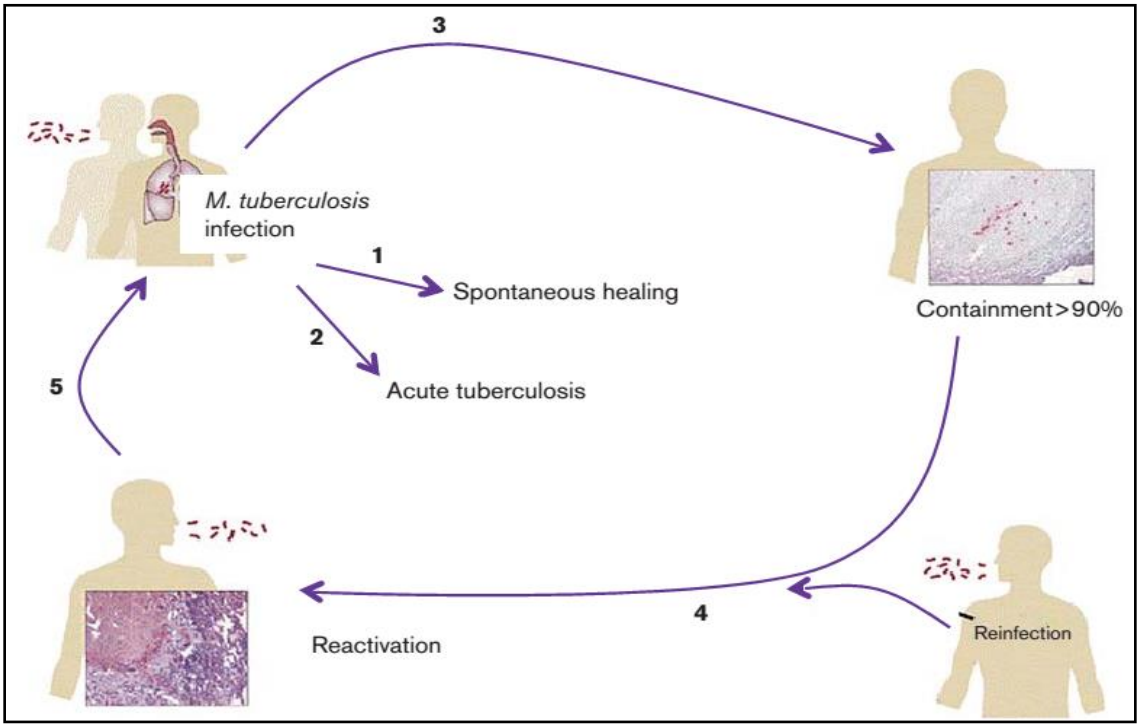


Figure 1.5: Infection Cycle of TB based on Banuls *et al*. (2015). *M. tuberculosis* enters the host by inhalation of aerosols. Different scenarios are likely to occur (1) Pulmonary immune system immediately eliminates the organism; (2) Progression to active tuberculosis; (3) *M. tuberculosis* enters a latency phase; (4) Endogenous reactivation of a latent focus or a new exogenous infection progresses to active disease (5) Following progression to active infection, *M. tuberculosis* can be disseminated and transmitted to another host.

This ability to remain alive within host macrophages is mediated through a complex and synchronized process under control of a special bacterial protein secretion system named ESX-1 (Raghavan *et al*., 2008; Stanley *et al*., 2003; MacGurn *et al*., 2005; Porcelli, 2008).

ESAT-6 and CFP-10 are two proteins produced by the ESX-1 system. These proteins are the outstanding targets of the immune system in hosts infected with *M. tuberculosis* (Sorensen *et al*., 1995). Moreover, animal experiment reports have shown that ESX-1 mutants grow poorly inside macrophages (DiGiuseppe and Cox, 2007; Stanley *et al*., 2003). ESAT-6 and CFP-10 are believed to be involved in the cytolysis of alveolar epithelial cells and macrophages (Gao *et al*., 2004; Van der Wel *et al*., 2007; Xie *et al*., 2016). ESAT-6 suppresses T cell IFN-γ secretion and IL-12 production by monocytes, supporting the intercellular spread of M. *tuberculosis* (Wang *et al*., 2009; Vander Wel *et al*., 2007; Kinhikar *et al*., 2010; Xie *et al*., 2016).

1.7.1. Cell wall composition and pathogenicity of *M. tuberculosis*.

The mycobacterial cell wall (Figure 1.6) has unusually high lipid content compared to other bacterial cell walls. This explains the large number of genes involved in lipid biosynthesis in their genomes (Cole *et al*., 1998; Cole, 1999). The cell wall of mycobacteria consists of inner and outer layers (Brennan, 2003). The outer layer consists of lipo-polysaccharides and proteins. Lipoarabinomannan (LAM) and dimycolyl trehalose (cord factor) are specific outer cell wall components of *M. tuberculosis* associated with its virulence (Nigou *et al*., 2003; Brennan, 2003; Hett and Rubin, 2008; Bansal-Mutalik and Nikaido, 2014). The inner layer consists of covalently linked complex of MA-AG-PG (MAPC) which is composed of mycolic acids (MA) arabinogalactan (AG) and peptidoglycan (PG). This complex extends from the cell membrane outwards in layers, beginning with PG and terminating with MAs (Figure 1.6) (Brennan, 2003; Hett and Rubin, 2008; Kieser and Rubin *et al*., 2014). The presence of the thick, lipid-rich structure is one of the distinguishing characteristics of the mycobacterial cell wall. This feature gives the mycobacteria a natural resistance to host harsh environments (Brennan and Nikaido, 1994;

Barry and Mdluli, 1996). It is mainly due to the highly hydrophobic cell envelope elements that lead to a decrease in the permeability to many extra-cellular compounds, including antibiotics, making therapy difficult (Jarlier and Nikaido, 1994; Clemens, 1997; Hett and Rubin, 2008).

Mycolic acid is one of the important components of mycobacterial cell wall which forms a lipid coat around the bacterium and thus affects its permeability. It is an essential virulence factor, since it protects mycobacteria from the effect of lysozyme, cationic proteins and oxygen radicals inside the host cells. It is also known to protect extracellular mycobacteria in serum from deposition of complement (Alderwick *et al*., 2007; Hett and Rubin, 2008; Vendar Beken *et al*., 2010). Another component of the cell wall is the cord factor which is toxic to mammalian cells and inhibits migration of polymorphonuclear neutrophil (PMN). It is most commonly produced by virulent strains of *M. tuberculosis* while avirulent strains do not produce cord factor (Asano *et al*., 1993).

Lipoarabinomannan (LAM) is also an important constituent of mycobacterial cell wall that has been found to be involved in phagocytosis of MTB (Brennan, 2003). The unique structure of the cell wall impedes the host primary immune response mechanisms and establishes the survival of the bacilli within the phagocytic granules (Tyagi *et al*., 2015; Alderwick *et al*., 2007; Brennan, 2003; Vendar Beken *et al*., 2010; Kieser and Rubin *et al*., 2014).

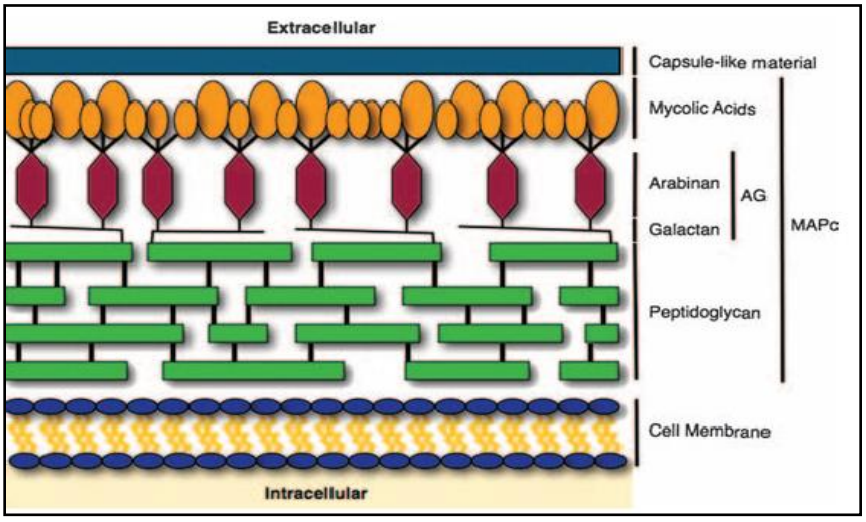


Figure 1.6: Schematic diagram of mycobacterial cell wall (Hett and Rubin, 2008).

1.7.2. The immune response to *M. tuberculosis* and clinical progression of tuberculosis.

M. tuberculosis stimulates a variety of host immune responses (Flynn *et al*., 1995). CD4 and CD8 T-cells and macrophages have been shown to play the major role for bacterial containment and protection against the infection. They are essential in initiating the innate immune defence in tuberculosis infection. Activation of macrophages is an essential process to increase their capacity to phagocytosis (Grange, 1988; Nau *et al*., 2002; Clark-Curtiss and Haydel, 2003; Meena and Rajni, 2011; Russell 2011; Lin *et al*., 2014).

Stages of the immune response to *M. tuberculosis* start with the phagocytic interaction between the pathogen and macrophages. After the initial infection with *M. tuberculosis* (Daley *et al*., 2003), droplets containing tubercle bacilli are able to reach the alveoli of the lung of the uninfected individual (Grang, 1988; Daley, *et al*., 2003; Clark-Curtiss and Haydel, 2003). In humans, the complement receptor CR3 on macrophages is used by *M. tuberculosis* as one of its major phagocytic receptors (Hu *et al*., 2000; Schlesinger *et al*., 1990). *M. tuberculosis* can also bind directly via the macrophage mannose receptors (MMR) (Fenton *et al*., 1996; Malik *et al*., 2000). Alveolar macrophages engulf the bacteria and enclose them in phagosomes (Clark-Curtiss and Haydel, 2003; Russell 2001). The tubercle bacilli have the ability to survive and replicate inside the phagosomes until the macrophages burst (Section 1.7.2.1). Consequently, the bacilli are released into the lung tissue, where they are phagocytosed again by macrophages (Clark-Curtiss and Haydel, 2003; Russell 2001).

After 2 to 14 weeks the infected host begins to generate an immune response (American Thoracic Society, 2000), because some of the macrophages succeed in phagocytosing and partially digesting the mycobacteria by enzymes within lysosomes, and present the digested products with major histocompatibility complex (MHC) class II molecules (Grange, 1988; Russell 2001). These macrophages are now called antigen presenting cells (APCs) and present the antigen to T-cells (CD4 and CD8). The sensitized T-cells then proliferate and encounter their antigenic target, they release different cytokines to attract other macrophages/T-cells and activate them in the infected area (Galdwin and

Trattler, 1997; Clark-Curtiss and Haydel, 2003; Grange, 1988; Smith, 2003). Eventually, the activated macrophages and T-cells surrounding the mycobacteria (Smith, 2003; Lin *et al*., 2014) and the necrosed tissue within this region looks like a granular creamy cheese and is called caseous necrosis. The centre of this soft caseous material is surrounded by macrophages, multinucleated giant cells, fibroblasts and collagen and the whole structure is called a granuloma (Figure 1.7) (Meena and Rajni, 2011; Galdwin and Trattler, 1997; Vignery, 2005; Lin *et al*., 2014). Calcification may occur, which is the deposition of calcium salts in tissues of the granuloma (Chan, 2002). This case of infection is known as primary tuberculosis (Galdwin and Trattler, 1997; Vignery, 2005).

In the case of primary tuberculosis, the potency of the host cell mediated immune response determines whether the organisms are killed and the infected person heals spontaneously, or the organisms are contained and go into a dormant state (latent tuberculosis) or the infection progresses to active tuberculosis (Galdwin and Trattler, 1997; Godreuil *et al*., 2007; Lin *et al*., 2014; Vignery, 2005; Russell 2011; Smith, 2003; Kaufmann *et al*., 2006; Banuls *et al*., 2015). In individuals with an efficient immune system, the infection may be stopped permanently at this point (Smith, 2003; Russell 2011; Banuls *et al*., 2015), and the granuloma consequently heal, with small fibrous or calcified lesions remaining that may not be shown on X-Ray assessment after healing (Smith, 2003; Clark-Curtiss and Haydel, 2003). This is the case with 90% of primary tuberculosis cases (American Thoracic Society, 2000; Banuls *et al*., 2015).

In the case of latent tuberculosis, the numbers of organisms are decreased but remain viable and go into a dormant state, because they are incapable of replicating within the caseous tissue as a result of its acidic pH, hypoxia and the existence of toxic fatty acids (Smith, 2003; Clark-Curtiss and Haydel, 2003; Banuls *et al*., 2015; Lin *et al*., 2014). Latent tuberculosis may continue for years, decades or even until the end of the individual's life, however individuals with latent tuberculosis are not infectious and cannot transmit the organisms (American Thoracic Society, 2000; smith, 2003; Grange, 1988; Banuls *et al*., 2015). Thus, not everyone who gets infected with tubercle bacilli develops active disease at once, but the likelihood of developing active TB is about 5% - 10% in one's lifetime for most infected persons (Dye, 1999).

Active tuberculosis may occur in two circumstances (Daley *et al*., 2003; Galdwin and Trattler 1997; Smith, 2003; American Thoracic Society, 2000; Banuls *et al*., 2015). First circumstance occurs after primary infection if an infected individual's immune system failed to contain the infection at the stage of primary tuberculosis that may lead to progress of the situation to active tuberculosis directly (Daley *et al*., 2003; Banuls *et al*., 2015; Lin *et al*., 2014). This situation represents about 5% of infected individuals. The active infection in this situation develops to active tuberculosis within one to two years after initial infection (American Thoracic Society, 2000; Smith, 2003). This type of tuberculosis occurs more commonly in children and immunocompromised individuals (Galdwin and Trattler, 1997; American Thoracic Society, 2000; Smith, 2003).

In the second circumstance, active tuberculosis may occur later in life in some individuals who have latent tuberculosis after reactivation of dormant bacilli and here it is called post-primary tuberculosis (Daley *et al*., 2003; Russell, 2011) or secondary tuberculosis (Galdwin and Trattler, 1997; Smith, 2003). This situation arises in 5-10 % of individuals with latent infection (American Thoracic Society, 2000; Clark-Curtiss and Haydel, 2003) and it is common in adults especially those with immune systems weakened by immunosuppressive drugs, HIV infection, malnutrition or age (Galdwin and Trattler, 1997; Smith, 2003; American Thoracic Society, 2000; Meena and Rajni, 2011; Banuls *et al*., 2015; Daley *et al*., 2003; Lin *et al*., 2014).

Active tuberculosis ensues when the granuloma lesions become liquefied (Smith, 2003), serving as a rich medium in which *M. tuberculosis* can replicate in an uncontrolled manner. This rich environment benefits *M. tuberculosis* by providing a constant supply of susceptible host cells to infect and, in the presence of a high concentration of oxygen, leads to an escalation in local tissue damage causing an extensive necrosis (Peyron *et al*., 2008; Egen *et al*., 2008; Davis and Ramakrishnan, 2009; Bold and Ernst, 2009; Smith, 2003; Russell, 2011). At this stage, viable MTB bacilli can escape from the liquefied caseous granuloma and disseminate to the surrounding area. Also, the bronchial walls may be injured causing progression of the disease and formation of the cavities. Cavities occur as a result of caseous necrosis and usually contain the highest concentration of tubercle bacilli lesion in the lungs. Consequently, tubercle bacilli are released and disseminate to the rest

of the lungs (Daley *et al*., 2003; Clark-Curtiss and Haydel, 2003; Agarwal *et al*., 2009; Axelrod *et al*., 2008; Galdwin and Trattler, 1997; Smith, 2003; Russell 2011; Lin *et al*., 2014). The active disease is characterized by developing symptoms (Section 1.2.1). In this case, the disease will continue unless the patients are given treatment (Section 1.3) (Smith, 2003; Nahid *et al*., 2016; Zumla *et al*., 2015).

Several extra-cellular inducers (cytokines) of macrophage activation have been identified (Smith, 2003; Ragno *et al*., 2001; Nau *et al*., 2002; Grange, 1988; Cavalcanti *et al*., 2012). For example, interleukin 1 (IL-1) secreted from APCs induces T-cells to secrete interleukin-2 (IL-2) which in turn, stimulates T-cell proliferation and secretion of interferon gamma (IFN-γ). The latter is an important macrophage stimulating cytokine (Grange, 1988; Flesch and Kaufmann, 1999; Flesch *et al*., 1994). IFN-γ activated macrophages (Figure 1.7) show augmented expression of MHC class II molecules, which enhance antigen presentation (Orme *et al*., 1992; Flory *et al*., 1992; Meena and Rajni, 2011; Cavalcanti *et al*., 2012).

Another macrophage stimulating cytokine is the tumour necrosis factor alpha (TNF-α). TNF-α (Figure 1.7) plays a major role in regulation of immune cells and systemic inflammation. The crucial role of TNF-α during *M. tuberculosis* infection has been reported, however its over secretion is associated with harmful effects (Flynn *et al*., 1995; Meena and Rajni, 2011; Cavalcanti *et al*., 2012; Russell 2011; Lin *et al*., 2014). Many cell types are known to generate TNF-α including macrophages. TNF-α is produced when macrophages are exposed to Lipopolysaccharide (LPS) or pathogen like mycobacterial bacilli (Flynn *et al*., 1995; Wang *et al*., 2003; Volpe *et al*., 2006). IFN-γ activated macrophages produce TNF-α in association with interleukin-1 (IL-1) which acts as a pyrogen (Rook *et al*., 1986; Cavalcanti *et al*., 2012; Dinarello *et al*., 1986; Russell, 2011; Lin *et al*., 2014). TNF-α compound facilitates maturation of macrophages, and enhances the anti-microbial efficiency of host monocytes during *M. tuberculosis* infection (Figure 1.7) (Rook *et al*., 1986; Mangelsdorf *et al*., 1984; Meena and Rajni, 2011). TNFα and IFN-γ, both induce the release of microbiocidal reactive nitrogen intermediate (RNI) by macrophages which represents an essential host defence mechanism that manages intracellular infections (Section 1.7.2.1) (Flynn *et al*., 1995; Flesch and Kaufmann, 1990).

Tuberculosis is a remarkable example of TNF-α acting as a 'double-edged sword', because in spite of its role in counteracting the *M. tuberculosis* infection, it can also cause severe damage of affected tissue (Mootoo *et al*., 2009). Therefore, the overproduction of TNF-α may have severe pathologic effects, such as fever, noticeable loss of fat, muscle weakness, fatigue, night-sweats, lung necrosis and weight loss (Figure 1.7) (Tracey *et al*., 1988; Bevilacqua *et al*., 1986; Meena and Rajni, 2011; Lin *et al*., 2014).

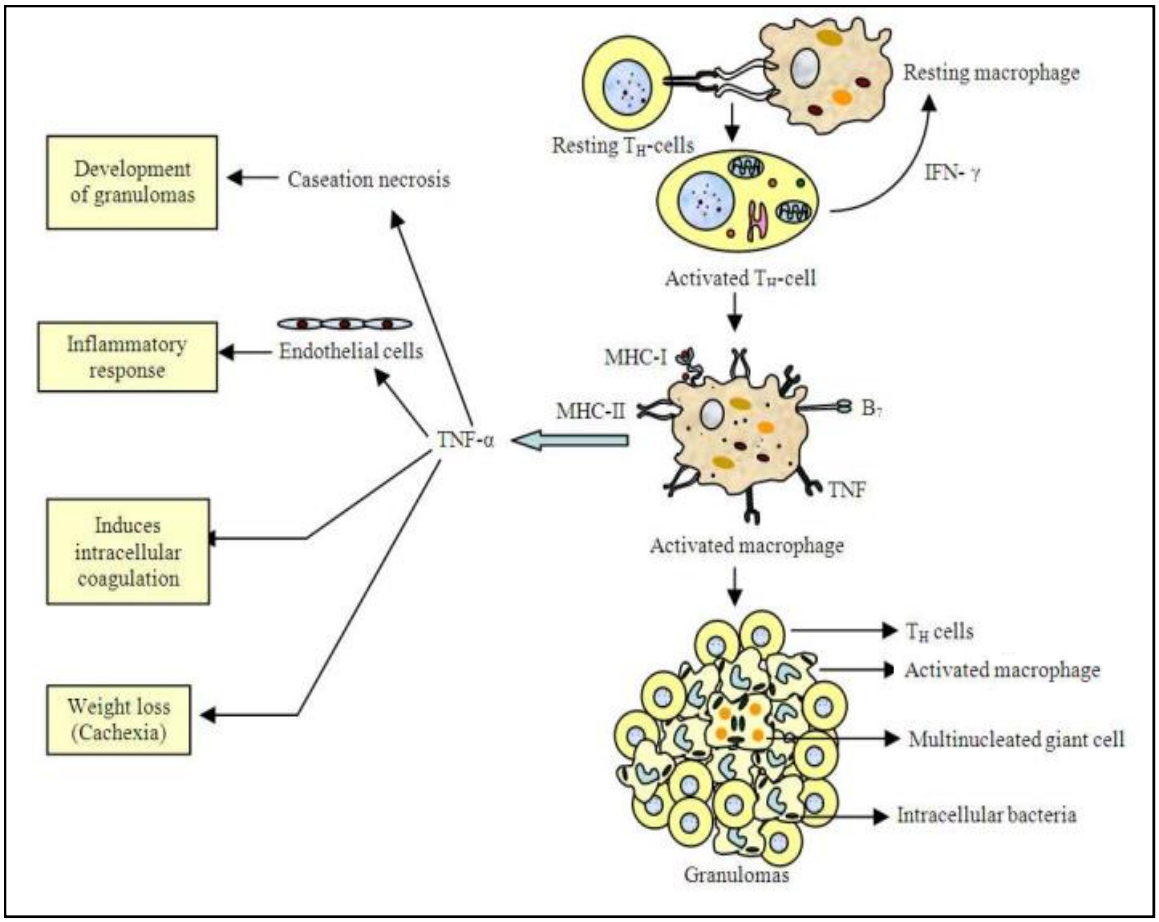


Figure 1.7: Schematic diagram showing the role of TNF-α in the formation of a granuloma and the immunopathology of tuberculosis (Meena and Rajni, 2011).

1.7.2.1. Mycobacteria and oxidative and acid stress.

The key event in the interaction between the host and MTB is what happens inside macrophages. When bacteria are inside macrophages, they encounter reactive oxygen

intermediates (ROI), reactive nitrogen intermediates (RNI) and acid stresses (low pH). This host response represents the initial approach for protection against bacteria (Foster and Hall, 1990; Wei *et al*., 2000; Saviola *et al*., 2003; Pagan-Ramos *et al*., 2006; Firmani and Riley, 2002). ROI and RNI stress agents have an ability to destroy membranes, proteins and DNA of the bacterial cells (Gutteridge, 1995; Moncada *et al*., 1997; Miller and Britigan, 1997). H_2O_2 may affect bacteria in two ways, with a direct toxic effect (Imlay and Linn, 1986), or in an indirect way via formation of $OH^{\bullet}$ radicals either by the reduction of hydrogen peroxide (H_2O_2) to yield hydroxyl radicals ($HO^{\bullet}$) as shown in formula 1 (Gutteridge, 1995; Miller and Britigan, 1997), or as a result of the Fenton pathway (Storz and Imlay, 1999; Benov, 2000) as it can be seen in formula 2.

Formula (1) $H_2O_2 \xrightarrow{e^-} OH^- + HO^{\bullet}$

Formula (2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$

H_2O_2 plays a vital role in the killing of bacteria during phagocytosis by macrophages and neutrophils (Miller and Britigan, 1997). $OH^{\bullet}$ is described as a highly toxic radical on cells since it has the ability to damage DNA causing mutation, and protein damage leading to death of the bacterial cell (Storz and Imlay, 1999; Keyer and Imlay, 1996).

As for nitric oxide (NO), nitric oxide synthase (NOS) catalyzes the alteration of L-arginine to create NO and citrulline. It has been reported that NO toxic action results from its ability to inhibit the action of enzymes involved in the respiratory reactions and to inhibit the DNA synthesis in the target cells (Nguyen *et al*., 1992; Moncada *et al*., 1997). The NO produced by macrophages and neutrophils can react with superoxide to produce peroxynitrite $ONOO^-$ (Formula 3), a highly efficient substance in killing bacteria (Moncada *et al*., 1997; Beckman *et al*., 1990; Miller and Britigan, 1997).

Formula (3) $NO^{\bullet} + O_2^{\bullet -} \rightarrow ONOO^-$

Subsequently, generated peroxynitrite ($ONOO^-$) compound can be decomposed (protonated) at acid pH to release hydroxyl radicals and nitrogen oxide ($NO_2^{\bullet}$) (Beckman *et al*., 1990; Gutteridge, 1995) as it can be seen in formula (4).

Formula (4) $ONOO^- + H^+ \rightarrow OH^{\bullet} + NO_2$

Low pH, in phagosomes for instance, provides favourable conditions for enzymatic hydrolysis, since this acidic environment obviously optimizes the activity of several lysosomal enzymes including lysozyme, phospholipases, glycosylases and nucleases. Also it has been suggested that it facilitates the microbial killing process by inducing spontaneous generation of hydrogen peroxide (H_2O_2) from superoxide (O_2^-) (Buchmeier and Heffron, 1991; Andrew *et al*., 1987; Tapper *et al*., 1990; Clemens, 1996).

Numerous studies have demonstrated that strains of *Mycobacterium* differ in susceptibility to ROI and RNI. A variety of mechanisms that contribute to resistance to these stress agents have been proposed. The low permeability of the mycobacterial cell envelope for many toxic molecules plays a role. In addition, the ability for detoxification of ROI and RNI molecules using enzymatic antioxidants (Haas and Goebel, 1992; Zahrt and Deretic, 2002), the repair of the damage caused by these molecules and its ability to maintain a neutral intra-bacterial pH within acidic environments are the major factors to resist intracellular killing (Ehrt and Schnappinger, 2009). *Mycobacteria* can avoid the low pH of the phagosome by blocking H^+-ATPases that are responsible for the acidification of phagosome vacuoles (Mellman *et al*., 1986; Sturgill-Koszyck *et al*., 1994; Malik *et al*., 2001). Another method is the ability of the virulent strain to block phagosome-lysosome (P-L) fusion to establish its residence in macrophages (Kang *et al*., 2005). *M. tuberculosis* has been reported also to resist harmful ROI by the phenolic glycolipid (PGL) compounds of the cell wall that are highly effective in scavenging $OH^{\bullet}$ and $O_2^{\bullet -}$ and may enhance the intracellular survival and prevent bacterial killing by phagocytes (Chan *et al*., 1989; Neill and Klebanoff, 1988).

Many studies revealed that mutations or deletions change the susceptibility of the bacteria to these stresses. Superoxide dismutases (SODs) are a group of enzymatic antioxidants involved in detoxifying these radicals (Formula 5) (Neill and Klebanoff,

1988). *M. tuberculosis* harbours two superoxide dismutase genes, named *sodA* and *sodC* (Wu *et al*., 1998). Mutations of these genes compromise the ability of the organism to combat different stress conditions. For example, lack of *sodC* was found to increase susceptibility of *M. tuberculosis* to superoxide with or without nitric oxide and to be killed by IFNγ activated macrophages (Piddington *et al*., 2001).

Formula (5) $O_2^{\bullet -} + O_2^{\bullet -} \xrightarrow[\text{Superoxide dismutase}]{2H^+} H_2O_2 + O_2$

Another example of enzymatic antioxidants is the Catalase peroxidase (formula 6) (Gutteridge, 1995; Miller and Britigan, 1997). *katG* mutated *M. tuberculosis* showed no catalase production and was hypersusceptible to H_2O_2 in culture (Ng *et al*., 2004).

Formula (6) $2H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2$

M. tuberculosis expresses also an NADH-dependent peroxidase and peroxynitrite reductase which have a role in detoxifying RNI and ROI. Alkylhydroperoxide reductase subunit C (AhpC) is one of the components of Peroxynitrite reductase (Bryk *et al*., 2000). It has been found that disruption of *ahpC* made the mutant hyper-susceptible to RNI (Chen *et al*., 1998).

The mycobacterial defences include antioxidant compounds present in/on the microorganism itself. For example, *M. tuberculosis* contains mycothiol (MSH) that helps to maintain the reducing conditions inside the cytoplasm (Newton *et al*., 2008). MTB mutants with decreased cellular levels of mycothiol showed increased susceptibility to oxidative stress (Buchmeier *et al*., 2006; Buchmeier and Fahey, 2006).

Mycobacterial adaptation may also play an important role in its resistance to acid stress. O'Brien *et al*. (1996) found that *M. smegmatis* exposure to a pH of 5.0, which is an adaptive acidic level, conferred a notable level of protection against consequent exposure to a lethal pH of 3.5 using phosphoric acid or hydrochloric acid (O'Brien *et al*., 1996).

1.8. Genomic evolutionary analysis of *M. tuberculosis* complex.

The *M. tuberculosis* complex is a set of mycobacterial species (Section 1.6.1) which are grouped together genetically by virtue of being similar at the nucleotide level at more than 95 % (Table 1.2) (Muttucumaru *et al*., 2004). Genomic analysis of the species of the MTBC has the potential to define the genetic basis of their phenotypes (Brosch *et al*., 2000; Cole *et al*., 1998; Pym *et al*., 2003).

Species	Genome size (bp)	Number of Genes	References
M. tuberculosis (H37Rv)	4411529	3966	Cole *et al*., 1998
M. africanum	4493502	4069	Hurtado *et al*., 2016
M. bovis	4345492	3952	Brosch *et al*., 2000; Garnier *et al*., 2003;
M. microti	4370115	4369	Zhu *et al*., 2016
M. canettii	4482060	4137	Supply *et al*., 2013
M. marinum	6636827	5424	Stinear *et al*., 2008

Table 1.2: Genetic composition of *M. tuberculosis* complex members

The complex originated from a common ancestor strain which underwent irreversible genetic events including DNA loss or polymorphisms (Figure1.8). The region of difference 9 (RD 9) and *M. tuberculosis* specific deletion (TbD 1) deletions were the major phylogenetic markers for *M. tuberculosis* and other members of MTBC complex. The first substantial evolutionary event was the loss of RD 9, which was followed by successive deletions. The RD 9 and subsequent deletions identified mainly the lineages of *M. africanum*, *M. microti*, and *M. bovis*. The *M. canettii* and *M. tuberculosis* strains lack the RD 9 deletion and all the subsequent deletions. Thus, it was concluded that *M. canettii* and ancestral *M. tuberculosis* strains are direct descendants of the common progenitor that

existed before the *M. africanum*, and *M. bovis* lineages (Brosch *et al.*, 2002; Nebenzahl-Guimaraes *et al*., 2016).

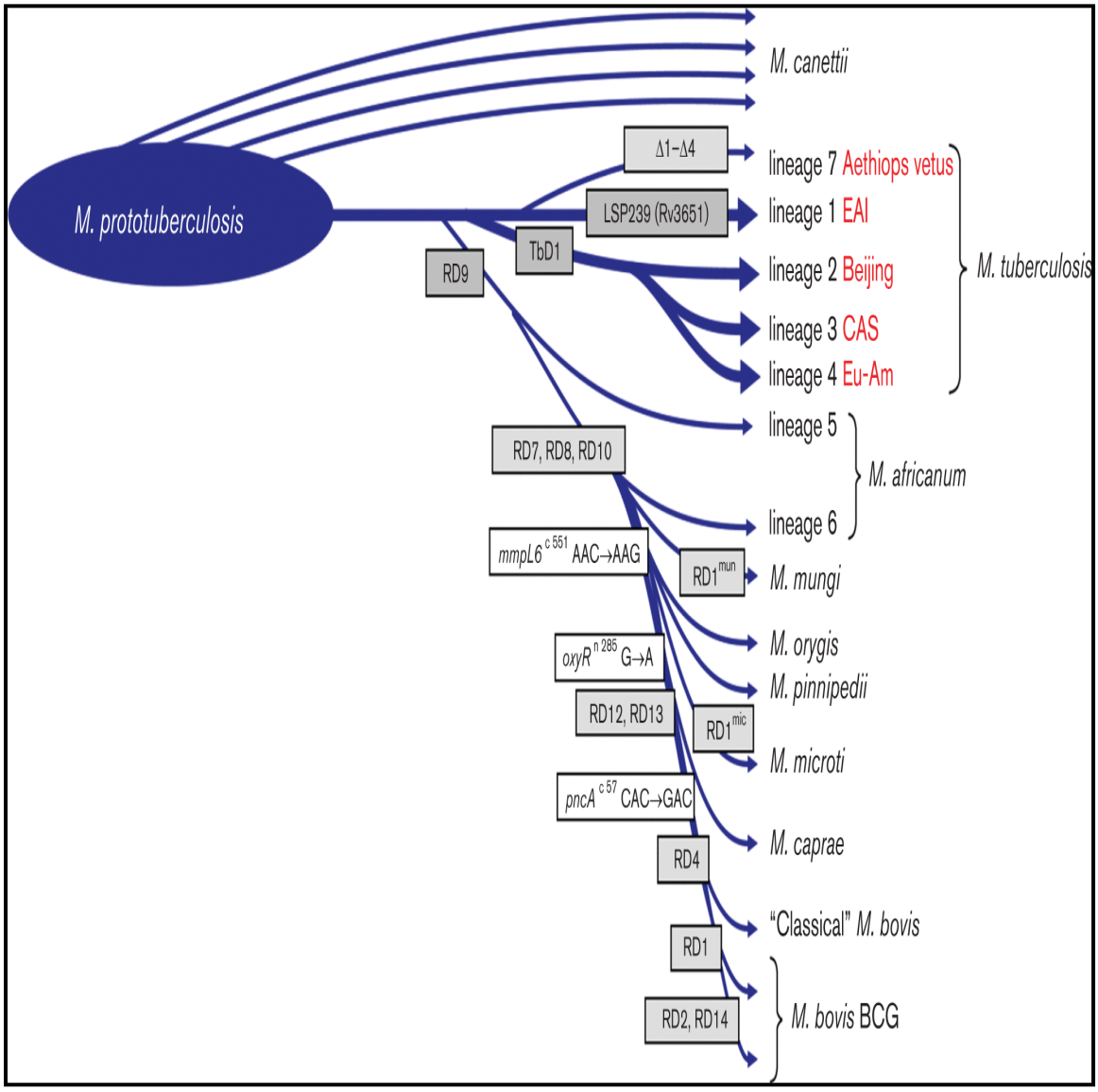


Figure 1.8: Schematic diagram illustrates evolutionary events of *M. tuberculosis* complex following successive deletions and polymorphisms and demonstrates the origin of modern and ancient *M. tuberculosis* lineages (Nebenzahl-Guimaraes *et al*., 2016).

1.8.1. Lineages of *M. tuberculosis*.

The evolutionary analysis of *M. tuberculosis* was associated with another notable polymorphism, the *M. tuberculosis* specific deletion (TbD1), which followed the RD9 deletion (Figure 1.8). Based on this deletion, the strains were grouped into ancient (ancestral) strains having the TbD1 region and ‘‘modern’’ that have lost TbD1 (Table 1.3) (Brosch *et al.*, 2002; Nebenzahl-Guimaraes *et al*., 2016).

It was reported that Gagneux *et al*. (2006) classified *M. tuberculosis* into six major lineages related to particular geographical distributions. They suggested that each lineage may be preferentially adapted to the unique genetic background of a specific human host population (Gagneux *et al*., 2006). Further, Gagneaux and Small (Table 1.3) were able to confirm these six lineages, lineages 1 to 4 of *M. tuberculosis* and lineages 5 and 6 of *M. africanum*, by combining certain target regions of difference (RDs) and a small 7bp polymorhism in the *pks*15/1 gene (Gagneaux and Small, 2007; Gagneux, 2012). Recently, a novel lineage 7 designated as *Aethiops vetus* was reported in Ethiopia. Genome sequencing localized the strains of this lineage between ancient and modern lineages. The TbD1 region was conserved in the new lineage (Firdessa *et al*., 2013; Banuls *et al*., 2015; Nebenzahl-Guimaraes *et al*., 2016). The Ethiopian strains were found to have a characteristic four specific deletions (D1-D4) (Nebenzahl-Guimaraes *et al*., 2016).

The ancestral strains fit into a genetic group ($TbD1^{+}$) that includes *M. africanum* (West African lineages) (lineages 5 and 6) and the strains of the Indo-Oceanic lineage (lineage 1), in addition to the new *Aethiops vetus* (lineage 7). The modern strains comprise genetic group ($TbD1^{-}$) that includes the East Asian lineage, the East-African Indian lineage and the Euro-American lineage (lineage 2, lineage 3, lineage 4) respectively (Brosch *et al*., 2002; Gagneux *et al*., 2006; Gagneaux and Small, 2007; Gagneux, 2012; Comas *et al*., 2013; Banuls *et al*., 2015; Firdessa *et al*., 2013; Nebenzahl-Guimaraes *et al*., 2016). The ‘modern’ strains are more successful than ‘ancient’ strains in terms of their geographical distribution. Remarkably, the universal success of ‘modern strains’ is attributed to a hypo-inflammatory phenotype character, that may reflect higher virulence in humans (Vellarikkal

et al., 2013; Sarkar *et al*., 2012; Gagneux, 2013; Newton *et al*., 2006; Reed *et al*., 2004; Comas *et al*., 2013).

Lineages	Name of lineage	Presence of TbD1	Presence of Target RD	Ancientness
lineage 1	Indo-Oceanic lineage	$TbD1^{+}$	$RD239^{-}$	Ancient
lineage 2	East Asian lineage 'Beijing'	$TbD1^{-}$	$RD105^{-}$	Modern
lineage 3	East African-Indian 'CAS'	$TbD1^{-}$	$RD750^{-}$	Modern
lineage 4	Euro-American lineage	$TbD1^{-}$	*Pks15*/1-7bp^{-}	Modern
lineage 5	West African lineage I	$TbD1^{+}$	$RD711^{-}$	Ancient
lineage 6	West African lineage II	$TbD1^{+}$	$RD702^{-}$	Ancient
lineage 7	*Aethiops vetus*	$TbD1^{+}$	$(D1\text{-}D4)^{-}$	Ancient

Table 1.3: Comparison of molecular markers for seven major lineages of *M. tuberculosis* and *M. africanum* (Brosch *et al*., 2002; Gagneux *et al*., 2006; Gagneaux and Small, 2007; Gagneux, 2012; Comas *et al*., 2013; Banuls *et al*., 2015; Firdessa *et al*., 2013; Nebenzahl-Guimaraes *et al*., 2016).

The six main lineages (Figure 1.9) are highly predominant in specific geographic areas and named according to their geographical distribution (Gagneux *et al*., 2006; Shabbeer *et al*., 2012). Consequently, different *M. tuberculosis* lineages may have adapted to different human host populations (Gagneux and Small, 2007; Shabbeer *et al*., 2012). The Indo-Oceanic lineage includes a group of strains that predominate in the Indian subcontinent, South East Asia and all around the Indian Ocean (Gagneux *et al*., 2006; Gagneux and Small, 2007). The West-African lineages 1 and 2 correspond to strains that traditionally have been known as *M. africanum* which occur almost exclusively in West Africa (Gagneux *et al*., 2006; Mostowy *et al*., 2004). The Euro-American lineage dominates in Europe and America. The strains H37Rv, H37Ra, Erdman, and the clinical strain CDC1551 all belong to the Euro-American lineage (Gagneux *et al*., 2006; Gagneux

and Small, 2007). CDC 1551 is a strain that was responsible for a large outbreak of tuberculosis in a rural area near the Kentucky-Tennesse border in USA (Valway *et al*., 1998; Behr *et al*., 1999). The East-Asian lineage which predominates in many Far East countries includes strains of the "Beijing family" that is most frequent in East Asia. *M. tuberculosis* strain HN878 belongs to the East Asian strain lineage (Gagneux *et al*., 2006). This strain caused a large outbreak in a prison in Houston in the USA (Sreevatsan *et al*., 1997; Manca *et al*., 1999). The East-African Indian lineage 3 occurs mainly on the Indian subcontinent. Also, this lineage is common in the United Kingdom amongst the Asian ethnic group, which is a major ethnic group in Leicester city (Gascoyne-Binzi, 2002; Newton *et al*., 2006). The lineage is known also as Central Asian (CAS)/Delhi family (Gagneux *et al*., 2006; Gagneux and Small, 2007).

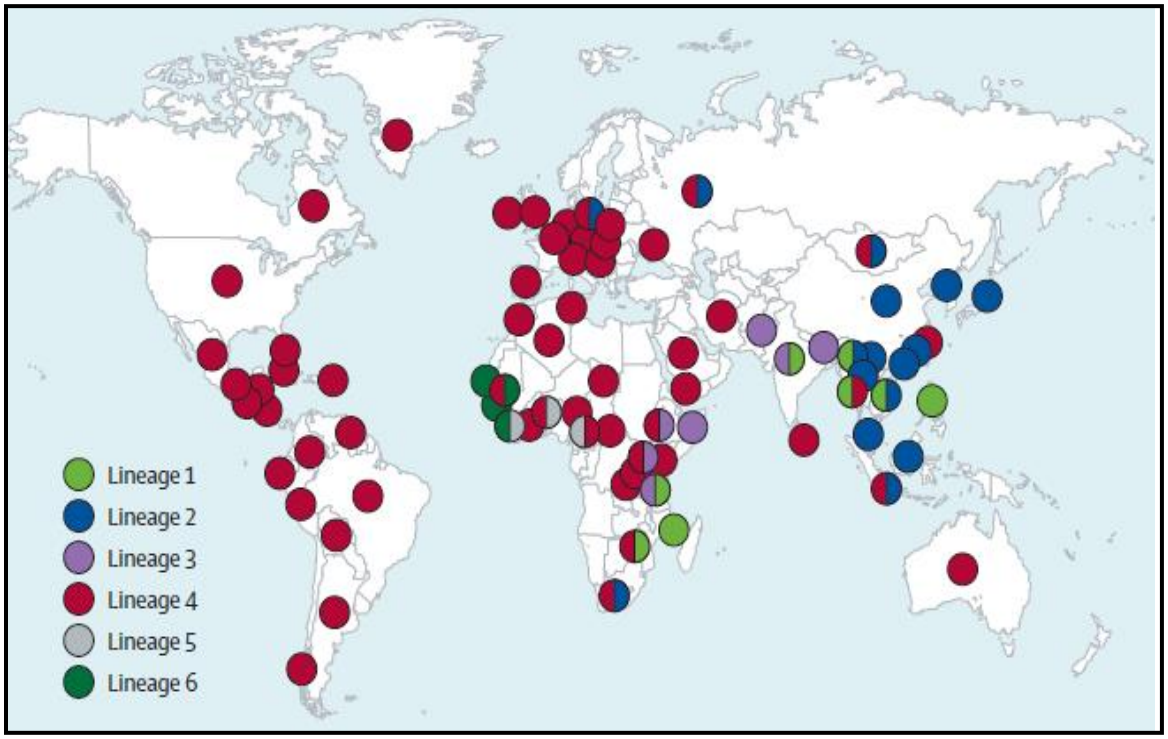


Figure 1.9: Global geographical distribution of different lineages of *M. tuberculosis* (Gagneux and Small, 2007).

1.8.2. Genome of *M. tuberculosis*.

The complete genome sequence of the virulent *M. tuberculosis* H37Rv laboratory strain was first established in 1998 (Cole *et al.*, 1998). The genome is a single circular chromosome with no plasmids (Veyrier *et al*., 2009; Becq *et al*., 2009) comprised of 4,411,529 base pairs, containing about 4000 genes which encode proteins and about 50 genes that encode for stable, functional RNA molecules (Cole *et al*., 1998). The *M. tuberculosis* genome has a high number of repetitive DNA insertion sequence (IS) elements; with up to 56 copies of eight different families. These families are *IS3, IS5, IS21, IS30, IS110, IS256, IS1535* and *IS6110*. The 1355-bp length *IS6110* element was found to be the most abundant one and is unique to *M. tuberculosis* strains (Thierry *et al*., 1990; Gordon *et al*., 1999). Insertion sequences are small-sized (usually < 2.5 kb) genetic elements which have the ability to move from a location to another within the genome. They were recognized as sources of genomic variation among *M. tuberculosis* strains (Fang *et al*., 1998; Gordon *et al*., 1999). For example, the IS*6110* element's existence at differing sites in the genome with many copies, has offered an outstanding method by which strains can be genotyped. For these reasons, IS*6110* has been used widely for epidemiological purposes (Coros *et al*., 2008; McEvoy *et al*., 2007; Alonso *et al*., 2013). Natural variation of *M. tuberculosis* strains is largely due to the mobile insertion sequence IS*6110* which can cause spontaneous mutations either by insertional inactivation (Sampson *et al*., 1999) or by deletion of genes (Fang *et al*., 1999; Alland *et al*., 2006).

Another remarkable feature of the *M. tuberculosis* genome is that it has an abundance of genes encoding enzymes that are involved in fatty acid metabolism. It has been reported that there are more than 250 enzymes in *M. tuberculosis* responsible for fatty acid metabolism in comparison with only 50 in *E. coli* (Cole *et al*., 1998). The *M. tuberculosis* genome also contains a large number of genes encoding proteins that can be considered as potential targets for anti-tuberculosis drugs (Table 1.1), such as transcription factors, enzymes responsible for fatty acid metabolism and cell wall biosynthesis involved enzymes (Lamrabet and Drancourt, 2012).

ESAT-6 (early secretory antigenic target of 6 kDa) and CFP-10 (culture filtrate protein of 10 kDa) (Table 1.4) are the two-major T-cell stimulating antigens that are

encoded by the *rv3875* and *rv3874* genes respectively (Brodin *et al*., 2004; Berthet *et al*., 1998; Mustafa, 2014; Gcebe *et al*., 2016). The ESAT-6 and CFP-10 proteins belong to the Esx family that comprises 23 small secreted proteins encoded by genes *esxA*-*esxW* (Bitter *et al*., 2009). The prototypes of the Esx family are EsxA (ESAT-6) and EsxB (CFP-10). The *esxA* and *esxB* genes encoding ESAT-6 and CFP-10 respectively are located adjacent to each other in the conserved ESX1 locus and reported to be co-transcribed (Andersen *et al*., 1995; Uplekar *et al*., 2011). These secretory proteins have been extensively investigated as potential targets for development of new vaccines and immunodiagnostic tools, as they are believed to have the ability to stimulate immune response of diagnostic and protective value (Mustafa, 2014; Gcebe *et al*., 2016; Etna *et al*., 2015).

One of the most distinguished results of the *M. tuberculosis* genome project was the discovery that about 9% of the genome encodes two extensive families of new glycine rich proteins, PE (glycine-alanine-rich) and PPE (glycine-aspargine-rich) (Table 1.4). The genome of *M. tuberculosis* has a very high content of guanine and cytosine (65%) that is reflected in the unique amino-acid composition of the proteins (Cole *et al*., 1998). In comparison to other bacteria, GTG initiation codons in *M. tuberculosis* genome are more frequently found (35%) than *Bacillus subtilis* (9%) and *E. coli* (14%), though ATG (61%) is still the most frequently found initiation codon (Cole *et al*., 1998). The high G+C content of the genome was reflected on the abundance of the amino acids Gly, Ala, Arg, Pro, and Trp that are encoded by G+C-rich codons. In contrast, the amino acids that are encoded by A+T rich codons such as Tyr, Asn, Lys, Ile, and Phe, are reduced (Cole *et al*., 1998; Domenech *et al*., 2001). This analysis explained the abundance of the two groups of proteins that belong to new families, PE and PPE (Cole *et al*., 1998; Domenech *et al*., 2001; Ahmed *et al*., 2015).

PE and PPE are thought to be a mycobacterial cell envelope protein (Sampson *et al*., 2001; Lamrabet and Drancourt, 2012). Also, it has been reported to have a potent T-cell antigen activity like ESAT-6 and CFP-10. It stimulates secretion of high levels of interferon-γ from peripheral blood monocytes isolated from *M. tuberculosis*-infected patients (Demangel *et al*., 2003). PE is thought to be involved in mycolic and fatty acid biosynthesis and is preferentially expressed by bacilli in macrophages and granulomas

(Ramakrishnan *et al*, 2000; Lamichhane *et al*, 2003). Members of the PE and PPE families are characterized by the presence of a conserved Pro-Glu (PE) and Pro-Pro-Glu (PPE) motif in their N-terminal region, and by the existence of GC-rich stretches in their C terminals, which are believed to be hotspots for recombination events and other mutations (Sampson, 2011). This leads to a high chance for development of polymorphism and sequence variation in these proteins (McEvoy *et al*., 2009). In addition, this variability may enhance antigenic variation which assists the pathogen to escape host immune responses (Ahmed *et al*., 2015).

1.8.3. Genomic deletions and virulence of *M. tuberculosis*.

Many studies have demonstrated the role of different genes in the virulence of *M. tuberculosis*. However, gene deletion/absence also may be important. Although a large proportion of studies indicated that deletions appear to be deleterious, some may be linked with an increased probability of transmission and virulence (McLaughlin *et al*., 2007; Pym *et al*., 2002; Tsolaki *et al*, 2004; Rajakumar *et al*., 2004; Alland *et al*., 2007). Mutations have been hypothesised to occur at random, producing genetically different individuals when microorganisms live under harsh conditions, such as DNA damage agents, starvation, pH stress, oxidative stress or high temperatures (Lamrabet and Drancourt, 2012; Foster, 2007; Cabiscol *et al*., 2010). While adapting to host conditions, microorganisms gather mutations that improve their fitness and, in some cases, they lose genes whose functions mismatch with what pathogens do. In losing these anti-virulence genes, a microorganism may delete large DNA segments (Maurelli, 2007; Kimberl *et al*., 2012).

The effect of gene deletions on pathogenicity and host specificity of bacteria has been demonstrated in many bacteria. For example, Parkhill *et al*. (2003) compared the genome sequence of 3 closely related gram positive bacteria; *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. They concluded that the virulent strain *B. pertussis*, the one of recent evolutionary origin and the main aetiologic agent of whooping cough, adapted itself by large scale gene loss or inactivation rather than gene gain. Another example, deletions of 317 genes from virulent *Yersinia pestis* (the causative agent of plague) were detected compared with *Yersinia pseudotuberculosis*, indicating that

approximately 13% of *Y. pseudotuberculosis* genes are certainly not necessary for *Y. pestis* virulence, but they may have a function. These deletions provide a natural example of how a highly virulent bacterium can evolve from one which is less virulent (Chain *et al*., 2004). Also, in *Shigella* it has been found that certain gene deletions, for example *cadA* and *ampT* could potentiate the virulence of these pathogenic species in the host (Day *et al*., 2001). Furthermore, in *Salmonella enterica*, over 200-fold deletion rates were found between different genome regions. Around 25% of the investigated deletions resulted in an improvement of fitness under one or more of different growth conditions (Koskiniemi *et al*., 2012).

M. tuberculosis does exhibit genetic variability, both in the form of single nucleotide polymorphism (SNPs) (Fleischmann *et al*., 2002), and in chromosomal gene content (Tsolaki *et al*., 2004). In the *M. tuberculosis* complex, LSPs (Large sequence polymorphisms) are defined as regions of difference (RDs) or gene deletions that occur in the genome (Fleischmann *et al*., 2002; Tsolaki *et al*., 2004; Hirsh *et al*., 2004; Alland *et al*., 2007).

Different RDs were previously identified using genomic approaches to compare several genetic regions of difference between virulent *M. bovis*, *M. tuberculosis* and avirulent *M. bovis* BCG (Mahairas *et al*., 1996; Behr *et al*., 1999; Gordon *et al*., 1999). Mahairas *et al*. (1996) identified three genomic regions of difference which are RD1, RD2 and RD3. Also, Behr *et al*. (1999) using DNA-microarray technology identified other regions of *M. tuberculosis* DNA that are deleted in some or all strains of BCG. 16 large deletions were identified, including RD1, RD2 and RD3, which have been identified previously by Mahairas *et al*. (1996). They found that eleven of these 16 deletions were present in *M. tuberculosis* H37Rv but deleted from virulent *M. bovis* strains. These RDs represent unique deletions specific to the virulent *M. bovis* are RD3 -RD7, RD9 - RD13 and RD15. The remaining 5 RDs were present in the virulent *M. bovis*, but absent from some or all BCG strains. However, one of these 5 deletions, identified as RD1, was a region absent from all of the BCG strains that are currently used as tuberculosis vaccines, but present in all virulent strains of *M. tuberculosis* and *M. bovis* and other *M. tuberculosis*

complex members, suggesting that the deletion of RD1 was an original attenuating mutation which was established during the derivation of BCG (Behr *et al*., 1999).

Later studies confirmed that RD1 encoded gene product(s) (Table 1.4) are involved in a universal regulatory system associated with virulence (Pym *et al*., 2002; Lewis *et al*., 2003: Mustafa, 2014; Etna *et al*., 2015; Gcebe *et al*., 2016). Many *in vitro* and *in vivo* experiments have been performed to support this hypothesis, using the strategies of gene knock-in (complementation/introduction) and gene knock out (inactivation/disruption). For example, a knock-in study was done by Pym *et al*. (2002) who introduced the RD-1 region of *M. tuberculosis* into *M. bovis* BCG and *M. microti*. These studies revealed that the RD1 complemented strains were much more virulent, since they grew more vigorously, than their wildtype parents in SCID mice (Pym *et al*., 2002). In another study, BCG complemented with RD1 (BCG::RD1) displayed markedly increased virulence compared with parental BCG, but without reaching to the level of virulence of *M. tuberculosis* H37Rv (Majlessi *et al*., 2005).

Further study by Lewis *et al*. (2003) through knock out illustrated how deletion of RD1 affected *M. tuberculosis* H37Rv growth and survival in human monocyte-derived macrophages and in mice. The phenotype of H37Rv:ΔRD1 was very similar to the attenuated phenotype of *M. bovis* BCG. They suggested that the H37Rv:ΔRD1 strain could participate in developing a better TB vaccine because it derives from *M. tuberculosis* and not *M. bovis*. Further, Guinn *et al*. (2004) investigated the mechanism by which RD1 affects *M. tuberculosis* pathogenesis. They constructed five mutants disrupted at individual RD1 genes namely, *rv3870*, *rv3871*, *rv3874* (CFP-10), *rv3875* (ESAT-6) and *rv3876*. Interestingly, all the single-gene RD1 mutants showed an attenuation phenotype in C57BL/6 mice, equivalent to the *M. tuberculosis* H37Rv RD1 region mutant (H37Rv:ΔRD1) (Guinn *et al*., 2004).

Another substantial deletion is the deletion of a 7-bp segment in the *pks*15/1 gene from the reference strain *M. tuberculosis* H37Rv and its presence in Beijing strain *M. tuberculosis* HN878. Presence of this segment was potentially associated with the tendency of Beijing strain HN878 to generate an immunosuppressive phenolic glycolipid (Reed *et*

al., 2004) that contributes, via immunosuppressive cytokine production, to the greater virulence of this strain (Reed *et al*., 2004; Constant *et al*., 2002; Chaiprasert *et al*., 2006).

Locus No.	ORFs	Salient functions
1	*rv3871*	Hypothetical protein
2	*rv3872*	PE-family protein
3	*rv3873*	PPE-family protein
4	*rv3874*	CFP-10, Culture filtrate protein.
5	*rv3875*	ESAT-6, Early secretory antigen target.
6	*rv3876*	Hypothetical protein
7	*rv3877*	Hypothetical protein
8	*rv3878*	Hypothetical protein
9	*rv3879*	Hypothetical protein

Table 1.4: Genetic composition of RD1 in *M. tuberculosis*; ORFs proposed by Cole *et al*. (1998), and suggested functions were described by Cole *et al*. (1998); Domenech *et al*. (2001) and Cole, (2002).

More genomic deletions have been defined over time and used for designation and evolutionary analysis of the strains they were found in. For example, within the lineage of *M. tuberculosis* of Latin-American Mediterranean (LAM) family, some of its branches became highlighted by their RDs and were named after their geographic origin as RD-Rio, a predominant genotype in Rio de Janeiro which is defined by a large-scale 26.3 kb deletion (Lazzarini *et al*., 2007). This genomic deletion was considered to be linked with another deletion, RD174 and has been associated with higher levels of transmission and of multi-drug resistance but the data about their association with disease severity are contradictory (Lazzarini *et al*., 2007; Vasconcellos *et al*., 2014). Following the RD distribution and evolution in LAM lineage of *M. tuberculosis*, Mokrousov and his colleagues concluded that human mass migration was the main factor responsible for the phylogeography of *M. tuberculosis* over long periods of time (Mokrousov *et al*., 2016). RD149 and RD152 are other examples of RDs which were identified with high frequencies in Central Asian Strain

1 (CAS1) isolates and linked to host phenotypic variations (Kanji *et al*., 2011a). All CAS1 strains provoked higher levels of IL-10 and TNF-α secretion in monocytic cells than H37Rv. However, RD149 deleted CAS1 strains provoked more secretion of TNF-α in host cells than those without deletions with important clinical consequences and were associated with reduced growth (Kanji *et al*., 2011b).

Experimentally, some studies reported that gene deletions may account for increasing the virulence of pathogenic mycobacteria. For example, the deletion of the *pknH* gene from *M. tuberculosis* was associated with a higher bacterial load in BALB/c mice (Papavinasasundaram *et al*., 2005). Likewise, deletion of *hspX* (*acr*, *rv2031c*), that encodes a 16-kDa α-crystallin-like protein had markedly increased bacillary growth following infection of BALB/c mice compared to the wildtype strain (Hu *et al*., 2006). Also, it has been reported that deletion of *dosR* (also called *devR*), the gene controlling *hspX*, was associated with more rapid deaths among the SCID mice than those infected with the wildtype *M. tuberculosis* H37Rv strain (Parish *et al*., 2003).

One of the most important naturally occurring gene deletions within the genome of *M. tuberculosis* was reported in an outbreak strain in Leicester city in 2001. Eight genes were confirmed to be deleted from the strain, which was named as *M. tuberculosis* CH (Shafi *et al*., 2002; Rajakumar *et al*., 2004) and the strain is described in the next section (Section 1.9).

1.9. *Mycobacterium tuberculosis* CH, tuberculosis outbreak strain in Leicester city, 2001.

M. tuberculosis CH strain has caused the largest school tuberculosis outbreak ever in the UK. The index patient was a 14 year-old student who was diagnosed with sputum smear-positive tuberculosis only after complaining of a chronic cough for 9 months (Ewer *et al*., 2003). Subsequently, a survey and diagnostic procedure by the health authorities of the whole school attendants and relatives was done by TST (Section 1.2.3). Furthermore, the exposure to *M. tuberculosis* was confirmed using investigation based on ELISPOT

assay analysis (Section 1.2.4.3) that provided more accuracy than TST for diagnosis of persons who had latent TB infection (Ewer *et al*., 2003). 1128 school attendants, and family contacts with the index patient (Ewer *et al*., 2003; Rajakumar *et al*., 2004) were tested. The investigations identified 77 cases of active disease diagnosed with primary TB within one year and 254 cases of latent tuberculosis (Rajakumar *et al*., 2004). Out of 77 diagnosed as primary tuberculosis, 17 were culture-positive with the remaining diagnosed using standard clinical criteria (Newton *et al*., 2006). The number (77 cases of active disease) represented 23.3% of the total diagnosed by the TST as TB infected. Thus, development of active primary disease in the Leicester city outbreak seemed noticeably higher than the usually reported 5-10% risk (Ewer *et al*., 2003).

1.9.1. Genotypic characteristics of *M. tuberculosis* CH strain.

Molecular analysis of the outbreak strain initially used DNA hybridisation microarray to determine the differences from the H37Rv reference strain (Shafi *et al*., 2002). Results of DNA microarray analysis identified 11 open reading frames (ORFs) that were possibly deleted from the sequence of strain CH compared with the H37Rv sequence. These potential deletions included 3 singles and 4 adjacent pairs of ORFs distributed in 7 different loci on the CH chromosome (Shafi *et al*., 2002).

Accordingly, 5 loci of the 7 likely genomic deletions identified by microarray in CH were confirmed by carrying out PCR analysis using primers flanking the deletions. The primers used were chosen from those previously designated with ORFs on the *M. tuberculosis* H37Rv microarray (Rajakumar *et al*., 2004; Stewart *et al*., 2002). PCR analyses carried out for both H37Rv and CH DNA using these primers confirmed the existence of five deletions ranging in size from 0.8 to 2.0 kb. These loci were two singles (*rv0180* and *rv1519*) and three pairs (*rv1995-rv1996*, *rv3516-rv3517* and *rv3738-rv3739*) (Table 1.5) (Rajakumar *et al*., 2004).

Investigation of the 6th potentially deleted locus harbouring the *qor* gene (*rv1454*) showed the same size fragment of 2.4 kb with both CH and H37Rv sequences, indicating

that this locus was present and not deleted from CH strain (Rajakumar *et al*., 2004). Also, PCR examination of the 7th locus having the *esxR-esxS* tandem genes (*rv3019-rv3020*) revealed fragments of about 7 kb, with both CH and H37Rv sequences; this failed to verify the deletion (Rajakumar *et al*., 2004).

The five PCR panel used to individually investigate these loci was together named as the genome level-informed PCR (GLIP) analysis. The results of the GLIP assay were confirmed by sequencing analysis of both CH and H37Rv amplicons. The expected change in PCR product size for each deletion was consistent with the sizes showed by gel electrophoresis (Rajakumar *et al*., 2004).

Genomic deletions in CH and present in H37Rv		Proposed gene products
Single	*rv0180*	Probable trans-membrane protein, cell wall and cell processes
Single	*rv1519*	Hypothetical protein
Pair	*rv1995*	Hypothetical protein
	rv1996	Conserved hypothetical protein. Similar to universal stress protein (USP)
Pair	*rv3516 (echA19)*	Possible enoyl-coA hydratase (echA19), Lipid metabolism
	rv3517	Conserved hypothetical proteins
Pair	*rv3738 (PPE66)*	PPE-Family protein
	rv3739 (PPE67)	PPE-Family protein

Table 1.5: Genomic deletions in the *M. tuberculosis CH* strain compared with the reference strain *M. tuberculosis* H37Rv that confirmed by GLIP analysis (Rajakumar *et al*., 2004; Cole *et al*., 1998).

Complete genotype investigations identified that *M. tuberculosis* CH strain is characterised by the presence of a deletion region (*rv1519*) within its genome. This large polymorphism was coincident with RD750 (Table 1.3), which is a relatively ancient polymorphic region that defines the East African-Indian lineage or the Central Asian lineage (CAS) (Rajakumar *et al*., 2004; Newton *et al*., 2006; Cheah *et al*., 2010; Gagneux

et al., 2006; Gagneaux and Small, 2007). Hence, such an overview is important to give an indication of the history and evolutionary steps of the *M. tuberculosis* CH strain.

1.9.1.1. Evolutionary steps of *M. tuberculosis* "CH strain'' and evidence that *rv1995* and *rv1996* were the most recent gene deletions of this strain.

Although, horizontal gene transfer plays an important role in the evolution of bacteria, *M. tuberculosis* exhibits almost no horizontal gene exchange (Supply *et al*., 2003; Gutacker *et al*., 2006; Veyrier *et al*., 2009). Therefore, genomic regions that have been lost from a particular strain cannot be reacquired, which indicates that genomic deletions will be maintained clonally. Consequently, genomic deletions (i.e. LSPs) can be used to type and determine strain lineages of *M. tuberculosis* (Brosch *et al*., 2002; Gagneaux and Small, 2007; Hirsh *et al*., 2004; Mostowy *et al*., 2004; Rajakumar *et al*., 2004; Alland *et al*., 2007; Nebenzahl-Guimaraes *et a*l., 2016). Unique genomic deletions have been exploited to provide accurate genetic markers for molecular epidemiological, evolutionary and phylogenetic analysis of global *M. tuberculosis* complex (Brosch *et al*., 2002; Rajakumar *et al*., 2004; Gagneaux and Small, 2007; Nebenzahl-Guimaraes *et al*., 2016).

The previously mentioned GLIP assays that identified the CH strain deletions were used for subsequent analysis of other *M. tuberculosis* isolates (Rajakumar *et al*., 2004). Fifty six isolates were investigated including the CH outbreak index patient isolate, 12 other outbreak associated isolates**,** and 43 non-outbreak associated isolates obtained from patients over the preceding 2 years of the outbreak (Rajakumar *et al*., 2004).

The final outcomes of the GLIP analysis classified the investigated isolates according to presence or absence of deletion loci into five groups from DT1 to DT5 (deletion type, DT) (Table 1.6). DT1 was characterised by the absence of all five deletions. DT2, DT3 and DT5 contained variable numbers of the deletions on their genomes. DT3 was found to contain all deletions of interest except the *rv1995-rv1996* locus. However, the 13 isolates including the index patient isolate, and those from patients with strong contact to the index patient revealed existence of all five deletions. This group was assigned as deletion type 4 (DT4). These findings indicated that no one of the 43 non-outbreak

isolates displayed all of the 5 deletions. Consequently, GLIP analysis revealed that the five loci deletions (DT4) were unique to the CH outbreak strain. Furthermore, this technique revealed that the *rv1995-rv1996* locus (Figure 1.10) was unique to the CH outbreak strain and the most recent gene deletions acquired by this strain (Rajakumar *et al*., 2004).

Presence or absence of target ORF						No. of initial outbreak-associated and retrospective isolates
DT	*rv1519*	*rv0180*	*rv3516-rv3517*	*rv3738-rv3739*	*rv1995-rv1996*	
DT1	+	+	+	+	+	23
DT2	–	+	–	–	+	15
DT3	–	–	–	–	+	4
DT4	–	–	–	–	–	13
DT5	–	+	–	+	+	1

Table 1.6: GLIP assay of *M. tuberculosis* strains isolated from Leicester area patients. The (+) symbol indicates the presence and (–) symbol indicates the absence of the investigated locus in the corresponding isolate (Rajakumar *et al*., 2004).

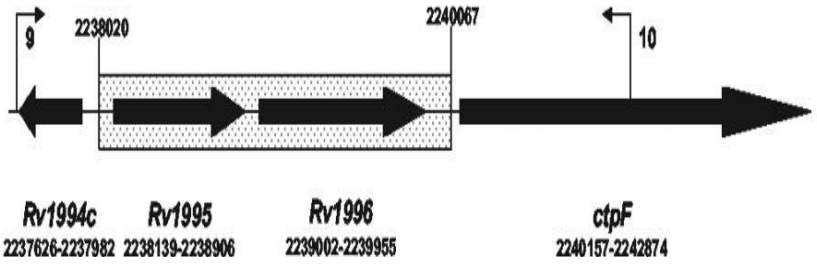


Figure 1.10: Locus of the *rv1995* and *rv1996* genes on the *M. tuberculosis* strain H37Rv. The extensions of each ORF are represented below the corresponding gene according to the accession number of H37Rv complete genome AL123456.1. The shaded box points to the deleted region in CH. The curved 9 and 10 arrows show the approximate position of the locus specific forward and reverse GLIP primer binding sites respectively (Rajakumar *et al*., 2004).

Since these polymorphisms represent key points in population-specific adaptations of the pathogen, it implies that they also may have important phenotypic effects. The discovery of deletions in the CH outbreak isolates highlighted that gene deletions may represent a major driver of genome diversity in this species that may lead to increase virulence and transmissibility of *M. tuberculosis* (Rajakumar *et al*., 2004; Newton *et al*., 2006; Reed *et al*., 2004).

1.9.2. Phenotypic characteristics of *M. tuberculosis* CH strain.

Virulence of *M. tuberculosis* strains generally is verified by the variation in their survival inside experimentally infected guinea pigs or mice (North *et al*., 199; Palanisamy *et al*., 2008; Reiling *et al*., 2013). With *M. tuberculosis* CH, animal studies using severe combined immunodeficiency (SCID) mice have revealed that it was highly aggressive in animal infection. It killed SCID mice almost twice as rapidly when compared to H37Rv indicating that CH strain is more virulent and rapidly lethal to SCID mice (UK Research and Innovation Project, G0300403).

Newton and colleagues (2006) investigated virulence associated *in vitro* traits of the CH strain that might explain its capacity to cause the outbreak. In broth medium, they found that the *M. tuberculosis* CH strain grew less rapidly than the reference strains *M. tuberculosis* H37Rv and *M. tuberculosis* CDC 1551. The CH strain was less resistant to low pH and to hydrogen peroxide (H_2O_2) than CDC1551 and H37Rv. However, there were no differences observed in susceptibility of CH, CDC1551 and H37Rv to nitric oxide (NO•) (Newton *et al*., 2006). Also, it has been found that CH was comparable to H37Rv and CDC1551 in its capability to grow in human monocyte-derived macrophages (Newton *et al*., 2006). Furthermore, it was found that the CH strain stimulated less production of IL-12p40 (Inflammation-inducing cytokine) (Newton *et al*., 2006) in comparison with H37Rv. In contrast, CH strain stimulated more production of anti-inflammatory IL-10 and IL-6 from MDMs than H37Rv (Newton *et al*., 2006). Although, the CH strain was less resistant to hydrogen peroxide and acid stresses that may decrease its fitness, it could transmit

extensively within the school environment in Leicester, in 2001. One possibility was that the CH strain compensated for the deduced microbial attenuation by skewing the innate immune response via suppression of phagocytes (Newton *et al*., 2006; Rajakumar *et al*., 2004). The most interesting observation of the CH strain was the implication of the gene deletions in increasing anti-inflammatory IL-10 and IL-6 secretion. However; which gene deletion/deletions was/were behind the apparent microbiological attenuation and the increase of IL-10 secretion was unknown.

1.9.3. The *rv1995* and *rv1996* genes.

The *rv1995* gene is 768 bp in size and encodes a hypothetical protein product with unknown function (Cole *et al*., 1998). Very few data are available about the *rv1995* gene and its protein. However, it was reported that it is likely to be a membrane protein and annotation reveals similarity with Hemerythrin-like proteins (Veyrier *et al*., 2009). In a study performed by Veyrier *et al*. (2009) using bioinformatics tools, they found that 137 genes showed evidence of horizontal gene transfer (HGT) acquisition such as presence of phage or plasmid sequences at different evolutionary stages. The *rv1995* gene was one of these genes predicted to show evidence of HGT (Veyrier *et al*., 2009).

Regarding the *rv1996* gene, it was described as a latency gene (Park *et al*., 2003; Ohno *et al*., 2003) with a size of 954 bp (Cole *et al*., 1998), which encodes a universal stress protein (USP) in *M. tuberculosis* (Ohno *et al*., 2003) and is located in a deletion hot spot, suggesting a potential association with drug resistance (Tsolaki *et al*. 2004; Hu *et al*., 2015). USPs were first identified in *E. coli* and are up-regulated under several stress conditions to perform undefined functions (Kvint *et al*., 2003; Nachin *et al*., 2005, Hingley-Wilson *et al*., 2010). USPs are a group of proteins whose expression is enhanced several folds when cell survival is challenged by stressful circumstances such as heat shock, nutrient starvation, oxidants, acids, and antibiotics or DNA damaging agents (O'Toole and Williams, 2003; Siegele, 2005). This class of proteins is suggested to be important in the resistance of bacteria to stress and prolonged arrested growth in mycobacteria (O'Toole and Williams, 2003).

In the *M. tuberculosis* genome, 8 genes are predicted to encode USPs and, of these, six (including the *rv1996* gene) belong to the *dosR* dormancy regulon suggesting that USPs play important roles in *M. tuberculosis* persistence and latency (Park *et al*., 2003; Ohno *et al*., 2003; Boon and Dick, 2012). The *dosR* regulon genes are stimulated under stress conditions such as hypoxia and nitrogen intermediates (Park *et al*., 2003; Voskuil *et al*., 2008; Shiloh *et al*., 2008; Boon and Dick, 2012). It is also induced in IFNγ-stimulated macrophages (Schnappinger *et al*., 2003; Lin and Ottenhoff, 2008). The induction of this regulon has an essential role in the dormancy stage of *M. tuberculosis* under low oxygen tension and other stress conditions (Rustad *et al*., 2008).

In a recent study, the relationship between the latency-related gene, *bcg2013*, and Isoniazid (INH) resistance was investigated. The *bcg2013* region in *M. bovis* BCG was identical to the *rv1996* region of *M. tuberculosis* H37Rv. It was found that *bcg2013* overexpression raised sensitivity to INH in comparison to the wildtype strain as a result of an increase in KatG protein levels under the effect of an increase in protein levels of mycobacterial USP (Hu *et al*., 2015).

1.10. The aim of the present study.

Mycobacterium tuberculosis strain CH caused the largest recorded school outbreak of tuberculosis ever documented in the UK (Shafi *et al*., 2002; Rajakumar *et al*., 2004). This outbreak strain was reported previously to possess five confirmed genomic deletions (*rv0180, rv1519, r1995-rv1996, rv3516-rv3517* and *rv3738-rv3739*) compared with the reference sequenced strain H37Rv (Rajakumar *et al*., 2004). A large project ensued to study the functional properties of these deletions and to determine which of these deletions was associated with the propensity of the outbreak strain (UK Research and Innovation Project, G0300403).

The research described here focused on the *rv1995-1996* locus as a part of this major project. While the other loci were found in different frequencies in the non-outbreak strains, the *rv1995/1996* locus deletion was unique to the CH strain and was not found in any of the non-outbreak strains (Rajakumar *et al*., 2004). This could be an evolutionary

clue that these two genes were the last deletions within the genome of the *M. tuberculosis* CH outbreak strain.

The main aim of the current study was to investigate if the hypothesis that the *rv1995* and *rv1996* gene deletions, particularly the *rv1996* gene which encodes a universal stress protein, contribute to the virulence of the Leicester area strain is correct. Therefore, a series of experiments related to knocking-in and knocking out of the individual target genes was carried out.

This study was undertaken to achieve the following objectives:

(1) To construct expression vectors carrying the target genes (*rv1995* and *rv1996*) separately. Since direct study with *M. tuberculosis* requires a biosafety level 3 laboratory (Category 3 laboratory), the expression vectors constructed in this study were sent to Imperial College in order to be introduced into the CH strain.

(2) To construct suicide vectors carrying the disrupted genes (*rv1995* and *rv1996*) separately.

(3) To investigate whether the *rv1995* and *rv1996* genes are present in *M. bovis* BCG and *M. smegmatis*.

(4) To introduce the suicide vectors carrying the disrupted genes into *M. tuberculosis* H37Rv and into *M. bovis* BCG in order to obtain recombinants with disrupted genes.

(5) Since the structural organization of the *bcg2012/bcg2013* region in *M. bovis* BCG was identical to the *rv1995/rv1996* region of *M. tuberculosis* H37Rv, the low pathogenicity strain *M. bovis* BCG was used as a model for studying the *rv1995* and *rv1996* through knock-out experiments. The study aimed to construct a new mutant from the obtained BCG recombinants with the disrupted genes replacing the intact genes of the mycobacterial chromosomes by homologous recombination.

(6) To investigate whether or not the disruption (knock out) of the target gene resulted in altered *in vitro* growth rate or sensitivity to hydrogen peroxide (H_2O_2), sodium nitrite ($NaNO_2$) and acid stress (low pH).

CHAPTER 2:

MATERIALS AND METHODS

2.1. Basic microbiology laboratory techniques.

2.1.1. Chemicals.

Except where indicated, chemicals and materials were purchased from Sigma Biochemical Company Ltd, UK, BDH Limited, UK or Fisher Scientific, UK. Growth media were purchased from Oxoid Limited, England and Difco (BD), USA. Enzymes, enrichment supplements and antibiotics were purchased from Sigma Biochemical Company Ltd, UK, Invitrogen UK, and New England Biolabs, UK. Primers were purchased from Tagn Newcastle Ltd, England and MWG Biotech, UK. All, except where indicated, were stored at room temperature.

2.1.2. Sterilisation.

Sterilisation of culture media, glass wares and all materials which can resist heating were performed by steam under pressure using autoclave at 121^{o}C for 20 min. All materials which can be degraded by autoclaving were sterilised by mechanical sterilisation using filtration by 0.2 μm Milipore filters; Acrodisc Syringe Filters (Pall Life Sciences, USA) or funnel shaped filtration units (Nalgene Nunc International, USA).

2.1.3. Preparation of growth media.

Media used in the current study were prepared as described by the manufacturer's instructions or as previously described (Hatfull and Jacobs, 2000), using distilled water. After sterilisation, all media were kept at room temperature, except those that contained enrichment supplements or antibiotics: they were stored at 4^{o}C. The compositions of media are given in tables 2.1 - 2.4.

2.1.4. Preparation of enrichment solutions.

Middlebrook albumin-dextrose-catalase (ADC) and albumin-oleic acid-dextrose-catalase (OADC) enrichment supplements that were used for growth of mycobacterial cells were prepared as described in table 2.5. Enrichment solutions were incubated at 37^{o}C for overnight to detect possible contamination, followed by storing at 4^{o}C (Hatfull and Jacobs, 2000).

Table 2.1: Recipes of Luria media (Hatfull and Jacobs, 2000).

Luria medium	Contents
Luria broth (LB)	5 g tryptone (Oxoid), 2.5 g yeast extract (Oxoid), 2.5 g NaCl and, then distilled H_2O was added to make up a final volume of 500 ml.
Luria broth with Tween 80	After autoclaving and cooling of Luria broth, 1.25 ml of sterile 20% (v/v) Tween 80 (Section 2.1.5) was added to give a final concentration of 0.05% (v/v).
Luria agar (LA)	7.5 g Bacto-agar (Oxoid) was added to the Luria broth contents and then distilled H_2O was added to make up a final volume of 500 ml a final concentration of 1.5% (w/v) agar.
Luria agar with sucrose	After autoclaving and cooling to 46-50^oC, 100 ml containing 50 g sucrose, sterilised by filtration (Section 2.1.2) was added to make up the final volume of 500 ml to give a concentration of 10% (w/v) sucrose.

Table 2.2: Recipes of Middlebrook media.

Middlebrook media	Contents
Middlebrook 7H9 broth	2.4 g Middlebrook 7H9 broth powder (Difco), 1 ml glycerol and, then distilled H_2O was added to make up to 450 ml. After autoclaving and cooling to 45^oC or less, 50 ml of filtered albumin-dextrose-catalase (ADC), (Section 2.1.4) was added to make up a final volume to 500 ml, and then 1.25 ml of sterile 20% (v/v) Tween 80 (Section 2.1.5) was added for a final concentration of 0.05% (v/v).
Middlebrook 7H11 agar	10.5 g Middlebrook 7H11 agar powder (Difco), 2.5 ml glycerol. Distilled H_2O was added to 450 ml. After autoclaving and cooling to 46-50^oC, 50 ml of filtered albumin-oleic acid-dextrose-catalase (OADC), (Section 2.1.4) was added to make up a final volume to 500 ml.

Table 2.3: Recipe of Sauton liquid medium (Hatfull and Jacobs, 2000).

Medium name	Contents
Sauton Liquid Medium	0.5 g KH_2PO_4 0.5 g $MgSO_4$ 4.0 g L-Asparagine 0.05 g Ferric ammonium citrate 2.0 g Citric acid 0.1 ml 1% (w/v) $ZnSO_4$ 60 ml Glycerol Distilled H_2O was added to make up to 900 ml, and then the pH was adjusted to 7.0. After autoclaving and cooling, 2.5 ml of sterile 20% (v/v) Tween 80 was added for a final concentration of 0.05% (v/v).

Table 2.4: Recipe of SOC medium (Promega, 2003).

Medium name	Contents
SOC medium	4 g Tryptone (Oxoid) 1 g Yeast extract (Oxoid) 0.1 g NaCl 2 ml of 250 mM KCl 40 μl of 5M NaOH Distilled water was added to make up to 200 ml. After autoclaving (before use), 200 μl of filtered 1M glucose and 50 μl of autoclaved 2M $MgCl_2$ were added to 10 ml of broth.

Table 2.5: Recipes of the enrichment supplements (Hatfull and Jacobs, 2000).

Enrichment supplements	Contents
Albumin-dextrose-catalase supplement (ADC)	5 g Bovine albumin fraction. 2 g D^+ Glucose 3 mg Beef catalase Distilled H_2O was added to make up to 100 ml. The solution was sterilised by filtration through 0.2 μm Milipore filter.
Oleic acid-albumin-dextrose-Catalase supplement (OADC)	5 g Bovine albumin fraction 2 g D^+ Glucose 4 mg Beef catalase 0.85 g NaCl 0.02 g Oleic acid Distilled water was added to make up to 100 ml. The solution was sterilised by filtration through 0.2 μm Milipore filter.

2.1.5. Preparation of Tween 80.

Tween 80 at 20% (v/v) was prepared as described previously (Hatfull and Jacobs, 2000). The solution was mixed by swirling to dispense the Tween 80 completely. After that, the solution was sterilised with 0.2 μm Acrodisc Syringe Filter (Section 2.1.2). 2.5 ml of 20% (v/v) Tween 80 was used to be added to 1000 ml of mycbacteria liquid medium to obtain a final concentration of about 0.05% (v/v). Tween 80 was added to prevent clumping of mycobacterial cells in liquid medium.

2.1.6. Acid fast staining.

Acid fast staining was used to confirm the presence of mycobacteria. First a drop of water was placed on a clean slide. A small amount of the bacterium was removed from the surface of agar media and mixed with the water or a drop from fresh culture. Using a loop, the mixture was spread over the entire slide to form a thin film. The preparation was

allowed to dry. The slide was passed through the flame of the Bunsen burner 3 or 4 times to do heat fixation. The bacterial smear was flooded with carbol-fuchsin solution (0.3 g of basic fuchsin dissolved in 10 ml of absolute ethanol (90-95%) was added to 90 ml of a 5% (v/v) aqueous solution of phenol) for 5-7 min and then the slide was washed with tap water. The slide was decolourised twice with 3% (v/v) acid-alcohol (3 ml of absolute HCl was added to 97 ml of absolute ethanol (90-95%)). After completing the decolourisation, the slide was washed well with water. The smear was counterstained by adding methylene blue (0.3 g of methylene blue chloride in 100 ml distilled water) for 1 min. Finally, the slide was washed carefully with running water and then was left for air drying, without blotting. Observation was done using a 100X oil immersion objective. Acid-fast cell wall stained red (Hatfull and Jacobs, 2000; Somoskovi *et al*., 2001).

2.1.7. Preparation of antibiotics.

Antibiotics used in the current study were prepared as described in the supplier's instructions (Sigma Biochemical Company Ltd, UK) using nanopure H_2O. All the prepared antibiotics were sterilised using 0.2 μm Acrodisc Syringe Filters. Antibiotics used and their concentrations are given in table 2.6.

Table 2.6: Concentrations of antibiotics.

Antibiotic name	Stock solution (Concentration in nanopure H_2O)	Concentration in medium	Bacterium used
Hygromycin	150 mg/ml	150 μg/ml	*E. coli*
		150 μg/ml	*M. tuberculosis*
		150 μg/ml	*M. bovis BCG*
Kanamycin	20 mg/ml	20 μg/ml	*E. coli* DH5α
		20 μg/ml	*M. tuberculosis*
		20 μg/ml	*M. bovis BCG*
Ampicillin	100 mg/ml	100 μg/ml	*E. coli*

2.1.8. Preparation of bacterial stock cultures.

Bacterial cultures for short term storage were maintained onto agar plates or onto agar slopes. For longer term storage, *E. coli* DH5α cultures were maintained by adding 1 volume of 15% sterile glycerol to a log phase culture with an OD of 0.5 at 600 nm (Sambrook *et al*., 1989). Alternatively, 10 ml of *E. coli* DH5α broth culture was centrifuged and then the pellet was re-suspended in 1 ml of 10% (v/v) sterilised glycerol. Mycobacterial cells were maintained by adding 1 volume of a sterile solution consisting of 65% (v/v) glycerol, 0.1 M $MgSO_4$ and 0.025 M Tris-HCL (pH 8.0) to an equal amount of log phase culture with an OD of ~0.6 at 600 nm. All cultures containing glycerol as a cryo-protectant were prepared in microcentrifuge tubes and then were stored at –70ºC (Hatfull and Jacobs, 2000).

2.1.9. Cultivation of *Mycobacterium bovis* BCG.

Before starting experiments with *M. bovis* BCG, stock cultures that had been stored in glycerol solution at -70ºC or in cultures with no glycerol up to 3 weeks at 4ºC, were cultured by streaking onto Middlebrook 7H11 agar supplemented with OADC and incubated at 37ºC for 3 to 4 weeks until single colonies were visible. Then, experiments were started via transfer of colonies to 7H11 agar plates supplemented with OADC or 7H9 supplemented with ADC and 0.05% (v/v) Tween 80 and incubated at 37ºC as before.

2.1.10. Optical density measurements.

To measure optical density of bacterial growth culture, the optical density (OD) of 1 ml of culture was measured at 600 nm (Hatfull and Jacobs, 2000) using an Ultrospec spectrophotometer (Pharmacia Biotech). 1 ml of fresh medium was used as a blank control.

2.1.11. Viable count enumeration of bacteria.

Viable count (CFU/ml) of mycobacterial cells was determined using the drop-plate method of Hoben and Somasegaran (1982). Ten-fold serial dilutions of 20 μl of culture in

180 µl of 0.01% (v/v) Tween 80 were done in sterile 96 well microtitre plates. 50 µl of each bacterial dilution was then cultured by pipetting onto 7H11 agar plates supplemented with OADC. All plates were left to dry at room temperature, then sealed with nescofilm tape and incubated at 37^{o}C from 3 to 4 weeks. The number of colonies was counted at an appropriate dilution and colony forming units (CFU/ml) were calculated using the following formula (Hoben and Somasegaran, 1982):

CFU/ml = number of bacterial colonies x 20 x dilution factor

2.1.12. Growth rate calculations.

Growth rate constant calculation of bacteria was done based on the formula adopted by Madigan *et al*. (2003).

$$g = \frac{t}{n} = \frac{t}{3.3\,(\log N - \log N0)}\ hr$$

$$K = \frac{In}{g} = \frac{0.693}{g}\ hr^{-1}$$

Where,

g = generation time (doubling time).

t = time interval units.

n = number of generations in t.

N = final cell number.

N0 = initial number.

K= growth rate constant.

In = natural log.

L =log

2.2. Category III microbiology laboratory techniques.

Mycobacterium tuberculosis is a hazardous pathogen and consequently all work related to this micro-organism was carried out in a containment level 3 laboratory. For the same reason, the Category 3 suite was supplied with dedicated equipment.

2.2.1. Centrifugation.

When *M. tuberculosis* cultures where centrifuged, only polypropylene tubes were used (Hatfull and Jacobs, 2000). Not more than 30 ml of bacterial culture in each 50 ml polypropylene tubes (Simport, Canada) were used. They were balanced, sealed with parafilm tapes and then swabbed with fresh 2% (v/v) stericol, before the tubes were placed gently in appropriate buckets. The buckets were closed and the outside swabbed with fresh 2% (v/v) stericol, before removal from the Class 1 cabinet to the centrifuge. All centrifugation processes were done at not more than 2000 g (Hatfull and Jacobs, 2000). After centrifugation, the buckets were transferred from the centrifuge into the Class 1 cabinet, taking care when opening the buckets and collecting the tubes. Centrifugation was done using a bench top Micro-Centaur Centrifuge (MSE, UK).

2.2.2. Incubation of mycobacteria.

30 ml universal tubes, with no more than 10 ml of culture, and 500 ml polycarbonate flasks with no more than 100 ml of liquid medium, were used to grow *M. tuberculosis*. Tubes and flasks, as well as agar plates, were sealed with parafilm tapes. All were doubled-bagged in sealed polythene bags and their surfaces were decontaminated by swabbing with 2% (v/v) stericol before removing from the Class 1 cabinet to an incubator for incubation or to a fridge for storing.

2.2.3. DNA techniques.

All techniques related to DNA preparations, including genomic DNA extraction (Section 2.3.4.2.2) and plasmid DNA electroporation (Section 2.3.6.2) were done in Class 1 cabinet of the category 3. For electroporation, the shock pod was placed in the cabinet with the power pack outside of the cabinet. After pipetting the mixture of competent cells and the desired plasmid into the electroporator cuvette, it was sealed with autoclave tape and then parafilm, before placing into the electroporator shock pod stage. Also, when electroporating of DNA molecules, it was not able to do more than two attempts in order to prevent the equipment (electroporator) from heating. This was due to the cell wall thickness of mycobacterial cells. After electroporation, cuvettes were discarded in pots containing 2% (v/v) stericol.

2.3. Molecular microbiology tools and techniques.

2.3.1. Bacterial strains.

The bacterial strains used in the present study are listed in table 2.7. *Escherichia coli* DH5α and *Mycobacterium smegmatis* MC2 155 were grown in Luria broth and onto Luria agar at 37ºC. *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* (all strains) were grown in 7H9 broth supplemented with ADC and 0.05% (v/v) Tween 80, and onto 7H11 agar supplemented with OADC at 37ºC.

2.3.1.1. *Escherichia coli* DH5α (*E. coli* DH5α).

E. coli DH5α is a mutant of wildtype *E. coli*. It was developed for laboratory purposes as a cloning strain that enable high-efficiency transformations, which means its ability to take up extra-cellular DNA and express genes encoded by it. *E. coli* DH5α carries the mutated *lacZ* gene with deleted sequence (*lacZ*ΔM15). Therefore, it allows for blue/white selection for recombinant cells. Consequently, it was used as a cloning strain in the present study (Taylor *et al*., 1993; Sambrook *et al*., 1989; De Togni *et al*., 1988).

Table 2.7. List of bacteria.

Bacteria	Relevant features	Reference or source
E. coli DH5α	A laboratory strain	Hanahan, (1983)
M. smegmatis MC2 155	A laboratory strain	Snapper *et al*. (1990)
M. tuberculosis H37Rv	A laboratory strain	NCTC 7416
M. tuberculosis CH	A tuberculosis outbreak strain. Leicester 2001.	Rajkumar *et al.* (2004)
M. bovis BCG	An attenuated vaccine strain	Evans Medical Ltd.U.K.
M. bovis BCG *rv1996*::hyg^R	A mutant derivative of *M. bovis* BCG contains an insertion mutation of hyg^R in the *rv1996* gene.	Current study

2.3.2. Plasmids used in cloning of mycobacterial genes.

Plasmids used within the present study are listed in table 2.8. *E. coli* and mycobactera, harbouring vectors containing a hygromycin resistance cassette, were grown in the presence of 150 μg/ml of hygromycin B. Bacteria harbouring vectors containing a kanamycin resistance cassette were grown in the presence of 20 μg/ml kanamycin. Bacteria harbouring vectors containing an ampicillin resistance cassette were grown in the presence of 100 μg/ml ampicillin.

Table 2.8: List of plasmids.

Plasmid	Relevant features	Reference or source
pSMT3	*E. coli*-mycobacteria shuttle vector with a promoter of the mycobacterial heat shock protein gene (*hsp60*), and carrying the hygromycin resistance gene.	O'Gaora *et al*. (1997)
pAAO1	Developed from pSMT3 for expressing the *rv1995* gene PCR product, downstream from *hsp60* promoter, containing hygromycin resistance gene. This plasmid was sent to Imperial College to be inserted into *M. tuberculosis* CH to make a complemented strain of *M. tuberculosis* CH with *rv1995*::hyg^R.	Current study
pAAO2	Developed from pSMT3 for expressing the *rv1996* gene PCR product, downstream from *hsp60* promoter, containing hygromycin resistance gene. *rv1996* with this plasmid was not able to express in *M. tuberculosis* CH (Imperial College). Therefore, an alternative one was constructed (pAAO3).	Current study
pAAO3	Developed from pSMT3 for expressing the *rv1996* gene PCR product, downstream from *hsp60* promoter, containing hygromycin resistance gene. This plasmid was also sent to Imperial College to be inserted into *M. tuberculosis* CH to make a complemented strain of *M. tuberculosis* CH with *rv1996*::hyg^R.	Current study

Table 2.8: List of plasmids (continued).

Plasmid	Relevant features	Reference or source
pGAOL17	A vector carrying *sacB* cassette (sucrose sensitivity) flanked by *Pac*I, and ampicillin marker, containing resistance to ampicillin.	Parish and Stocker, (2000)
pGEM-T Easy	PCR product cloning vector containing a *lacZ* promoter for gene expression, blue-white selection, and carrying the ampicillin resistance gene.	Promega
pASO1	359 bp PCR product of upstream region (fragment A) of *rv1995* was cloned into pGEM-T Easy, containing the ampicillin resistance gene.	Current study
pASO2	359 bp PCR product of downstream region (fragment B) from *rv1995* was cloned into pGEM-T Easy, containing the ampicillin resistance gene.	Current study
pASO3	1356 bp PCR product of complete hygromycin resistance gene from pSMT3 was cloned into pGEM-T Easy, containing the ampicillin resistance gene.	Current study
p1NIL	Suicide vector for gene manipulation carrying kanamycin cassette conferring resistance to kanamycin.	Parish and Stocker, (2000)
pASO5	Cloned PCR products of both fragments A and B (*rv1995*) were cloned into p1NIL, containing the kanamycin resistance gene.	Current study

Table 2.8: List of plasmids (continued).

Plasmid	Relevant features	Reference or source
pHSO1	718 bp PCR product of ligated fragment AB of *rv1995* was cloned into pGEM-T Easy, containing the ampicillin resistance gene.	Current study
pASO6	The cloned 718 bp ligated fragment AB of *rv1995* from pHSO1 was cloned into p1NIL, containing the kanamycin resistance gene.	Current study
pANO1	Suicide vector was developed from pASO6 to knock out the intact chromosomal *rv1995*. The hygromycin resistance cassette of pSMT3 cloned by pGEM-T Easy was inserted between the two fragments A and B from the *rv1995* gene and cloned into pASO6 conferring the kanamycin resistance gene.	Current study
pHSO2	904 bp PCR product of ligated fragment AB of *rv1996* was cloned into pGEM-T Easy, containing the ampicillin resistance gene.	Current study
pASO7	The cloned 904 bp ligated fragments AB of *rv1996* from pHSO2 was cloned into p1NIL, containing the kanamycin resistance gene.	Current study
pANO2	Suicide vector was developed from pASO7 to knock out the intact chromosomal *rv1996*. The hygromycin resistance cassette of pSMT3 cloned by pGEM-T Easy was inserted between the two fragments A and B from the *rv1996* gene and cloned into pASO7 conferring the kanamycin resistance gene.	Current study

2.3.3. Design of primers used in cloning of mycobacterial genes.

All primers were chosen manually from the corresponding DNA sequences and then they were checked for the correct alignment of sequences using www.ncbi.nlm.nih.gov-BLAST Sequence (Altschul and Gish, 1990). Proper selection of the PCR primers was critical. The designed primers were long enough, with different sequences except the matching area to avoid matching in other sites of the DNA (Perez-Pinera *et al*., 2006; Sambrook *et al*., 1989).

The annealing temperature (*Ta*) of each primer pair in each PCR reaction was estimated by determining the melting temperature (*Tm*) using the following formula (Brown, 2001): *Tm* = 4 x (G + C) + 2 x (A + T), since (G + C) is the total number of G and C nucleotides, and (A + T) is the total number of A and T nucleotides in the primer sequence. The *Tm* of the two primers was balanced by adding or removing some nucleotides. Annealing temperature must be low enough (but also high enough) to allow for hybridization of the primer to the DNA strand. If the temperature is too low, the primer might bind imperfectly, and if it is too high the primer might not bind (Saiki *et al*., 1988; Brown, 2001; Perez-Pinera *et al*., 2006). Consequently, the annealing temperature (*Ta*) for the primer pair was calculated as 4^{o}C lower than the estimated melting temperature of lowest primer *Tm* (Rychlik, 1995). Also, the annealing temperature (*Ta*) was determined using gradient PCR when needed (Section 2.3.5.5).

Primers that were used for DNA fragments cloning purposes had an appropriate restriction digestion sequences, also there were a number of A nucleotides were included at the start of designing primers for ligation with T tail cloning vector (Pinera, 2006; Promega). These restriction sequences, as well as the starting nucleotides were not involved in the calculation of melting temperature of the primers.

Primers were purchased from TAGN Ltd or MWG Bio Technology U.K. All purchased primers were diluted to a concentration of 100 pmol/µl (Stock solution). The primer concentration to be used in the PCR reactions was prepared by diluting 10 µl of the stock solution ten-times, using 90 µl of sterilised nanopure water, to obtain a final concentration of 10 pmol/µl. All diluted primers were stored at -20^{o}C. The nucleotide sequences of primers used in the current study are given in table 2.9.

Table 2.9: List of PCR primers and sequences in bold letters represent restriction enzyme target sequences.

Primer name	Primer sequence (5' to 3')	No of bases
	Primers to amplify the *rv1995* gene	
For-1	5`AAAA**AAGCTT**CGCATCTCACACGTCAGC 3`	18
Rev-1	5`AAAA**ATCGAT**AGGTTCGTTTGTTGGGC 3`	17
	Primers to amplify the *rv1996* gene	
For-2	5`AAAA**AAGCTT**GGCATCTACGACCTGAGCC 3`	19
Rev-2	5`AAAA**ATCGAT**GGCAGACACTGACGCCG 3`	17
	Alternative primers to amplify the *rv1996* gene	
For-3	5`AAA**GGATCC**GGGACGCCAATGTCA 3`	15
Rev-3	5`AAA**CTGCAG**TCAGGCCGGCACTGC 3`	15
	Primers to amplify the *rv1995* Fragment A.	
ForA-5	5`AAAA**AGTAGT**GTGGTGGCCAGCGGCGCTG 3`	19
RevA-5	5`AAAA**GGCGCGCC**TGTCCGTGGTAGTCCTCGA TGA 3`	19
	Primers to amplify the *rv1995* Fragment B.	
ForB-5	5`AAAA**GGCGCGCC**CGGACATCACCTCGGTC TTGC 3`	21
RevB-5	5`AAAA**AAGCTT**TTAGCTGGGGGTGAACTGGC 3`	20
	Primers to amplify the *rv1996* Fragment A.	
ForA-6	5`AAAA**AAGCTT**ATGTCAGCCCAACAAACG3`	18
ForA-6.6	5`AAAA**AAGCTT**ATGTCAGCCCAACAAACGAA CCT 3` (Alternative)	23
RevA-6	5`AAAA**GGCGCGCC**GCGTGGCGAACCAGGCTTG 3`	19

Table 2.9: List of PCR primers (continued).

Primer name	Primer sequence (5' to 3')	No of bases
	Primers to amplify the *rv1996* Fragment B	
ForB-6	5`AAAA**GGCGCGCC**CGCACGCGCCGGTTG 3`	16
RevB-6	5`AAAA**GGTACC**TCAGGCCGGCACTGCCGGA TCTT 3`	23
	Primers to amplify the Hygromycin Resistance Gene from pSMT3 sequence.	
For- Hyg	5`AAAA**GGCGCGCC**AAGAACTTCGTCGTGCG ACTG 3`	21
Rev- Hyg	5`AAAA**GGCGCGCC**TTCATCCATAGTTGCCTG ACTCC 3`	23
	Primers to amplify the ligated *rv1995* Fragments AB from p1NIL sequence.	
For-A-1	5' ATCGTTGTCAGAAGTAAGTTGG 3'	22
Rev-A-1	5' ATACGACTCACTATAGGGAGAC 3'	22
Rev-T-2	5' ACTGATGAATGTTCCGTTGC3'	20
	Primers to amplify the disrupted *bcg2012* (*rv1995*) and *bcg2013* (*rv1996*) genes.	
For- BCG 2011	5` TTCCCGCATCTCACACGTCAG 3`	21
Rev- BCG 2013	5` TCCGAGGTTCGTTTGTTGGGC 3`	21
For- BCG 2012	5` TTGGCATCTACGACCTGAGCC 3`	21
Rev- BCG 2014	5` TCCGAGGTTCGTTTGTTGGGC 3`	21
For- BCG Hyg	5` AAGAACTTCGTCGTGCGACTGT 3`	22
Rev-BCG Hyg	5` TCATCCATAGTTGCCTGACTCC 3`	22

Table 2.10: Nucleotide sequences of the primers and Taq Man probes (TMP) used to detect mRNA from *M. bovis* BCG genes.

Primer name	Primer sequence (5' to 3')	No of bases
	Primers and Taq Man probes used to detect mRNA of *bcg2013* in *M. bovis* BCG.	
For -13 BCG	5` ACGCGTGGAGCGACATG 3`	17
Rev- 13 BCG	5`CTTCGAGGTTTCTCCATTCGAT 3`	18
TMP- 13 BCG	5`CCTCGACTTTCCTAGGCTCAATTGGGC 3`	27
	* Primers and Taq Man probes used to detect mRNA of *sigA* in *M. bovis* BCG Housekeeping gene	
For- SigA BCG	5` AAACCATCTGCTGGAAGCCA 3`	20
Rev- SigA BCG	5` CGGCCGGTGTAGCGC 3`	15
TMP- SigA BCG	5` CCTGCGCCTGGTGGTTTCGC 3`	20

* Sequences for *SigA* primers and Taq Man probe were kindly provided by Professor Philip Butcher, St. George's Hospital Medical School, London.

Table 2.11: List of Restriction enzymes and target sequences.

Restriction enzyme	Restriction enzyme sequence (5' to 3')
*Hin*dIII	**AAGCTT**
*Cla*I	**ATCGAT**
*Sca*I	**AGTACT**
*Asc*I	**GGCGCGCC**
*Bam*HI	**GGATCC**
*Pst*I	**CTGCAG**
*Kpn*I	**GGTACC**
*Pac*I	**TTAATTAA**

2.3.4. Extraction of DNA from bacterial cells.

2.3.4.1. Extraction of plasmid DNA from *E. coli*.

Plasmid DNA extractions (Kado and Liu, 1981) using alkaline lysis was performed as described previously (Sambrook *et al*., 1989). The solutions used are shown in table 2.12.

Table 2.12: Solutions were used for plasmid DNA extraction.

Solutions (Stock solution)	Components	
Solution I	Glucose	50 mM
	Tris HCL pH 8.0	25 mM
	EDTA pH 8.0	10 mM
Solution II	NaOH	1 ml of 0.2 M
	SDS	0.5 ml of 10% (w/v)
	Distilled H_2O	3.5 ml
Solution III	Potassium acetate	60 ml
	Glacial acetic acid	11.5 ml
	Distilled H_2O	28.5 ml
Purification Solution	Phenol	25 ml
	Chloroform	24 ml
	Iso-amyl alcohol	1 ml
TE buffer	Tris-HCL pH 8.0	10 mM
	EDTA pH 8.0	1 mM

- Glucose solution was filtered through 0.2 μm funnel-shaped filtration units and then added to autoclaved solution I.
- Solution II was prepared fresh before use from a 2 M NaOH stock solution and 10% (w/v) SDS.
- All solutions were stored at room temperature, except the purification solutions which were stored at 4^{o}C.

2.3.4.1.1. Extraction of small scale plasmid DNA from *E. coli*.

Small scale plasmid DNA extraction using alkaline lysis was done as described by Sambrook *et al.* (1989) using the solutions given in table 2.12.

5 ml of L-broth was inoculated with a fresh single bacterial colony that contained the desired plasmid. The culture was incubated overnight at 37^{o}C with shaking at 200 rpm. 1.5 ml culture was harvested by centrifuging at 10000g for 1 minute using a microcentrifuge. The supernatant was discarded and the resulting bacterial pellet was re-suspended in 100 µl of ice-cold solution I and vortexed to disperse the bacterial pellet. The suspension was incubated at room temperature for 5 min, then 200 µl of fresh solution II were added and mixed well by inverting the tube 6-10 times gently. This was followed by incubation for 5 min on ice. 150 µl of ice-cold solution III was added and mixed gently by inverting the tubes for 10 seconds and immediately incubated on ice for at least 5 min. The tube was centrifuged in a microcentrifuge at 17000g for 10 min to remove the particular material. The supernatant was transferred to a fresh tube and an equal volume (450 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed slowly for 1 min to precipitate the DNA. The tube was incubated at room temperature for 10 min and centrifuged in a microcentrifuge at 17000g for 10 min. Then the upper layer was removed into a fresh tube and 1volume of chloroform: isopropanol (1:1) was added and centrifuged for 1 min at 17000g to remove the phenol. The supernatant was removed into fresh tube and 2 volumes of 100% ethanol were added, mixed well, then incubated at room temperature for 2 min. The plasmid DNA was collected by centrifugation at 17000g for 25 min in a centrifuge. The supernatant was decanted and the DNA pellet was washed in 500 µl of fresh 70% (v/v) ethanol, and then centrifuged at 17000g for 5 min. After decanting the supernatant, the pellet was left to dry at room temperature or incubated at 37^{o}C for 5 min to remove the residual ethanol. The DNA pellet was dissolved in 50 µl of TE buffer and then the RNA debris was removed by adding 2 µl of 20 µg/ml RNase and incubated for one hr at 37^{o}C.

2.3.4.1.2. Extraction of large scale plasmid DNA from *E. coli*.

Large scale plasmid DNA extraction using alkaline lysis was done as described by Sambrook *et al.* (1989) using the solutions are given in table 2.12.

A single colony of *E. coli* DH5α from a fresh overnight culture on L- agar was inoculated into 5 ml of L- broth and incubated overnight at 37ºC with shaking at 200 rpm. 500 ml fresh Luria broth in a 2 litre flask was inoculated with the 5 ml overnight culture and incubated overnight at 37ºC with shaking at 200 rpm. The bacterial cells were harvested by centrifugation at 7000g for 10 min in a Sorvall GSA centrifuge, the supernatant was decanted and the pellet was re-suspended in 25 ml of solution I containing 10 mg lysozyme and then incubated for 10 min at room temperature. 50 ml of solution II was added and mixed gently by inversion, to produce a clear lysate. Then 37.5 ml of solution III was added and mixed gently and incubated on ice for 10 min.

The preparation was centrifuged at 10000g for 30 min at 4ºC in a Sorvall GSA centrifuge to remove the cell debris. The supernatant was filtered through a 5 ml Gilson tip, plugged with polymer wool, into 0.6 volume isopropanol and mixed by inversion and incubated for 10 min at room temperature to precipitate the DNA. DNA was then collected by centrifugation at 10000g for 10 min in a Sorvall GSA centrifuge, at room temperature to prevent precipitation of salt. The supernatant was decanted carefully and the DNA pellet was washed in approximately 5 ml of fresh 70% (v/v) ethanol, dried and then, dissolved in 3 ml of TE buffer. 3 ml of cold 5 M LiCl was added to the 3 ml of DNA solution and mixed to precipitate the RNA. The RNA was pelleted by centrifugation at 17000g in a Sorvall SS-34 centrifuge for 10 min. The supernatant was added to an equal volume of isopropanol, mixed by inversion to precipitate the DNA again and pelleted by centrifugation at 17000g in a Sorvall SS-34 centrifuge then washed in 2 ml of 70% (v/v) ethanol and dried.

The resulting dried DNA pellet was dissolved in 500 µl of TE buffer containing 10 µg DNAse-free RNAse. The solution was incubated at 37ºC for 30 min including 10 min in water bath at 45ºC. After that, 1.6 M NaCl containing 13% (w/v) polyethylene glycol (PEG) was added and incubated on ice for 10 minutes and then centrifuged for 10 min at 17000g in a microcentrifuge. The supernatant was carefully poured off and the DNA pellet

was dissolved in 400 µl of TE buffer by incubating for 10 min in water bath at 45^{o}C. DNA was extracted by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Extractions were repeated until no white material could be seen. The phases were separated by centrifugation at 17000g for 5 min in a micro centrifuge. The aqueous upper layer was transferred to fresh tube and the residual phenol was removed by adding an equal volume of chloroform: isoamyl alcohol (24:1). DNA was precipitated by addition of 100 µl of 10 M ammonium acetate and 1 ml of 100% absolute alcohol and incubated at –20^{o}C for 30 min then pelleted by centrifugation at 17000g for 10 min. The pelleted DNA was washed in 70% (v/v) ethanol, then it was dried and dissolved in 100-200 µl of TE buffer.

2.3.4.1.3. Extraction of plasmid DNA from *E. coli* using QIA prep Spin Miniprep Kit Protocol.

A QIA prep Spin Miniprep Kit was obtained from Qiagen. Steps and centrifugation processes of this protocol were carried out according to the manufacturer's instruction at room temperature using a bench microcentrifuge.

This protocol was used when plasmid DNA extraction was to identify the recombinant cells that contained the desired plasmid or to extract a plasmid with a high level of purification for further DNA reactions; for example, ligation or restriction digestion.

Before use, the provided RNase A solution was added to buffer P1, mixed and stored at 4^{o}C. Absolute ethanol was added to buffer PE as advised on the bottle label. 1 to 1.5 of 5 ml overnight culture of *E. coli* in L- broth was collected by centrifugation at 10000g for 2 min (For more than 1.5 ml of culture, the bacterial cells were collected using a universal tube). Then the pellet was re-suspended in 1 ml of LB medium and transferred to a 1.5 ml microcentrifuge tube for collection, as described above. The supernatant was discarded and the pellet was kept. The pelleted bacterial cells were re-suspended in 250 µl of buffer P1 provided with RNase A. 250 µl of buffer P2 was added and mixed by inverting the tube gently 4-6 times in less than 5 min. After that, 350 µl of buffer N3 was added and

the solution was mixed immediately by inverting the tube gently 4-6 times. The microcentrifuge tube was centrifuged for 10 min at 17000g in a bench-top microcentrifuge. The supernatant was transferred by pipetting to a QIAprep spin column after sitting on its collection tube. The column then was centrifuged for 1 minute at 17000g to bind the DNA into the QIAprep spin column membrane. The flow-through was discarded and the QIAprep spin column placed onto its collection tube. 750 µl of buffer PE was added and centrifuged for 1 min at 17000g to wash the QIAprep spin column. The flow-through was discarded and the QIAprep spin column was placed onto its collection tube once for doing an additional round of centrifugation for 1 min at 17000g to remove any residual wash buffer. To elute the DNA, QIAprep column was placed into a fresh 1.5 ml microcentrifuge tube and 50 µl of nanopure H_2O was added to the centre of a QIAprep spin column. The column was left to stand for 1 min and centrifuged for 1 min at 17000g. The eluted DNA was used or kept at –20^{o}C until use.

2.3.4.1.4. Extraction of Plasmid DNA from *E. coli* using HiSpeed Plasmid Maxi Kit Protocol.

The HiSpeed Plasmid Maxi Kit (Qiagen) was used for preparing low copy plasmid DNA or when the plasmid DNA was required for sequencing or cloning processes. The kit was used according to the supplier's instructions (Qiagen) at room temperature.

First, the provided RNase A was added to solution buffer P1. One vial of RNase A solution per one bottle of buffer P1 to give RNase of 100 µg/ml. The solution was mixed and used or stored at 4^{o}C until use. Before use, buffer P2 was checked for SDS precipitation due to low storage temperature; occasionally it needed to be warmed to 37^{o}C to dissolve. Buffer P3 was prechilled to 4^{o}C.

A single colony from a freshly streaked selective plate was picked and inoculated into a starter culture of 5 ml L-broth medium containing the appropriate selective antibiotic. The inoculated medium was incubated for 8 hr at 37^{o}C, with vigorous shaking at 300 rpm. 250 ml of a selective L-broth medium was inoculated with 2.5 ml of the starter culture and incubated at 37^{o}C, overnight, with vigorous shaking at 200 rpm. The overnight culture was

harvested by centrifugation at 6000g for 15 min at 4ºC. The supernatant was removed and the bacterial pellet was frozen at - 20ºC for 24 h. After that, the pellet was re-suspended in 10 ml of buffer P1 containing RNase A, using a vessel that allowed complete mixing by swirling quickly. 10 ml of buffer P2 was added and mixed by swirling the vessel very gently 4-6 times before incubation at room temperature for 5 min. 10 µl of chilled buffer P3 were added to the lysate and mixed immediately, but very gently, 4-6 times. The mixture was transferred to Sorvall GSA tube and centrifuged for 10 min at 7000g. The supernatant was filtered from the residual cell debris using the barrelled QIAfilter Maxi Cartridge. To bind the DNA, the filtered solution was poured into the HiSpeed Maxi Tip and left to pass through the Tip membrane by gravity flow. 60 ml of buffer QC was applied to wash the HiSpeed Maxi Tip and the buffer allowed moving through the Tip membrane by gravity flow. The DNA was eluted with 15 ml of QF buffer into 30 ml propylene tube. Then it was precipitated by adding 10.5 ml (0.7 volumes) of isopropanol, mixed and incubated at room temperature for 5 min. The elute/isopropanol mixture was transferred into the QIAprecipitator Maxi Module attached to a 30 ml syringe. The DNA was bound to the QIAprecipitator filter by pulling in the solution with the syringe. 2 ml of 70% (v/v) ethanol was added to QIAprecipitator 30 ml syringe and the DNA washed as before. The DNA was dried by expelling the air from the syringe through the QIAprecipitator quickly and forcibly, twice. The QIAprecipitator was removed from the 30 ml syringe and re-attached to a new 5 ml syringe. 1 ml of nanopure H_2O was added and the DNA was eluted as before in to a 1.5 ml microcentrifuge tube. To obtain the maximum amount of DNA, DNA was re-eluted. The eluted DNA was used or kept at –20ºC until use.

2.3.4.2. Extraction of mycobacterial genomic DNA.

2.3.4.2.1. Extraction of genomic DNA from *M. smegmatis* MC 155.

This protocol was based on the method described by Davis *et al*. (1991) for fast growing mycobacteria. The following solutions that were used are given in table 2.13.

Table 2.13: Solutions used in extraction of chromosomal DNA from *M. smegmatis* MC 155.

Solutions	Contents
Wash solution	0.3 M Sucrose 50 mM Tris-HCL pH 8.0 10 mM EDTA pH 8.0
GSE Solution	6 M Guanidium chloride 1% (v/v) Sarkosyl 20 mM EDTA pH 8.0

M. smegmatis MC 155 was grown in 50 ml L-broth for 36 hr (shaking at 180 rpm) at 37°C. The culture was harvested by centrifugation at 7000g for 15 min. The supernatant was removed and the pellet was retained. The pellet was washed twice in 5 ml wash solution and the pellet was then re-suspended in 2 ml of wash solution. 4 mg of lysozyme and 4 mg of lipase were added to the suspension which then was incubated at 37°C for 12-18 hr. After incubation, 4 ml of GSE solution was added to the suspension which was incubated again for 2 hr at 37°C. An equal volume of chloroform was added to extract DNA, and then the (7 ml) upper, DNA, layer was transferred to a centrifuge tube. DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of absolute ethanol. The preparation was centrifuged at 17000g for 10 min to collect the DNA. The collected pellet was washed in 5 ml of 70% (v/v) ethanol, and then centrifuged as before and the pellet was dissolved in 600 μl of TE buffer. 10 μl of 5 g/ml proteinase K, 10 μl of 5mg/ml RNase and 30 μl of 10% (w/v) SDS were added and incubated at 37^{o}C for 2 hr. DNA was extracted twice to the upper phase by adding an equal amount (700 μl) of phenol: chloroform: isoamyl alcohol (25:24:1). DNA was extracted again with an equal amount (700 μl) of chloroform: isomyl alcohol 24:1. 0.1 volume (100 μl) 3 M sodium acetate and 2 volumes (2 ml) absolute ethanol were added to precipitate the chromosomal DNA. The DNA divided between three Eppendorf tubes and DNA was collected by centrifugation in a microcentrifuge at 17000g. The supernatants were removed and the DNA pellets were washed in 70% (v/v) ethanol, then they left to dry in air and dissolved in 70 μl TE buffer for each tube. The mycobacterial chromosomal DNA was stored at 4^{o}C.

2.3.4.2.2. Extraction of genomic DNA from *M. tuberculosis* and *M. bovis* BCG.

The method of extraction was based on the method described by Belisle *et al*. (1998). 90 ml of Middlebrook 7H9 broth supplemented with ADC and 0.05 % (v/v) Tween 80 were inoculated with 10 ml of a two weeks old culture and incubated for four weeks at 37ºC with shaking. Four 25 ml tubes of each culture were harvested by centrifugation at 2000g for 15 min and the pellets were re-suspended in 10 ml of TE buffer. The suspension was centrifuged at 2000g for 15 min, the supernatant was decanted and the cell pellet was frozen at –20ºC for a minimum of 4 hr, to give more efficient lysis of the cell wall. Then the pellet was re-suspended in 5 ml TE buffer and rocked on a platform rocker for 5 min. An equal volume (5 ml) of chloroform: methanol (2:1) was added and bacterial cell suspension was centrifuged at 2000g for 20 min to obtain the separate phases. The separated phases were removed to another fresh uncapped tube and then incubated in a water bath at 55ºC for 10-15 min to remove the traces of organic solvent. After incubation, 5 ml of TE buffer was added and the cells were re-suspended by vortexing vigorously. 0.1 volume of 1 M Tris-HCL (pH 9.0) was added to increase the pH of the cell suspension, 55 µl of 20 mg/ml lysozyme and 10 µl of 10 mg/ml RNase were added and incubated at 37ºC for 12-16 hr. After incubation, 0.1 ml volume of 10% (w/v) SDS and 0.01 volume of 20 mg/ml proteinase K was added and mixed gently and then incubated at 37ºC for 3 hr. Another 0.01 volume of proteinase K was added, mixed and incubated for an additional 1 hr. An equal volume of phenol/chloroform:isoamyl alcohol (25:24:1) was added and rocked gently on a platform rocker for 30 min and then centrifuged at 2000g for 10 min to sediment the contaminating proteins. Carefully the aqueous upper layer was removed to another tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added and incubated for 5 min with gentle rocking at room temperature, then centrifuged at 2000g for 30 min to remove the residual phenol. The aqueous upper layer was removed carefully to a fresh tube and an equal volume of chloroform:isoamyl alcohol (24:1) was added and then centrifuged again. The upper layer was removed to a fresh tube and 0.1 volume of 3 M sodium acetate pH 5.2 and 1 volume of isopropanol were added. The tube was mixed slowly and placed at -20°C for one hr to precipitate the DNA. The solution was centrifuged at 17000g for 10 min to pellet the DNA. The supernatant was removed and the DNA pellet

washed twice with 5 ml of cold 70% (v/v) ethanol and centrifuged at 17000g again. The supernatant was decanted and the DNA pellet was left to dry at room temperature. The DNA was dissolved in 500 µl of TE buffer pH 8.0 by incubation at 65ºC for 10 min and then placed at 4ºC for storage.

To purify DNA, 500 µl of TE buffer was added and the DNA was incubated overnight at room temperature. 1 volume of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 17000g for 1 min in a microcentrifuge. The upper layer was removed to a fresh tube, 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH5.2) were added as before centrifuging at 17000g for 10 min. The supernatant was decanted and the DNA pellet washed with 1 ml of cold fresh 70% (v/v) ethanol and centrifuged at 17000g for 5 min in a microcentrifuge. The supernatant was decanted and the DNA left to dry, then the DNA was dissolved in 100 µl of TE. Then the concentration of DNA was quantified (Section 2.3.10).

2.3.5. Polymerase chain reaction techniques.

When setting up PCR (Saiki *et al*., 1988), all reagents and reaction mixtures were kept in an area separated from that used for DNA preparation and sterile disposable tips containing hydrophobic filters were used to minimize cross-contamination. It was important to put the DNA polymerase used in PCR reaction on ice to keep its activity, also other reagents and PCR tubes (Qiagen). In addition, it was important to mix the solutions well before use to avoid localised concentration of salts (Qiagen).

Buffer solution was used in PCR reaction to provide suitable chemical conditions for optimum activity and stability of the DNA polymerase as a result of nearly resisting changing in its pH. Taq DNA polymerase is a magnesium-dependent enzyme. Therefore, magnesium acts as a co-factor for activity of polymerase of the PCR reaction (Qiagen; Brown, 2001; Perez-Pinera *et al*., 2006).

Q-Solution was used in all PCR reactions either with Taq or Proof DNA Polymerase (Qiagen). Addition of Q-Solution to the PCR reaction mixture had no effect on PCR efficacy (Qiagen; Mielke and Machl, 2001). On the other hand, it has many

advantages; for example, it is able to improve a suboptimal PCR caused by templates that are GC–rich (Qiagen), such as mycobacterial genomic DNA or the hygromycin resistance cassette. Also, it minimises nonspecific PCR products. Overall, the addition of Q-Solution to the PCR reaction can significantly increase specificity of PCR reactions (Mielke and Machl, 2001; Qiagen). All PCR reactions were carried out using the Peltier Thermal Cycler (MJ Research). For analysis, the PCR products were run through 0.8% (w/v) agarose gel, as described in section 2.3.8.1.

2.3.5.1. PCR using HotStarTaq DNA Polymerase and Q-Solution.

PCR with HotStarTaq DNA Polymerase and Q-Solution were used when normal PCR amplification was required; for example, to identify the presence of a DNA fragment on a bacterial genome or after cloning. Also, it was used to confirm the presence of the deletion mutations in the mycobacterial genome.

Reagents for PCR with HotStarTaq DNA Polymerase and Q-Solution were obtained from Qiagen, except dNTP from Advanced Biotechnologies Ltd and primers from TAGN Ltd and MWG Bio Technology U.K. PCR reactions were done in 10 µl or 100 µl volumes according to the manufacturer's recommendations (Qiagen).

PCR reactions in a volume of 10 µl were done using 1 µl of 10x PCR buffer contains 15 mM $MgCl_2$, 2 µl of 5x Q-solution, 0.2 µl (1 unit) of HotStarTaq DNA polymerase or (ProofStart DNA polymerase), 0.2 µl of 10 mM solutions of each of the following deoxynucleotides: dATP, dTTP, dGTP and dCTP (dNTPs) dissolved in sterilised nanopure H_2O, 1 µl of solutions (10 pmol/µl) of each of the forward and reverse primers dissolved in sterilised nanopure H_2O, 4.6 µl of sterilised nanopure H_2O and then 1 µl of template DNA at approximately 10 ng - 50 ng was added to make up the final volume of 10 µl. The PCR sample tubes were placed in the PCR machine for running the PCR thermal cycling programme according to the manufacturer's instructions and as described in section 2.3.5.3 and as it can be seen in table 2.14.

2.3.5.2. PCR using ProofStart DNA Polymerase and Q-Solution.

PCR with ProofStart DNA Polymerase and Q-Solution was mainly used when high-fidelity PCR amplification was required; for example, to obtain DNA fragments for cloning in further experiments or DNA fragments for sequencing (Qiagen). The advantage of this enzyme is to generate PCR products with more 10 times the accuracy of Taq DNA Polymerase (Qiagen). This is because it has exonuclease activity that enables it to excise any miss-incorporated nucleotide that does not match with the opposite one in the DNA template used for extending DNA strand. This activity followed by re-inserting the correct nucleotide and continuing amplification. The disadvantage of lacking exonuclease activity in HotStarTaq DNA Polymerase (no error-proof-reading activity) results in relatively low amplification fidelity (mutations at nucleotides) sometimes, which affects the fidelity of the PCR products (Eckert and Kunkel, 1991; Lawyer *et al*., 1993; Khare and Eckert, 2002).

In order to set up a PCR reaction using this enzyme, a reaction volume of 50 µl was used (Qiagen). The reaction included 5 µl of 10x ProofStart PCR Buffer (15 mM $MgCl_2$), 10 µl of 5x Q-Solution, 1.5 µl of dNTP mixture (10 mM of each), 5 µl of solutions of each of the forward and reverse primers dissolved in sterilised nanopure H_2O (10 pmol/µl), 1 µl of ProofStart DNA Polymerase (2.5 units) then sterilised nanopure H_2O and approximately of 100 ng - 1000 ng template genomic DNA or 10 ng - 100 ng template plasmid DNA were added to make up the final volume to 50 µl. The PCR sample tubes were placed in the PCR machine for running as described in section 2.3.5.3 and table 2.14.

2.3.5.3. PCR thermal cycles programme.

The programme of PCR thermal cycles was designed as recommended before (Qiagen; Brown, 2001; Saiki *et al*., 1988). PCR tubes were incubated at 95^{o}C for 15 min for the initial activation step of HotStarTaq DNA Polymerase or for 5 min for ProofStart DNA Polymerase.

The DNA template was denatured at 94^{o}C for 30-60 sec. The temperature was cooled to 56^{o}C-62^{o}C for 30-60 sec for annealing of the oligonucleotide primers. The degree

of annealing temperature (*Ta*) was calculated based on the melting temperature (*Tm*) of each pair of primers of each reaction (Section 2.3.3).

The temperature was raised to 72^{o}C for 1-3 min for extension of the PCR products. The extension temperature used of all PCR reactions was fixed at 72^{o}C (Brown, 2001; Parsons *et al*., 2002). However, the extension time depended on the size of DNA fragment be amplified; 1 min per kb DNA fragment (Qiagen; Brown, 2001). The amplification cycle consisting of denaturation, annealing and extension was repeated for approximately 30 times. Then, a final extension single step at 72^{o}C for 10 min was performed.

The number of cycles in PCR reactions depended on the amount of the starting DNA template used. However, a typical cycling programme consisted of approximately 30 to 40 cycles and the ideal number of cycles was 30 cycles (Rychlik, 1995; Qiagen). An increased number of cycles do not dramatically change the amount of PCR product (Qiagen; Brown, 2001).

Finally, the temperature was held at 15^{o}C until the PCR tubes were removed. After collecting the tubes, PCR samples were used either for further work immediately or stored at 4^{o}C for a short time or at -20^{o}C before use. The programme of PCR thermal cycles is shown in table 2.14.

2.3.5.4. Purification of PCR product.

All PCR reaction products that were used for cloning were purified using the QIAquick PCR Purification Kit Protocol obtained from Qiagen. Also, this kit was used to purify other DNA fragments; for example, digestion reactions, ligation reactions and plasmid DNA extracted by alkaline lysis methods. The protocol was performed as described by the manufacturer's instructions, at room temperature. First 100% ethanol was added to buffer PE, as instructed by the manufacturer, before use. 5 volumes of buffer PB were added to 1 volume of sample before adding to a QIAquick spin column. The column then was placed in 2 ml collection tube. The mixture was centrifuged using a microcentrifuge at 17000g for 1 min to bind the DNA to the column membrane. The flow-through was discarded and the QIAquick column was placed back into the same collection

tube. For washing, 750 µl buffer PE was added to the QIAquick column and centrifuged at 17000 g for 1min. The flow-through was discarded and the QIAquick column was placed back into the same collection tube to re-centrifuge for an additional 1 min. The QIAquick column was placed in a clean 1.5 ml microcentrifuge (Eppendorf) tube. For elution of DNA, 30-50 µl of sterile nanopure H_2O was added to the centre of the column membrane. The column was left to stand for 1 min, and then centrifuged at 17000g for 1min. The eluted DNA then kept at –20^oC until use.

Table 2.14: The thermal cycles programme of HotStarTaq or ProofStart DNA Polymerase and Q-Solution, according to the manufacturer's instructions (Qiagen).

PCR cycles	Incubation time	Temperature degree	Notes
First stage: Activation of 1 cycle	15 min or 5 min	95^oC	This initial heating cycle was used to activate HotStarTaq for 15 min and 5 min for ProofStart DNA Polymerase.
Second stage: Amplification of approximately 25- 35 cycles consist of 3 steps of each Step 1: Denaturation Step 2: Annealing Step 3: Extension	 50 sec 50 sec 1-3 min	 94^oC 56-68^oC 72^oC	-*Ta* was calculated with approximately 4^oC below *Tm* of primers - Extension time used was approximately of 1 min per kb DNA fragment - Number of cycles was based on the amount of DNA.
Third stage: Final extension of 1 Cycle	10 min	72^oC	This cycle was used to complete the non-completed PCR products
End of PCR cycling	Indefinite	15^oC	Until collecting the samples to use or store

2.3.5.5. Gradient PCR.

This form of PCR was used in order to determine the optimum annealing temperature for the designed primers when using the annealing temperature determined by using the formula in section 2.3.3, did not give a specific product (Lopez and Prezioso, 2001). Reaction preparation for this PCR was done typically as described in the method of preparation of PCR when using HotStar Taq DNA Polymerase and Q-Solution (Section 2.3.5.1).

Exactly twelve PCR tubes were used. The tubes were placed horizontally into their specific wells of the PCR machine. The thermal cycling programme was the same as described before (Table 2.14), except the annealing temperature (Step 2) from the second stage was changed to a variety of annealing temperatures, between 58^{o}C, and 66^{o}C. In the available PCR machine, the number of annealing temperatures for assay was 12. These were between 58^{o}C and 66^{o}C. Consequently, each PCR reaction tube had an annealing temperature different from the others. After analysing DNA of PCR products by 0.8% (w/v) agarose gel electrophoresis (Section 2.3.8.1), the temperature giving the clearest band was chosen to be the optimum annealing temperature for the primers pair of interest for further PCRs.

2.3.6. Transformation of plasmid DNA into bacterial cells.

2.3.6.1. Electroporation of *E. coli* DH5α.

2.3.6.1.1. Preparation of *E. coli* DH5α for electropoation.

Electrocompetent *E. coli* DH5α was prepared using the method of Dower *et al*. (1988). Initially, bacterial cells were grown on L-Agar by plating overnight at 37^{o}C. A fresh 5 ml of L-broth was inoculated using a loop from the overnight culture and incubated at 37^{o}C overnight. The 5 ml of overnight culture was inoculated into 95 ml of L-broth to make up a final volume of 100 ml in 500 ml flask and incubated at 200 rpm shaking until an OD of 0.6-0.75 at 600 nm was reached (mid log phase). Approximately 3 hr incubation

was needed to reach an OD 600 of 0.6-0.75. The culture was incubated on ice for 30 min and centrifuged at 4^{o}C at 7000g for 15 min in a Sorvall GSA centrifuge. The supernatant was discarded and the pellet was washed two times using 20 ml of ice-cold sterilised nanopure water, then centrifuged at 5000g for 10 min at 4^{o}C in a Sorvall SS-34 centrifuge. The bacterial cell pellet was washed three times using 20 ml of ice-cold sterilised 10% (v/v) glycerol and centrifuged at 5000g for 10 min at 4^{o}C in a Sorvall SS-35 centrifuge. Finally, the pellet was resuspended in 1 ml ice-cold 10% (v/v) glycerol and stored at –70^{o}C until use.

2.3.6.1.2. Electro-transformation of plasmid DNA into *E. coli* DH5α.

Electro-transformation of plasmid DNA into *E. coli* was done using the method of Dower *et al*. (1988). 40 µl of fresh or frozen electrocompetent cell suspension was placed in a cold 0.2 cm electroporation cuvette (Bio-Rad electroporator). 5 µl ligation mix (plasmid DNA and insert) was added and mixed with the cell suspension and placed on ice for 1 min. The Bio-Rad Gene Pulser Apparatus was set to 1,500 volts, 25 µFD capacitance and 1000 Ω *(OHMS)* resistance. The cold electroporation cuvette was placed in the chamber slide and pushed in until the cuvette was seated in between the contacts at the base of the chamber. The cuvette was given a single pulse and immediately 1 ml of cold SOC medium was added to the treated cell suspension and mixed gently. The mixture was transferred to a sterile tube and incubated for 1 hr at 37^{o}C shaking at 180 rpm. The transformation reaction (50 µl, 100 µl and 200 µl) was plated onto LB agar containing the appropriate antibiotic. The plates were stored at room temperature until the liquid was absorbed, followed by incubation at 37^{o}C. The plates were incubated overnight at 37^{o}C for the selection of transformants.

2.3.6.2. Electroporation of *M. tuberculosis* H37Rv and *M. bovis* BCG.

2.3.6.2.1 Preparation of *M. tuberculosis* H37Rv and *M. bovis* BCG for electroporation.

The protocol for preparing mycobacterial electrocompetent cells was adapted from the method of Hatfull and Jacobs, (2000). Washing and electroporation of cells were done at room temperature.

M. tuberculosis H37Rv or *M. bovis* BCG was grown on 7H11 agar supplemented with OADC and incubated for four weeks at 37°C. One colony was cultured in 10 ml 7H9 medium supplemented with ADC and Tween 80 and incubated for two weeks shaking at 150 rpm at 37°C. 5 ml of the two-week culture was inoculated into 100 ml 7H9 medium supplemented with ADC and Tween 80 and incubated with shaking at 150 rpm at 37°C until the OD 600 was between 0.6 and 0.8. This took approximately 5-7 days. 100 ml culture was transferred to 2 x 50 ml conical tubes and the cells were pelleted by centrifugation at 2500g for 15 min. The supernatant was discarded and cells were washed with an equal volume of sterile 10% (v/v) glycerol at room-temperature. Then the cells were pelleted by centrifugation at 2500g for 15 min. The cells were washed twice and centrifuged as described before. The cells were resuspended in 1 ml of sterile 10% (v/v) glycerol at room temperature.

2.3.6.2.2. Electro-transformation of plasmid DNA into *M. tuberculosis* H37Rv and *M. bovis* BCG.

The protocol was modified from Hatfull and Jacobs (2000). 200 µl of the prepared cells (Section 2.3.6.2.1) were transferred to a microcentrifuge tube. Approximately 500 ng of plasmid DNA dissolved in 8 µl sterile nanopure H_2O was added and mixed. The mixture was incubated at room temperature for 10 min. Then the mixture was placed in 0.2 cm cuvette and electroporated at 2,500 V, 1000 Ω, 25µF. Only two transformations at a time were done to avoid overheating the electroporator. 1 ml of Middlebrook 7H9 medium

supplemented with ADC and 0.05 % (v/v) Tween 80 was added to each reaction. After that, the transformed cell suspension was transferred to a universal tube to incubate for 24 hr at 37°C in order to allow for expression of antibiotic resistance. In the next day, the cells were plated on 7H11 agar containing the required antibiotic, and then incubated at 37°C for 4 weeks for the selection of transformants (recombinants).

2.3.6.3. Heat-shock transformation of *E. coli* DH5α.

2.3.6.3.1. Preparation of *E. coli* DH5α for heat-shock transformation.

The method of preparing *E. coli* DH5α competent cells using calcium chloride (Weston *et al*., 1981) and DNA transformation by the Heat-Shock technique was based on Cohen *et al*. (1972), as modified by Sambrook *et al*. (1989). This method was used in the present study to transform *E. coli* DH5α with the insert DNA ligated to pGEM T-Easy plasmid, because there were some problems with electroporator equipment.

A single bacterial colony was picked from a plate that had been incubated overnight at 37ºC. The colony was transferred into a 10 ml of L-broth medium and the culture was incubated overnight at 37ºC shaking. The next day, a 100 ml of L-broth medium was inoculated with one ml of the 10 ml overnight culture using 500 ml flask and the culture was incubated with vigorous shaking at 200 rpm at 37ºC for 3-4 hr to an OD 600 of 0.4-0.5. The bacterial cells were transferred to sterile, ice-cold 50 ml polypropylene tubes. The culture was cooled to 0ºC by placing the tubes on ice for 10 min. The cells were recovered by centrifugation at 2700g for 10 min at 4ºC. The medium was decanted from the cell pellets. Each pellet was re-suspended by swirling in 30 ml of ice-cold 0.1 M $CaCl_2$ solution and the solution was stored on ice for 30-60 min. The cells were recovered by centrifugation at 2700g for 10 min at 4ºC. The supernatants were decanted from the cell pellets. The pellet was re-suspended by pipetting gently in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. At this point, the cells either were used directly for transformation with 200 µl for each reaction or they were dispensed into 200 µl aliquots and frozen at -70ºC.

2.3.6.3.2. Heat-shock transformation of plasmid DNA into *E. coli* DH5α.

To transform the $CaCl_2$ - treated cells directly, 200 µl of the *E. coli* DH5α competent cells suspension was transferred to a sterile, chilled microcentrifuge tube using a micropipette tip. Plasmid DNA (approximately 50 ng), in a volume not more than of 10 µl, was added to each tube. The contents of the tubes were mixed by pipetting gently, then the tubes were stored on ice for 30 min. The tubes were transferred to a rack placed in a preheated 42°C water bath and incubated for exactly 90 sec. Later on, without shaking or swirling, the tubes were rapidly transferred to an ice bath to allow the cells to chill for 1-2 min. 800 µl of SOC medium was added to each tube and incubated for 45 min at 37°C shaking at 200 rpm to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. An appropriate volume, between 50 µl and 200 µl of transformed competent cell, was plated onto L- agar containing the appropriate antibiotic. The plates were stored at room temperature until the liquid was absorbed, followed by incubation at 37°C. The transformed colonies were visualised after 12-16 hr incubation.

2.3.7. Ligation of DNA into pGEM-T Easy Cloning Vector.

The ligation of PCR products and T-tailed pGEM-T Easy Amp Cloning Vector was done as described in the supplier's protocol (Promega) and the ratio of insert: vector was calculated from the following formula obtained from the technical manual of pGEM-T Easy Vector Systems (Promega). The calculation was based on using 50 ng of pGEM-T Easy vector.

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \quad \text{x} \quad \text{insert: vector molar ratio} = \text{ng of insert}$$

The ligation reaction was performed in a 0.5 ml microcentrifuge tube in a volume of 10 µl using 1 µl (50 ng) of the pGEM-T Easy Amp Cloning Vector, 5 µl of 2x Rapid Ligation Buffer, 1 µl (3 units) of T4 DNA ligase, and the amount of DNA insert according to the size of PCR product, after calculation using the above formula. Sterile nanopure water was added to make up a final volume of 10 µl. The ligation reaction was mixed gently

and incubated at room temperature for one hour firstly. Then, the mixture was incubated for overnight at 4ºC to obtain maximum efficiency of ligation reaction (maximum number of transformants) (Promega). All ligation reactions were precipitated by 2 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) (Section 2.3.8.9) washed in 70% (v/v) ethanol, dried and dissolved in 5 µl for each of sterile nanopure water. Ligated DNA was transformed into *E. coli* DH5α by electroporator (2.3.6.1.2) or by heat shock as described in section 2.3.6.3.2.

2.3.8. DNA manipulation techniques.

2.3.8.1. Agarose gel electrophoresis.

Agarose gel electrophoresis was used to separate or/and to purify DNA molecules. Also, it could be used to measure the approximate DNA concentration of the sample when loaded beside a NEB electrophoresis ladder (New England Biolabs). The method of separating DNA fragments used was according to Sambrook *et al*. (1989) and Brown, (2001). A 0.8% (w/v) agarose gel was prepared to separate DNA fragments more than 500 bp, whereas to separate DNA fragments less than 500 bp or to determine the presence of the RNA molecules in the samples, a 1.8% (w/v) agarose gel was used. The solutions used for preparing the agarose gel are shown in table 2.15.

To prepare the agarose gel, the desired amount of multipurpose agarose (Bioline) was dissolved in TAE buffer pH 7.7 by heating, until boiling, in a microwave oven. The solution was left to cool to approximately 60ºC. Then, a 1 µl of 0.5 µg/ml ethidium bormide (EtBr) was added. While the agarose gel was cooling, a plastic gel electrophoresis tray was prepared by sealing its edges with tape and putting an appropriate comb into the tray.

The warm agarose gel solution was poured into the prepared tray (a volume to give approximately 6 mm thickness was used) and left to set and to cool at room temperature. After that, the tapes and the comb were removed gently and the gel in its tray was placed on a platform in an electrophoresis tank containing TAE buffer. Care was taken to ensure the TAE buffer was sufficient to cover the surface of the gel before running. 2 µl of loading

buffer was added to each DNA sample and mixed. The loading buffer was prepared using 0.25 % (w/v) bromophenol blue, 0.25 % (w/v) xylene cyanol FF, 15 % (w/v) Ficoll Type 400 (Pharmacia) in water. Also, the loading dye solution consisted of 0.09 % (w/v) bromophenol blue, 0.09 % (w/v) xylene cyanol FF, 60 % (v/v) glycerol, 60 mM EDTA (MBI Fermentas) was used with the same amount. Finally, the samples then were loaded into the wells in the agarose gel.

Subsequently, electrophoresis at a constant voltage of 80 mV–120 mV was done. On completion of electrophoresis, DNA bands were visualised by placing the gel without its tray under a long wave length UV light transilluminator at 300 nm (Gel Doc Mega from Biosystematica). The size of the DNA fragments was determined by comparison with a 1kb GeneRulerTM ladder (MBI Fermentas) or 1kb DNA ladder (New England Biolabs).

Table 2.15: Preparation of solutions for gel electrophoresis.

Solutions	Contents
Stock solution (50x) TAE	242 g Tris-base, 57.1 ml Glacial acetic acid, EDTA 73.2 g (pH 8.0). Distilled water was added to make up a final volume to 1000 ml
Working solution (1x) TAE	20 ml of 50x stock solution was added to 980 distilled water to make up a final volume of 1000 ml.
Agarose gel solution for use	Multipurpose agarose (Bioline) was dissolved in 1x TAE buffer by heating to give 0.8% (w/v). 1 μl of 0.5 μg/ml Ethidium bromide (Sigma) was added after cooling

2.3.8.2. Purification of DNA after agarose gel electrophoresis.

DNA fragments, were purified from agarose gels using the QIAquick Gel Extraction Kit Protocol (Qiagen). When the DNA was visualised using a UV light transilluminator, the desired DNA fragment size band was excised using a clean and sharp scalpel. The agarose gel containing the DNA band was weighed in a pre-weighed colourless tube. Three volumes of Gel Solubilizer (Buffer QG) were added to 1 volume of gel (300 µl of Buffer QG to each 100 mg of gel), mixed and incubated at 50ºC for 10 min (or until the gel slice has completely dissolved), mixing every 2-3 min during the incubation. If the colour of the solution changed, 10 µl of 3 M sodium acetate, pH 5.0, was added to return the colour to yellow. 1 gel volume of isopropanol was added to the sample and mixed gently. To bind the DNA, the sample was applied to a QLAquick column and placed in a 2 ml collection tube, then centrifuged in a microcentrifuge for 1 min at 17000g. 500 µl of Buffer QG was added to the QLAquick column and centrifuged for 1 min at 17000g to remove all traces of agarose. To wash, 750 µl of Buffer PE was added to the QLAquick column and centrifuged for 1 min at 17000g. The flow-through was discarded and the QLAquick column was centrifuged for an additional 1 min at 17000g in a microcentrifuge. The QLAquick column was placed into a clean 1.5 ml microcentrifuge tube and DNA eluted by adding 30 µl of Buffer EB to the centre of the QLAquick membrane and incubated at room temperature for 5 minutes and then the column was centrifuged in a microcentrifuge for 1 min at 17000g.

2.3.8.3. Irradiation of plasmid DNA molecules with Ultraviolet (UV) for electro-transformation.

Irradiation of plasmid DNA molecules with Ultraviolet (UV) for electro-transformation was done using the protocol of Hinds *et al*. (1999). 5 µg-10 µg of plasmid DNA molecules was precipitated with two volume 100% ethanol and 0.1 volume 3 M sodium acetate pH 5.2. The DNA was pelleted by centrifugation at 17000g and then washed twice with 70% (v/v) ethanol to remove all traces of salts. The DNA pellet was dried and re-suspended in 10 µl of sterile nanopure H_2O. The 10 µl DNA sample was placed

in a well of a sterile microwell plate. With the cover of the microtitre plate removed, the DNA was subjected to 100 mJ/cm^2 UV light given with an UV Stratalinker 1800. 100 mJ /cm^2 was chosen, because it had been reported to give a marked increase in the number of homologous recombination transformants (Hinds *et al*., 1999). Finally, the irradiated DNA was transferred into a microtube and stored at -20 °C until required.

2.3.8.4. Denaturation of plasmid DNA with NaOH for electro-transformation.

Denaturation of plasmid DNA for transformation using NaOH was done using the protocol of Hinds *et al*. (1999). 5 µg - 10 µg of plasmid DNA was precipitated by two volume 100% ethanol and 0.1 volume 3 M sodium acetate pH 5.2. The DNA was pelleted by centrifugation at 17000g and, then washed twice with 70% (v/v) ethanol to remove all traces of salts. The DNA pellet was dried and re-suspended in 10 µl sterile nanopure H_2O. 20 µl of the solution consisted of 0.2 mM EDTA and 0.2 M NaOH were added to the DNA sample and mixed then, incubated at 37°C for one hr. The denaturated DNA was precipitated by adding 2.5 volume of 100% ethanol and 0.1 volume of 3M sodium acetate pH 5.2 and incubated at -20°C for 30 min. Then, DNA was collected by centrifugation for 20 min at 17000g in a bench microcentrifuge. After that, DNA was washed twice with 70% (v/v) ethanol to remove all traces of salts, dried and re-suspended in 10 µl of sterile nanopure H_2O to be ready for electroporation methods.

2.3.8.5. Digestion reactions of DNA molecules.

Restriction enzymes and their buffers were obtained from Gibco-BRL, Promega, New England BioLabs (NEB) and Invitrogen. The DNA digestion reaction was done according to the manufacturer's recommendations, using 0.5 µl (3-5 units) of endonuclease, 2.0 µl 10x reaction buffer supplied by the manufacturer, approximately 1 µg of DNA, and then sterile nanopure water was added to make up a final volume of 20 µl of the reaction. A reaction tube contained the same reagents except endonuclease was used as a control. Digestion reaction tubes including control was incubated at 37°C for 2 hr. The

volume of the digestion reaction depended on the amount of DNA to be digested; for example, when a high amount of DNA (4-5 μg) was needed to be digested a 100 μl volume was used. When the amount of DNA was approximately 500 ng or less it was digested using 0.2 μl (1-3 units) of enzyme in a 20 μl volume (Ausubel *et a*l., 1995; Brown, 2001; Sambrook *et al*., 1989).

When restriction digestion with two different enzymes was required and their buffers were different, the DNA was digested with the two enzymes in two different reactions. The second reaction was done after precipitation of the DNA with 100% ethanol following first reaction. However, if the buffer for the two enzymes was the same, the two enzymes were used in the one reaction.

When incubation was completed, samples of the digestion reactions were taken for analysis by agarose gel electrophoresis (Section 2.3.8.1). 1kb ladder and undigested DNA control were used when running the samples on agarose gel to confirm both the digestion and the target DNA size. If the DNA was not digested or was not completely digested, more restriction enzyme was added to the reaction or the time of reaction was extended. However, adding more enzyme to the reaction or extending the incubation time was found to be non-beneficial. After confirming the digestion and the size of the DNA fragments by agarose gel electrophoresis (Section 2.3.8.1), the restricted DNA was precipitated with 100% ethanol (Section 2.3.8.9) or purified with a QIAquick PCR purification kit (Section 2.3.5.4) and stored at -20^{o}C for further work.

2.3.8.6. Ligation reactions of DNA molecules.

For ligation of DNA fragments, whether sticky or blunt ends, T4 DNA ligase (Invitrogn) was used. In order to prepare the ligation reaction, plasmid DNA and the DNA fragment to be ligated were digested with the same restriction enzymes (Section 2.3.8.5). To obtain the optimal ratio of plasmid and fragment in the ligation reaction, a ratio of 1:3 of plasmid to fragment DNA was recommended using 100 ng of plasmid (Promega; Ausubel *et al*., 1995). On occasion, ratios of 1:1 and 1:6 were used when difficulties with ligation were found.

The ligation reaction was prepared in a 0.5 ml microcentrifuge tube using 2 µl (2 units) of T4 DNA ligase, 4 µl of 5x T4 DNA ligase buffer supplied by the manufacturer, 100 ng of digested purified vector, and the required amount of purified insert DNA according to the insert to vector ratio. Sterile nanopure water was added to make up a final reaction volume of 20 µl. The ligation mixture was incubated at 16ºC overnight (Sambrook *et al*., 1989; Ausubel *et al*., 1995). Following incubation, the ligation reaction was precipitated with 2 volumes of 100% ethanol, washed in 70% (v/v) ethanol, dried and dissolved in 8 µl of sterile nanopure water. 4 µl of the ligation solution was then transformed into electrocompetent *E. coli* DH5α cells by electro-transformation (Section 2.3.6.1.2) or by heat shock transformation (Section 2.3.6.3.2). In the case of *M. tuberculosis* H37Rv and *M. bovis* BCG cells, the whole volume (8 µl) of ligation reaction was used for electro-transformation (Section 2.3.6.2).

2.3.8.7. Dephosphorylation of plasmid DNA molecules.

Dephosphorylation of DNA molecules was done to remove the terminal 5'-phosphate group to suppress self-ligation of plasmid DNA. To set up the reaction, plasmid DNA was first digested completely using the appropriate restriction edonuclease. The dephosphorylation reaction was performed according to the manufacturer's recommendations (BioLabs). 2 µl of 10x NE Buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol at pH 7.9) and 0.5 units of calf intestinal alkaline phosphatase (CIP) per 1 µg plasmid DNA were mixed and then the reaction was made up to a final volume of 20 µl by adding sterile nanopure H_2O. The reaction was incubated at 37ºC for 60 min. To stop the reaction, the DNA was extracted with phenol:chloroform:isoamyl alcohol (Section 2.3.8.9), then it was precipitated by ethanol (Section 2.3.8.9). The DNA was left to dry and then re-suspended in sterile nanopure H_2O.

2.3.8.8. Phenol: chloroform extraction.

In order to purify the DNA fragments after an enzymatic reaction, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA sample and the solution was mixed for 1 min to form a suspension. The suspension was centrifuged at

17000g for 5 min in a microcentrifuge. The upper aqueous phase was transferred to a fresh tube. To remove residual phenol, an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous phase, mixed for 1 min and centrifuged for 2 min as mentioned before. The final upper aqueous phase was transferred to a fresh tube.

2.3.8.9. Precipitation of DNA.

DNA was precipitated from the final upper aqueous phase with 2 volumes of 100% ethanol or 1 volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The DNA was incubated at -20°C for 30 min. The DNA was collected by centrifugation at 17000g for 20 min in a microcentrifuge at 4°C. The resultant pellet was washed by adding 1 ml of 70% (v/v) ethanol and centrifuged once again at 17000g for 2 min in a microcentrifuge at 25°C to prevent precipitating salts. The supernatant was discarded and the DNA was dried at room temperature or by incubating at 37°C, and then it was dissolved in a suitable amount of TE or nanopure H_2O. 100% ethanol was used to precipitate a small amount of DNA. For example, DNA digestion reaction, PCR products, DNA ligation reaction, whereas isopropanol was used to precipitate a large amount of DNA, such as genomic DNA preparations or when a large volume of solution containing DNA was required to be precipitated in a Sorvall SS-34 centrifuge using glass cortex tubes; for instance, when precipitation of plasmid DNA using large scale preparation protocol.

2.3.9. Sequencing of DNA.

DNA sequencing was performed by MWG BioTechnology using the chain termination method. 5 µg of purified double strand recombinant plasmid DNA or 5 µg of PCR products and 20 µl (10 pmol/1µl) of each appropriate forward and reverse primers (Table 2.9) were needed for this purpose. Automated DNA sequencing (Sanger *et al*., 1977; Biotech) is based on chain termination where trace amounts of four fluorescent dye-labeled dideoxynucleotides (d-dNTPs) terminators d-dATP (green), d-dGTP (black), d-dCTP (blue) and d-dTTP (red) replace the usual deoxynucleotides (dNTPs) in the enzymatic reaction of DNA polymerase.

The basis of the method was that the DNA sequence reaction components were similar to PCR reaction except for addition of dideoxynucleotides. The incorporation of dye-labelled dideoxynucleotides into the DNA blocks further chain synthesis, because the dideoxynucleotide lacks a hydroxyl group at the 3' position of the sugar component and this results in to a fluorescent dye-labelled reaction product (Brown, 2001).

Analysis of the sequence data obtained from MWG BioTechnology was done using www.ncbi.nlm.nih.gov/-BLAST (Altschul and Gish, 1990) to find similarity with known DNA nucleotide sequences from *M. tuberculosis* H37Rv.

2.3.10. Quantitation of DNA.

DNA quantitation was performed by measurement of the absorption at 260 nm, or by agarose gel analysis. For DNA to be quantified by spectrophotometry, the DNA was prepared by diluting in sterile TE buffer or sterile nanopure H_2O at a ratio of 1:20 in a 100 µl microcuvette or 1:100 in a 1 ml cuvette.

The concentration was calculated by the following formula (Sauer *et al*., 1998; Qiagen; Ahn *et al*., 1996):

A_{260} of 1 corresponds to 50 micrograms/ml for dsDNA.

Therefore, (DNA) µg/ml = A_{260} × Dilution factor × 50.

Absorbance readings at 260 and 280 nm were taken against a blank of H_2O or TE buffer. The A_{260} allowed calculation of the concentration of nucleic acid in the sample, whereas the A_{280} gave an estimate of the protein contamination of the sample. The ratio of A_{260}/A_{280} gave an estimate of the purity of the DNA: values of 1.7-2.0 predict a clean DNA free from residual protein (Ahn *et al*., 1996).

Since the spectrophotometric measurement did not differentiate between DNA and RNA, RNA in the sample could lead to over-estimation of DNA value (Giagen and Sauer, 1998). In order to obtain an accurate reading at A_{260} nm, the DNA samples were pre-treated with RNase A (Section 2.3.4.1.1) followed by purification either using PCR purification kit (Section 2.3.5.4) or alcohol precipitation (Section 2.3.8.9). When working with small amounts of DNA preparations, such as purified PCR products, purified digestion reaction

or DNA fragments extracted from agarose gels, DNA concentration was estimated using agarose gel analysis (Sauer *et al*., 1998). The DNA sample was run on a 0.8% (w/v) agarose gel alongside a 1kb NEB ladder (New England Biolabs). The concentration of the DNA sample loaded was estimated by comparing its band with the marker bands after visualising with UV light (Sauer *et al*., 1998; Ahn *et al*., 1991; New England Biolabs).

2.3.11. RNA tools and techniques.

2.3.11.1. Extraction of RNA from *M. bovis* BCG.

This protocol was based on the methods of Stewart *et al*. (2002). *M. bovis* BCG was grown in 100 ml of 7H9 broth supplemented with ADC and Tween 80 for 7 days shaking at 37°C to reach an optical density at 600 nm of approximately 0.6-0.7 (~ $6x10^6$ CFU/ml). Four volumes of GTC lysis solution (Table 2.16) were added to 1 volume bacterial culture and mixed rapidly by swirling. The bacterial solution was transferred into a 250 ml Sorvall GSA centrifuge tube and centrifuged at 8000g for 20 min. The supernatant was discarded and the pellet was re-suspended in 1 ml GTC solution. After that the bacterial suspension was centrifuged at 17000g for 20 sec. The supernatant was discarded again and the pellet was resuspended in 1.2 ml of trizol and immediately transferred to a ribolyser blue matrix tube and processed in a RiboLyser (Hybaid RiboLyser) at 6.5 power setting for 45 sec. The tube was left at room temperature for 5 min, then 0.2 ml of chloroform was added and vortexed for 15 sec and allowed to stand at room temperature for a further 2-3 min. Later on, the bacterial solution was centrifuged at 12000g for 15 min at 4ºC to separate the solution into a lower red organic phase and upper clear aqueous phase containing the RNA.

The upper layer was transferred carefully to a fresh microcentrifuge tube. Extraction with chloroform was repeated once, then 0.5 ml of isopropanol was added and then incubated at room temperature for 15 minutes in order to precipitate the RNA. After that, the solution was centrifuged at 4ºC at 12000 g for 10 min. The supernatant was discarded and the pellet re-suspended in 100 µl of RNase free water (Qiagen, 2002).

Table 2.16: Contents of solution used to extract RNA from *M. bovis* BCG.

Solution	Contents	
GTC Solution	Guanidine thiocyanate	5 M
	Sodium N-Lauryl Sarcosine	0.5% (w/v)
	Tri-sodium citrate, pH 7.0	25 mM
	2-Mercaptoethanol	0.1 M
	Tween 80	0.5% (v/v)
	Nanopure water was added to make up a final volume of 300 ml. The solution was sterilised by filtration through a 0.2 μm filtration unit.	

2.3.11.2. Purification of the RNA samples from DNA.

All RNA samples that were used for reverse transcriptase PCR or quantitative RT-PCR were treated with DNAseI in order to purify the RNA sample from DNA. Deoxyribonuclease I (Amplification Grade) was obtained from Invitrogen. The reaction was performed according to the manufacturer's recommendations using sterilised 0.5 ml microcentrifuge tube on ice. 10 μl of RNA sample was added to 5 μl of 10x DNase I Reaction Buffer, 5 μl of Amplification Grade DNase I (1unit/μl) and 30 μl of RNase-free water to make up a final reaction volume of 50 μl. The reaction tube was mixed and incubated at room temperature for 15 min. After that, 5 μl of 25 mM EDTA solution (pH 8.0) was added to the reaction mixture and heated for 10 min at 65^{o}C in order to inactivate the DNase I. The reaction solution was transferred immediately to -70^{o}C to prevent the RNA from degradation.

2.3.11.3. Clean up of the RNA samples using RNeasy Mini Protocol.

The RNeasy Mini Protocol for RNA Clean up from Qiagen was used to clean up RNA previously isolated. Before starting this protocol, the RNA sample was adjusted with

RNase-free water to a volume of 100 µl. 10 µl of ß-mercaptoethanol (ß-ME) was added to 1 ml of buffer RLT. Also, 4 volumes of absolute ethanol (96-100%) were added to buffer RPE as indicated by the manufacturer.

350 µl of the B-Mercaptoethanol solution was added to the 100 µl RNA solution and mixed using a pipette. 250 µl of absolute ethanol was also added to the diluted RNA and mixed thoroughly by pipetting. The whole 700 µl mixture was applied to RNeasy mini column which was placed in the supplied 2 ml collection tube. The tube was closed gently, then centrifuged for 15 sec at 8000g. The supernatant was discarded and the column was transferred to another fresh 2 ml collection tube (supplied with the kit). To wash the sample, 500 µl of buffer RPE (prepared previously) was added onto the RNeasy column. The tube was closed gently, and then centrifuged for 15 sec at 8000g. The supernatant was removed. To wash again, 500 µl of RPE buffer was added to the same column which was placed onto the same collection tube as before and then centrifuged for 2 min at 8000g and the supernatant was removed. To eliminate any RPE buffer, the RNeasy column was placed onto a fresh 2 ml collection tube and centrifuged for 1 min at 9000g. The supernatant and the collection tube were discarded, whereas the RNeasy column was transferred carefully to a fresh 1.5 ml RNase-free microcentrifuge tube (supplied with the kit). To elute the RNA, 50 µl of RNase-free water (supplied with the kit) was directly pipetted into the centre of the column. The column was left to stand for 1 min. After that, it was closed gently and centrifuged for 1 min at 8000g. Subsequently, the column was discarded and the eluted RNA was kept at -70^{o}C until use.

2.3.11.4. Quantitation of RNA.

The RNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer as described in the RNeasy Mini Handbook (Qiagen). 1/50 dilution was obtained by adding 10 µl RNA sample to 490 µl sterilised nanopure H_2O. A 1 ml cuvette (washed with 0.1 M NaOH and 1 mM EDTA pH 7.4, then RNAse-free water) was used to measure the absorbance of the diluted RNA sample.

The concentration of the RNA sample was calculated using the following formula from Qiagen:

An A_{260} of 1 unit corresponds to 40 micrograms/ml for RNA.

Therefore, (RNA) μg/ml = A_{260} × Dilution factor × 40.

Absorbance readings at 260 and 280 nm were taken against a blank of H_2O or TE buffer. The A_{260} allowed, calculation of the concentration of RNA in the sample, whereas, the A_{280} gave an estimate of the protein contamination of the sample. The ratio of A_{260}/A_{280} gave an estimate of the purity of the RNA: values of 1.9-2.1 predict a clean RNA free from protein residuals (Qiagen).

2.3.11.5. Reverse Transcriptase reaction.

In order to synthesise the first complimentary DNA strand (cDNA), reverse transcriptase (RT) reaction was done (Desjardin *et al*., 1996). The reaction was performed according to the manufacturer's recommendations (Invitrogen) in a total volume of 20 μl when using SuperScriptTM III Reverse Transcriptase. 2 μl (250 ng) of random primers (Invitrogen), 10 μl (5 μg) of total purified RNA (Section 2.3.11.3), 1 μl (10 mM) of dNTP mix and 1 μl of sterilised nanopure H_2O were pipetted into a 0.5 ml autoclaved microcentrifuge tube to a final volume 14 μl.

The mixture was mixed well by pipetting. The reaction was heated to 65^oC for 5 min in a water bath, and then it was transferred immediately to incubate on ice for 3 min. The reaction was centrifuged briefly at 1000 rpm in a bench microcentrifuge to collect the contents. 4 μl of 5x First-Strand Buffer, 1 μl of 0.1 M DTT, 1μl of SuperScriptTM III reverse transcriptase (200 units/μl) were added. The reaction mixture was mixed by pipetting gently up and down. After that, the reaction tube was transferred to the thermal cycler to incubate at 25^oC for 5 min, followed by incubating at 50^oC for 60 min. Finally, the reaction mixture was incubated at 70^oC for 15 min to inactivate the reaction by heating. To confirm the success of conversion of RNA to cDNA, 3 μl of the RT product was used for amplification using PCR (Section 2.3.5.1). The resultant cDNA was kept at -20^oC until use.

2.3.11.6. Quantitative Real Time PCR (qRT-PCR).

For quantification of *rv1996* gene expression, quantitative PCR (qRT- PCR) was performed using internal fluorescent hybridisation-Taq Man probes. All probes were dually labelled with 5-carboxyfluorescein (FAM) at the 5' end and N, N, N, N'-tetramethyl-6-carborhodamine (TAMRA) at the 3' end. The qRT-PCR primers and probe sequences shown in table 2.10 were designed and sent to Biotech-MWG to be made.

To correct for the possibility of DNA contamination of the extracted RNA samples, a crude DNase treated RNA sample with no reverse transcriptase enzyme was included. The crude DNase treated RNA samples were diluted 1:1 with nanopure H_2O. For setting up the reaction, 0.2 ml strip tubes were used. 3 µl of each cDNA or diluted RNA was assayed in a total reaction volume of 25 µl containing 12.5 µl of the AB soluteTM qPCR Mix which contains reaction buffer and Thermo-Start DNA Polymerase (ABgene), 12.5 pmol of each forward and reverse TaqMan primers for either *rv1996* gene or *sigA* gene, 5pmol of TaqMan probe for either the *rv1996* gene or the *sigA* gene according to the corresponding reaction for each gene.

The final reaction volume was made up to 25 µl by adding nanopure H_2O, then the tubes were closed tightly. Reaction tubes and the reagents were kept on ice during the work and protected from light before they were transferred immediately to the machine. The tubes for both reactions were placed in the real-time thermal circler (R Corbett Research, RG-6000). Reaction incubation conditions are given in table 2.17.

PCR amplification for the expression of *sigA*-specific mRNA was performed on the cDNA templates from the parental strain and mutant as a normalising housekeeping gene and as a control to confirm that the cDNAs from the two strains served as templates for PCR [Papavinasasundaram *et al*., 2005]. The s*igA* gene was used because its mRNA level does not change in the cell during exposure to different stress conditions [Gomez *et al*., 1997; Stahlberg *et al*., 2004; Singh and Singh, 2009]. The copy number of target cDNA in each sample was determined by comparing the cycle threshold (Ct) value to standards with known amounts of wildtype BCG genomic DNA.

The copy number values of each sample target gene were normalised by calculating relative to the copy number value of cDNA of *sigA* gene, and the result was expressed as a ratio according to the following formula [Pfaffi, 2003; Newton *et al*., 2006]

$$\text{Ratio} = \frac{\text{Value obtained for target gene}}{\text{Value obtained for } sigA \text{ gene}}$$

Table 2.17: The thermal cycles programme for Quantitative Real Time PCR (qRT-PCR) of *rv1996* (Test gene) and *sigA* (Reference gene) according to the manufacturer's instructions (ABgene, 2006).

PCR cycles	Incubation Time	Temperature degree	Notes
Initial Start 1 cycle	2 min	50°C	Start of reaction
First stage: Activation of 1cycle	15 min	95°C	This initial heating cycle was used to activate Thermo-Start DNA Polymerase.
Second stage: Amplification of 40 cycles consist of 3 steps of each Step 1: Denaturation Step 2: Annealing Step 3: Extension	 20/15 sec 60 sec 50/20 sec	 95°C 60°C 72°C	*Ta* was calculated with approximately 4°C below *Tm* of primers. - Denaturation time was 20 sec for the test gene and 15 sec for the house keeping gene. - Extension time used was adjusted to 50 sec for the test gene and 20 sec for the house keeping gene.

2.4. *In vitro* experiments to determine the effect of gene disruption *bcg2013* (*rv1996*) on *M. bovis* BCG.

2.4.1. Preparation of *M. bovis* BCG cells.

From fresh cultures, *M. bovis* BCG cells were grown in 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 from 6 to 7 days. The cultures were harvested by centrifugation at 3000g in a Bench Top centrifuge for 15 min. The supernatants were discarded and the cell pellets were re-suspended in 1 or 2 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80. After preparing the bacterial cells, the incubation time of the bacterial cultures depended on the purpose of the experiment.

2.4.2. Measurement of growth of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant in 7H9 broth.

Tissue culture flasks containing 18 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 were inoculated by 2 ml of 7 days old cultures with OD of 0.6-0.7 at 600 nm (~ $6x10^6$ CFU/ml) of bacteria to give 1:10 dilution. Cultures were grown for 10 days (240 hr) with shaking at 37^{o}C. The OD 600 nm was measured and colony forming units per millilitre (CFU) were determined as described in sections 2.1.10 and 2.1.11.

2.4.3. Determination of Acid resistance of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant.

To establish the range of acid tolerance response of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant, they were incubated at several pH levels between 3.5 and 6.8 to assess the lethal, the optimum and the tolerated pH levels for growth and survival. This experiment was performed, with some modifications based on the methods described by

O'Brien *et al*. (1996) and Newton *et al*. (2006). 10 ml of 7 days old cultures, with an OD of 0.6-0.7 at 600 nm, were centrifuged and re-suspended in 2 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80. 100 µl of the bacterial suspension was inoculated into each tissue culture flask containing 20 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) tween 80 to obtain an OD of 0.06 at 600 nm with ~ $4x10^6$ cfu/ml. The bacterial growth medium was adjusted with 2 M hydrochloric acid (HCl) or 2 M NaOH to obtain the desired pH levels and filtered through a 0.2 µm filter on the day of use. Cultures were grown for 240 hr with shaking at 37ºC. The OD was measured at 600 nm and colony forming units per millilitre (CFU) were determined as in sections 2.1.10 and 2.1.11 respectively.

2.4.4. Investigating the effect of acid adaptation on acid resistance of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant.

The protocol applied to determine the effect of exposing the strains to acid adaptation conditions on their resistance to acid, was based with some modifications on the methods of Foster *et al*. (1991), O'Brien *et al*. (1996) and Tosun *et al*. (2005) using acidified 7H9 broth. Cultures were grown for 7 days in universal bottles containing 10 ml 7H9 broth (Standard pH 6.8) supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 to an OD of 0.7 at 600 nm (~ $6x10^6$ CFU/ml). The cultures were centrifuged at 3000g in a bench centrifuge and resuspended in 1 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80.

50 µl of the bacterial suspensions was inoculated into 10 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 adjusted to pH 5.5 and into broth at standard pH 6.8 for 72 hr or for 96 hr at 37ºC. Before inoculation, all media were sterilised by filtration (Section 2.1.2). After incubation, cultures were centrifuged at 3000g in a bench centrifuge and resuspended in 1 ml of 7H9 broth supplemented with 10% (v/v) and 0.05% (v/v) Tween 80 once again.

2.4.4.1. Acid challenge investigation.

To investigate the effect of acid adaptation on the acid resistance of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R, 50 µl of the bacterial suspensions of acid adapted and control cultures (Section 2.4.4) were shifted to universal bottles containing 10 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80, to obtain approximately ~ 2x10^4 CFU/ml. Before inoculation, all media were acidified with HCl to pH 3.5 or 4.5 as well as control with standard pH 6.8 and sterilised by filtration (Section 2.1.2). All cultures were incubated at 37^{o}C for 24 hr or for 14 hr. CFU of acid adapted and control cultures were enumerated as described in section 2.1.11.

2.4.5. Investigating the effect of $NaNO_2$ on viability of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant.

This experiment was done to assess the effect of sodium nitrite ($NaNO_2$) on the viability of the bacteria based on the methods described by Newton *et al*. (2006), Firmani and Rily, (2002a) and Firmani and Rily, (2002b) with some modifications. 10 ml of 7 days old cultures with an optical density of 0.6 - 0.7 at 600 nm, were centrifuged and resuspended in 2 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80. Before inoculation, an appropriate volume of the 7H9 broth medium supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 was prepared and adjusted to pH 5.4. A stock of this medium with 32 mM $NaNO_2$ concentration was prepared and then filtered through a 0.2 µm filter. 10 ml of the 32 mM $NaNO_2$ 7H9 broth were serially diluted to concentrations of 16 mM, 8 mM, 4 mM, 2 mM and 1 mM using the same previously used acidic medium. After that, 50 µl of the bacterial suspensions was inoculated into the universal bottles containing the different concentrations of $NaNO_2$ to obtain a concentration of ~ 2x10^6 CFU/ml. A free $NaNO_2$ bottle with acidic medium was used as a control.

All treated bacterial cultures were incubated at 37^{o}C for 48 hr. Colony forming units per millilitre (CFU) were determined after 90 min, 14 hr, 24 hr and then after 48 hr as described in section 2.1.11. All plates were incubated at 37^{o}C for 3 to 4 weeks.

2.4.6. Investigating the effect of H_2O_2 on viability of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant.

This experiment was done as described before by Newton *et al*. (2006), Firmani and Rily, (2002a) and Firmani and Rily, (2002b) with some modifications. 10 ml of 7 days old cultures with an optical density of approximately 0.7 at 600 nm were centrifuged and re-suspended in 2 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 (Section 2.4.2). To assay the effect of hydrogen peroxide (H_2O_2) on the viability of the bacteria, 50 µl of the bacterial suspensions was inoculated into universal bottles containing 10 ml of 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 with serial dilutions of H_2O_2 concentration starting at 32 mM as stock solution and serially diluted to concentrations of 16 mM, 8 mM, 4 mM, 2 mM and 1 mM after filtration through a 0.2 µm filter. A H_2O_2 free medium bottle was used as a control.

All treated bacterial cultures were incubated at 37^{o}C for 48 hours. Colony forming units per millilitre (CFU/ml) were determined after 90 min, 14 hr, 24 hr and then after 48 hr as described in section 2.1.11. All plates were incubated at 37^{o}C for 3 to 4 weeks.

2.5. Statistical analysis.

For analysing data of the experiments in the current study, the data were obtained from two independent experiments and each experiment was conducted in duplicates. Statistical analyses were done by the student t-test and confirmed by SPSS softwares. Significant difference was assumed for p values less than 0.05 ($p<0.05$).

CHAPTER 3

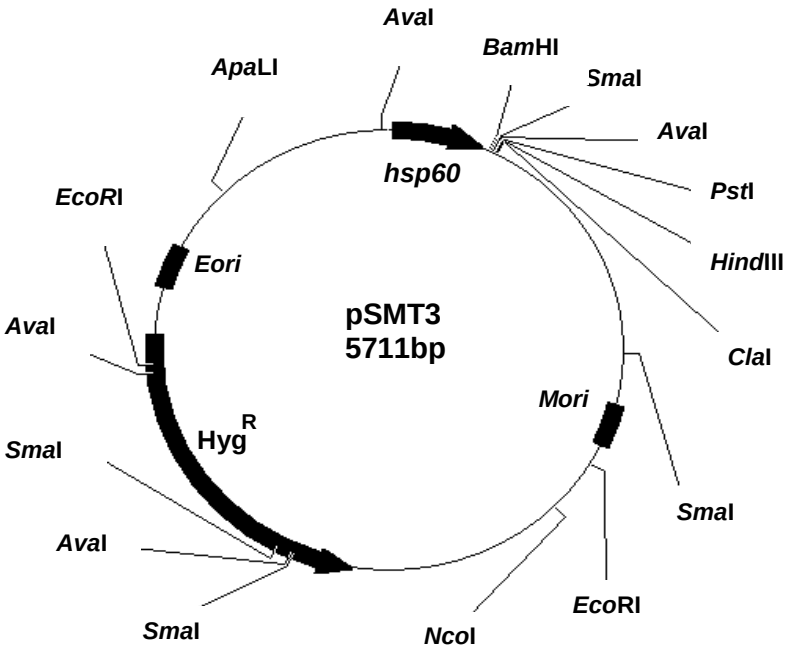
RESULTS

3.1. Construction of shuttle expression vectors.

The aim of the work described in this chapter was to construct *E. coli-Mycobacterium* shuttle expression vectors containing the *rv1995* and *rv1996* genes to be available for introduction into *Mycobacterium tuberculosis* CH which was the causative strain of the 2001 tuberculosis outbreak in Leicester. The CH strain was confirmed to have deletions in 5 loci including the locus of *rv1995* and *rv1996* (Section 1.8.).

In order to construct such shuttle expression vectors, PCR products containing the *rv1995* and *rv1996* genes were individually cloned into the pSMT3 expression vector and transformed into *E. coli* DH5α. This plasmid was used due to its ability to replicate in *E. coli* and also to be maintained in recipient mycobacterial cells (O'Gaora *et al*., 1997). It is useful for the exploration of effective delivery of heterogeneous molecules by mycobacteria (O'Gaora *et al*., 1997; Golanska *et al*., 1989). The pSMT3 vector (Figure 3.1) is characterized by the presence of a hygromycin resistance gene of *Streptomyces hygroscopicus*, both *E. coli* and mycobacterial origins of replication, and a strong *hsp60* (heat shock protein) promoter from *M. tuberculosis* (O'Gaora *et al*., 1997; Golanska *et al*., 1989).

The hsp60 protein is essential under all growth conditions, but is expressed at higher amounts in response to stress (Stover *et al*., 1991) such as heat and oxidative stress (Cabiscol *et al*., 2002; Shi *et al*., 2015). Therefore, the *hsp60* gene promoter was chosen to drive the expression of the *rv1995* and *rv1996* genes in the *M. tuberculosis* CH strain (Newton *et al*., 2006). Using the multiple cloning site (MCS) downstream of the mycobacterial *hsp60* gene promoter in pSMT3, the expression of *rv1995* and/or *rv1996* would be under *hsp60* promoter control (Stover *et al*., 1991; Newton *et al*., 2006).



Hsp60 Promoter *Bam*HI *Sma*I *Pst*I *Eco*RI *Hin*dIII *Cla*I

----------GGATCC CCCGGG CTGCAG GAATTC GATATC **AAGCTT ATCGAT**

Figure 3.1: Map of the pSMT3 plasmid, position of *hsp60* promoter and multiple cloning site downstream of the *hsp60* promoter. The sequence of the *hsp60* promoter is shown as (cut lines) and the multiple cloning site sequences are shown downstream of it. The hygromycin resistance gene (Hyg^R), the mycobacterial origin of replication (*Mori)*, the *E. coli* origin of replication (*Eori*) and multiple cloning site (MCS) are shown on the pSMT3 plasmid.

3.1.1. Construction of shuttle expression vectors for *rv1995* and *rv1996* genes.

3.1.1.1. PCR to amplify the *rv1995* and *rv1996* gene sequences.

Initially, in order to clone the 768 bp *rv1995* and 954 bp *rv1996* gene sequences, they were amplified by PCR from *M. tuberculosis* H37Rv DNA which is a reference strain known to contain both genes (Cole *et al*., 1998). The same strain was the reference used to

detect the deleted genes from the CH strain in the studies done by Shafi *et al*. (2002) and Rajakumar *et al*. (2004) and it was also used to compare the sequence identity of these two genes with genes from other mycobacterial strains used in the present study. Forward primer (For-1) and reverse primer (Rev-1) were designed to amplify the *rv1995* gene (Table 2.9). Forward primer (For-2) and reverse primer (Rev-2) were designed to amplify the *rv1996* gene (Table 2.9). After designing the primers, they were checked for the correct alignment of sequences using NCBI BLAST tool (Section 2.3.3). The primers initially were designed to include the amplification of the intergenic sequences in addition to part of the neighbouring upstream gene. This was done to include the cognate promoters of the target genes if available and to facilitate the process of sequence validation. The positions of primers for target genes (*rv1995* and *rv1996*) in the *M. tuberculosis* H37Rv strain (accession no. AL123456.3), are shown in Figure 3.2 and Figure 3.3, respectively.

To enable direct ligation of the PCR products into the multiple cloning sites of pSMT3, restriction endonuclease sites were incorporated into the primers. The restriction enzyme target sequence was incorporated into 5' sequence of each primer. A *Hin*dIII restriction site was introduced into the forward primers (For-1 and For-2) and a *Cla*I into the reverse primer (Rev-1 and Rev-2). According to analysis using the Cutter Section from www.neb.com, each restriction enzyme was chosen after confirming that it only digests the vector in the MCS and the DNA insert in the corresponding cloning site incorporated into the primers. Also, four additional A bases (Table 2.9) were added to the 5' end of the restriction enzyme site in the primer to facilitate efficient cutting (Newton *et al*., 2006; Perez-Pinera *et al*., 2006; Allemando *et al*., 2002).

The PCR reactions of the *rv1995* and *rv1996* genes were carried out using proof start DNA polymerase and Q-Solution to obtain high-fidelity PCR amplification as described in Materials and Methods (Section 2.3.5.2). The PCR products were visualised after running through a 0.8 % (w/v) agarose gel (Figure 3.4). The contents of PCR products for the *rv1995* gene (1062 bp) and the *rv1996* gene (1307 bp) are diagrammed in Figure 3.2 and Figure 3.3 respectively. The size of PCR product was as expected from the data in the published *M. tuberculosis* H37Rv genome sequence (Cole *et al*., 1998).

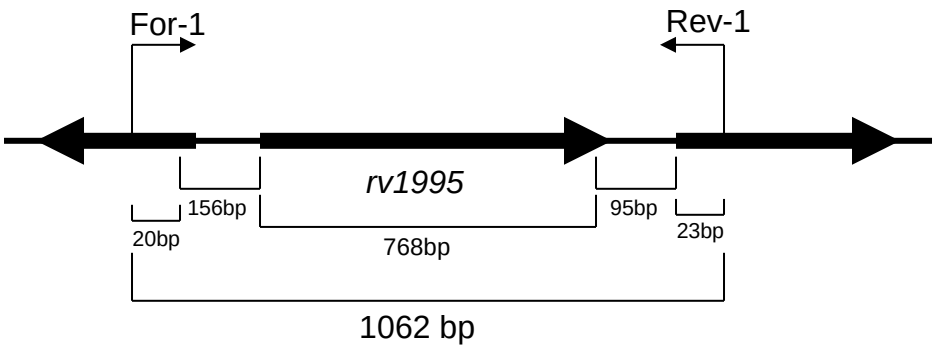


Figure 3.2: Schematic map to show the positions of the PCR primers on *M. tuberculosis* H37Rv DNA to amplify the *rv1995* gene and constituents of the resultant PCR product.

Positions of For-1 and Rev-1 primers for the amplification of the *rv1995* gene are shown. For-1 primer is at the start of the *rv1994* sequence and Rev-1 primer is at the start of the *rv1996* sequence. The resultant PCR product using For-1 and Rev-1 primers (1062 bp) consists of the end of *rv1994* (20 bp), the intergenic sequence between *rv1994*/*rv1995* genes (156 bp), the *rv1995* gene (768 bp), the intergenic sequence between *rv1995*/*rv1996* genes (95 bp) and the start of *rv1996* gene (23 bp).

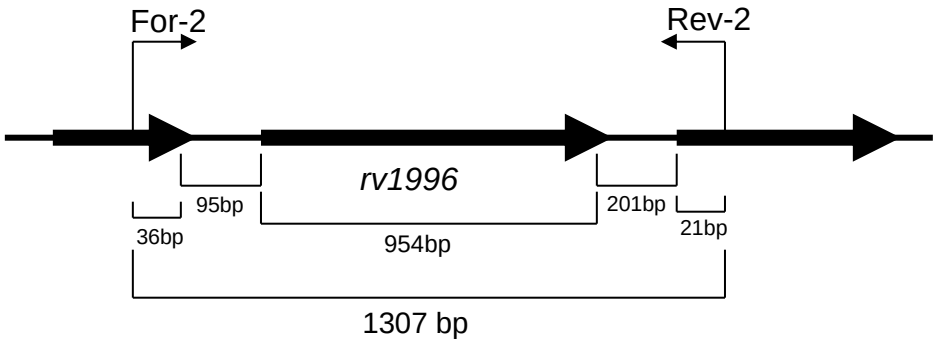


Figure 3.3: Schematic map to show the positions of the PCR primers on *M. tuberculosis* H37Rv DNA to amplify the *rv1996* gene and constituents of the resultant PCR product.

Positions of For-2 and Rev-2 primers for the amplification of the *rv1996* gene are shown. For-2 is at the end of the *rv1995* sequence and Rev-2 is at the start of the *rv1997* sequence. The resultant PCR product using For-2 and Rev-2 primers (1307 bp) consists of the end of *rv1995* gene (36 bp), the intergenic sequence between the *rv1995*/*rv1996* genes (95 bp), the *rv1996* gene (954 bp), the intergenic sequence between the *rv1996*/*rv1997* genes (201 bp) and the start of *rv1997* (21 bp).

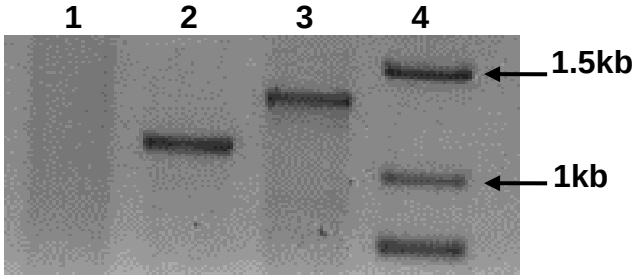


Figure 3.4: PCR to amplify the *rv1995* and *rv1996* genes from *M. tuberculosis* H37Rv DNA

Amplification of the *rv1995* and *rv1996* genes sequences were performed using For-1/ Rev-1 and For-2/ Rev-2 primers, respectively. Lane 4 contains DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases. Lane 1 contains the negative control with all PCR reagents except DNA templates. Lanes 2 and 3 contain *rv1995* PCR product at approximately 1070 bp and *rv1996* PCR product at approximately 1300 bp, respectively.

3.1.1.2. Cloning of PCR products containing the *rv1995* and *rv1996* genes into pSMT3 to produce the required expression vectors.

To construct the required expression vectors, first the 1062 bp PCR product containing the *rv1995* gene and the 1307 bp PCR product containing the *rv1996* gene were purified from the PCR mixtures using QIAquick PCR Purification Kit Protocol (Section 2.3.5.4). The purified 1062 bp and 1307 bp fragments were digested with *Hin*dIII and *Cla*I. Also, in order to linearise pSMT3, the plasmid was digested with the same restriction enzymes as used for each digested amplicon, for three hours at 37^{o}C as described in Materials and Methods (Section 2.3.8.5), followed by purification by precipitation using 100% ethanol (Section 2.3.8.9). Subsequently the PCR fragments were ligated into the digested pSMT3 using T4 ligase with a vector: insert ratio of 1:3 (Section 2.3.8.6).

The ligation products were precipitated with 100% ethanol and 0.1 volume of 3 M sodium acetate pH 5.2 (Section 2.3.8.9), washed in 70% (v/v) ethanol, dried and re-

suspended in 8 µl of sterile nano-pure water. The ligated DNAs were individually introduced into *E. coli* DH5α by electroporation (Section 2.3.6.1). After electroporation, bacterial cells were plated onto L-agar containing 150 µg/ml hygromycin and incubated overnight at 37^{o}C. Afterwards, transformant colonies were individually picked into L-broth containing 150µg/ml hygromycin (Section 2.1.3.) and incubated overnight at 37^{o}C with shaking. Cultures were centrifuged at 2000 ×g and cell pellets were collected.

3.1.1.2.1. Identification of pSMT3::*rv1995* and pSMT3::*rv1996* clones.

After incubation and collection of cells, transformants were identified by small scale preparation of plasmid DNA (Section 2.3.4.1.1). Plasmid DNAs (pSMT3::*rv1995* and pSMT3::*rv1996*) were digested (Section 2.3.8.5) with *Hin*dIII (the single digest with *Hin*dIII was done to identify the recombinant plasmids compared with pSMT3 control), followed by electrophoresis through 0.8% (w/v) agarose gel.

Three colonies out of seventeen tested were identified as having recombinant plasmid for the *rv1995* gene with a fragment of approximately 6770 bp after digestion with *Hin*dIII (Figure 3.5-A lanes 3, 9, and 13) compared with pSMT3 with a size of approximately 5700 bp after digesting with *Hin*dIII. One of the three clones was picked and the plasmid named pAAO1.

Six colonies out of nine tested were identified as having recombinant plasmid for the *rv1996* gene with a fragment of approximately 7000 bp after digestion with *Hin*dIII (Figure 3.5-B lanes 1, 2, 3, 6, 9 and 10) compared with pSMT3 with a size of approximately 5700 bp after digesting with *Hin*dIII. One was picked and the plasmid named pAAO2.

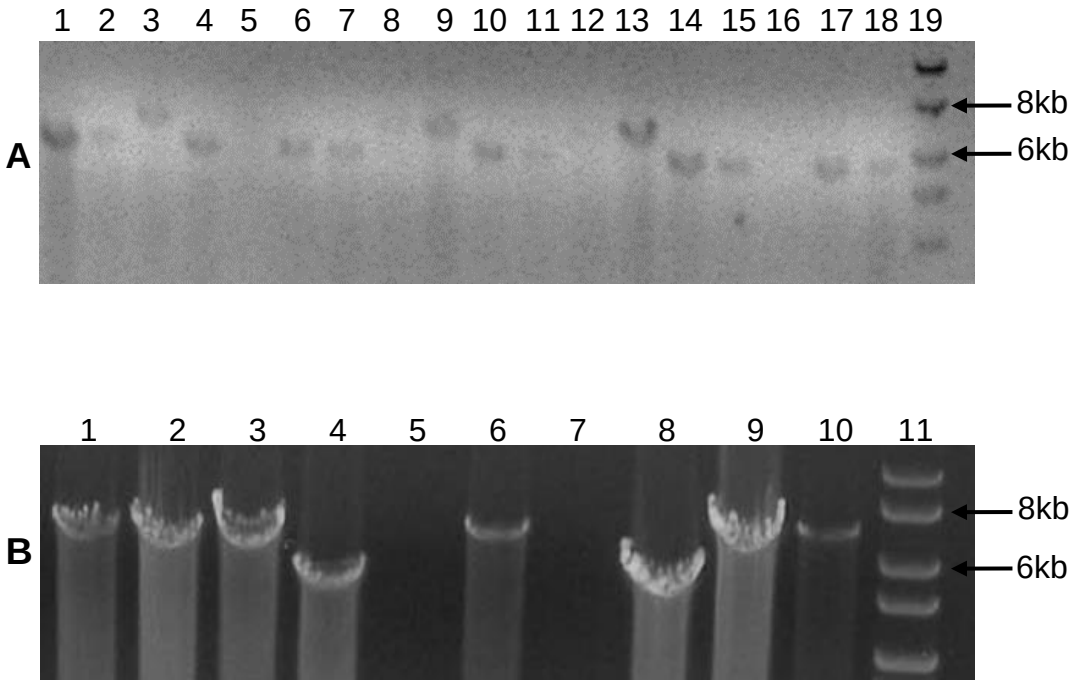


Figure 3.5: Identification of the recombinant plasmids; (A) pSMT3::*rv1995* and (B) pSMT3::*rv1996*.

To identify the recombinant *E. coli* containing pSMT3::*rv1995* (A) and pSMT3::*rv1996* (B), the plasmid DNAs were digested with *Hin*dIII. Lanes 19 (A) and 11 (B) contain DNA size marker (1Kb ladder, NEB), with fragment sizes shown in kilobases (kb). Restriction digestion with *Hin*dIII for pSMT3::*rv1995* (A) resulted in one fragment of approximately 6770 bp (lanes 3, 9, and 13), whereas lanes 2, 4, 6, 7, 10, 14, 15, 17 (Non-recombinant) and 18 (control) had one fragment of pSMT3 at approximately 5700 bp. In the remaining lanes, there were no bands. Restriction digestion with *Hin*dIII of pSMT3::*rv1996* (B) resulted in one fragment of approximately 7000 bp (lanes 1, 2, 3, 6, 9 and 10), whereas lanes 8 (Non-recombinant) and 4 (control) had one fragment of pSMT3 at approximately 5700 bp. In the remaining lanes, there were no bands.

3.1.1.2.2. Confirmation of the presence of the *rv1995* and *rv1996* genes in pAAO1 and pAAO2 expression vectors.

To confirm the presence of the fragments containing the *rv1995* and the *rv1996* genes in the recombinant plasmids, DNA was prepared from one transformant colony of each plasmid (pAAO1 and pAAO2 respectively) using the large scale protocol (Section 2.3.4.1.2). pAAO1 and pAAO2 plasmids were digested with *Hin*dIII and *Cla*I (the double digest was done to confirm the presence of insert fragment).

Digestion of pAAO1 DNA with *Hin*dIII and *Cla*I after electrophoresis through a 0.8% (w/v) agarose gel resulted in two fragments of approximately 5700 bp and 1070 bp (Figure 3.6-A lane 2) that represented the sizes of pSMT3 (5711bp) and fragment containing the *rv1995* gene (1062 bp) respectively. Digestion of pAAO1 DNA with either *Hin*dIII or *Cla*I alone resulted in one fragment of approximately 6770 bp (Figure 3.6-A lanes 1 and 4) that represented the linearised size of pAAO1 (6773 bp). The map of pAAO1 plasmid construction is shown in Figure 3.7.

Digestion of pAAO2 DNA with *Hin*dIII and *Cla*I after electrophoresis through a 0.8% (w/v) agarose gel resulted in two fragments of approximately 5700 bp and 1300 bp (Figure 3.6-B lane 2) that represented the sizes of pSMT3 (5711 bp) and fragment containing the *rv1996* gene (1307 bp) respectively. Digestion of pAAO2 DNA with either *Hin*dIII or *Cla*I alone resulted in one fragment of approximately 7000 bp (Figure 3.6-B lanes 1 and 4) that represented the size of pAAO2 (7018 bp). The map of pAAO2 plasmid construction is shown in Figure 3.8.

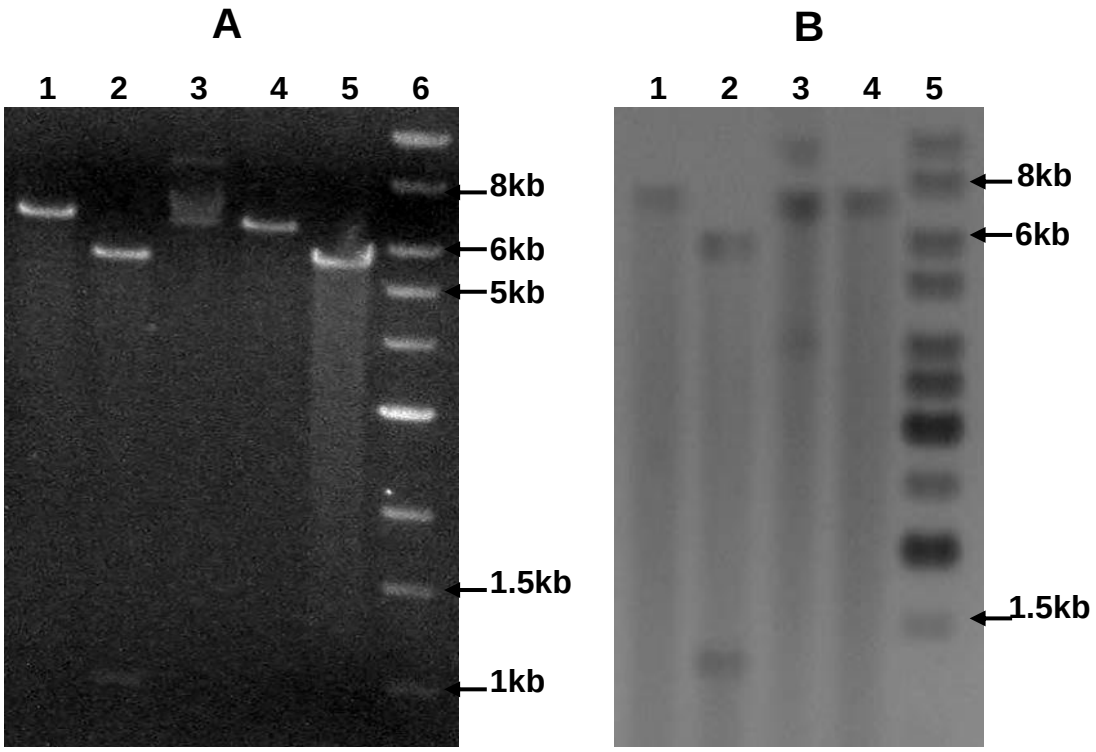


Figure 3.6: Confirmation of the presence of *rv1995* gene in pAAO1 (A) and *rv1996* gene in pAAO2 (B).

To confirm the presence of the 1062 bp fragment containing the *rv1995* gene in pAAO1 (A), and the presence of the 1307 bp fragment containing the *rv1996* gene in pAAO2 (B), the DNAs were digested with *Hin*dIII and/or *Cla*I. (A) Lane 6 contain DNA size marker (1 Kb ladder, NEB), with fragment sizes shown in kilobases (kb). 5700 bp pSMT3 digested with *Hin*dIII (plasmid control-lane 5). Restriction digestion with either *Cla*I (lane 1) or *Hind*III (lane 4) resulted in one fragment of approximately 6770 bp. Restriction digestion with *Cla*I and *Hin*dIII resulted in two fragments of approximately 5700 bp and 1070 bp (the plasmid fragment containing the *rv1995* gene) (lane 2). Lane 3 contains (control) undigested pSMT3. (B) Lane 5 contain DNA size marker (1 Kb ladder, MBI Fermentas) with fragment sizes shown in kilobases. Restriction digestion with either *Cla*I (lane 1) or *Hin*dIII (lane 4) resulted in one fragment of approximately 7000 bp. Lane 2 shows two fragments of approximately 5700 bp and 1300 bp (the plasmid fragment containing the *rv1996* gene) on digestion with both *Cla*I and *Hin*dIII. Lane 3 contains (control) undigested pSMT3.

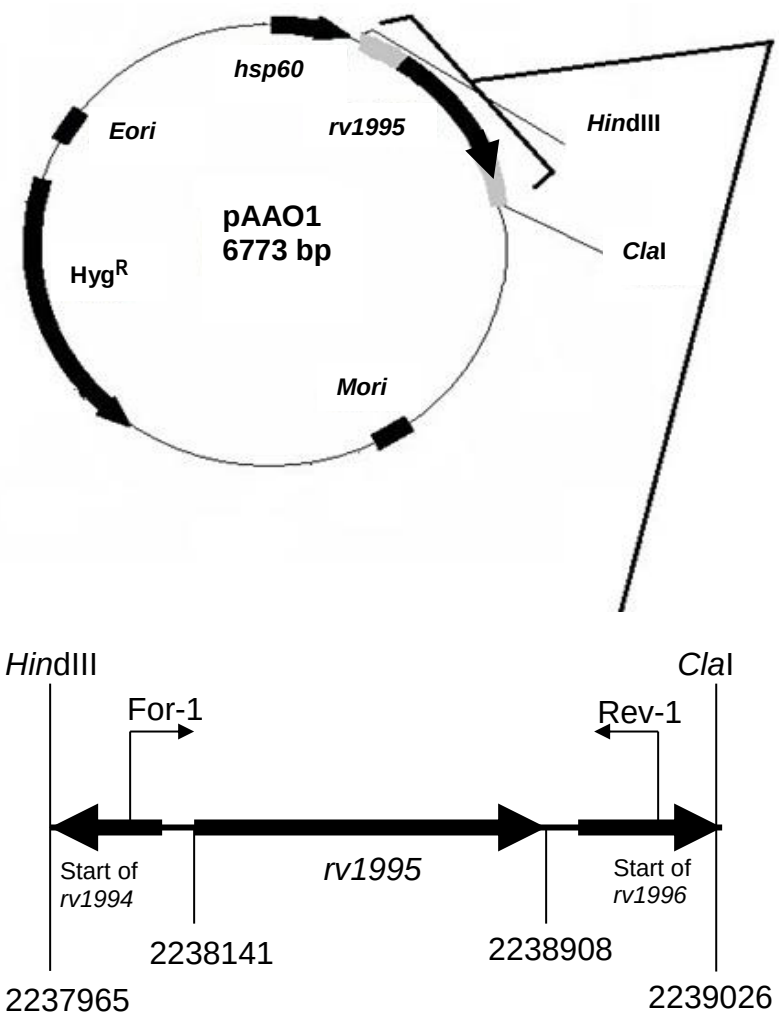


Figure 3.7: Diagrammatic map of pAAO1 plasmid construction.

The amplified PCR product sequence contained the *rv1995* gene from *M. tuberculosis* H37Rv (1062 bp) drawn to scale, digested with *Hin*dIII and *Cla*I and ligated between the *Hin*dIII and *Cla*I sites of pSMT3 (Figure 3.1) to produce pAAO1. The bent arrows represent the approximate positions of the For-1 and Rev-1 primers. Abbreviations are mycobacterial origin of replication (*Mori)*, *E. coli* origin of replication (*Eori*), mycobacterial promoter, heat shock protein (*hsp60*), hygromycin resistance gene (Hyg^R). The coordinates of *rv1995* ORF (2238141- 2238908) and the beginning and the end of the PCR product (2237965-2239026) are shown in big numbers.

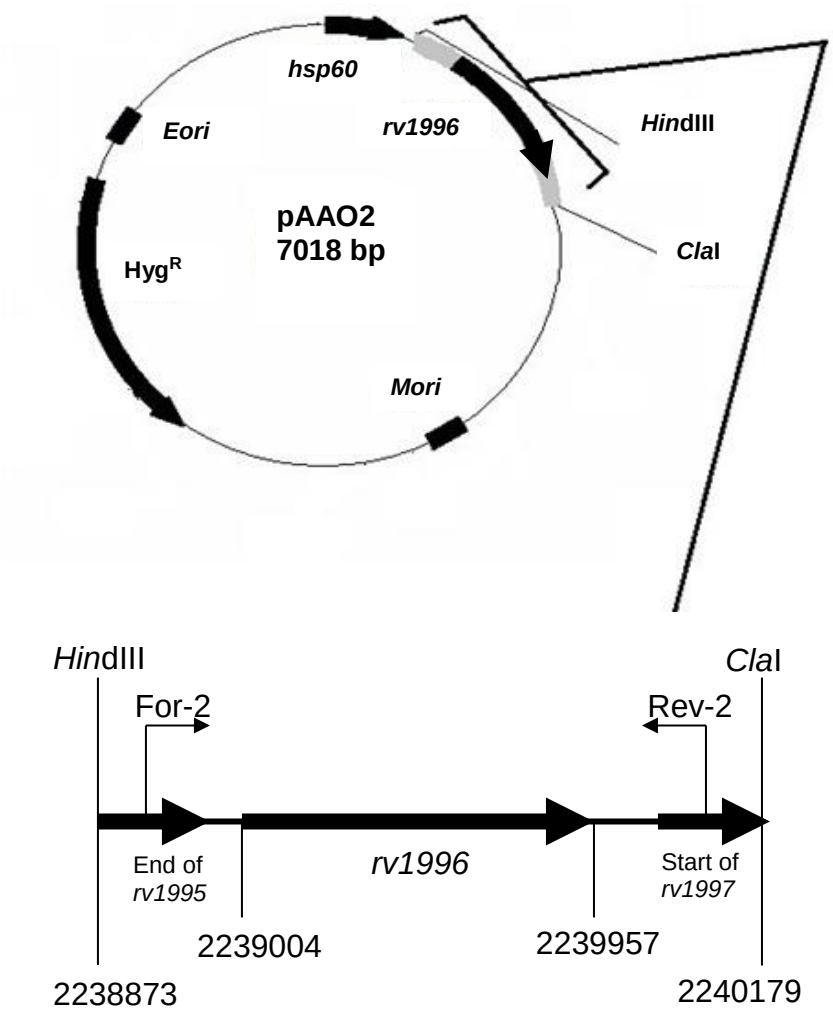


Figure 3.8: Diagrammatic map of pAAO2 plasmid construction.

The amplified PCR product sequence contained the *rv1996* gene from *M. tuberculosis* H37Rv (1307 bp) drawn to scale, digested with *Hin*dIII and *Cla*I and ligated between the *Hin*dIII and *Cla*I sites of pSMT3 (Figure 3.1) to produce pAAO2. The bent arrows represent the approximate positions of the For-2 and Rev-2 primers. Abbreviations are mycobacterial origin of replication (*Mori)*, *E. coli* origin of replication (*Eori*), mycobacterial promoter, heat shock protein (*hsp60*), hygromycin resistance gene (Hyg^R). The coordinates of *rv1996* ORF (2239004- 2239957) and the beginning and the end of the PCR product (2238873-2240179) are shown in big numbers.

3.1.1.3. Sequencing of *rv1995* and *rv1996* PCR fragments from pAAO1 and pAAO2, and referral of constructs to Imperial College.

After cloning, DNA molecules (pAAO1 and pAAO2) were stored at -20^{o}C at the laboratories of the University of Leicester until further use in other experiments. Samples of the prepared recombinant plasmids were sent to MWG for sequencing. The 1062 bp insert containing *rv1995* from pAAO1 and the 1307 bp insert containing *rv1996* from pAAO2 were both sequenced using the automated method as described in Material and Methods (Section 2.3.9). Sequences of the inserted clones were analysed using NCBI BLAST tool (Section 2.3.9), compared with those contained in the H37Rv reference strain and found to be 100% identical to that found in reference strain.

After confirming the success of cloning of the target genes into the plasmids, samples from the prepared DNA constructs (pAAO1 and pAAO2) were sent to Imperial College (London) for further investigation including introduction of the DNA constructs into the *M. tuberculosis* CH strain, measuring the expression of the target genes (*rv1995* and *rv1996*) and phenotyping experiments on the recombinant CH strains (Newton *et al*, 2006). This work was carried out externally as the University of Leicester Category 3 laboratory was not available to work on the CH strain at that time.

Initial data obtained from Imperial College team (2004) which included assessment of recombinant CH strains' gene expression using Quantitative RT-PCR (Newton *et al*., 2006) confirmed that *rv1995* gene carried by the pAAO1 plasmid was expressed in the CH strain successfully, whereas the *rv1996* gene carried by pAAO2 was not able to be expressed when introduced into CH. Therefore, a second attempt to clone the *rv1996* gene into pSMT3 was done.

3.1.2. Construction of a second shuttle expression vector for the *rv1996* gene.

Given the first gene expression attempt of the *rv1996* gene was unsuccessful (Section 3.1.1.3), a second attempt was carried out to construct another expression vector for the *rv1996* gene. The same steps done to construct pAAO1 and pAAO2 were followed to construct another expression vector for the *rv1996* gene using different restriction enzyme sites (*Bam*HI and *Pst*I) and different primer annealing positions on the H37Rv genome, upstream Rv1996-forward (For-3) and downstream Rv1996-reverse (Rev-3) (Table 2.9), to make the *rv1996* gene directly downstream to the *hsp60* promoter. The positions of primers for the target gene (*rv1996*) in *M. tuberculosis* H37Rv (accession no. AL123456.3), are shown in Figure 3.9. A *Bam*HI restriction site was introduced into the forward primer (For-3) and a *Pst*I site into the reverse primer (Rev-3) (Table 2.11).

The PCR reaction of the *rv1996* gene was carried out as described in Materials and Methods (Section 2.3.5.2). The PCR product was visualised (Figure 3.10). The contents of PCR product for *rv1996* gene (963 bp) is diagrammed in Figure 3.9.

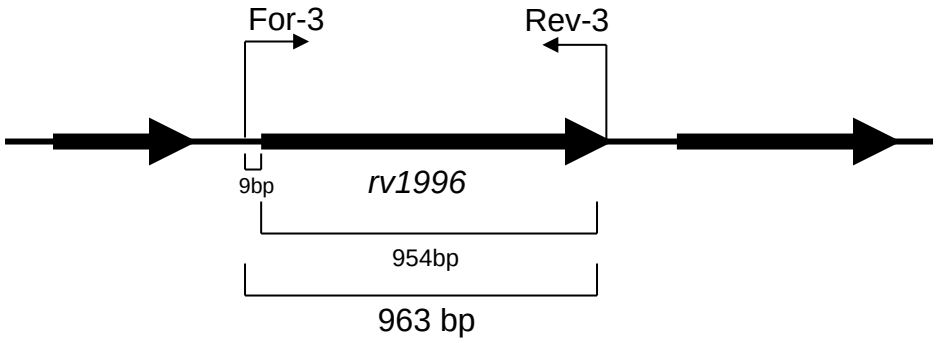


Figure 3.9: Schematic map to show the positions of the PCR primers on *M. tuberculosis* H37Rv DNA to amplify the *rv1996* gene and constituents of the resultant PCR product.

Positions of For-3 and Rev-3 primers for the amplification of the *rv1996* gene are shown. The resultant PCR product using For-3 and Rev-3 primers (963 bp) consists of the *rv1996* gene (954 bp) and 9 base pairs of the intergenic region included in the forward primer.

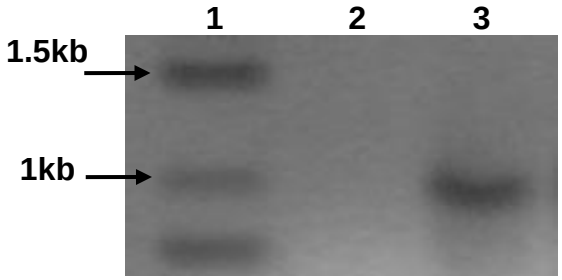


Figure 3.10: PCR to amplify the *rv1996* gene from *M. tuberculosis* H37Rv DNA.

Amplification of the *rv1996* gene sequence was performed using For-3 and Rev-3 primers. Lane 1 contain DNA size marker (1 Kb ladder, MBI Fermentas) with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control. Lane 3 contains the PCR product of *rv1996* at approximately 1000 bp.

To construct the required expression vector, the 963 bp PCR product containing the *rv1996* gene was purified from the PCR mixtures (Section 2.3.5.4). Both purified PCR product and pSMT3 plasmid were digested with *Bam*HI and *Pst*I as described before. Then the PCR fragment was ligated into the digested pSMT3 using T4 ligase (Section 2.3.8.6).

The ligated DNAs were introduced into *E. coli* DH5α by electroporation (Section 2.3.6.1) and then transformants were identified (Section 2.3.4.1.1). Three colonies out of eight tested were identified as containing the recombinant plasmid (pSMT3::*rv1996*) with a fragment of approximately 6700 bp after digesting with *Bam*HI, (Figure 3.11. lanes 5, 6 and 9) compared with pSMT3 with a size of approximately 5700 bp after digesting with *Bam*HI. One colony was picked and the plasmid named pAAO3.

3.1.2.1. Confirmation of the presence of the *rv1996* gene in pAAO3.

To confirm the presence of the fragment containing the *rv1996* gene in the recombinant plasmid, DNA was prepared from one transformant colony obtained for pAAO3 (Section 2.3.4.1.2). pAAO3 was digested with *Bam*HI and *Pst*I. Digestion of

pAAO3 DNA with *Bam*HI and *Pst*I after electrophoresis through a 0.8% (w/v) agarose gel resulted in two fragments of approximately 5700 bp and 1000 bp (Figure 3.12. lane 2) that represented the sizes of pSMT3 (5711 bp) and fragment of *rv1996* gene (963 bp) respectively. Digestion with either *Bam*HI or *Pst*I alone resulted in one fragment of approximately 6700 bp (Figure 3.12. lanes 1 and 4) that represented the size of pAAO3 (6674bp). A map of the pAAO3 plasmid is shown in Figure 3.13.

After construction pAAO3, it was sent to Imperial College in London where data obtained confirmed that the *rv1996* gene carried by pAAO3 was successfully expressed in the CH strain.

The recombinant strains for the *rv1995* and *rv1996* genes were named CH::hyg^R*1995* and CH::hyg^R*1996* respectively. The expression of the *rv1995* and *rv1996* genes in recombinant strains, and their phenotypic effects were investigated and published by Newton and others (2006).

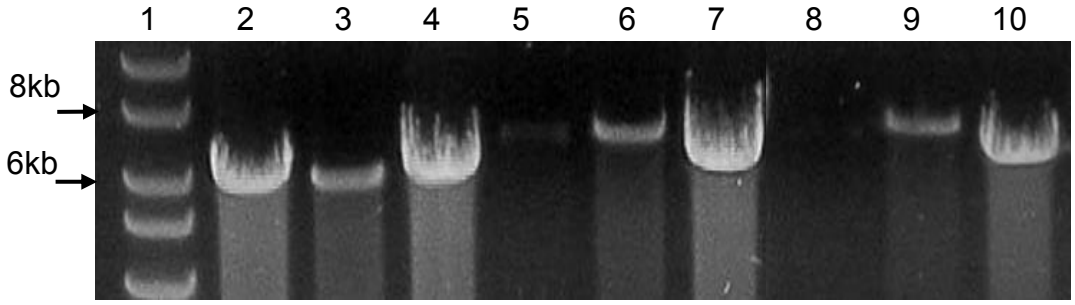


Figure 3.11: Identification of the recombinant plasmid pSMT3::*rv1996*.

To identify the recombinant *E. coli* as containing pAAO3, the DNA was digested with *Bam*HI. Lane 1 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Restriction digestion with *Bam*HI resulted in one fragment of approximately 6700 bp (lanes 5, 6 and 9), whereas lanes 3, 4, 7 and 10 shown one fragment of pSMT3 at approximately 5700 bp (non-recombinant). Lane 2 contains control pSMT3 with a size of approximately 5700 bp. In lane 8, no plasmid fragments were evident.

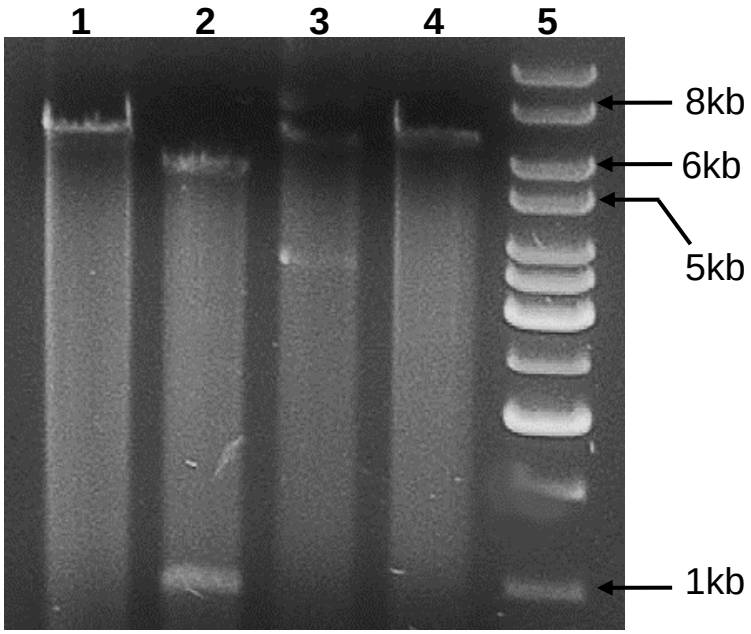


Figure 3.12: Confirmation of the presence of *rv1996* gene in pAAO3.

To confirm the presence of the 945 bp *rv1996* fragment in recombinant pAAO3, the DNA was digested with *Bam*HI and/or *Pst*I. Lane 5 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Restriction digestion with *Bam*HI and *Pst*I resulted in two fragments of approximately 5700 bp and 1000 bp (lane 2). Restriction digestion with *Bam*HI or *Pst*I separately resulted in one fragment of approximately 6700 bp (lanes 1 and 4 respectively). Lane 3 contains undigested pSMT3 plasmid (control).

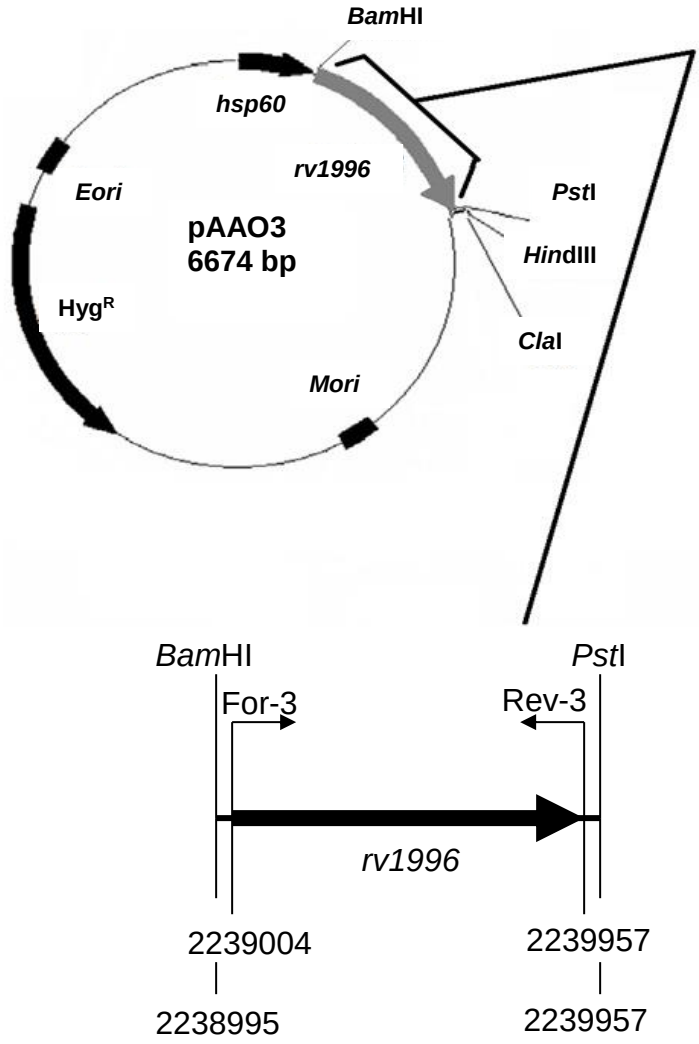


Figure 3.13: Diagrammatic map of pAAO3 plasmid construction.

The amplified PCR product sequence represented the *rv1996* gene from *M. tuberculosis* H37Rv (963 bp) drawn to scale, digested with *Bam*HI and *Pst*I before ligation between the *Bam*HI and *Pst*I sites of pSMT3 (Figure 3.1) to produce pAAO3. The bent arrows represent the approximate positions of the For-3 and Rev-3 primers. Abbreviations are mycobacterial origin of replication (*Mori)*, *E. coli* origin of replication (*Eori*), mycobacterial promoter, heat shock protein (*hsp60*), hygromycin resistance gene (Hyg^R). The coordinates of *rv1995* ORF (2239004- 2239957) and the beginning and the end of the PCR product (2238995-2239957) are shown in big numbers.

3.2. Discussion.

The plasmid based "knock-in" experimental strategy involved in the current study has also been used in mycobacteria in other situations. The same plasmid, pSMT3 used for *rv1995* and *rv1996*, was used in the study performed by Newton *et al*. (2006) to also reintroduce the *rv1519* and *rv0180* genes into the CH strain to regain the function of these genes. The strategy could be used also to introduce a gene of known function to add a desired feature to a certain strain. For example, it was used to introduce ESAT-6 encoding gene *rv3875* into BCG for enhancement of the immunogenicity and improving the efficacy of the BCG vaccine (Pym *et al*., 2003). Similarly, a gene of a known function can be introduced into a strain which is known to harbour the same gene for the purpose of overexpressing its function. Wang *et al*. (2012) reported that rBCG strains overexpressing immunodominant antigens such as Ag85A, which is encoded by *rv3804c* (BCG::*rv3804c*), showed more protection against *M. tuberculosis* than the BCG control. Overexpression of a particular gene also was used by Hu *et al*. (2015) who constructed a strain of *M. bovis* BCG that overexpresses the *bcg2013* (*rv1996*) gene. They found that overexpression of *bcg2013* increased susceptibility of the recombinant strain to INH compared with the wildtype strain. Furthermore, the approach of a knock in strategy using expression vectors was used to investigate the function of many mycobacterial genes. For instance, Li *et al*. (2014a) used the pNIT-1 plasmid to investigate the function of the *rv3402c* gene via delivering the gene into *Mycobacterium smegmatis* and it was found to enhance the persistence of the microorganism within the host cell.

3.2.1. Introduction of DNA constructs pAAO1 and pAAO2 (pAAO3) into CH and investigation of the *rv1995* and *rv1996* gene expression.

After sending the vectors to Imperial College, they were individually electroporated into competent log phase CH cells, and transformants were selected on 7H11 agar containing hygromycin. RNA was extracted and mRNA expression of both the *rv1995* and *rv1996* genes was measured by qRT-PCR and compared to the corresponding genes in

H37Rv (Newton *et al*., 2006). The PCR primer and probe sequences were shown in Newton *et al*. (2006) supplementary material. The data provided by the Imperial College team indicated that *rv1995* of pAAO1 was expressed in the CH strain. However, the target gene *rv1996* was not expressed in the case of pAAO2 (data not available) but was successfully expressed in pAAO3. The reasons of failure of expression in the case of pAAO2 was assumed to be that transcription was terminated before starting the *rv1996* sequence in pAAO2 due to presence of a transcriptional terminator sequence or transcription terminator pause site in the intergenic sequence between the *rv1995* and *rv1996* genes (Section 3.2.1.1).

The level of mRNA expression of the *rv1995* and *rv1996* genes carried by pAAO1 and pAAO3 of the recombinant strains was calculated as ratios normalized to the 16S cDNA. The ratio of the *rv1995* relative to 16S was 2.5×10^{-6} for H37Rv, whereas it was 3.7×10^{-4} for the complemented CH strain. In the case of *rv1996*, relative to 16S the ratio was 2.1×10^{-3} for the H37Rv and 2.6×10^{-4} for the recombinant one (copy number of mRNA for each gene is not available) (Newton *et al*., 2006a). The successful expression of the *rv1996* gene carried by pAAO3 might be attributed to the absence of intergenic sequence from the insert and thus the *rv1996* sequence falls directly downstream of the *hsp60* promoter.

In the case of pAAO1, though the whole length of intergenic sequence between the *rv1994* and *rv1995* genes was included in the insert, it seems that there was no terminator sequence in the area between the *rv1994* and *rv1995* genes, hence the *rv1995* gene had the chance to be successfully transcribed. This assumption was supported by a bioinformatic analysis using an algorithm for identification of Rho-independent transcription terminators (Naville *et al*., 2011) which revealed no predicted terminator in the intergenic sequence between the *rv1994* and *rv1995* genes. Since the afore mentioned database could be searching for canonical terminators only (Lesnik *et al*., 2001), another database (WebGeSTer DB-a transcription terminator database) (Mitra *et al*., 2011) was accessed and among terminators predicted for the H37Rv strain, there was no predicted terminator between *rv1994* and *rv1995*. To check the analytical process followed, the WebGeSTer database was accessed to detect an already known terminator sequence

(5'GCGAUUCUG3') which was identified by Czyz *et al*. (2014) for *rv1324* and the sequence was successfully identified.

The finding that there was no terminator sequence between *rv1994* and *rv1995* is also supported by the fact that the orientation of the *rv1994* gene is opposite to that of the *rv1995* gene which makes the two genes divergently transcribed (Figures 3.2 and 3.7). Haller *et al*. (2010) reported that transcriptional terminator sequences are mostly plentiful between two genes which are convergently transcribed and uncommon or absent between divergently transcribed genes. In the same work, Haller *et al*. (2010) expected that promoters are commonly observed between the divergently transcribed genes, which could be another interpretation of the *rv1995* gene expression in the case of pAAO1, since the intergenic sequence between the *rv1994* and *rv1995* genes could have its own promoter in this sequence. Therefore, a further bioinformatic analysis using the BProm program SoftBerry algorithm for promoter detection (Solovyev and Salamov, 2011) was performed on the intergenic sequence between *rv1994* and *rv1995*. A single promoter with a transcription start site at position 76 relative to the start of the intergenic sequence provided, a -10 box at position 61 (5'-agatatgat) and a -35 box at position 41 (5'-ctgaat), was found which was expected to be for the *rv1995* gene. Regarding the *rv1994c* gene, the complementary sequence for the same intergenic sequence was also checked using the same algorithm for another promoter. A single promoter with a transcription start site at position 123, a -10 box at position 104 (5'-atgtacaat) and a -35 box at position 88 (5'-atcata) was found. This promoter was suggested to be for the *rv1994*c gene. The -35 and -10 boxes are required for interaction with the σ^{70} subunit of RNA polymerase (Dekhtyar *et al*., 2008). The optimally-separated patterns should be at -10 and -35 from the transcription start site however this is not always present (Dekhtyar *et al*., 2008). The existence of both promoters for *rv1994c* and *rv1995* and lack of a terminator sequence in the intergenic region between the two divergently transcribed genes (*rv1994c* and *rv1995*) is consistent with the previously mentioned report by Haller *et al*. (2010).

A similar bioinformatic analysis for detecting a promoter sequence in the intergenic sequence between the *rv1995* and *rv1996* genes was performed and revealed a single promoter sequence with a transcription start site at position 72, a -10 box at position 57

(5’-ggctaacgt) and a -35 box at position 38 (5’-ctgacg). However, in spite of the potential presence of a *rv1996* promoter in pAAO2, the gene was not expressed. Thus, another possible reason for the absence of gene expression in the case of pAAO2 is that the promoter could not work properly within the insert (Park *et al*., 2003; Voskuil *et al*., 2003; Isaza *et al*., 2011). Voskuil *et al*. (2003) reported that the 48 genes under *dosR* control are not dispersed randomly across the *M. tuberculosis* chromosome, but they are clustered in a unique manner suggestive of a supra-operonic organisation to facilitate a coordinated transcriptional response. Therefore, the complementation of the CH strain with the *rv1996* gene in pAAO2 may have left the promoter of the *rv1996* gene outside this network of *dosR* regulation.

3.2.1.1. Transcription termination in mycobacteria and possible role in non-expression of *rv1996* in pAAO2.

Mycobacteria like other bacteria use two main means of transcription termination; the first is intrinsic, Rho-independent, and second is Rho-dependent termination. Intrinsic terminators, encode GC-rich RNA hairpins which is usually followed by more than 6 Us-successive nucleotides (Griffith *et al*., 2005). Analysis of the intergenic sequences between the *rv1995* and *rv1996* genes revealed that U rich terminators are lacking (data not shown). However, Mitra *et al*. (2008) reported that these U- rich terminators are underrepresented in mycobacteria, hence mycobacterial RNA polymerases may stop at intrinsic terminators lacking U-rich sequences. Most of these terminator sequences are located within fifty base pairs downstream of the stop codon (Mitra *et al*., 2008; Czyz *et al*., 2014). As an example, a candidate sequence which could terminate transcription in spite of lacking a U-rich sequence, is 5'GCGAUUCUG3' that was predicted by Czyz *et al*. (2014) in the case of the *rv1324* gene. They reported also that mycobacterial RNA polymerase terminates more efficiently than *E. coli* RNA polymerase at canonical terminators with imperfect U-tracts.

The second possible mechanism which could be involved in transcription termination and hence in failure of pAAO2 expression is Rho dependent. Rho is a helicase that binds to a transcription terminator pause site which is usually an exposed region of single stranded RNA after the open reading frame at C-rich sequences that do not have

neither the hairpin loops nor the U residues (Mitra *et al*., 2014; Hinde *et al*., 2005). Transcription terminator pause site includes an upstream part called the *rut* (*r*ho *ut*ilization) site. Rho binds the newly formed RNA chain at the *rut* site just upstream from sequences at which the RNA polymerase tends to pause. After binding, rho facilitates the release of the RNA from RNA polymerase (Griffith *et al*., 2005; Peters *et al*., 2006; D'Heyg`ere *et al*., 2015).

CHAPTER 4

RESULTS

4.1. Construction of suicide vectors.

Aim of the work described in this chapter was to construct suicide vectors harbouring the disrupted *rv1995* and *rv1996* genes separately in order to be introduced into *M. tuberculosis* H37Rv (reference strain) and *M. bovis* BCG (vaccine strain) to obtain isogenic mutants via homologous recombination. The mutation strategy was that of insertional mutagenesis using a hygromycin resistance gene. This was initiated by PCR amplification of the sequences flanking the point of insertion. Then the plan was to use a directional cloning approach of flanking regions and hygromycin resistance cassette into a mycobacterial suicide vector, p1NIL.

Construction of a suicide delivery vector is often a difficult process as several cloning steps are required to include the relevant markers and finding appropriate restriction sites for inserting these genes is another limiting factor (Parish and Stoker, 2000). The 4525 bp plasmid p1NIL was used as a suicide vector in *M. tuberculosis* according to the approach described by Parish and Stocker (2000). The p1NIL vector lacks a mycobacterial origin of replication and is, therefore, unable to replicate in mycobacteria, but it has an *E. coli* origin of replication that enables it to replicate in *E. coli* for cloning purposes. In addition, this vector is characterized by harbouring a kanamycin resistance gene for selection of both *E. coli* and mycobacterial cells on media provided with kanamycin to enable selection for the presence of the plasmid. Furthermore, it contains multiple cloning sites for ligation of target DNA inserts (Parish and Stoker, 2000; Ailenberg *et al*., 2005).

4.1.1. Construction of a suicide vector for *rv1995*.

4.1.1.1. PCR to amplify the *rv1995* fragment A, *rv1995* fragment B and the hygromycin resistance cassette.

For disruption of the *rv1995* gene, two pairs of primers (Table 2.9) were used to amplify two fragments (designated as fragment 95-A and fragment 95-B) of the *rv1995* gene derived from *M. tuberculosis* H37Rv DNA (Figure 4.1) by PCR. The forward primer (ForA-5) and the reverse primer (RevA-5) were designed (Section 2.3.3) to amplify

fragment 95-A which extends from the 1st nucleotide in the gene to the nucleotide no. 359. The forward and reverse primers of fragment B (ForB-5; RevB-5) respectively were used to amplify fragment 95-B which starts from the nucleotide no. 409 to the last nucleotide of the *rv1995* gene no. 768. The 50 bp segment between the two fragments was not included in the amplification process, hence it will be lost and may be used as another marker of mutation. For PCR amplification of the 1356 bp hygromycin resistance gene, pSMT3 was used as a template. The forward primer (For-Hyg) and the reverse primer (Rev-Hyg) from pSMT3 DNA were designed to amplify the whole gene with its own promoter and transcriptional terminator.

Primers of all fragments were designed to contain restriction enzyme target sequences. A *Sca*I restriction site was incorporated into the forward primer and an *Asc*I restriction site was incorporated into the reverse primer of the fragment 95-A. An *Asc*I restriction site was incorporated into the forward primer and a *Hin*dIII restriction site was incorporated into the reverse primer of the fragment 95-B. An *Asc*I restriction site was incorporated into the forward and reverse primer of the hygromycin resistance gene. PCR reactions were done as described in Materials and Methods (Section 2.3.5.2). The PCR product results were visualised at approximately 360 bp for the fragment 95-A, 360 bp for fragment 95-B and 1360 bp for the hygromycin resistance gene after running on a 0.8 % (w/v) agarose gel (Figure 4.2). These PCR product sizes closely agreed with the deduced sizes of fragment 95-A (359 bp), fragment 95-B (359 bp) and the hygromycin resistance gene (1356 bp) respectively.

4.1.1.2. Sequencing of the *rv1995* PCR product fragments.

The two PCR product fragments A and B and their corresponding forward and reverse primers were sent to MWG Biotech for sequencing. The sequencing was done as described in Material and Methods (Section 2.3.9). The two primers were used to enable overlapping sequence data to be obtained from the whole amplified fragment. Sequence analysis was done by comparing the sequences of amplified fragments against known sequences of the H37Rv reference strain using NCBI BLAST tool (Section 2.3.9). The analysis confirmed that the query fragments were amplified successfully with 100% identity to H37Rv.

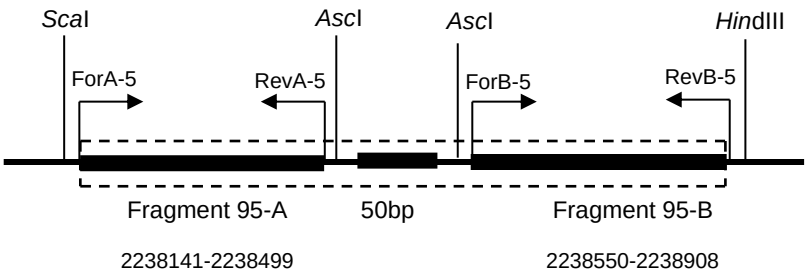


Figure 4.1: Schematic map to show position of the PCR primers to amplify the fragments 95-A and 95-B on *M. tuberculosis* H37Rv DNA, the positions of restriction enzymes cutting sequences and the relative positions of the amplified fragments in relation to ORF of *rv1995*.

The coordinates of fragments 95-A and 95-B on *M. tuberculosis* H37Rv DNA are shown below each. The amplified fragments and the 50 bp segment in-between (black thick lines), ORF of *rv1995* (cut lines box), the forward ForA-5 and reverse RevA-5 primers for the amplification of 95-A, and the forward ForB-5 and reverse RevB-5 primers for the amplification of 95-B (bent arrows) and restriction enzyme cutting sequences are shown.

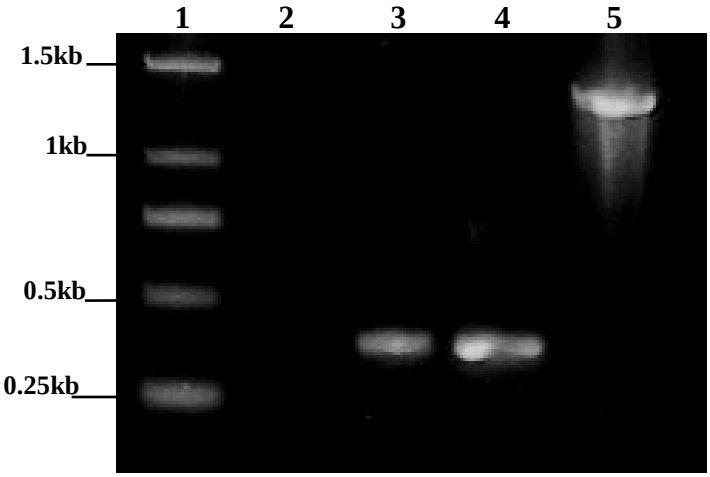


Figure 4.2: PCR to amplify fragments 95-A and 95-B and the hygromycin resistance cassette.

Amplification of the fragments 95-A, 95-B and hygromycin resistance cassette was performed using ForA-5/RevA-5, ForB-5/RevB-5 and For-Hyg/Rev-Hyg respectively. Lane 1 contains DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control (No template control). Lane 3, 4 and 5 contain the PCR products of fragment 95-A, fragment 95-B and hygromycin resistance cassette, at approximately 360 bp, 360 bp and 1360 bp, respectively.

4.1.1.3. Cloning of the fragment 95-A, fragment 95-B and hygromycin resistance cassette into pGEM-T Easy to produce pASO1, pASO2 and pASO3, respectively.

To clone the individual amplicons as a step in the process of construction of required suicide vectors, the amplified fragments 95-A, 95-B and hygromycin resistance gene were individually cloned into the pGEM-T Easy plasmid (Ailenberg, 2005; Promega, 2003). The pGEM-T Easy plasmid is a 3015 bp cloning vector, which has an origin of replication, *lacZ* promoter and gene, ampicillin resistance gene, and multiple cloning sites (Promega, 2003; Brown, 2001). The transformants with white colour, not expressing β-galactosidase are selected. B-galactosidase expression phenomenon for selecting transformants depended on the presence of X-gal (Dimethylformamide), IPTG and DNA insert. Insertion of DNA fragment into the *lacZ* gene (between the T terminals) inactivates the synthesis of β-galactosidase, which is responsible for breaking down the lactose analogue (X-gal) leading to blue coloured colonies. IPTG was added to the growth medium to induce the *lacZ* promoter for transcription the *lacZ* gene (Promega 2003: Brown, 2001). The PCR products were ligated between T tailed sites of pGEM-T Easy. When designing primers, it was preferred to use restriction digestion sites that are not found on pGEM-T Easy to avoid multiple cutting of the plasmid, in order to find only the DNA fragment of interest after digestion reactions.

After amplifying the 359 bp fragment 95-A, the 359 bp fragment 95-B, and the 1356 bp hygromycin resistance gene by PCR as described in section 3.2.5.2, the PCR product of each reaction was purified using a PCR purification kit (Section 2.3.5.4) then each was ligated with a T tailed pGEM-T Easy (Section 2.3.7). Afterwards, ligation products were introduced into *E. coli* DH5α by electroporation (Section 2.3.6.1.2), and transformants were selected on agar media containing ampicillin, X-gal and IPTG. One white colony of each reaction was picked, propagated in L-broth containing ampicillin and incubated at 37^{o}C overnight. Cells were centrifuged and cell pellets were collected. The plasmids were extracted (Section 2.3.4.1.1), and digested using the following restriction enzyme combinations *Sca*I and *Asc*I (pGEM-T::95-A), *Hin*dIII and *Asc*I (pGEM-T::95-B)

and *Asc*I alone (pGEM-T::hyg^R) respectively, to confirm the presence of the planned plasmid constructs. Restriction digestions were run through a 0.8 % (w/v) agarose gel (Section 2.3.8.1). Restriction digestions resulted in two fragments of approximately 360 bp that represented fragment 95-A (359 bp), 360 bp that represented fragment 95-B (359 bp), and 1350 bp that represented the hygromycin resistance gene (1356 bp), whereas the 3000 bp represented the size of pGEM-T Easy (3015 bp) respectively for each reaction. The recombinant plasmids carrying the fragments 95-A, 95-B, and the hygromycin resistance gene were named pASO1, pASO2 and pASO3 respectively.

4.1.1.4. Sub-cloning of the fragment 95-A, fragment 95-B, and hygromycin resistance cassette into p1NIL to produce pASO5.

In order to construct the desired suicide vector to knock out the *rv1995* gene by homologous recombination, first the PCR products of fragment 95-A, fragment 95-B, and hygromycin resistance gene were isolated from pASO1, pASO2 and pASO3 respectively using their corresponding restriction enzymes. This was followed by purification of the target fragments from agarose gel as described in Material and Methods section (2.3.8.2). The 4525 bp plasmid p1NIL was digested with *Sca*I and *Hin*dIII resulting in two fragments of 4200 bp and 325 bp. The 325 bp fragment was discarded whereas the 4200 bp fragment was retained. Thereafter, all DNA fragments were simultaneously included in a single reaction with the *Sca*I and *Hin*dIII digested p1NIL to be ligated together for production of a recombinant suicide vector for the mutated *rv1995* gene. The reaction contents were transformed into *E. coli* DH5α (Section 2.3.6.1.2). No growth was obtained after growing on kanamycin and hygromycin containing media. Two attempts were done for this purpose and both were unsuccessful.

Another approach was tried to attain the same construct in which the fragments 95-A and 95-B were put together with p1NIL. The ligation reaction was introduced into *E. coli* DH5α by electroporation as previously described (Section 2.3.6.1.2). After growing on kanamycin, five colonies were obtained, however, they were only visible after 48 hr to

72 hr incubation at 37°C. The control *E. coli* DH5α containing only p1NIL plasmid grew overnight as expected (from 8 to 12 hr incubation).

To confirm whether the experiment was proceeding in the correct way, and the fragments were correctly ligated and cloned, plasmid DNA was prepared from one of the 5 colonies previously described using the mini prep protocol from Qiagen (Section 2.3.4.1.3). The extracted DNA was digested with *Sca*I, *Asc*I and *Hin*dIII, then run through a 0.8 % (w/v) agarose gel. As it can be seen in Figure 4.3, digestion with *Asc*I or *Hin*dIII resulted in one fragment of approximately 5500 bp (Lane 5 and 6) compared with p1NIL (control) that digested with *Hin*dIII had an approximate size of 4500 bp (Lane 2). Digestion with *Sca*I resulted in no digestion of plasmid (Lane 3). Digestion with both *Asc*I and *Sca*I resulted in one fragment of approximately 5500 bp (Lane 4). Digestion with both *Hin*dIII and *Asc*I resulted in two fragments of approximately 4400 bp and 1000 bp (Lane 7). The recombinant plasmid with the unexpected size in this experiment was named as pASO5. According to the restriction digestion results, including the lack of digestion with *Sca*I and the unexpected size of *rv1995* fragment B after digesting with *Asc*I and *Hin*dIII (Approximately 1000 bp), it was concluded that there was a problem with the ligation reaction. The recombinant plasmids from the other four transformants were extracted by mini-prep and each was incubated with *Sca*I. Unfortunately, only uncut plasmid was obtained with all of them after incubation with *Sca*I. Also, a sample of the recombinant plasmid using primers ForA-1, RevA-1 and RevT-2 from p1NIL sequence was sent to MWG BioTech for sequencing which was also unable to be sequenced. It was also clear that this attempt to clone the *rv1995* fragments into p1NIL had failed.

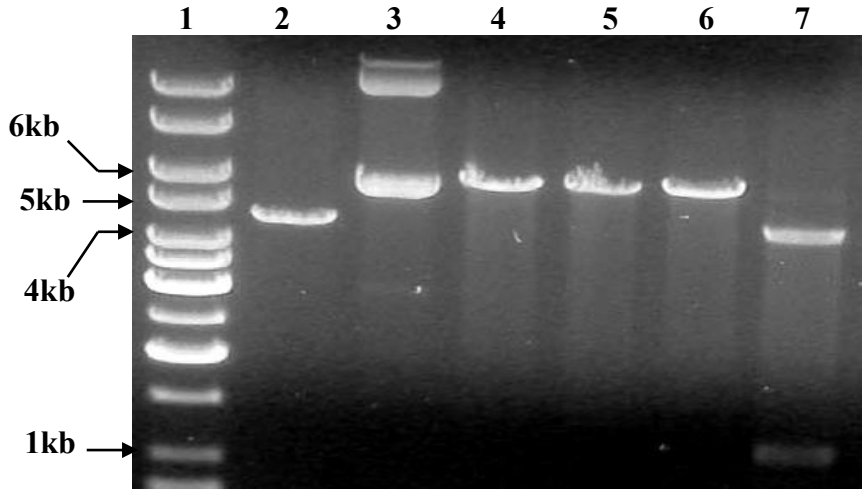


Figure 4.3: Digestion of pASO5 using restriction enzymes *Sca*I, *Asc*I and *Hind*III.

To confirm or exclude the presence of the fragments 95-A and 95-B in pASO5, the DNA was digested with *Sca*I, *Asc*I and *Hind*III. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Restriction digestion of p1NIL (control) (lane 2). Restriction digestion with *Sca*I resulted in no digestion (lane 3). Restriction digestion with either *Asc*I and *Sca*I (lane 4), *Asc*I (lane 5) or *Hind*III (lane 6) resulted in one fragment of the same size; approximately 5500 bp. Restriction digestion with *Hind*III and *Asc*I resulted in two fragments of approximately 4400 bp and 1000 bp (lane 7).

4.1.2. A new alternative strategy for construction of *rv1995* and *rv1996* suicide vectors.

To overcome the difficulties encountered by cloning of multiple DNA inserts, a new alternative strategy was developed in the present study to make a suicide vector for disruption of the *rv1995* and *rv1996* genes. This strategy involved ligation (Section 2.3.8.6) of fragments A and B obtained previously (Section 4.1.1.1) using ends digested by *Asc*I, (Section 2.3.8.5). This was followed by a second round of PCR, cloning processes and insertional mutagenesis to obtain suicide vectors for the disrupted *rv1995* and *rv1996* (Figure 4.4). This approach effectively introduced a solution for introduction of multiple inserts inside a vector.

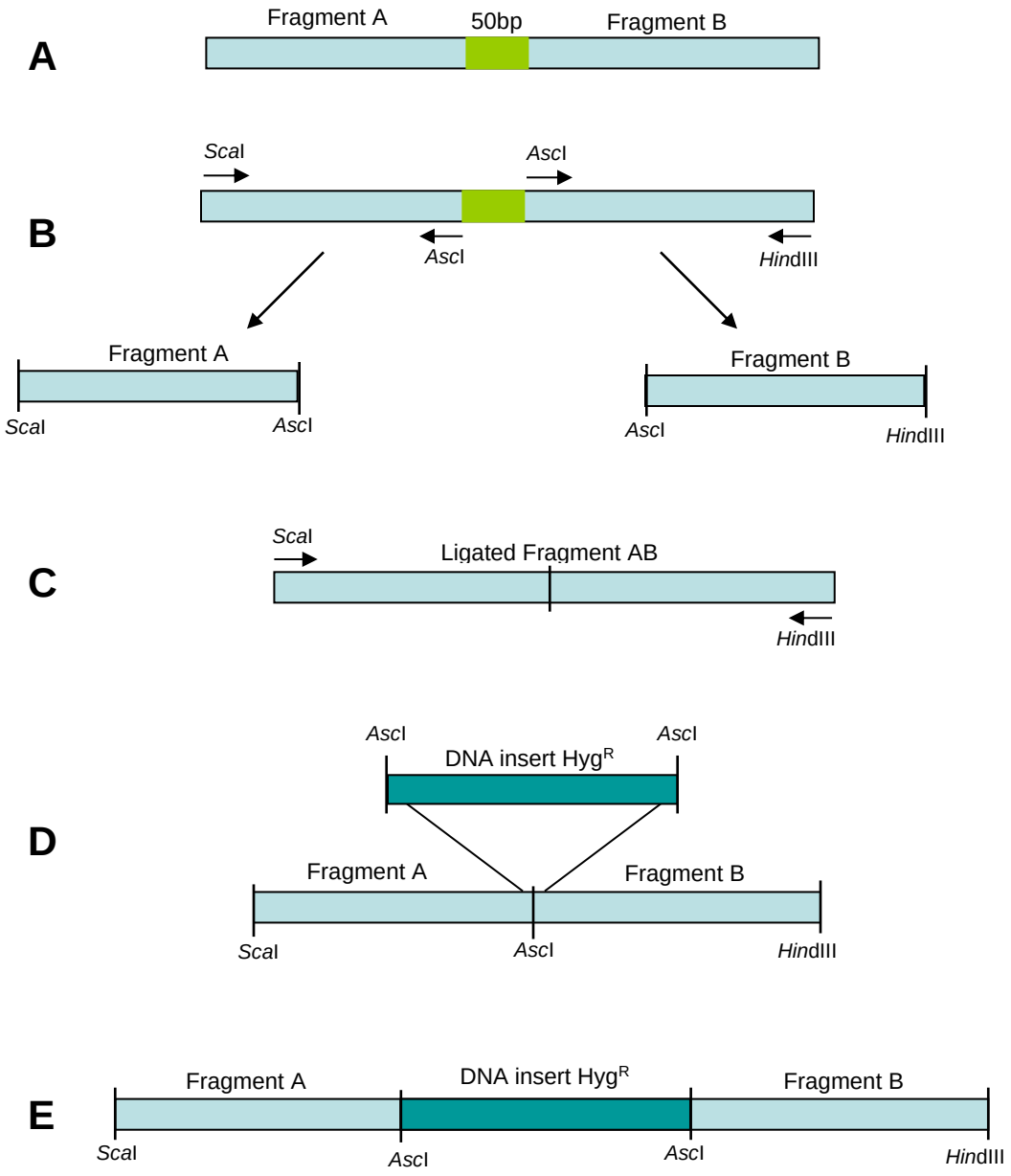


Figure 4.4: Schematic diagram to illustrate the strategy of disrupted gene construction. The directions of primers represented by arrows and restriction enzyme sites are shown in the figure (REs mentioned in the figure were used in the case of *rv1995* gene).

(A) Gene sequence to be disrupted; (B) First round of PCR to produce two separate fragments A and B; (C) Ligation of the PCR products of Fragment A and Fragment B followed by second round of PCR to amplify fragment AB; (D) Insertion of hyg^R; (E) Gene disrupted by Insertion of hyg^R.

4.1.2.1. Construction of *rv1995* suicide vector.

4.1.2.1.1. PCR to obtain combined fragment 95-AB.

PCR amplicons for fragments 95-A and 95-B obtained previously (Section 4.1.1.1) were digested by *Asc*I (Section 2.3.8.5), purified (Section 2.3.4.5) and ligated together by T4 ligase using *Asc*I digested ends to obtain ligated fragment 95-AB (Section 2.3.8.6). Then, a second round of PCR was performed for the purified fragment 95-AB using primers (ForA-5) and (RevB-5). A sample of the PCR product was run through a 0.8% (w/v) agarose gel and gave the expected band with a size of approximately 700 bp representing the ligated fragment 95-AB (718 bp) (Figure 4.5 -A).

To confirm the successful ligation of the fragments 95-AB, the PCR product was purified using a PCR purification kit (Section 2.3.5.4), and then digested with *Asc*I (Section 2.3.8.5). The restriction digestion reaction was run through a 1.8% (w/v) agarose gel (Section 2.3.8.1) to give double bands representing fragments 95-A and 95-B of approximately 350 bp (Figure 4.5 -B).

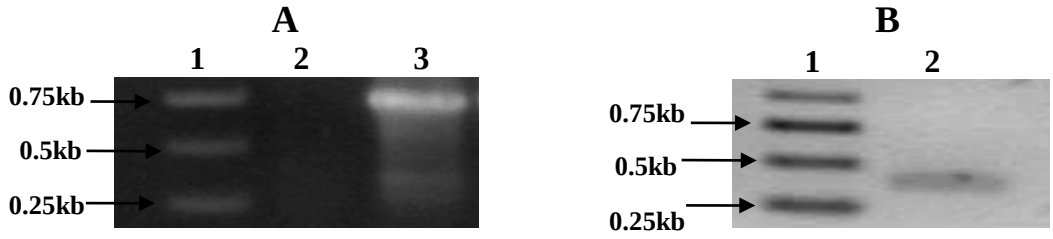


Figure 4.5: (A) PCR to amplify the fragment 95-AB. (B) Digestion reaction of the fragment 95-AB PCR product with *Asc*I.

(A) Amplification of the ligated fragment 95-AB sequence was performed using ForA-5 and RevB-5 primers. Lane 2 contains negative control. Lane 3 contains expected PCR product at 700 bp. (B) Lane 2 contains expected double fragments of approximately 350 bp after digestion with *Asc*I. Lane 1 in both (A) and (B) contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb).

4.1.2.1.2. Cloning of the fragment 95-AB into pGEM-T Easy to produce pHSO1.

After confirming the identity of the fragment 95-AB, it was ligated into the T-tailed site of pGEM-T Easy, as described in Material and Methods (Section 2.3.7). The ligation product was introduced into *E. coli* DH5α by heat shock transformation as described in Material and Methods (Section 2.3.6.3.2). To select the transformants containing the recombinant plasmid, the reaction contents were plated onto L- agar containing ampicillin, X-gal and IPTG to select white colonies (Ampicillin selects transformants and X-gal is a screen for recombinants). Twenty-three white colonies were obtained.

4.1.2.1.2.1. Identification of pHSO1 clones.

In order to identify the colonies containing the recombinant plasmid, the twenty-three white colonies were grown in L-broth with ampicillin at 37^{o}C overnight. After that, a PCR reaction (Section 2.3.5.1) was carried out with a sub-set with fourteen separate white colonies being chosen to be tested further. PCR was done as a quick screen prior to more detailed verification to amplify the fragment 95-AB from the recombinant plasmid using primers (ForA-5) and (RevB-5). Four colonies, containing the recombinant plasmid carrying the target fragment were identified. The predicted 700 bp fragment can be seen in Figure 4.6 (lanes 2, 8, 11 and 13). The resulting plasmid was named pHSO1.

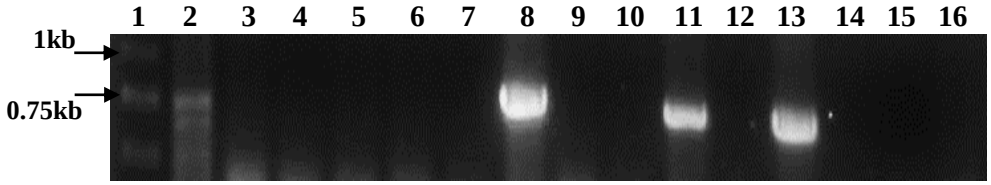


Figure 4.6: PCR to amplify the fragment 95-AB from recombinants.

Amplification of the fragment 95-AB sequence was done using ForA-5 and RevB-5 primers to identify transformants harbouring the fragment AB. Lane 1 contains DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 16 contains the negative control. Lanes 2, 8, 11 and 13 contain the target fragment of approximately 700 bp. In the remaining lanes, no DNA was amplified.

4.1.2.1.2.2. Confirmation of the presence of fragment 95-AB in pHSO1.

To confirm the presence of the fragment 95-AB in pHSO1, one of the three recombinant colonies identified previously by PCR was picked, propagated in L-broth with ampicillin at 37^{o}C overnight and the plasmid DNA was prepared (Section 2.3.4.1.4). Following this, samples of the plasmid were digested (Section 2.3.8.5) with *Sca*I, *Asc*I and *Hin*dIII and run through a 0.8% (w/v) agarose gel (Figure 4.7). Digestion with *Asc*I or *Hin*dIII resulted in one fragment representing the linearised pHSO1 of approximately 3700 bp (Lanes 6 and 7). This size (3700 bp) represented the combination of pGEM-T Easy vector (3015 bp) and fragment 95-AB (718 bp). Digestion with *Sca*I resulted in two fragments of approximately 1850 bp (doublet bands in Lane 3). The two fragments resulted from a *Sca*I restriction site at the end of the inserted amplicon and the other resulted from a *Sca*I restriction site in the vector. Digestion with both *Asc*I and *Hin*dIII resulted in two fragments of approximately 3350 bp and 350 bp. The 3350 bp represented the combination of pGEM-T Easy vector (3015 bp) and fragment 95-A (359 bp), whereas the 350 bp represented 95-B (359 bp) (Lane 8). Digestion with both *Asc*I and *Sca*I resulted in three fragments of approximately 1850 bp, 1500 bp and 350 bp. The 1850 bp and 1500 bp bands appeared because of the two restriction sites of *Sca*I in pHSO1. The 350 bp represented 95-

A (359 bp) (Lane 4). Digestion with both *Sca*I and *Hin*dIII resulted in three fragments of approximately 1850 bp, 1150 bp and 700 bp. The 700 bp represented the required fragment 95-AB (718 bp) (Lane 5). A map describing the development of pHSO1 is shown in Figure 4.8.

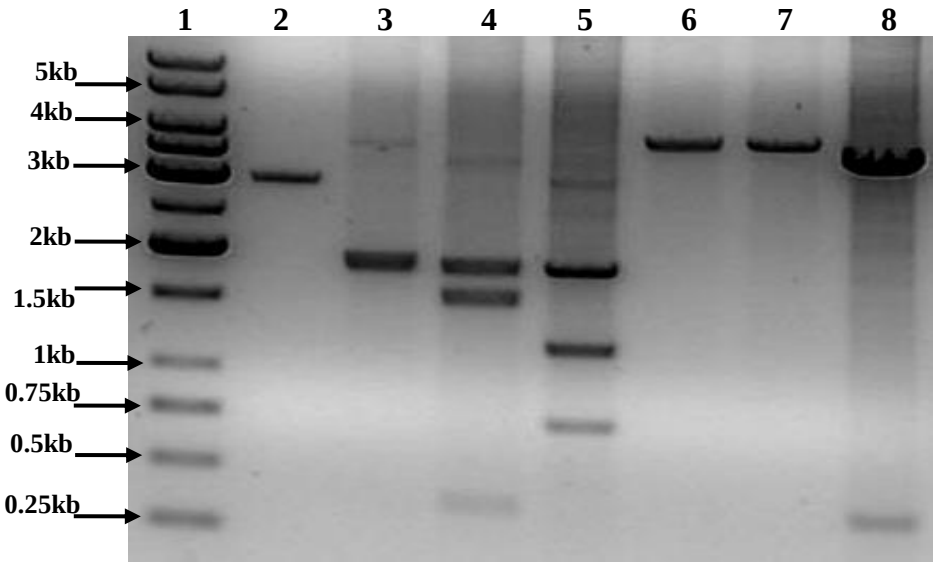


Figure 4.7: Conformation of the fragment 95-AB in pHSO1.

For confirmation of the presence of the ligated fragment 95-AB in pHSO1, the DNA was digested with combinations of *Sca*I, *Asc*I and *Hin*dIII. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). pGEM-T easy (control) (lane 2). Restriction digestion with *Sca*I resulted in two double fragments (lane 3). *Asc*I and *Sca*I digestion resulted in three fragments (lane 4). *Sca*I and *Hin*dIII digestion produced three fragments (lane 5). Digestion with *Asc*I (lane 6) or *Hin*dIII (lane 7) resulted in one fragment of the same size (approximately 3700 bp). Lane 8 shows two fragments from digestion with *Asc*I and *Hin*dIII.

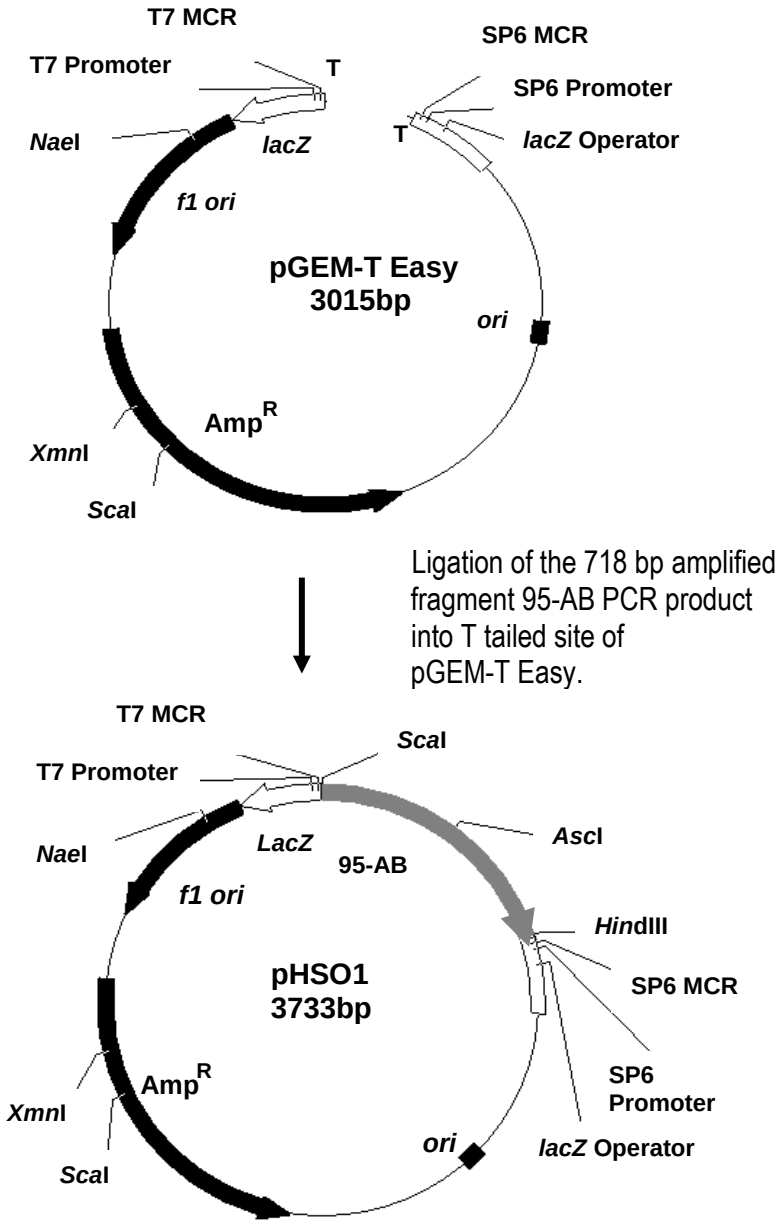


Figure 4.8: Development of pHSO1.

The 718 bp amplified fragment 95-AB was ligated into the T tailed sites of pGEM-T Easy to produce pHSO1. Abbreviations are; origin of replication (*ori*), Ampicillin resistance gene (Amp^R), β-galactosidase for selecting blue and white colonies (*lacZ*), multiple cloning site (MCS), phage origin of replication (*f1 ori*) and *rv1995* fragment AB (95-AB).

4.1.2.1.3. Sub-cloning of fragment 95-AB into p1NIL to produce pASO6.

The fragment 95-AB digested with *Sca*I and *Hin*dIII from pHSO1 (Section 4.1.2.1.2.2.) was extracted from the gel (Section 2.3.8.2) and ligated with p1NIL digested with the same enzymes (Section 2.3.8.6). This was performed as a step prior to insertion of the hygromycin resistance gene in the *Asc*I site between the two ligated fragments A and B. Three plasmid DNA: insert ratios were used in the ligation reactions 1:1, 1:3 and 1:6. The ligation products were purified and then introduced into *E. coli* DH5α by electroporation. The reaction components were grown on L-agar containing kanamycin. Twenty colonies were selected after incubation overnight at 37^{o}C.

4.1.2.1.3.1. Identification of pASO6 clones.

To identify the transformants having the desired recombinant plasmid, the twenty colonies obtained were grown in L-broth with kanamycin at 37^{o}C overnight, and then a PCR amplification (Section 2.3.5.1) of the 718 bp fragment 95-AB using primers (ForA-5) and (RevB-5) was done. Nine colonies were identified as containing the recombinant plasmid (Figure 4.9. lanes 3, 4, 6, 7, 9, 17, 18, 21 and 22). The plasmid was designated as pASO6.

4.1.2.1.3.2. Confirmation of the presence of fragment 95-AB in pASO6.

To confirm the presence of the sub-cloned fragment 95-AB in pASO6, one colony of the nine putative recombinants in *E. coli* DH5α was picked, grown in L-broth with kanamycin at 37^{o}C overnight prior to a maxi preparation to obtain purified plasmid (Section 2.3.4.1.4). An amount of the plasmid DNA was digested (Section 2.3.8.5) with *Sca*I, *Asc*I and *Hin*dIII and run through a 0.8 % (w/v) agarose gel (Figure 4.10). Digestion with *Sca*I, *Asc*I or *Hin*dIII alone resulted in one fragment of approximately 4900 bp that represented linearised pASO6 (Lanes 3, 6 and 7) compared with the original p1NIL (control) of approximately 4500 bp digested with *Hin*dIII (Lane 2). The fragment of approximately 4900 bp represented the 700 bp from the fragment 95-AB (718 bp) together

with the size of digested p1NIL (4200 bp). The size difference between the original p1NIL and the digested 4200 bp one is due to the 325 bp segment previously removed from the original p1NIL for cloning purpose. Digestion with both *Sca*I and *Hin*dIII resulted in two fragments of approximately 4200 bp that represented the size of p1NIL, and 700 bp from the target fragment 95-AB (718 bp) (Lane 5). Digestion with both *Sca*I and *Asc*I resulted in two fragments of approximately 4550 bp (p1NIL and fragment 95-B), and 350 bp (fragment 95-A) (Lane 4). Digestion with both *Asc*I and *Hin*dIII resulted in two fragments of approximately 4550 bp (p1NIL and fragment 95-A), and 350 bp (fragment 95-B) (Lane 8). Obtaining the 700 bp from pASO6 confirmed the successful cloning of the fragment 95-AB into p1NIL.

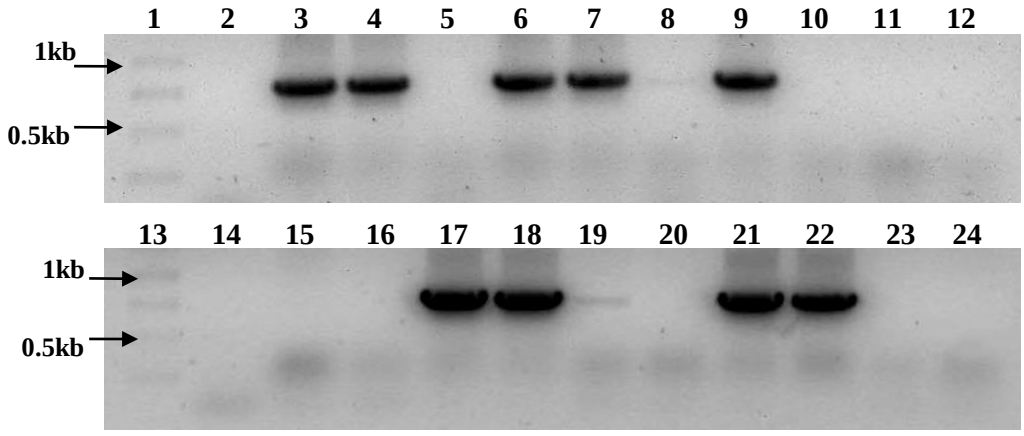


Figure 4.9: PCR to amplify the fragment 95-AB from pASO6.

Amplification of the fragment 95-AB sequence was done using ForA-5 forward primer of 95-A and RevB-5 reverse primer of 95-B. Lanes 1 and 13 contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 14 contain the negative control. Lanes 3, 4, 6, 7, 9, 17, 18, 21, and 22 contain the target fragment of approximately 700 bp. The remaining lanes had no amplified bands.

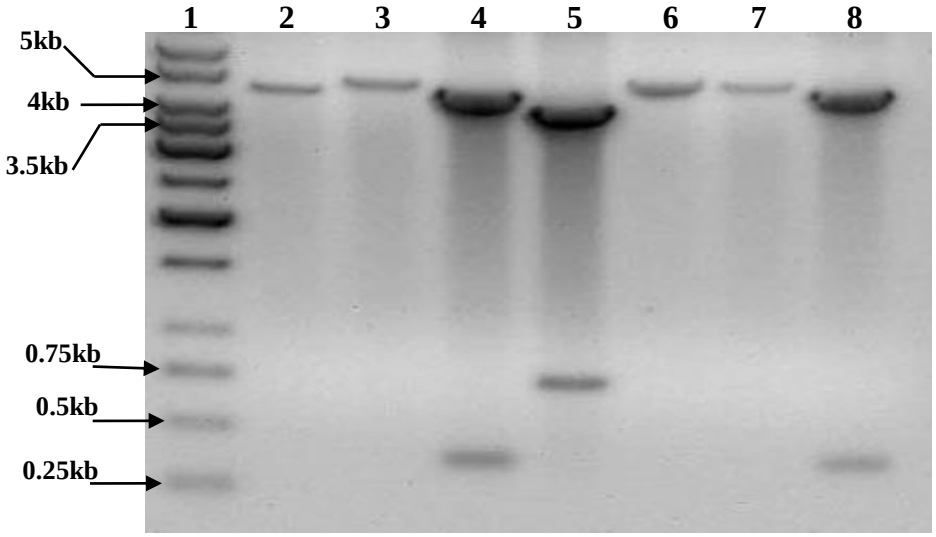


Figure 4.10: Confirmation of the presence of the fragment 95-AB in pASO6.

The DNA was digested with *Sca*I, *Asc*I and *Hin*dIII. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). The p1NIL (control) (lane 2). Restriction digestion with either *Sca*I (lane 3), *Asc*I (lane 6) or *Hin*dIII (lane 7) resulted in one fragment. Digestion with *Asc*I and *Sca*I resulted in two fragments (lane 4). *Sca*I and *Hin*dIII digestion produced two fragments (lane 5). Two fragments are shown in lane 8 on digestion with *Asc*I and *Hin*dIII.

4.1.2.1.4. Sub-cloning of the hygromycin resistance cassette into pASO6 to produce the mutated *rv1995* gene in the suicide vector, pANO1.

The plasmid pASO6 was digested (Section 2.3.8.5) with *Asc*I resulting in one fragment of approximately 4900 bp. Subsequently, the 1356 bp hygromycin resistance cassette flanked with *Asc*I sites that was obtained from pASO3 (Section 4.1.1.3.) was ligated into the *Asc*I site of pASO6. The resultant ligation product was transformed into *E. coli* DH5α by electroporation (Section 2.3.6.1.2). After incubation, overnight at 37^{o}C, there

were a large number of colonies (360 colonies) after selection on hygromycin B and kanamycin.

4.1.2.1.4.1. Identification of pANO1 clones.

In order to identify transformants containing the desired plasmid, twelve separate colonies were picked, grown in L-broth with kanamycin and hygromycin overnight at 37^{o}C. Then, PCR (Section 2.3.5.1) was done with the twelve colonies using primers (ForA-5) and (RevB-5). The PCR products were run on a 0.8 % (w/v) agarose gel. Three colonies were found to contain the recombinant plasmid carrying the target fragment of approximately 2050 bp (Figure 4.11. lanes 5, 6, 7 and 9). The obtained plasmid was named pANO1.

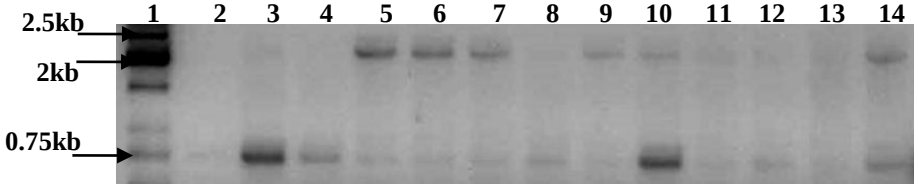


Figure 4.11: PCR to amplify the *rv1995* gene disrupted with the hygromycin resistance gene in pANO1.

Amplification was done using ForA-5 and RevB-5 primers. Lanes 1 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control. Lanes 5, 6, 7 and 9 contain the target fragment of the disrupted *rv1995* with a size of approximately 2050 bp, whereas the other lanes do not have the desired bands.

4.1.2.1.4.2. Confirmation of the presence of the *rv1995* gene disrupted by hygromycin resistance cassette in pANO1.

To confirm the presence of the mutated *rv1995* gene in pANO1 (6274 bp), the plasmid DNA from one of the three recombinant colonies identified by PCR was prepared using the maxi prep protocol (Section 2.3.4.1.4) after growing the cells in L-broth with kanamycin and hygromycin at 37ºC overnight. The plasmid DNA was then digested (Section 2.3.8.5) with combinations of *Sca*I, *Asc*I and *Hin*dIII and analysed on a 0.8 % (w/v) agarose gel (Figure 4.12).

Digestion using one of the enzymes, *Sca*I or *Hin*dIII, resulted in a fragment of 6250 bp (linearised pANO1) which consisted of pASO6 (4918 bp), and the hygromycin resistance cassette (1356 bp) (Lane 3 and 7). Using both *Sca*I and *Hin*dIII enzyme digestion produced two fragments of approximately 4200 bp (p1NIL) and 2050 bp of the *rv1995* gene disrupted with the hygromycin resistance gene. The 2050 bp disrupted *rv1995* gene consisted of the hygromycin resistance gene (1356 bp) flanked with fragment 95-A (359 bp) and fragment 95-B (359 bp) (Lane 4). Two fragments were obtained when pANO1 was digested with *Asc*I at approximately 4900 bp and 1350 bp that represented the pASO6 (4918 bp) and the hygromycin resistance cassette (1356 bp) respectively (Lane 5). Cutting the plasmid with both *Sca*I and *Asc*I resulted in three fragments with approximate sizes of 4550 bp (4200 bp p1NIL and 359 bp 95-B fragment), 1350 bp (1356 bp hygromycin resistance gene) and 350 bp (359 bp 95-A) (Lane 6). Three fragments were also obtained on digestion using both *Asc*I and *Hin*dIII; 4550 bp (p1NIL and 95-A together), 1350 bp (hygromycin resistance gene) and 350 bp (95-B) (Lane 8). The original 4525 bp p1NIL digested with *Hin*dIII was used as a control with approximate size of 4500 bp at Lane 2.

Obtaining the fragment of 1350 bp representing the hygromycin resistance gene from the constructed plasmid confirmed the successful ligation and cloning of the hygromycin resistance cassette into the *rv1995* gene. Consequently, the construction of the suicide vector pANO1 in the current study was successful. A map of the resultant pANO1 (developed from p1NIL) carrying the *rv1995* gene mutated by inserting the hygromycin resistance cassette is shown in Figure 4.13.

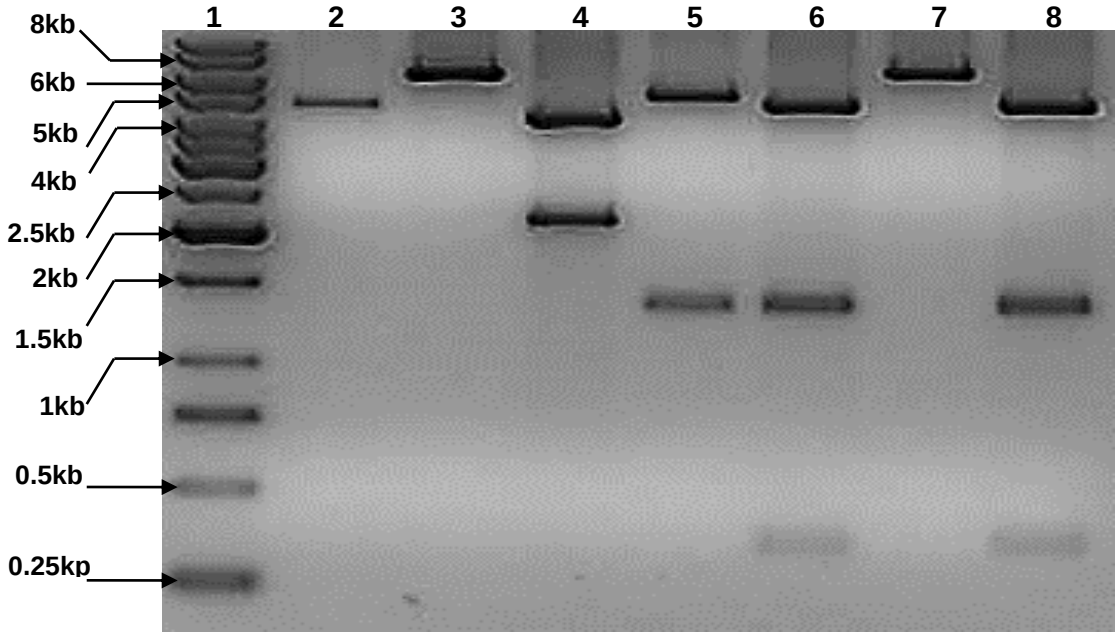


Figure 4.12: Confirmation the presence of hygromycin resistance gene in the suicide vector pANO1.

In order to confirm the presence of the hygromycin resistance gene between fragments A and B of *rv1995* in pANO1, the DNA was digested with *Sca*I, *Asc*I and *Hin*dIII. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). The p1NIL (control) (lane 2). One same size fragment on digestion with *Sca*I (lane 3) or *Hin*dIII (lane 7). Restriction digestion with *Sca*I and *Hin*dIII resulted in two fragments (lane 4). *Asc*I restriction digestion resulted in two fragments (lane 5). Lane 6 shows three fragments on digestion with both *Asc*I and *Sca*I. Restriction digestion with both *Asc*I and *Hin*dIII resulted in three fragments (lane 8).

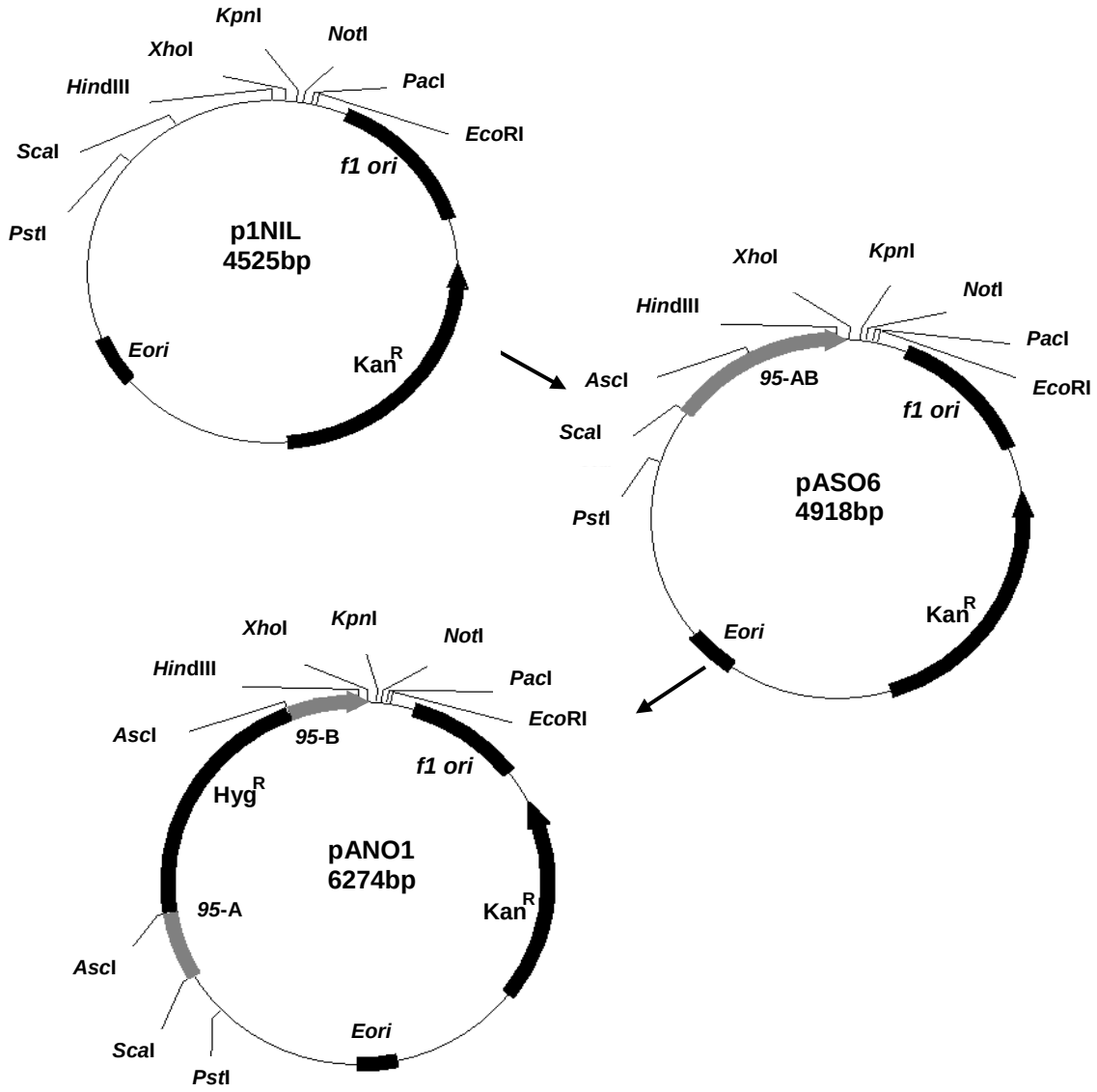


Figure 4.13: Schematic map showing the construction of pANO1 containing the disrupted *rv1995* gene from pASO6 developed from p1NIL.

The 718 bp fragment 95-AB was obtained from pHSO1 (Section 4.1.2.1.2.2.) by digesting with *Sca*I and *Hin*dIII and ligated into the *Sca*I/*Hin*dIII position of p1NIL from which 325 bp were removed by digestion, to produce pASO6. The 1356 bp hygromycin resistance cassette was obtained from pASO3 (Section 4.1.1.3) by digesting with *Asc*I and ligated into the *Asc*I position of pASO6 to construct pANO1 suicide vector. Abbreviations are; *rv1995* fragment-A (*95*-A), *rv1995* fragment-B (*95*-B), *Escherichia coli* origin of replication (*Eori*), kanamycin resistance gene (Kan^R), hygromycin resistance gene (Hyg^R), ligated *rv1995* fragment AB (*95*-AB) and phage origin of replication (*f1 ori*).

4.1.2.2. Construction of *rv1996* suicide vector.

The strategy used to construct a suicide vector for the *rv1996* gene was the same as that used to construct a suicide vector for the *rv1995* gene except for changing the restriction sites to be *Hin*dIII and *Kpn*I instead of *Sca*I and *Hin*dIII. This was done to avoid the technical problems occurred with digestion using *Sca*I described in section 4.1.1.4. Although, the technical problems were resolved (Section 4.1.2.1), it was preferred to use different restriction enzymes.

4.1.2.2.1. Two steps PCR to obtain combined fragment 96-AB.

In order to disrupt the 954 bp *rv1996* gene, two separate fragments (A and B) of the gene were individually amplified by PCR, with the intention of ligating a hygromycin resistance gene between them. Forward primer (ForA-6) and reverse primer (RevA-6), and forward primer (ForB-6) and reverse primer (RevB-6) were designed (Section 2.3.3) to amplify fragments 96-A and 96-B respectively using the gene sequence of *rv1996* of *M. tuberculosis* H37Rv (Figure 4.14). The fragment 96-A starts from the first nucleotide in the *rv1996* gene to the nucleotide no 452. The fragment 96-B starts from nucleotide 502 to the last nucleotide 954 in the *rv1996* gene. As described with the *rv1995* gene, a 50 bp segment between the two fragments was not included in the amplification process. A *Hin*dIII restriction site was incorporated into primer (ForA-6) and an *Asc*I restriction site was incorporated into primer (RevA-6) of fragment 96-A, and a *Asc*I restriction site was incorporated into primer (ForB-6) and a *Kpn*I restriction site was incorporated into the primer (RevB-6) of fragment 96-B.

Fragment 96-A and fragment 96-B of *rv1996* gene were individually amplified in the first round of PCR using primers (ForA-6) and (RevA-6) for fragment 96-A and, primers (ForB-6) and (RevB-6) for fragment 96-B. PCR reactions were done as described in Material and Methods (Section 2.3.5.2). The PCR products were visualized at approximately 450 bp for both fragments after running on a 0.8 % (w/v) agarose gel (Figure 4.15). As previously described (Section 4.1.2.1.1) the two amplified PCR products were purified using a PCR purification kit (Section 2.3.5.4) followed by digestion with *Asc*I

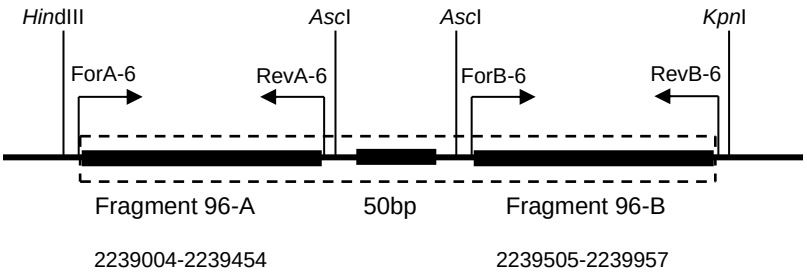


Figure 4.14: Schematic map to show position of the PCR primers to amplify the fragments 96-A and 96-B on *M. tuberculosis* H37Rv DNA, the positions of restriction enzymes cutting sequences and the positions of the amplified fragments in relation to ORF of *rv1996*.

The coordinates of fragments 96-A and 96-B on *M. tuberculosis* H37Rv DNA are shown below each. The amplified fragments and the 50 bp segment in-between (black thick lines), ORF of *rv1996* (cut lines box), the forward ForA-6 and reverse RevA-6 primers for the amplification of 96-A, and the forward ForB-6 and reverse RevB-6 primers for the amplification of 96-B (bent arrows) and restriction enzyme cutting sequences are shown.

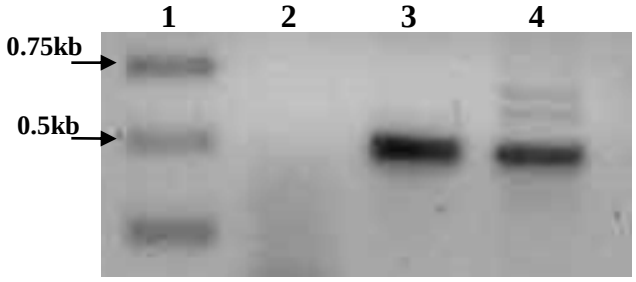


Figure 4.15: PCR to amplify the fragment 96-A and fragment 96-B.

Amplification of the 452 bp fragment 96-A and 452 bp fragment 96- B sequences was done using ForA-6 and RevA-6 primers for fragment 96-A and ForB-6 and RevB-6 primers for fragment 96-B, respectively. Lane 1 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control. Lanes 3 and 4 contain the PCR products of fragment 96-A and fragment 96-B, respectively, at approximately 450 bp for both.

(Section 2.3.8.5). The two fragments were ligated (Section 2.3.8.6), then the ligation product was amplified by a second round of PCR using primers ForA-6 and RevB-6 (Table 2.9). After that, the PCR product was analysed on a 0.8% (w/v) agarose gel (Section 2.3.8.1) but an unexpected amplicon size of ~ 700 bp was seen in addition to the expected fragment of approximately 900 bp (Figure 4.16-A). To confirm whether or not the ligation and PCR reactions were correct, the PCR product was purified as before (Section 2.3.5.4) and digested with *Asc*I followed by analysis on a 1.8% (w/v) agarose gel. This analysis gave the same unexpected band at ~ 700 bp in addition to the expected band size of ~ 450 bp corresponding to the 2 fragments A and B (Figure 4.16-B). The unexpected size band possibly occurred because of nonspecific amplification since one or both of the primers used was a small size or because there was a similarity between the primer sequences and the ligated DNA fragments, so the primers bound to different places on the template DNA (Rychlik, 1995; Brown, 2001: Primrose *et al*, 2001).

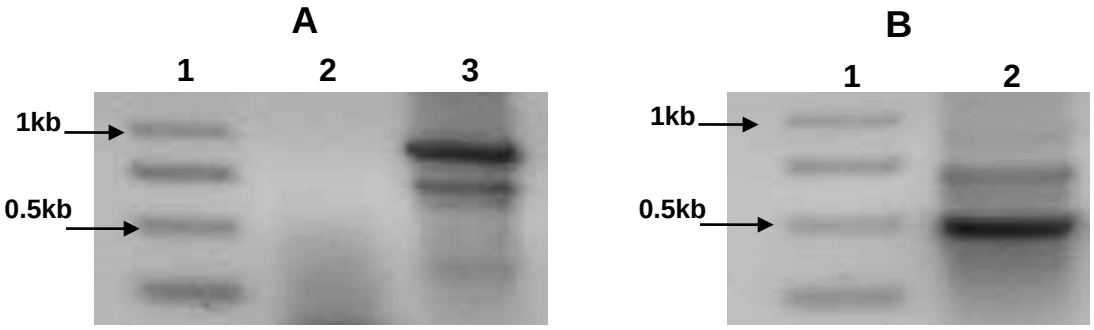


Figure 4.16: (A) PCR to amplify the fragment 96-AB. (B) Digestion reaction of the fragment 96-AB with *Asc*I.

(A) Amplification of the ligated fragment 96-AB sequence was performed using ForA-6 and RevB-6 primers. Lane 2 contains the negative control. Lane 3 contains the PCR product at approximately 900 bp and the unexpected band at ~ 700 bp. (B) Restriction digestion with *Asc*I resulted in two fragments of approximately 450 bp in addition to the band at 700 bp. Lane 1 in both (A) and (B) contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb).

In order to find a solution for this problem, the forward primer of fragment 96-A was made a little longer and named ForA-6.6 (Table 2.9). The annealing temperature was determined by the gradient PCR (Section 2.3.5.5). After that, the PCR reaction was repeated using primers (ForA-6.6) and (RevB-6) to amplify the 904 bp fragment 96-AB (Figure 4.17-A). The resultant band was visualized at approximately 900 bp as was expected. After that, the 904 bp PCR product was purified and digested with *Asc*I. The expected band of approximately 450 bp was obtained (Figure 4.17-B). Then the two PCR products of fragments 96-A and 96-B were sent with their corresponding primers to MWG Biotech for sequencing. Sequence analysis against corresponding sequences from H37Rv reference strain using NCBI BLAST tool (Section 2.3.9) confirmed that fragment A and fragment B of *rv1996* had been successfully amplified with 100% identity to H37Rv strain.

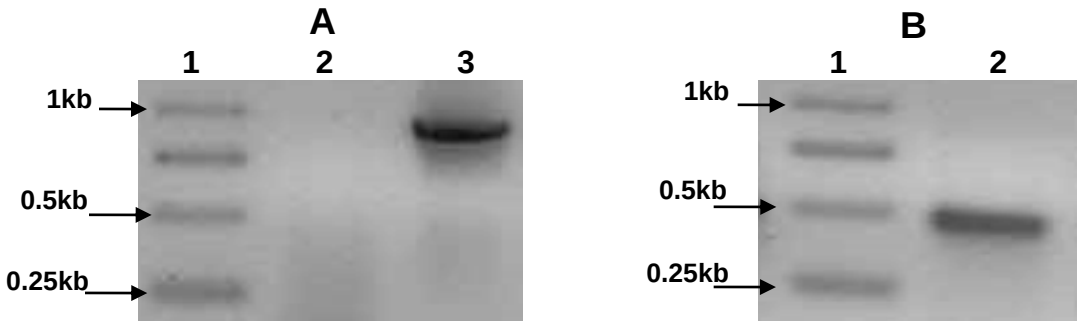


Figure 4.17: (A) PCR to amplify the fragment 96-AB. (B) Digestion reaction of the fragment 96-AB with *Asc*I.

(A) Amplification of the ligated fragment 96-AB sequence was performed using ForA-6.6 and RevB-6 primers. Lane 2 contains the negative control. Lane 3 contains the expected PCR products at approximately 900 bp. (B) Restriction digestion with *Asc*I resulted in a double fragment of approximately 450 bp. Lane 1 in both (A) and (B) contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb).

4.1.2.2.2. Cloning of fragment 96-AB into pGEM-T Easy to produce pHSO2.

In order to construct the *M. tuberculosis rv1996* gene knock out, several steps were carried out. pGEM-T Easy was used as the basis for the construction of plasmid pHSO2. The 904 bp PCR product of the ligated fragment 96-AB was ligated with pGEM-T Easy as described in Material and Methods (Section 2.3.7). The ligation product was transformed into *E. coli* DH5α (Section 2.3.6.3.2). The bacterial suspension was plated onto L- agar containing ampicillin, X-gal and IPTG to select for transformants. After incubation, twenty nine white colonies were obtained.

4.1.2.2.2.1. Identification of pHSO2 clones.

In order to identify the recombinant colonies containing the desired plasmid, PCR was performed on twenty four separate white colonies using (ForA-6.6) and (RevB-6) primers (Section 2.3.5.1). Two colonies were identified as containing the recombinant plasmid with a fragment of approximately 900 bp after running on a 0.8 % (w/v) agarose gel (Figure 4.18 lanes 7 and 21). The resultant plasmid was named pHSO2.

4.1.2.2.2.2. Confirmation of the presence of fragment 96-AB in pHSO2.

To confirm the presence of the fragment 96-AB in pHSO2, plasmid DNA was prepared from one of the two transformants identified, using a maxi prep protocol (Section 2.3.4.1.4). The plasmid DNA was digested with *Hin*dIII, *Asc*I and *Kpn*I followed by analysis on a 0.8 % (w/v) agarose gel (Figure 4.19). Digestion with *Hin*dIII, *Asc*I or *Kpn*I resulted in a fragment of approximately 3900 bp, representing the linearised pHSO2 (Lanes 3, 5 and 8) compared with the linearised 3000 bp pGEM-T Easy (Lane 2). The size of pHSO2 consisted of pGEM-T Easy (3015 bp) and ligated fragment 96-AB (904 bp). Digestion with *Hin*dIII and *Kpn*I together resulted in two fragments of approximately 3000 bp and 900 bp. The 3000 bp represented the pGEM-T Easy vector, whereas the 900 bp represented the ligated fragment 96-AB. The 900 bp cloned fragment AB of *rv1996* was the required fragment needed for further work (Lane 4). Digestion with *Hin*dIII and *Asc*I

together resulted in two fragments of approximately 3450 bp and 450 bp. The 3450 bp represented the pGEM-T Easy and fragment 96-B together, whereas the 450 bp represented the fragment 96-A (Lane 6). Digestion with both *Kpn*I and *Asc*I together resulted in two fragments of approximately 3450 bp and 450 bp. The 3450 bp represented the pGEM-T Easy and fragment 96-A together, whereas the 450 bp represented the fragment 96-B (lane 7). A map of pHSO2 carrying the fragment 96-AB is shown in Figure 4.20. The next stage was to sub-clone the *rv1996* fragment AB into p1NIL to produce pASO7.

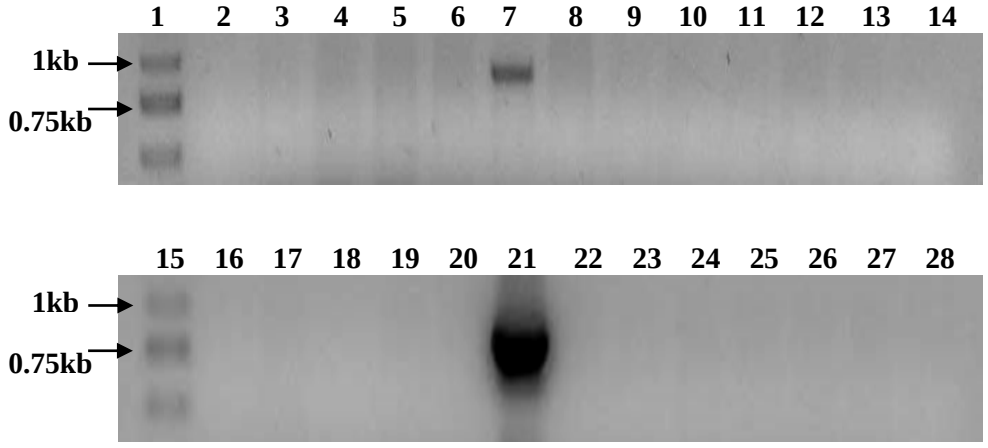


Figure 4.18: PCR to identify recombinants with the fragment 96-AB in pHSO2.

Amplification of the ligated fragment 96-AB sequence in pHSO2 was done using ForA-6.6 and RevB-6 primers. Lanes 1 and 15 contain DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 and 16 contain the negative control. Lanes 7 and 21 contain the target fragment of approximately 900 bp. No product was seen in the remaining lanes.

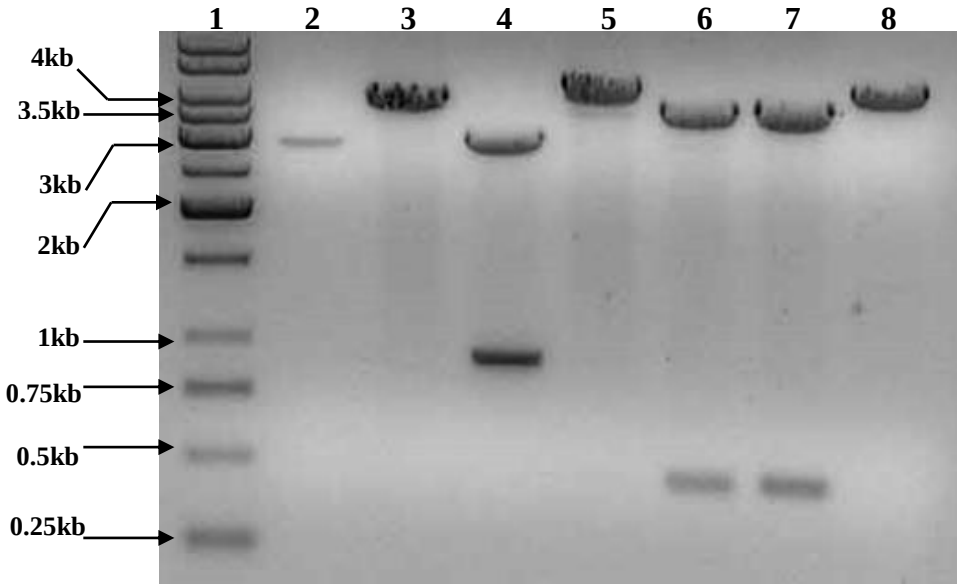


Figure 4.19: Conformation of the fragment 96-AB in pHSO2.

The DNA was digested with *Hin*dIII, *Asc*I and *Kpn*I. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). The pGEM-T Easy control (lane 2). Restriction digestion with either *Hin*dIII (lane 3), *Asc*I (lane 5) or *Kpn*I (lane 8) resulted in one fragment at approximately 3900 bp. Restriction digestion with *Hin*dIII and *Kpn*I, together, resulted in two fragments (lane 4). Restriction digestion with both *Hin*dIII and *Asc*I, resulted in two fragments (lane 6). Similar size two fragments are shown in lane 7 on digestion with both *Kpn*I and *Asc*I (lane 7).

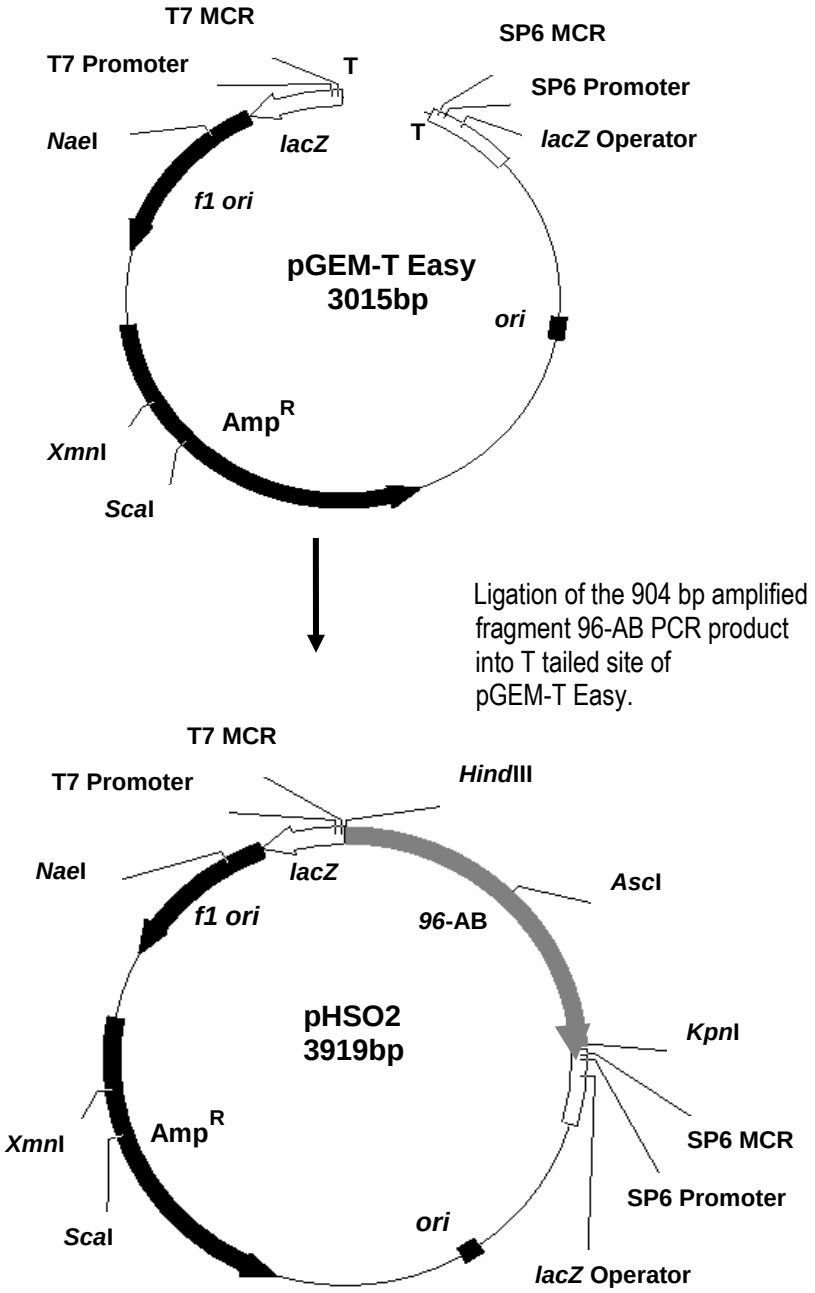


Figure 4.20: Diagram of development of pHSO2.

The 904 bp amplified fragment 96-AB was ligated into the T tailed site of pGEM-T Easy to produce pHSO2. Abbreviations are: origin of replication (*ori*), ampicillin resistance gene (Amp^R), *rv1996* fragment AB (96-AB), β-galactosidase (*lacZ*), multiple cloning region (MCR) and phage origin of replication (*f1 ori*).

4.1.2.2.3. Sub-cloning of the fragment 96-AB into p1NIL to produce pASO7.

In order to sub-clone the fragment 96-AB into p1NIL, the 904 bp fragment 96-AB digested with *Hin*dIII and *Kpn*I from pHSO2 (Section 4.1.2.2.2.2) was extracted from the gel (Section 2.3.8.2). Also, the plasmid p1NIL (4525 bp) was digested with the *Hin*dIII and *Kpn*I resulting in two fragments of 4433 bp and 92 bp. The 4433 bp fragment was used to construct a suicide vector containing the *rv1996* gene. Then, the 904 bp fragment 96-AB was ligated into the *Hin*dIII and *Kpn*I sites of the digested plasmid p1NIL (4433 bp) (Section 2.3.8.6). Three ratios of plasmid DNA to insert were used in the ligation reaction, 1:1, 1:3 and 1:6. The ligation products were transformed into *E. coli* DH5α by electroporation and transformants were selected on L-agar containing kanamycin.

4.1.2.2.3.1. Identification of pASO7 clones.

Forty seven potential transformants were obtained after selection on kanamycin. Twenty four separate colonies were chosen to be scanned by PCR for identifying the transformants containing the required plasmid. PCR (Section 2.3.5.1) was done using primers (ForA-6.6) and (RevB-6) to amplify the 904 bp fragment 96-AB from the recombinant plasmid. Twenty one colonies from the twenty four tested were identified as having the desired recombinant plasmid after running on a 0.8% (w/v) agarose gel (Figure 4.21 lanes 3-7, 10-14 and 18-28). The resultant plasmid was named pASO7.

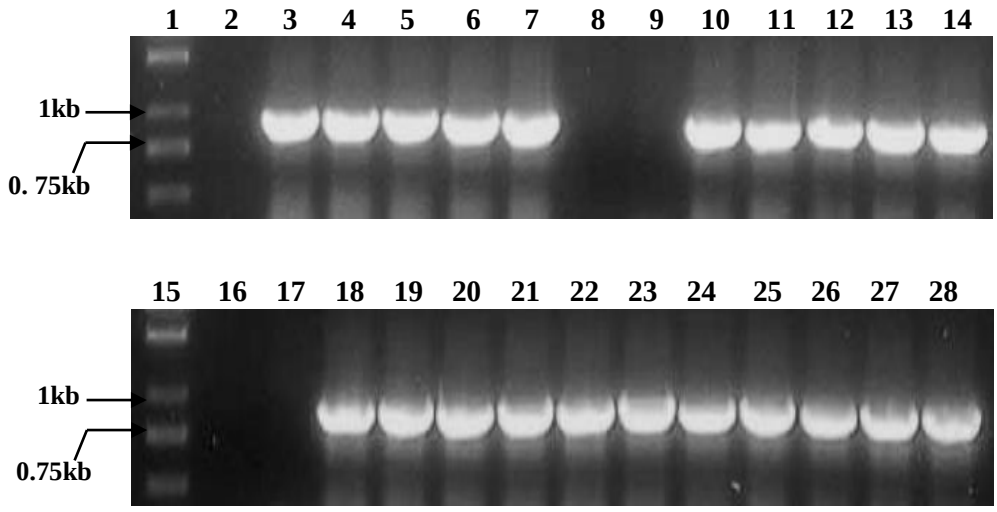


Figure 4.21: PCR to identify recombinants with the fragment 96-AB in pASO7.

Amplification of the ligated fragment 96-AB sequence of pASO7 was done using ForA-6.6 and RevB-6 primers. Lanes 1 and 15 contain DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 16 contain the negative control. All the bands in the remaining lanes represent the target fragment of approximately 900 bp except lanes 8, 9 and 17 which are free from bands.

4.1.2.2.3.2. Confirmation of the presence of the fragment 96-AB in pASO7.

To confirm the presence of the fragment 96-AB in the recombinant plasmid, plasmid DNA was extracted from one of the colonies using the Maxi prep protocol (Section 2.3.4.1.4). Samples of the prepared DNA were digested with *Hin*dIII, *Asc*I and *Kpn*I and run through a 0.8% (w/v) agarose gel (Figure 4.22). Digestion with *Hin*dIII, *Asc*I or *Kpn*I resulted in a fragment of approximately 5350 bp that represented the linearised pASO7 (Lanes 3, 5 and 8), compared with the 4500 bp linearised p1NIL (Lane 2), which was digested with *Hin*dIII. The size of pASO7 (5337 bp) consisted of p1NIL (4433 bp) and fragment 96-AB (904 bp) that all together represented the approximate 5350 bp band on

the agarose gel. Digestion with *Hin*dIII and *Kpn*I together, resulted in two fragments of approximately 4450 bp and 900 bp. The 4450 bp fragment represented p1NIL (4433 bp), whereas, the 900 bp one represented the required fragment of the cloned fragment 96-AB (904 bp) (Lane 4). Digestion with *Hin*dIII and *Asc*I, together, resulted in two fragments of approximately 4900 bp and 450 bp. The 4900 bp represented p1NIL and fragment 96-B together, whereas the 450 bp represented the fragment 96-A (lane 6). Digestion with *Kpn*I and *Asc*I resulted in two fragments of approximately 4900 bp and 450 bp. The 4900 bp represented p1NIL and fragment 96-A together, whereas the 450 bp represented the fragment 96-B (lane 7). Obtaining the 900 bp fragment from pASO7 (lane 4) confirmed the successful cloning of the fragment 96-AB.

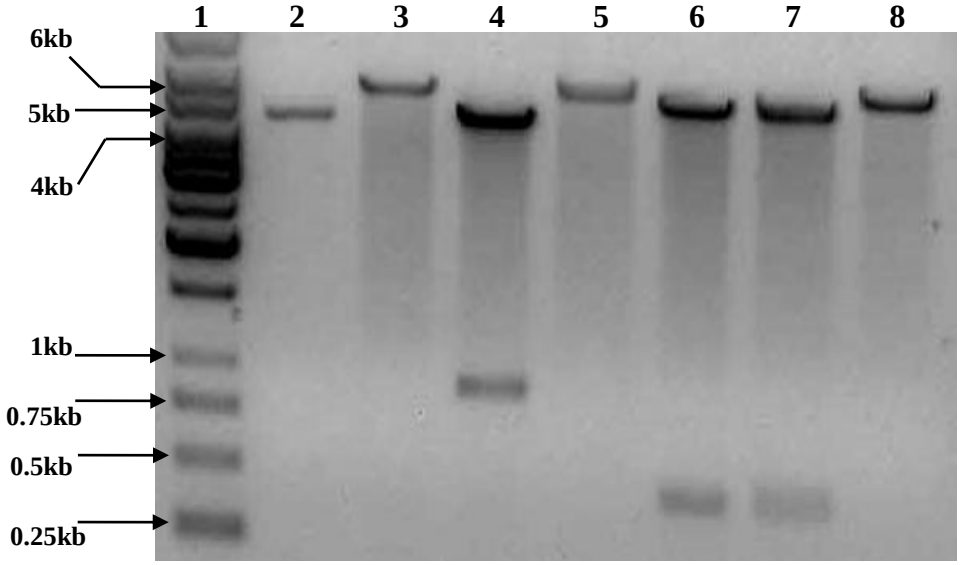


Figure 4.22: Insertion of the fragment 96-AB into p1NIL to produce pASO7.

To confirm the presence of the fragment 96-AB in pASO7, the DNA was digested with *Hin*dIII, *Asc*I and *Kpn*I. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). p1NIL (control, lane 2) and pASO7 (lane 3) digested with *Hin*dIII. Lane 4 pASO7 digested with *Hin*dIII and *Kpn*I. Lane 5 pASO7 digested with *Asc*I. Restriction digestion of pASO7 with both *Hin*dIII and *Asc*I (lane 6) or *Kpn*I and *Asc*I (lane 7). Lane 8 pASO7 digested with *Kpn*I.

4.1.2.2.4. Sub-cloning of the hygromycin resistance cassette into pASO7 to construct the mutated *rv1996* gene in the suicide vector, pANO2.

A sample of pASO7 (Section 4.1.2.2.3.2) was digested (Section 2.3.8.5) with *Asc*I. After that, the 1356 bp hygromycin resistance cassette obtained from pASO3 (Section 4.1.1.3) was ligated into the *Asc*I site of pASO7. The ligation product was introduced into *E. coli* DH5α by electroporation.

4.1.2.2.4.1. Identification of pANO2 clones.

In order to select the transformants containing the recombinant plasmid, the electroporated bacterial suspension was plated onto L-agar containing hygromycin B and kanamycin. After incubation, there were thirty six potential transformants. To identify if they contained the recombinant plasmid carrying the *rv1996* gene disrupted by inserting the hygromycin resistance gene, PCR (Section 2.3.5.1) was performed for twenty four separate colonies using the primers (ForA-6.6) and (RevB-6). Results showed that there were fifteen colonies among the twenty four tested that contained the recombinant plasmid with PCR products of approximately 2250 bp (Figure 4.23. Lanes 3, 5, 8, 10, 11, 12, 13, 14, 17, 18, 19, 20, 23, 26 and 28). On this basis, the plasmid was named pANO2.

4.1.2.2.4.2. Confirmation of the presence of the *rv1996* gene inactivated by hygromycin resistance cassette in pANO2.

To confirm the presence of the disrupted *rv1996* gene in pANO2, DNA was prepared from one of the recombinant colonies using the maxi prep protocol (Section 2.3.4.1.4). Samples of the prepared plasmid DNA were individually digested with combinations of *Hin*dIII, *Asc*I, and *Kpn*I followed by separation using a 0.8 % (w/v) agarose gel (Figure 4.24) and the remaining DNA construct was kept at -20^{o}C until required. Digestion with *Hin*dIII or *Kpn*I resulted in one fragment of approximately 6700 bp that represented the size of pANO2 (Lanes 3 and 7), compared with the size of p1NIL

control (approximately 4500 bp) digested with *Hin*dIII (Lane 2). The exact size of pANO2 (6693 bp) consisted of the 5337 bp pASO7 plasmid with the 1356 bp hygromycin resistance gene. Digestion with *Hin*dIII and *Kpn*I together, resulted in two fragments of approximately 4450 bp that consisted of p1NIL and 2250 bp from the *rv1996* gene disrupted with the hygromycin resistance gene. The 2250 bp band was the required fragment (Lane 4). The disrupted *rv1996* gene consisted of hygromycin resistance gene (1356 bp) flanked with fragment 96-A (452 bp) and fragment 96-B (452 bp). Digestion with *Asc*I resulted in two fragments of approximately 5350 bp (pASO7) and 1350 bp (hygromycin resistance gene) (Lane 5). Digestion with both *Hin*dIII and *Asc*I resulted in three fragments of approximately 4900 bp (p1NIL and fragment 96-B together), 1350 bp (hygromycin resistance gene), and 450 bp that represented the fragment 96-A (Lane 6). *Kpn*I and *Asc*I digestion resulted also in three fragments of approximately 4900 bp (p1NIL and fragment 96-A together), 1350 bp (hygromycin resistance gene), and 450 bp for fragment 96-B (Lane 8).

Obtaining the 1356 bp from pANO2 confirmed the successful ligation and cloning of the hygromycin resistance cassette into pASO7, and consequently the successful construction of the suicide vector, pANO2. A map of pANO2 (developed from p1NIL) carrying the *rv1996* gene mutated by inserting the hygromycin resistance cassette is shown in Figure 4.25.

The suicide vectors pANO1 and pANO2 carrying the target disrupted genes of *rv1995* and *rv1996* respectively became available to be transformed into the target mycobacterial strains to obtain recombinant cells by homologous recombination for both *rv1995* and *rv1996* genes. Therefore, samples of the prepared DNA molecules (pANO1 and pANO2) were used to complete the experiments in the current study. The remaining DNA molecules were stored at -20^{o}C at the laboratories of the University of Leicester for further future work.

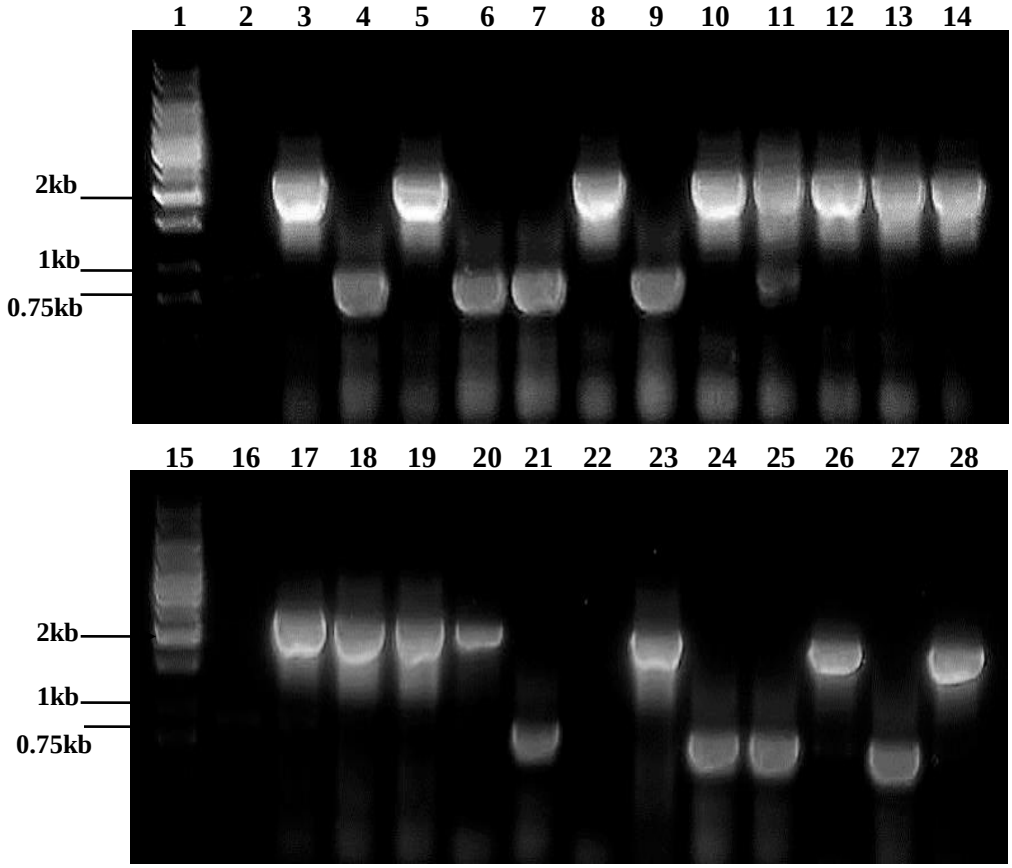


Figure 4.23: PCR to identify recombinants with the *rv1996* disrupted with hygromycin resistance gene, in pANO2.

Amplification was done using ForA-6.6 and RevB-6 primers. Lanes 1 and 15 contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 16 contain the negative control (no bands). Lanes 3, 5, 8, 10, 11, 12, 13, 14, 17, 18, 19, 20, 23, 26 and 28 contain the target fragment of approximately 2250 bp whereas the other bands contain the fragment 96-AB.

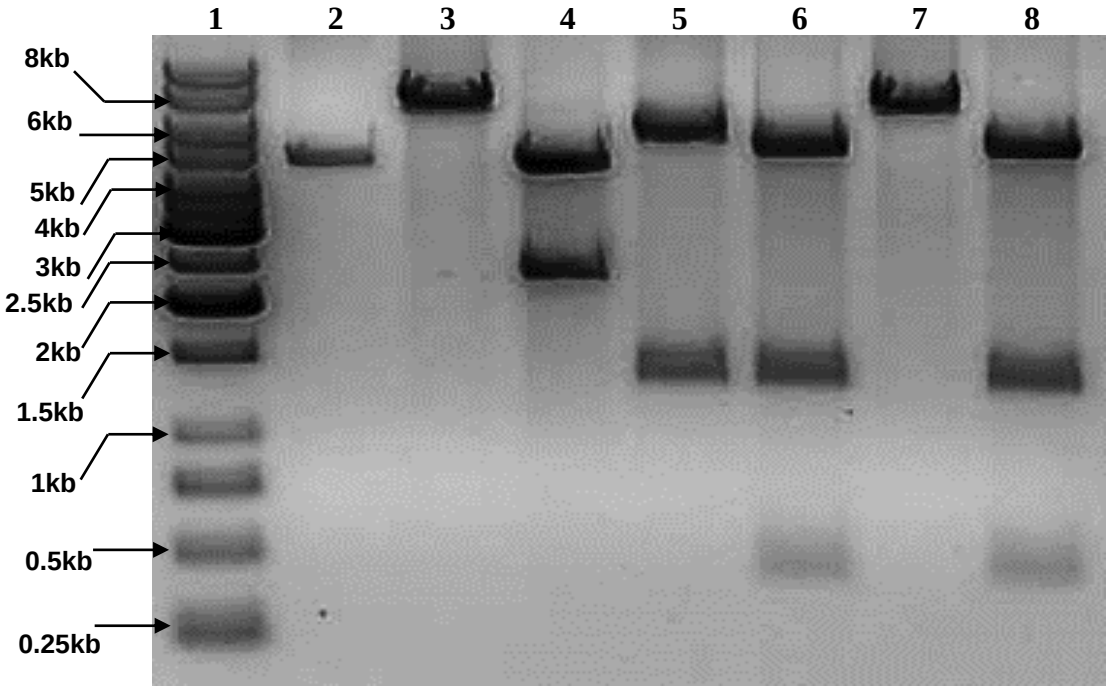


Figure 4.24: Confirmation the insertion of the hygromycin resistance cassette into *rv1996* to produce pANO2 suicide vector.

To confirm the presence of the *rv1996* disrupted by hygromycin resistant gene between the fragments AB of *rv1996* in pANO2, the DNA was digested with *Hin*dIII, *Asc*I and *Kpn*I. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 shows p1NIL (control digested with *Hin*dIII). pANO2 digested with *Hin*dIII in lane 3. *Hin*dIII and *Kpn*I digestion resulted in two fragments (lane 4). Two fragments in (lane 5) resulted from digestion with *Asc*I. Restriction digestion with *Hin*dIII and *Asc*I resulted in three fragments (lane 6). pANO2 digested with *Kpn*I (lane 7). Digestion with *Kpn*I and *Asc*I (lane 8).

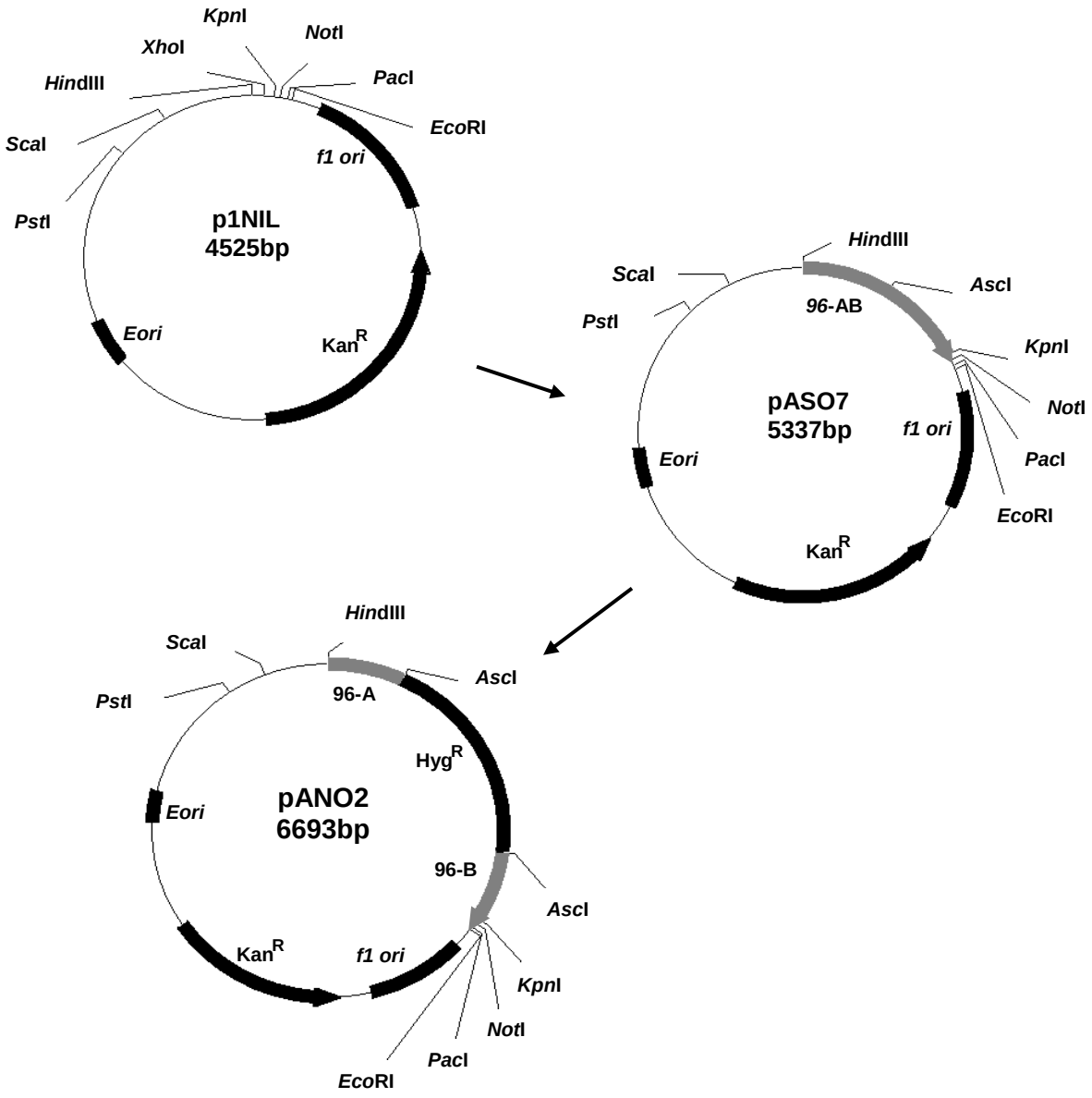


Figure 4.25: Schematic map showing the construction of pANO2 containing the disrupted *rv1996* gene from pASO7 developed from p1NIL.

The 904 bp fragment 96-AB was obtained from pHSO2 (Section 4.1.2.2.2.2) by digesting with *Hin*dIII and *kpn*I and ligated into the *Hin*dIII/*kpn*I position of p1NIL to produce pASO7. The 1356 bp hygromycin resistance cassette was obtained from pASO3 (Section 4.1.1.3) by digesting with *Asc*I and ligated into the *Asc*I position of pASO7 to construct pANO2 suicide vector (6693 bp). Abbreviations are; *rv1996* fragment-A (*rv1996* fra-A), *rv1996* fragment-B (96-B), *Escherichia coli* origin of replication (*Eori*), kanamycin resistance gene (Kan^R), hygromycin resistance gene (Hyg^R), ligated *rv1996* fragment AB (96-AB) and phage origin of replication (*f1 ori*).

4.2. Discussion.

One of the most essential strategies for mutagenesis of a particular gene is the use of an efficient delivery system, in which the disrupted gene is introduced in a vector and then homologous recombination occurs between the disrupted gene and the intact gene in the mycobacterial chromosome after which the vector is lost. This has been performed in mycobacteria by the use of suicide (non-replicating) plasmids (Parish and Stoker, 2000; Stewart *et al*., 2005; Balhana *et al*., 2010). As described in chapter 4.1 an insertional mutagenesis strategy using a hygromycin resistance gene cassette was followed for disruption of the target genes (*rv1995* or *rv1996*). One of the difficulties found in the development of a suicide vector harbouring the mutated gene is to ligate multiple inserts into the desired vector. In the current study, two attempts to ligate more than one insert into p1NIL in a single reaction were done. Unfortunately, they were unsuccessful. The unsuccessful ligation in these experiments was attributed to using several DNA fragments in one ligation reaction. The efficiency of ligation reaction depends on many parameters including DNA concentration, vector/insert ratio, and the ligation buffer (Cranenburgh, 2004; Topcu, 2000). These parameters were adjusted and validated to be efficient in the present study for a single reaction, however ligation of multiple DNA fragments was reported to be commonly inefficient even after optimisation of the reaction conditions (An *et al*., 2010; Christ *et al*., 2006; Legerski and Robberson, 1985). An *et al*. (2010) reported that in such cases most of the products obtained are either non-full-length DNA fragments or self-ligation inverted repeat fragments since small fragments are easier to form than the full-length DNA fragment. They also reported that increasing the number of DNA inserts decrease the possibility of correct plasmid construction.

In the current study, the construction of the desired suicide vector succeeded after introduction of one insert (Fragment AB) of either *rv1995* or *rv1996* into the p1NIL vector in a single reaction. Then, hygromycin resistance gene cassette was ligated in-between fragment A and B using *Asc*I ends in a separate reaction to develop the desired suicide vectors (pANO1 and pANO2) for *rv1995* and *rv1996*, respectively. In conclusion, the use of sequential ligation steps with no more than two fragments (DNA insert + Vector) in each reaction was the resolution of the unsuccessful ligation problem.

CHAPTER 5

RESULTS

5.1. Knock out experiments.

The aim of work described in this chapter was to obtain *M. tuberculosis* H37Rv and *M. bovis* BCG recombinants having the insertionally disrupted *rv1995* and *rv1996* genes that are carried by pANO1 and pANO2 vectors respectively, obtained in the previous chapter. *M. tuberculosis* H37Rv (Cole *et al*., 1998) and *M. bovis* BCG (Section 5.3.1) were chosen for this purpose as they were known to have the target gene sequences within their genomes. This was followed by knocking out the *rv1996* gene in BCG through homologous recombination to investigate the phenotypic effects of the *rv1996* gene.

5.1.1. Choice of mycobacterial strains for knocking out experiments.

In order to choose a knockout model for the *rv1995* and *rv1996* genes, in addition to the *M. tuberculosis* H37Rv, *M. bovis* BCG and *M. smegmatis* both were tested for presence of both genes via PCR amplification using the same primers used in previous sections (Sections 4.1.1.1 and 4.1.2.1).

Genomic DNA was extracted from both *M. bovis* BCG and *M. smegmatis* strains (Section 2.3.4.2.1 and 2.3.4.2.2). This was followed by PCR amplification of each gene using forward primer (ForA-5) and reverse primer (RevB-5) to amplify the *rv1995* gene, and forward primer (ForA-6.6) and reverse primer (RevB-6) (Table 2.9) to amplify the *rv1996* gene (Section 2.3.5.2). *M. tuberculosis* H37Rv DNA was used as a positive control. After separation on a 0.8 % (w/v) agarose gel (Section 2.3.8.1) the PCR products were observed (Figure 5.1) to have a size of approximately 750 bp from H37Rv DNA (Positive control) and BCG (Lanes 3 and 5) that represented the calculated size of *rv1995* (768 bp), and 950 bp for *rv1996* from H37Rv DNA (positive control) and BCG (Lanes 6 and 8) that represented the calculated size of *rv1996* (954 bp) respectively. However, there were no bands generated for either *rv1995* or *rv1996* from the DNA template of *M. smegmatis* (Lanes 4 and 7). This indicated that the *rv1995* and *rv1996* gene sequences were present in *M. bovis* BCG, as well as *M. tuberculosis* (Positive control) and absent from *M. smegmatis*

or another possibility was that the sequence where one or both primers anneal was sufficiently different to limit amplification. Thus, it was necessary to exclude the possibility of primer sequence variation as a reason for negative PCR and to confirm the absence of the *rv1995* and *rv1996* gene sequences from *M. smegmatis.* BLAST searching using the NCBI BLAST tool (Altschul and Gish, 1990) was done at the time when the experimental work was carried out in 2006. The *rv1995* and *rv1996* gene sequences were only present in *M. bovis* BCG and *M. tuberculosis* CDC 1551 as well as *M. tuberculosis* H37Rv. The searches performed in 2006 confirmed that the *rv1995* and *rv1996* sequences were not found in *M. smegmatis* strain MC2 155 genome used in my study. For this reason, *M. smegmatis* was excluded from the next knockout experiments. In BCG, the names of *rv1995* and *rv1996* sequences were found to have the names *bcg2012* and *bcg2013*, respectively. The coordinates of *bcg2012* and *bcg2013* on *M. bovis* BCG Pasteur strain (accession no. NC_008769.1) were (2216796-2217563) and (2217659 - 2218612) respectively.

To confirm the identity of PCR products of *rv1995* and *rv1996* obtained from *M. bovis* BCG against that of H37Rv, both products were sequenced by MWG Bio-technology (Section 2.3.9) and confirmed to be 100% identical with no sequence variation between PCR products from both strains.

After PCR confirmation of the presence of the target genes in *M. bovis* BCG in addition to the H37Rv strain and DNA sequence homology analysis, the knock out experiments were suggested initially to be started using both strains.

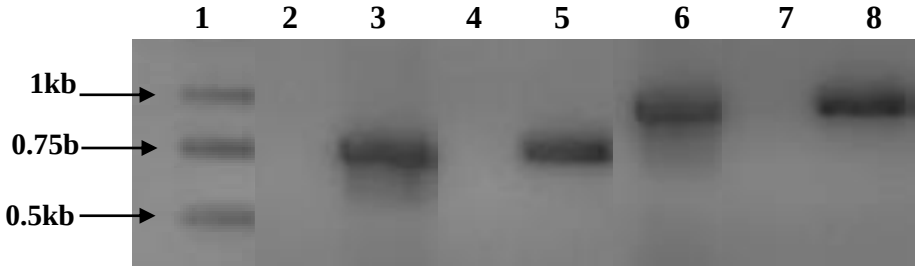


Figure 5.1: PCR to amplify the *rv1995* and *rv1996* gene sequences from *M. tuberculosis* H37Rv and *M. bovis* BCG genomes.

Amplification of the *rv1995* and *rv1996* genes was done from *M. tuberculosis* and from *M. bovis* BCG genomes using ForA-5 and RevB-5 primers for *rv1995* gene sequence and ForA-6.6 and RevB-6 primers for *rv1996* gene sequence. Lane 1 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment size shown in kilobases (kb). Lane 2 contains the negative control (no bands). Lanes 4 and 7 there were no bands represent the *rv1995* and *rv1996* genes from *M. smegmatis* DNA template respectively. Lanes 3 and 6 contain PCR product of the *rv1995* and *rv1996* genes from *M. tuberculosis* H37Rv DNA template (positive control). Lanes 5 and 8 contain PCR product of the *rv1995* and *rv1996* genes from *M. bovis* BCG DNA template. The photo has been edited to remove lanes containing unrelated samples.

5.1.2. Construction of the sucrose counter-selectable suicide vector for the positive selection of recombinants.

The homologous recombination has the possibility of double and single cross over, however; single cross over is far more likely. For this reason, the *sacB* cassette counter-selectable marker was proposed to be used to distinguish between both cases and to directly select for the product of a double cross over (DCO). The *sacB* gene of *Bacillus subtilis* encodes the enzyme levansucrase that converts sucrose to levan, which is toxic to cells such as mycobacteria that lack a functional levanase (Pelicic *et al*., 1996a; Pelicic *et al*., 1996b). Thus *SacB*+, but not *sacB*- cells are killed upon addition of sucrose. The *sacB* cassette as a counter selectable marker was previously investigated in many bacteria

including *M. tuberculosis*, *M. bovis* BCG (Pelicic *et al*., 1996; Parish and Stocker, 2000) and *E. coli* (Gay *et al*., 1983; Steinmetz *et al*., 1983; Reyrat *et al*., 1998; Li *et al*., 2013).

The *sacB* marker was planned to be inserted in both pANO1 and pANO2 before introducing them into H37Rv and BCG. In the case of a single cross over event, the presence of *sacB* leads to cell death. However, in the case of double cross over event, cells survive as a result of eliminating the whole plasmid including the *sacB* cassette since the plasmid has no *Mori* (Pelicic *et al*., 1996b; Parish and Stoker, 2000; Muttucumaru *et al*., 2004).

In order to insert the *sacB* counter-selective marker gene in the pANO1 and pANO2 vectors, the two plasmids were digested (Section 2.3.8.5) at the *Pac*I site (Figure 5.2. lane 2 and 3) and purified from the digestion reaction mixtures (Section 2.3.5.4). The *sacB* cassette is present in plasmid pGAOL17 flanked by the two *Pac*I sites (Parish and Stocker, 2000). The plasmid pGAOL17 was completely digested after three attempts using *Pac*I enzyme resulting in two fragments of approximately 6200 bp and 2300 bp (Figure 5.2. lane 4. The 2300 bp fragment containing the desired *sacB* marker gene was ligated into the *Pac*I site of linearised pANO1 and pANO2. The ligation products were then transferred into *E. coli* DH5α by electroporation (Section 2.3.6.1.2). The integration of the *sacB* cassette into the plasmids was checked after plating transformants on 10% (w/v) sucrose-containing L-agar and sucrose-free L-agar. This attempt failed to clone the *sacB* into pANO1 or pANO2. Equivalent levels of heavy growth were observed on both medium containing sucrose as on control plates lacking sucrose. To resolve this problem, pANO1 and pANO2 were digested with *Pac*I and then dephosphorylated (Section 2.3.8.7) to prevent self-ligation. After that, the *sacB* cassette was ligated into the digested pANO1 and pANO2 from *Pac*I site. This attempt was also unsuccessful. At the third attempt, 100 colonies of both pANO1 and pANO2 were chosen from the sucrose-free L-agar and streaked on L-agar with and without sucrose. All of the streaked colonies grew on sucrose. Nevertheless, 25 colonies were randomly picked by loop and grown in L-broth medium. After overnight incubation at 37^{o}C, mini preps were done for all cultures (Section 2.3.4.1.3). The extracted plasmids were digested with *Pac*I restriction enzyme. The DNAs were analysed by running through 0.8% agarose gel. Unfortunately, all resultant bands

gave the same size as the pANO1 and pANO2 controls (data not shown), indicating that cloning of *sacB* cassette into the vectors had failed.

Failure of this experiment could be attributed to self-ligation of either the insert or the vector despite dephosphorylation of the vector, or there was a difficulty with ligation at the *Pac*I site, or possibly because of the large size of *sacB* cassette (2300 bp) that prevents it from ligating with the suicide vectors in the restriction position of *Pac*I.

As a result of unsuccessful attempt of *sacB* cloning into both vectors, an alternative PCR based approach was carried out described in the next sections to identify and confirm the occurrence of homologous recombination in the recombinants after introducing the suicide vectors into the target mycobacterial cells.

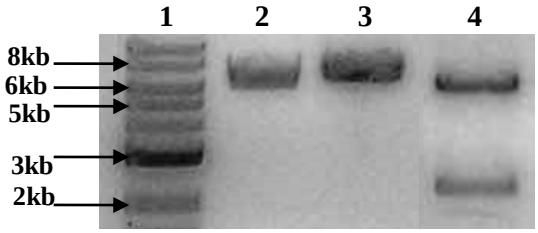


Figure 5.2: Preparation of pANO1, pANO2 and *sacB* by digesting with *Pac*I restriction enzyme.

In order to clone the *sacB* cassette into pANO1 and pANO2, the plasmid DNAs were digested with *Pac*I restriction enzyme. Lane 1 contains DNA size markers (1Kb ladder, NEB), with fragment sizes shown in kilobases (kb). Restriction digestion of pANO1 resulted in one fragment of approximately 6250 bp (lane 2). Restriction digestion of pANO2 resulted in one fragment of approximately 6700 bp (lane 3). Restriction digestion of pGAOL17 resulted in two fragments of approximately 6200 bp and 2300 bp (lane 4).

5.1.3. Experiments for knocking out the *rv1995* and *rv1996* genes from *M. bovis* BCG and *M. tuberculosis* H37Rv.

In order to construct the *rv1995* and *rv1996* knock out mutants of *M. tuberculosis* H37Rv and *M. bovis* BCG, a homologous recombination strategy was attempted using pANO1 and pANO2 suicide vectors carrying *rv1995* and *rv1996* fragments that were disrupted by a hygromycin resistance cassette. The vectors were individually electroporated into BCG and H37Rv. After the electroporation process, reaction contents were plated onto 7H11 L-agar supplemented with hygromycin and OADC and incubated for 4 to 6 weeks at 37^{o}C in order to select the recombinants. There were no pANO1 and pANO2 recombinants obtained. The process was repeated again using both vectors as well as pSMT3 plasmid as a control for the electroporation process. Again, there were no pANO1 and pANO2 recombinants obtained whereas there was an uncountable number of transformants with pSMT3. This result indicated that the electroporation process was successful, but homologous recombination for target DNA molecules was not obtained in this attempt.

5.1.3.1. Transformation of pANO1 and pANO2 into *M. tuberculosis* H37Rv and *M. bovis* BCG after treatment with ultraviolet irradiation.

In order to overcome the obstacle of homologous recombination for the target genes, both plasmids DNA (pANO1 and pANO2) were pre-treated with either ultraviolet irradiation (UV) (Section 2.3.8.3) and/or alkali denaturation (NaOH) (Section 2.3.8.4). The plasmids, pANO1 and pANO2, were subjected to 100 mJ UV irradiation (Section 2.3.8.3) before transforming into the mycobacterial cells. The treated DNAs were then transformed into BCG and H37Rv by electropration (Section 2.3.6.2). pSMT3, carrying the hygromycin resistance gene was electroprated into *M. bovis* BCG as a control to check the electroporation processes.

After electroporation, the transformed cell suspensions were plated onto 7H11 agar plates containing 150μg/ml hygromycin B and supplemented with OADC. The plates were incubated at 37ºC for 3 to 5 weeks. The results obtained illustrated that this attempt after treatment of the plasmid DNA with UV light was successful, since potential recombinants were counted for both strains H37Rv and BCG after electroporation with both plasmids pANO1 and pANO2 (Table 5.1) and the homologous recombination theoretically occurred as pANO1 and pANO2 do not contain a *Mori*. Because of the experiments with UV treated plasmids were successful, there was no need to use the plasmids treated with NaOH.

Plasmids	Recipients	Transformants
pANO1	*M. tuberculosis* H37Rv	22
	M. bovis BCG	17
pSMT3	*M. bovis* BCG	Uncountable
pANO2	*M. tuberculosis* H37Rv	18
	M. bovis BCG	22
pSMT3	*M. bovis* BCG	Uncountable

Table 5.1: Number of recombinants for *M. tuberculosis* H37Rv and *M. bovis* BCG after treating plasmid with UV light.

After obtaining the recombinants for BCG and for H37Rv, work with H37Rv recombinants was stopped due to lack of Cat 3 facilities in the laboratories of the university at that time and recombinants were stored at the laboratories of the university until use in further future experiments. Therefore, the recombinants of low pathogenicity strain BCG were used to complete the knocking out experiments of the *rv1995* and *rv1996* genes. The *bcg2012* and *bcg2013* genes are orthologues of the *rv1995* and *rv1996* genes respectively. For clarity, it was preferred to stick to the names *rv1995* and *rv1996* instead of *bcg2012* and *bcg2013* as the focus of the work of the current study was on the genes in *M. tuberculosis*.

5.1.3.2. PCR to detect the mutated *rv1995* and *rv1996* genes in *M. bovis* BCG cells.

To detect whether recombinants of BCG contained mutated *rv1995* and mutated *rv1996* sequences, PCR was done as described in Material and Methods (Section 2.3.5.1) to amplify the 1356 bp hygromycin resistance cassette using a forward primer (For-BCG Hyg) and a reverse primer (Rev-BCG Hyg) (Table 2.9). All the *rv1995* BCG recombinants tested were found to contain the hygromycin resistance gene with an amplicon size of approximately 1350 bp after running on except the reactions run in lanes 12 and 20 of a 0.8% (w/v) agarose gel (Figure 5.3). Similarly, the hygromycin resistance gene was found in all the *rv1996* BCG recombinants that were tested (Figure 5.4).

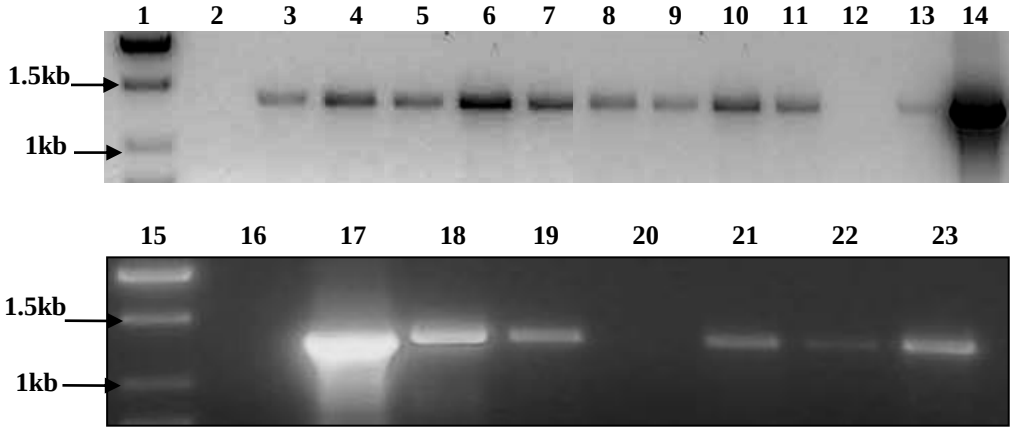


Figure 5.3: PCR to amplify the hygromycin resistance gene from the *rv1995 M. bovis* BCG recombinants.

Amplification of the hygromycin resistance gene sequence was done using For-BCG-Hyg and Rev-BCG Hyg primers. Lanes 1 and 15 contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 16 contain the negative controls with no bands. Lane 14 and 17 show the hygromycin resistance gene from pANO1 at approximately 1350 bp (positive controls). The other lanes are the PCR products of the hygromycin resistance gene from *rv1995 M. bovis* BGG recombinants.

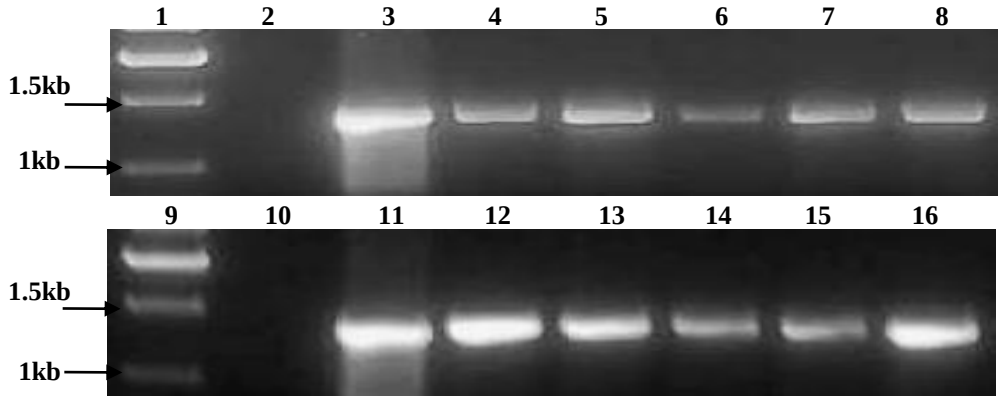


Figure 5.4: PCR to amplify the hygromycin resistance gene from the *rv1996 M. bovis* BGG recombinants.

Amplification of the hygromycin resistance gene sequence was done using For-BCG Hyg and Rev-BCG Hyg primers. Lanes 1 and 9 contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 10 contain the negative controls. Lanes 3 and 11 show the hygromycin resistance gene from pANO2 at approximately 1350 bp (positive control). The other lanes show the PCR products of the hygromycin resistance gene from *rv1996* BGG recombinants.

To confirm the integration of the mutated *rv1995* and *rv1996* fragments into the intact genome of the BCG cells, another PCR was done using the upstream forward primer, For-BCG 2011, and the downstream reverse primer, Rev-BCG 2013, to amplify the disrupted *rv1995* gene sequence (Table 2.9). To amplify the disrupted *rv1996* gene sequence, the upstream For-BCG 2012 and downstream Rev-BCG 2014 primers (Table 2.9) were used. A band of 2300 bp was expected for the disrupted *rv1995* gene, however; all bands obtained for the recombinants tested were found to be approximately 1000 bp, which represented the size of the intact gene (Figure 5.5). This could be due to a single cross over event in which the plasmid is still a merodiploid and the smaller amplicon of the original size was favoured in the PCR.

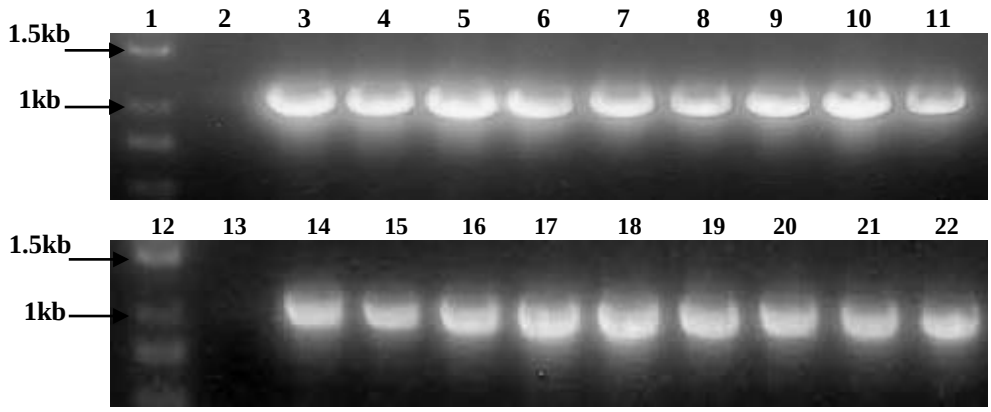


Figure 5.5: PCR to amplify the disrupted *rv1995* gene sequence in *M. bovis* BCG recombinants.

Amplification of the disrupted *rv1995* gene sequence was done using For-BCG 2011 and Rev-BCG 2013 primers. Lanes 1 and 12 contain DNA size marker (1 Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 13 contain the negative controls with no bands. Lane 22 shows the *rv1995* gene from BCG wildtype strain DNA at approximately 1000 bp (positive control).

For the *rv1996* disrupted gene sequence, a PCR product of approximately 2650 bp was expected to be obtained. Fortunately, two recombinants were identified as containing *rv1996* disrupted by the hygromycin resistance gene. A PCR product of approximately 2650 bp was successfully amplified (Figure 5.6. lanes 14 and 25). Appearance of the band at approximately 2650 bp indicated that a double cross over event had occurred. This represented the desired disrupted fragment and the mutant was named *M. bovis* BCG *rv1996*::hyg^R.

To continue the investigation of *rv1995* gene recombinants, a further PCR (Section 2.3.5.1) was done to investigate the possibility of occurrence of homologous recombination by a single cross over using forward primer (For-BCG 2011) upstream the *rv1995* fragment A outside the cloned DNA and hygromycin reverse primer (Rev-BCG Hyg) to amplify the *rv1995* fragment A and hygromycin resistance gene together. Similarly, to amplify the *rv1995* fragment B and hygromycin resistance gene together, hygromycin forward primer (For-BCG Hyg) and reverse primer (Rev-BCG 2013) downstream the *rv1995* fragment B outside the cloned DNA were used. Unfortunately, there were no bands representing the desired fragments (data not shown). Consequently, based on the initial result of the PCR, it appeared that there was no knock out obtained for

the *rv1995* gene sequence in BCG in spite of showing bands indicating the presence of hygromycin resistance gene in the recombinants. Due to restriction of time, the decision was taken to complete the work on *rv1996* and stop working on the *rv1995* gene.

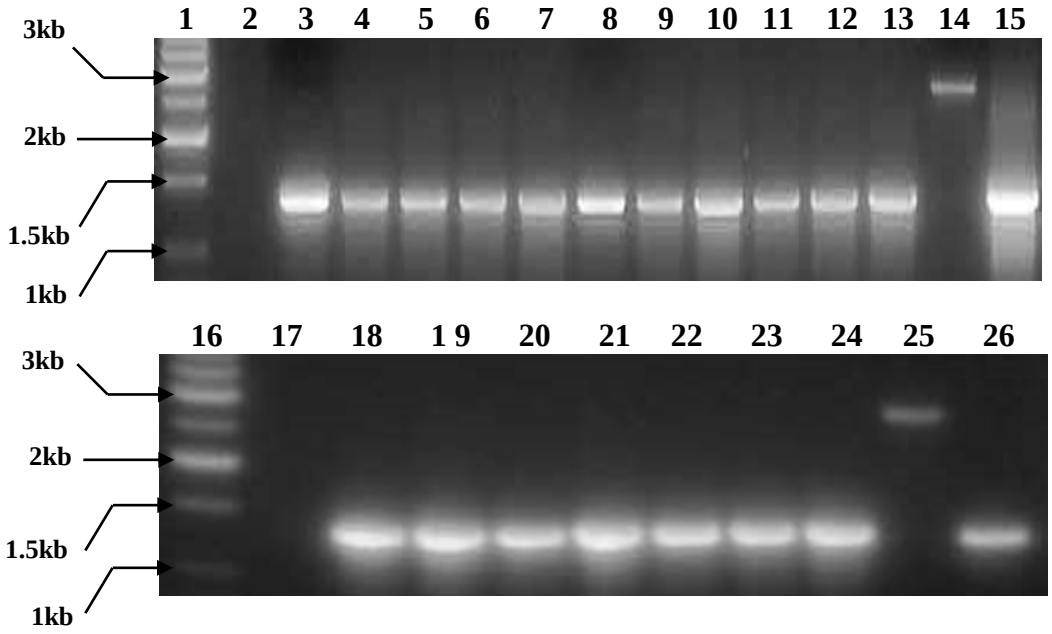


Figure 5.6: PCR to amplify the disrupted *rv1996* gene sequence in BCG recombinants.

Amplification of the disrupted *rv1996* gene sequence was done using For-BCG 2012 and For-BCG 2014 primers. Lanes 1 and 16 contain DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lanes 2 and 17 contain the negative control with no bands. Lanes 3 and 18 show the *rv1996* gene from wildtype BCG strain DNA (positive control). Lanes 14 and 25 show the expected bands of the disrupted *rv1996* gene. The remaining lanes show bands with size of the intact *rv1996* gene.

5.1.3.3. Confirmation of the double cross over recombination in the mutated *rv1996* in *M. bovis* BCG genome.

5.1.3.3.1. PCR to confirm the mutated *rv1996* in *M. bovis* BCG genome.

To confirm the insertional inactivation of the *rv1996* gene sequence in BCG genome, a PCR was done as described in Material and Methods (Section 2.3.5.1) using purified extracted chromosomal DNA (Section 2.3.4.2.2) from one of the two recombinant

BCG colonies (Figure 5.6, a colony represented by lane 14). PCR products were analysed after using 0.8% (w/v) agarose gel as it can be seen in Figure 5.7. Use of forward primer (For-BCG 2012) and reverse primer (Rev-BCG 2014) gave a PCR product of approximately 2650 bp (Lane 3). This was consistent with insertion of the hygromycin resistance gene by a double cross over event. The forward primer (For-BCG 2012) and hygromycin reverse primer (Rev-BCG Hyg) were used to amplify the *rv1996* fragment A and hygromycin resistance gene together and gave a PCR product of approximately 1900 bp (Lane 4). The reverse primer (Rev-BCG 2014) and hygromycin forward primer (For-BCG Hyg) were used to amplify the *rv1996* fragment B and hygromycin resistance gene together and gave a PCR product of approximately 2000 bp (Lane 5). Again, both were consistent with insertion of the hygromycin resistance gene by a double crossover event. The hygromycin forward primer (For-BCG Hyg) and hygromycin reverse primer (Rev-BCG Hyg) were used to amplify the hygromycin resistance gene and gave the expected PCR product of approximately 1350 bp (Lane 6 and 8). Homologous recombination through double cross over occurs via two steps as can be seen in Figure 5.8.

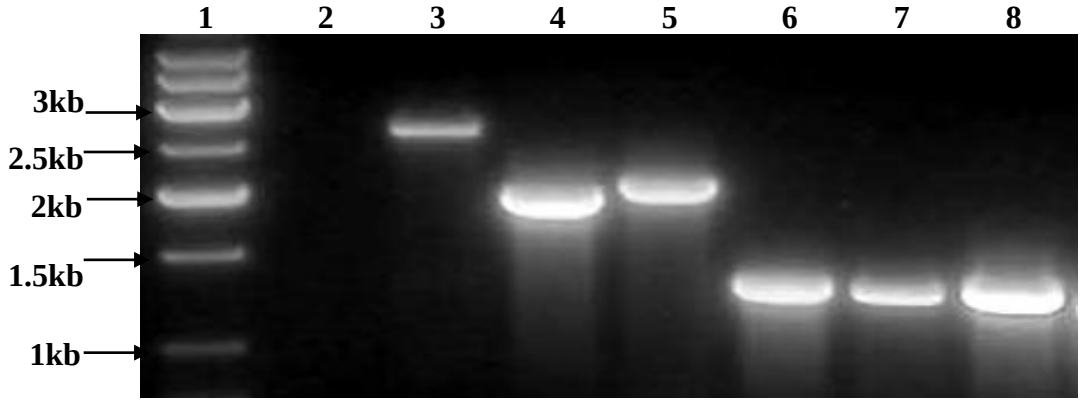


Figure 5.7: PCR to confirm the knock out of *rv1996* sequence in *M. bovis* BCG genome.

To confirm the presence of the disrupted *rv1996* gene sequence in the genomic DNA of a mutated BCG, PCR amplification was done. Lane 1 contains DNA size marker (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control. Lane 3 shows the products of the disrupted *rv1996* gene. Lane 4 shows the products of the *rv1996* fragment A and hyg^R gene together. Lane 5 shows the products of the *rv1996* fragment B and hyg^R gene together. Lane 6 shows the products of the hyg^R gene in *M. bovis* mutant BCG. Lane 7 shows the products of the *rv1996* gene in *M. bovis* wildtype BCG (positive control). Lane 8 shows the products of the hyg^R gene in pSMT3 (positive control).

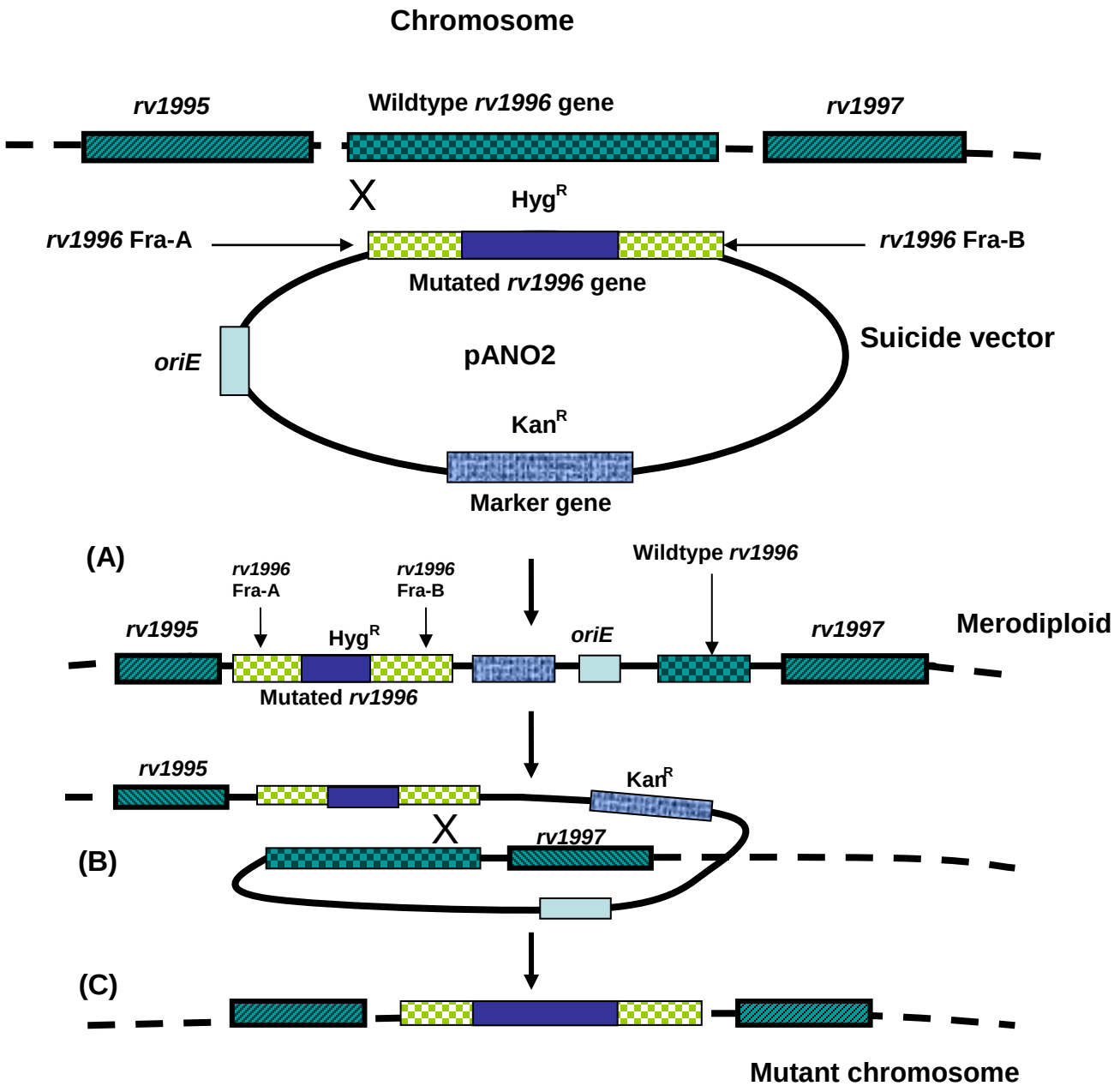


Figure 5.8: Schematic diagram represents a possible two-step event of single cross over for gene replacement by homologous recombination.

A suicide plasmid carrying a mutated copy of the target gene is introduced into the bacterium. Homologous recombination between the plasmid and the intact chromosome (indicated by X), in the case of two-step recombination, is based on a single cross-over event as a first step at which the whole delivery vector is integrated in the chromosome as shown in A. In the second step, another single cross-over event occurs where allelic replacement replaces the wildtype gene with the mutated allele as shown in B. This is followed by the loss of the whole delivery vector integrated due to the lack of a functional origin of replication and formation of the mutated target gene as shown in C.

5.1.3.3.2. Reverse transcriptase PCR (RT-PCR) analysis of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mRNA.

To verify that the *rv1996* gene sequence was disrupted in BCG genome, the expression of the gene was investigated by detection of its mRNA via reverse transcriptase PCR.

Total RNA first was extracted from exponentially growing cultures of parental BCG and mutant BCG *rv1996*::hyg^R (Section 2.3.11.1). Before converting the RNA to cDNA by reverse transcriptase, residual genomic DNA was removed by treating the samples with DNase (Section 2.3.11.2). To confirm success of the DNase reaction, cut pSMT3 plasmid DNA was added to two extracted BCG RNA samples before DNase treatment. One of them was not treated with DNAse as a negative control. This was followed by running through a 0.8 (w/v) agarose gel (Figure 5.9).

RNA samples then were purified using RNeasy Mini Protocol for RNA cleanup (Section 2.3.11.3). Then, an aliquot of total RNA for each strain (BCG and mutant) from the purified preparations was used as templates to synthesise cDNAs by reverse transcriptase, using random hexamer primers as described in Material and Methods (Section 2.3.11.5). After completion of the reverse transcriptase reaction, the cDNA samples of wildtype BCG and BCG mutant were amplified by PCR (Section 2.3.5.1) using forward primer (ForA-6.6) and reverse primer (RevB-6) to amplify the *rv1996* gene cDNA. Another PCR reaction using forward primer (ForA-5) and reverse primer (RevB-5) to amplify *rv1995* gene cDNA was done as a control (Table 2.9). As it can be seen in Figure 5.10, analysis of the PCR products on a 0.8% agarose gel confirmed the presence of the *rv1996* gene cDNA in BCG wildtype with a band of approximately 1000 bp (Lane 6) and its absence in the BCG mutant (Lane 4). This result confirmed that the mRNA expressed in BCG wildtype strain successfully, but not in the BCG mutant strain. Also, amplification of *rv1995* gene sequence from the cDNAs of the BCG strain (Lane 3) and the BCG mutant strain (Lane 5) confirmed that the lack of *rv1996* expression in the BCG mutant strain was not due to degradation of the isolated RNA samples.

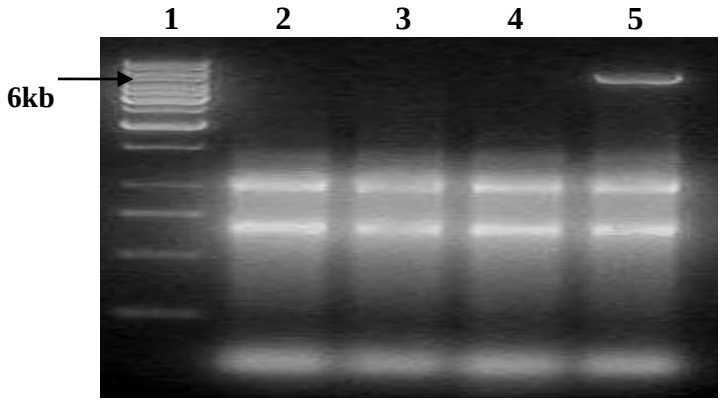


Figure 5.9: Agarose gel to confirm the removing of residual DNA from RNA samples by treating with DNase.

To prepare DNA-free RNA samples in order to use as templates in RT-PCR and qRT-PCR reactions, the RNA samples were treated with DNAse. Lane 1 contains DNA size markers (1Kb ladder, MBI Fermentas), with fragment sizes shown in kilobases (kb). Lane 2 contains BCG RNA sample and DNase. Lane 3 contains mutant *rv1996*::hyg^R BCG RNA sample and DNase. Lane 4 contains BCG RNA sample with adding cut pSMT3 DNA and DNase (positive control). Lane 5 contains BCG RNA with adding cut pSMT3 plasmid DNA and no DNase (negative control). Cut pSMT3 plasmid DNA is approximately of 5700 bp.

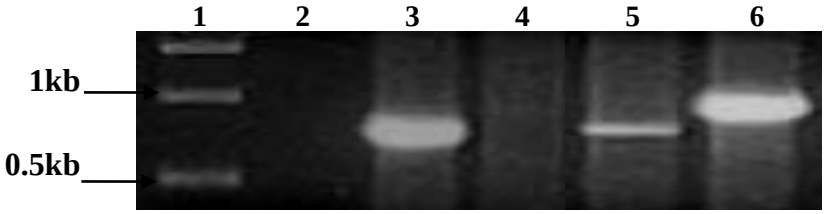


Figure 5.10: PCR to amplify the *rv1996* sequence from the cDNAs of BCG wildtype and BCG *rv1996*::hyg^R mutant.

Amplification of the *rv1996* sequence from the cDNAs of BCG and mutant strains was done using ForA-6.6 and RevB-6 primers. Lanes 1 contains DNA size marker (1 Kb ladder, NBE), with fragment sizes shown in kilobases (kb). Lane 2 contains the negative control. Lanes 3 and 5 shows the size of *rv1995* for both wildtype and mutant strains respectively (Assay controls). Lane 6 shows the *rv1996* presence in BCG wildtype strain. Lane 4 shows the absence of a band from BCG mutant strain.

5.1.3.3.3. Real time PCR (qRT-PCR) analysis of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mRNA.

To estimate the levels of *rv1996* mRNA expression for the knocked out *rv1996* sequence in the mutated BCG strain before phenotype experiments, qRT-PCR was done as described in Material and Methods (Section 2.3.11.6). First, total RNA was extracted from exponentially growing cultures of both BCG wildtype and mutant strains (Section 2.3.11.1) after growing the strains for six days. The number of cells for both strains was estimated to be 10^7 cells/ml. This was followed by treatment with DNase (Section 2.3.11.2). Two aliquots of DNase treated total RNA from independent preparations of BCG wildtype strain and BCG mutant strain were subjected to reverse transcription (Section 2.3.11.5) to produce cDNA. The resultant cDNA concentration was measured and the same quantity for each sample (750 ng) was subjected to the reaction of qRT-PCR using the primers (For-13 BCG) and (Rev-13 BCG), and a Taq Man probe (TMP-13 BCG) (Table 2.10) to quantify the cDNA from *rv1996* in the wildtype and mutant strains (Section 2.3.11.6). Another separate reaction using a forward primer (For- SigA BCG), a reverse primer (Rev-SigA BCG) and a Taq Man probe (TMP-SigA BCG) were used (Table 2.10) to quantify the cDNA of *sigA* in wildtype and mutant BCG. The s*igA* gene was used as an internal standard control and a normalizing housekeeping gene (Papavinasasundaram *et al*., 2005; Stahlberg *et al*., 2004; Singh and Singh, 2009). The *sigA* amplicon was approximately the same size as the target *rv1996* gene sequence. Crude DNase treated RNA samples from both strains not treated with reverse transcriptase were also subjected to qRT-PCR as a negative control in order to measure the level of contamination with chromosomal DNA.

A 1:10 dilution series of the BCG wildtype strain genomic DNA was done to be used as a standard mycobacterial genomic DNA (10^7 to 10^2 copies/µl) to estimate the mRNA levels for the test samples. The standards were used in order to provide a standard curve using the cycle threshold (Ct) for each sample against its estimated concentration.

Expression of the housekeeping gene *sigA* was found to be similar in the two strains (5.2 x10^7 copies for the wildtype and 8 x10^6 copies for the mutant). In contrast, the cDNA in the mutant was extremely low (7.9x10^2 copies) in comparison with *rv1996* cDNA in the wildtype (2.1 x 10^6 copies). The low copy number of the target gene in the mutated

strain could be a background reaction or a truncated transcript. The amount of mRNA for each sample was calculated as a ratio normalised to the amount of *sigA* cDNA as described in Material and Methods (Section 2.3.11.6). The ratio of the *rv1996* to *sigA* was 0.04 (4 x 10^{-2}) for the wildtype whereas it was 0.0001 (1 x 10^{-4}) for the mutant. The real time PCR reaction was done twice. The first attempt was done as a trial. Results provided were for the last experiment.

The mutant BCG *rv1996*::hyg^{R} constructed in the current study, which contained the target disrupted gene sequence (*rv1996*) became available for further investigation. Aliquots of the knock out strain were kept and stored at the laboratories of the University of Leicester for future experiments, whereas other aliquots were used to carry out the *in vitro* experiments described in the next sections of the current study.

5.2. *In vitro* investigations of phenotypic properties of *M. bovis* BCG wildtype strain compared with *M. bovis* BCG *rv1996*::hyg^{R} mutant strain.

As *rv1996* is one of the gene deletions reported in *M. tuberculosis* CH outbreak strain (Rajakumar *et al*., 2004), the aim of the work described in this chapter was to evaluate the effect of the insertional mutation of this gene using the *M. bovis* BCG strain as a model for MTB H37Rv. This current study was done as part of a larger project to investigate the gene deletions detected in the CH strain to determine if any of these deletions increased the virulence of the CH strain. The hypothesis was that one or several gene deletions were responsible for the increased propensity of the strain that caused the tuberculosis outbreak in Leicester, in 2001 (Shafi *et al*., 2002; Rajakumar *et al*., 2004; Newton *et al*., 2006). *In vitro* modelling provides an important experimental path to determine phenotypic virulence traits associated with gene polymorphisms in *M. tuberculosis.* The Rv1996 conserved hypothetical protein is known to be a universal stress protein homologue, therefore the effect of the *rv1996* knock out was investigated for resistance to different types of stresses such as acid stress, reactive oxygen intermediate

(represented by hydrogen peroxide) and reactive nitrogen intermediate (represented by nitric oxide) stresses, which are involved in the host defence against MTB.

5.2.1. Growth curves of *M. bovis* BCG wildtype and *M. bovis* BCG *rv1996*::hyg^R mutant.

In order to assess whether the disruption of *rv1996* sequence has an effect on the growth of *M. bovis* BCG *in vitro*, both BCG wildtype strain and BCG *rv1996*::hyg^R mutant strain were grown in 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 at a standard pH of 6.8. After appropriate time intervals, optical density readings at 600 nm and viable counts (CFU/ml) were determined as described in Material and Methods (Section 2.1.10 and section 2.1.11), and then the growth curves were done. The growth rate of each strain was calculated as described in Material and Methods (Section 2.1.12).

The results obtained demonstrated that there was no significant difference ($p>0.05$) regarding the growth rates between the wildtype (BCG) and the mutant (BCG *rv1996*::hyg^R). Both strains reached similar growth yields with similar values of optical densities of approximately 1.85 for BCG wildtype and 1.75 for BCG mutant (Figure 5.11-A and Figure 5.15). This was manifested by similar values at approximately ($1.4x10^9$ CFU/ml) for BCG wildtype and ($1.3x10^9$ CFU/ml) BCG mutant after 192 hr (Figure 5.11-B and Table 5.2). The obtained data further indicated that the growth rate recorded of the BCG wildtype strain was 0.035 hr^{-1} and that of the mutant strain was 0.036 hr^{-1}, and the generation time recorded was 19.7 hr and 19.4 hr respectively (Figure 5.15).

Although the data shown in figures 5.11-A and B, tables 5.2-A and B, and tables 5.3 suggest an effect of the mutation on growth of BCG *rv1996*::hyg^R mutant, there was no significant difference compared with BCG wildtype ($p>0.05$). Therefore, it can be concluded that the inactivation of the *rv1996* gene sequence from BCG strain had a minor or had no effect on the ability of the bacterium to grow in rich medium with a standard pH (6.8).

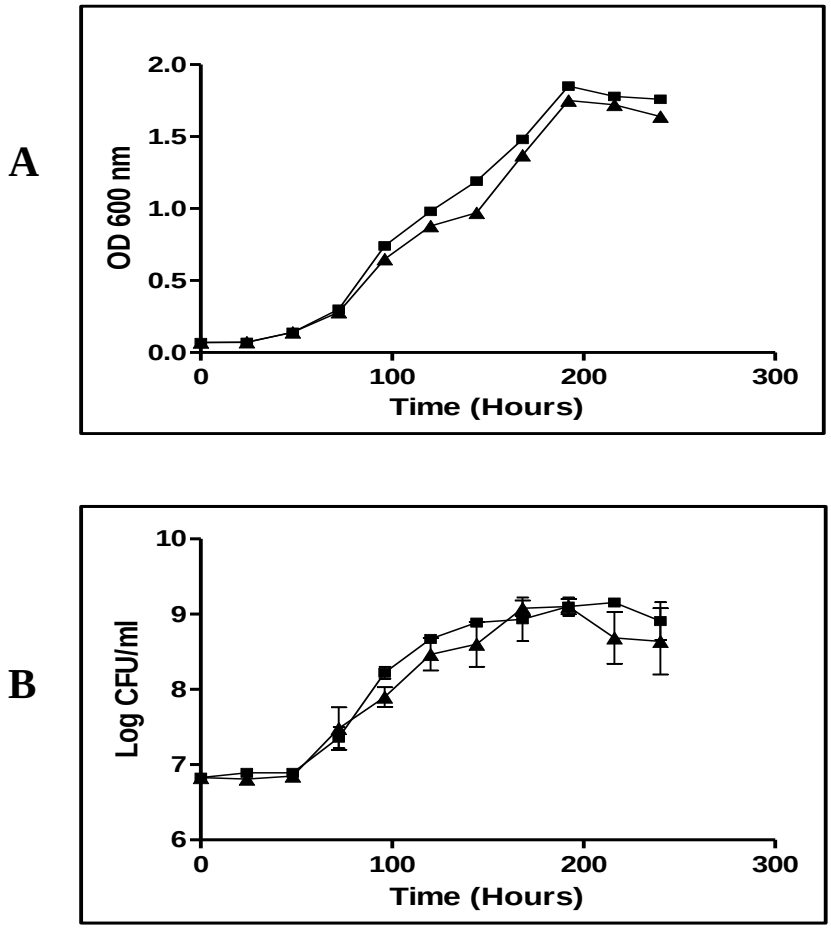


Figure 5.11: Growth curves of *M. bovis* BCG wildtype strain and *M. bovis* BCG *rv1996*::hyg^R mutant strain measuring by optical density (OD 600) (Graph A) and viable count (CFU/ml) (Graph B).

Mycobacterium bovis BCG wildtype strain and *M. bovis* BCG *rv1996*::hyg^R mutant were grown in 7H9 supplemented with ADC. The experiments were done at pH 6.8. Data were obtained from two experiments and each experiment was replicated (n=4) for each culture strain. Cultures were incubated at 37^{o}C. ■ represents the growth curve of BCG (control); ▲ represents the growth curve of BCG *rv1996*::hyg^R (mutant). Note: Panel A was drawn for illustration of the mean OD_{600} of experiments only.

5.2.2. Susceptibility of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant to $NaNO_2$.

In order to investigate whether mutation of *rv1996* altered susceptibility to reactive nitrogen intermediates (NO), the *in vitro* resistance of BCG wildtype strain and BCG *rv1996*::hyg^R mutant strain to nitric oxide was assessed. As described in Material and Methods (Section 2.4.5), cultures were set up in acidified (pH 5.4) 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80, containing concentrations of $NaNO_2$ from 0 to 32 mM. The presence of $NaNO_2$ in acidified media leads to the generation of reactive nitrogen intermediates and converts it to nitric oxide (Rhee *et al*., 2004; Rhee *et al*., 2005). Cultures were incubated at 37^{o}C with shaking for 48 hr and viable counts determined at intervals as described in Material and Methods (Section 2.1.11).

Results revealed that after an incubation period of 90 minutes, there was no significant difference in viable counts of BCG and BCG *rv1996*::hyg^R ($p>0.05$) at all concentrations of $NaNO_2$ used (Figure 5.12-A). After 14 hr of incubation, at the higher concentrations of $NaNO_2$ (16 mM and 32 mM), the viable counts of both strains of mycobacteria were decreased dramatically ($p<0.05$) compared to the control, but there was no significant difference ($p>0.05$) between both strains tested. At the other concentrations, there was no effect on the viability of the two strains (Figure 5.12-B). After 24 hr of incubation, at the highest concentration of $NaNO_2$ (32 mM), no bacteria were recovered, while at 16 mM concentration a reduction ($p<0.05$) in viable counts was observed. At the other concentrations of $NaNO_2$, there was no change in viable count. There was no significant difference ($p>0.05$) between the two strains tested (Figure 5.13-A). After 48 hr of incubation, no bacteria were recovered from cultures incubated at 16 mM. The viable counts of both strains showed a significant decrease ($p<0.05$) in viability in a dose-dependent manner at 4 and 8 mM $NaNO_2$, but there was no significant difference between the strains ($p>0.05$). At 1 and 2 mM there was no effect on viability of the two strains (Figure 5.13-B). From these results, it was demonstrated that over 48 hr of incubation BCG *rv1996*::hyg^R mutant strain did not differ from the BCG wildtype strain in susceptibility to nitric oxide.

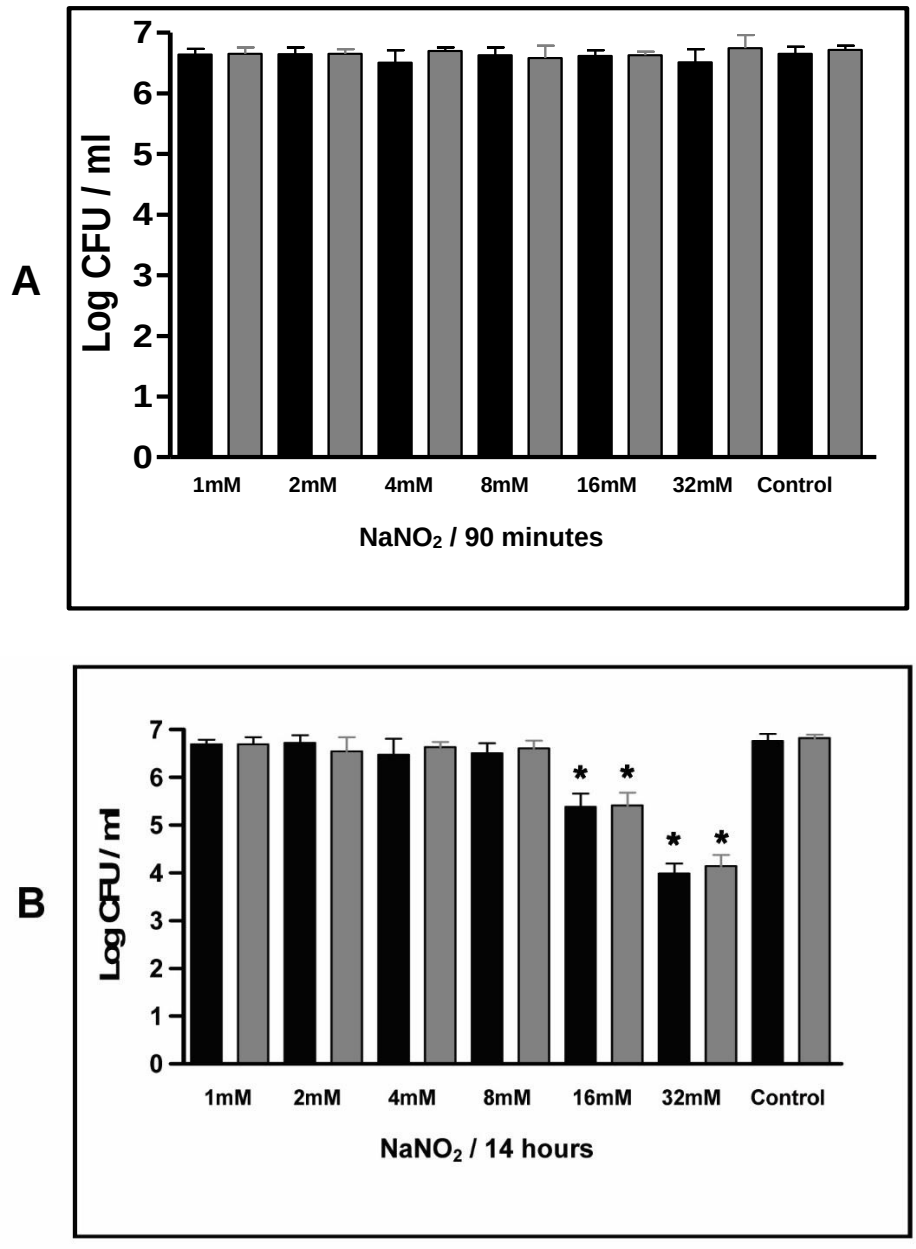


Figure 5.12: Susceptibility of *M. bovis* BCG wildtype strain (black bars) and *M. bovis* BCG *rv1996*::hyg^R mutant strain (grey bars) to different concentrations of $NaNO_2$ over 90 minutes (A) and 14 hours (B).

Data obtained are of 2 replicates of two independent experiments (n=4) of each strain that were inoculated into 7H9 broth containing the shown concentrations of acidified $NaNO_2$ in the figure at pH 5.4. Cultures were incubated at 37^{o}C for 90 minutes (A) and for 14 hours (B). In B a star (*) indicates a significant difference from control ($p<0.05$). Error bars represent standard deviations.

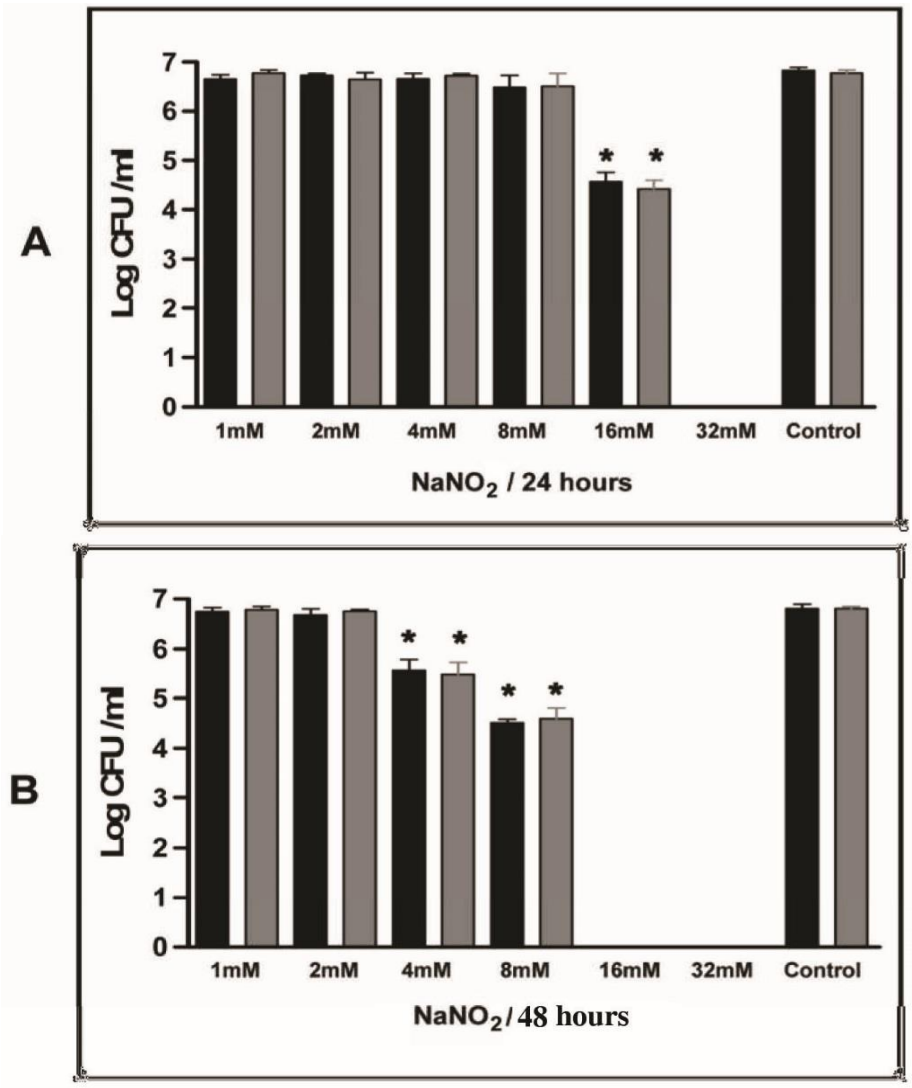


Figure 5.13: Susceptibility of *M. bovis* BCG wildtype strain (black bars) and *M. bovis* BCG *rv1996*::hyg^R mutant strain (grey bars) to different concentrations of $NaNO_2$ over 24 hours (A) and 48 hours (B).

Data obtained are of 2 replicates of two independent experiments (n=4) of each strain that were inoculated into 7H9 broth containing the shown concentrations of acidified $NaNO_2$ in the figure at pH 5.4. Cultures were incubated at 37^{o}C for 24 hours (A) and for 48 hours (B). In both A and B a star (*) indicates a significant difference from control ($p<0.05$). Error bars represent standard deviations.

5.2.3. Susceptibility of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant to H_2O_2.

The resistance to oxidative stress (H_2O_2) and the effect of the disruption of *rv1996* on resistance of both BCG wildtype strain and its mutant BCG *rv1996*::hyg^R also was assayed. Both strains were set up in 7H9 broth lacking catalase and contained concentrations of H_2O_2 between 0 and 32 mM, as described in Materials and Methods (Section 2.4.6). Cultures were incubated by shaking at 37^{o}C for 48 hr and viable counts were determined at intervals as described in Materials and Methods (Section 2.1.11).

It was clear that after only 90 min of incubation in H_2O_2, at the standard pH 6.8, no bacteria were recovered from 2 mM H_2O_2 or above with both the wildtype BCG and BCG *rv1996*::hyg^R mutant. The viable counts of both strains showed a significant decrease ($p<0.05$) in viability at 1mM H_2O_2 compared with the control without H_2O_2, but there was no significant difference between both strains ($p>0.05$) (Figure 5.14). After incubation periods for 14, 24 and 48 hr no viable counts were shown at all concentrations used (data not shown).

From these data it was observed that both strains *M. bovis* BCG wildtype strain and its mutant BCG *rv1996*::hyg^R were highly sensitive to the lowest concentration of H_2O_2. This observation is in contrast to $NaNO_2$ where both strains were found to be completely resistant *in vitro*.

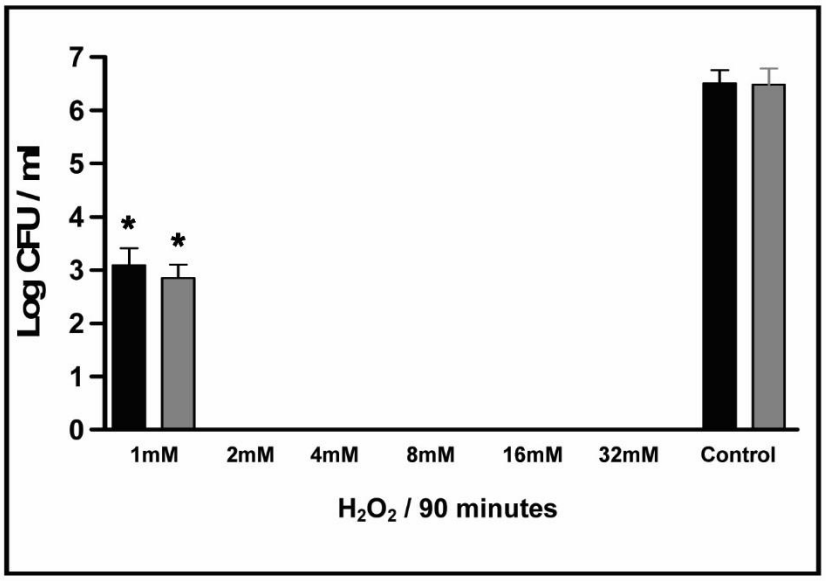


Figure 5.14: Susceptibility of *M. bovis* BCG wildtype strain (black bars) and *M. bovis* BCG *rv1996*::hyg^R mutant strain (gray bars) to different concentrations of H_2O_2 over 90 minutes.

Data obtained are of 2 replicates of two independent experiments (n=4) of each strain that were inoculated into 7H9 broth containing the shown concentrations of H_2O_2. Cultures were incubated at 37^{o}C for 90 minutes. A star (*) indicates a significant difference from control ($p<0.05$). Error bars represent standard deviations.

5.2.4. Viability of *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^{R} mutant over different pH levels.

In order to determine the lethal and the adaptive pH values for BCG (wildtype strain) and BCG (mutant strain), viability of both strains was investigated at different pH values (3.5, 4.5, 5.5, 6.5, and 6.8) using 7H9 broth supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80. The lethal pH level was determined as the highest level that resulted in a sharp decrease in viable count. The adaptive pH level was determined as the lowest level that allowed bacteria to continue replicating, but slowly.

Results given in table 5.2 showed that the lethal pH of both strains was 3.5, and the adaptive pH level was 5.5. The level pH of 5.5 was investigated as to whether it conferred the possibility of observing an acid tolerance response. As seen in the data in table 5.2 and figure 5.15, viability values for both strains decreased with declining external pH levels in a standard manner. Also, at pH 4.5, although the data suggested an effect on the mutant BCG to pH 4.5, there was no significant difference ($p>0.05$) compared with BCG wildtype. However, at pH 3.5 significant differences ($p<0.05$) in viability of both strains were found after incubation periods for 24 and 48 hr. These data obtained at pH 3.5 revealed that BCG *rv1996*::hyg^{R} (mutant strain) was less resistant to acid stress than BCG (wildtype strain).

***M. bovis* BCG**

pH / Time	Mean log CFU/ml at different pH levels				
	3.5	4.5	5.5	6.5	6.8
t = 0	6.81	6.78	6.79	6.79	6.83
t = 24	4.99	6.79	6.83	6.86	6.89
t = 48	2.68	6.84	6.90	7.19	6.89
t = 72	0	7.2	7.56	7.69	7.41
t = 96	0	7.41	7.86	8.34	8.22
t = 120	0	7.45	8.50	8.56	8.67
t = 144	0	7.70	8.79	8.60	8.89
t = 168	0	7.75	8.82	8.92	8.93
t = 192	0	7.52	8.79	8.84	9.10
t = 216	0	7.12	8.80	9.82	9.15
t = 240	0	6.92	8.77	8.92	8.91

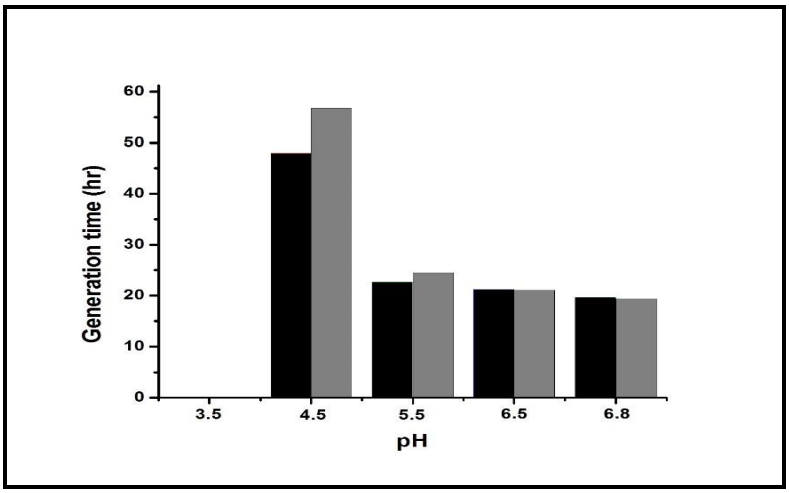
(B) *rv1996*::hyg^R *M. bovis* BCG

pH / Time	Mean log CFU/ml at different pH levels				
	3.5	4.5	5.5	6.5	6.8
t = 0	6.78	6.80	6.82	6.80	6.83
T = 24	3.53	6.78	6.81	6.85	6.81
T = 48	0	6.83	6.86	7.34	6.85
T = 72	0	7.5	7.36	7.58	7.48
t = 96	0	7.35	8.10	7.77	7.99
T = 120	0	7.48	8.57	8.47	8.65
T = 144	0	7.55	8.78	8.80	8.60
T = 168	0	7.60	8.84	8.88	9.08
T = 192	0	7.14	8.87	8.92	9.10
T = 216	0	6.71	8.57	8.79	8.69
T = 240	0	6.24	8.57	8.55	9.64

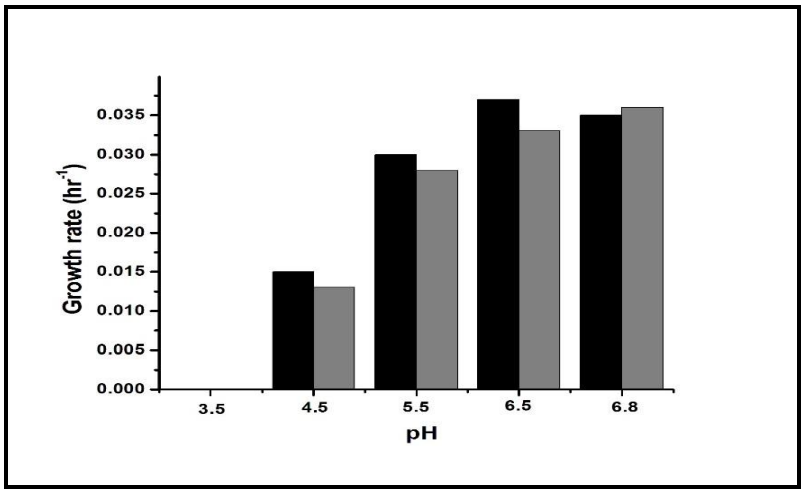
Table 5.2: Survival of *M. bovis* BCG wildtype strain (A) and *M. bovis* BCG *rv1996*::hyg^R mutant (B) over a range of pH.

Values of the CFU/ml for *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R are shown over 240 hours incubation at each pH. Cultures were acidified with hydrochloric acid. The data represent the mean of two independent experiments and each experiment was replicated.

A



B



C

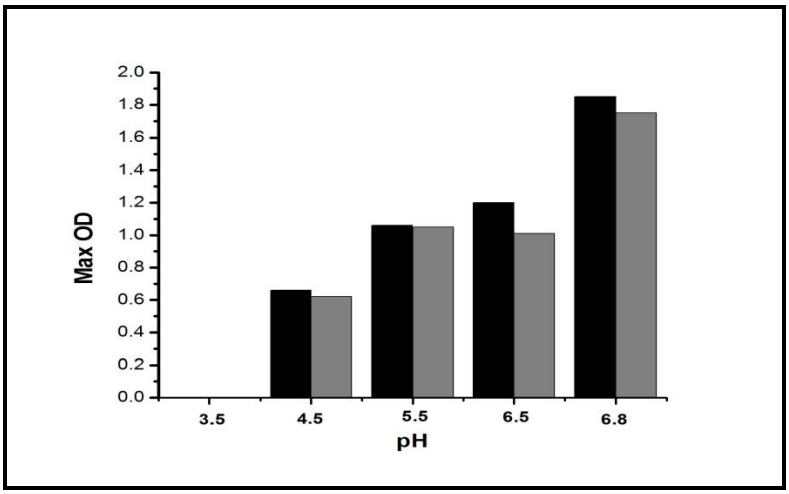


Figure 5.15: Effect of different pH levels on generation time (A), growth rate (B) and max OD (C) of *M. bovis* BCG wildtype strain (black bars) and *M. bovis* BCG *rv1996*::hyg^R mutant strain (grey bars) over 240 hours.

5.2.5. Acid challenge of adapted *M. bovis* BCG and *M. bovis* BCG *rv1996*::hyg^R mutant.

The ability of BCG wildtype strain and BCG *rv1996*::hyg^R mutant strain to resist the lethal pH 3.5 after adaptation at pH 5.5 was investigated.

To assess the effect of adaptation at pH 5.5, aliquots of fresh bacterial suspensions of both strains were transferred into fresh 7H9 broth media supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80, and adjusted to pH 5.5 using hydrochloric acid in order to adapt, as well as into fresh 7H9 at pH 6.8 (non-adapted control, for comparison). All inoculated media were incubated for 72 hr at 37^{o}C as described in Material and Methods (Section 2.4.4). After the adaptation period, the bacterial cells were collected by centrifugation, re-suspended into 7H9 broth adjusted to lethal pH 3.5 for acid challenge and incubated at 37^{o}C for 24 hr as described in Material and Methods (Section 2.4.4.1). Other aliquots of the bacterial suspensions were re-suspended in culture media at pH 4.5 and pH 6.8. The viable count of each culture was done as described in Material and Methods (Section 2.1.11).

The obtained results revealed that no colonies were recovered from adapted BCG and BCG *rv1996*::hyg^R after incubation for 24 hr at challenge pH 3.5 (data not shown). This means that the BCG cells of both strains could not adapt themselves at pH 5.5 for 72 hr. When the adaptation period at pH 5.5 was extended to 96 hr and the exposure of the bacteria to the challenge of pH 3.5 was decreased to 14 hr, no significant difference ($p>0.05$) in viability between adapted BCG and non-adapted BCG cells was recorded. The colony forming units of adapted bacteria decreased dramatically within 14 hr in a similar mode to that of non-adapted cells when compared to controles. Also, there was no significant difference ($p>0.05$) in viability between adapted BCG and non-adapted BCG at pH 4.5 with no loss of viability after 14 hr incubation of cultures (Figure 5.16). Furthermore, with adapted BCG *rv1996*::hyg^R, the result was similar to those of adapted BCG, with no significant difference ($p>0.05$) between adapted and non-adapted after shifting either to pH 3.5 or to 4.5, as it can be seen in Figure 5.16.

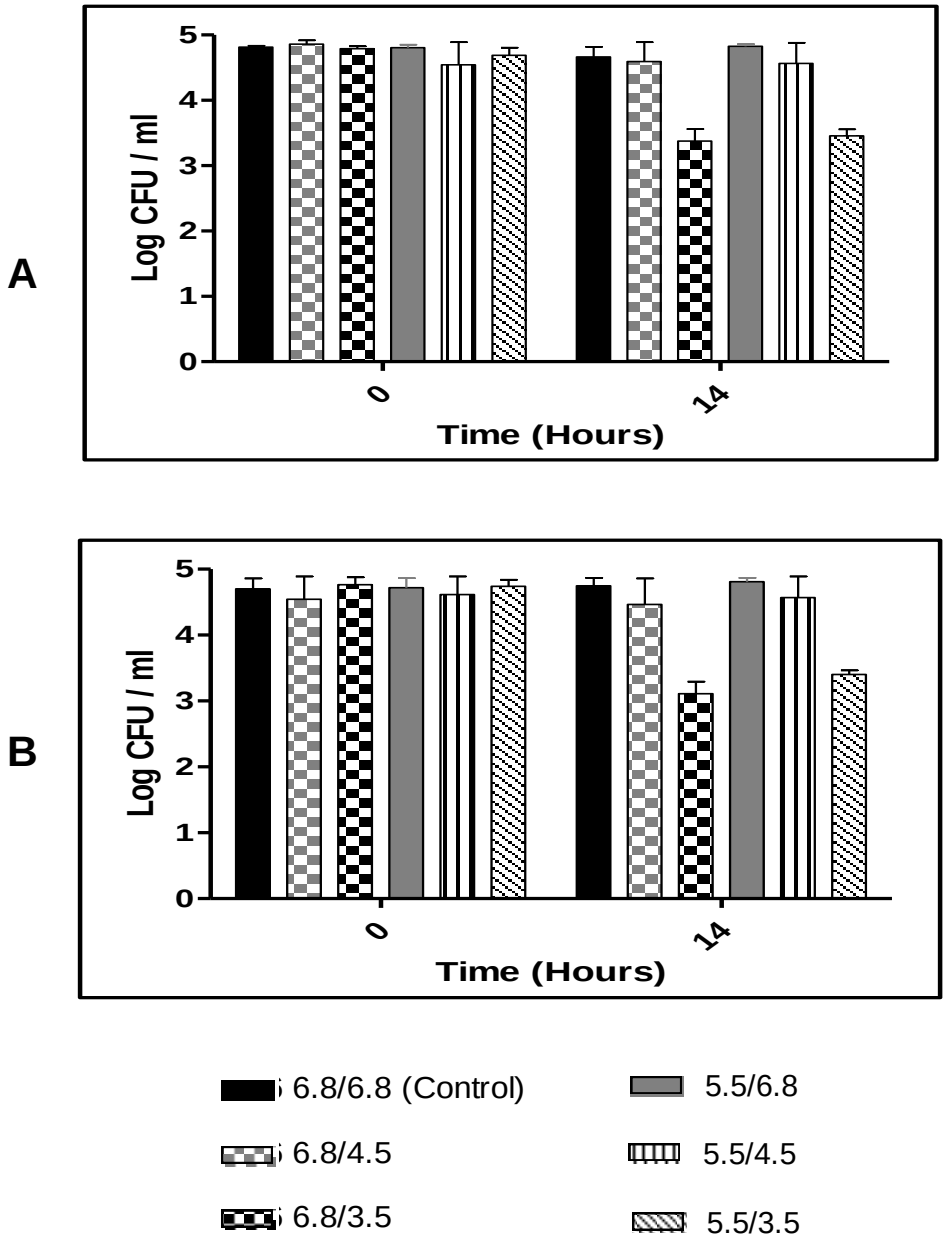


Figure 5.16: Survival of *M. bovis* BCG wildtype strain (A) and *M. bovis* BCG *rv1996*::hyg^R mutant strain (B) at different pH levels after adaptation at pH 5.5.

M. bovis BCG cultures (A) and *M. bovis* BCG *rv1996*::hyg^R cultures (B) were adapted at pH 5.5 or they were grown at 6.8 (non-adapted) for 96 hours before exposing to pH 3.5, 4.5 or 6.8. The chart shows the viability of bacteria after 14 hours incubation. Values obtained represent the mean of 2 replicates of three independent experiments (n=6) at pH 3.5 and pH 6.8, and 2 replicates of two independent experiments (n=4) at pH 4.5. Culture medium was acidified using hydrochloric acid. Error bars represent standard deviations.

5.3. Discussion.

Before beginning the *rv1995* and *rv1996* gene knock out steps in the H37Rv reference strain, two model systems, *M. bovis* BCG and *M. smegmatis* strains, were tested for the presence of both genes using PCR. *M. smegmatis* revealed a negative PCR result that was most probably due to gene absence and this was confirmed by BLAST analysis (Altschul and Gish, 1990) at the time when the work was carried out (2006). For further confirmation, a new BLAST search (Altschul and Gish, 1990) for both *rv1995* and *rv1996* gene sequences was done against *M. smegmatis* (taxid:1772) including *M. smegmatis* strain MC2 155 (taxid:246196) that revealed no significant similarities between the *rv1995* and *rv1996* gene sequences with any *M. smegmatis* strains on Genbank (Benson *et al*., 2012).

To exclude the presence of the *rv1995* and *rv1996* genes with low homology in the *M. smegmatis* genome, another BLAST search using the BLASTX algorithm (Johnson *et al*., 2008) for comparing translated sequences of both genes with proteins of the *M. smegmatis* was done for each gene separately. Regarding the *rv1995* gene, no similarity was found with any *M. smegmatis* strains in the Genbank (Benson *et al*., 2012). However, BLASTX for the *rv1996* gene showed a degree of similarity with some *M. smegmatis* USPs with up to 46% identity.

5.3.1. Conservation of the *rv1995* and *rv1996* genes among mycobacterial species.

To study the conservation of the *rv1995* and *rv1996* genes among different mycobacterial species strains, a recent bioinformatic analysis using BLAST search (Altschul and Gish, 1990) was performed. The sequences of the *rv1995* and *rv1996* genes were blasted individually against *M. tuberculosis* (taxid:1773) and *M. bovis* (taxid:1765) and were found to be conserved amongst almost all available *M. tuberculosis* (more than 100 strains) and *M. bovis* (17 strains) completely sequenced genomes submitted to Genbank (Benson *et al*., 2012). In addition, both gene sequences were found to be conserved in whole genome sequences of *M. africanum*, *M. microti* and *M. canettii* strains

with 100% identity against the original H37Rv strain which indicates the highly-conserved sequence of both genes among the mentioned strains.

Bioinformatic analysis confirmed the conserved nature of the *rv1995* and *rv1996* genes among different *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canettii* strains with 100% identity against the original H37Rv strain. Unfortunately, the CH strain whole genome sequence is not available on Genbank (Benson *et al*., 2012). Furthermore, to my knowledge, there is no report about any other strain in recent whole genome sequence (WGS) databases that show the same pattern of deletions as CH. Although both genes were found to be highly conserved, both of them were considered to be non-essential genes for neither *in vitro* nor *in vivo* growth (Sassetti *et al*. 2003; Griffin *et al*., 2011). This is indicated by the fact that deletion of both genes (*rv1995* and *rv1996*) in addition to other CH gene deletions did not much reduce their ability to grow in culture media (Newton *et al*., 2006) and to cause the outbreak in Leicester (Ewer *et al*., 2003).

5.3.2. Success of homologous recombination after UV pre-treatment.

The process of homologous recombination in mycobacteria is known to be a non-straightforward process (Hinds *et al*., 1999; Parish and Stoker, 2000; Muttucumaru and Parish, 2004; Tufariello *et al*., 2014). The need for its enhancement was reported in many studies (Hinds *et al*., 1999; Parish and Stoker, 2000; Muttucumaru and Parish, 2004; Balhana *et al*., 2010). Hinds *et al*. (1999) used DNA pre-treated with alkali or UV and found that pre-treatment facilitated and greatly enhanced successful homologous recombination in mycobacteria. For this reason, experiments were repeated after pre-treating both plasmids DNA (pANO1 and pANO2) with ultraviolet irradiation (UV). Potential pANO1 and pANO2 recombinants for both strains H37Rv and BCG were obtained successfully at this time.

The improvement in homologous recombination shown may be due to stimulation of RecA or other DNA repair systems (Hinds *et al*., 1999). The key player in

the homologous recombination process is the RecA protein (Clark and Margulies, 1995; Parish and Stocker, 2000; Gupta *et al*., 2013). The RecA is essential for repair of damaged DNA and maintenance of DNA, but also it has a major role in DNA exchange between homologous regions (Clark and Margulies, 1995; Hinds *et al*., 1999; Balhana *et al*., 2010).

5.3.2.1. DCO, SCO and illegitimate recombination events after knocking out of *rv1995* and *rv1996* genes.

Almost all the *rv1995* BCG and the *rv1996* BCG recombinants tested were found to contain the hygromycin resistance gene, however PCR for confirming the integration of the disrupted *rv1995* gene revealed persistence of the intact gene inside the cells in all tested recombinants. The reasoning for absence of PCR amplicon of the desired size in spite of presence of hygromycin gene cassette could be either due to a single cross over (SCO) event and the smaller amplicon of the original size was favoured in the PCR or due to illegitimate (non-homologous) recombination by which bacteria acquired the hygromycin gene cassette but in an incorrect location, during the experiment. Similar findings reported by Parish *et al*. (1999) who electroporated suicide vectors into *M. tuberculosis*, each carrying one of nine amino acid biosynthesis genes. They confirmed by southern blotting that most recombinants analysed were spontaneous hyg^R mutants, while DCO was scarcely achieved and the remaining were SCOs without even a single case of illegitimate recombination. This supported that the development of DCO event is a difficult one with a low probability in slowly growing mycobacteria such as *M. tuberculosis* and *M. bovis* BCG. However, against their findings, in the present study only two cases which were negative for hyg^R gene by PCR can be assumed to be spontaneous mutants. The detailed data of southern blotting in Parish *et al*. (1999) were not shown. However, Hinds *et al*. (1999) reported among many trials for enhancement of delivery of suicide vectors into mycobacteria that illegitimate recombination was a common troublesome event in addition to SCO.

To investigate for SCO event in the *rv1995* gene recombinants, a further two PCR reactions using primers that flank one of the fragment in addition to hyg^R gene were

performed separately for each recombinant. Results revealed absence of SCO events in any of the recombinants obtained. The existence of hygromycin resistance gene in absence of either a DCO or SCO event was possibly attributed to illegitimate recombination (IR) which is a process by which two non-homologous DNA molecules are joined (Hinds *et al*., 1999; Muttucumaru and Parish, 2004; Khattak *et al*., 2013). Illegitimate recombination has been reported to occur with a high frequency in slowly growing mycobacteria such as *M. tuberculosis* (Kablana *et al*., 1991; Tufariello *et al*., 2014). A high level of illegitimate recombination might suppress the RecA activity reducing the possibility of homologous recombination (Hinds *et al*., 1999; Parish and Stoker, 2000; Muttucumaru and Parish, 2004; Gupta *et al*., 2013). In spite of its natural deletion in the case of the CH strain and many other clinical isolates, another explanation of the *rv1995* gene knockout failure is the possibility that the *rv1995* mutation is lethal or too detrimental to find when constructed in this particular background.

Unlike the results of the *rv1995* gene knockout, the *rv1996* gene knockout was successful. Fortunately, two recombinants were identified as containing the *rv1996* disrupted gene by the hygromycin resistance cassette. The success of *rv1996* knock out and failure in the case of *rv1995* could be attributed to the fact that different genes behave differently and some genes are knocked out easily and others are refractory (Hinds *et al*., 1999).

5.3.3. *In vitro* investigations; points for discussion.

It was reported in some studies that exposing some bacteria to a mild pH level (pH 5.5 to 6.0) may induce an acid tolerance response mechanism (ATR) which ultimately protects the cell from more severe acid stress (Foster and Hall, 1990; O'Brien *et al*., 1996; Tosun and Gonul, 2005). The acid adaptation period of bacterial cells required protein synthesis. These proteins are suggested to prevent and/or repair damaged molecules caused by acid shock of the cell and involved in pH regulation, inducing bacteria to resist an acid environment and to be able to survive even at low pH level. Hence, it is necessary to differentiate between acid resistance and acid tolerance. Acid resistance is defined as the ability of a microorganism to withstand an acid challenge, whereas acid tolerance is a

phenomenon by which a microorganism shows an increase in resistance to acid environmental stress after exposure to mild acid stress (Foster, 1993; O'Brien *et al*., 1996; Foster, 2000; Foster and Hall, 1991; Tosun and Gonul, 2005; Muller *et al*., 2009).

O'Brien *et al*. (1996) reported that *M. smegmatis* has the ability to create an acid tolerance system (O'Brien *et al*., 1996). However, it is unknown whether *M. tuberculosis* and *M. bovis* BCG strains have the ability to develop an acid tolerance system or not as it is observed in *M. smegmatis* (Safiola *et al*., 2012). In the current study, an attempt was made to investigate whether *M. bovis* BCG (wildtype) and *M. bovis* BCG *rv1996*::hyg^{R} have the ability to generate an acid tolerance system by exposing them to acidic external pH medium with hydrochloric acid (HCl).

5.3.3.1. Exposure to adaptive pH could not increase the resistance of BCG strains to acid stress.

Before starting the experiments associated with the adaptation the lethal and adaptive pHs have been determined to be 3.5 pH and at pH 5.5 respectively. No difference was found between the wildtype and mutant BCG strains regarding lethal and adaptive pHs, however, it was observed that they were relatively different from those of other mycobacterial and bacterial species reported by several investigators (O'Brien *et al*., 1996; Foster and Hall, 1990; Gorden and Small, 1993; Small *et al*., 1994; Tosun and Aktuggonul, 2005). The variations in the adaptive and lethal pH values may reflect the environments these bacteria normally inhabit. For example, *S. enterica* sv Typhimurium, *E. coli* or *Sh. flexneri* can withstand the acidic nature of the stomach as enteric organisms (Foster and Hall, 1990; Foster, 1995; Richard and Foster, 2004; Muller *et al*., 2009). Also, the differences in the experimental strategies may contribute. For example, the method of acidification or other variables that are used to acidify the medium may have an effect (O'Brien *et al*., 1996; Deng *et al*., 1999).

It was expected that the disruption of the *rv1996* gene would lead the non-adapted mutant to be less tolerant to acid challenge than adapted wildtype. However, both wildtype and mutant strains were unable to adapt themselves remarkably when grown at pH 5.5 for

72 hr since wildtype cells of BCG showed a decreased survival similar to that of mutant after exposing to acid challenge at pH 3.5. Furthermore, expanding the adaptation period at 5.5 to 96 hr did not induce an acid tolerance response in either strains. These results demonstrated that the *rv1996* gene might be not involved in the acid tolerance response of *M. bovis* BCG on using HCl as acidulant in the medium, although there was a minor difference in the viability between adapted and non-adapted *M. bovis* BCG strains at pH 3.5, but this was not significant.

The failure of adaptation to confer protection to *M. bovis* BCG against acid challenge may be attributed to a variety of reasons. For example, the level of adaptive pH used (5.5) possibly was not sufficiently low to stimulate the synthesis of acid shock proteins (ASPs) (Foster and Hall, 1990; O'Brien *et al*., 1996; Tosun and Aktuggonul, 2005). Tosun and Aktuggonul, (2005) reported that exposure of *E. coli* O157: H7 cells to acid at pH 4.5 for 2 hours for acid adaptation resulted in the maximum increase in acid tolerance following acid challenge at pH 3.0, better than when the cells were exposed to acid at pH 5.0 or 5.5 for acid adaptation (Tosun and Aktuggonul, 2005). Also, acidulant material, HCl was used in the current study. O'Brien *et al*. (1996) demonstrated that adapted *M. smegmatis* cells with H_3PO_4 at pH 5.0 conferred a better protection than those adapted with HCl at the same pH when exposed to a lethal pH of 3.0 (O'Brien *et al*., 1996). Furthermore, the composition of culture medium may have a remarkable impact on bacteria to acid resistance. For example, Sung and Collins, (2003) found that the *M. paratuberculosis* cells growing in glycerol-containing medium were more resistant to low pH than others grown with no glycerol.

5.3.3.1.1. Future directions for investigating acid tolerance of BCG.

The actual ability of *M. tuberculosis* and *M. bovis* BCG to adapt itself to lethal pH is yet to be further evaluated (Saviola, 2012). In the current study, an attempt to evaluate adaptation ability of BCG wildtype and mutant strains was performed. However, both wildtype and mutant strains failed to adapt under the test conditions. Therefore, the used test conditions need to be modified. In particular, the adaptive pH level sufficient to induce tolerance to lethal pH should be accurately determined. For this purpose, two groups of

BCG bacterial cells can be investigated during the acid tolerance response stage, first group is adapted to acid stress and the other one is non-adapted (mutant and wildtype cells). The conditions of adaptation should be modified such as modification of pH ranges to be 4, 4.5, 5 and 5.5 instead of 4.5, 5.5 and 6.5. The time of exposure to lethal pH could also be reduced to 0, 2, 4, 6, 8 and 10 hours instead of 14 and 24 hours done in the current study. The relative longer time might have led the adapted cells to be killed, since, loss of viability does not require long incubation time unlike the time required to develop tolerance by exposure to adaptive pH. After exposure of mycobacterial cells to the predetermined adaptive pH, both adapted and non-adapted groups would be challenged with lethal pH. Other samples of adapted and non-adapted cells would be investigated for the *rv1996* gene expression using RT-qPCR or RNA-sequencing to compare its expressions under both conditions.

CHAPTER 6:

GENERAL DISCUSSION

6.1. Rationale of the present study.

Tuberculosis remains a major challenge to medicine and public health. In 2015, the WHO estimated that the number of new cases increased to reach 10.4 million new (incident) TB cases worldwide (WHO Global Report, 2016). Although the tuberculosis outbreaks are most commonly encountered in developing countries, the evolution of new strains of *M. tuberculosis*, that have the ability to skew the immune defence mechanisms, makes it possible to have tuberculosis outbreaks even in developed countries (Newton *et al*, 2006; Reed *et al*, 2007; Sarkar *et al*, 2012). This clearly demonstrates the need to continue research on tuberculosis for a better understanding of the nature of infection, the virulence factors of the causal pathogens and their mode of action, the actual relationship between the causal pathogen and the human immune system and to find out effective protocols for disease protection and treatment.

6.1.1. Outbreak of tuberculosis in Leicester city, 2001 had unusual clinical features.

The tuberculosis outbreak caused by *M. tuberculosis* CH reported in Leicester city in 2001 was described as the largest tuberculosis outbreak ever in the United Kingdom and since that time no outbreak has been reported in UK with a similar pattern of spread and unusual clinical features (Section 1.9). The large number of active disease cases (77) represented a percentage of 23.3% of the total outbreak identified cases (Ewer *et al*., 2003; Rajakumar *et al*., 2004). This proportion appeared to be higher than that usually observed, which is 5-10% (Newton *et al*., 2006). In animal studies, the CH strain was highly aggressive in immunodeficient (SCID) mice which were killed almost twice as rapidly when compared to another group infected with H37Rv (UK Research and Innovation Project, G0300403).

6.1.2. The unique genomic markers of *M. tuberculosis* CH and the role of *rv1995/rv1996* locus deletion as a phylogenetic marker for the strain.

On the genomic level, the CH strain had characteristic large sequence polymorphisms which defined it as a unique strain among the East African-Indian lineage 3 (Section 1.9.1) (Rajakumar *et al*., 2004; Newton *et al*., 2006; Gagneux and Small, 2007). Five gene deletions dispersed on the CH strain chromosome were identified and confirmed. They were two singles (*rv0180, rv1519)* and three adjacent pairs (*rv1995-rv1996, rv3516-rv3517* and *rv3738-rv3739*). These deletions were unique genomic markers to the outbreak associated CH strain and presented one of the most important natural events of gene deletions (Rajakumar *et al*., 2004). It was suggested that one or more of these deletions increased the virulence of *M. tuberculosis* CH strain in comparison with *M. tuberculosis* H37Rv, the reference strain (Rajakumar *et al*., 2004; Newton *et al*., 2006).

In the study done by Rajakumar and his colleagues (2004) (Section 1.9.1.1), they observed that most of the investigated non-outbreak isolates were found to have 1 or more of the five deletions except for the deletion of the *rv1995/rv1996* locus which was exclusively found in the outbreak CH isolates; a clue to its recent loss relative to other loci deleted from the CH strain (Rajakumar *et al*., 2004). A study done by Menendez *et al*. (2007) involved non-CH clinical strains isolated from the South Asian population in the UK. They found that three of the deleted regions in these strains were identical to some CH strain deleted genes, namely *rv1519*, *rv3516-rv3517* and *rv3738c-rv3739c*. A noteworthy observation was that also the *rv1995/rv1996* locus was not deleted from these isolates, unlike the CH strain (Menendez *et al*., 2007). The previous findings indicated the importance of the *rv1995/rv1996* locus deletion (LSP) in the genomic characterisation of the CH strain that can be used as a phylogenetic marker for the strain and its progeny. The relative recent loss of these genes raises the possibility of being involved somehow in the clinical and epidemiological pattern of the outbreak.

The isolated deletion of the *rv1995/rv1996* locus was also reported in other non-CH clinical strains (Tsolaki *et al*., 2004; Isaza *et al*, 2012). A further up to date *in silico*

analysis was performed to investigate the existence of the *rv1995* and *rv1996* deletion in other mycobacterial strains in the Genbank database (Benson *et al*., 2012). The *M. tuberculosis* strains with complete genome sequences on Genbank were searched and 5905 strains with more than 4 million bp sequence length were found. To check for the incidence of *rv1995* and *rv1996* deletions among the available strains, the first 200 strains were chosen to be blasted against. The *rv1995* and *rv1996* sequences were individually blasted (Altschul and Gish, 1990) against these 200 strains using their accession numbers. Among the 200 strains, only three strains were found to lack both genes (NZ_AP017901.1, NZ_CP018305.1, NZ_CP018302.1). None of the two genes was deleted alone. Interestingly, *rv1997* was partially deleted with only 80% identity in these strains. The combined deletion of the *rv1995* and *rv1996* genes in these strains with partial deletion of *rv1997* indicates a potential cofunction for the three genes (Section 6.4.2).

In conclusion, although gene deletion is usually associated with decreased virulence or even loss of virulence of mycobacteria, as in the case of RD1 in BCG, this is not always the case. In many cases, loss of genes was found to be associated with increasing pathogenicity (Section 1.8.3).

In the present study, the hypothesis was to investigate whether the loss of the two recently deleted genes, namely *rv1995* and *rv1996*, could have a role in the infection pattern caused by the outbreak strain, *M. tuberculosis* CH.

Two molecular approaches were involved in the present study; knock in and knock out, to investigate the role of the *rv1995* and *rv1996* genes in the phenotype and hypervirulence of *M. tuberculosis* CH strain. The knock in strategy is used to investigate the role of a particular gene(s) through construction of recombinant strains expressing the target gene/s (Pym *et al*., 2003; Newton *et al*., 2006; Zhang *et al*., 2010; Li *et al*., 2014a). The other approach for discovering the function of genes and to understand the basis of pathogenicity and virulence, is the knock out of a particular gene by homologous recombination using suicide vectors (Pashley, 2003; Parish and Stoker, 2000; Drumm *et al*., 2009). The knock in approach would not regain the lost function(s) of downstream gene(s). Therefore, in the case of CH, using an expression vector has the advantage of

restoring the function of the target gene only without regaining function of downstream genes under its polar effect, thus it gives a better knowledge about the exact function of studied gene, whereas individual knock out of each gene would disrupt the gene of interest and may affect also the downstream genes via its polar effect, hence it is a better strategy to simulate the situation of gene deletion in mycobacteria.

The knock in experiments performed in the current study was a part of a wider project involved research teams in Imperial College and Leicester University (UK Research and Innovation Project, G0300403). The proposal was essentially to reintroduce the deleted genes into CH through knock in experiments followed by testing their phenotype characteristics.

Expression vectors; pAAO1 and pAAO3 were constructed harbouring the *rv1995* and *rv1996* genes respectively. After introducing these vectors into *M. tuberculosis* CH and confirming expression of the genes, the recombinant strains for the *rv1995* and *rv1996* genes were named CH::hyg^{R}*1995* and CH::hyg^{R}*1996* respectively (Newton *et al*., 2006).

The suicide vectors pANO1 and pANO2, were developed and used as delivery vectors for the disrupted *rv1995* and *rv1996* genes into both *M. bovis* BCG and *M. tuberculosis* H37Rv. The recombination event in *M. bovis* BCG strain via homologous recombination was successful in the case of *rv1996* and unsuccessful in the case of *rv1995* as discussed previously (Section 5.3.2). Before phenotypic experiments the absence of the expression of the disrupted *rv1996* gene from the BCG recombinants was confirmed by reverse transcriptase PCR (RT-PCR) and Real time PCR (qRT-PCR) techniques. The knocked out BCG mutant was named *M. bovis* BCG *rv1996*::hyg^{R}.

6.2. *In vitro* phenotypic characterisation of knocked in CH and knocked out BCG strains.

The antimicrobial products of macrophages, such as hydrogen peroxide (H_2O_2), nitric oxide (NO), and acidification (low pH) of the phagolysosome are known to be host defence factors against intracellular pathogens and the ability to resist one or all, is a virulence attribute. Nitric oxide and hydrogen peroxide play crucial roles in controlling growth and survival of *M. tuberculosis* in macrophages. The differences in virulence among strains may relate to their ability to detoxify and resist ROI and RNI. These phenotypic characters associated with virulence could be investigated via *in vitro* experiments (O'Brien *et al*., 1994; Rhee *et al*., 2005; MacMicking *et al*., 1997; Firmani and Riley, 2002a; Newton *et al*., 2006; Subbian *et al*., 2007). Hence, investigations to compare the growth rate, and resistance to reactive nitrogen intermediates (RNI) represented with $NaNO_2$, reactive oxygen intermediates (ROI) represented with H_2O_2, and acid stress between strains were performed.

Although the CH strain, on the microbiological level, seems to be phenotypically attenuated regarding slow growth, less resistance to low pH and hydrogen peroxide in comparison to H37Rv and CDC1551, however on the clinical (Ewer *et al*., 2003) and cellular (Newton *et al*., 2006) level, this was not the case. Investigating the possible function of each deleted gene was expected to give reasons for such phenotypic findings. Particularly the *rv1996* gene was expected to have a role in the microbiological attenuation of the CH strain since it encodes for a conserved hypothetical universal stress protein that is induced by *dosR* regulator under the stress of nitric oxide, carbon monoxide and hypoxia (Voskuil *et al*., 2004; Park *et al*., 2003; Boon and Dick, 2012).

In the case of a knock in approach, after confirming the expression of both *rv1995* and *rv1996* genes carried by pAAO1 and pAAO3 vectors in strain CH, the complemented strains (CH::hyg^R*1995* and CH::hyg^R*1996*), were compared to the parental CH strain. The reintroduction of the *rv1995* and *rv1996* genes carried by pAAO1 and pAAO3 in CH, performed by the Imperial College, indicated that the recombinant CH strain either with

the *rv1995* or *rv1996* genes remained indistinguishable from the parental CH regarding growth rate, effect of $NaNO_2$ and H_2O_2 (Data obtained from Imperial College team, 2006) as well as cytokine secretion pattern (Newton *et al*., 2006). However, the CH::hyg^R*1996* recombinant strain was found to be more resistant to pH 5.5 than parental CH strain (Data obtained from Imperial College team, 2006).

In the case of the knock out approach performed in the present study, almost the same conclusion was obtained. Both wildtype BCG and mutant BCG, *rv1996*::hyg^R strains displayed similar growth rates under standard conditions, similar levels of resistance to various concentrations of $NaNO_2$ and sensitivity to H_2O_2, however the mutant BCG *rv1996*::hyg^R strain revealed less resistance than wildtype BCG at pH 3.5.

The CH::hyg^R*1995* complementation was not found to have any change in relation to the parenteral CH strain. Some genes of unknown function, when lost, give an advantage for the strain and may play a role in the ability of strain to become virulent (Alland *et al*., 2007). Further discussion for this point in section 6.4.

6.2.1. The *rv1996* gene showed no role in growth rate under standard conditions.

In both knock in and knock out experiments, growth rate under standard conditions was not significantly changed on reintroduction of the *rv1996* genes to CH, or on disruption of the *rv1996* gene from *M. bovis* BCG.

These results were consistent with many reports of other USP genes deletion such as the results obtained by Hu *et al*. (2006) that showed no significant difference in growth rates between *M. tuberculosis* H37Rv wildtype and Δ*hspX* mutant grown in 7H9 broth. Similarly, Parish *et al*. (2003) found that there was no significant difference between the H37Rv wildtype and the Δ*dosR* mutant after growing both strains in 7H9 broth. A relatively recent study performed by Hingley-Wilson *et al*. (2010) reported that individual deletion of four USP genes was not associated with any phenotypic change in the mutated strains including the growth rate. Interestingly, the *rv1996* gene was one of the genes investigated.

The redundant nature of the USP genes in which gene/s compensate/s for loss of function of others could be a reason for the similarity in growth rates between the wildtype and mutant strains.

6.2.2. Knocking in and out of the *rv1996* gene did not affect mycobacterial resistance to ROI and RNI.

Differences in virulence between strains of *M. tuberculosis* may relate to their capacity to detoxify and resist ROI and RNI through which host defence against MTB is essentially mediated (Wilkinson *et al*., 2005; Ng *et al*., 2004). In addition, NO was found to upregulate expression of many USP genes including the *rv1996* gene in the study of Ohno *et al*. (2003). Thus, it was expected that deletion of the *rv1996* gene would affect the resistance of mycobacteria to RNI stress. However, this was not the case. The deletion of *rv1996* from the CH strain did not affect the resistance of CH to NO in comparison to H37Rv. It was also observed that the complemented strain, CH::hyg^R*1996* showed a similar level of NO resistance compared with the parental CH strain (Newton *et al*., 2006). Similarly, disruption of the *rv1996* gene in BCG *rv1996*::hyg^R displayed no change in resistance to various concentrations of $NaNO_2$.

Regarding ROI, it was reported that H_2O_2 exposure stimulated a similar transcriptional response as Nitric oxide. However, unlike Nitric oxide, H_2O_2 exposure did not induce dormancy-regulated genes including the *rv1996* gene (Voskuil *et al*., 2011). Thus, the decreased resistance of CH to H_2O_2 in comparison to H37Rv was mostly not attributed to the deletion of *rv1996*. This was confirmed by similar level of resistance shown by CH::hyg^R*1996* compared with the parental CH strain and similar sensitivity of BCG *rv1996*::hyg^R to H_2O_2 compared to wildtype BCG.

The findings provided by Imperial College team, (2006) regarding ROI and RNI resistance of CH::hyg^R*1995* and CH::hyg^R*1996* and the findings of the current study were consistent with what was observed in the work done by Hingley-Wilson *et al*. (2010) that knock out of the *rv1996* gene was not associated with any effect on resistance to H_2O_2 or

NO stress *in vitro* (Hingley-Wilson *et al*., 2010). Hu *et al*. (2006) found that there were no significant differences in survival between the wildtype and the *hspX* mutant upon treatment with NO, H_2O_2, or low acid (Hu *et al*., 2006). The *hspX* gene belongs to the stress gene family that is upregulated by *dosR* as is *rv1996* (Ohno *et al*., 2003; Drumm *et al*., 2009). Drumm and his colleagues (2009) found that the *rv2623* gene deficient mutant, which is another USP gene regulated by *dosR*, was comparable to the wildtype strain growth rate under different stresses *in vitro* including oxidative stress, acid stress, heat shock, antibiotic and DNA damage stresses.

6.2.2.1. Variation between different studies in mycobacterial resistance to ROI and RNI.

Although, the disruption of the *rv1996* gene showed no effect on BCG cells in the presence of $NaNO_2$ and/or H_2O_2, there were variations in the sensitivity between BCG strain used in the current study, and BCG and other mycobacterial strains investigated in previous studies. The obtained result of the current study indicated that the decrease in the viability of BCG cells after incubation for 48 hr in the presence of $NaNO_2$ at 1 to 32 mM was a dose dependent, compared to unexposed cells (control). At low concentrations of $NaNO_2$ (1, 2 and 4 mM), both strains were resistant after 24 hr of incubation. This result is almost consistent with those of Firmani and Rily (2002a) who reported that *M. bovis* BCG and other mycobacterial strains including *M. tuberculosis* H37Rv were resistant to 3 mM $NaNO_2$ for 16 hr. In addition, Rhoades and Orme (1997) demonstrated that mycobacterial strains could resist less than 5 mM $NaNO_2$ in acidified medium for 24 hr incubation. Furthermore, on exposing the bacteria to $NaNO_2$ above 10 mM in the present study, they lost their viability after 24 hr. Almost the same finding was obtained by Rhoades and Orme (1997) when exposing mycobacteria to 10 mM $NaNO_2$ concentration. However, Firmani and Rily (2002a) found that BCG cells decreased dramatically in their viability within 16 hr when grown on 7H9 medium at 8 mM $NaNO_2$ concentration.

Regarding the response to hydrogen peroxide (H_2O_2), results obtained revealed that both BCG and BCG *rv1996*::hyg^R strains could not resist H_2O_2 at all concentrations used

except at 1 mM for only 90 min. These results were inconsistent with those of Firamni and Rily (2002a) and Rhoades and Orme (1997) who found that *M. bovis* BCG, and *M. tuberculosis* H37Rv and other clinical strains were resistant to 2 mM H_2O_2 after 16 hr of incubation. Newton *et al*. (2006) investigated *M. tuberculosis* H37Rv and CH strain to H_2O_2 at 1, 2, 4 or 8 mM for 90 min. They reported resistance of both strains in a dose dependent manner but the CH strain was less resistant than H37Rv. The variation in the studies could be attributed to technical differences such as starting concentration of the organism or difference in the chemicals and media composition or volume.

6.2.3. The *rv1996* gene and possible role in resistance to acid stress.

The finding consistent with the annotation of the *rv1996* gene as a universal stress protein encoding gene was its ability to increase the recombinant strain (CH::hyg^R*1996*) resistance to low pH compared to the parental CH strain at pH 5.5 (Data obtained from Imperial College team, 2006), whereas its disruption made the mutant BCG *rv1996*::hyg^R strain less resistant than wildtype BCG at pH 3.5. There was a significant difference in the viable counts of *M. bovis* BCG *rv1996*::hyg^R (mutant) compared with the *M. bovis* BCG (wildtype), where the viable count of the mutant reduced dramatically within 24 hours whereas the wildtype resist for up to 48 hours post exposure to 3.5 pH. However, in the knock out study of Hingley-Wilson *et al*. (2010), they reported that individual knockout of *rv1996,* has no major *in vitro* effect on the growth of the mutated strain under acid stress at pH 4 and pH 5. The technical differences of acid stress experiments may be a reason for the discrepancy between the results.

A microarray analysis was performed on *M. tuberculosis* transcriptome after exposure to acid stress. Interestingly, among 23 ORFs found to be significantly induced, none of the USP genes, including the *rv1996* gene, were upregulated (Fisher *et al*., 2002). This discrepancy could be interpreted by the limited time of exposure to acid stress in Fisher and his colleagues' work which was only 15 and 30-minute at pH of 5.5 exposure, unlike the 4-day exposure performed by Newton *et al*. (2006) after which CFU were counted.

Another study performed by microarray analysis to investigate gene expression generated by assaying the MTB transcriptome at 20 min intervals up to 2 hr post infection and compared *in vivo* and *in vitro* acid-responsive genes (Rohde *et al*., 2007). Again, the *rv1996* gene was not reported to be upregulated in the work done by Rohde *et al*. (2007). However, they observed limited overlap with previous microarray results performed by Fisher *et al*,. 2003 regarding the pH-regulated genes. They attributed this difference to variation in MTB strains, different growth conditions, and duration of acid stress (Rohde *et al*., 2007). Also, Kim *et al*. (2008) identified a different set of genes which are expressed under mild acidic conditions in *M. tuberculosis* H37Rv. The *rv1996* gene was not among them.

To my knowledge, no study comprehensively reported all the genes which could be affected by acid stress. Thus, absence of upregulation of the *rv1996* gene in these studies does not mean that it lacks a role in acid stress resistance. The current work could be the first to implicate the *rv1996* gene in a role in resistance to acid stress which is supported by regaining of low pH resistance of the CH strain after its complementation with the *rv1996* gene using pAAO3 expression vector. These findings might justify the low acid resistance of the CH strain in comparison to H37Rv and CDC1551 strains reported by Newton *et al*. (2006).

Overall, the observations obtained in the current study suggest that the *rv1996* gene individually does not have a significant effect on viability of mycobacterial cells in the presence of H_2O_2 and $NaNO_2$ *in vitro,* although *rv1996* was described as one of the genes upregulated on exposure to RNI (Ohno *et al*., 2003). However, the *rv1996* gene was not the only one of the stress genes induced under this stress (Ohno *et al*., 2003). Therefore, absence of observable phenotypic changes on disruption of the gene from BCG or its deletion as in CH, may be interpreted by compensation for its absence by other genes. Nevertheless, it is important to point out that the loss of effect of a protein encoded by a particular gene in a specific condition does not necessary exclude its importance under this condition or stress (Boon and Dick, 2002; Hu *et al*., 2006; Hingley-Wilson *et al*., 2010).

It has also to be mentioned that although phenotypic virulence traits could be investigated under adjusted conditions *in vitro* (Rhee *et al*., 2005; O'Brien *et al*., 1994;

Firmani *et al*., 2002a), but many studies reported that some genes such as genes encoding USPs were non-essential for growth under different stresses in *in vitro* experiments, however their loss caused remarkable changes when tested *in vivo* (Hu *et al*., 2006; Hingley-Wilson *et al*., 2010; Parish *et al*, 2003; Drumm *et al*., 2009). Hence, another interpretation for absence of phenotypic changes after the *rv1996* gene knocking in or knocking out is that USP genes might work essentially *in vivo* rather than *in vitro*.

The failure of the CH::hyg^R*1996* to reacquire completely the phenotype of H37Rv may be attributed to another factor which is the potential functional loss of the *rv1997* gene which was not a target to be investigated in the present study. A worthy report by Isaza *et al*. (2012) indicated that *rv1996* and *rv1997* are working as an operon with a common promoter upstream *rv1996*. Deletion of the *rv1996* gene in the case of CH was associated with deletion of its promoter which left *rv1997* as a pseudogene. The *rv1997* gene (*ctpF*) is also one of the *dosR* regulated genes which encodes a putative metal cation transporter of the plasma membrane (Ohno *et al*., 2003; Pulido *et al*., 2014). Further work should be done to confirm this finding about the *rv1997* gene such as measuring mRNA of *rv1997* in CH and reintroduction of the whole operon into the CH strain.

6.2.4. Cytokine pattern of CH was not changed on *rv1996* complementation.

It was expected that deletion of *rv1996* may reduce the capacity of CH to survive in activated macrophages, since it was previously reported that *rv1996* is moderately upregulated in activated, but not resting, murine bone marrow derived macrophages (Schnappinger *et al*., 2003). However, CH was almost equal in its ability to grow in monocytes and monocyte-derived macrophages (MDM) compared to H37Rv (Newton *et al*., 2006). This was attributed to the ability of CH to induce more anti-inflammatory cytokines IL-10 and IL-6 (Newton *et al*., 2006). Individual reintroduction of the *rv1996* gene revealed no observable change on the cytokine secretions pattern of complemented CH strain (Newton *et al*., 2006). Similarly, Hingley-Wilson *et al*. (2010) found that knock

out of the *rv1996* gene was not responsible for any change on growth or survival of the TB bacillus in pre-activated human monocyte derived cell line THP-1.

6.3. Deletion of the *rv1996* gene as a dormancy regulated USP gene and its potential role in the hypervirulence phenotype of the CH outbreak strain.

The capacity of *M. tuberculosis* to go into dormancy is a very important aspect in its pathogenesis. The *M. tuberculosis* dormancy response features a dramatic decrease in metabolic activity, resulting in a rapid decrease in bacterial replication. The mechanisms and pathways by which the microbe initiates and maintains dormancy is not yet well defined (Hingley-Wilson *et al*., 2003; Boon and Dick, 2012). The mycobacterial dormancy survival regulator (*dosR*) is a key for adaptation of the *M. tuberculosis* under hypoxic conditions which regulates the genes referred to as the dormancy regulon. *rv3133* (*dosR*), is a gene known to be required in long-term hypoxic survival of *M. tuberculosis* under the effect of nitric oxide and hypoxia challenge; conditions thought to promote latency (Voskuil *et al*., 2004; Park *et al*., 2003; Via *et al*., 2008; Ohno *et al*., 2003; Magombdze *et al*., 2013).

6.3.1. Mycobacterial USP domain based structure and potential function.

Among the eight genes encoding USP proteins in *M. tuberculosis* H37Rv (O'Toole and Williams, 2003), six are under *dosR* regulation including the *rv1996* gene (Magombedze *et al*., 2013; Hingley-Wilson *et al*., 2010).

Based on their USP domain organisation, mycobacterial USPs are divided into 3 classes. One USP encoded by the *rv1636* gene is included in the first class and possesses a single conserved USP domain. The second class includes 3 USPs encoded by the *rv3134c*, *rv2028c* and *rv2624c* genes. This class contains a single USP domain, in addition to a

second C-terminal domain. The third class possesses two USP domains and encompasses four USPs encoded by the following genes; *rv1996*, *rv2005c*, *rv2026c* and *rv2623c*. All the *M. tuberculosis* USPs possess a conserved (G-2X-G-9X-G-S/T) ATP binding motif in each of their respective USP domains which is present in the *Methanococcus jannaschii* (MJ0577) protein. The corresponding region of the *uspA* gene of *H. influenzae* (identical in *E. coli*) is lacking an ATP binding motif (O'Toole and Williams, 2003; Florczyk *et al*., 2001; Hingley-Wilson *et al*., 2010; Banerjee *et al*., 2015).

The effect of USP genes has been reported in several studies; for example, in *E. coli*, it has been shown that expression of *uspA* is enhanced as a response to exposure to stress agents that arrest cell growth including H_2O_2. In contrast, *uspA* mutants were found to have impaired ability to survive growth arresting conditions (Nystrom and Neidhardt, 1994; Nachin *et al*., 2005). Also, the universal stress protein of *E. coli* has been shown to be required for resistance to DNA-damaging agents, such as UV irradiation (Diez *et al*., 2000; Gustavsson *et al*., 2002).

In Mycobacteria, USPs play an important role during infection in intracellular survival and adaptation of mycobacteria. The upregulated expression of the USPs when the availability of oxygen decreases as the pulmonary lesion calcifies, may enable *M. tuberculosis* to survive within the granuloma with prolonged growth arrest and against different stress factors encountered during infection of macrophages (Wayne and Sohaskey, 2001; O'Toole and Williams, 2003; Boon and Dick, 2012; Jia *et al*., 2016).

6.3.2. *dosR* regulated gene deletion and role in hypervirulence phenotype.

Previous studies reported that lack of one or more members of the dormancy regulon (*dosR*) might result in failure of the bacillus to initiate and establish dormancy inside the host, leading to uncontrolled growth and hence, a hypervirulence phenotype (Drumm *et al*., 2009; Parish *et al*., 2003; Hu *et al*., 2006).

In a study performed by Drumm *et al*. (2009), they reported that an *rv2623*-deficient mutant failed to establish chronic tuberculous infection in guinea pigs and mice, exhibiting

a hypervirulence phenotype associated with increased bacterial burden and mortality. Interestingly, they found that a *rv2623*-deficient mutant was comparable to wildtype strain growth rate under different stresses *in vitro*. This was similar to the *in vitro* phenotypic results obtained by knocking out of the *rv1996* gene which was found to be indistinguishable from the wildtype BCG strain in the current study except for a small difference in acid resistance.

Another *dosR* regulated gene, namely *hspX* (*acr*, *rv2031c*), when mutated was reported to promote increased mutant growth following infection of BALB/c mice *in vivo* and in both resting and activated macrophages (as measured by the number of CFU) and increased pathology in lungs of mice after infection (Hu *et al*., 2006; Stewart *et al*., 2006). Drumm *et al*. (2009) reported that both proteins encoded by the *rv2623* and *hspX* genes are required for the development of a chronic persistent infection suggesting that these two "stress" proteins might have comparable regulatory roles during dormancy. A similar function could be attributed to the *rv1996* gene which similarly encodes for a "stress" protein under *dosR* regulation. The *dosR* mutant itself is another reported example of hypervirulence associated with its deletion. Hypervirulence was reported for a *dosR* mutant in mice (Parish *et al*., 2003).

Natural deletion of the *rv1996* gene in clinical isolates other than CH strain outbreak was also reported. Tsolaki *et al*. (2004) have described deletion of the *rv1996* gene from ten clinical isolates from San Francisco in cluster with *rv1997 (ctpF), rv1992* (*ctpG), rv1993c, rv1994c* and *rv1995*. They attributed the repeated infection by this strain, to the deletion of hypoxia induced regulon genes which hindered latency making development of active disease more likely (Tsolaki *et al*., 2004).

Surprisingly, another clinical Latin American strain reported and fully sequenced in Colombia (Isaza *et al*., 2012) was found to possess the same deletion previously reported by Tsolaki *et al*. (2004) having a large genomic deletion of 3.6 kb, leading to the loss of *rv1996* and *rv1997* in addition to *rv1993c*, *rv1994c* and *rv1995* genes. Interestingly, in both cases reported by Tsolaki *et al*. (2004) and Isaza *et al*. (2012) *rv1997* (*ctpF*) was also deleted. The repetition of this deletion in many clinical isolates including the CH strain

suggests a potential role in increasing the virulence of *M. tuberculosis* and development of active disease.

Therefore, in the case of the CH outbreak strain it is highly possible that deletion of the *rv1996* gene which is the most recent deletion in the outbreak strain associated with potential *rv1997* loss of function, hindered latency development and hence facilitated progress into active disease and dissemination to new hosts. The *rv1996* and *rv1997* genes are among the most highly induced genes in the dormancy regulon on exposure to hypoxia and nitrosative stress (Ohno *et al*., 2003). The afore mentioned observations highly imply that the *rv1996* gene deletion had a role in the hypervirulence phenotype of the CH strain. A "fitness cost" compensatory mutation elsewhere in the genome might be involved, which allowed the bacteria to be better transmitted.

Another conclusion about the *dosR* regulated USP genes in mycobacteria could be observed that these genes have a role in establishment of latency rather than defence against stresses inside the host. It can be assumed that the *rv1996* gene as a USP gene, may enhance *M. tuberculosis* transition into latency and its loss deprives the bacilli from this advantage. The latency allows persistence of tubercle bacillus inside the host which enables it to be reactivated and cause active disease when the immune status is compromised. The success of the bacillus to be alive over all these decades is mainly attributed to this character.

6.4. Importance of the *rv1995* gene deletion in the CH outbreak strain.

The *rv1995* gene, as encoding a hypothetical protein of unknown function, could be one of the genes whose loss was almost neutral for the bacillus. Alland *et al*. (2007) hypothesised that some LSP deletions could be highly unstable genomic regions that are frequently deleted since the genes included by these LSPs are non-functional. Therefore, loss of these genes might be advantageous for the bacilli via loss of non-functional DNA, giving the bacteria great genetic variability and a method for adaptation among different populations.

The deletion of the *rv1995* gene was also associated with loss of the promoter of the *rv1996/rv1997* operon causing potential loss of their function. Another issue is that loss of its hypothetical protein may be associated with immune modulation. However, this could be applied to almost all genes encoding hypothetical proteins that are deleted from the CH strain. Another issue associated with the deletion of the *rv1995* gene in addition to the *rv1996* gene is that they represent a phylogenetic marker unique for the CH strain as discussed in section 6.1.2.

6.4.1. The Rv1995 as a Hemerythrin-like protein.

The Rv1995 protein was identified as a Hemerythrin-like protein in a previous report (Veyrier *et al*., 2009). Bioinformatic analysis of the *rv1995* gene showed its conserved nature in species in the *M. tuberculosis* complex (Section 5.3.1). As a tool to predict the function of the Rv1995 protein and its homologues in other mycobacterial and non-mycobacterial species, a new bioinformatic analysis was performed using BLASTP (protein-protein BLAST) (Altschul *et al*., 1997; McGinnis and Madden, 2004) using the "Exclude" tool for the *M. tuberculosis* complex (taxid:77643) (data not shown). This analysis revealed similar structure with up to 77% identity in many mycobacterial and non-mycobacterial species. Similar to *rv1995*, its homologues in all the found species were annotated as hemerythrin domain containing proteins with iron binding sites.

A recent report by Li *et al*. (2015b) identified 3 hemerythrin like proteins in *M. smegmatis*. However, *M. smegmatis* was not one of the strains which had similarities to the Rv1995 protein in the afore mentioned protein blast search. Therefore, the Rv1995 protein was compared to *M. smegmatis* using the same protein blast search tool to detect any part with similarity with the Rv1995 protein sequence. This revealed only a 20% identity with a transporter protein which was not defined as a hemerythrin like (Hr like) protein in the work performed by Li *et al*. (2015b).

Depending on this bioinformatic analysis and the already saved data on Genbank, the potential function of the Rv1995 protein mostly depends on its hemerythrin-like domain. Hemerythrin-like proteins appear to participate in many different biological processes. Via their binuclear non-heme iron centres, they are known to be able to

reversibly bind oxygen. The non-heme iron centre consists of two oxygen-bridged iron atoms bound to a set of conserved histidine, aspartate and glutamate residues with the motifs H–HxxxE–HxxxH–HxxxxD (French *et al*., 2008; Alvarez-Carreño *et al*., 2018).

The putative function of binding oxygen in Hemerythrin-like proteins was proposed to be a storage mechanism or for delivery to oxygen-requiring enzymes (Karlsen *et al*., 2005), a sensory mechanism (Xiong *et al*., 2000; Isaza *et al*., 2006) or as a detoxification mechanism (French *et al*., 2008). An interesting study on *Desulfovibrio vulgaris*, an anaerobic bacterium, reported that it uses a hemerythrin-like domain as an aerotaxis receptor protein (Isaza *et al*., 2006).

In *M. smegmatis*, a recent study by Li *et al*. (2015a) reported a Hr-like protein; msmHr which was proposed to regulate the expression of a sigma factor (SigF) in response to exposure to H_2O_2 rich environments (Li *et al*., 2015a). The sigma factor F (σ^F factor) is one of the sigma proteins needed for initiation of transcription in *M. tuberculosis* and is induced on exposure to several stresses such as anaerobiosis, oxidative stress, nutrient depletion and after entry into stationary phase (Rodrigue *et al*., 2006).

In *M. tuberculosis*, a recent study on the Rv2633 protein of *M. tuberculosis* which is another Hemerythrin-like protein, reported a possible role for this protein in defence against oxidative stress (Ma *et al*., 2018). Being a hemerythrin-like protein, a similar function can be proposed for the Rv1995 protein to be an O_2 sensor which responds to hypoxia and/or oxidative stress. This could implicate the *rv1995* gene in a possible role in induction of dormancy. An interesting bioinformatic analysis performed on 290 mycobacterial hypothetical proteins, including Rv1995, revealed that expression of this protein is increased in dormancy by 80-100% (Al-Khafaji, 2014; Murphy and Brown, 2007). The *rv2633c* gene which codes for the afore mentioned Rv2633 hemerythrin-like protein was also found to be highly expressed in dormancy (Al-Khafaji, 2014). Taken together, this may link the *rv1995* gene function to the proposed role of the *rv1996* and *rv1997* genes in the potential for dormancy.

6.4.2. Do the *rv1995/96/97* genes work as a functional regulon in *M. tuberculosis*?

M. tuberculosis harbours a large family of proteins that function as a flexible switch on exposure to oxidative and reductive conditions that causes an imbalance in intracellular redox homeostasis (Chinta *et al*., 2016). These bacterial iron-based sensor proteins utilize the redox chemistry of heme to sense surrounding gases and the intracellular redox state (Farhana *et al*., 2012; Chinta *et al*., 2016). The *M. tuberculosis* two sensor kinases, DosS (Rv3132c) and DosT (Rv2027c), of the DosR (Rv3133c) regulon are model examples of heme-based sensors capable of sensing multiple gases including NO, CO, and O_2 and their heme iron plays a major role in their response to these gases (Bhat *et al*., 2012; Imlay, 2013). Similar to the two regulator sensor kinases of the *dosR* gene, the Rv1995 protein could be one of these proteins which act as a switch sensor for the *dosR* regulated *rv1996* and *rv1997* genes.

The proposal that the *rv1995* gene encodes a sensor protein for *rv1996/97* could raise the possibility that each *dosR* regulated gene has another local repression or de-repression mechanism by a nearby gene and that the *rv1995/96/97* genes act as a regulon under the control of *dosR*. The actual role of the *dosR* regulon and its subunits could be to maintain the survival of bacteria under unfavourable stress conditions by making changes in different physiological, metabolic and cell processes needed during the dormancy of the organism. These include changes in the cell wall and its permeability, decreased growth rate and shutting down protein synthesis and other metabolic functions (Cunningham and Spreadbury, 1998; Hu *et al*., 1998; Voskuil *et al*., 2004; Boon and Dick, 2012; Glass *et al*., 2017).

The detection of a promoter by *in silico* analysis (Solovyev V, Salamov, 2011) of the intergenic sequence between *rv1996* and *rv1997* revealed that the *rv1997* gene lacks a specific promoter and is co-transcribed using the *rv1996* promoter. This also supports the possibility that the 3 genes are working as a functional unit in which *rv1995* works as a sensor for the adjacent *dosR* regulated genes.

The Rv1997 protein (CtpF) is known to be a metal cation transporter of the P-ATPase superfamily (Niederweis *et al*., 2015). P-ATPases are membrane proteins which transport a variety of ions against electrochemical concentrations using the energy of ATP hydrolysis. Therefore, it was assumed that CtpF is involved in cell wall processes and permeability and has a role in cell homeostasis (Novoa-Aponte and Soto Ospina, 2014). The *M. tuberculosis* genome encodes a variety of P-ATPases. Among the known *M. tuberculosis* P-ATPases encoding genes, the *ctpF* gene is the only one that is regulated by the global latency regulator, *dosR*, and it is highly expressed under hypoxic stresses (Bespyatykh *et al*., 2016).

In conclusion, the *rv1995/96* locus deletion which was the last to occur in the CH strain, in addition to the potential functional loss of *rv1997* being a pseudogene may have led to the loss of an important functional unit (*rv1995/96/97*) of the *dosR* regulon and that event may have resulted in the hypervirulent phenotype of the strain due to a failure of latency as observed in the Leicester tuberculosis outbreak in 2001.

6.5. Discoveries in tuberculosis field related to the outbreak strain, *M. tuberculosis* CH and the findings of the *rv1519* gene published in 2006.

The study performed by Newton and other colleagues including the author of this thesis (2006) to investigate the relationship of genotype to phenotype of *M. tuberculosis* CH involved the CH strain in comparison to a second CH outbreak isolate, CAS2, as well as H37Rv and CDC1551. It was concluded that in spite of the attenuated phenotype of both CH and CAS2 against antibacterial agents (lower pH and H_2O_2) and lower growth rate reported by the authors in comparison to other strains, CH led to the largest tuberculosis outbreak ever documented in a school in the UK. Reduction of the ability of the CH strain to resist intracellular killing agents should have decreased its replication in human monocytes and monocyte-derived macrophages (MDMs) (Fermani *et al*., 2002a; Newton *et al*., 2006). However, it was found to be similar to H37Rv in its ability to replicate in

these cells (Newton *et al*., 2006). Cytokine production pattern in response to CH strain revealed less inflammation-inducing cytokine (IL-12p40), but more production of anti-inflammatory cytokines (IL-10 and IL-6) from MDMs compared with H37Rv. These findings suggested that this strain skewed the innate cytokine response through phagocyte deactivation. CAS2, the second outbreak isolate, was found to be indistinguishable from the CH strain regarding the investigated phenotypic, in addition to genotypic characters (Newton *et al*., 2006).

Further investigation revealed that none of the gene deletions from the CH strain (Rajakumar *et al*., 2004) including the *rv1995*/*rv1996* genes, but only the reintroduction of the *rv1519* gene into the CH strain led to the suppression of IL-10 secretion from MDMs to the level induced by H37Rv, whereas there was no effect on the secretion of IL-12p40 that indicated that the reduction of IL-10 secretion was not mediated via the increase of IL-12p40 secretion. This implicated the deletion of *rv1519* (RD750), earlier described to define the East African Lineage (CAS), in modulation of the primary immune response (Newton *et al*., 2006). The *rv1519* gene was reported as a hypothetical protein (Cole *et al*., 1998) which appeared to be dispensable (Sassetti and Rubin, 2003). However, in homology analysis studies, the protein encoded by the *rv1519* gene did not appear strongly homologous to proteins with known functions (Newton *et al*., 2006).

The immuno-suppressive activity of IL-10 may explain the ability of *M. tuberculosis* to survive intracellularly on its production. Several reports have indicated that the presence of IL-10 leads to down-regulation of macrophage activation (Boussiotis *et al*., 2000; Murray *et al*., 1997; Abdalla *et al*., 2016) via its ability to inhibit NO secretion by monocytes, and MHC class-II expression by monocytes (Fiorentino *et al*., 1991; De Waal Malefyt *et al*., 1991; Turner *et al*., 2002). Also, it was characterised as a factor that could inhibit Th1 cell proliferation and IFN-γ secretion from Th1 cell clones (Sendide *et al*., 2005; Redford *et al*., 2011). By contrast, IL-12 is believed to play an important role in cell-mediated immunity against the intracellular micro-organism, *M. tuberculosis*, since it represents an essential factor in developing monocytes into antigen-presenting cells (APC) that are also known as activation or maturation macrophages. Also, it induces the release

of IFN-γ and TNF-α from activated T-cells and natural killer (NK) cells (Xing *et al*., 2000; Hamza *et al*., 2010; Mendez-Samperio *et al*., 2010).

Skewing the primary immune response via downregulation of pro-inflammatory cytokines or upregulation of anti-inflammatory cytokines observed in the case of CH outbreak strain was previously reported in the Beijing strain HN878; another outbreak strain, but with a different mechanism. The latter down-regulated the human cytokine response via the production of an immuno-suppressive phenolic glycolipid (PGL) that reduces the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-12 and INFγ) leading to a greater virulence of this strain compared with CDC1551 and H37Rv in mice (Reed *et al*., 2004; Manca *et al*., 2004) and in rabbits (Tsenova *et al*., 2005). The presence of an intact *pks15/1* gene was incriminated in the hypervirulence phenotype of this strain (Reed *et al*., 2004; Manca *et al*., 2004; Tsenova *et al*., 2005). Interestingly the *pks15/1* gene was intact in CH however, the immuno-suppressive phenolic glycolipid (PGL) was not produced (Newton *et al*., 2006). This was consistent with what Reed *et al.* (2007) reported, that not all strains with an intact *pks15/1* gene produce detectable levels of PGL. A finding indicates that the *pks15/1* gene was not involved in the hypervirulence of CH.

One of the most important observations in the case of the CH strain is that gene deletion events occurred naturally and led the CH strain to be less resistant to hydrogen peroxide and acid stresses that may decrease its fitness. However, overall the gene deletions increased, rather than decreased the propensity of CH to cause an unusual outbreak in Crown Hill community (Newton *et al*., 2006). This may be attributed to the fitness costs associated with the deletion events which was compensated by mutations elsewhere in the genome that could take place and produced the suppression of phagocytes via cytokine modified secretion pattern (zur Wiesch *et al*., 2010; Farhat *et al*., 2013; Abdalla *et al*., 2016). A hypervirulent phenotype was observed with loss of a class of genes called anti-virulence genes (Foreman-Wykert and Miller, 2003; Bliven and Maurelli, 2012). Another interpretation of the immunomodulatory phenotype observed with the CH strain is that the *rv1519* gene could be one of these anti-virulence genes.

6.5.1. Non-CH CAS lineage strains phenotype and virulence.

More recent studies that included the CH strain in addition to another CAS lineage strains reported that all investigated East African Lineage (CAS) strains share similar phenotypic features including lower growth rate *in vitro*, higher IL-10 and TNF induction when compared to H37Rv (Tanveer *et al*., 2009; Kanji *et al*., 2011; Sarkar *et al*., 2012). Interestingly, Kanji *et al*. (2011) reported some strains with extra deletions other than RD750, namely RD149 and RD152 were associated with lower growth rate and more induction of IL-10 and TNF than those without extra deletions in comparison to H37Rv. The later deletion (RD152) included, *plcD* (*rv1755c*) that was previously reported by Yesilkaya *et al.* (2006) to be disrupted by insertion in CH. Furthermore, Sarkar *et al*. (2012) compared strains of East-Asian and Euro-American as well as CAS/Delhi lineages including the CH strain as an example of CAS lineage. It can be observed from their results that there were phenotypic differences in-between CH strain and the other non-CH CAS strain represented by lower growth rate, more uptake by MDM cells and higher generation time in MDM cells, in addition to more induction of TNF-α.

The phenotypic differences between CH and other CAS lineage strains suggested that gene deletions other than the *rv1519* gene deletion (RD750), might have a role in the CH exceptional and distinct phenotype and hypervirulence.

6.6. Other CH gene mutations, could they have a role?

The deleted genes from CH strain belong to different functional gene classes (Section 1.9.1) (Yesilkaya *et al*., 2006; Rajakumar *et al*., 2004). Previous studies showed some disagreement concerning the importance of the genes affected by deletions in the CH strain. The deleted genes *rv3516* (*echA19*), *rv0180*, *rv0395*, *rv3738* (*PPE66*) and *rv3739* (*PPE67*) have been previously reported as essential for *in vitro* growth of *M. tuberculosis* (Yesilkaya *et al*., 2006) which is supported by decreased growth rate of the CH strain *in vitro* when compared to H37Rv (Newton *et al*., 2006). In contrast, a more recent work by Griffin *et al*. (2011) reported that the genes (*echA19*, *rv0395*, *rv3517*) were non-essential for *in vitro* growth of H37Rv and no data were available about *rv0180*, *rv3738* (*PPE66*)

and *rv3739* (*PPE67*) which was almost the same as reported by Sassetti *et al*. (2003). Consistent with this finding is that their individual complementation into CH produced almost no *in vitro* change in the growth rate of the complemented strains (Newton *et al*., 2006). This contradiction might be explained by the fact that complementation was performed for individual genes and not by the whole set of deleted genes. Also, the presence of other genetic factors which were not corrected by complementation could be another reason.

In contrast, none of the *in vivo* essential genes previously identified in a mouse infection model (Sassetti and Rubin, 2003), were deleted in the CH strain (Yesilkaya *et al*., 2006). The likely non-essential function of deleted genes *in vivo* is supported by the fact that their deletion in CH was not associated with impact on the capacity of the strain to cause the outbreak. Also, the ability of the CH strain to grow even more rapidly than H37Rv in a SCID model is more evidence of the non-essential function of the deleted genes.

Two PPE (glycine-rich proteins) genes were deleted in the outbreak strain, namely *rv3738c* and *rv3739*. The PPE gene class is reported to encode mycobacterial cell envelope proteins and is known as a preferential target for InDel and SNPs events. They have been proposed to play a role in antigenic variation of *M. tuberculosis* (Fleischmann *et al*., 2002; Yesilkaya *et al*., 2006; McEvoy *et al*., 2009; Sampson, 2011; Lamrabet and Drancourt, 2012). Thus, it could be possible that CH genomic mutations in the PPE genes played a role in the unique antigenic characteristics of the CH strain and might support the infecting organism escape the host immune response, and facilitate ongoing transmission (Yesilkaya *et al*., 2006; McEvoy *et al*., 2009; Sampson, 2011).

The deletion of the *rv3516* (*echA19*) gene was reported to encode enoyl-coenzyme A hydratase that has a link to lipid metabolism in *M. tuberculosis* (Dubnau *et al*., 2002). It was described as a highly-transcribed gene in macrophages which is involved in degradation of fatty acids in *M. tuberculosis*. Deletion of such a highly transcribed gene could be a means to escape the immune response (Bernaschi *et al*., 2002; Yesilkaya *et al*. 2006).

6.6.1. *IS6110* based molecular analysis of CH genome.

A possible reason for an apparently virulent strain being not affected by deletions is that these mutations were compensated by another gene/s (Newton *et al*., 2006). This observation should focus attention on many factors that were not investigated but which could account for the hypervirulence of the CH strain. For example, insertions and SNPs which could be present in the strain and had a role in the unique phenotype should be highlighted.

In another molecular study using *IS6110* based hemi-nested inverse PCR done by Yesilkaya *et al*. (2006) to analyse the CH genome, they determined a total of 16 genes mutated either by deletions (ten), including those previously described by Rajakumar *et al*. (2004), or by *IS6110* insertions (six) compared to H37Rv (Yesilkaya *et al*., 2006). The genes mutated by *IS6110* insertions were (*rv0395*, *rv1504*, *rv1755*, *rv1917*, *rv2735* and *rv2787*) in addition to 3 intergenic sequences (*rv2815-16, rv3018-19, rv3187-88*). These insertions and two of the deletions namely (*rv3737* and *rv1520*) were firstly reported by Yesilkaya *et al*. (2006) and not previously investigated. However, all of the genes mutated by insertion, were classified as hypothetical proteins except for *rv1755 (plcD)* which encodes a probable phospholipase C and *rv1917* which encodes PPE (Yesilkaya *et al*. (2006). Interestingly, deletion of the *rv1755 (plcD)* gene from some clinical *M. tuberculosis* isolates was observed to be associated with a twofold increased risk of extrapulmonary tuberculosis (Yang *et al*, 2005). Hence, disruption of *plcD* gene by insertion could have a role in hypervirulence of CH outbreak strain.

The mutation of these genes by deletion (Rajakumar *et al*., 2004; Yesilkaya *et al*., 2006) or insertion (Yesilkaya *et al*., 2006) in the CH strain could be associated also with loss of function of downstream genes whose expression may be affected by their mutation. This might have a role in the phenotypic characteristics of the CH strain including its slow growth, less resistance to acid stress and H_2O_2 *in vitro* compared with H37Rv and CDC1551 strains that was not regained by complementation of the deleted genes in the study performed by Newton *et al*. (2006).

6.7. Potential study weaknesses.

Every study has its strength and weakness points which should be considered to put the experimental design in a biological context, thus it would be appropriate to mention some considerations about possible points of weakness in the experimental approach used in the study described here.

6.7.1. Choice of the avirulent *M. bovis* BCG strain as a model for gene knock out experiments instead of *M. tuberculosis*.

Studying gene function of a virulent clinical strain of *M. tuberculosis* in the non-virulent *M. bovis* BCG strain might be a weakness of the experimental approach. The main motive for working with *M. bovis* BCG instead of *M. tuberculosis* was that at the time working with a hazard group 3 pathogen was not possible, however, there is biological justification for taking this approach.

The similarities between physiological, molecular, and metabolic responses of *M. bovis* BCG and those of the tubercle bacillus have been reported in several studies. Hence, *M. bovis* BCG as a safe strain has been extensively used both *in vitro* and *in vivo* to investigate several phenotypic characteristics of *M. tuberculosis* (Lim *et al*., 1999; Fritz *et al*., 2002; Pym *et al*., 2003, Beste *et al*., 2004; Minassian *et al*., 2012; Hu *et al*., 2015). *M. bovis* BCG is derived from a virulent *M. bovis* strain and has 99.95% sequence homology relative to live *M. bovis* (Minassian *et al*., 2012) and shares more than 95% of its genetic composition with that of H37Rv (Muttucumaru *et al*., 2004).

The sequence of the genes (*rv1995*, *rv1996* and *rv1997*) that are core for the current study are present in the genome of BCG strain with 100% identity to that of *M. tuberculosis* (Section 3.1.1.3; Hu *et al*., 2015), and the organisation of these *dosR* regulated genes are conserved between *M. bovis* BCG and *M. tuberculosis* (Boon and Dick, 2002; Alvarez *et al*., 2009). Therefore, it was justifiable to investigate the function of *rv1995/96/97* in the non-virulent, slow growing BCG. Indeed, the relationship between *rv1996* and acid resistance identified using a knock out mutation in *M. bovis* BCG is consistent with the

phenotypic results of the knock in mutation of the virulent CH strain (Newton *et al*., 2006; Data obtained from Imperial College team, 2006).

Other studies have demonstrated that BCG shows similar types of stress responses to *M. tuberculosis*. For example, Firmani and Riley (2002a and b) reported no significant difference between different mycobacterial strains, including BCG, at a low concentration of reactive nitrogen and oxygen intermediates. However, in a high concentration of ROI and RNI, CDC1551 showed more resistance than the other mycobacterial strains investigated; this significant difference was not observed between BCG and H37Rv (Firmani and Riley 2002a and b). A relatively recent report demonstrated comparable levels of nitrite production in response to mycobacterial BCG and H37Rv infection of macrophages (Jung *et al*., 2013). They also reported a similar level of growth and viability for both strains after 48 hours of macrophage infection with induction of maximal NO production inside the macrophages (Jung *et al*., 2013). Another explanation for using *M. bovis* BCG to investigate a role of a gene in stress response encountered by MTB, is that the BCG strain is reported to have the ability to inhibit the fusion of phagosomes with lysosomes in macrophages, like many other species of mycobacteria including *M. tuberculosis* (Via *et al*., 1997; Vandal *et al*., 2009).

Although *M. bovis* infection of cattle usually results in a slowly progressive disease in which latency and reactivation are uncommon (Whelan *et al*., 2010; Waters and Palmer, 2015), strong evidence of latency in humans with occupational exposure to *M. bovis* has been postulated by Vayr *et al*. (2018), and cattle was the main reservoir of infection in the majority of studies (Vayr *et al*., 2018). In humans, *M. bovis* BCG can form granulomas and abscesses in several tissues similar to *M. tuberculosis*. In immune-competent individuals, *M. bovis* BCG may persist for long periods after vaccination (Trevenen and Pagtakhan, 1982). The survival and persistence of the live BCG vaccine are necessary to elicit protective immunity. Nevertheless, BCG mimics *M. tuberculosis* in its ability to cause reactivation disease in the immunocompromised host (Talbot *et al*., 1997; Fritz *et al*., 2002; Santos *et al*., 2010). *M. bovis* BCG has been also used as a human challenge model for *M. tuberculosis* since it causes a self-contained limited infection in immunocompetent animals and humans (Minassian *et al*., 2012).

6.7.2. Stress conditions investigated in the experimental approach.

Investigating phenotypic changes in response to stress conditions such as low pH, H_2O_2 and NO might be considered irrelevant to environmental cues encountered by *M. tuberculosis in vivo.* One of the main virulence mechanisms developed by MTB is to prevent phagolysosome fusion, thus they block respiratory burst and prevent acidification of the vacuole. This allows the bacterium to replicate within macrophages without being exposed to the oxidative, nitrosative and acid stresses of the phagolysosome (Section 1.7.2.1). However, this is not always the case. Several MTB mutants lack the ability to block phagolysosome fusion, nevertheless, many of them were able to survive in phagolysosomes and even to replicate intracellularly similar to wildtype MTB (MacGurn and Cox, 2007; Pethe *et al*., 2004; Ehrt and Schnappinger, 2009).

Also, unlike naive macrophages, the block in phagosome lysosome maturation by mycobacteria is overcome in IFN-γ activated macrophages leading to an acidic environment where hydrolases, reactive nitrogen and reactive oxygen operate most effectively (Russel, 2001; Ehrt *et al*., 2009; Stallings and Glickman, 2010). In addition, many reports showed that *in vivo* studies on mutant animals such as mice, which are defective in the production of ROI or RNI were more susceptible to infection by mycobacteria (MacMicking *et al*., 1997; Cooper *et al*., 2000; Stallings and Glickman, 2010). Taken together, these reports provide strong evidence that resisting acid, oxidative and nitrosative stresses is important for *M. tuberculosis* growth and persistence in macrophages and different niches inside the host (Stallings and Glickman, 2010). One of the important niches in which the mycobacteria live, is the granuloma. Granuloma is the hallmark of TB infection. *M. tuberculosis* has the ability to survive in a non-replicating state in oxygen limited environments, such as fibrotic granulomatous lesions *in vivo*. In addition, being acid-resistant, this allows mycobacteria to survive in acidic, inflamed lesions (Tan *et al*., 2010; Weiss and Schaible, 2015). Different stages of granuloma in latent and active phases of disease were described. These are solid granuloma which is common during latent phases of disease, necrotic granuloma in the early stages of active infection

and caseous granuloma during end-stage or severe TB (Gengenbacher and Kaufmann, 2012; Ruggiero *et al*., 2017).

The solid granuloma is a composed of highly structured tissue reactions of macrophages, dendritic cells, as well as T and B lymphocytes and typically encircled by a fibrotic wall that separates it from surrounding tissue. Within a solid (cellular) granuloma, the pH is estimated to be less than 6 (Iacobino *et al*., 2016). An interesting report that blocking the drop in pH interferes with the upregulation of around 80% of the genes which are normally upregulated in *M. tuberculosis* inside the macrophages. This is evidence that acidity plays a key role in the environmental stresses and signals encountered by *M. tuberculosis in vivo* (Rhode *et al*., 2007; Saviola, 2012). Inside the solid type of granuloma, MTB bacilli are most likely in a stage of dormancy with a low metabolic activity and a non to low replication activity. The adaption to changes in oxygen availability inside this type of granuloma is mediated by the *dosR* regulatory complex. The *dosR* regulon governs metabolic shift of MTB from aerobic to anaerobic functioning, ensures survival of the bacillus during hypoxia and controls reversal to replication upon re-exposure to oxygen (Gengenbacher and Kaufmann, 2012; Pulido *et al*., 2014; Glass *et al*., 2017). Inside the necrotic liquefying granuloma, the situation is different. Unlike the common belief that the pH of liquefied caseous material is acidic, it was reported that pH of a caseous material has a range from 6.1 to 7.5 in mice, guinea pigs, rabbits, and humans (Iacobino *et al*., 2016, Lanoix *et al*., 2015). In caseous granuloma, the centre liquefies leading to formation of a cavity which contains the cellular debris in addition to the bacilli. The high oxygen content and the caseous material provides a rich source of nutrient and promotes bacterial growth. Actually, during active stages of TB, different forms of granulomas coexist with a gathering of various microenvironments to which the pathogen has to adapt (Gengenbacher and Kaufmann, 2012).

6.7.3. Composition of culture media used in the phenotypic experiments.

Culture medium composition clearly affects the physiology of mycobacteria and influences stress resistance *in vitro* (Sung and Collins, 2003; Venkataswamy *et al*., 2012;

Palange *et al*., 2016). The experimental design of the current study to investigate the possible stress responses of MTB *in vitro* was dependant on the use of different culture media to which the source of the stress was added (acidulent in the case of low pH, H_2O_2 in the case of oxidative stress and $NaNO_2$ in the case of nitrosative stress). The basic culture medium used in my work was 7H9 broth medium in which glycerol was used as a source of carbon. The same experimental design was followed in the work performed on the CH strain (Newton *et al*., 2006) and in the vast majority of other *in vitro* studies performed on MTB (Sung and Collins, 2003; Firmani and Riley 2002a and b; Hingley-Wilson *et al*., 2010; Venkataswamy *et al*., 2012; Jung *et al*., 2013; Glass *et al*., 2017). However, a point which should be acknowledged is that *M. tuberculosis* needs cholesterol as a carbon source *in vivo*. In the lungs of chronically infected animals, cholesterol, which is a major component of host cell membranes, is essential for persistence of *M. tuberculosis* and for its growth within the IFN-γ-activated macrophages that prevail at this stage of infection (Brown *et al*., 2008; Gatfield and Pieters, 2000; Pandey and Sassetti, 2008; Miner *et al*., 2009). A *M. tuberculosis* mutant which is deficient for uptake of cholesterol revealed impaired replication only inside IFN-γ activated macrophages but not resting macrophages. Hence, it was concluded that cholesterol metabolism is mainly required for growth of bacteria in restrictive intracellular conditions induced by IFN-γ (Brown *et al*., 2008, Pandey and Sassetti, 2008). The afore mentioned notes about the need for cholesterol *in vivo* in the restrictive environment such as inside IFN-γ activated macrophages, may have influenced the results of the *in vitro* experimental approach used here in terms of its applicability for evaluating a virulence trait such as growth and survival on exposure to different stresses, since it is not mimicking precisely the *in vivo* environment. However, a relatively recent report concluded that cholesterol is not required as a nutritional source during infection (Yang *et al*., 2011). More work is needed to evaluate the validity of such experimental approaches in assessment of the different stresses encountered by MTB *in vivo*.

In conclusion, although the *in vitro* experiments represent the first approach for investigating phenotypic changes under stresses encountered by mycobacteria *in vivo*, they are indispensable in order to obtain a full picture for understanding the pathogenesis of MTB and for better simulation of what happens inside the host.

6.8. Future work and alternative approaches.

Research on *Mycobacterium tuberculosis* as a whole and on *M. tuberculosis* CH strain as an example of unique outbreak strain should be continued to better understand the behaviour and genotype to phenotype correlation of this important pathogen.

The difficulties in experimental work on MTB are well-known. The fastidious growth requirements necessitate strictly obligate aerobic and highly nutritious growth conditions. In addition, the tubercle bacillus is slow growing and requires more than 18 hrs to duplicate. Comparing the proliferative potential of MTB against *E. coli* which needs only 18-20 minutes to duplicate, clarifies many of these difficulties. As a result of this long generation time, the mycobacterial cells need to be incubated from one to two weeks in liquid medium, and from three to five weeks on solid medium to obtain desired growth. For this reason, different experiments should be done simultaneously and changing the conditions of one experiment requires 1-2 months extra work. Furthermore, the known biohazard of the organism necessitates working in a Category 3 laboratory most of the time which needs special authorisation and strict precautions during each experiment. All these difficulties in performing experiments on the organism make it necessary to build new ideas and concepts for future complementation of work on *M. tuberculosis* complex.

The results of the current study have demonstrated that both *rv1995* and *rv1996* genes had a minor or no role on exposure of *M. tuberculosis* to H_2O_2 and NO stress under the experimental conditions followed in this work. However, in the case of *rv1996*, a difference on exposure to acid stress was found either on disruption of the gene from BCG or complementation of the gene into CH strain. The role of this gene in acid stress needs to be further addressed and confirmed in future work. Another hypothesis was developed in the current study is that *dosR* regulated stress genes, such as *rv1996* and *rv1997*, have a role in initiation and maintenance of dormancy on exposure to stress rather than defense against such stress. Therefore, deletion of these dormancy stress genes might increase the likelihood of active disease and outbreak development. A series of *in vivo* experiments need to be performed to confirm the role of deletion of the *rv1996* gene in hypervirulence of CH. These future aspects and others will be discussed in the following sections.

6.8.1. Further genomic analysis for the CH strain.

While standard genotyping analysis of *M. tuberculosis* outbreak investigation is increasingly anticipated to have limited resolution for effective public health surveillance and intervention, whole genome sequencing (WGS) based assays has been proven to provide the best resolution to detect microevolution within the *M. tuberculosis* genome and to identify subtle differences that might influence transmission of the organism and to trace possible scenarios in outbreak transmission (Witney *et al*., 2016; Cole *et al*., 1998; Roetzer *et al*., 2013). In addition, WGS enables a reliable prediction of the drug susceptibility of the strain and its use could be the first step toward a systematic approach to achieve tuberculosis control (Witney *et al*., 2016).

The already performed work on the CH strain needs further explanation of many of its unique characteristics. The methods used for identification and genetic characterisation of the strain was by using a whole genome microarray of the CH strain DNA in comparison to H37Rv (Shafi *et al*., 2002; Rajakumar *et al*., 2006), PCR GLIP assay to identify and confirm the deletions assumed by microarray (Rajakumar *et al*., 2006) and lastly reverse hemi-nested PCR which identified many insertions not previously reported (Yesilkaya *et al*., 2006). However, many of the extra genes, deletions, insertions and SNPs which probably affected the phenotype of the strain could be missed by using these methods. An important approach which could cover all these objectives is the WGS of the strain, which was difficult in the context of the time the work was undertaken and, to my knowledge, it has not been done so far. The WGS technique is necessary to fully identify the genetic composition of the CH strain and to trace the fine differences in the genome which could be associated with the evolutionary steps of CH that possibly led to the development of the outbreak with its unique phenotypic characters.

6.8.2. Transcriptomic analysis.

The deletion of the *rv1996* gene was most probably associated with loss of *rv1997* function since they are working as an operon (Isaza *et al*, 2012). Similar to *rv1996*, the *rv1997* gene is another *dosR* regulated gene which is induced under stress agents such as

hypoxia and NO exposure (Ohno *et al*, 2003; Isaza *et al*, 2012; Hingley-Wilson *et al*, 2012), hence it may have a role in the phenotype and hypervirulence of CH. Therefore, the lost function of *rv1997* should be confirmed by RT-PCR and qPCR quantitation of *rv1997* mRNA from CH in comparison to H37Rv. Likewise, other gene deletions in CH could affect downstream genes especially if they share a common promoter. Furthermore, many other genes might be inactivated due to *IS6110* insertions such as the case of *plcD* (*rv1755c*) (Yesilkaya *et al.*, 2006). Gene expression profiling using RNA sequencing from exponential phase growth of CH and H37Rv in Middlebrook 7H9 broth under standard growth conditions should be performed to highlight those genes which are not expressed in the CH strain in spite of their presence in the genome.

6.8.3. Investigating the role of *rv1996* gene in acid stress.

Although several microarray studies were done to analyse differential gene expression in response to acid stress (Fisher *et al*., 2002; Rohde *et al*., 2007; Kim *et al*., 2008), none of them reported the *rv1996* gene. However, in the current work, disruption of the gene was associated with decreased resistance to acid. Thus, the potential role of the *rv1996* gene against acid stress needs further investigation. One approach is to measure *rv1996* gene expression using RT-qPCR in a BCG or H37Rv strain under acid stress in comparison to a non-stressed strain. Another approach would be to perform transcriptome analysis for mycobacterial cells under standard and low pH using RNA-sequencing which has a wider scope to analyse which genes can be co-expressed under acid stress along with the *rv1996* gene.

6.8.4. Knock in approaches and related phenotypic studies.

6.8.4.1. Introduction of pAAO1 and pAAO3 into *M. smegmatis*.

The expression vectors pAAO1 and pAAO3 containing the *rv1995* and *rv1996* genes constructed in the current study, can be introduced into *M. smegmatis* as an alternative approach to evaluate the proposed function of both genes *in vitro*, since *M. smegmatis* is a rapidly growing mycobacterial strain which was confirmed to lack the

sequence of both genes (Section 5.1.1). This could help to overcome the slow growing nature and the difficulties of working on *M. tuberculosis* and *M. bovis* BCG strains. The complemented strains could be investigated against different stresses especially those which represent antimicrobial products of macrophages, such as hydrogen peroxide (H_2O_2), nitric oxide (NO), and acidification (low pH) of the phagolysosome.

6.8.4.2. Construction of expression vector for *rv1996/97* operon.

In the work performed by Newton *et al*. (2006), the *rv1996* gene was individually introduced into CH using expression vector which could regain the function of the gene only without downstream genes. An alternative approach for regaining the function of the whole operon of *rv1996*/*rv1997* if lost transcription of the *rv1997* gene was confirmed (Section 6.7.2), is reintroduction of the genes within an intact operon. The same method described for pAAO3 construction could be performed using the insert containing the whole *rv1996*/*rv1997* sequence. This should be followed by reintroduction of the insert into CH and testing the complemented strain *in vitro* and *in vivo* in comparison to the parental CH to define the role of these two genes in the phenotype and hypervirulence of CH.

6.8.4.3. Integration of candidate genes into CH chromosome.

One major disadvantage of the expression vectors used is that mRNA levels of the inserted genes in CH were greater than found in H37Rv (Newton *et al*., 2006). This over expression may have led to non-specific effects. Therefore, an integration-proficient vector such as pKINTA (Stewart *et al*., 2002) should be used to reintroduce the candidate genes (*rv1995*, *rv1996*) individually into the CH strain, each under the control of its own promoter. This approach has the advantage of integrating a single copy of the gene of interest or genes within the bacterial chromosome which make expression experiments performed under conditions that mimic those for chromosomal genes or operons. Also, the integration of such vectors would not affect the viability of the transformed mycobacteria, since it occurs in nonessential chromosomal *attB* sites (Stewart *et al*., 2002; Yang *et al*., 2002; Beste *et al*., 2009).

The proposed knock in constructs should be introduced correspondingly to the intended strain (*M. smegmatis* or CH strain) then expression of the inserted genes should be confirmed by RT-qPCR. Thereafter, each recombinant strain of *M. smegmatis* or CH, would be phenotypically investigated against its wildtype in comparison to H37Rv.

6.8.5. Knock out approaches and *in vivo* phenotypic studies.

The knock out strategy is characterised by being a better simulation of the natural gene deletion event. Thus, it is necessary to confirm the results of the current study and that obtained by Newton *et al*. (2006) regarding the candidate genes *rv1996* and *rv1519* via knocking out these genes from H37Rv and/or BCG. This should be followed by testing the knocked-out mutants for phenotypic differences in animal models in addition to MDM.

In vitro modelling provides an important experimental path to determine phenotypic virulence traits associated with gene polymorphisms in *M. tuberculosis.* However, the *in vivo* model is not dispensable for interpretation and confirmation of many suppositions. A phenotype that affects innate immunity may be detected more in a susceptible mouse model and to measure the virulence of *M. tuberculosis* strains, immunocompromised e.g. SCID, immunocompetent e.g. BALB/c models could be used (Guinn *et al*., 2004; Palanisamy *et al*., 2008). This could be applied through determination of the difference in survival in experimentally infected animals or by counting CFU in infected tissues (Pym *et al*., 2002; Polanis *et al*., 2008; Papavinasasundaram *et al*., 2005; Guinn *et al*., 2004; Palanisamy *et al*., 2008).

6.8.5.1. Potential role of the USP *rv1996* gene in induction of latency in *M. tuberculosis* rather than resistance to different stresses.

It was reported that lack of one or more members of the *dosR* regulon might lead to failure of *M. tuberculosis* to establish dormancy inside the host, resulting in uncontrolled growth which could be a reason for the hypervirulence phenotype (Drumm *et al*., 2009; Parish *et al*., 2003; Hu *et al*., 2006). Therefore, one of the most important points that needs to be further evaluated in future work based on the results of the current study, is to confirm

or exclude the role of the *rv1996* deletion in the hypervirulence phenotype of the CH strain given that it is a *dosR* regulated USP gene.

This can be achieved via infecting SCID mice with the knock out mutants obtained in the current study (BCG mutant, *rv1996*::hyg^R and/or the kept H37Rv recombinant) in comparison to their wildtypes respectively. Then, survival screen of the infected mice would be analysed. If a non-significant difference is found, confirmation via CFU count of the tissues would be done. Another approach is to use the CH complemented strain (CH::hyg^R*1996*) in comparison to CH wildtype strain and H37Rv to infect the same *in vivo* model used before.

6.8.5.2. Disruption of the *rv1519* gene from H37Rv and/or BCG by homologous recombination followed by *in vivo* experiments.

Regarding the *rv1519* gene, the unique finding implicated this gene in the induction of a protective innate response to *M. tuberculosis* (Newton *et al*., 2006) need to be further investigated in an *in vivo* model. It should be determined whether or not the disruption of *rv1519* from H37Rv or BCG strains will increase secretion of IL-10 from MDMs. This could be followed by infecting the immunocompromised (SCID) mice with the *rv1519* mutant to investigate its virulence in comparison to the wildtype H37Rv, CH and the recombinant CH strains (CH:: hyg^R*rv1519*). Construction of a *rv1519* mutant from H37Rv or BCG can be done following the same strategy performed to obtain BCG *rv1996*::hyg^R mutant described in the current study.

6.8.5.3. Measurement of cytokines in MDM infected with proposed *rv1519* knock out mutant.

The *rv1519* role in the immune subversion of the CH strain via increasing production of anti-inflammatory IL-10 and IL-6 from MDM could be further investigated. After knocking out the *rv1519* gene from H37Rv, the mutant would be grown on MDM, then IL-12, IL-6 and IL-10 cytokine measurement should be done in comparison to wildtype strain with considering that IL-12 production in CH was less than H37Rv and it

was produced in similar level in-between CH parental strain and complementary CH strain with the *rv1519* gene. This is useful to compare between the knock-in and knock out of this gene in MDMs.

6.9. Conclusion.

The molecular studies involving the genomes of the *M. tuberculosis* complex has opened new areas of tuberculosis research. Extensive progress has been made in understanding the basis of pathogenesis through the applications of molecular technology.

This project presented two of the most important strategies of gene manipulation techniques in microorganism to characterise the functional role of genes; expression vectors (pAAO1 and pAAO3) that have the ability to express the genes of interest (*rv1995* and *rv1996*) in mycobacteria and, suicide vectors (pANO1 and pANO2) that have the ability to replace the intact target genes with the disrupted genes in the mycobacterial genomes via homologous recombination. Both may contribute to understanding pathogenesis and virulence in *M. tuberculosis*.

Individual introduction of the *rv1995* and *rv1996* genes into the CH strain revealed no change on the recombinant strains regarding the effect of $NaNO_2$, H_2O_2 and cytokine secretion compared with the CH parental one. However, the CH::hyg^R*1996* recombinant strain was found to be more resistant to pH 5.5 than the parental CH strain (Newton *et al*.,2006; Data obtained from Imperial College team, 2006).

Both *M. bovis* BCG wildtype strain and its mutant, *M. bovis* BCG *rv1996*::hyg^R showed correlated growth curves, similar viability at standard pH level (6.8), similar adaptation to mild acidic pH value (5.5) and similar susceptibility to $NaNO_2$ and to H_2O_2, but the mutant *M. bovis* BCG *rv1996*::hyg^R showed less resistance to acid stress at pH level 3.5. This indicates that disruption of the *rv1996* sequence in *M. bovis* BCG had little or no effect on the resistance of the mutant in the presence of H_2O_2 and $NaNO_2$. It was hypothesised that the disruption of the *rv1996* sequence from *M. bovis* BCG would make the mutant more sensitive to H_2O_2, and $NaNO_2$, compared with the wildtype strain.

However, based on the results obtained in this project, the universal stress protein encoded by the *rv1996* gene seems to play a minor role in resistance to H_2O_2 and $NaNO_2$, but it has an effect against low pH.

The results obtained in the current study are considered to be the first report, to my knowledge, that implicates the universal stress gene *rv1996* in resistance against acid stress in mycobacteria.

It could be also hypothesised that the *rv1996* gene, as a *dosR* regulated gene, plays a role in the initiation and maintenance of latency rather than protecting the mycobacterial cell from some environmental stress conditions. Deletion of the *rv1996* gene with loss of its polar effect on the *dosR* regulated *rv1997* gene might be associated with failure of latency development which might lead to establishment of another mutation as a fitness cost. The latter could be responsible for the hypervirulence propensity of the CH strain. This could implicate the *rv1996* gene deletion in increasing the likelihood of active disease and outbreak development.

One important observation regarding the *rv1995/rv1996* locus deletion is that evolutionary analysis of the CH strain endorses this deletion as a phylogenetic marker for the strain since it indicates that the *rv1995/rv1996* deletion was the last to occur and was unique among CH deletions when compared with previously reported non-outbreak strains.

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