The interrelationship of strain diversity, virulence and patient ethnicity for tuberculosis in the Midlands, UK

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Abstract

This study examined the relationship between *Mycobacterium tuberculosis* global clades and patient origin. In the UK, the rate of tuberculosis is higher amongst patients who originated from the Indian subcontinent (ISC), where two dominant lineages are present, the Central Asian Strain (CAS) and the East African Indian (EAI) lineage. Mycobacterial interspersed units containing variable number of tandem repeats DNA fingerprinting of *M. tuberculosis* strains isolated from UK patients who originated from the ISC, as defined by novel software, identified that CAS was the most prevalent clade (39%) and EAI was the third most prevalent clade (15%). To further elucidate the relationship between host origin and strain lineage, two rigorous new models of infection were developed which assessed mycobacterial growth and host cell response. The monocyte-derived macrophage model was more appropriate for measuring cytototoxicity than the THP-1 cell model as in the absence of infection, 50% of THP-1 cells died compared to 2% of macrophages. CAS strains caused 1.5 fold more cell necrosis and their growth was four fold higher than EAI strains in the monocyte-derived macrophage model. Finally, the response of polarised monocyte-derived macrophages from Asian and Caucasian donors to different *M. tuberculosis* lineages was compared. CAS strains grew preferentially better in M2 macrophages from Asian donors. The prevalence of CAS in the Midlands is likely to be due to a combination of specific strain importation and increased ability of this strain to transmit to the population present in the Midlands.

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List of Abbreviations

AE	ancient epidemiological (ancient strain from an epidemiological cluster)
AS	ancient single (unique ancient strain, not from an epidemiological cluster)
ATCC	American Type Culture Collection
BCG	Bacille Calmette-Guérin
CAS	Central Asian Spoligotype
CD	cluster of differentiation
CDC	Centers for Disease Control
CE	CAS epidemiological (CAS strain from an epidemiological cluster)
CEL	cultural, ethnic, and linguistic
CFP10	culture filtrate protein 10
CFU	colony forming unit
CH	Crown Hills
CMI	cell mediated immunity
CS	CAS single (unique CAS strain, not from an epidemiological cluster)
DNA	deoxyribose nucleic acid
DR	direct repeat
DTH	delayed type hypersensitivity
EAI	East African Indian
EDC	1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ESAT-6	early secretory antigen-6
ETSN	enhanced tuberculosis surveillance network
ETR	exact tandem repeat
FBS	fetal bovine serum
IgG	immunoglobulin G
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
HPA	Health Protection Agency
IFN-γ	interferon-gamma
IGRA	interferon gamma release assay

LDH	lactate dehydrogenase
IL	interleukin
ISC	Indian subcontinent
LAM	Latin American Mediterranean
LSP	large sequence polymorphism
MDM	monocyte-derived macrophage
MDR	multi-drug resistance
MGIT™	mycobacterial growth indicator tube
MHC	major histocompatibility complex
MIRU	mycobacterial interspersed units
MOI	mulitiplicity of infection
MRCM	Midlands Regional Centre for Mycobacteriology
NCTC	National Collection of Type Cultures
NHS	National Health Service
OADC	oleic acid, albumin, dextrose and catalase
ONS	Office for National Statistics
PANTA™	polymyxin B, amphotericin B, nalidixic Acid, trimethoprim, azlocillin
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCT	primary care trust
PGG	principal genetic group
PMA	phorbol 12-myristate 13-acetate
RD	region of difference
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
SIT	spoligotype international types
SITVIT	an *M. tuberculosis* molecular markers database
SNP	single nucleotide polymorphism
SpolDB4	spoligotyping database 4
TB	tuberculosis
TbD1	*M. tuberculosis* specific deletion 1

Tm	melting temperature
TNF	tumor necrosis factor
TST	tuberculin skin test
TTP	time to positivity
VNTR	variable number of tandem repeats
WGS	whole genome sequence
WHO	World Health Organisation
ZN	Ziehl-Neelsen

1 INTRODUCTION

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease that can affect every organ of the body, although the most common form of the disease occurs in the lungs. TB can occur in humans and animals; the disease is caused by bacteria belonging to the *Mycobacteriaceae* family. There are over 120 species of mycobacteria, most of which are saphrophytic organisms that inhabit water and soil (Tortoli, 2006).

The species of mycobacteria that are capable of causing TB belong to the *Mycobacterium tuberculosis* complex, and include *Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microtti* and "*Mycobacterium canettii*" (Nicol and Wilkinson, 2008; Aranaz *et al*., 2003). Members of the complex have comparable genomes, with 99.9% nucleotide similarity and identical 16S rRNA sequences (Brosch *et al*., 2002). Despite the nucleotide similarity shared by members of the complex, there are wide variations in pathogenicity and host range (Table 1-1) (Gordon *et al*., 2009).

Skeletal abnormalities associated with TB disease have been identified in prehistoric human remains, indicating that TB affected ancient humans. This evidence has been corroborated by the detection and molecular characterisation of *M. tuberculosis* from 9000-year-old skeletons (Hershkovitz *et al*., 2008). In 1700 approximately one tenth of deaths registered in the London Bills of Mortality were caused by TB and by the end of the 18th century TB was estimated to cause a quarter of all deaths in Europe (Porter, 1989). TB can be contracted through the ingestion of infected cow's milk and

so the introduction of Pasteurisation helped to reduce the spread of the disease. Public health measures, including vaccination and contact tracing, improved diagnostic techniques and the introduction of antibiotic therapy have all been instrumental in reducing the incidence of TB. However, the disease has not been eradicated and by the 1980s the number of cases of TB had begun to increase.

Table 1-1 Phenotypic differences between five members of the *M. tuberculosis* complex (Nicol and Wilkinson, 2008; Goh *et al*., 2001)

Organism	Habitat	Primary route of transmission	Usual colony morphology
M. tuberculosis	Humans	Airborne transmission from person to person	Rough, eugonic growth
M. bovis	Wide host range: humans, cattle, cats, badgers etc.	Airborne transmission, ingestion of contaminated cow's milk	Intermediate rough, dysgenic growth
M. africanum	Humans	Airborne transmission from person to person	Rough, dysgenic growth
M. microtti	Rodents	Airborne transmission	Rough or smooth
M canetti	Humans	Airborne transmission	Smooth, eugonic growth

Cases of drug resistant TB due to poor compliance with treatment regimens (Shah *et al*., 2007) and the spread of human immunodeficiency virus (HIV) which leaves people susceptible to TB infection due to depletion of CD4+ T cells (Kaufmann and McMichael, 2005) have contributed to the rise in the number of cases of TB. In 1993, the World Health Organisation (WHO) declared that the rising incidence of TB was a global health emergency (WHO, 1993).

1.1.1 Global incidence of TB in humans

The WHO estimates that *M. tuberculosis* currently infects one third of the world's population, although the number of new TB cases has begun to decline. In 2007, it

was estimated that there were 9.3 million incident TB cases and 1.32 million deaths caused by the disease. Current data suggest that there were 8.7 million incident cases of TB in 2011 (equivalent to 125 cases per 100,000 population) and 1.4 million deaths (WHO, 2012c; WHO, 2010). The highest burden of TB is in developing countries with 59% of cases in 2011 occurring in Asia and 26% in Africa (WHO, 2012c). The country with the largest number of new TB cases in 2011 was India, which had over 20% of all cases (2 million cases).

1.1.2 Incidence of TB in the UK

The number of TB cases in the UK has been gradually increasing since 1987, when 5,085 cases were reported. By 2011, the number of cases had increased to 8,963 with a rate of 14.4 cases per 100,000 population. The most recent data for the UK show that 433 people who were diagnosed with TB died in 2010 (HPA, 2012). In order to reduce the incidence of TB in the UK it is important to understand the reason for the increasing number of cases.

Multi-drug resistant (MDR) TB, where bacteria are resistant to two of the three antibiotics used to treat the disease, accounted for nearly 4% of global TB cases in 2011 (WHO, 2012b). There has been a gradual increase in the number of MDR-TB cases in the UK, 0.9% of culture confirmed cases in 2000 were MDR, increasing to 1.6% in 2011 (HPA, 2012). However, the number of MDR cases in the UK is not increasing more quickly than the total number of cases suggesting that a rise in MDR is not driving the increase in TB.

People who are co-infected with HIV and TB accounted for 13% of global TB cases in 2010 (WHO, 2012a). The number of HIV and TB co-infections in England, Wales and Northern Ireland peaked in 2004 at 9% and according to the most recent data, less than 5% of TB patients also had HIV in 2010. (HPA, 2011b; Crofts *et al*., 2008). The downwards trend in cases of HIV and TB co-infections indicates that the prevalence of HIV is not responsible for the increase of TB in the UK.

The number of TB cases amongst the UK-born population has remained stable over the past decade, but there has been an increase in the number of cases amongst the non-UK born population (Figure 1-1) (HPA, 2012). Nearly three quarters of the cases reported in 2011 occurred in people who were not born in the UK and the rate of TB among this group (84 cases per 100,000 population) was more than 20 times the rate among people born in the UK (4 cases per 100,000 population) (HPA, 2012). The highest caseload of TB in the UK is amongst people born in the Indian subcontinent (ISC) and Africa (Figure 1-2). Immigration from countries where TB is endemic is therefore likely to be the major cause of increased TB cases in the UK (Gilbert *et al*., 2009).

There are also hot spots of TB infection, which occur in large urban areas such as London and the Midlands, UK where rates of TB are much higher than the national average and can reach those seen countries with high TB prevalence such as India and Africa (HPA, 2012).

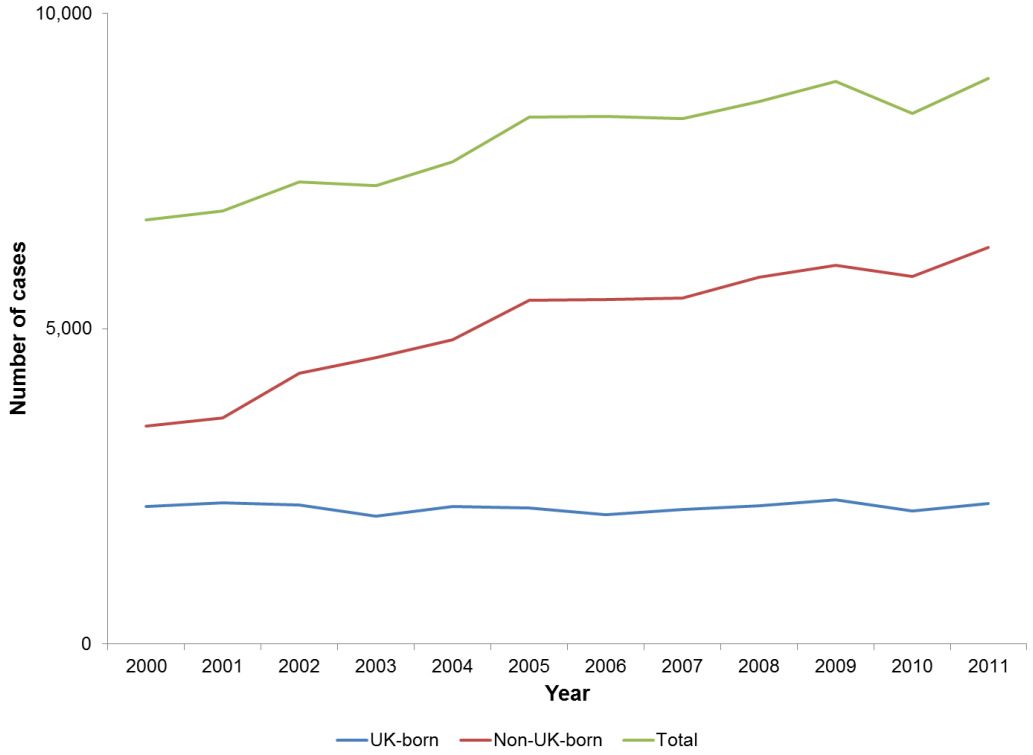


Figure 1-1 Tuberculosis case reports by place of birth, UK, 2000 to 2011

(HPA, 2012)

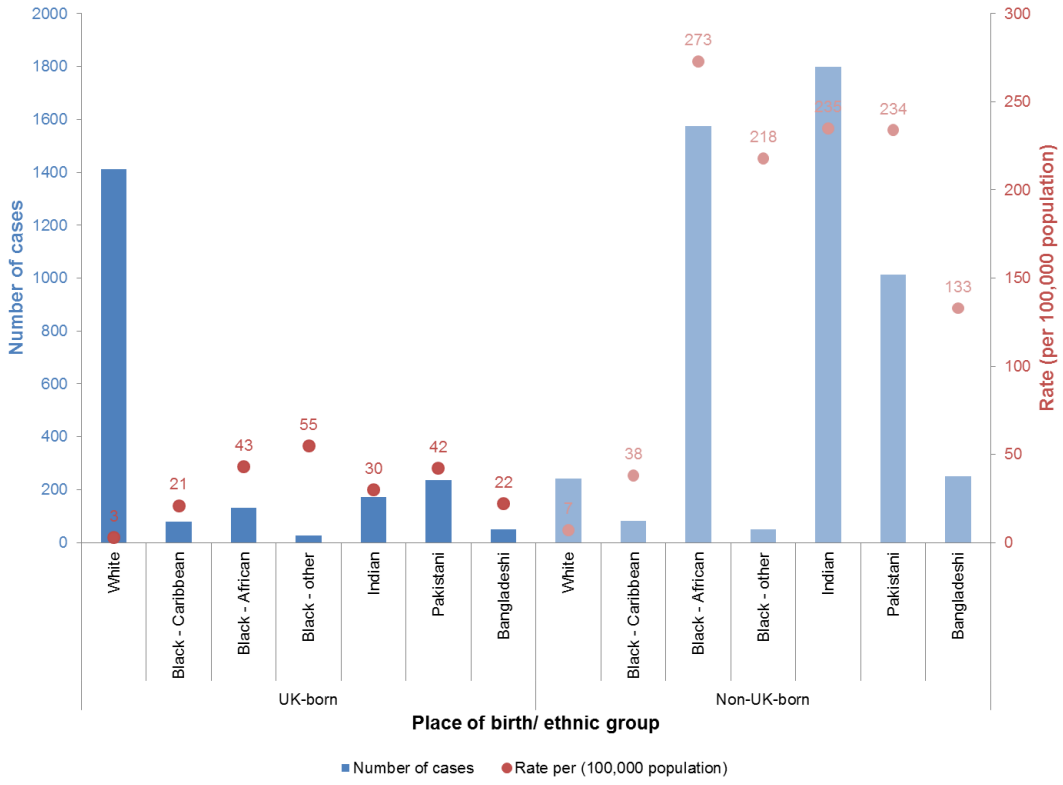


Figure 1-2 Tuberculosis case reports by place of birth and ethnic group, UK, 2009

(HPA, 2010)

The number of TB patients born in India has been increasing and India was the most common country of birth amongst non-UK born TB patients in 2009 and 2010 (Figure 1-3) (HPA, 2012). According to the 2001 census Indians and people born to Indian families were the largest ethnic minority group in the UK, constituting 2% of the total population and numbering just over a million people (ONS, 2003). The 2011 census shows that Indians are still the largest ethnic minority group, constituting 2.5% of the population and numbering 1.4 million people (ONS, 2012) (Figure 1-4). People from the Indian ethnic group accounted for a quarter of UK TB cases in 2010, the largest proportion of cases amongst a single ethnic group and the rate of TB was the second highest in this group (151 cases per 100,000 population) (HPA, 2011b).

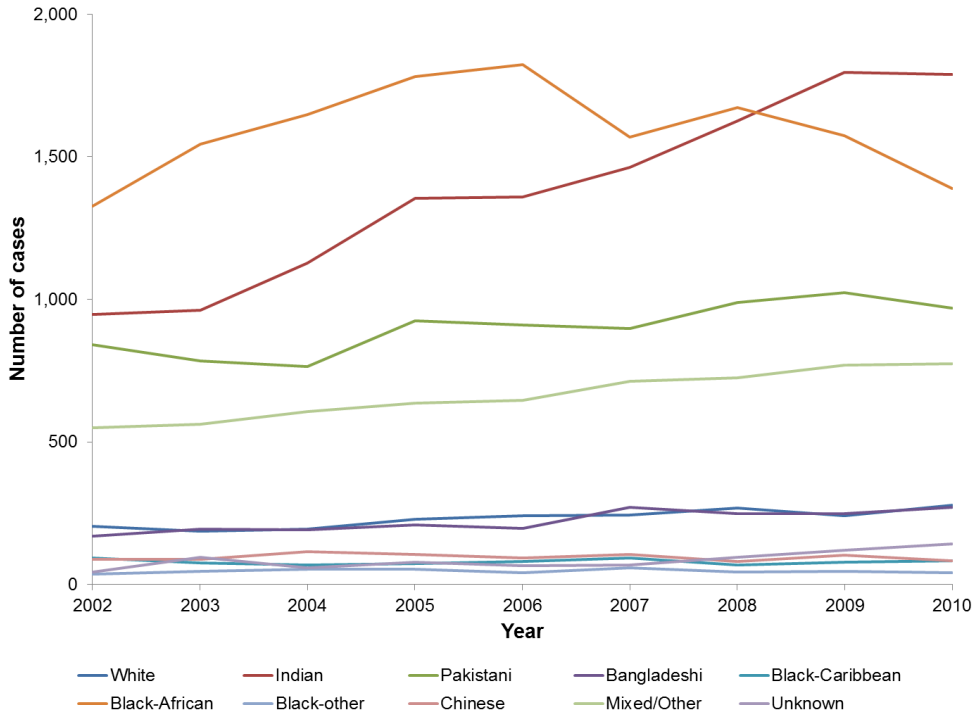


Figure 1-3 Tuberculosis case reports amongst non-UK born patients by ethnic group, UK, 2002 to 2010

(HPA, 2011b)

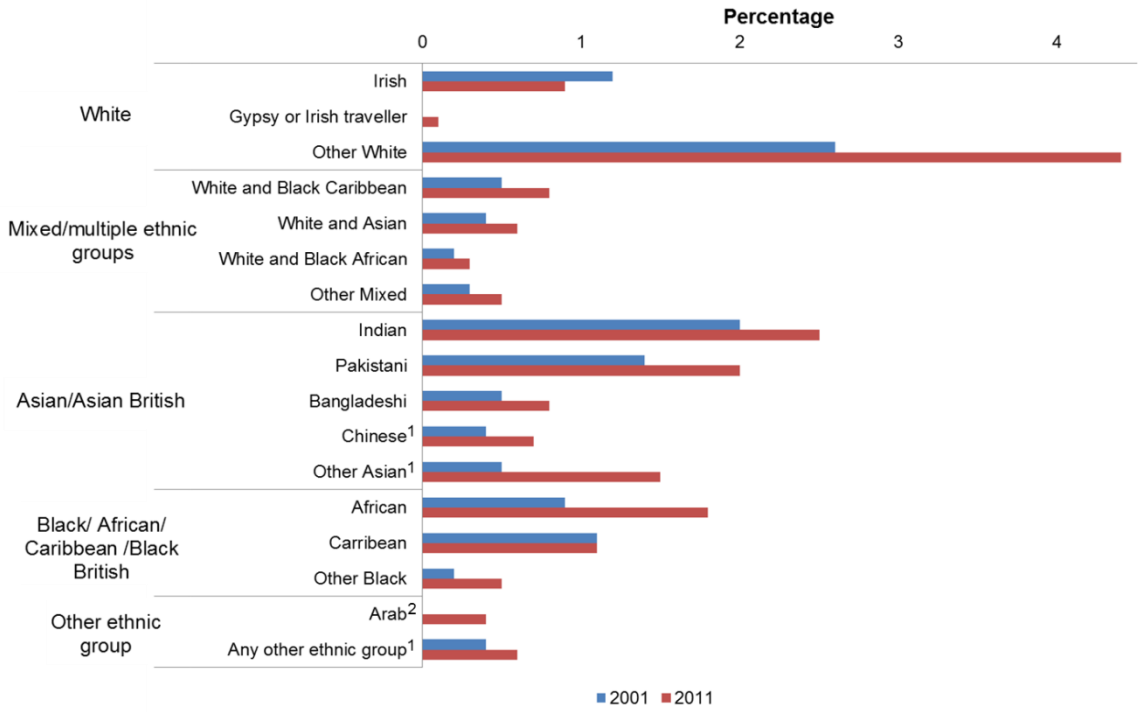


Figure 1-4 Ethnic groups in England and Wales, 2001 and 2011

(ONS, 2012; ONS, 2003)**.** 1. Comparability issues exist between these ethnic groups for the 2001 and 2011 Census. 2. No comparable data exists for these ethnic groups in the 2001 Census

1.2 Transmission of TB

TB can be contracted when *M. tuberculosis* is ingested (Rua-Domenech, 2006) or when it is introduced through the skin (Kramer *et al*., 1993). However, *M. tuberculosis* is predominately an airborne pathogen that spreads between hosts by inhalation.

1.2.1 Respiratory transmission of TB

Droplets of moisture from the respiratory tract are expectorated when people cough, sneeze or talk. Xie and colleagues found that when a person is talking, 60% of the droplets that are expelled from the mouth are smaller than 100 µm in diameter, which means that they evaporate rapidly (Xie *et al*., 2009; Xie *et al*., 2007; Wells, 1934). Droplets produced by people with active pulmonary TB contain bacilli and so an infectious core is left behind, following evaporation, which is known as a droplet

nucleus. Droplet nuclei can travel on air currents and remain airborne for several hours.

It is estimated that eight hours of exposure to an infectious person are required for TB transmission to occur (Musher, 2003; Riley *et al*., 1962). Table 1-2 shows the risk of TB infection associated with different types of exposure.

Table 1-2 Risk of infection with TB derived from different types of exposure to an infected person (Musher, 2003)

Nature of contact	Risk of infection*
None known	1 in 100,000
Casual social contact	1 in 100,000
School, workplace	1 in 50 to 1 in 3
Nursing home	1 in 20
Bar, social club	Up to 1 in 10
Dormitory	1 in 5
Home	1 in 3

* values are estimates, reflecting the number of bacilli that are aerosolised by the index patient, the proximity to that patient (ventilation, size of space) and the duration of the exposure.

The droplet nuclei that are produced when a person with active TB coughs, range in size between 1 and 7 µm (Fennelly *et al*., 2004). If another person inhales the droplet nuclei then the fate of the nuclei depends upon its size. The largest droplet nuclei fall out of airflow and become trapped in the mucus that coats the upper airways. The airway epithelium is lined with cilia, which move the mucus towards the mouth, thus removing the trapped particles from the lung.

Droplet nuclei that are smaller than 5 µm in diameter are carried directly to the alveoli by diffusion (Musher, 2003). The alveoli have no cilia (Figure 1-5) and so particles that are deposited here cannot be removed by mucociliary clearance.

Figure 1-5 Structure of the alveoli

Image taken from http://www.mmi.mcgill.ca/mmimediasampler/

1.3 Immune response to TB

1.3.1 Initial infection

Free macrophages are present in the alveoli. Their function is to ingest particles and destroy bacteria. Ingestion occurs when receptor molecules on the surface of the macrophage bind to the surface of a bacterium. *M. tuberculosis* is a facultative intracellular pathogen, which primarily inhabits macrophages. The outcome of the deposition of *M. tuberculosis* in the alveoli depends on the virulence of the bacteria and the microbicidal capacity of the alveolar macrophage (Dannenberg, 1991). The response of the alveolar macrophage can prevent the host from succumbing to TB. However, if the response is excessive the inflammation caused can lead to permanent tissue damage (Tomlinson *et al*., 2012).

It has been estimated that 70% of people who are exposed to *M. tuberculosis* are able to destroy the bacteria without specific macrophage activation. This estimate is based on the number of close contacts of TB patients who develop disease after exposure. There is also anecdotal evidence of natural resistance to *M. tuberculosis*. One such example is the Lübeck disaster, which occurred in 1929 when 251 new-borns were mistakenly given virulent *M. tuberculosis* instead of the Bacille Calmette-Guérin (BCG) vaccine (discussed in section 1.4). Within a year of inoculation, 72 infants had died and 135 had developed active TB, although they later recovered. However 44 infants did not develop any clinical signs of infection during a 12-year follow up period (Rivero-Lezcano, 2012). Cobat and colleagues carried out tuberculin skin tests (TST) on a sample of 128 families in Cape Town, where the annual rate of TB is 940 cases per 100,000 population and found that over a period of 5 years, 40% of the people tested remained TST negative (Cobat *et al*., 2009).

The macrophages of people who are exposed to TB but do not become infected are able to produce toxic reactive nitrogen and oxygen intermediates in response to ingestion of *M. tuberculosis*. The phagosome, containing the ingested bacteria, fuses with lysosomes, which introduces digestive enzymes that destroy the bacteria (Hope *et al*., 2004). Proteins from dead bacteria are displayed on the surface of the macrophage, in association with major histocompatibility complex (MHC) class II. This allows the antigen to be presented to T cell receptors on CD4+ T cells. Lipids can be presented to different sets of T cells, including CD8+ T cells by CD1 molecules (Figure 1-6). This causes stimulation of the T cells and the macrophage

which presented the antigen and results in further activation of the immune response (Pieters, 2001).

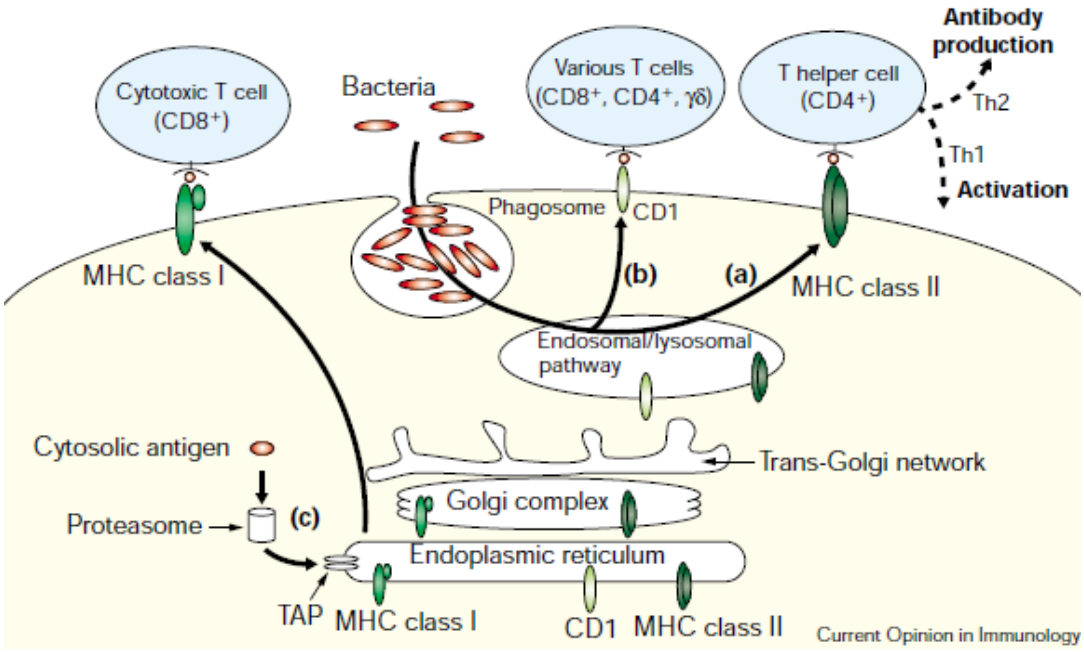


Figure 1-6 Interaction of the phagosomal/endosomal pathways with the adaptive immune system

(a) Antigens that are degraded in the endosomal/lysosomal system can be presented by MHC class II molecules. Peptides in complex with MHC class II can be recognized by T helper cells, that can either activate the presenting cell (this is done by Th1 cells) or stimulate the production of antibodies by B cells (this is done by Th2 cells).
(b) In addition, lipid moieties derived from microbes are presented to various T cell subsets (CD4+, CD8+ or γδ T cells) by CD1 molecules.
(c) MHC class I molecules present peptides - derived from antigens located in the cytosol and processed by the proteasome and TAP (transporter associated with antigen processing) - at the cell surface to CD8+ cytotoxic T cells. Activation of cytotoxic T cells leads to rapid lysis of the presenting cell (Pieters, 2001).

In 30% of individuals, the actions of the alveolar macrophage are not sufficient to kill the *M. tuberculosis*. It has been suggested that the type of macrophage receptor that a bacterium binds can determine the response that is generated by the macrophage (Le Cabec *et al*., 2000; Astarie-Dequeker *et al*., 1999). Another study suggests that when reactive nitrogen species, produced by the macrophage, are at a sub-lethal concentration they signal the bacteria to enter a quiescent state by up-regulating the

expression of a set of 48 genes, which are associated with dormancy (Voskuil *et al*., 2003). It has also been shown that when macrophages are unable to destroy *M. tuberculosis* the bacteria prevent the normal maturation of the phagolysosome. Some studies have shown that mycobacteria prevent the fusion of the phagosome with the lysosome (Vergne *et al*., 2005; Clemens and Horwitz, 1995; Armstrong and Hart, 1971), whereas others have reported that *M. tuberculosis* is able to escape from the phagolysosome (McDonough *et al*., 1993). Ingested *M. tuberculosis* remains viable and bacterial antigens are not presented on the surface of the infected macrophage. Macrophages burst when the number of intracellular bacteria becomes too large. The content of the burst macrophage is discharged into the alveoli, releasing the bacteria, which are ingested by other alveolar macrophages, or monocytes that migrate from the blood stream. These cells are not activated and so the bacteria continue to divide and more monocytes are recruited to the area. A primary complex forms, which consists of a small lesion at the site of infection and enlarged regional lymph nodes.

Dendritic cells are a small population of cells that line the trachea and can also phagocytose mycobacteria. Once mycobacteria have been ingested, the dendritic cell travels to the draining lymph node and displays mycobacterial antigens on the cell surface. Dendritic cells are able to activate T cells and can polarise the T cell response towards Th1 or Th2 phenotypes (Hope *et al*., 2004). Th1 and Th2 phenotypes are discussed in section 1.3.2.

1.3.2 Cell mediated immunity

A cell mediated immune (CMI) response is generated following the formation of a primary complex, which leads to an influx of inflammatory cells such as macrophages and T cells. There are subsets of T cells, which can be classified by the presence of cell surface molecules. T cells that express the cell surface molecule CD4 are known as helper T cells. T helper cells are further classified into T helper 1 (Th1) and T helper 2 (Th2) subsets. Th1 and Th2 cells produce cytokines, which are cell-signalling molecules that modulate the immune system (Berger, 2000). Th1 cells, which have been activated by dendritic cells, recognise the antigen displayed by infected macrophages and respond by secreting pro-inflammatory cytokines such as interferon-gamma (IFN-γ). IFN-γ activates macrophages, increasing production of nitrogen and oxygen metabolites, which are toxic to microorganisms. Macrophage activation also increases the rate at which phagosomes and lysosomes fuse. Phagosomes contain the ingested bacteria and lysosomes contain digestive enzymes so an increase in their rate of fusion increases the capacity of the macrophage to kill intracellular bacteria.

If the activated macrophage is still unable to kill the mycobacteria, it becomes successively less responsive to activation. The persistently infected macrophage will be surrounded by lymphocytes and macrophages, which encase it, forming a granuloma or tubercle. The infected macrophages are destroyed by natural killer cells and later by cytotoxic T cells, in order to eliminate the intracellular environment that favours bacterial growth. The centre of the granuloma undergoes caseous

necrosis, which forms a solid structure that is presumed to inhibit the growth of extra-cellular bacteria.

Approximately 10% of people who form a primary complex develop primary active TB. Primary TB usually occurs in children and immunocompromised adults because the CMI response is inadequate. Bacilli can escape from the edge of the caseous area and begin multiplying in non-activated macrophages, which are present at the edge of the caseous area. Cytotoxic T cells, which express the cell surface molecule CD8, destroy the infected macrophages and the caseous area is enlarged. This process destroys lung tissue and the bacteria are able to spread by the lymphatic system to other sites, where they may cause extra-pulmonary TB. If the lungs sustain enough injury, it will result in death (Ottenhoff, 2012; Davies, 1999).

Immunocompetent hosts develop good CMI and the macrophages that surround the caseum are highly activated. This means that any bacilli that escape from the caseous centre are ingested and destroyed. The lesions do not enlarge and the patient is unaware of the infection. These tubercles can calcify and become visible on X-rays (Janeway, 1999).

The infection is contained in such individuals by the CMI response but it is not eliminated and so people are described as being latently infected. *M. tuberculosis* is able to persist within granulomata for decades without causing any sign of illness in the host. Latently infected people are not able to transmit the infection to other people but they have a 10% risk of developing active TB over their lifetime

(Comstock, 1982), with this risk increasing if accompanied by HIV infection (Corbett and De Cock, 1996).

A weakening of the host immune system, which could be caused by malnutrition, stress, old age or the administration of therapeutic immunosuppressants, causes the caseum of the granulomata to liquefy, although the mechanism by which this occurs is unknown. The growth of bacteria was inhibited by the solid caseum but once the caseum is liquefied, the bacilli are able to multiply extracellularly. A large quantity of bacteria builds up and the granuloma breaks down, releasing the bacteria and leading to active post-primary TB. The large numbers of bacilli that are released often overwhelm the CMI response. A delayed type hypersensitivity (DTH) reaction destroys the lung tissue and discharges the bacteria into the airways where they can infect other areas of the lung and spread to other people (Figure 1-7).

1.4 Vaccination

The only established vaccine available against *M. tuberculosis* is BCG, which is a live attenuated vaccine that was produced by repeated passage of an *M. bovis* isolate using a medium containing bile (Calmette, 1931). After 230 passages it was shown that the strain was no longer able to cause disease in animals or humans. Administration of the BCG vaccine to humans began in 1921 and since then it has been given to over three billion people. It was not possible to store the vaccine strain in 1921 so its repeated passage continued so that there are now several different vaccine strains (Behr, 2002).

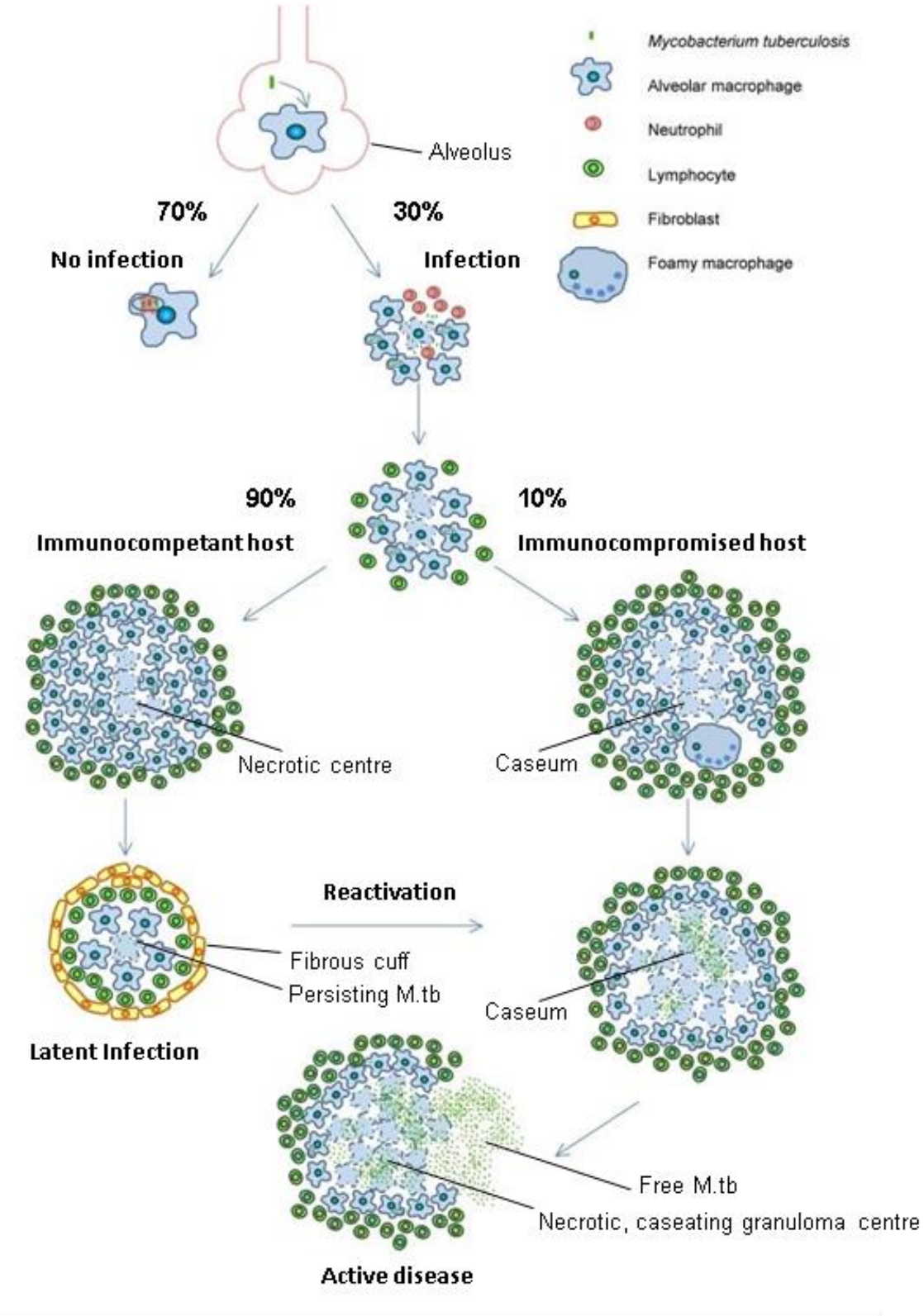


Figure 1-7 Result of infection with *M. tuberculosis*

Adapted from (Sasindran and Torrelles, 2011)

The efficacy of the BCG vaccine has been questioned; several studies have shown that the protective immunity varies in different human populations, which may be due in part to the use of different vaccine strains. Datta and colleagues reported that BCG had no protective immunity in adults in south India (Tuberculosis Research Centre, 1999), whereas 80% protection has been reported in adults in the UK (Colditz *et al*., 1994). The vaccine appears to be more efficacious against TB meningitis in young children than it is against pulmonary TB in adults (Fine, 2001; Colditz *et al*., 1995; Colditz *et al*., 1994; Comstock, 1994; Rodrigues *et al*., 1993). Routine BCG vaccination of UK schoolchildren ended in 2005 as the risks associated with the vaccine, including infection of immune-compromised people were deemed to outweigh the protective benefits of the vaccine. The vaccine is now only recommended in the UK for babies born in areas with high TB rates, health workers, recent immigrants from countries where TB is endemic and people who have come into close contact with a TB patient.

1.5 Diagnosis of TB

The symptoms of pulmonary TB in humans include a persistent cough, weight loss, fever and night sweats. People feel generally tired and unwell and suffer a loss of appetite. In the later stages of the disease, there may be blood present in the mucus or sputum that is coughed up from the airways.

1.5.1 Clinical diagnosis of TB

A sputum sample and a chest X-ray are taken from a person who shows signs of pulmonary TB. Extra-pulmonary TB is diagnosed with computerised tomography (CT)

or magnetic resonance imaging (MRI) scans. Blood and urine samples are collected for identification of systemic mycobacterial infection and a tissue biopsy may be taken from the infected area when extra-pulmonary TB is suspected.

1.5.2 Immunological diagnosis of TB

People who may be infected with TB but are asymptomatic (e.g. contacts of index patients or immigrants from areas where TB is endemic) can be tested for latent TB. A person who has been exposed to TB will have memory T cells that are specific for TB antigens. Subsequent intra-dermal or subcutaneous injection of protein antigen causes a local T cell-mediated inflammatory reaction, which evolves over 24-72 hours. The reaction results in a palpable, raised and hardened area (known as an induration) that indicates a positive TST. If a person has been vaccinated with BCG or has been in contact with non-TB mycobacteria then this will also cause a reaction when the TST is administered. The sensitivity of TST in immunocompromised people is low. The problems associated with TST have led to the development of blood tests for TB, which are called IFN-γ release assays (IGRAs).

An IGRA measures the amount of IFN-γ that is released by CD4+ T cells. Blood samples are mixed with two *M. tuberculosis* specific protein antigens, which are not present in BCG or in non-TB mycobacteria (Brosch and Vincent, 2007). If a person has been in contact with *M. tuberculosis*, their T cells will react to the antigen and release IFN-γ. The amount of IFN- γ that is released can be measured using an enzyme linked immunosorbent assay (ELISA) (Mazurek *et al*., 2001).

1.5.3 Laboratory diagnosis of TB

Samples from patients including sputum, blood, urine or tissue biopsies are sent for bacteriological identification. Samples from non-sterile sites may contain commensal flora so they are decontaminated with 4% sodium hydroxide. Decontamination is necessary because *M. tuberculosis* has a slow growth rate, which means that cultures are quickly dominated by contaminants, making it difficult to isolate the mycobacteria. Rapidly growing microorganisms have a lower resistance to sodium hydroxide than mycobacteria, so a controlled exposure to sodium hydroxide can kill the contaminants without destroying all of the mycobacteria.

1.5.3.1 Microscopic observation of acid fast bacilli

The cell wall of mycobacteria is rich in lipids, which makes them resistant to acid-alcohol decolourisation when certain staining techniques are employed; this is described as being acid-fast. Two staining techniques are routinely used for identification of TB, the Ziehl-Neelsen (ZN) method, which stains mycobacteria pink and a fluorescent procedure using auramine, which stains mycobacteria yellow-green (Figure 1-8).

Microscopic analysis is inexpensive and provides rapid results, although there is a requirement for experienced staff who can distinguish between mycobacteria and other acid-fast bacteria. A lack of observable acid-fast bacteria in a stained sample does not always mean that a patient is not infected and so in the UK further tests are carried out. In countries where there is a high burden of disease, microscopy may be the only diagnostic test that is performed.

1.5.3.2 Mycobacterial culture

Culture of patient samples is more sensitive than microscopy for the detection of *M. tuberculosis* (Yeager, Jr. *et al*., 1967). In the UK, patient samples are inoculated into mycobacterial growth indicator tubes (MGITs™) which contain Middlebrook 7H9 liquid broth and an oxygen-quenched fluorophore. Bacteria deplete oxygen as they respire until there is insufficient oxygen to quench the fluorophore, which causes an increase in fluorescence. Tubes are placed in a BACTEC™ MGIT™ 960 System (BD Biosciences, Oxford, UK), which incubates them at 37°C and monitors for increasing fluorescence. Once the fluorescence from a tube reaches 75 units it is declared positive. Tubes that show no fluorescence for a minimum of 34 days are declared negative.

MGITs™ also contain an antimicrobial mixture called PANTA™ (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) which reduces contamination from non-mycobacterial organisms and an enrichment, OADC (Oleic acid, Albumin, Dextrose and Catalase) which is essential for growth of mycobacteria.

1.5.3.3 Genotyping using DNA amplification

Bacteria from positive MGIT™ cultures are identified with a species specific *M. tuberculosis* complex DNAstrip® test (Hain Lifescience, Nehren, Germany) (Richter *et al*., 2004). The test can differentiate between *M. tuberculosis*, *M. africanum*, *M. bovis*, *Mycobacterium caprae*, *M. microti* and *M. bovis* BCG.

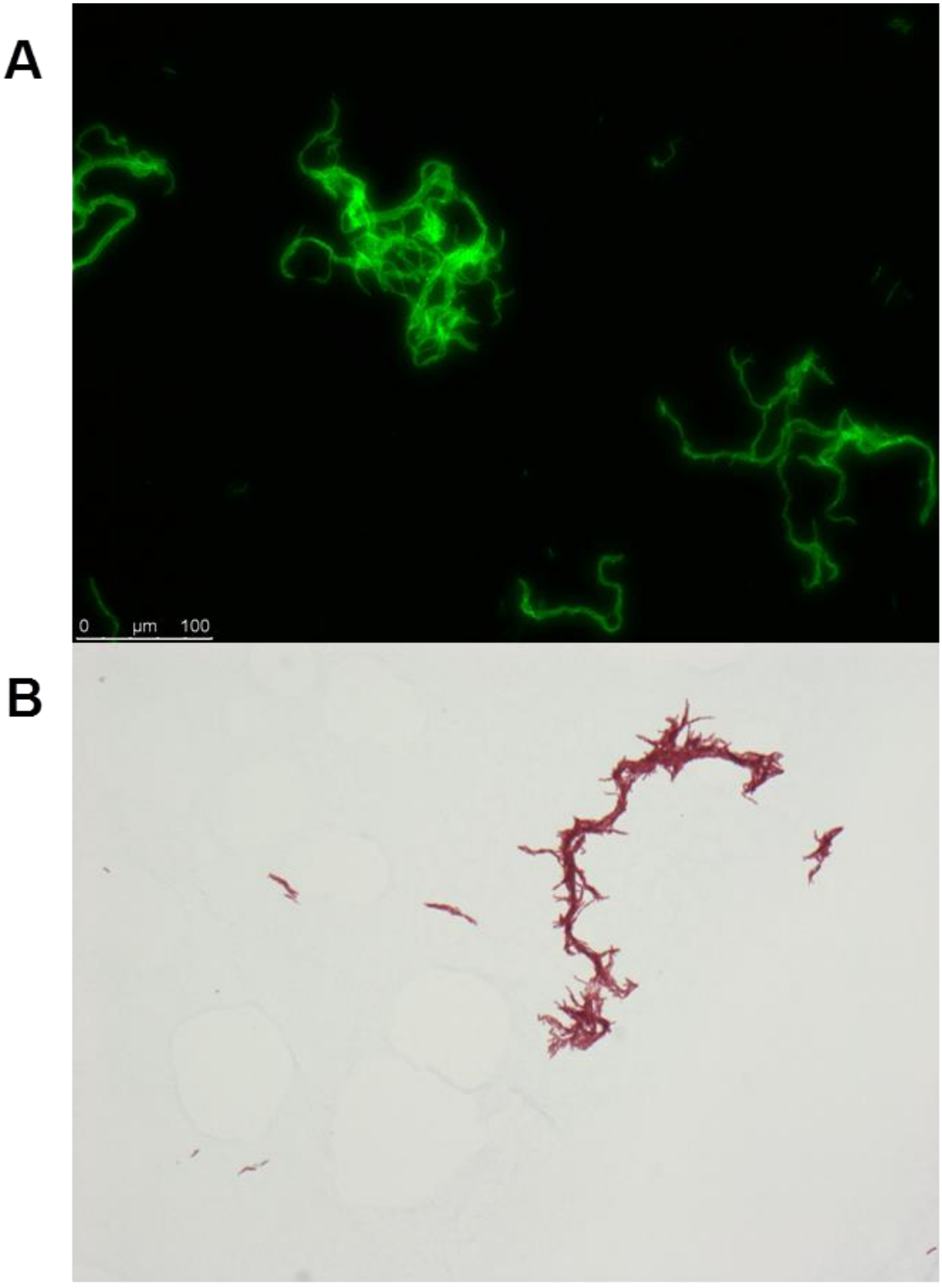


Figure 1-8 Examples of different acid fast stains

A. Auramine stained *M. tuberculosis* H37Rv (yellow/ green)
B. Ziehl-Neelsen stained *M. tuberculosis* H37Rv (pink)
Images were taken during the current study.

DNA is extracted from positive cultures and amplified by PCR using several primers each of which are specific to a different species. The amplicons are denatured and placed on a DNAstrip® that is coated with complementary probes. The amplicon binds to its complementary probe and a conjugation reaction produces a colour that appears as a band. The specific banding pattern is used to identify the species (Figure 1-9).

1.5.3.4 Antibiotic sensitivity testing

Liquid culture is used to test the susceptibility of a patient isolate to isoniazid, rifampicin, ethambutol and pyrazinamide, which are the four drugs used in routine treatment of TB. The test is based on the growth of an *M. tuberculosis* isolate in a MGIT™ containing a drug compared to a MGIT™ that does not contain the drug.

If there is resistance to rifampicin or isoniazid then it is confirmed using a test that is similar to the Hain Lifescience genotype test, which can identify the presence of a DNA mutation conferring drug-resistance.

1.6 Treatment of TB

Patients with drug sensitive TB are treated with a combination of the antibiotics rifampicin, isoniazid, pyrazinamide and ethambutol for two months, followed by rifampicin and isoniazid for a further four months (Brosch and Vincent, 2007; Gillespie and Hawkey, 2006).

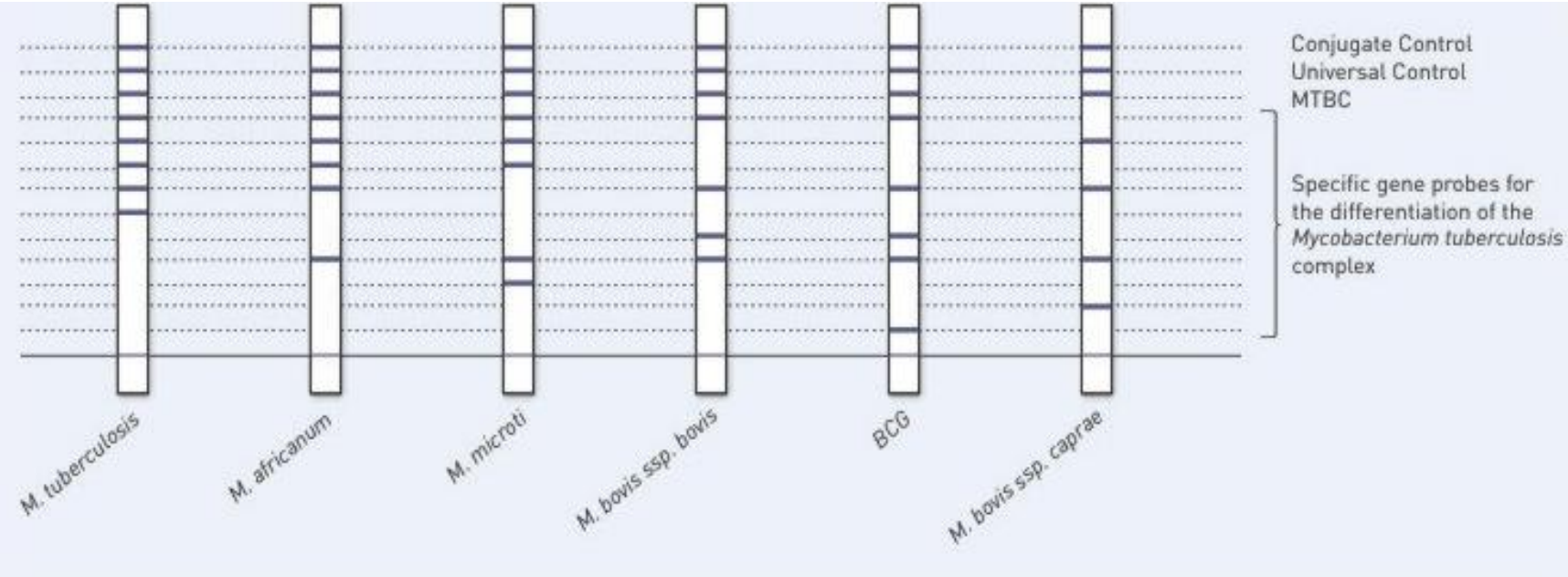


Figure 1-9 Differentiation of *M. tuberculosis* complex organisms using the Hain Lifescience genotype test

Image taken from www.hain-lifescience.de

Rifampicin inhibits mycobacterial transcription by binding to DNA-dependent RNA polymerase and blocking the path of the elongating RNA transcript. Mutation in a specific region of a gene, which encodes the β-subunit of RNA polymerase (*rpoB*), prevents rifampicin from binding, leading to rifampicin resistance (Campbell *et al*., 2001). More than 96% of rifampicin resistant strains have a mutation in an 81 base pair region of *rpoB* (Telenti *et al*., 1993).

Isoniazid is believed to block the synthesis of components of the mycobacterial cell envelope. Isoniazid is a pro-drug that is activated by a mycobacterial catalase-peroxidase. Approximately half of the clinical isolates that are resistant to isoniazid have mutations in the *katG* gene, which codes for the primary catalase-peroxidase (Somoskovi *et al*., 2001).

Pyrazinamide is also a pro-drug, which is activated by a mycobacterial enzyme. Pyrazinamide is thought to disrupt fatty acid synthesis and resistance can arise when the action of the activating enzyme is ablated by mutation. Up regulation of efflux pumps that extrude pyrazinamide can also lead to drug resistance (Louw *et al*., 2009; Somoskovi *et al*., 2001).

Patients who have drug resistant TB can be treated with second line antibiotics, which are prescribed on an individual basis, although cases of extensively drug resistant TB (XDR-TB) have been described which are resistant to all known antibiotics (Velayati *et al*., 2009).

1.7 Evidence for *M. tuberculosis* phenotypic strain diversity

TB disease results from the interaction of a favourable environment for transmission, a susceptible host, and a virulent strain of *M. tuberculosis*. Virulence can be defined as the ability to cause disease, mortality and morbidity, or the capacity of a pathogen to overcome host defences (Thomas and Elkinton, 2004). Several strains of *M. tuberculosis* are described as having increased virulence. There are also examples of attenuated strains of *M. tuberculosis*, which have lost their virulence. Some of the observations of strains with altered virulence are described below.

1.7.1 Laboratory strains

In 1905, a strain of *M. tuberculosis* was isolated from the sputum of a patient with pulmonary TB (Steenken *et al*., 1934). This isolate was called H37 and it was used extensively for laboratory studies of TB. In 1926, Petroff and Steenken found that H37 occasionally failed to infect guinea pigs (Petroff and Steenken, 1930). Different types of culture media were used to 'dissociate' H37 into two distinct variants. One was termed H37Rv as it was virulent in guinea pigs and the other was termed H37Ra as it was avirulent (Steenken, 1935; Steenken *et al*., 1934). H37Ra and H37Rv are available from the American Type Culture Collection (ATCC) but the original H37 strain was lost.

1.7.2 Epidemiological strain variation

A number of TB outbreaks have provided evidence that some strains of *M. tuberculosis* possess characteristics that make them better able to spread between hosts and more proficient at causing disease (Thwaites *et al*., 2008). The strain CDC1551 caused an outbreak of TB near Kentucky, USA in 1995, which was

characterised by a high rate of transmission to patient contacts. Patient contacts also had a strong response to TST (Valway *et al*., 1998). Initial investigators described CDC1551 as hypervirulent because transmission occurred in a low risk population and there was evidence of transmission occurring in an outdoor setting. The authors suggested that transmission was due to a characteristic of the strain rather than susceptibility of the patients or environmental factors that may have favoured the spread of the disease. CDC1551 belongs to the X3 spoligotype clade (spoligotype international type 549) (Mathema *et al*., 2012).

A study by Zhang and colleagues identified five unique isolates from patients with pulmonary TB in Los Angeles, USA. They were described as unique because each strain was only isolated once during the study period, none of them had caused disease in another person prior to their identification and they were not found in any other patient in the four-year follow up study. The isolates were all taken from sputum samples that contained acid-fast bacilli and could be grown in culture. All five patients had spent time at homeless shelters before they were diagnosed with TB. The authors concluded that each of these strains had caused disease in only one person, despite being present in an environment where transmission of TB was common. The people in the homeless shelters who were exposed to these isolates had high risk factors for TB development, which implies that the reason that these strains did not spread to others was due to the characteristics of the strains (Zhang *et al*., 1999).

In 2001, the Crown Hills (CH) strain caused an outbreak of TB in a school in Leicester, UK. Tuberculin skin testing was performed on 1,128 people who attended

the school which identified a further 69 cases of active TB and 254 cases of latent infection (Ewer *et al*., 2003). The rate of transmission was unusually high in this outbreak and there was a high rate of active disease (Rajakumar *et al*., 2004). The CH strain is a member of the Central Asian Spoligotype (CAS) clade.

1.8 Evidence for *M. tuberculosis* genotypic strain diversity

Variation between strains of the same bacterial species can have a large impact on the disease that they cause in humans. The influence of strain variation is well established for a number of pathogens, including *Escherichia coli*, *Haemophylus influenzae* and *Neisseria*, *Bordetella* and *Streptococcus* species.

Infection with *M. tuberculosis* can lead to active or latent TB, as discussed in section 1.3. There are several well-known risk factors that increase the probability of developing active TB, including behaviours such as substance abuse (Oeltmann *et al*., 2009; Reichman *et al*., 1979), underlying medical conditions and socioeconomic factors including poor diet and housing (Goh *et al*., 2001; Terris, 1948).

Several human gene polymorphisms, which increase the risk of contracting TB disease have been identified (Bellamy *et al*., 1998), for example those leading to altered serum levels of mannose binding protein (Soborg *et al*., 2003; Selvaraj *et al*., 1999). There is a polymorphism in the Toll-like receptor 2 gene, which was found more significantly associated with TB patients than with healthy control subjects (Ogus *et al*., 2004). Polymorphisms in the gene encoding the vitamin D receptor have been linked to an increased likelihood of developing active TB (Wilbur *et al*., 2007; Wilkinson *et al*., 2000). However a meta-analysis carried out in 2005 that looked at

vitamin D receptor polymorphisms found that results were inconclusive and studies were underpowered (Lewis *et al*., 2005). More recently, a study compared the interleukin-23 (IL-23) receptor gene of 168 TB patients with 150 healthy people in Tunisia and found an association between a functional single nucleotide polymorphism (SNP) and the severity of pulmonary disease (Ben-Selma and Boukadida, 2012). These observations have focussed attention on host factors however, it is also important to consider the effect that bacterial variation has on the outcome of infection with *M. tuberculosis*.

In order to investigate the genetic diversity of *M. tuberculosis* strains and the impact that this has on human disease, molecular strain typing techniques are required, which can identify genotypic differences between strains. The sampling frameworks that are used to discriminate between strains exploit the unique ways in which *M. tuberculosis* undergoes genetic rearrangements (Gagneux and Small, 2007; Tanaka *et al*., 2000).

The advent of molecular strain typing techniques that analyse the genome of bacterial isolates has revolutionised outbreak investigations by detecting or refuting epidemiological links. Effective control of TB transmission relies on tracing the contacts of people with active disease and treatment of latent cases before they become active. However because *M. tuberculosis* can exist in a dormant state, strains that belong to the same outbreak can be temporally separated and become difficult to link by common case factors such as location and time.

1.8.1 Typing methods giving insight into clinical outbreaks

Clinical isolates of *M. tuberculosis* are usually typed by analysing chromosomal regions that are associated with mobile insertion elements or repetitive DNA sequences (Barnes and Cave, 2003). In order to be clinically applicable, the rate at which polymorphism in the chosen chromosomal region takes place, must be fast enough to distinguish between strains that are not related by epidemiology, but must also occur slowly enough to allow related cases to be linked reliably (Mathema *et al*., 2006). Three methods have been widely used for typing clinical isolates, they are summarised in Table 1-3 and discussed below in detail.

Table 1-3 Comparison of the three most commonly used *M. tuberculosis* strain-typing techniques in clinical practice (Malik and Godfrey-Faussett, 2005)

Method	Target	Culture required	Time	Output	Discrimination (compared with IS*6110* RFLP)	Cost
IS*6110*-RFLP[1]	IS*6110* insertion sequence	Yes	Weeks	Analog	Gold standard: limited in isolates with <6 copies	High
Spoligo-typing	43 DR[2] cluster; presence or absence	No	Days	Digital	Lower	Low
MIRU-VNTR[3]	12 MIRU used	No	Days	Digital	Slightly lower	Medium

[1] RFLP=restriction fragment length polymorphism
[2] DR=direct repeat
[3]MIRU-VNTR= mycobacterial interspersed units containing variable number of tandem repeats

1.8.1.1 IS*6110* restriction fragment length polymorphism

IS*6110* is an insertion element that is widely dispersed throughout the *M. tuberculosis* complex. The number of copies of IS*6110* in the genome and their

location cause variation between *M. tuberculosis* strains (Tanaka *et al*., 2000). Digestion of DNA from clinical isolates with a restriction enzyme results in different sized fragments that are detected by Southern hybridisation. Differences between strains are known as restriction fragment length polymorphisms (RFLP).

IS*6110* RFLP was the first genomic method used for typing of clinical *M. tuberculosis* isolates and it gives good discrimination between strains. However, the technique is labour intensive and requires large amounts of genomic DNA (Gascoyne-Binzi *et al*., 2002). Skilled workers are needed to identify patterns and the data is not digitised, making comparison of results difficult. Some strains, including many from the ISC, have less than six copies of IS*6110* and cannot be typed by IS*6110* RFLP, (Sola *et al*., 2003). The method also assumes that distribution of IS*6110* through the genome is random; however, IS*6110* is restricted to two-thirds of the genome and there are insertion hot spots (Gillespie *et al*., 2000). IS*6110* RFLP analysis of clinical isolates has been superseded by PCR-based methods, which are more suitable for high through put screening (Fang *et al*., 1998).

1.8.1.2 Spoligotyping

The DR locus of *M. tuberculosis* complex organisms contains a variable number of well-conserved 36 base pair direct repeats, interspersed with non-repetitive unique spacer sequences (Figure 1-10A). Uni-directional deletion of spacers and repeats causes variation between strains. In *M. tuberculosis* H37Rv, which has a genome of 4.4 Mb, the DR locus is 5,186 base pairs in length. PCR amplification of the locus, using primers targeting DRs, produces multiple DNA fragments of different sizes

(Figure 1-10B). Hybridisation of the fragments to 43 spacer oligonucleotides (37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG) allows detection.

If a strain is positive for a spacer it is represented by the number 1 and if it is negative, it is represented by a 0, which produces a 43 digit binary code known as a spoligotype. Spoligotypes can be compared to an international database called SpolDB4 (Brudey *et al*., 2006), which can be queried online using SITVIT (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). The database contained 39,609 strains from 122 countries when it was accessed in January 2013. Bioinformatics and a number of visual rules are used to classify spoligotype patterns into 62 clades or lineages, for example, members of the Beijing clade have retained spacers 35 to 43 but spacers 1 to 34 are absent (Filliol *et al*., 2003). Clades are generally named after the area where a spoligotype was first identified however groups of poorly defined strains that have been isolated from several areas have been named using letters (Table 1-4). Spoligotype clades can be sub-divided into shared-types (STs) which are also known as spoligotype international types (SITs).

The existence of the SpolDB4 database means that spoligotypes can be used to make global comparisons between strains. However, spoligotyping does not discriminate between strains as well as IS*6110* RFLP and is therefore not as useful for outbreak investigation (Nguyen *et al*., 2004).

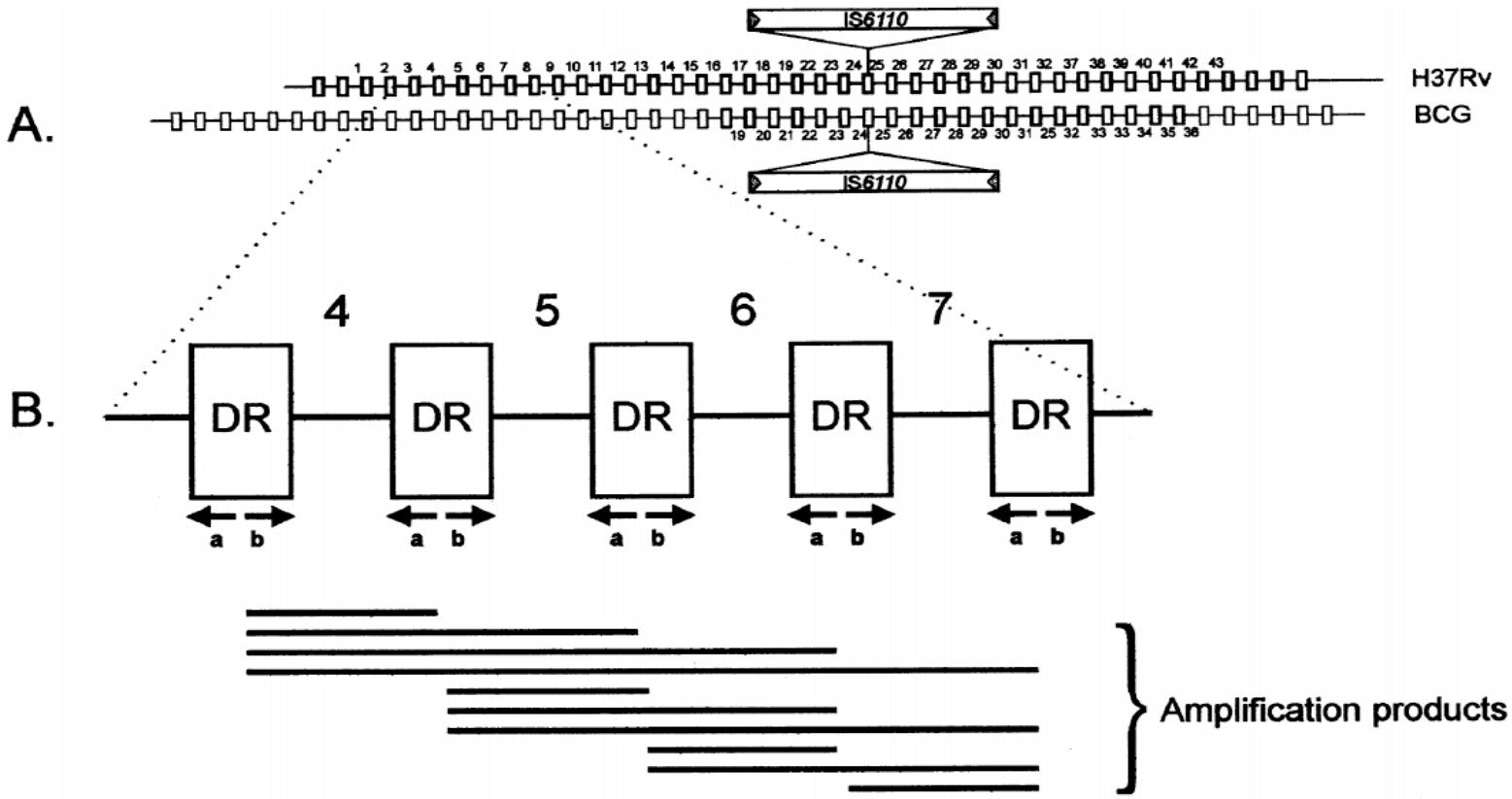


Figure 1-10 Structure of the direct repeat locus

A. Structure of the DR locus, DRs are depicted by rectangles and numbers correspond to unique spacer regions.
B. PCR amplification of the DR region. All DRs in the region can act as targets for the primers so that DNA is a mixture of fragments of different sizes. The amplification products shown would be produced from amplification of a region containing only five contiguous DRs (Kamerbeek *et al*., 1997).

Spoligotyping was carried out by reverse line blot hybridisation, which was labour intensive and relied on skilled workers to visually assign the designation of each spacer. The introduction of a partially automated multi-analyte bead based system has reduced the amount of time taken to produce a spoligotype and the designation of positive or negative spacers is more accurate.

Table 1-4 Definition of major clades present in the SpolDB4 database (Brudey *et al*., 2006)

Clade name	Derivation of clade name	Geographical regions where the clade is commonly isolated
CAS	Central Asian Spoligotype	Far east Asia, ISC and Tanzania
EAI	East African Indian	Originated in east Africa and India
U	Unknown	Isolated in Bangladesh
Beijing	Beijing	China with recent global spread
LAM	Latin American Mediterranean	Mediterranean basin, and the Caribbean
S	Sicilian	Sicily and Sardinia
T	Poorly defined	World wide
Haarlem	Haarlem	Europe
X	Poorly defined	UK, USA and former British colonies

1.8.1.3 Mycobacterial interspersed repetitive unit (MIRU) typing

Variable number of tandem repeats (VNTRs) are locations in the genome where a short nucleotide sequence is organised as a tandem repeat. Two or more nucleotides are repeated and the repetitions are directly adjacent e.g. GATCG|GATCG|GATCG. VNTRs can increase and decrease by rare strand slippage of DNA polymerase, recombination and looping out of sections of DNA.

Mycobacterial interspersed repetitive units (MIRU) containing VNTRs (MIRU-VNTR) are mini-satellite sequences of 53-111 base pairs in length. They occur throughout the genome as single or multiple repeats. MIRU-VNTR profiles are generated using a PCR-based method where multiple independent loci are amplified and then differentiated by size, which can be used to calculate the number of repeats at each locus as shown in Figure 1-11 (Cowan *et al*., 2002).

MIRU-VNTR can discriminate between strains better than spoligotyping and IS*6110* RFLP for low IS*6110* copy number strains, although it does not discriminate as well as IS*6110* RFLP for high IS*6110* copy number strains (Nguyen *et al*., 2004). However, the method is widely used for clinical investigation, as it is robust and easy to perform. The process has been partially automated and results can be digitised for comparison of data (Evans *et al*., 2004).

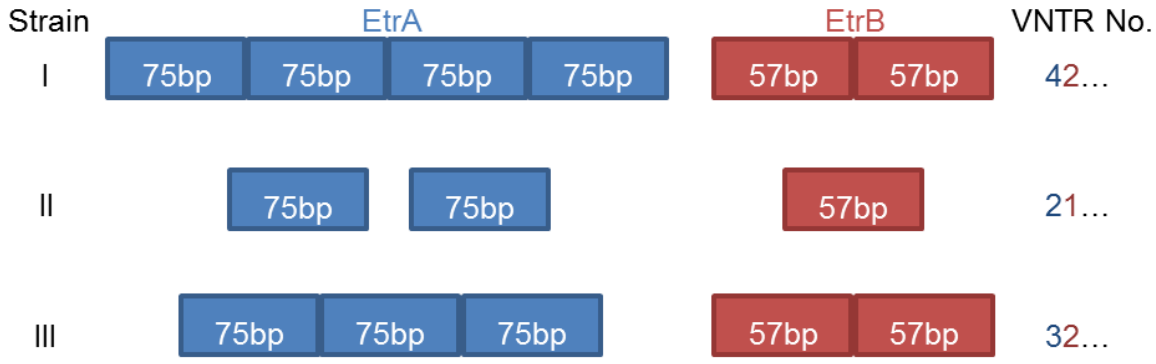


Figure 1-11 Principle of MIRU-VNTR typing

Two MIRU-VNTR loci, exact tandem repeat A (Etr-A) and exact tandem repeat B (Etr-B) are shown in three example strains (I, II and III). Primers that are specific for the DNA up and downstream of each loci are used to produce PCR amplicon. The number of repeats at each locus can be calculated based on the size of the PCR amplicon and used to produce the complete MIRU-VNTR profile.

1.8.2 Typing methods giving insight into the evolutionary origins of *M. tuberculosis* strains

In order to identify the evolutionary origin of strains, a typing method must differentiate between evolutionary distinct strains. This means that typing methods that give insight into the evolutionary origin of strains can be less stringent when discriminating between strains than typing methods used for clinical strains, as chains of transmission do not need to be identified.

1.8.2.1 Single nucleotide polymorphisms

Generation of genetic diversity in *M. tuberculosis* complex organisms can occur through single nucleotide polymorphisms (SNPs). Few spontaneous mutations become fixed within the population structure if there is no selective pressure. SNPs that are established in a clonal lineage are relatively stable, and are often used to group strains into phylogenies (Huard *et al*., 2006). Sreevatsan and colleagues carried out the first major study using SNPs for strain classification. They found that SNPs in the *katG* codon 463 and the *gyrA* codon 95 were phylogenetically informative and used them to assign strains of *M. tuberculosis* to three principal genetic groups (PGGs) as shown in Figure 1-12 (Sreevatsan *et al*., 1997). PGG1 organisms are the oldest in evolutionary terms and are described as ancient. Genetic diversity is greatest in PGG1 because organisms have evolved over a longer period. PGG2 organisms are evolutionary more modern than PGG1 organisms, followed by PGG3 organisms, which are the most modern strains and show the least genetic diversity.

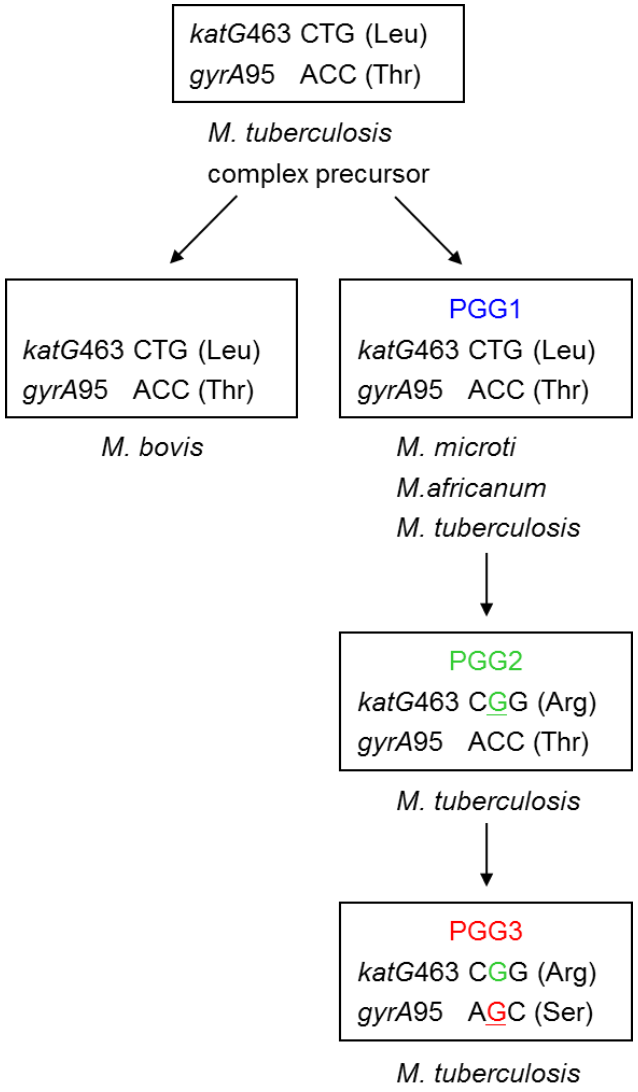


Figure 1-12 SNP based evolutionary scenario for *M. tuberculosis* complex organisms

Lineage defining SNPs in *katG* codon 463 *and gyrA* codon 95 for principal genetic group (PGG) 1, 2 and 3 organisms are shown with the DNA triplet code and the corresponding amino acid three-letter code (Sreevatsan *et al*., 1997).

1.8.2.2 Large sequence polymorphisms

Comparison of *M. tuberculosis* complex sequenced genomes has revealed the loss of segments of chromosomal DNA (Brosch *et al*., 2002). These losses are called large sequence polymorphisms (LSPs) and they represent irreversible, unique event polymorphisms (Gagneux *et al*., 2006).

When the genome of *M. tuberculosis* H37Rv was compared to BCG, 14 major regions of difference (RD) were discovered (Gordon *et al*., 1999). The regions of DNA, which are absent from the genome of BCG, are denoted as RD1 to RD14. RD1 is a 9.5 Kb region that deletes seven genes and truncates a further two. The region is absent from all BCG strains but present in other strains of *M. bovis.* The deletion of RD1 is believed to be an important event in the loss of virulence associated with the attenuation of the vaccine strain (Lewis *et al*., 2003). The functions of the deleted genes are unknown; however, two of the genes encode early secretory antigen-6 (ESAT-6) and culture filtrate protein-10 (CFP10). Experiments that delete the RD1 region from *M. tuberculosis* H37Rv give rise to mutants that have reduced virulence (Lewis *et al*., 2003).

Genomic comparison of *M. tuberculosis* H37Rv with other members of the *M. tuberculosis* complex revealed a deletion specific to *M. tuberculosis* strains, named *M. tuberculosis* specific deletion 1 (TbD1). A large set of strains from geographically diverse settings have been analysed and the regions of DNA that border the TbD1 region, were conserved. This indicates that deletion of the TbD1 region occurred in a common progenitor. The same study found that a small number of strains retained the TbD1 region. These strains clustered together when analysed with different typing techniques and it was noted that they had low copy numbers of IS*6110* (Brosch *et al*., 2002). Subsequent analysis of PGG typing has indicated that strains belonging to PGG1 may have retained or lost TbD1, but none of the strains belonging to PGG2 or PGG3 have retained the region. Therefore, presence of the TbD1 region is a marker for ancient strains and the absence of tbD1 is a marker for

modern strains. There are no repetitive DNA sequences bordering the TbD1 or RD1 regions so the mechanism of DNA elimination is unknown. It is possible that rare strand slippage errors of DNA polymerase are responsible for the deletions.

Subsequent studies have supported the division of *M. tuberculosis* strains into modern and ancient lineages based on the absence or presence of the TbD1 region respectively. Hershberg and colleagues produced a phylogenetic tree based on the DNA sequences of 89 genes for a collection of 108 *M. tuberculosis* complex strains (Hershberg *et al*., 2008). The authors found that the primary branches of their phylogenetic tree grouped modern and ancient strains which corresponded with the presence or absence of TbD1. The authors also showed that the difference between modern and ancient strains was greater than previously assumed and was not restricted to the TbD1 region. Wirth and colleagues produced a phylogenetic tree based on MIRU data for 355 isolates and showed that strains can be divided into two major lineages (Wirth *et al*., 2008). The authors describe lineage one, which includes the spoligotype clades T, Haarlem, LAM, S, X, Beijing and CAS all of which are TbD1 negative strains and lineage two that includes the spoligotype clade EAI and *M. africanum.* All the strains in lineage 2 have retained the TbD1 region.

1.8.3 Whole genome sequencing

The first *M. tuberculosis* whole genome sequence (WGS) was published in 1998 (Cole *et al*., 1998). Since then improvements in technology have reduced the cost and time required to produce a WGS so that there are currently over 600 *M. tuberculosis* genomes available (http://tinyurl.com/b3bowce - accessed 2013).

In 2011, Gardy and colleagues used WGS to compare 36 *M. tuberculosis* clinical isolates. They found that two distinct groups were present in an outbreak in which all of the strains had the same MIRU-VNTR profile (Gardy *et al*., 2011). When the sequence data was combined with social-network analysis, the authors were able to identify the chain of transmission that had occurred in the outbreak, which was not revealed by conventional genotyping and contact tracing.

The improvements in WGS technology and studies such as the one carried out by Gardy and colleagues show that WGS is a powerful tool that could improve clinical outbreak investigations. WGS will undoubtedly lead to greater understanding of the evolution of *M. tuberculosis* and the epidemiology of the disease. Pallen and colleagues report that WGS can cost less than £1,000 per strain (Pallen *et al*., 2010). The cost of 24 loci MIRU-VNTR typing is approximately £75 per strain so whilst the cost of WGS is constantly falling, it may be some time before it replaces the conventional typing methods described in sections 1.8.1 and 1.8.2.

1.8.4 Evolution of the *M. tuberculosis* complex

Several studies have used a combination of typing techniques to construct an evolutionary scenario for *M. tuberculosis* organisms (Gagneux, 2012). Allelic variations in bacteria can occur by random mutation, horizontal gene transfer (HGT) or recombination events. Microorganisms can acquire genetic diversity by HGT between different species. New genes obtained by HGT may confer an advantage to the recipient under certain selective pressures. Sequences that have been acquired by HGT have defining characteristics such as aberrant GC (guanine-cytosine) content, which allow them to be identified (Jang *et al*., 2008; Becq *et al*., 2007).

There is evidence of HGT and recombination in *M. tuberculosis* complex organisms, but the exchange of genetic material appears to have occurred only in the distant past (Dos Vultos *et al*., 2008; Gutierrez *et al*., 2005). Streevatsen and colleagues noted that the number of SNPs in *M. tuberculosis* complex organisms is lower than in other pathogenic organisms, meaning that there is a high degree of conservation in the housekeeping genes of members of the complex (Hirsh *et al*., 2004; Sreevatsan *et al*., 1997).

The high degree of conservation in housekeeping genes and the lack of HGT signifies that members of the *M. tuberculosis* complex are the clonal progeny of a single successful ancestor. This progenitor strain resulted from an evolutionary bottleneck at the time of speciation, which occurred between 20,000 and 35,000 years ago (Ernst *et al*., 2007; Gutierrez *et al*., 2005; Supply *et al*., 2003). Figure 1-13 shows a scheme for the proposed evolution of the *M. tuberculosis* complex. The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes.

1.8.5 Geographical partitioning of *M. tuberculosis* strains

In 1961, Bhatia and colleagues compared 281 TB isolates from patients in Madras, India with 93 isolates from patients in the UK (Bhatia *et al*., 1961). The isolates were injected into guinea pigs and the progression of the disease was monitored. The study found that Indian isolates were less virulent than UK isolates as they caused lower mortality in the guinea pig model. This was one of the earliest observations of phenotypic differences between strains of *M. tuberculosis* from different regions.

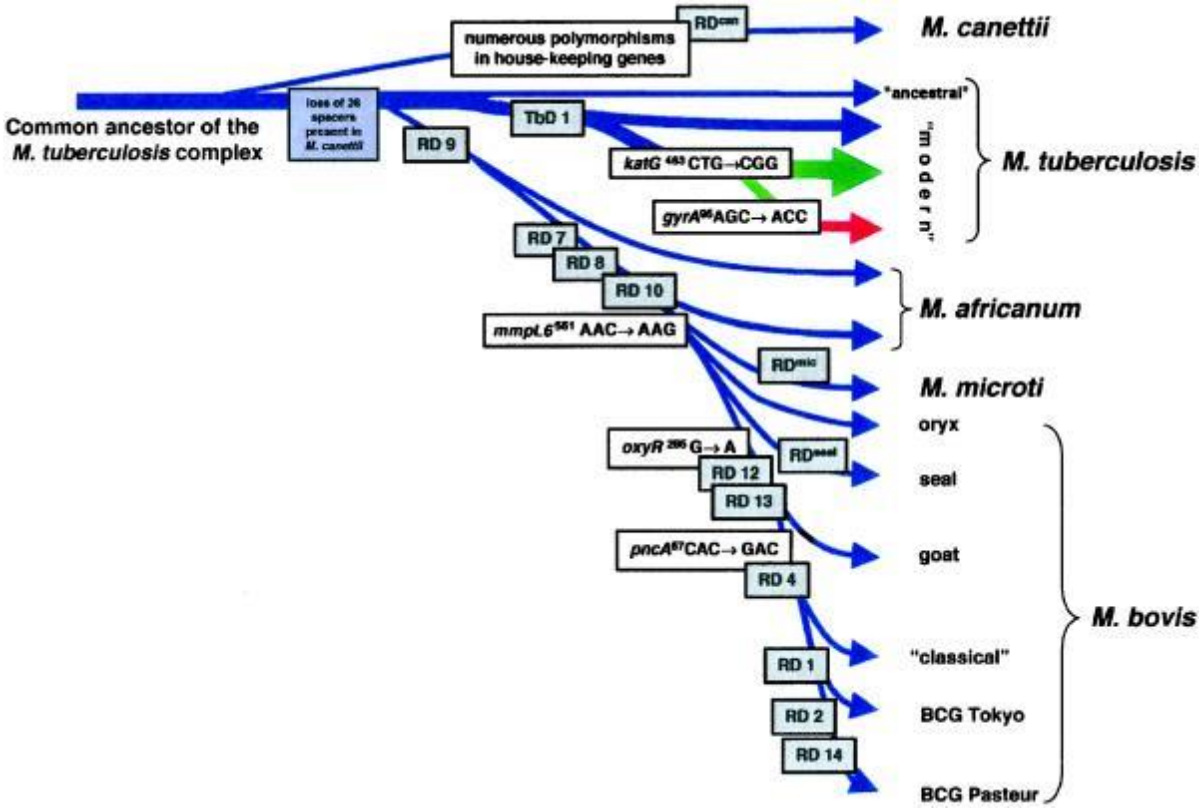


Figure 1-13 Proposed evolutionary pathway of members of the *M. tuberculosis* complex

The distances between branches are for illustration and may not be consistent with calculated phylogenetic differences.

Blue boxes depict successive loss of DNA in certain lineages,
blue arrows show ancient strains belonging to PGG1,
green arrows indicate that strains belong to PGG2 and
the red arrow indicates that strains belong PGG3,
according to Sreevatsan's definitions (Brosch *et al*., 2002).

Molecular typing methods, which assess variation in mobile genetic elements or repetitive DNA sequences (as discussed in sections 1.8.1 and 1.8.2), have identified groups of strains that are closely related and can be assembled into strain families (Bhanu *et al*., 2002; van Soolingen *et al*., 1995). Members of the same family share several independent genetic characteristics and are believed to have recently diverged from a common ancestor.

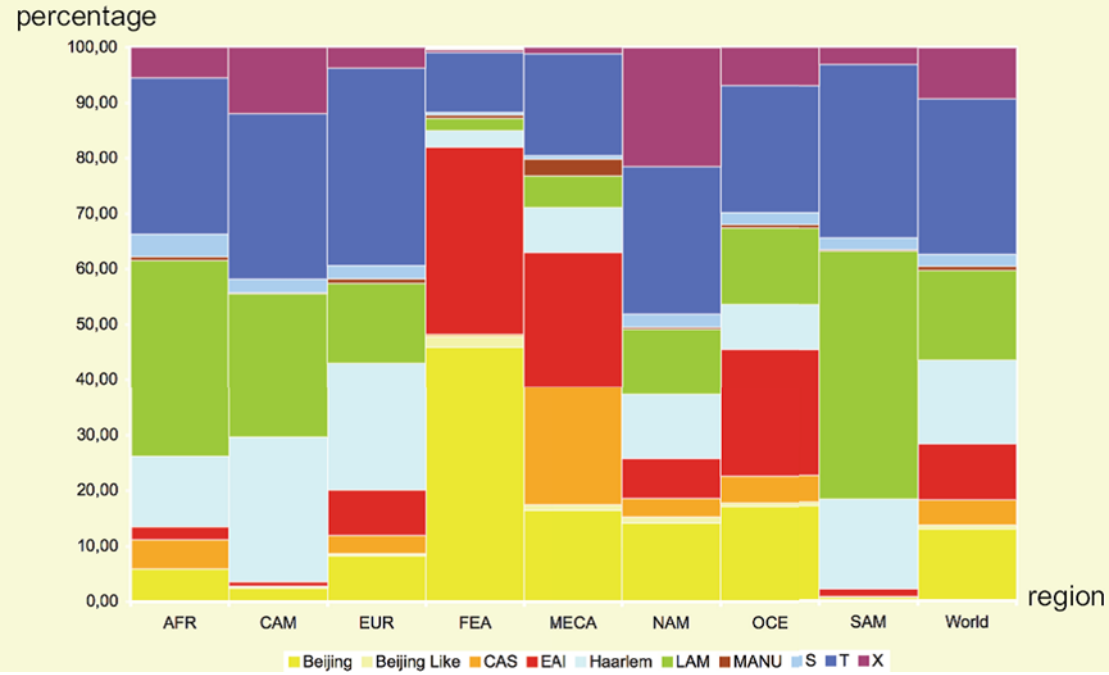
There are different classification systems for the identification of phylogeographical lineages. The most widely recognised lineages were identified using spoligotyping or

LSP analysis (Garzelli *et al*., 2010). LSP-based analysis carried out by Gagneux and colleagues identified six major lineages that were associated with specific human populations and geographical areas (Gagneux *et al*., 2006). Spoligotyping has divided strains into a larger number of families, the majority of which are geographically partitioned (Brudey *et al*., 2006). There is good correlation between the two classification systems so that spoligotype clades correspond to LSP-based lineages proposed by Gagneux and colleagues (Table 1-5).

Table 1-5 Association between major spoligotype clades and LSP lineages, adapted from (Gagneux and Small, 2007)

LSP lineage	Spoligotype clade	Geographical area
Indo-oceanic	EAI	All around the Indian Ocean
East Asian	Beijing	East and south east Asia, rapid spread in eastern Europe
East African-Indian	CAS	East Africa, north India and Pakistan
Euro-American	T, Haarlem, LAM, S, X	Europe and the Americas, Africa and the Middle East
West African I and West African II	*M. africanum*	Africa

Early studies identified that certain *M. tuberculosis* strain families were dominant in particular geographical regions, for example the Beijing family was found to be the principal strain in China, Mongolia, South Korea, and Thailand, but was not prevalent in The Netherlands. The global distribution of 11 of the major spoligotype clades is shown in Figure 1-14.



AFR = Africa
CAM = central America
EUR = Europe
FEA = far east Asia
MECA = Middle East & central Asia
NAM = North America
OCE = Oceania
SAM = South America

Figure 1-14 Percentage of main spoligotyping-defined *M. tuberculosis* complex genotype families within the international spoligotype database (SpolDB4)

(Brudey et al., 2006; van Soolingen et al., 1995).

It is hypothesised that *M. tuberculosis* emerged in Africa (Gagneux, 2012), before an evolutionary bottleneck occurred approximately 35,000 years ago that allowed the expansion of a successful clone (Brosch *et al*., 2002). Gutierrez and colleagues have suggested that the successful clone was dispersed globally at the same time as ancient humans migrated out of Africa (Gutierrez *et al*., 2005). Following the global dispersal of *M. tuberculosis,* it is believed that different regions became isolated, with little movement of human population between areas. It is thought that co-evolution of the pathogen and the host occurred in each geographically isolated area, allowing *M. tuberculosis* to adapt to specific human populations (Chapman and Hill, 2012). This co-evolution has led to distinct families of *M. tuberculosis* strains that are seen to be closely associated with different global regions (Baker *et al*., 2004).

Gagneux and colleagues have shown that the association between pathogen and host is stable by studying a migrant population living in the large multicultural city of San Francisco. The results from 875 isolates indicate that when a person relocates to a different geographical area, they are more likely to become infected with an *M. tuberculosis* strain that is associated with the region where they were born, despite being exposed to other, more prevalent strains. For example, TB patients born in the USA who declared themselves to be of Filipino ethnicity were infected with the same strains as TB patients who were born in the Philippines. Whilst the authors concede that these observations could be caused by social factors such as lack of social mixing due to the presence of ethnic enclaves, they go on to state further evidence in support of their theory of a stable host pathogen association. For example patients born in the USA who were not of Filipino ethnicity rarely became infected with *M. tuberculosis* strains associated with the Philippines, however when this did occur the patients were HIV positive or homeless. The authors suggest that Filipino *M. tuberculosis* strains are not adapted to cause disease in immunocompetant non-Filipino's and are only able to do so when the host immune system is weakened (Gagneux *et al*., 2006). The study carried out by Gagneux and colleagues was replicated using 798 isolates from Montreal. The conclusions reached by the two studies were similar in spite of differences between the human population structures in the two cities (Reed *et al*., 2009).

1.9 Investigation of virulence

If strains of TB have adapted to host ethnicity then the genotype of a patient isolate could be used to identify at risk populations that are more susceptible to that strain. This would enable more efficient contact tracing and help to reduce the spread of TB.

M. tuberculosis strains that are able to infect people from different ethnic backgrounds may exist which could be an indication of altered virulence. There is no definitive measure of virulence, however a number of studies have assessed the propensity of different strains to cause disease by using epidemiological data or models. The systems, which have been used to compare the virulence of different strains of *M. tuberculosis*, are described below.

1.9.1 Cohort studies

Several groups have followed outbreaks of TB to assess links between the strain of *M. tuberculosis* and the symptoms of the patient. Thwaites and colleagues showed a correlation between mycobacterial genotype and features of chest X-rays, but were not able to adjust their analysis for variation in the duration of illness prior to diagnosis. They were able to show some correlation between genotype and progression of the disease in the case of TB meningitis and between lineage and drug resistance (Thwaites *et al*., 2008).

Sun and colleagues compared 21 people infected with *M. tuberculosis* strains from the Beijing spoligotype clade with 20 people infected with non-Beijing strains. They found that people infected with Beijing strains had a longer duration of cough before diagnosis than those infected with non-Beijing strains but were less symptomatic as there was a lower frequency of night sweats, fever and pulmonary cavitation (Sun *et al*., 2006).

De Jong and colleagues followed the household contacts of TB patients in The Gambia. The percentage of contacts with incident TB during the two-year follow-up

varied five-fold between contacts exposed to different strains. Only 1% of contacts in households exposed to *M. africanum* went on to develop active disease, whereas nearly 6% developed active disease when exposed to Beijing strains from the *M. tuberculosis* lineage (de Jong *et al*., 2008).

Some studies have identified genes that act as virulence factors. These studies have compared the clinical characteristics of disease caused by strains that are positive or negative for the virulence factor. Kong and colleagues found an involvement between a mycobacterial gene mutation and extra-thoracic TB, which remained significant after controlling for host related risk factors such as ethnicity, sex and HIV infection (Kong *et al*., 2005).

Talarico and colleagues looked at polymorphisms in mycobacterial lipase genes, which may be involved in *M. tuberculosis* survival in macrophages. They found that one group of mutations was over-represented among TB patients with no lung cavitation compared to patients with lung cavitation (Talarico *et al*., 2007).

Cohort studies provide evidence that *M. tuberculosis* strain variation can lead to altered disease progression. However, large numbers of patients are required for these studies and patients must be followed up over several years (Thwaites *et al*., 2008). Treatment of patients will unavoidably change the epidemiology, so the true rate of transmission cannot be measured. Measurement of clinical characteristics such as fever, lung cavitation and duration of cough before diagnosis can be subjective, which makes it difficult to compare data between studies. These issues

have led investigators to develop model systems for studying mycobacterial infections.

1.9.2 Model systems for studying mycobacterial infections

Model systems are useful for the study of *M. tuberculosis* virulence because host genetics and behaviour can be standardised.

1.9.2.1 Animal models

Replication of *M. tuberculosis* within specific organs of experimental animals and the length of time than an experimental animal survives once it has been infected are both used as measures of virulence (Palanisamy *et al*., 2008; Theus *et al*., 2004).

Rabbits, guinea pigs, mice and non-human primates are all susceptible to infection with *M. tuberculosis* when it is artificially induced and have been used to model different aspects of TB, including active disease and latency (Dharmadhikari and Nardell, 2008). *M. tuberculosis* is categorised by the Advisory Committee on Dangerous Pathogens as a hazard group three organism because it can cause severe disease in humans. Special containment facilities are required in order to work with a hazard group three organism, so animal models of TB infection are only carried out at a limited number of centres. The use of animal-based models to assess the virulence of *M. tuberculosis* strains is discussed in more detail in section 4.1.1.1.

1.9.2.2 *In vitro* assays

In vitro assays that use cells in tissue culture are cheaper and more accessible than animal models for assessment of *M. tuberculosis* virulence. Most assays of mycobacterial virulence use phagocytic cells, although some research has been

carried out with lung epithelial cells (Danelishvili *et al*., 2003; Dobos *et al*., 2000). Models of macrophage infection can use either primary cells or a cell line, which has been immortalised. Cells can be obtained from experimental animals or from human donors. The use of cell-based models to study the virulence of *M. tuberculosis* strains is discussed in more detail in section 4.1.1.2.

2 AIMS AND HYPOTHESIS

2.1 Assessment of *M. tuberculosis* genotypes and patient ethnicity in the Midlands, UK

2.1.1 Hypothesis

Strains of TB that are prevalent in the ISC will also be prevalent in the Midlands.

2.1.2 Aim

To determine whether there is a correlation between the ethnicity of TB patients in the Midlands and the genotype of the TB strains with which they are infected.

2.2 Assessment of virulence of the most prevalent TB strains in the Midlands, UK using *in vitro* models

2.2.1 Hypothesis

Prevalent TB strains from the Midlands will be more virulent in a cellular model of infection than strains that are not prevalent.

2.2.2 Aim

To assess the impact of *M. tuberculosis* strain genotypic variation on virulence in cellular models of infection.

3 ASSESSMENT OF *M. TUBERCULOSIS* GENOTYPES AND PATIENT ETHNICITY IN THE MIDLANDS, UK

3.1 Introduction

Within the UK, the West Midlands region has the second largest proportion of people with Indian ethnicity (ONS, 2003). The region has a total population of 9.5 million and contains 24% of the UK's south Asian population (ONS, 2003). Approximately 1.5% of the people in the West Midlands were born in India and in some regions such as Wolverhampton, the percentage is over 5%; the national average is 0.9%. According to the 2001 census over 50% of the population served by the Heart of Birmingham Primary Care Trust (PCT), which provides services for approximately 300,000 people in the centre of Birmingham, had ISC ethnicity (Figure 3-1). The 2011 census data has not yet been analysed according to PCT, however regional data shows that the proportion of people with ISC ethnicity has increased (Figure 3-2). The West Midlands also has the second highest proportion of TB cases in the UK, with 11% of the case load in 2009 and the second highest rate of TB at 18.7 cases per 100,000 (95% CI 17.6-19.9) (Figure 3-3).

A recent study suggested that 20% of immigrants entering the UK are latently infected with TB and the majority of these infections are missed by current screening practices (Pareek *et al*., 2011). Data from the HPA shows that only 21% of TB cases amongst immigrants are diagnosed within two years of arriving in the country (HPA, 2010). The increase in the number of UK TB cases amongst people born in India coupled with the delay in presentation of active disease and the failure to detect all latent infections means that strains of TB that are being imported from India are likely

to account for a significant amount of disease in the UK in the coming years. There is also evidence that immigrants are more likely to contract TB from bacteria that are endemic in their home country, rather than from bacteria that are endemic in the country to which they have emigrated (Gagneux *et al*., 2006). It has been suggested that immigrants become infected with *M. tuberculosis* strains that are endemic in their home country because they are genetically more susceptible to these strains, the pathogen having co-evolved with the host. However, a lack of social mixing amongst immigrants, who will therefore not be frequently exposed to strains that are endemic in the indigenous population, could also explain the association.

The West Midlands has a large population of people who were born in India or have ISC ethnicity and it has a high rate of TB. The rate of TB in the UK has been linked with the number of immigrants from countries where TB is endemic (Gilbert *et al*., 2009). It is therefore hypothesised that the most prevalent TB strains in the Midlands will also be those that are commonly found in the ISC. In order to test this hypothesis it is necessary to identify the ethnicity of the TB patients in the Midlands and to identify the prevalent lineages of TB.

3.1.1 Identification of patient ethnic origin

Studies that are concerned with the pattern of change in the conveyance of TB from one person to another have analysed population groups. Population groups are usually based on declarations made by the patient about their ethnic origin or country of birth. Country of birth information is useful for classifying the origin of an immigrant, but is less useful for identifying people who were born in the UK but whose parents or grandparents were immigrants. Patient declared ethnicity data is collected using a

limited number of ethnic group classifications, which masks the complexity found within a group (Mateos, 2007). Ethnicity can be interpreted in different ways for example Asian ethnicity can mean continental Asia or ISC (Aspinall, 2003). The analysis of name origins is one method of identifying a person's ethnicity and can overcome some of the problems associated with the ethnic group classifications that are used to collect health care data.

In the current study, Origins software (Origins; Experian, Nottingham, UK) was used to predict the ethnicity of TB patients in the Midlands. Origins software partitions people by cultural, ethnic or religious origin based on their name. Origins was developed to identify UK consumers who were born abroad or to recent immigrants, so that they could be sent targeted advertising (Webber, 2007). The software was first used in healthcare to identify how migration of people from Poland to the UK affected the use of an National Health Service (NHS) hospital emergency department (Leaman *et al*., 2006). It has also been used to identify the global origins of TB in the Midlands, UK (Evans *et al*., 2010).

Origins software uses a database of names with records from 12 countries making it more reliable than other databases, such as Nam Pehchan and SANGRA, which are based on UK data (Webber, 2007; Nanchahal *et al*., 2001; Cummins *et al*., 1999). The Origins database contains 1,600,000 family names and 600,000 given names. Expert knowledge and data mining algorithms were used to place each name into one of 200 origin types based on cultural, ethnic and linguistic (CEL) factors.

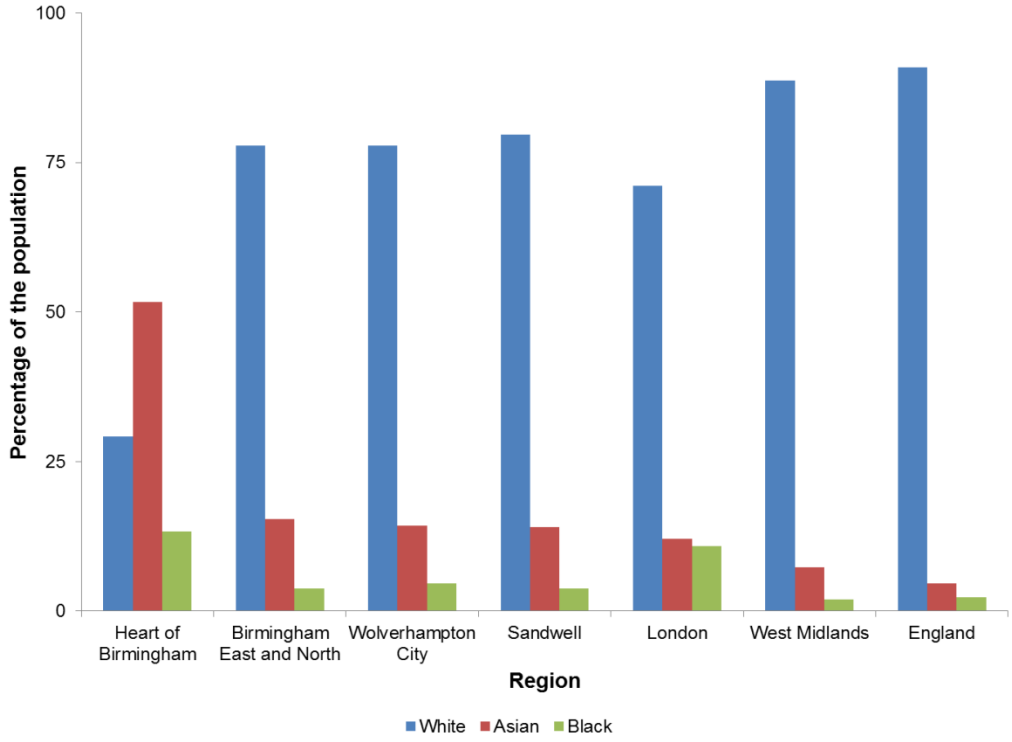


Figure 3-1 Proportion of ethnic groups in selected West Midlands primary care trusts, and aggregated data for London, the West Midlands and England

(ONS, 2003)

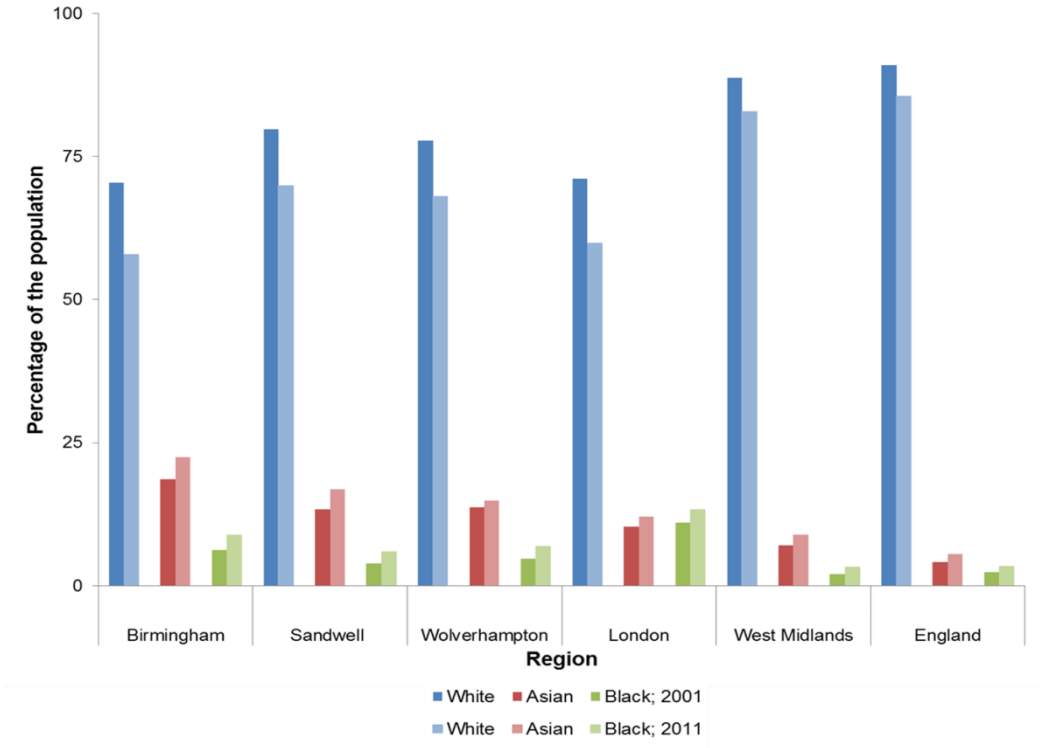


Figure 3-2 Proportion of ethnic groups in selected local authorities in the West Midlands, and aggregated data for London, the West Midlands and England

(ONS, 2012; ONS, 2003)

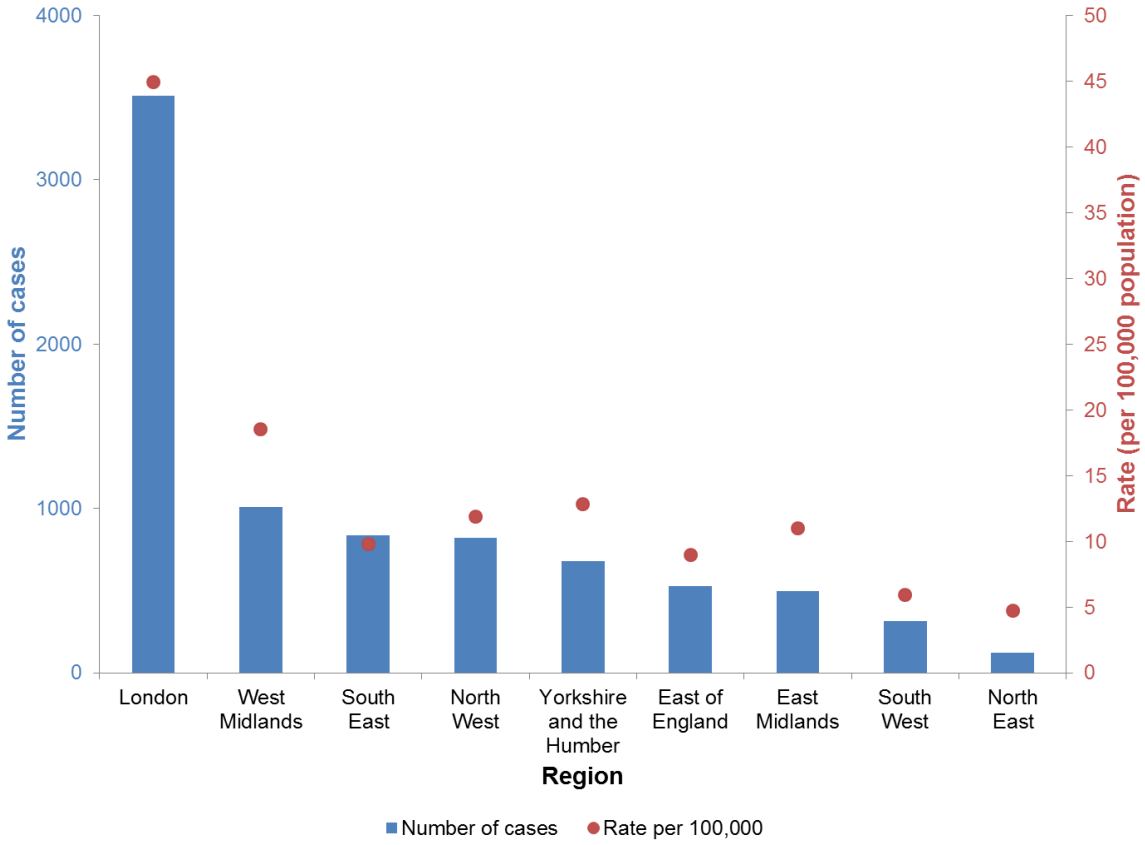


Figure 3-3 Rate of TB and the number of TB cases by region, England, 2011

(HPA, 2012)

The origin type can correspond to a particular country or more than one origin type can come from the same country, as is the case for India where there are several different religions. In some cases, countries can share the same origin type, for example Germany and Austria, which have similar naming practices because of the use of the same language.

To infer the origin of a name the origin type of both the given and the family name are identified, which in most cases will be the same. Occasionally the given and family name will have different origin types and so a set of rules, based on confidence scores, is used to determine which name is the most faithful indicator of the origin of

that person. The 200 origin types can then be grouped depending on how the data will be analysed. The groups could be based on broad geographical regions, religion or language. For example the surname, 'Mascarenhas' would identify a person as having Goan ancestry. This would mean that the geographical region that they come from is south Asia. Their language and culture is likely to be Portuguese and their religion is likely to be Catholic (Webber, 2007).

The use of a person's name to infer their origin is useful in cases where ethnicity or country of birth has not been recorded or where data protection regulations mean that the information can only be used for the original purpose for which it was recorded. In the current study, the use of origins software meant that data could be analysed immediately rather than waiting for the ethnic origin of a patient to be collated by the enhanced tuberculosis surveillance network (ETSN). It also allows more detailed analysis to be carried out as it can be used to give an indication of the region of a country that a name originates from, a parameter that is not recorded by the ETSN.

3.1.2 Molecular typing of *M. tuberculosis* isolates in the Midlands

The HPA Midlands Regional Centre for Mycobacteriology (MRCM) at Birmingham Heartlands Hospital receives approximately 1,300 specimens and isolates per year from 35 laboratories in the East and West Midlands of the UK. The MRCM undertakes mycobacterial culture, identification, drug susceptibility testing and DNA fingerprinting for these samples (Evans *et al*., 2007).

Most databases that are publicly available for the identification of TB strain lineages are based on spoligotyping or LSP typing. However, the MRCM uses MIRU-VNTR to type all clinical isolates, as it is gives better discrimination of strains. Allix-Beguec and colleagues developed a database called MIRU-VNTR*plus*, which combines data from several typing methods and allows correlations between methods to be identified (Allix-Beguec *et al*., 2008b). Spoligotyping has been widely used for typing TB strains across the globe and the spolDB4 database, which can be accessed online via SITVIT, was the largest publicly available database of TB typing data (Brudey *et al*., 2006). MIRU-VNTR*plus* was therefore used to match MIRU-VNTR profiles to their most likely spoligotype. TB isolates from the Midlands, UK could then be compared with the spolDB4 database, without having to carry out the spoligotyping procedure, in turn allowing global strain lineages to be identified.

MIRU-VNTR*plus* contains 186 reference isolates for which the MIRU-VNTR profile, spoligotype, SNP and LSP profile has been recorded, some of these data are shown in Table 3-1 and Table 3-2. The reference isolates are representative of the main TB lineages as assessed by spoligotyping and LSP analysis. Other members of the MTB complex and a number of ATCC type strains are also included in the database (*M. tuberculosis* H37Rv; ATCC 27294, *M. bovis*; ATCC 19210 and *M. africanum*; ATCC 25420). To identify the spoligotype lineage that a test isolate belongs to a categorical distance is calculated by scoring the number of markers where there is a difference divided by the total number of markers used, best matching then identifies the reference genotypes with the closest distance to the test isolate. A categorical distance of 0.17 was proposed by the designers of MIRU-VNTR*plus* as a cut off,

which corresponds to a difference in 4 loci when using 24 loci MIRU-VNTR typing or a difference of 7 spacers when using spoligotyping (Allix-Beguec *et al*., 2008b).

Table 3-1 Spoligotype data for 186 reference strains present in the MIRU-VNTR*plus* database (Allix-Beguec *et al*., 2008b)

SITVIT spoligotype clade	No. of different SITs	Percentage of isolates (No.)	Most prevalent SIT (No. of isolates)	No. of other SITs (No. of isolates)	No. of isolates with no SIT
AFRI	6	17 (31)	326 (7)	5 (11)	(13)
Beijing	1	5 (10)	1 (10)	0 (0)	(0)
BOV	7	6 (11)	482 (3)	6 (6)	(2)
CANETTI	1	1 (2)	592 (2)	0 (0)	(0)
CAP	4	6 (11)	647 (7)	3 (3)	(1)
CAS	4	5 (10)	1,092 (5)	3 (4)	(1)
EAI	8	6 (12)	11 (2)	7 (8)	(2)
H37Rv	1	1 (1)	451 (1)	0 (0)	(0)
Haarlem	8	11 (20)	50 (5)	7 (14)	(1)
LAM	9	13 (25)	61 (8)	8 (15)	(2)
MICROTI	2	3 (6)	642 (4)	1 (2)	(0)
PIN	0	1 (2)	N/A (0)	0 (0)	(2)
S	4	6 (12)	34 (9)	3 (3)	(0)
T	6	16 (30)	53 (10)	5 (11)	(9)
X	1	2 (3)	119 (2)	0 (0)	(1)
Total	**62**	**100 (186)**	**(75)**	**(77)**	**(34)**

Table 3-2 VNTR data for 186 reference strains present in the MIRU-VNTR*plus* database (Allix-Beguec *et al*., 2008b)

SITVIT spoligotype clade	No. of isolates (%)	Most prevalent VNTR profile	No. of isolates	No. of other VNTR profiles	No. of isolates	Total no. of VNTR profiles
AFRI	31 (17)	42432	15	11	16	12
Beijing	10 (5)	42435	9	1	1	2
BOV	11 (6)	45543	2	9	9	10
CANETTI	2 (1)	N/A	0	2	2	2
CAP	11 (6)	N/A	0	11	11	11
CAS	10 (5)	42235	8	*1	2	2
EAI	12 (6)	46464	2	10	10	11
H37Rv	1 (1)	33443	1	0	0	1
Haarlem	20 (11)	32333	11	7	9	8
LAM	25 (13)	22232	5	7	20	8
MICROTI	6 (3)	93571	2	4	4	5
PIN	2 (1)	93463	2	0	0	1
S	12 (6)	32443	4	3	8	4
T	30 (16)	32333	9	8	21	9
X	3 (2)	32333	2	1	1	2
Total	**186 (100)**					**88**

* the other CAS VNTR profile is 42234, N/A indicates not applicable

3.2 Aims and hypothesis

3.2.1 Hypothesis

CAS and EAI strains will be the most prevalent spoligotypes clades in the Midlands and will be isolated from TB patients with ISC ethnicity.

3.2.2 Aims

- Use Origins software to select 100 *M. tuberculosis* isolates predicted to be from patients of ISA ethnicity and genotype the isolates to identify the most prevalent spoligotype clades
- Compare patient declared ethnicity for 100 strains with predictions made by Origins software to assess the accuracy of the predictions
- Compare MIRU-VNTR profiles from 100 selected isolates to the MIRU-VNTR*plus* database in order to test the accuracy of spoligotype predictions made by the MIRU-VNTR*plus* database
- To use Origins software to predict the most prevalent ethnic groups amongst TB patients in the Midlands from 2007 to 2008
- To use the MIRU-VNTR*plus* database to predict the spoligotype of Midlands isolates from 2007 to 2008 in order to identify the most prevalent spoligotypes in the Midlands and to make global comparisons

3.3 Materials and methods

3.3.1 Study population

The study took place in the Midlands region of the UK. The study population included all *M. tuberculosis* isolates submitted to the MRCM between 1st January 2007 and 31st December 2008 (n = 2,185). The MRCM undertook mycobacterial culture,

identification, drug susceptibility testing and DNA fingerprinting for these isolates (Evans *et al*., 2007). Ethical approval was obtained according to local guidelines.

Origins software version 1 (Professor Richard Webber, King's College London, UK) identified that 1,186 of 2,185 (54%) isolates came from patients who originated in the ISC. A sample of 100 isolates was selected from amongst these patients (Table 3-3). These isolates represented the range of MIRU-VNTR profiles, weighted so that the most common strains were represented proportionally. Once all commonly isolated strains had been included in the sample in the correct proportion, strains that had only been isolated once but had been typed in 2008 were randomly selected until the set included 100 isolates.

Control organisms *M. bovis* NCTC 10772, *M. tuberculosis* H37Rv NCTC 7416 and *M. tuberculosis* H37Ra NCTC 7417 were obtained from the National Collection of Type Cultures, Colindale, London, UK. *M. bovis* BCG Danish Vaccine strain 1331 was obtained from the Statens Serum Institute, Copenhagen.

3.3.2 Bacterial growth conditions

TB isolates were incubated in liquid culture in a BACTEC™ MGIT™ 960 System (BD Biosciences, Oxford, UK) at 37°C until a positive growth index was detected by fluorescence. Positive cultures were identified with the HAIN GenoType species-specific *M. tuberculosis* complex DNAstrip test (Hain Lifescience, Nehren, Germany) (Richter *et al*., 2004).

Table 3-3 MIRU-VNTR profiles in the Midlands from patients with ISC* ethnicity, 2007-2008

VNTR profile	MIRU profile	No. typed	%	No. selected
42235	2542517333	120	4.6	5
42235	2642515333	50	1.9	2
42234	2642517323	45	1.7	2
42235	2642517333	40	1.5	2
42234	2742511334	38	1.5	2
32333	2432515314	37	1.4	2
-2235	2542517333	33	1.3	2
42235	2642516333	31	1.2	2
32433	2512511322	27	1.0	2
61464	2432622334	26	1.0	2
32333	2532515323	21	0.8	1
21433	2412615221	16	0.6	1
42235	2642513333	14	0.5	1
32433	2312515324	13	0.5	1
42435	2332517333	13	0.5	1
42235	2442517333	12	0.5	1
42235	2542516333	12	0.5	1
42435	2332515333	11	0.4	1
42235	2532517333	10	0.4	1
42235	2742517333	10	0.4	1
-2544	2742414322	9	0.3	1
32433	2512511323	9	0.3	1
42235	2541517333	9	0.3	1
61466	2432622333	9	0.3	1
32333	2532315323	8	0.3	1
42235	2342517333	8	0.3	1
42235	2532517323	8	0.3	1
42235	2542518333	8	0.3	1
31433	2232515323	7	0.3	1
32235	2642517333	7	0.3	1
32333	2531315323	7	0.3	1
42235	2642517332	7	0.3	1
42235	2642517334	7	0.3	1
42235	2642518333	7	0.3	1
42435	2232517343	7	0.3	1
32235	2542517333	6	0.2	1
32433	2332514327	6	0.2	1
32433	2432515324	6	0.2	1
42234	2542517333	6	0.2	1
42234	2742511324	6	0.2	1
42235	2542517323	6	0.2	1
42235	2632517333	6	0.2	1
42443	2332616324	6	0.2	1
61464	2432722334	6	0.2	1
61466	2432622334	6	0.2	1
-2235	2642517333	5	0.2	1
-2235	2542517332	5	0.2	1
21433	2232515323	5	0.2	1
32433	2312515322	5	0.2	1
42234	2742511323	5	0.2	1
42235	2542515333	5	0.2	1
42235	2542517334	5	0.2	1
42235	2632515333	5	0.2	1
76466	2422622322	5	0.2	1
-5265	2632622313	4	0.2	1
-2235	2542516333	4	0.2	1
-2235	2442517333	4	0.2	1
21433	2312615221	4	0.2	1
22333	2532315323	4	0.2	1
22433	2322615321	4	0.2	1
22433	2442615221	4	0.2	1
31533	2222513323	4	0.2	1
32432	2322615321	4	0.2	1
42232	2542517333	4	0.2	1
42234	2342517323	4	0.2	1
42234	2642517324	4	0.2	1
42235	1542517333	4	0.2	1
42235	2432517333	4	0.2	1
42235	2442517323	4	0.2	1
42235	2452517333	4	0.2	1
42235	2542517331	4	0.2	1
42235	2642515332	4	0.2	1
42235	2642517331	4	0.2	1
32334	2632516323	3	0.1	1
32433	2332615321	3	0.1	1
42234	2842511334	3	0.1	1
42235	2642516332	3	0.1	1
42236	2542517333	3	0.1	1
61456	2432622334	3	0.1	1
614-4	2432722334	3	0.1	1
-4455	2432622132	2	0.1	1
-2445	2332522133	2	0.1	1
22233	2662117333	2	0.1	1
31233	2222514323	2	0.1	1
42234	2632517331	2	0.1	1
	Total	**908**	**35**	**98**

* prediction of patient ethnicity was made using Origins software Version 1

3.3.3 Bacterial DNA extraction

Bacteria were sonicated for 15 minutes in a bath sonicator then heated to 100°C for 30 minutes. The resulting lysate was centrifuged at 10,000 × *g* to remove cellular debris before removal of the supernatant from the category 3 containment facility and storage at -20°C. Lysate was used as template for PCR reactions.

3.3.4 General PCR amplification primers and conditions

Target gene loci, their primer names and sequences are listed in Table 3-4. All oligonucleotides were obtained from MWG, Germany. PCR reaction mixtures contained 0.5 µM of forward and reverse primer, 200 µM dNTP mix, 3 mM $MgCl_2$, 5 µl reaction buffer, 1 µl template DNA, 1 U Amplitaq Gold polymerase (Applied Biosystems, Warrington, UK), and sterile water to 50 µl. Amplification was performed in a Multi-Block System Thermal Cycler (ThermoHybaid, Ashford, UK). An initial denaturation of 10 minutes at 95°C was followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing between 54 and 70°C (Table 3-4) for 1 minute, and extension at 72°C for 1 minute, followed by a final extension step of 5 minutes at 72°C.

PCR products and 50 base pair step ladder (Promega, London, UK) were separated by agarose gel (1% wt/vol) electrophoresis at 150 V and visualised with ethidium bromide. All PCR reactions included a positive control (*M. tuberculosis* H37Rv, *M. bovis* or *M. bovis* BCG) for amplification of the target locus as well as a negative control, containing water but no template DNA.

Table 3-4 Published primer sequences

Locus	Sequence (5' to 3')	Tm (°C)	Source
RD1 internal	GTC AGC CAA GTC AGG CTA CC CAA CGT TGT GGT TGT TGA GG	55.0	(Rao *et al*., 2005)
RD1 flanking	GAA ACA GTC CCC AGC AGG T TTC AAC GGG TTA CTG CGA AT	54.0	(Rao *et al*., 2005)
katG	GAC GAG GTC GGC GAA GGG ACA TTT TGA GGG CCG CTG GTC CCC AAG CAG AC	70.0	(Huard *et al*., 2006)
gyrA	GGA GGT GCG CGA CGG GCT CAA G ACC CGG CCG TCG TAG TTA GGG ATG AAA TC	69.6	(Huard *et al*., 2006)
TbD1 internal	CGT TCA ACC CCA AAC AGG TA AAT CGA ACT CGT GGA ACA CC	57.3	(Brosch *et al*., 2002)
TbD1 flanking	CTA CCT CAT CTT CCG GTC CA CAT AGA TCC CGG ACA TGG TG	59.4	(Brosch *et al*., 2002)
DR	GGT TTT GGG TCT GAC GAC CCG AGA GGG GAC GGA AAC	58.0	(Kamerbeek *et al*., 1997)

3.3.5 DNA fingerprinting

Isolates were analysed by MIRU-VNTR typing using 15 loci as previously described (ETR-A, -B, -C, -D, -E and MIRU-02, -10, -16, -20, -23, -24, -26, -27, -39, -40) (Evans *et al*., 2007) using non-denaturing high performance liquid chromatography with a WAVE microbial analysis system (Transgenomic, Elancourt, France) (Evans *et al*., 2004).

3.3.6 Spoligotyping

Spoligotyping was performed using the Luminex Multianalyte Profiling System (Cowan *et al*., 2004).

3.3.6.1 Amplification of the DR locus

The primer sequences for the DR locus are listed in Table 3-4. Primer DRa is biotinylated at the 5' terminus to allow subsequent detection using a streptavidin conjugate. The primers hybridise to the 36 base pair DR between each spacer and simultaneously amplify all of the present spacers (Cowan *et al*., 2004).

3.3.6.2 Bead coupling

The sequences of the 43-oligonucleotide probes are listed in Table 3-5. Probes were synthesised with a 5'-terminal amino group with a 6-carbon spacer to allow covalent attachment to carboxyl group (COOH) linked beads.

A mixture of 200 pmol probe, 2.5 X 10^6 beads (Biorad, Bath, UK), and 25 µg 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific, UK) was prepared in 25 µl of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 4.5 (Sigma, UK). The reaction mixture was incubated at room temperature in the dark for 30 minutes. The addition of EDC and subsequent incubation were repeated once. After coupling the beads were washed with 0.5 ml of 0.02% Tween 20 followed by 0.5 ml of 0.1% sodium dodecyl sulfate. Coupled beads were suspended in 50 µl of Tris-EDTA, pH 8 and quantified using a haemocytometer. There was an average recovery of 45,000 beads/µl. Coupled beads were stored at 4°C in the dark. A mixture of all 43 bead-sets was prepared by combining equal volumes of each.

3.3.6.3 Hybridisation of coupled beads to PCR amplicons

Bead mix was diluted by addition of 1.5 X hybridization buffer (1.5 M tetramethylammonium chloride (Sigma, UK), 75 mM Tris, pH 8.0, 6 mM EDTA, 0.15% Sarkosyl) to a final concentration of approximately 150 beads of each set/ µl. PCR amplicon (5 µl), diluted bead mix (33 µl) and TE (12 µl) were combined in a Thermowell 96-well plate (VWR International) and incubated for 10 min at 94°C, followed by incubation for 30 min at 52°C, in a GeneAmp 9700 PCR System (Perkin-Elmer, UK). The plate was centrifuged at 2,250 × *g* for 3 minutes and beads

were resuspended in 75 µl detection buffer (R-phycoerythrin-conjugated streptavidin (Invitrogen, Paisley, UK) diluted to 4 µg/ml with 1X hybridization buffer). Samples were then incubated at 52°C for 5 minutes.

3.3.6.4 Analysis

Samples were analysed using a Bioplex 100, version 1.7; with a minimum of 100 events per bead set. Final spoligotypes were generated with a Microsoft Excel spreadsheet designed by the Centre for Disease Control (CDC). Spoligotype profiles were submitted to the SpolDB4 database for clade assignation

3.3.7 RD1 and TbD1 analysis

To test for the presence of LSPs in the genome of *M. tuberculosis* isolates, two sets of primers were used, one set complementary to sequence flanking the target region and one set complementary to the internal sequence of the target region. If a region is deleted amplicon is produced with the flanking primers but not the internal primers and vice versa (Gutierrez *et al*., 2006).

3.3.8 Principal Genetic Grouping

PGG was determined by PCR-RFLP of *katG* and *gyrA* amplicon with restriction enzymes *Bst*NI and *Ale*I (New England Biolabs, UK) according to the manufactures instructions. *Bst*NI digestion of *katG* PCR amplicon from group 1 isolates produced four DNA fragments (12, 61, 104 and 174 base pairs), while that from groups 2 and 3 produced three DNA fragments (12, 61 and 278 base pairs). *Ale*I digestion of *gyrA* PCR amplicon produced two DNA fragments (161 and 193 base pairs) from group 1 and 2 isolates, while that from group 3 strains remained uncut (354 base pairs). Digest products were separated by 2 hours agarose (2%) gel electrophoresis.

Table 3-5 Oligonucleotides used for Spoligotyping (Kamerbeek *et al*., 1997)

Name	Sequence (5' to 3')	Tm (°C)
SPF01	MMT ATA GAG GGT CGC CGG TTC TGG ATC A	66.3
SPF02	MMT CCT CAT AAT TGG GCG ACA GCT TTT G	63.0
SPF03	MMT CCG TGC TTC CAG TGA TCG CCT TCT A	66.3
SPF04	MMT ACG TCA TAC GCC GAC CAA TCA TCA G	64.6
SPF05	MMT TTT TCT GAC CAC TTG TGC GGG ATT A	61.3
SPF06	MMT CGT CGT CAT TTC CGG CTT CAA TTT C	63.0
SPF07	MMT GAG GAG AGC GAG TAC TCG GGG CTG C	71.2
SPF08	MMT CGT GAA ACC GCC CCC AGC CTC GCC G	74.5
SPF09	MMT ACT CGG AAT CCC ATG TGC TGA CAG C	66.3
SPF10	MMT TCG ACA CCC GCT CTA GTT GAC TTC C	66.3
SPF11	MMT GTG AGC AAC GGC GGC GGC AAC CTG G	72.8
SPF12	MMT ATA TCT GCT GCC CGC CCG GGG AGA T	69.5
SPF13	MMT GAC CAT CAT TGC CAT TCC CTC TCC C	66.3
SPF14	MMT GGT GTG ATG CGG ATG GTC GGC TCG G	71.2
SPF15	MMT CTT GAA TAA CGC GCA GTG AAT TTC G	61.3
SPF16	MMT CGA GTT CCC GTC AGC GTC GTA AAT C	66.3
SPF17	MMT GCG CCG GCC CGC GCG GAT GAC TCC G	77.7
SPF18	MMT CAT GGA CCC GGG CGA GCT GCA GAT G	71.2
SPF19	MMT TAA CTG GCT TGG CGC TGA TCC TGG T	66.3
SPF20	MMT TTG ACC TCG CCA GGA GAG AAG ATC A	64.6
SPF21	MMT TCG ATG TCG ATG TCC CAA TCG TCG A	64.6
SPF22	MMT ACC GCA GAC GGC ACG ATT GAG ACA A	66.3
SPF23	MMT AGC ATC GCT GAT GCG GTC CAG CTC G	69.5
SPF24	MMT CCG CCT GCT GGG TGA GAC GTG CTC G	72.8
SPF25	MMT GAT CAG CGA CCA CCG CAC CCT GTC A	69.5
SPF26	MMT CTT CAG CAC CAC CAT CAT CCG GCG C	69.5
SPF27	MMT GGA TTC GTG ATC TCT TCC CGC GGA T	66.3
SPF28	MMT TGC CCC GGC GTT TAG CGA TCA CAA C	67.9
SPF29	MMT AAA TAC AGG CTC CAC GAC ACG ACC A	64.6
SPF30	MMT GGT TGC CCC GCG CCC TTT TCC AGC C	72.8
SPF31	MMT TCA GAC AGG TTC GCG TCG ATC AAG T	64.6
SPF32	MMT GAC CAA ATA GGT ATC GGC GTG TTC A	63.0
SPF33	MMT GAC ATG ACG GCG GTG CCG CAC TTG A	69.5
SPF34	MMT AAG TCA CCT CGC CCA CAC CGT CGA A	67.9
SPF35	MMT TCC GTA CGC TCG AAA CGC TTC CAA C	66.3
SPF36	MMT CGA AAT CCA GCA CCA CAT CCG CAG C	67.9
SPF37	MMT CGC GAA CTC GTC CAC AGT CCC CCT T	69.5
SPF38	MMT CGT GGA TGG CGG ATG CGT TGT GCG C	71.2
SPF39	MMT GAC GAT GGC CAG TAA ATC GGC GTG G	67.9
SPF40	MMT CGC CAT CTG TGC CTC ATA CAG GTC C	67.9
SPF41	MMT GGA GCT TTC CGG CTT CTA TCA GGT A	64.6
SPF42	MMT ATG GTG GGA CAT GGA CGA GCG CGA C	69.5
SPF43	MMT CGC AGA ATC GCA CCG GGT GCG GGA G	72.8

3.3.9 Identification of patient ethnic origin

The given and family names of 2,185 patients were entered into Origins software Version 6 (Origins; Experian, Nottingham, UK) to obtain a CEL group for each, which was then assigned a continent on the basis of the United Nations Standard Country and Area Coded Classification Scheme as previously described (Evans *et al*., 2010).

3.3.10 Strain lineage assignment

MIRU-VNTR data for each isolate was compared to the MIRU-VNTR*plus* database (Allix-Beguec *et al*., 2008b). A categorical co-efficient which generates a match distance was used to identify strains from the database which are related to the MIRU-VNTR profile for each Midlands isolate. A match value of 0 indicates an absolute match and a value of 1 indicates that there was no match at any of the 15 MIRU-VNTR. A cut off value of 0.17 indicates differences at no more than four loci and a cut off value of 0.3 indicates differences at no more than seven loci.

3.3.11 Identification of patient location

Postcodes were matched to known PCTs in the Midlands. PCTs are healthcare organisations based on population and geographical boundaries, which are responsible for 80% of the NHS budget. There are 17 PCTs in the West Midlands and 9 in the East Midlands. Postcode data was missing for 291 isolates which were excluded from the study and a further 211 isolates were excluded as the patient did not reside in the Midlands region. A summary of the strains that were selected is shown in Figure 3-4.

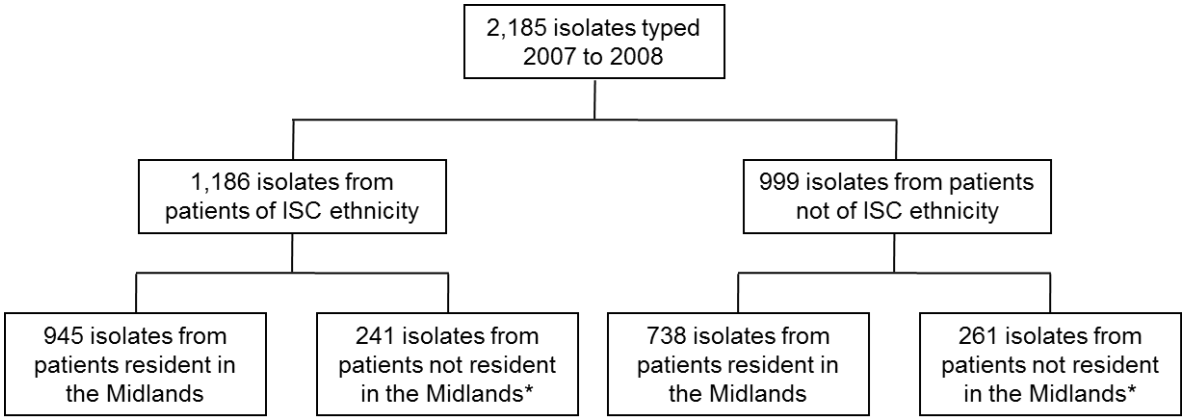


Figure 3-4 Strains selected for analysis of phylogeographic lineages of *M. tuberculosis* present in the Midlands

* patients not residing in the Midlands or for whom postcode data was not available were excluded from further study

3.3.12 Geographical distribution of spoligotype clades

Geographical distribution of spoligotype clade and TB patient ethnicity was analysed as a whole and within the Midlands region, which is made up of 11 counties and within the West Midlands county, which consists of nine Primary Care Trusts (PCT).

3.4 Results

3.4.1 Phylogeographic lineages of *M. tuberculosis* amongst 100 patients of ISC ethnicity in the Midlands, UK

3.4.1.1 Spoligotype clade assignation

A selection of 100 *M. tuberculosis* isolates from the most prevalent MIRU-VNTR profiles identified in patients who had ISC ethnicity were analysed by spoligotyping. Spoligotype interrogation was successful for 96 of 100 isolates and resulted in 60 different spoligotype patterns. Spoligotype patterns were not obtained in four cases as DNA amplification was unsuccessful. Comparison of the 60 spoligotype patterns to SITVIT showed that 40 of the patterns were SITs and 20 were unique patterns that are described as orphan profiles, (one pattern was not compared to SITVIT). Nearly 80% (75 of 96) of the isolates belonged to a SIT.

Of the 40 SITs that were found the largest group was SIT 26 (CAS1_DELHI), which contained 18 isolates. SIT 11 (EAI3_IND) contained five isolates and SIT 21 (CAS1_KILI) contained four isolates. SIT 53 (T1), SIT 2696 (CAS1_Delhi) and SIT 1 (BEIJING) each had three isolates. SIT 2993 (CAS2), SIT 288 (CAS2), SIT 485 (CAS1_DELHI), SIT 25 (CAS1_DELHI), SIT 490 (X1) and SIT 50 (H3) each contained two isolates. The 28 other SITs contained one isolate each. When the SITs and orphan profiles were grouped into clades, the largest clade was CAS, which contained 57 isolates. EAI was the second largest clade and contained 10 isolates. Clades T and H contained eight and seven isolates respectively. The rest of the clades contained three isolates or fewer (Table 3-6).

Table 3-6 Number of *M. tuberculosis* isolates belonging to different spoligotype clades

Clade	No. of isolates	Sub-clade	No. of isolates	No. of different SITs	No. of orphan patterns
CAS	57	CAS1-Delhi	45	15	10
		CAS1-Kili	5	1	1
		CAS2	6	2	2
		CAS	1	1	0
EAI	10	EAI	3	1	2
		EAI3-IND	5	1	0
		EAI6-BGD1	2	2	0
T	8	T1	7	5	0
		T5	1	1	0
H	7	H2	1	1	0
		H3	3	1	1
		H4	3	2	1
Beijing	3	Beijing	3	1	0
U	3	U	3	1	2
LAM	2	LAM11-ZWE	2	1	1
X	2	X1	2	1	0
AFRI	1	AFRI	1	1	0
MANU	1	MANU2	1	1	0
S	1	S	1	1	0
Unclassified	1	Unclassified	1	0	1
Total	**96**		**96**	**40**	**21**

3.4.1.2 Principal Genetic Grouping

Figure 3-5 shows example PCR-RFLP patterns from PGG1, PGG2 and PGG3 strains. These strains were determined to belong to each group by sequencing of PCR amplicon for the *katG* and *gyrA* regions. *Ale*I digestion of amplicon from the *gyrA* loci of PGG1 and PGG2 strains produced two DNA fragments (161 and 193 base pairs) (Figure 3-5, lanes two and three), while amplicon from PGG3 strains remained uncut (354 base pairs) (Figure 3-5, lane 4). *Bst*NI digestion of amplicon from the *katG* loci of PGG1 strains produced four DNA fragments (12, 61, 104 and 174 base pairs), three of these fragments were visible on a 2% agarose gel (Figure 3-5, lane six); the 12 base pair fragment was too small to be resolved. *Bst*NI digestion of amplicon from the *katG* loci of PGG2 and PGG3 strains produced three DNA fragments (12, 61 and 278 base pairs), two of which were visible on a 2% agarose gel (Figure 3-5, lanes seven and eight) again the 12 base pair fragment was not resolved. PGG typing identified that 72 of 96 (75%) isolates belonged to PGG1, 18 of 96 (19%) belonged to PGG2 and 6 of 96 (6%) belonged to PGG3.

3.4.1.3 RD1 and TBD1 analysis

The RD1 region was present in all 96 selected isolates (100%). The TbD1 region was retained (TbD1+) in 11 of 96 (12%) isolates and the region was deleted (TbD1-) in 84 of 96 (88%) isolates. DNA from one isolate failed to amplify when PCR was carried out using primers that were complementary to the internal sequence of the TbD1 region or with primers that were complementary to the sequence that flanks the TbD1 region. DNA from this isolate was re-extracted but PCR was still unsuccessful and so the presence or absence of the region in this isolate could not be determined.

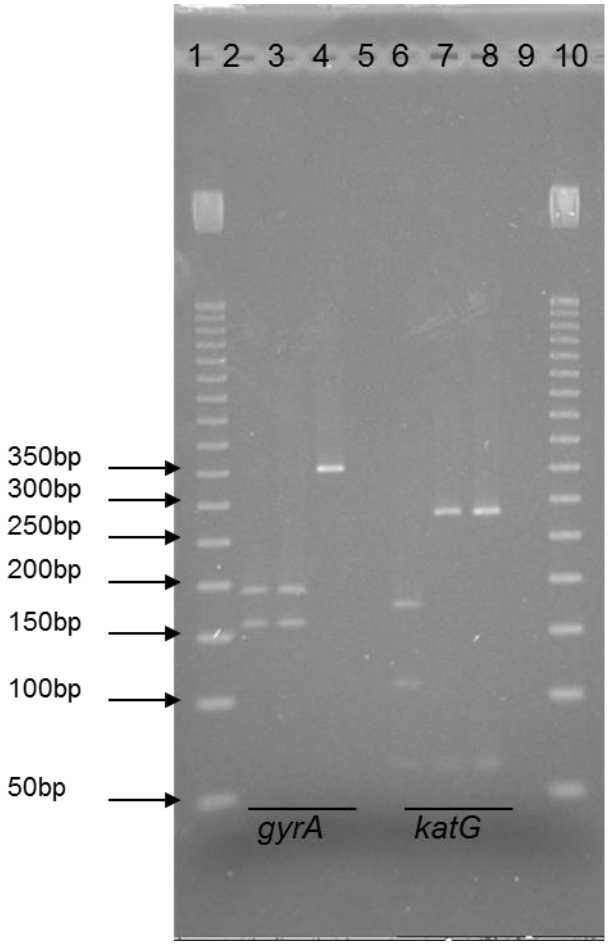


Figure 3-5 Ethidium bromide staining of PCR products for *katG* and *gyrA* loci

Loci were amplified from total genomic DNA of PGG 1, 2 and 3 organisms and digested with *Ale*I (*gyrA* amplicon), and *Bst*NI (*katG* amplicon), separated on a 2% agarose gel by electrophoresis, then stained with ethidium bromide, bp stands for base pairs

Lane 1; 50 base pair ladder

Lane 2; digested *gyrA* amplicon from PGG1 organism

Lane 3; digested *gyrA* amplicon from PGG2 organism

Lane 4; digested *gyrA* amplicon from PGG3 organism

Lane 5; negative control containing no PCR amplicon

Lane 6; digested *katG* amplicon from PGG1 organism

Lane 7; digested *katG* amplicon from PGG2 organism

Lane 8; digested *katG* amplicon from PGG3 organism

Lane 9; negative control containing no PCR amplicon

Lane10; 50 base pair ladder

3.4.1.4 Summary of genotyping data

Of the 96 isolates selected for analysis there were 11 PGG1 strains that had retained the TbD1 region (TbD1+) and were ancient in terms of evolution. Ten of the TbD1+ isolates belonged to the EAI spoligotype and one isolate belonged to the AFRI clade (Table 3-7). There were 60 PGG1 isolates in which the TbD1 region was deleted (TbD1-), meaning they were modern compared to the PGG1 isolates that have retained the TbD1 region. Of these 60 isolates, 56 belonged to the CAS spoligotype clade, three isolates belonged to the Beijing clade and one was not analysed. There were 18 PGG2 TbD1- isolates, which were more modern than the PGG1 isolates. There were six PGG3 TbD1- isolates, which were the most modern strains. PGG2 and PGG3 isolates belonged to various modern spoligotype clades.

Table 3-7 Distribution of ancient, and modern strains of *M. tuberculosis* amongst the spoligotype clades

	Time →				
Spoligotype	**TbD1+ PGG1**	**TbD1- PGG1**	**TbD1- PGG2**	**TbD1- PGG3**	**Total strains**
CAS	0	56	0	0	56
EAI	11	0	0	0	11
T	0	0	3	5	8
Haarlem	0	0	7	0	7
Beijing	0	3	0	0	3
U	0	0	3	0	3
LAM	0	0	2	0	2
X	0	0	2	0	2
AFRI	1	0	0	0	1
S	0	0	0	1	1
MANU	0	0	1	0	1
Not defined	0	1	0	0	1
Total strains	**12**	**64**	**18**	**6**	**96**

3.4.1.5 Identification of patient country of birth

Information about country of birth was available for 69 of the 96 patients from whom TB isolates were selected for further analysis. Eighteen of the 69 patients (26%) were born in India, which was the most common country of birth, followed by Pakistan,

where 11 of the patients (16%) were born. There were 10 patients (15%) born in Somalia and 10 patients born in the UK respectively. Country of birth was recorded as unknown for 9 of the 69 patients (13%). There were two patients (3%) born in Kenya and there was one patient (2%) born in each of the following countries; Afghanistan, Bangladesh, Congo, Iraq, Malaysia, Saudi Arabia, Sri Lanka, Sudan and Zimbabwe.

When countries were grouped by continent, it was found that more than half of the patients were born in Asia (35 of 69), a fifth were born in Africa (15 of 69) and less than a sixth of patients were born in Europe (10 of 69), of the patients that data was available for.

3.4.1.6 Verification of population groups assigned by Origins software

Self-declared population group data for 69 patients identified as originating from the ISC was assessed to determine the accuracy of ethnicity predictions made by Origins software Version 1. Nearly three quarters (74%) of the predictions made by Origins Version 1 were concordant with patient self-declared ethnicity. The predications made for 9% of the patients (6 of 69) could not be confirmed because the ethnicity of the patient was not recorded. If these patients are removed from the analysis, the concordance is increased to 83% (52 of 63). In the 17% of cases (11 of 69) where the ethnicity of a patient was incorrectly predicted by Origins Version 1, the patient was always assigned to the Black African CEL group.

Of the 69 patients for whom self-declared ethnic origin data was available, over 40% (29 of 69) had Indian ethnicity. More than 20% of the patients (15 of 69) had

Pakistani ethnicity and 17% (12 of 69) had Black African ethnicity. Mixed ethnicity was recorded for one patient (<2%) and ethnic origin was recorded as not known for five patients (7%). There were five patients listed as having other Asian ethnicity and two patients (3%) with Bangladeshi ethnicity. When patient ethnicity was grouped by continent, nearly 74% (51 of 69) of the patients had Asian ethnicity, 17% (12 of 69) had African ethnicity and the ethnicity of the remaining patients was not recorded.

Origins software Version 6 (Experian, Nottingham, UK) was released after spoligotyping had been carried out on the 96 selected strains. The ethnicity of the patients was reanalysed using the updated version of Origins. Origins software Version 6 predicted that 2 of the 96 patients had African ethnicity, 2 had east Asian ethnicity, 16 had Middle Eastern ethnicity, 72 had south Asian ethnicity, 3 had south European ethnicity and there was 1 patient for whom no match was found.

The predictions made by Origins software Version 6 about the ethnicity of 69 patients were concordant with patient self-declared ethnicity in 74% of cases (51 of 69). The predictions made for 9% of the patients (6 of 69) could not be confirmed, as patient declared ethnicity was not recorded. If these patients are removed from the analysis, the concordance is increased to 85% (51 of 60). In 17% of cases (11 of 69), the ethnicity of a patient predicted by Origins software Version 6 was discordant with patient declared ethnicity (Table 3-8).

3.4.1.7 Evaluation of spoligotype clade assignation using MIRU-VNTR*plus*

MIRU-VNTR profiles using 15 loci were recorded between 2007 and 2008 for 2,185 Midlands *M. tuberculosis* isolates. However, an international database of

MIRU-VNTR profiles was not available at the time of analysis to allow identification of phylogeographical lineages in the Midlands. In order to study phylogeographical lineages the MIRU-VNTR*plus* database was used to assign spoligotype clades based on MIRU-VNTR data, which could then be compared to SITVIT. In order to evaluate the accuracy of spoligotype assignations made using MIRU-VNTR*plus*, the spoligotype clade assignments made by SITVIT for the 96 isolates for which experimental spoligotype profiles have been generated were compared to spoligotype assignations made by MIRU-VNTR*plus.* A categorical co-efficient which generates a matching distance was used to identify strains from the MIRU-VNTR*plus* database which are related to the MIRU-VNTR profiles obtained for the Midlands strains.

Table 3-8 Comparison of patient declared ethnicity with predictions made by Origins software Version 6 for 69 patients for whom ethnicity data was available

		Patient declared ethnicity (%)			
		African	**Asian**	**European**	**Unknown**
Ethnicity predicted by Origins	**African**	1 (8)	0 (0)	0 (0)	1 (17)
	Asian	8 (67)	50 (98)	0 (0)	5 (83)
	European	2 (17)	1 (2)	0 (0)	0 (0)
	Unknown	1 (8)	0 (0)	0 (0)	0 (0)

The spoligotype clades assigned by SITVIT and MIRU-VNTR*plus* were concordant for 75 of 95 isolates (79%). One isolate was not compared to SITVIT and so the spoligotype pattern was not assigned to a clade. There was 91% (52 of 57) concordance between the assignations made for the CAS spoligotype clade. However, MIRU-VNTR*plus* incorrectly assigned four CAS isolates to the Beijing clade (matching distance for each isolate = 0.133) and one to the T clade (matching distance = 0.200).

There was 90% concordance between the assignations made by SITVIT and MIRU-VNTR*plus* for the EAI clade (9 of 10). However, one EAI isolate was incorrectly assigned to the Beijing clade by MIRU-VNTR*plus* (matching distance = 0.286). There was 13% concordance between assignations made by SITVIT and MIRU-VNTR*plus* for clade T (1 of 8). MIRU-VNTR*plus* predicted that three isolates belonged to clade T, however only one of these predictions was correct, the other two isolates actually belonged to the CAS and AFRI clades. Six T clade isolates were assigned to the LAM clade by MIRU-VNTR*plus* and one to the Haarlem clade (Table 3-9). The positive predictive power of MIRU-VNTR*plus* for spoligotype clades that are prevalent in the ISC, such as CAS and EAI was high. The predictive power of MIRU-VNTR*plus* for modern spoligotype clades such as T, Haarlem and LAM was much lower.

3.4.2 Phylogeographic lineages of *M. tuberculosis* present in the Midlands, UK

3.4.2.1 Identification of patient ethnic origin

Of the 2,185 TB patients from whom *M. tuberculosis* isolates were assessed by the MRCM between 2007 and 2008, Origins was able to assign a CEL group in 2,143 cases (98%). A CEL group could not be assigned for 42 patients (2%) as either the first or the second name was not recognised by the Origins database (40 cases) or only the first initial had been recorded (two cases). When CEL groups were arranged by continent, Asia was the most common continent of origin amongst TB patients (1,186 of 2,185; 54%) and Europe was the second most common continent of origin (709 of 2,185; 32%). Patients who originated from Africa (212 of 2,185; 10%), the Americas (28 of 2,185; 1%), and Oceania (eight of 2,185; <1%) accounted for 12% of the TB cases.

Table 3-9 Comparison of the spoligotype assignments made by the SITVIT and MIRU-VNTR*plus* databases

Clade	SITVIT assignment*	MIRU-VNTR*plus* prediction	MIRU-VNTR*plus* average match distance (all matches)	No. of correct MIRU-VNTR*plus* predictions**	MIRU-VNTR*plus* average match distance (correct matches)	SITVIT assignment of MIRU-VNTR*plus* under predictions	SITVIT assignment of MIRU-VNTR*plus* over predictions
CAS	57	52	0.15	52	0.15	4 Beijing, 1 T	-
EAI	10	9	0.17	9	0.17	1 Beijing	-
T	8	3	0.18	1	0.13	6 LAM, 1 Haarlem	1 CAS, 1 AFRI
Haarlem	7	11	0.07	7	0.05	-	2 X, 1 T 1 orphan
Beijing	3	8	0.14	3	0.09	-	1 EAI, 4 CAS
Orphan	3	0	-	0	-	1 Haarlem, 2 LAM	-
LAM	2	11	0.15	2	0.17	-	6 T, 2 orphan, 1 MANU
X	2	0	-	0	-	2 Haarlem	-
AFRI	1	0	-	0	-	1 T	-
MANU	1	0	-	0	-	1 LAM	-
S	1	1	0.13	1	0.13	-	-
Total	**95**	**95**	**-**	**75**	**-**		

* SITVIT assignments were made by experts at the Institute Pasteur

** correct assignments are where MIRU-VNTR*plus* predictions correlate with SITVIT assignments

The majority of TB patients were assigned to the Asian continental group. When this group was examined in more detail it was found that 79% of patients within the Asian continental CEL group could be further classified as belonging to the south Asian group (933 of 1,186), meaning that 43% of all of TB patients could be assigned to the south Asian group (933 of 2,185). There were three other groups within the Asian CEL group; Middle Eastern, which contained 18% of the patients (212 of 1,186), east Asian which contained 3% of the patients (38 of 1,186) and diasporic which contained less than 1% of the patients (3 of 1,186).

3.4.2.2 Spoligotype assignation using MIRU-VNTR*plus*

MIRU-VNTR profiles for 1,683 Midlands TB isolates obtained between 2007 and 2008 were compared to the MIRU-VNTR*plus* database. The numbers of matches made at different specificities are shown in Table 3-10.

An exact match to a database isolate was made for 6% of the Midlands isolates (97 of 1,683). When 0.17 was selected as the matching distance cut off, which corresponds to differences at no more than four MIRU-VNTR loci, a match was found for 61% of the Midlands isolates (1,025 of 1,683; average matching distance 0.10). Of the 1,025 isolates for which a match was found, 54% (911 of 1,683; average matching distance 0.10) were assigned to a single spoligotype clade but a spoligotype could not be assigned for 7% (114 of 1,683: average matching distance 0.13) of the isolates as they had a mixed assignation. Mixed assignations occurred because a test isolate matched to two or more database isolates with the same matching distance but the database strains belonged to different spoligotype clades, meaning that the test isolate was equally likely to belong to either spoligotype clade.

When the matching distance cut off was relaxed to 0.3, which corresponds to differences at no more than seven MIRU-VNTR loci a spoligotype was identified for 97% of the isolates (1,628 of 1,683; average matching distance 0.15). Of the 1,628 matches that were identified, 85% (1,437 of 1,683; average matching distance 0.14) were assigned to a single spoligotype clade and a mixed assignation was made for 11% of the isolates (191 of 1,683; average matching distance 0.16).

Using the least stringent matching criteria to permit a match to be found for all of the Midlands isolates, 88% of the isolates (1,483 of 1,683; average matching distance 0.15) were assigned to a single spoligotype clade. However, 12% of the isolates (200 of 1,683, average matching distance 0.17) could not be assigned to a single spoligotype clade as they had a mixed assignation. The 200 isolates with a mixed spoligotype assignation were excluded from further analysis, leaving 1,483 isolates. Matching distances ranged from 0 (exact match) to a maximum of 0.4, the average matching distance was 0.15. The average matching data for all strains, which were assigned to a single spoligotype clade with no ambiguity by matching to the MIRU-VNTR*plus* database, are shown in Table 3-11.

The most prevalent spoligotype clade in the Midlands was the CAS clade, which contained 39% of the isolates that were assigned to a single spoligotype clade (574 of 1,483: average matching distance 0.15). LAM, EAI and Haarlem contained 16%, 15% and 14% of the isolates respectively (LAM; 239 of 1,483, average matching distance 0.16.,EAI; 225 of 1,483, average matching distance 0.19, Haarlem; 209 of 1,483, average matching distance 0.10). The AFRI clade showed the lowest average

matching distance of 0.05 and the Caprae clade showed the highest average matching distance of 0.24.

Table 3-10 Number of Midlands isolates from 2007 to 2008 for which a spoligotype clade was identified using the MIRU-VNTR*plus* database at different levels of specificity

Matching distance cut off	Definition	Same 1st and 2nd match		Different 1st and 2nd match		Total (%)
		Different matching distance	Same matching distance	Different matching distance	Same matching distance	
0	Exact match	5	92	0	0	97 (6)
0.17	4 locus difference	325	415	74	114	928 (55)
0.3	7 locus difference	154	304	69	77	604 (36)
>0.3	More than 7 locus difference	5	36	4	9	54 (3)
Total		**489**	**847**	**147**	**200***	**1,683 (100)**

* these isolates were equally likely to belong to two different spoligotype clades

Table 3-11 Comparison of Midlands isolates from 2007 to 2008 that were accurately matched to the MIRU-VNTRplus database

Spoligotype clade	No. of isolates (%)		Average matching distance	Number of isolates with a MIRU-VNTR*plus* matching distance of					
				<0.17 (%)		0.18-0.3 (%)		>0.3 (%)	
CAS	574	(39)	0.15	374	(65)	193	(34)	7	(1)
LAM	239	(16)	0.16	111	(46)	118	(49)	10	(4)
EAI	225	(15)	0.19	97	(43)	105	(47)	23	(10)
Haarlem	209	(14)	0.10	167	(80)	39	(19)	3	(1)
Beijing	102	(7)	0.11	85	(83)	16	(16)	1	(1)
T	65	(4)	0.17	30	(46)	33	(51)	2	(3)
S	24	(2)	0.15	17	(71)	7	(29)	0	(0)
X	23	(2)	0.12	19	(83)	4	(17)	0	(0)
AFRI	10	(1)	0.05	10	(100)	0	(0)	0	(0)
Bovis	8	(1)	0.21	1	(13)	7	(88)	0	(0)
Caprae	4	(0)	0.24	0	(0)	4	(100)	0	(0)
Total	**1,483***	**(100)**	**0.15**	**911**	**(61)**	**526**	**(35)**	**46**	**(3)**

* 1,683 isolates from patients between 2007 and 2008 for which postcode data indicated residence within the Midlands, minus 200 isolates that could not be accurately assigned to a spoligotype clade by MIRU-VNTR*plus*

Mixed spoligotype assignations occurred for 12% of the isolates (200 of 1,683) (Table 3-12). Haarlem clade was the most likely to be present in a mixed assignation, 82% (164 of 200) of the isolates which had a mixed assignation could have belonged to Haarlem or another clade, 43% (86 of 200) were assigned to either Haarlem or X clade and 31% (62 of 200) could have belonged to Haarlem or LAM. Of the isolates with a mixed assignation 47% (94 of 200) belonged to X or another clade and 39% (77 of 200) belonged to LAM or another clade.

Table 3-12 Sub-analysis of 200 isolates which were unmatched by comparison to the VNTR-MIRU*plus* database

	Haarlem	X	LAM	T	CAS	Caprae	AFRI	Beijing	Bovis	S	EAI	Seal	Total
Haarlem	-	86	62	7	8	0	0	0	0	1	0	0	**164**
X	86	-	8	0	0	0	0	0	0	0	0	0	**94**
LAM	62	8	-	4	0	0	0	2	0	1	0	0	**77**
T	7	0	4	-	3	4	2	2	0	0	0	0	**22**
CAS	8	0	0	3	-	0	0	3	0	0	0	0	**14**
Caprae	0	0	0	4	0	-	3	0	2	0	0	0	**9**
AFRI	0	0	0	2	0	3	-	0	0	0	1	1	**7**
Beijing	0	0	2	2	3	0	0	-	0	0	0	0	**7**
Bovis	0	0	0	0	0	2	0	0	-	0	0	0	**2**
S	1	0	1	0	0	0	0	0	0	-	0	0	**2**
EAI	0	0	0	0	0	0	1	0	0	0	-	0	**1**
Seal	0	0	0	0	0	0	1	0	0	0	0	-	**1**
Total	**164**	**94**	**77**	**22**	**14**	**9**	**7**	**7**	**2**	**2**	**1**	**1**	

The MIRU-VNTR*plus* database contains the species assignation for each isolate. The species of each TB isolate in the Midlands is identified by the MRCM using a species specific *M. tuberculosis* complex DNAstrip®. Species assignations made by the MRCM were compared to the assignations made by MIRU-VNTR*plus* in order to check the accuracy of the matches at a species level. Nearly all of the species assignations made by MIRU-VNTR*plus* were the same as the assignations made by the MRCM (1,667 of 1,683; 99%) (Table 3-13). Less than 1% (16 of 1,683) of the species assignations did not match when the two methods were compared.

MIRU-VNTR*plus* assigned 12 isolates as *M. africanum* however, the MRCM identified 16 *M. africanum* isolates present in the sample. MIRU-VNTR*plus* correctly identified seven *M. africanum* isolates; but it assigned five *M. africanum* isolates as *M. caprae*, two *M. africanum* isolates as *M. bovis* and two *M. africanum* isolates as *M. tuberculosis*. MIRU-VNTR*plus* assigned nine isolates as *M. bovis* but the MRCM identified six *M. bovis* isolates present in the sample. MIRU-VNTR*plus* correctly identified six *M. bovis* isolates; however, it assigned two *M. africanum* isolates as *M. bovis* and one *M. tuberculosis* isolate as *M. bovis*. MIRU-VNTR*plus* assigned six isolates as *M. caprae* but the MRCM did not identify any *M. caprae* isolates. MIRU-VNTR*plus* assigned five *M. africanum* isolates and one *M. tuberculosis* isolate as *M. caprae*. MIRU-VNTR*plus* assigned 1,656 isolates as *M. tuberculosis* whereas the MRCM identified 1,661 *M. tuberculosis* isolates. MIRU-VNTR*plus* correctly assigned 1,654 *M. tuberculosis* isolates; but two of the isolates that it assigned as *M. tuberculosis* were *M. africanum*. MIRU-VNTR*plus* incorrectly assigned five *M. tuberculosis* isolates as *M. africanum*, one *M. tuberculosis* isolate as *M. bovis* and one *M. tuberculosis* isolate as *M. caprae*.

Table 3-13 Number of isolates that were assigned to different *M. tuberculosis* complex species by the MRCM and the MIRU-VNTR*plus* database

Species identified by DNAstrip® test	Species identified by MIRU-VNTR*plus*				Total
	M. africanum	*M. bovis*	*M. caprae*	*M. tuberculosis*	
M. africanum	7	2	5	2	**16**
M. bovis	0	6	0	0	**6**
M. caprae	0	0	0	0	**0**
M. tuberculosis	5	1	1	1,654	**1,661**
Total	**12**	**9**	**6**	**1,656**	**1,683**

3.4.2.3 Prominent CEL groups in the Midlands

There were a high proportion of *M. tuberculosis* strains from the CAS spoligotype clade in the Midlands. To determine whether the high proportion of CAS stains was

due to a high proportion of people from the ISC living in the Midlands, the population structure of the Midlands was assessed. The Origins database was used to assign patients to CEL groups, which were then grouped by geographical regions.

A CEL group was identified for 99% of the patients (1,659 of 1,683) who were diagnosed with TB between 2007 and 2008 (Table 3-14). When CEL groups were arranged by continent, Asia was the most common continent of origin amongst TB patients (945 of 1683, 56%) and Europe was the second most common continent of origin (538 of 1683, 32%). Patients who originated from Africa (151 of 1,683, 9%), the Americas (20 of 1,683, 1%), and Oceania (five of 1,683, <1%) were responsible for 10% of the TB cases.

When continental assignations were broken down into regions, nearly half of all patients were assigned to the south Asian region (783 of 1,683, 47%), with India being the most prevalent CEL group (397 of 1,683, 24%) from the Asian region. A quarter of all patients were assigned to the northern European region (432 of 1683, 26%), with England being the most prevalent CEL group (317 of 1683, 19%) from the region. There were 82 different CEL groups in the Midlands TB patient population. Europe was the most diverse region with 28 different CEL groups, followed by Asia, which had 25 different CEL groups. A quarter of the Midlands TB patients (417 of 1,659) that were assigned to a CEL group using origins had a UK CEL group, whereas three quarters of the patients (1,242 of 1,659) did not have a UK CEL group. Nearly two thirds of the people who did not have a UK CEL group (783 of 1,242, 63%) had a south Asian CEL group.

Table 3-14 Major Cultural, Ethnic, and Linguistic (CEL) groups present in typed *M. tuberculosis* isolates in the Midlands between 2007 and 2008

Continental origin of patient	Region	Most prevalent CEL group	n (%)	No. of other CEL groups	n (%)	Total isolates (%)
Asia	South	India	397 (24)	14	386 (23)	783 (47)
	West	West Asia	84 (5)	3	55 (3)	139 (8)
	East	Chinese Cantonese	6 (0)	3	9 (1)	15 (1)
	South east	Philippines	6 (0)	1	2 (0)	8 (0)
						945 (56)
Europe	North	England	318 (19)	10	114 (7)	432 (26)
	West	Germany	26 (2)	5	21 (1)	47 (3)
	South	Italy	17 (1)	6	27 (2)	44 (3)
	East	Poland	10 (1)	3	5 (0)	15 (1)
						538 (32)
Africa	East	Somalia	47 (3)	8	24 (1)	71 (4)
	South	Black S. Africa	21 (1)	3	8 (0)	29 (2)
	North	Morocco	16 (1)	0	0 (0)	16 (1)
	West	Nigeria	11 (1)	2	14 (1)	25 (1)
	Africa	Africa	5 (0)	1	1 (0)	6 (0)
	Middle	Congo	3 (0)	1	1 (0)	4 (0)
						151 (9)
Americas	Caribbean	Black Caribbean	5 (0)	1	1 (0)	6 (0)
	South	Brazil	5 (0)	0	0 (0)	5 (0)
	North	USA Black	4 (0)	2	5 (0)	9 (1)
						20 (1)
Oceania	Oceania	Australia	4 (0)	1	1 (0)	5 (0)
Not assigned			24 (1)	0	0 (0)	24 (1)
Total						**1,683 (100)**

3.4.2.4 Global distribution of *M. tuberculosis* lineages in CEL groups resident in the Midlands, UK

Two-thirds of the TB patients belonged to an Asian CEL group (895 of 1,483, 60%) so the most prevalent spoligotype clades amongst these patients strongly influenced the prevalence of clades within the Midlands. CAS was the most prevalent spoligotype clade in the Midlands (574 of 1,483, 39%) and it was the most prevalent spoligotype clade amongst TB patients from an Asian CEL group (Table 3-15). More than half of the TB strains from people belonging to an Asian CEL group belonged to

the CAS clade (463 of 895, 52%) (Figure 3-6). The second most prevalent CEL group was the European group (414 of 1,483, 28%). CAS was the third most prevalent clade (78 of 414, 19%) amongst patients from the European CEL group.

LAM was the second most prevalent spoligotype clade within the Midlands (239 of 1,483, 16%). LAM was the third most prevalent spoligotype clade amongst TB patients from an Asian CEL group (88 of 895, 10%) and the second most prevalent clade amongst TB patients from a European CEL group (88 of 414, 21%). The third most prevalent spoligotype clade in the Midlands was EAI (225 of 1,483, 15%). EAI was the second most prevalent spoligotype clade amongst TB patients from an Asian CEL group (157 of 895, 18%) and the fourth most prevalent clade amongst TB patients from a European CEL group (40 of 414, 10%). The fourth most prevalent spoligotype clade in the Midlands was Haarlem (209 of 1,483, 14%). Haarlem was the fifth most prevalent spoligotype clade amongst TB patients belonging to an Asian CEL group (66 of 895, 7%) but it was the most prevalent clade amongst TB patients belonging to a European CEL group (127 of 414, 31%).

Table 3-15 Distribution of *M. tuberculosis* isolates in the Midlands according to predicted spoligotype clade and continent of patient origin based on CEL group

	Spoligotype clade predicted by MIRU-VNTR*plus* (% of total by region)											
Region	**AFRI**	**Beijing**	**Bovis**	**Caprae**	**CAS**	**EAI**	**Haarlem**	**LAM**	**S**	**T**	**X**	**Total**
Asia	1 (0)	71 (8)	2 (0)	4 (0)	463 (52)	157 (18)	66 (7)	88 (10)	6 (1)	32 (4)	5 (1)	**895**
Europe	3 (1)	21 (5)	6 (1)	0 (0)	78 (19)	40 (10)	127 (31)	88 (21)	12 (3)	24 (6)	15 (4)	**414**
Africa	5 (4)	6 (4)	0 (0)	0 (0)	26 (19)	23 (17)	11 (8)	50 (37)	4 (3)	8 (6)	2 (2)	**135**
Others*	2 (5)	4 (10)	0 (0)	0 (0)	7 (18)	5 (13)	5 (13)	13 (33)	2 (5)	1 (3)	1 (3)	**39**
Total	**10**	**102**	**8**	**4**	**574**	**225**	**209**	**239**	**24**	**65**	**23**	**1,483**

* this group included Americas, Oceania and not identified

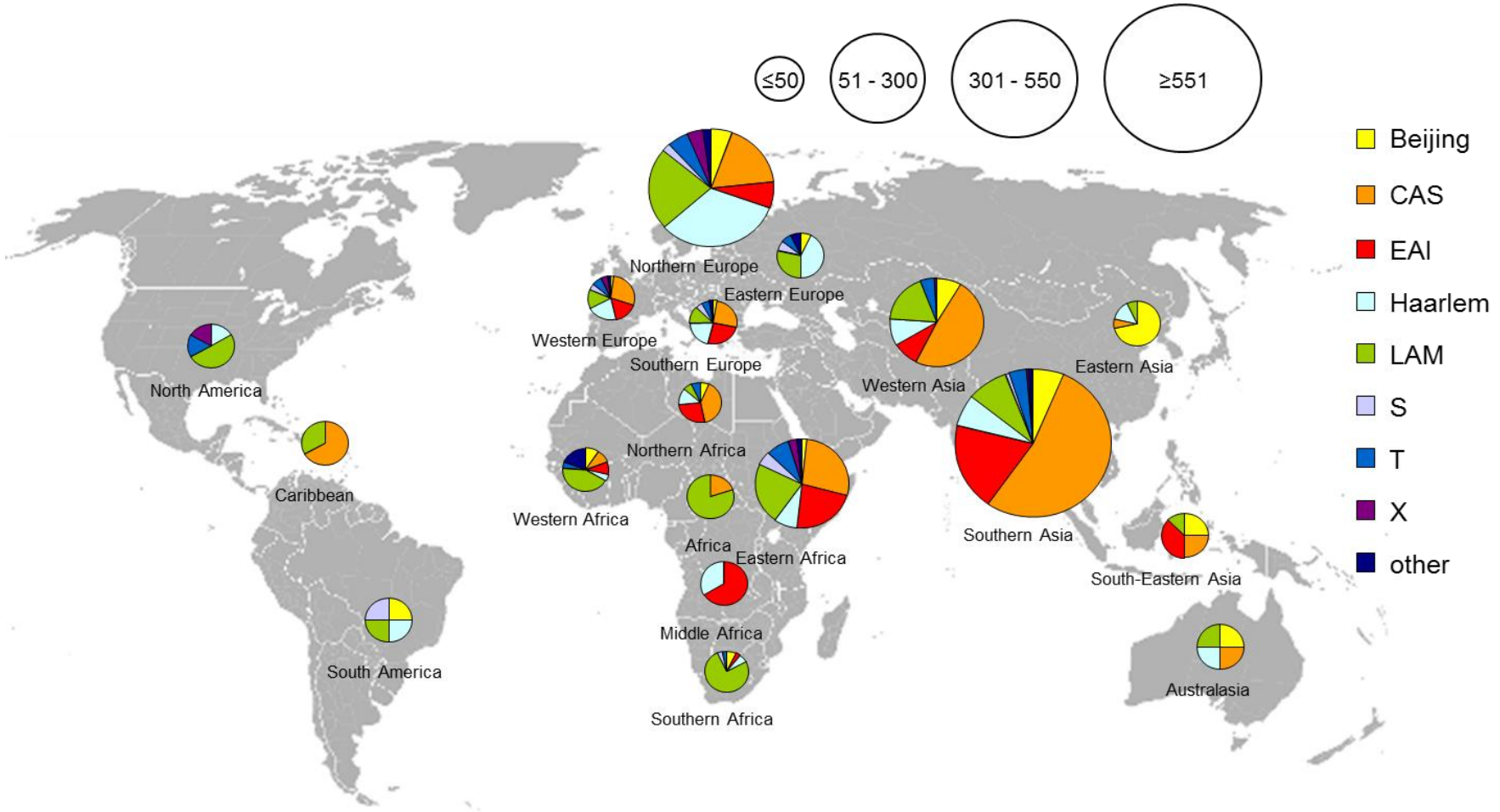


Figure 3-6 Global distribution of CEL groups and the eight major *M. tuberculosis* spoligotype clades

Each circle is located in the continental region where the CEL group originated. The size of the circle is proportional to the number of patients from each region. Map adapted from http://commons.wikimedia.org/wiki/File:BlankMap-World-2009.PNG.

3.4.2.5 The composition of *M. tuberculosis* lineages distributed across the Midlands, UK

The Midlands region of the UK consists of 11 counties, which are divided into the East Midlands (Derbyshire, Leicestershire, Lincolnshire, Northamptonshire and Nottinghamshire) and the West Midlands (Herefordshire, Shropshire, Staffordshire, Warwickshire, West Midlands county and Worcestershire). Nearly two thirds of the TB isolates from 2007 and 2008 came from patients who lived in the West Midlands (920 of 1,483, 62%), one third came from patients who lived in the East Midlands (563 of 1,483, 38%) (Figure 3-7). More than half of all these TB isolates came from patients who lived in the county of West Midlands (771 of 1,483, 52%) which is in the West Midlands region. Leicestershire had the second highest number of typed isolates (205 of 1,483, 17%) and Nottinghamshire had the third highest number of isolates (125 of 1,483, 8%). There were five counties that had less than 50 isolates (Herefordshire, Shropshire, Warwickshire, Lincolnshire and Worcestershire) and three counties with between 51 and 100 isolates (Staffordshire, Northamptonshire and Derbyshire). The lowest number of isolates came from the county of Herefordshire (5 of 1,483, <1%).

CAS was the most prevalent spoligotype in six counties (6 of 11, 55%), the highest proportion of CAS was in Staffordshire where it accounted for 32 of 67 (48%) isolates (Figure 3-7). The county of West Midlands had the highest number of CAS isolates with 340 of 771 (44%); followed by Leicestershire where CAS accounted for 98 of 250 isolates (39%).

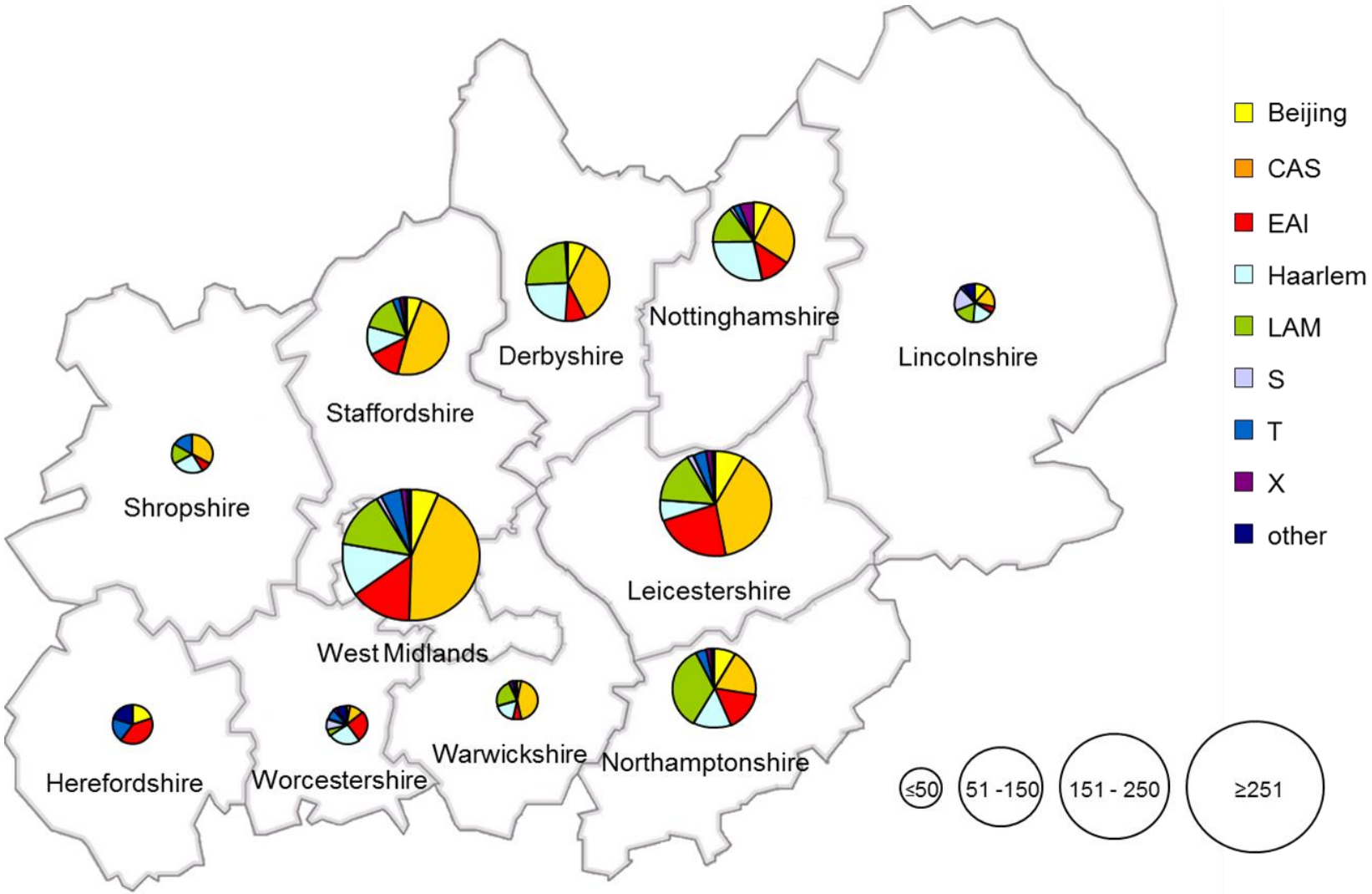


Figure 3-7 Distribution of the eight major *M. tuberculosis* spoligotype clades in the Midlands, UK

Each circle is located in the region that the TB patient resided in; the size of the circle is proportional to the number of patients from each region. Map adapted from http://en.wikipedia.org/wiki/File:Map_of_the_administrative_geography_of_the_ United_ Kingdom.png.

3.4.2.6 Distribution of CEL groups across eleven counties in the East and West Midlands

Nearly two thirds of the TB patients in the Midlands had a CEL code that belonged to the Asian continental group (895 of 1,483, 60%), which made Asia the most prevalent continental group (Figure 3-8). The Asian continental CEL group was the most prevalent group in five of 11 counties (45%). Leicestershire had the highest proportion with 182 of 250 patients (73%) originating from Asia. The Asian CEL group was also the most prevalent CEL group in the county of West Midlands (518 of 771, 67%), Warwickshire (17 of 30, 57%), Derbyshire (46 of 84, 55%) and Staffordshire (32 of 67, 48%). For comparison, the CEL structure of the total population of each county in the Midlands, UK is shown in Table 3-16.

The European continental CEL group was the second most prevalent group in the Midlands (414 of 1,483, 28%), and was the most prevalent group in 4 of 11 counties (36%). Worcestershire had the highest proportion with 25 of 35 patients (71%) originating from Europe. The European CEL group was also the most prevalent CEL group in Lincolnshire (24 of 35, 69%), Shropshire (8 of 12, 67%) and Herefordshire (3 of 5, 60%). In Nottinghamshire and Northamptonshire the proportions of patients from an Asian CEL group (Nottingham; 53 of 125, 42%, Northamptonshire; 27 of 69, 39%) was the same as those with a European CEL. The county with the highest proportion of patients from the African continental CEL group was Northamptonshire (12 of 69, 17%) and the county with the highest number of patients from this CEL group was the West Midlands county (83 of 771 11%). Patients from other continental CEL groups accounted for less than 3% of all patients (39 of 1,483).

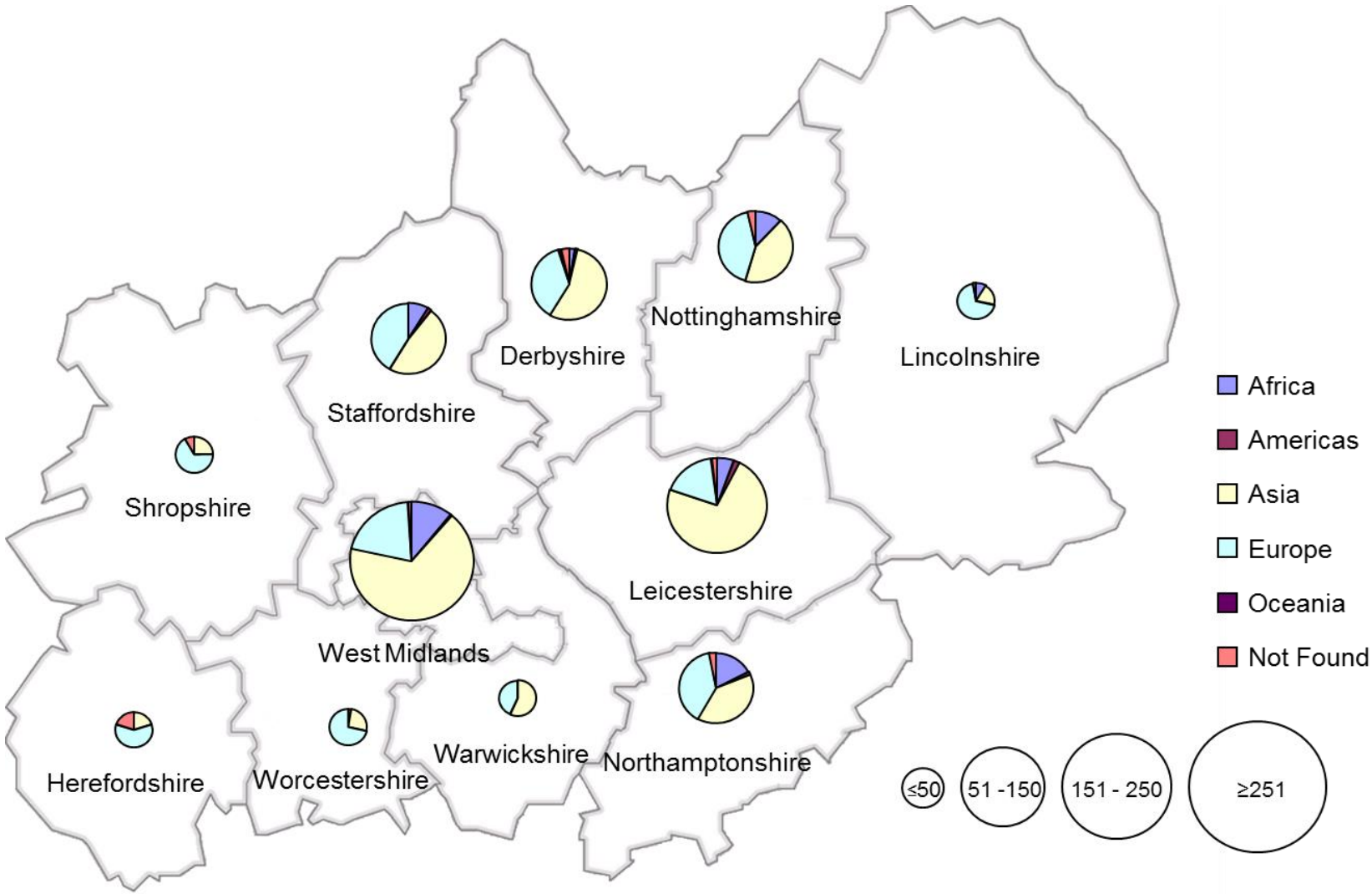


Figure 3-8 Distribution of five continental CEL groups amongst TB patients across the Midlands, UK

Each circle is located in the region that the TB patient resided in; the size of the circle is proportional to the number of patients from each region. Map adapted from http://en.wikipedia.org/wiki/File:Map_of_the_administrative_geography_of_the_ United_ Kingdom.png.

3.4.2.7 Distribution of *M. tuberculosis* lineages across Primary Care Trusts in the West Midlands county

There are nine Primary Care Trusts (PCT) in the West Midlands county. Nearly a third of the TB isolates from the West Midlands county came from the Heart of Birmingham PCT (240 of 771, 31%). Six PCTs had less than 100 isolates typed (Coventry Teaching, Wolverhampton City, Walsall Teaching, South Birmingham, Dudley and Solihull) and two PCTs had between 100 and 110 isolates (Birmingham East and North and Sandwell). The most prevalent spoligotype clade within the West Midlands county is the CAS clade (340 of 771, 44%). The CAS clade was also the most prevalent clade in every PCT within the West Midlands county (Figure 3-9).

3.4.2.8 Distribution of CEL groups across Primary Care Trusts in the West Midlands county

More than two thirds of the TB patients in the West Midlands county had a CEL code that belonged to the Asian continental group (518 of 771, 67%), which made Asia the most prevalent continental group. The Asian continental CEL group was the most prevalent group in all nine PCTs within the county (Figure 3-10). The Heart of Birmingham PCT had the highest proportion and the highest number of TB patients with an Asian continental CEL (175 of 240, 73%). Dudley PCT had the lowest proportion of TB patients with an Asian continental CEL (15 of 30, 50%) and Solihull PCT had the lowest number of TB patients with an Asian continental CEL (7 of 12, 58%). For comparison, the CEL structure of the total population of each PCT in the West Midlands county is shown in Table 3-17.

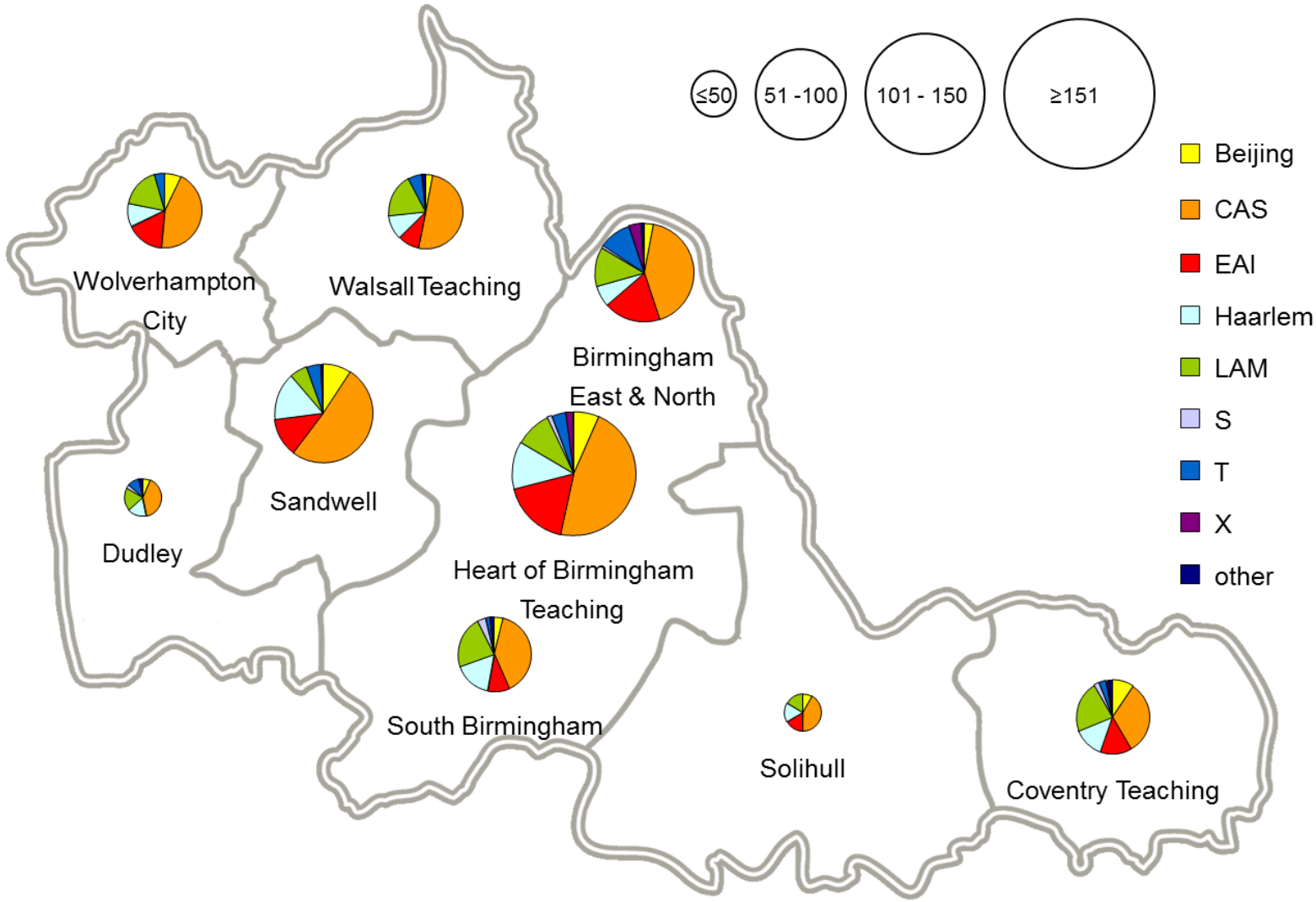


Figure 3-9 Distribution of the eight major *M. tuberculosis* spoligotype clades in the West Midlands county

Each circle is located in the region that the TB patient resided in; the size of the circle is proportional to the number of patients from each region. Map adapted from http://en.wikipedia.org/wiki/File:West_Midlands_County.png.

A fifth of the TB patients in the West Midlands county had a CEL code that belonged to the European continental group (158 of 771, 20%), which made Europe the second most prevalent continental group. The European continental CEL group was the second most prevalent group in all of the nine PCTs within the West Midlands county. Dudley PCT had the highest proportion of TB patients with a European continental CEL (13 of 40, 33%). The Heart of Birmingham PCT had the lowest proportion of TB patients with a European continental CEL (34 of 240, 14%), although it had the highest number of TB patients with a European continental CEL.

South Birmingham PCT had the highest proportion of TB patients with an African continental CEL group (9 of 53, 17%).The highest number of patients with an African continental CEL group were from the Heart of Birmingham Teaching PCT (29 of 240 12%). Patients from other continental CEL groups accounted for less than 2% of patients in the West Midlands county (12 of 771, <2%).

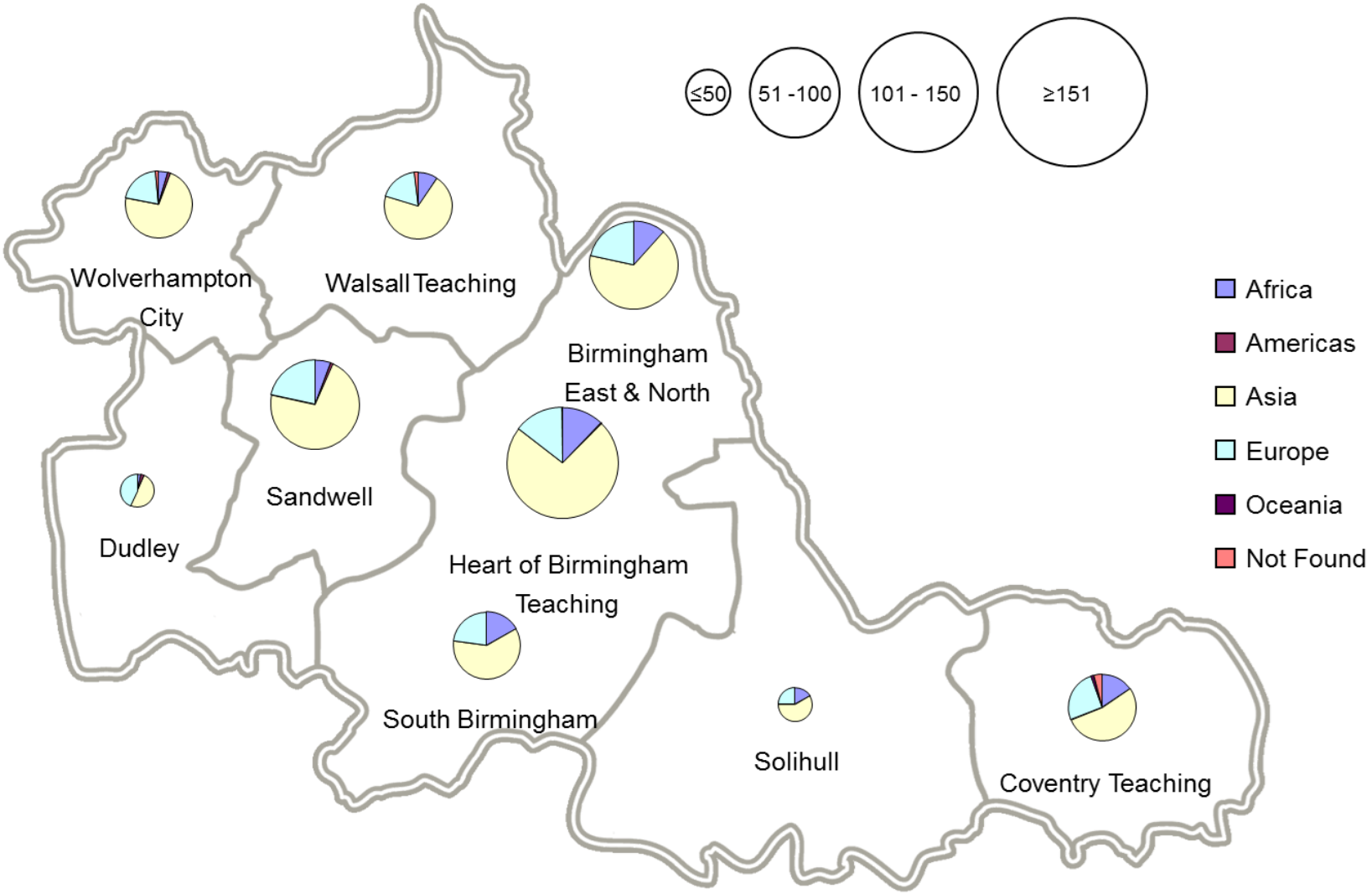


Figure 3-10 Distribution of the five continental CEL groups amongst TB patients in the West Midlands county

Each circle is located in the PCT that the TB patient resided in. The size of the circle is proportional to the number of patients from each region. Map adapted from http://en.wikipedia.org/wiki/File:West_Midlands_County.png.

Table 3-16 Distribution of the five continental CEL groups amongst TB patients in the Midlands, UK and amongst the total population

County	Percentage of TB patients belonging to different ethnic groups						Percentage of total population belonging to different ethnic groups*					
	Africa	Americas	Asia	Europe	Oceania	Not Found	Africa	Americas	Asia	Europe	Oceania	Mixed
Derbyshire	2.4	1.2	54.8	36.9	1.2	3.6	0.1	0.4	2.3	96.0	0.0	1.1
Leicestershire	5.6	2.0	72.8	17.6	0.4	1.6	0.5	0.6	11.5	85.5	0.0	1.8
Lincolnshire	8.6	0.0	20.0	68.6	0.0	2.9	0.1	0.1	0.4	98.7	0.0	0.8
Northamptonshire	17.4	1.4	39.1	39.1	0.0	2.9	0.4	0.8	2.0	95.1	0.0	1.7
Nottinghamshire	12.0	0.0	42.4	42.4	0.0	3.2	0.3	1.1	2.5	94.1	0.0	1.9
Shropshire	0.0	0.0	25.0	66.7	0.0	8.3	0.1	0.2	1.2	97.3	0.0	1.1
Staffordshire	9.0	1.5	47.8	41.8	0.0	0.0	0.1	0.2	1.7	97.0	0.0	1.0
Warwickshire	0.0	0.0	56.7	43.3	0.0	0.0	0.1	0.3	2.8	95.6	0.0	1.2
West Midlands	10.8	0.5	67.2	20.5	0.3	0.8	0.7	3.0	13.4	80.0	0.0	2.9
Worcestershire	0.0	2.9	25.7	71.4	0.0	0.0	0.1	0.2	1.1	97.5	0.0	1.1
Herefordshire	0.0	0.0	20.0	60.0	0.0	20.0	0.1	0.0	0.2	99.1	0.0	0.6

* Data obtained from the 2001 census (ONS, 2003)

Table 3-17 Distribution of the five continental CEL groups amongst TB patients in the West Midlands and amongst the total population

PCT	Percentage of TB patients belonging to different ethnic groups						Percentage of total population belonging to different ethnic groups					
	Africa	Americas	Asia	Europe	Oceania	Not Found	Africa	Americas	Asia	Europe	Oceania	Mixed
Birmingham East and North	11.8	0.0	66.7	21.6	0.0	0.0	0.7	3.1	15.4	77.8	0.0	3.0
Coventry Teaching	15.6	0.0	53.1	26.0	1.0	4.2	0.7	1.1	11.3	84.0	0.0	3.0
Dudley	3.3	3.3	50.0	43.3	0.0	0.0	0.2	0.8	4.0	93.7	0.0	1.4
HOB Teaching**	12.1	0.4	72.9	14.2	0.4	0.0	2.6	10.7	51.7	29.1	0.0	5.9
Sandwell	5.7	0.9	71.7	21.7	0.0	0.0	0.5	3.3	14.0	79.7	0.0	2.5
Solihull	16.7	0.0	58.3	25.0	0.0	0.0	0.2	0.8	2.5	94.6	0.0	1.9
South Birmingham	17.0	0.0	60.4	22.6	0.0	0.0	0.8	2.4	8.4	84.4	0.0	3.9
Walsall Teaching	9.4	0.0	70.3	18.8	0.0	1.6	0.3	1.1	10.4	86.4	0.0	1.8
Wolverhampton City	4.4	1.5	72.1	20.6	0.0	1.5	0.7	3.9	14.3	77.8	0.0	3.3

* Data obtained from the 2001 census (ONS, 2003), ** HOB = Heart of Birmingham

3.5 Discussion

The current study examined the spoligotype clade distribution of 1,483 *M. tuberculosis* strains isolated between 2007 and 2008 from patients in the Midlands region of the UK. This showed that CAS was the predominant spoligotype clade (39%), followed by LAM (16%) and EAI (15%). The CAS spoligotype clade is widespread in east Africa, northern India and Pakistan, the LAM clade is common in Europe and America and EAI is prevalent in east Africa and India (Gagneux and Small, 2007). The *M. tuberculosis* strain population was highly diverse with 11 clades identified.

The ethnicity of TB patients was predicted using a novel software programme (Origins) which assigned CEL groups based on a patient's name (Webber, 2007). More than half of the TB patients in the Midlands were predicted to have Asian ethnicity and one third were predicted to have European ethnicity. The prevalence of spoligotype clades from Asia and Europe corresponded with prevalence of patient ethnic groups, which is consistent with observations of an association between *M. tuberculosis* lineages and the geographical origin of TB patients (Reed *et al*., 2009; Gagneux *et al*., 2006).

In the current study, the population groups identified by Origins software were compared with self-declared population groups for a subset of patients that were predicted to have ISC ethnicity. There was a high degree of concordance (83%) between the two methods. This enabled population groups to be assigned to the rest of the patients in this study. Origins software is useful because self-declared

population data is not always available. A study in London, UK, which assessed *M. tuberculosis* genotyping and patient ethnicity, found that self-declared patient ethnicity data was missing in 40% of cases (Brown *et al*., 2010).

The MRCM receives *M. tuberculosis* strains from referring laboratories across the Midlands region of the UK and analyses them by MIRU-VNTR typing. The use of the MIRU-VNTR*plus* database to assign spoligotype clade based on MIRU-VNTR data was evaluated (Allix-Beguec *et al*., 2008b). Isolates that represented the most prevalent MIRU-VNTR profiles amongst patients who were predicted to have ISC ethnicity were selected and 98 isolates were spoligotyped. The spoligotype clades that were obtained from direct comparison of data to SITVIT were compared with inferred spoligotype clades from MIRU-VNTR*plus*. There was a high level of concordance (79%) between the two databases for all spoligotype clades. SITVIT and MIRU-VNTR*plus* were concordant for 91% of CAS strains spoligotyped in this study. This enabled spoligotype clades based on MIRU-VNTR data and comparison to MIRU-VNTR*plus* to be assigned to all *M. tuberculosis* isolates from the Midlands between 2007 and 2008 with a high degree of confidence.

The matching distance of all strains and spoligotype clades assigned by MIRU-VNTR*plus* were evaluated. Overall, there was a high degree of matching with 1,025 of 1,683 (61%) strains matched at less than 0.17 and 1,628 of 1,683 (97%) strains matched at less than 0.30. However, there were only 10 CAS strains present in the MIRU-VNTR*plus* database (Allix-Beguec *et al*., 2008b) whereas SITVIT contained 1,098 CAS strains (Brudey *et al*., 2006). This disparity of CAS isolates in

the MIRU-VNTR*plus* database could have resulted in incorrect assignment of spoligotype clades for some strains. Comparison to databases such as MIRU-VNTR*plus* and SITVIT is likely to introduce inaccuracies as there is no uniform international TB surveillance and so data that is submitted to global databases is heavily biased towards strains from countries where there are adequate resources to carry out surveillance.

When the spoligotype clades that were present in a subset of strains from patients with southern Asian ethnicity were analysed the most prevalent clade was CAS (59%) and the second most prevalent clade was EAI (10%). The CAS and EAI spoligotype clades are both prevalent in the ISC (Table 3-18). CAS and EAI clades differ in their evolutionary history, EAI an ancient clade of *M. tuberculosis* and CAS is a TbD1- modern clade (Brosch *et al*., 2002). These two clades have different geographical distributions within the ISC, CAS is predominant in the north and EAI is predominant in the south. This north, south geographical partitioning is seen most clearly in India (Table 3-18). The predominance of modern CAS strains amongst people of ISC ethnicity in the Midlands suggests that the majority of TB patients in the Midlands originated from regions in the north of the ISC. Origins software is constantly updated and a more in-depth understanding of the CEL group structure in the ISC is being constructed.

Typing of a subset of *M. tuberculosis* isolates from the Midlands using PGG and TbD1 showed good correlation with spoligotyping and MIRU-VNTR typing. This provides evidence to suggest that the clades of TB that are present in the Midlands

have not diverged from clades that are present in the SpolDB4 database, which indicates that local evolution of clades has not occurred in the Midlands (Lazzarini *et al*., 2007).

Table 3-18 Summary the most prevalent spoligotype clades present in different regions of India reported in published studies

Reference	No. of isolates	Region	Clade 1	Clade 2	Clade 3
			most prevalent →		least prevalent
(Kulkarni *et al*., 2005)	216	Central India	CAS (30%)	EAI (17%)	-
(Gutierrez *et al*., 2006)	12	Central India	EAI (52%)	CAS (24%)	-
(Singh *et al*., 2007)	85	Central India	CAS (41%)	EAI (18%)	-
(Chatterjee *et al*., 2010)	646	Central India	MANU (26%)	CAS (10%)	EAI (5%)
(Thomas *et al*., 2011)	101	Central India	CAS (40%)	EAI (38%)	MANU (8%)
(Bhanu *et al*., 2002)	83	North India	CAS (68%)	-	-
(Singh *et al*., 2004)	105	North India	CAS (33%)	EAI (9%)	-
(Gutierrez *et al*., 2006)	54	North India	CAS (32%)	EAI (32%)	Beijing (15%)
(Singh *et al*., 2007)	296	North India	CAS (45%)	EAI (24%)	-
(Mathuria *et al*., 2008)	83	North India	CAS (36%)	EAI (19%)	-
(Sharma *et al*., 2008)	97	North India	CAS (49%)	EAI (15%)	-
(Stavrum *et al*., 2009)	65	North India	CAS (55%)	EAI (22%)	-
(Varma-Basil *et al*., 2011)	101	North India	CAS (42%)	EAI (26%)	-
(Gutierrez *et al*., 2006)	25	South India	EAI (80%)	-	-
(Narayanan *et al*., 2008)	1215	South India	EAI (84%)	-	-
(Singh *et al*., 2007)	159	South India	EAI (66%)	CAS (5%)	-
(Narayanan *et al*., 2010)	23	South India	EAI (45%)	CAS (9%)	-
(Gutierrez et al., 2006)	91	India	EAI (45%)	CAS (26%)	Beijing (10%)
(Singh et al., 2007)	540	India	EAI (35%)	CAS (32%)	-

Gilbert and colleagues showed that between 1996 and 2005, the incidence of TB fell in 18 of the 21 European Organisation for Economic Co-operation and Development (OECD) member countries (Gilbert *et al*., 2009). However during the same period the incidence of TB in the UK increased by 31% (10.8 per 100,000 population in 1996 to 14.2 per 100,000 in 2005). The incidence of TB also increased in Norway (4.3 per 100,000 in 1996 to 6.3 per 100,000 in 2005; a 29% increase) and Sweden (5.6 per 100,000 in 1996 to 6.3 per 100,000 in 2005; a 13% increase). Gilbert estimated that

the increases in TB rate were equivalent to 75 extra cases in Norway and Sweden annually, and more than 2,200 in the UK in 2005. In all three countries, people born abroad accounted for 5% of the total population and approximately 75% of the TB cases. However, the number of foreign-nationals from countries with high incidence of TB was much greater for the UK than for Norway and Sweden. In the UK 10% of foreign-born nationals came from countries with a TB rate of more than 500 cases per 100,000 population, whereas none of the foreign-born nationals in Sweden and Norway came from countries with a TB rate of more than 250 cases per 100,000 population. The pattern of migration in the UK is likely to account for the increase in TB cases, although the authors concede that the provision of health services may also affect the trends in TB notification in Europe (Gilbert *et al*., 2009).

The current study showed that 39% of TB cases in the Midlands between 2007 and 2008 belonged to the CAS clade and 15% belonged to the EAI clade. Brown and colleagues spoligotyped 2,233 *M. tuberculosis* strains isolated in London, UK between 2005 and 2006. The authors showed that CAS and EAI were the most prevalent *M. tuberculosis* clades and patients originating from the ISC were the largest patient population group (Brown *et al*., 2010). There were 552 CAS strains (24%) and nearly 40% of them came from patients who had Asian ethnicity, the ethnicity of 40% of patients infected with CAS strains was unknown. There were 357 EAI strains (16%) and 40% came from patients with Asian ethnicity, the ethnicity of 35% of patients infected with EAI strains was unknown (Brown *et al*., 2010). Within the UK, the highest number of TB cases is in London. However, TB rates in London have been constant since 2006 (HPA, 2011a). The Midlands has the second highest

number of TB cases in the UK however, the proportion of CAS strains in the Midlands is much higher than in London. The importation of CAS strains to the Midlands may be driving the increase in TB. Comparison of the proportion of CAS and EAI strains in London and the Midlands during the same period would give a better understanding of how immigration is contributing to the *M. tuberculosis* population structure in different parts of the UK.

Strains from the CAS and EAI clades are not commonly isolated in other European countries. Oelemann and colleagues assessed 154 *M. tuberculosis* strains that were isolated from Hamburg, Germany in 2003 and found that Haarlem was the most prevalent clade (37%), only 8% of strains belonged to the CAS clade and 6% to the EAI clade (Oelemann *et al*., 2007). Allix-Beguec and colleagues assessed the population structure of 807 *M. tuberculosis* strains isolated in Brussels, Belgium between 2002 and 2005. The authors reported that nearly half of the TB patients (47%) had African ethnicity. The prevalent spoligotype clades were LAM (19.7%), followed by Haarlem (17%), CAS and EAI clades each accounted for 5% of the total (Allix-Beguec *et al*., 2008a). Viedma and colleagues did not find any *M. tuberculosis* strains from the CAS or EAI clades in a study of 233 strains isolated from Madrid, Spain between 2001 to 2002 (Garcia de Viedma *et al*., 2005a). However, Alonso and colleagues analysed 48 *M. tuberculosis* strains isolated from Madrid and Barcelona, Spain between 2003 and 2006. These strains were specifically selected from patients with Asian ethnicity, 12 isolates belonged to the CAS clade, and 13 isolates belonged to the EAI clade. CAS and EAI strains were not isolated from any patients with non-Asian ethnicity (Alonso *et al*., 2008). CAS was reported to comprise 9% of a sample

of 53 drug resistant *M. tuberculosis* strains isolated in Greece between 2007 to 2008 (Rovina *et al*., 2011). Ojo and colleagues analysed 171 *M. tuberculosis* strains isolated from Cork, Ireland between 2004 and 2006 and found that the most prevalent clade was X (29%). There were five EAI strains (3%) and four CAS strains (2%). Only four of the patients who were infected with CAS or EAI strains were nationals of Ireland, the other patients were born in India, Bangladesh, Pakistan or Zimbabwe (Ojo *et al*., 2010).

The current study used the five Exact Tandem Repeats (ETR) originally described by Frothingham and Meeker (Frothingham and Meeker-O'Connell, 1998) and twelve classical MIRU loci originally described by Supply and colleagues (Supply *et al*., 2001) to assign spoligotype clades. These 15 loci can be used to assign spoligotype clades using MIRU-VNTR*plus* as shown by the close matching distances identified in this study (Allix-Beguec *et al*., 2008b). However, accurate estimation of transmission rates cannot be obtained by using 15 MIRU-VNTR loci alone (Christianson *et al*., 2010; Garcia de Viedma *et al*., 2006; Supply *et al*., 2006). Routine analysis of the current internationally recommended set of 24 MIRU-VNTR loci is now carried out for all *M. tuberculosis* strains (Christianson *et al*., 2010; Garcia de Viedma *et al*., 2006; Supply *et al*., 2006).

The UK as a whole has low rates of TB; however, rates in migrant populations are far higher (HPA, 2012). The identification of clades present in the ISC and the UK combined with continued migration indicates that clades such as CAS are likely to

account for a significant proportion of disease in the UK in the future (Gilbert *et al*., 2009).

Previous studies indicated that the global origin of a patient is an important determinant in the associated *M. tuberculosis* genotype of TB (Gagneux *et al*., 2006). Whether this is due to bacterial adaptation to specific host populations or sociological and epidemiological factors is not clear. The mechanism and causes for the distribution of CAS in individuals resident in the Midlands and who have originated from the ISC warrant further investigation to examine the interaction between host and strain properties.

4 ASSESSMENT OF VIRULENCE OF THE MOST PREVALENT TB STRAINS IN THE MIDLANDS, UK USING *IN VITRO* MODELS

4.1 Introduction

4.1.1 Model systems for studying mycobacterial infections

Model systems are useful for the study of *M. tuberculosis* virulence because host genetics and behaviour can be standardised. For example, the CDC1551 strain caused an outbreak of TB in the USA; the strain was described as hypervirulent because of its high rate of transmission to patient contacts, which occurred in a low risk population. There was also evidence of transmission occurring outdoors where risk of infection should have been low (Valway *et al*., 1998). Initial investigators concluded that transmission was due to a characteristic of the strain rather than susceptibility of the patients or environmental factors that may have favoured the spread of the disease.

The finding that CDC1551 grew more rapidly in mice than the laboratory strain *M. tuberculosis* Erdman demonstrated the increased virulence of the strain in a more effective way than inferences made about transmission between human hosts (Valway *et al*., 1998). However, a study carried out by Manca and colleagues found that the rate of growth for CDC1551 was similar to H37Rv in the lungs of aerosol-infected mice and in human monocytes (Manca *et al*., 1999). Manca found that rather than CDC1551 having a higher growth rate in mice, the Erdman strain had a lower growth rate. Although CDC1551 did not exhibit increased growth *in vitro* or *in vivo*, the increased virulence that was suggested by the epidemiology of the outbreak

was demonstrated by the induction of a more rapid and vigorous immune response in a murine model of infection and a human monocytic model of infection. The study carried out by Manca highlights the importance of control strain selection for comparison with clinical isolates.

4.1.1.1 Animal models

There are no natural animal reservoirs for *M. tuberculosis*; however, a number of species are susceptible to infection when it is artificially induced. Robert Koch used the guinea pig in 1882 to demonstrate that *M. tuberculosis* is responsible for causing TB (Koch, 1882). In 1961 Bhatia and colleagues showed that clinical isolates from south Indian TB patients had a reduced ability to produce gross TB in a guinea pig model when compared to British isolates (Gagneux and Small, 2007; Smith and Wiegeshaus, 1989; Bhatia *et al*., 1961).

Guinea pigs are highly susceptible to infection with *M. tuberculosis* and infections can be established through the inhalation of relatively few bacteria. The course of disease in the guinea pig has many aspects in common with human disease, especially primary pulmonary disease. When guinea pigs develop TB, they form granulomata that can caseate and lead to necrosis (Dharmadhikari and Nardell, 2008). There are however fewer immunological tools available for the study of guinea pig models compared to other animal models and their larger size compared to mice is associated with higher rearing costs and requirement for more laboratory space.

Rabbits have also been used to study TB although they have a natural resistance to infection. When pulmonary infection is established, cavities may form but they often

regress and heal. Because of this, rabbits are often used to study latency, although spontaneous reactivation of disease will only occur if the immune system of the animal is suppressed (Dharmadhikari and Nardell, 2008).

Mice are frequently used for the study of TB because they have cheaper rearing costs than rabbits and guinea pigs. There is a wide range of immunological tools available for studying murine models and the development of selective gene knockout mice has enhanced the understanding of TB immunology. However, the course of disease in mice is different to humans. Mice form granulomata but these do not caseate and mice tend to enter a chronic phase of disease instead of latency, which can occur in humans (Dharmadhikari and Nardell, 2008).

The use of guinea pigs, rabbits and mice has been fundamental in studying many elements of TB including transmission of the disease and the immune response to it. Each animal model has advantages and disadvantages, some of which are summarised in Table 4-1. However, susceptibility to TB and the course of the disease in animal models differs from TB infection in humans. The immune response and the extent of organ involvement also vary considerably between species (Dharmadhikari and Nardell, 2008).

Modelling TB in non-human primates, such as the cynomolgus macaque replicates TB in humans more faithfully than using small animal models (Kaushal *et al*., 2012). However, non-human primates are not used frequently for TB research because of ethical considerations, high costs and their ability to transmit the disease to humans.

Humans can develop primary, latent, or reactivation TB, as described in section 1.3. Different animal models are therefore more applicable to different states of the disease. Animal models can provide information about the host response to TB and have also been used to show differences between strains of *M. tuberculosis*. The cost of keeping animals at containment level three is high and requires specialist facilities. Variation in host genetics also makes it difficult to compare subtle differences between strains of *M. tuberculosis*. This has led to the development of cell-based assays, which are cheaper than using animals and can reduce the influence of host genetics.

Table 4-1 Comparison of features of mouse, rabbit and guinea pig models of pulmonary TB with features of the disease in humans*

		Experimental animal model		
Feature	**Human host**	**Mouse**	**Rabbit**	**Guinea pig**
Susceptibility to *M. tuberculosis*	++	+	+	+++
Extrapulmonary dissemination	++	++	++	++
Hematogeous seeding of apical lobes	++	?	?	++
Typical mononuclear cell granulomas	++	++	++	++
Caseation necrosis	++	–	++	++
Liquifaction/cavitation	++	–	++	–
Delayed-type hypersensitivity	+/+	+/–	++	++
Protection by BCG vaccine	++/–	+	++	++
Biosafety level 3 space requirements	N/A	+	++++	++
Maintenance costs	N/A	+	++++	++
Immunological reagents	++++	++++	++	+

*adapted from (McMurray, 2001)
+ indicates the occurrence of this feature, multiple + symbols indicate the feature is more prevalent
– indicates that absence of a feature
+/- indicates that there are differences between individuals of the species
? indicates that there is insufficient data
N/A indicates not applicable

4.1.1.2 Cell based assays

Droplet nuclei that are deposited in the alveoli are phagocytosed by alveolar macrophages. *M. tuberculosis* is able to survive and replicate within macrophages, which ordinarily kill internalised microorganisms. For these reasons, most assays of *M. tuberculosis* virulence use phagocytic cells, although some research has been

carried out with lung epithelial cells (Danelishvili *et al*., 2003; Dobos *et al*., 2000). Models of macrophage infection use either primary cells or a cell line that has been immortalised.

4.1.1.2.1 Alveolar macrophages

Primary tissue macrophages are the most relevant cell type for modelling human infection with *M. tuberculosis*. Human alveolar macrophages are acquired by bronchoalveolar lavage, which is an invasive procedure that is usually only performed for diagnostic purposes. It is therefore difficult to obtain human alveolar macrophages from healthy donors.

Monocytes originate from a common precursor in the bone marrow and then travel via the blood to extravascular tissues. Monocytes can be recruited to sites of inflammation and injury. Monocytes can also differentiate into tissue macrophages in response to growth factors. Macrophages from different tissues have different phenotypes, suggesting that the unique conditions present in a particular tissue influence the development of the tissue macrophages (Rutherford *et al*., 1993). Alveolar macrophages are believed to exist in a primed or activated state, because of their constant exposure to inhaled antigen in the lung (Rutherford *et al*., 1993; Andreesen *et al*., 1990).

Several groups have shown that human alveolar macrophages are a heterogeneous population in which distinct subsets of cells can be distinguished (St-Laurent *et al*., 2009; Campbell *et al*., 1986). Due to the difficulty of obtaining alveolar macrophages from humans and the inherent heterogeneity of the cell population, the comparison of

virulence characteristics of *M. tuberculosis* strains in alveolar macrophages is technically challenging. Monocytes obtained from peripheral blood can be differentiated into macrophages *in vitro*. Most assays using primary human cells for the assessment of *M. tuberculosis* are performed with monocytes or monocyte-derived macrophages (MDMs) because of the ease with which blood can be collected. Several groups have compared MDMs to alveolar macrophages to determine differences and similarities between the cell types.

Li and colleagues used microarrays to assess the gene expression profiles of alveolar macrophages and MDMs from four healthy human volunteers. MDMs were generated by exposure to macrophage colony stimulating factor (M-CSF). The microarrays showed large differences between alveolar macrophages and MDMs, 16% of genes on the array were differentially expressed (Li *et al*., 2007). Taylor and colleagues showed that alveolar macrophages and MDMs phagocytosed *E. coli* and fluorescently labelled polystyrene beads at the same rate. MDMs were generated by exposure to granulocyte-macrophage colony stimulating factor (GM-CSF) (Taylor *et al*., 2010). Juarez and colleagues showed that mRNA levels of Toll-like receptors 2, 4 and 9 (which are receptors that recognise pathogen-associated molecular patterns) in human MDMs resembled the levels seen in alveolar macrophages obtained from the same person. MDMs were generated by plastic adherence; addition of cytokines was not listed in the methods (Juarez *et al*., 2010).

It is difficult to compare alveolar macrophages to MDMs because alveolar macrophages are a heterogeneous cell population and the phenotype of MDMs can

be altered by the methods used to purify and differentiate monocytes. Activation of MDMs with different cytokines can alter the gene expression profile and phenotype of the cells, which makes it harder to compare studies such as those carried out by Li, Taylor and Juarez (Taylor *et al*., 2010; Juarez *et al*., 2010; Li *et al*., 2007).

4.1.1.2.2 Monocyte-derived macrophages

The heterogeneity that is observed in human alveolar macrophage populations is due to the ability of macrophages to respond to activation signals. Macrophage activation occurs along a spectrum *in vivo*, with classical activation at one end of the scale and alternative activation at the opposite extreme. Macrophages are able to respond to activation in a reversible manner and are not permanently committed to develop into a particular lineage. Macrophage polarization can be simulated *in vitro* with MDMs and is also a reversible phenomenon (Ambarus *et al*., 2012; Stout *et al*., 2009).

GM-CSF and M-CSF can promote macrophage generation from human monocytes and aid macrophage survival. The use of GM-CSF to produce MDMs results in cells that are polarised towards a classically activated M1 phenotype and the use of M-CSF results in an alternatively activated M2 phenotype. The terms M1 and M2 are used because they mirror the nomenclature used to describe T helper cells, which can be polarised in a similar manner. M1 and M2 macrophages have different morphologies and express different cell surface markers (Figure 4-1).

M1 macrophages are able to produce reactive nitrogen intermediates and they secrete the pro-inflammatory cytokines IL-1, IL-12 and IL-23 (Sica and Mantovani, 2012). M1 macrophages show enhanced phagocytosis and facilitate resistance

against intracellular pathogens, including suppression of macrophage-tropic HIV-1 replication (Matsuda *et al*., 1995). M2 macrophage is a broad term used for several forms of activated macrophage that produce the anti-inflammatory cytokine IL-10 and have high levels of mannose and scavenger receptors. M2 macrophages have been shown to promote tissue repair and remodelling and have immunoregulatory functions, including involvement in anti-parasitic and allergic reactions. M2 macrophages are not able to suppress the replication of macrophage-tropic HIV-1 (Lacey *et al*., 2012; Martinez *et al*., 2006; Mantovani *et al*., 2005).

M1 macrophage morphology has been reported to resemble that of normal human alveolar macrophages and the expression of cell surface markers is similar. The phenotype of M2 macrophages is more closely related to peritoneal macrophages. Peritoneal macrophages come from a sterile anaerobic environment and are thought to be present in a quiescent, non-activated state (Komuro *et al*., 2001).

Raju and colleagues found that people with active TB could be divided into two groups based on the gene expression profile of their alveolar macrophages. One group had alveolar macrophages that showed an M1 gene expression profile and the other group had alveolar macrophages that showed an M2 expression profile. Raju also showed that alveolar macrophages isolated from one TB patient at the onset of antibiotic treatment showed an M2 gene expression profile, but as treatment progressed, the alveolar macrophages isolated from this patient showed an M1 gene expression profile. In addition, Raju found that alveolar macrophages from some individuals who were not infected with TB had gene expression profiles that were

indicative of an M2 profile (Raju *et al*., 2008). People who have M2 polarised alveolar macrophages may be more susceptible to infection with *M. tuberculosis* than people who have alveolar macrophages that have an M1 phenotype.

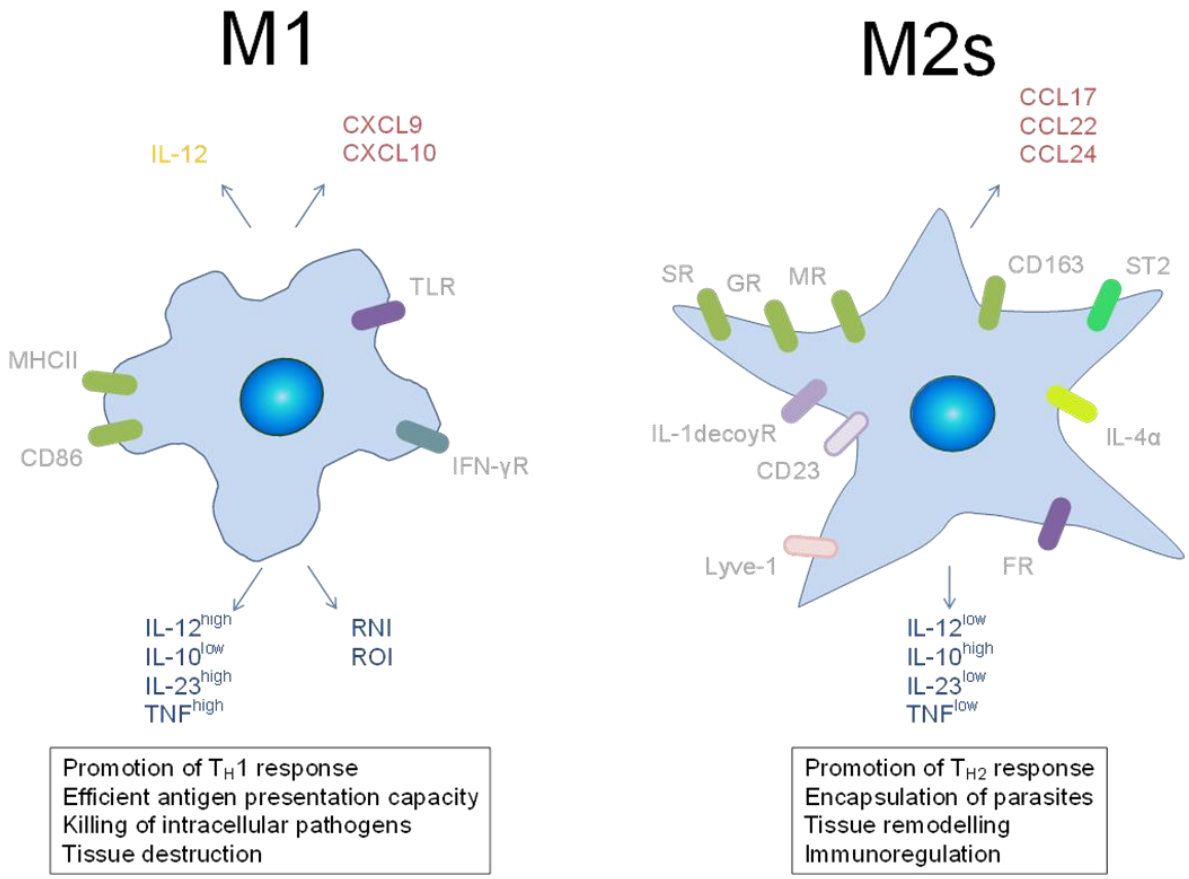


Figure 4-1 Features of M1 and M2 polarised macrophages

M1 macrophages exhibit classical 'fried egg morphology', whilst M2 macrophages have a stretched spindle like morphology. M2s refers to several diverse forms of M2 activation. Chemokines are shown in red, cytokines in orange, phenotype in dark blue, cell function in black and cell surface receptors in grey. Adapted from (Sica and Mantovani, 2012; Biswas and Mantovani, 2010; Verreck *et al*., 2006).

CCL = Cysteine-Cysteine motif chemokine ligand
CD = cluster of differentiation
CXCL = Cysteine-any-Cysteine motif chemokine ligand
FR = folate receptor
GR = galactose receptor
IFN-γR = interferon-gamma receptor
IL = interleukin
IL-1decoyR = interleukin-1 decoy receptor
Lyve-1 = lymphatic vessel endothelial receptor 1
MHCII = major histocompatibility complex class II
MR = mannose receptor
SR = scavenger receptor
ST2 = suppressor of tumorigenicity 2 receptor
TLR = Toll-like receptor
TNF = tumor necrosis factor
RNI = reactive nitrogen intermediate
ROI = reactive oxygen intermediate

A study by Chacón-Salinas and colleagues using murine bone-marrow derived macrophages showed that virulent strains of *M. tuberculosis* caused M2 macrophage

polarisation, whereas avirulent strains caused M1 macrophage polarisation (Chacon-Salinas *et al*., 2005). Redente and colleagues showed that upon initial infection of mice with *M. tuberculosis* strain Erdman, macrophages from bronchoalveolar lavage became M1 polarised but as inflammation progressed macrophages switched to an M2 phenotype. However macrophages that were part of a granuloma in the lung did not switch to an M2 phenotype, suggesting that free alveolar macrophages and macrophages associated with granulomata are exposed to different cytokines (Redente *et al*., 2010).

Almeida and colleagues showed that cells isolated by bronchoalveolar lavage from people who were infected with TB, had gene expression patterns that were consistent with an M2 macrophage phenotype, whereas people with other infectious lung diseases and healthy people had gene expression profiles consistent with an M1 macrophage phenotype. They also found that the M2 phenotype switched to an M1 phenotype with continuing antibiotic treatment for TB (Ho and Lape e Silva, 2010; Almeida *et al*., 2009).

The capacity of *M. tuberculosis* to alter the polarisation of macrophages may be an indication of virulence; strains that are more virulent may have an increased capacity to alter the activation state of macrophages.

Numerous studies have assessed the virulence of clinical isolates and laboratory strains of *M. tuberculosis* in MDMs. Studies have looked at the growth of bacteria within macrophages, the viability of infected macrophages and the levels of cytokines

produced by infected macrophages. The methods used in some of these studies and the results are summarised in Table 4-2, Table 4-3 and Table 4-4.

Despite the observation that *M. tuberculosis* is able to alter macrophage polarisation there is little evidence of whether polarised macrophages are able to control the growth of *M. tuberculosis*, especially clinical isolates. The virulence of *M. tuberculosis* strains in M1 and M2 macrophages has also not been assessed, although Portevin and colleagues assessed the cytokine response of M1 and M2 MDMs to *M. tuberculosis* clinical isolates from each of the six lineages defined by Gagneux and colleagues (Gagneux *et al*., 2006). The study by Portevin and colleagues concluded that modern *M. tuberculosis* strains induced a lower IL-6 response in M1 macrophages than ancient strains 24 hours after infection but not after 72 hours. This indicated that there was a delay in the immune response to modern strains (Portevin *et al*., 2011). Infection of M2 macrophages with the same isolates resulted in lower IL-6 production than in M1 macrophages.

Verreck and colleagues showed that uptake and growth of a luciferase-transfected *M. bovis* BCG reporter strain was higher in M2 human MDMs than in M1 MDMs, using a range of 2 to 20 bacilli per cell. There was no difference in the viability of M1 and M2 cells after six days of infection with the reporter strain and the phenotype of the cells was not altered by the infection. The authors also showed that M1 cells could efficiently present antigen and activate T cells but M2 cells could not (Verreck *et al*., 2004). Denis showed that growth of H37Rv was greater in M2 human MDMs compared to M1 MDMs when an infection ratio of 10 bacilli per cell was used (Denis,

1991). Kahnert looked at gene expression profiles of murine bone marrow-derived macrophages that had been polarised to an M1 or an M2 phenotype and then infected with *M. tuberculosis* strain Erdman. There was reduced expression of genes associated with production of nitric oxide and iron sequestration in M2 macrophages suggesting that M2 macrophages would support the intracellular growth of *M. tuberculosis* rather than inhibit it (Kahnert *et al*., 2006).

To address the question of whether clinical strains show a difference in virulence in polarised macrophages, human MDMs will be treated with M-CSF or GM-CSF and then infected with different strains of *M. tuberculosis.* As most studies of *M. tuberculosis* strain virulence in human cells have been carried out in monocytes or immortalised human cell lines, the virulence of the *M. tuberculosis* strains selected for the current study will first be assessed in one these models.

4.1.1.2.3 Immortalised human cell lines

In order to compare the virulence of *M. tuberculosis* strains in monocytes or MDMs, the same blood donor must be used which means only a limited number of strains can be compared, as cell number cannot be expanded in culture. The results are also indicative of the individual that the cells were obtained from, necessitating repeat experiments using different donors to ensure that results are due to *M. tuberculosis* strain variation and not natural variation of the donor cells.

Table 4-2 Summary of treatment of mycobacteria used in published infection models using MDMs

Reference	No. of bacteria per cell	Method of inoculum preparation	Were bacteria frozen before use?	Bacterial strains included in the study					No. of hours before extra cellular bacteria were washed out
				No. of clinical isolates tested	H37Ra	*M.bovis*	BCG	H37Rv	
(McDonough *et al*., 1993)	10	liquid	×	0	✓	×	✓	✓	6
(Paul *et al*., 1996)	1	liquid	×	0	✓	×	×	✓	6
(Zhang *et al*., 1998)	0.0125	liquid	×	4	✓	×	×	✓	24
(Silver *et al*., 1998)	1	liquid	✓	0	✓	×	✓	✓	1
(Zhang *et al*., 1999)	0.01	liquid	×	17	×	×	×	×	24
(Li *et al*., 2002)	1	liquid	✓	8	×	×	×	✓	1
(Wong *et al*., 2007)	-	solid	×	125	✓	×	×	✓	18
(Chen *et al*., 2006)	2 to 10	liquid	✓	0	✓	×	×	✓	4
(Denis, 1991)	10	liquid	✓	0	✓	×	×	×	6
(Portevin *et al*., 2011)	1	liquid	✓	26	×	×	✓	✓	-

✓ indicates yes
× indicates no
- indicates that the data was not included in the publication

Table 4-3 Summary of treatment of MDMs used in published mycobacterial infection models

Reference	No. of cells/ ml	Method used to isolate monocytes			Parameters tested				
		Plastic adherence	Positive bead selection	M1 or M2 polarised	Intracellular growth rate	Growth rate in broth	Macrophage apoptosis	Macrophage necrosis	Cytokine production
(McDonough *et al*., 1993)	-	✓	×	×	×	×	✓	×	×
(Paul *et al*., 1996)	2×10^5	✓	×	×	✓	✓	×	×	×
(Zhang *et al*., 1998)	4×10^5	✓	×	×	✓	✓	×	✓	✓
(Silver *et al*., 1998)	1×10^6	✓	×	×	✓	×	×	✓	✓
(Zhang *et al*., 1999)	5×10^5	✓	×	×	✓	×	×	✓	✓
(Li *et al*., 2002)	1×10^6	✓	×	×	✓	×	×	×	×
(Wong *et al*., 2007)	5×10^5	✓	×	×	✓	×	×	×	✓
(Chen *et al*., 2006)	5×10^5	✓	×	×	×	×	✓	✓	×
(Denis, 1991)	1×10^6	✓	×	✓	✓	×	×	×	×
(Portevin *et al*., 2011)	1×10^5	×	✓	✓	×	×	×	×	✓

✓ indicates yes
× indicates no
- indicates that the data was not included in the publication

Table 4-4 Summary of results from published mycobacterial infection models using MDMs

Reference	Growth in MDM		Growth in broth	MDM apoptosis	MDM necrosis	Cytokine production	
	Method used to assess growth (No. days post infection)	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Pro-inflammatory	Anti-inflammatory
(McDonough *et al*., 1993)	Electron microscopy (7)	Only H37Rv grew	-	-	Only with H37Rv	-	-
(Paul *et al*., 1996)	CFU / microscopy (6)	No difference	-	-	-	-	-
(Zhang *et al*., 1998)	CFU (10)	H37Ra, H37Rv, Clinical isolates	No difference	-	No difference	No difference for TNF-α or IL-12[1]	No difference for IL-10[1]
(Silver *et al*., 1998)	CFU (7)	H37Ra, BCG, H37Rv	-	-	BCG, H37Ra, H37Rv	High intracellular growth caused high TNF-α	No difference
(Zhang *et al*., 1999)	CFU (14)	Unique, Small cluster, Large cluster	No difference	-	No difference	No difference for TNF-α or IL-12[1]	No difference for IL-10[1]
(Li *et al*., 2002)	CFU (7)	EAI, H37Rv, Beijing	-	-	-	-	-
(Wong *et al*., 2007)	CFU (10)	Only H37Rv and 3 clinical isolates grew	-	-	-	High intracellular growth caused low TNF-α	-
(Chen *et al*., 2006)	-	-	-	H37Rv, H37Ra	H37Ra, H37Rv	-	-
(Denis, 1991)	CFU (8)	Lower growth in M1 cells	-	-	-	-	-
(Portevin *et al*., 2011)	-	-	-	-	-	Modern strains caused lowest IL-6[2]	-

[1] A higher number of bacteria was used obtain measurable cytokine concentrations.
[2] Modern and ancient as defined by LSP-based analysis (Gagneux *et al*., 2006).
- indicates that data was not included in the publication
CFU stands for colony forming unit.

The use of immortalised human cell lines that have phagocytic properties circumvents many of the problems associated with primary cells. Cell lines are advantageous because there is no donor variation and large numbers of cells can be grown. Macrophages from non-human species have been shown to differ from human macrophages. For example, murine macrophages produce large quantities of nitric oxide; the production of nitric oxide in human macrophages is much lower, although it can be stimulated by several conditions including infection with *M. tuberculosis* (MacMicking *et al*., 1997). The use of cell lines that are human in origin avoids many of the problems associated with primary cells or animal cells (Silver *et al*., 1998).

There are several human cell lines, which are commercially available, such as the monocytic cell lines Mono Mac 6 (Ziegler-Heitbrock *et al*., 1988) and HL-60 (Collins *et al*., 1978; Collins *et al*., 1977), and the promonocytic cell lines THP-1 and U-937. The Mono Mac 6 cell line was established from the peripheral blood of a patient with monoblastic leukemia in 1988 (Ziegler-Heitbrock *et al*., 1988). This cell line has been used to assess the antimicrobial activities of various compounds. The human leukemic cell line HL-60 was established from the peripheral blood leukocytes of an adult female with acute promyelocytic leukemia in 1977 (Collins *et al*., 1977). HL-60 cells have been used in a number of studies that look at the effect of vitamin D and the production of nitric oxide when *M. tuberculosis* infection is induced. The THP-1 and U-937 cell lines are commonly used to study the virulence of *M. tuberculosis* strains and will be discussed below.

The human macrophage-like cell line U-937 was derived from the pleural infusion of a 37 year old Caucasian male with diffuse histiocytic leukemia in 1976 (Sundstrom and Nilsson, 1976). U-937 cells can be converted from a non-adherent and weakly phagocytic phenotype to an adherent, actively phagocytic state with phorbol esters and other agents (Arcila *et al*., 2007). The U-937 cell line has been used extensively as a model system for studying the interaction between macrophages and several intracellular pathogens (Wei *et al*., 2000). Once U-937 cells have been activated, they can produce lysozyme, beta-2-microglobulin and tumor necrosis factor alpha (TNF-α). Beta-2-microglobulin is part of the MHC class I assembly, which displays protein antigens to T cells, and TNF-α is an inflammatory cytokine that is produced by activated macrophages.

THP-1 is a pro-monocytic leukaemia cell line that was isolated in 1980 from the blood of a 1-year-old male who had acute monocytic leukemia (Tsuchiya *et al*., 1980). THP-1 cells can be differentiated into a mature macrophage-like state with the addition of phorbol esters such as phorbol 12-myristate 13-acetate (PMA). Differentiation causes THP-1 cells to express several macrophage cell surface receptors such as FcγRI (CD64), FcγRII (CD32), complement receptor 3 A (CD11b) and the LPS receptor (CD14). CD64 and CD32 are both immunoglobulin G (IgG) receptors, CD11b mediates leukocyte adhesion and phagocytosis of complement-coated particles and CD14 is a co-receptor for pathogen-associated molecular patterns such as bacterial lipopolysaccharide (LPS) (Stokes and Doxsee, 1999). THP-1 cells have the same capacity as MDM to bind *M. tuberculosis* laboratory strain Erdman in the presence and absence of serum and are able to bind

IgG and complement coated particles. However, THP-1 cells differ from MDM in their expression of lectin-like receptors, which are capable of binding glycans (Stokes and Doxsee, 1999). Daigneault and colleagues carried out a comprehensive comparison of monocytes, MDMs and THP-1 cells. Daigneault concluded that when THP-1 cells were stimulated with PMA, then rested in the absence of PMA for 5 days, the phenotype of the resulting cells was similar to that of MDMs. Daigneault generated MDMs using a plastic adherence protocol and noted that the activation state of MDMs and differentiated THP-1 cells was different (Daigneault *et al*., 2010).

A review by Auwerx concluded that differentiated THP-1 cells behave more like native MDMs than HL-60 or U-937 cells (Auwerx, 1991). Like all models, THP-1 cells do not mimic MDM completely. However, the advantages of being able to grow large numbers of cells with no donor variability and the similarities they share with MDMs make THP-1 cells a good model to use for assessing bacterial variation. Macrophages are an extremely diverse population of cells and so any model is subject to limitations that must be accounted for when assessing results.

4.1.2 Studies using THP-1 cellular models of *M. tuberculosis* infection

THP-1 cells have been used in numerous studies that have assessed the virulence of clinical isolates and laboratory strains. Studies have looked at the growth of bacteria within macrophages, the viability of infected macrophages and the levels of cytokines produced by infected macrophages. The methods used in some of these studies are summarised in Table 4-5 and Table 4-6 and the results are summarised in Table 4-7.

Viability of infected macrophages has been studied because macrophage cell death is an important response to infection with *M. tuberculosis* (Duprez *et al*., 2009). Apoptosis is an energy dependant form of cell death that causes the controlled breakdown of the cell without induction of an inflammatory response (Figure 4-2), thus eliminating the intracellular environment that supports the growth of *M. tuberculosis* (Lammas *et al*., 1997). Apoptotic bodies are engulfed by other macrophages. The mycobacteria are sequestered within the apoptotic body and so may be unable to initiate the block on normal phagosome maturation resulting in the destruction of the pathogen (Fratazzi *et al*., 1997). Apoptosis can also facilitate antigen presentation to T cells (Winau *et al*., 2006; Schaible *et al*., 2003). There are several reports showing that virulent strains of *M. tuberculosis* prevent macrophage apoptosis, thus promoting their own survival (Riendeau and Kornfeld, 2003; Sly *et al*., 2003; Spira *et al*., 2003; Keane *et al*., 2000; Placido *et al*., 1997), whereas avirulent *M. tuberculosis* strains induce apoptosis. Higher induction of apoptosis by avirulent strains is not linked to increased intracellular growth as virulent strains were shown to have higher growth rates than avirulent strains (Keane *et al*., 2000).

Virulent strains of *M. tuberculosis* are reported to cause cellular necrosis (Keane *et al*., 2000). Necrosis is an energy independent form of cell death that causes disruption of the plasma membrane, releasing lysosomal enzymes and causing tissue damage (Figure 4-2). Infected macrophages that die by necrosis release bacteria, which can then infect other cells. Rapid growth of virulent strains of *M. tuberculosis* coincides with induction of cellular necrosis (Duprez *et al*., 2009; Park *et al*., 2006).

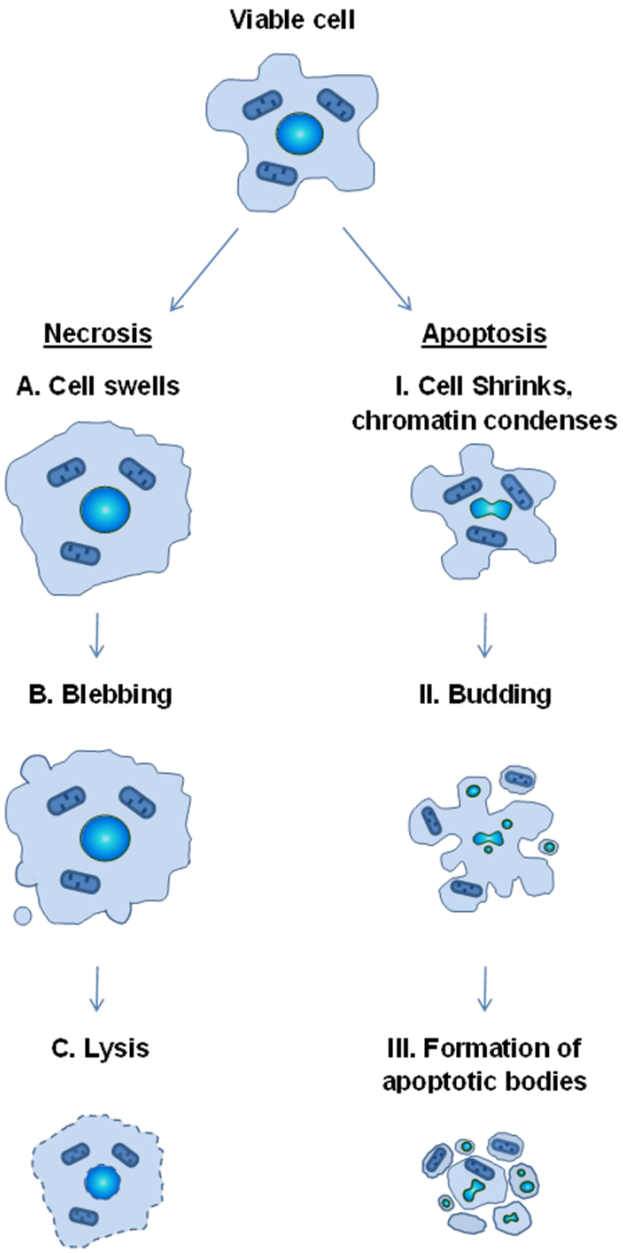


Figure 4-2 Features of apoptotic and necrotic cell death

Necrosis
A. The cell swells and small blebs begin to form.
B. Blebs become larger but do not contain organelles. The cell becomes leaky.
C. Cellular and nuclear lysis occur and organelles no longer function. The contents of the cell are released into the surrounding tissue causing inflammation.

Apoptosis
I. The cell shrinks and the chromatin condenses.
II. DNA becomes fragmented and membrane-bound apoptotic bodies that contain functional organelles, cytosol and DNA bud away from the cell.
III. The cell breaks into several apoptotic bodies, which are phagocytosed by other cells without triggering inflammation.
Adapted from (Wang *et al*., 2013) and (van Cruchten and van den Broeck, 2002)

Riendeau and colleagues used a THP-1 model to test the virulent laboratory strain H37Rv and the avirulent strain H37Ra as well as a virulent *M. bovis* strain and BCG which is avirulent (Riendeau and Kornfeld, 2003). They found that the avirulent strains caused a high level of apoptosis, whereas the virulent strains did not.

In 2003, Sharma and colleagues examined the intracellular growth rate of H37Rv, H37Ra and a strain designated as type 1 by IS*6110* RFLP, in THP-1 cells. Type 1 strains accounted for 24% of TB cases in Manitoba, Canada, and were prevalent among infected individuals of aboriginal decent. No difference in growth rate was found between the strains after 72 hours. Induction of IFN-γ, IL-1β, and TNF-α production was highest when THP-1 cells were infected with H37Rv. Levels of IL-10 were highest when THP-1 cells were infected with the type 1 strain (Sharma *et al*., 2003).

In 2004, Theus and colleagues carried out a study that used THP-1 cells to investigate H37Rv, TB282; which is a member of the Beijing clade and TB284, an isolate that was deemed to have a low rate of transmission based on epidemiological analysis. They found that the intracellular growth rates of H37Rv and TB282 were similar and significantly higher than TB284, despite all the strains having similar growth rates in 7H9 broth (Theus *et al*., 2004).

In 2005, Theus and colleagues tested 32 clinical isolates, including TB282 and TB284. TB284 and six other isolates were unique strains, deemed to have low rates of transmission. The other strains, termed cluster strains, had been identified from six

different epidemiologically linked clusters of disease. All strains were phagocytosed at a similar rate. After three days of infection, the intracellular growth rates began to differ between strains, and after seven days, the growth of the unique strains was significantly less than for the cluster strains. The THP-1 cells remained viable over the seven-day infection period regardless of the infecting isolate. Isolates that belonged to the same cluster of disease had similar intracellular growth rates. There was no difference in the amount of IL-10 produced as a result of infection with unique or cluster strains, regardless of the number of intracellular organisms. Induction of IL-10 production by infection with unique strains was delayed by four days compared to infection with cluster strains. High intracellular growth rate correlated with rapid induction of IL-10. Unique strains induced higher levels of TNF-α than cluster strains, showing a strong association between the ability to grow rapidly and the ability to suppress TNF-α secretion (Theus *et al*., 2005).

In 2007, Rajavelu and colleagues compared H37Rv, H37Ra and two clinical strains that were prevalent in south India. The clinical strains were able to infect more THP-1 cells with a lower number of bacilli, whereas the laboratory strains infected fewer cells but there were more bacilli per cell. H37Ra caused more apoptosis and there was a decrease in intracellular bacterial viability after seven days compared to the other strains (Rajavelu and Das, 2007).

Castro-Garza and colleagues looked at four clinical isolates that lacked the phospholipase C locus and used a THP-1 cell model to compare them to H37Rv. After 24 hours, 10% of the THP-1 cells that were infected with H37Rv had been

destroyed and after 72 hours, 50% had been destroyed. The clinical strains had a reduced cytotoxic effect; between 0% and 20% of infected THP-1 cells were destroyed after 72 hours.

In 2007, Theus and colleagues tested 15 genotypically distinct Beijing family strains. The rate of phagocytosis and the growth rate in 7H9 broth were similar for all strains, but the growth rate in THP-1 cells differed significantly between strains. Production of TNF-α by infected macrophages was inversely related to growth rate; macrophages infected with the three strains that had the slowest intracellular growth rates secreted more TNF-α than macrophages infected with the 12 strains that had fastest intracellular growth rates (Theus *et al*., 2007a).

In 2008, Sohn and colleagues compared cytokine release and macrophage death induced by infection with H37Rv and a Korean isolate termed K-strain, as well as the intracellular growth rates of the strains. K-strain is a member of the Beijing clade and was most commonly isolated from schoolchildren with pulmonary TB in Korea. Both strains had similar intracellular growth rates but K-strain induced less TNF-α, IL-6 and IL-12-p40 production from THP-1 cells compared to H37Rv. There was more cell death when cells were infected with K-strain and H37Rv compared to uninfected THP-1 cells, but K-strain induced the most cell death. K-strain induced less apoptosis and more necrosis in THP-1 cells than H37Rv (Sohn *et al*., 2009).

In 2009, Tanveer and colleagues compared H37Rv, BCG and 18 clinical isolates. Eight clinical isolates were from the CAS1 clade and eight were from the Beijing

clade, which were identified as the most prevalent clades in Pakistan. The clinical isolates had lower growth rates in broth and produced lower cytokine responses from THP-1 cells than H37Rv. There was a positive correlation between strain growth and induction of TNF-α and IFN-γ secretion for all strains.

These studies indicate that production of cytokines by infected THP-1 cells can vary according to mycobacterial strain both in the time it takes for production to be induced and in the level to which they are produced. The level of apoptosis versus necrosis induced in THP-1 cells by infection can also vary according to the mycobacterial strain. Some strains of *M. tuberculosis* suppress inflammatory responses causing early progression to active disease. Other strains induce a strong inflammatory response, possibly producing latent infection (Talarico *et al*., 2007).

It is difficult to compare data on different strains and their effects in THP-1 cells. There is wide variation in the number of bacteria that THP-1 cells have been infected with, ranging from one bacterium to ten cells, to fifty bacteria per cell. Studies have used bacteria that are in exponential growth phase or bacteria that have been frozen (Table 4-5). THP-1 cells have been differentiated overnight and rested for 3 days before use, or used directly after differentiation. Some studies add bacteria to the cells for a few hours before washing extra cellular bacteria away, and other studies do not wash away extracellular bacteria (Table 4-6). Studies have used laboratory strains and compared them to a limited number of clinical isolates. These clinical isolates have been typed by IS*6110* or spoligotyping, which are not used clinically to distinguish between strains, as they are not discriminatory enough.

Table 4-5 Summary of treatment of mycobacteria used in published infection models using THP-1 cells

Reference	No. of bacteria per cell	Method of inoculum preparation	Were bacteria frozen before use?	Bacterial strains included in the study					No. of hours before extra cellular bacteria were washed out
				No. of clinical isolates tested	H37Ra	*M. bovis*	BCG	H37Rv	
(Riendeau and Kornfeld, 2003)	10	liquid	×	0	✓	✓	✓	✓	4
(Sharma *et al*., 2008)	2	solid	×	1	✓	×	×	✓	N/A
(Theus *et al*., 2005)	50	liquid	✓	32	×	×	×	×	3
(Rajavelu and Das, 2007)	10	liquid	✓	2	✓	×	×	✓	3
(Castro-Garza *et al*., 2007)	0.1	liquid	✓	4	×	×	×	✓	N/A
(Theus *et al*., 2007b)	50	liquid	✓	15	×	×	×	×	3
(Sohn *et al*., 2009)	3	liquid	✓	1	×	×	×	✓	3
(Tanveer *et al*., 2009)	5	liquid	✓	16	×	×	✓	✓	N/A

✓ indicates yes
× indicates no
N/A indicates that no wash step was included in the protocol

Table 4-6 Summary of treatment of THP-1 cells used in published mycobacterial infection models

Reference	No. of THP-1 cells/ ml	Method used to differentiate cells			Parameters tested				
		Concentration of PMA	Concentration of IFN-γ	Length of treatment	Intracellular growth rate	Growth rate in broth	THP-1 cell apoptosis	THP-1 cell necrosis	Cytokine production
(Riendeau and Kornfeld, 2003)	5×10^5	5 nM	0	18 hours	×	×	✓	×	×
(Sharma *et al*., 2008)	2×10^6	N/A	N/A	N/A	✓	×	×	×	✓
(Theus *et al*., 2005)	-	100 nM	100 U/ml	3 days	✓	×	×	×	✓
(Rajavelu and Das, 2007)	1×10^5	20 nM	0	18 hours	✓	×	✓	×	×
(Castro-Garza *et al*., 2007)	5×10^5	10 nM	0	-	×	×	×	✓	×
(Theus *et al*., 2007b)	-	100 nM	100 U/ml	3 days	✓	✓	×	×	✓
(Sohn *et al*., 2009)	-	40 nM	0	18 hours	×	✓	✓	✓	×
(Tanveer *et al*., 2009)	-	32 nM	0	-	×	✓	×	×	✓

✓ indicates yes
× indicates no
N/A indicates that treatment was not performed; the cells were not differentiated and remained as monocytes
- indicates that the data not included in the publication.

Table 4-7 Summary of results from published mycobacterial infection models using THP-1 cells

Reference	Growth in THP-1 cells		Growth in broth	THP-1 cell apoptosis	THP-1 cell necrosis	Cytokine production	
	Test method (days post infection)	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Lowest rate ↓ Highest rate	Pro-inflammatory	Anti-inflammatory
(Riendeau and Kornfeld, 2003)	-	-	-	*M. bovis*, H37Rv, BCG, H37Ra,	-	-	-
(Sharma *et al*., 2008)	CFU counts (3)	No difference	-	-	-	H37Rv induced higher INF-γ, TNF-α secretion	Type I clinical isolate induced higher IL-10 secretion
(Theus *et al*., 2005)	CFU counts (7)	Unique isolates, clustered isolates	-	-	-	High intracellular growth correlated with suppression of TNF-α	High intracellular growth correlated with rapid IL-10 secretion
(Rajavelu and Das, 2007)	CFU counts (7)	H37Ra, S7, S10, H37Rv	-	S7, H37Rv, S10, H37Ra	-	-	-
(Castro-Garza *et al*., 2007)	-	-	-	-	Clinical isolates, H37Rv	-	-
(Theus *et al*., 2007b)	CFU counts (7)	Growth rate varied amongst 15 Beijing isolates	No difference	-	-	High intracellular growth correlated with suppression of TNF-α	-
(Sohn *et al*., 2009)	-	-	No difference	H37Rv, Clinical isolate	Clinical isolate, H73Rv	-	-
(Tanveer *et al*., 2009)	-	-	Clinical isolates, H37Rv	-	-	High growth in broth correlated with induction of TNF-α	-

- indicates that the data was not included in the publication

The effect of mycobacterial virulence on host cell apoptosis has also been studied. Host cell apoptosis in response to infection with an intracellular virus is a well-established phenomenon (Roulston *et al*., 1999). Some viral pathogens are able to prevent host cell apoptosis, thus securing a favourable environment in which to replicate. The first report of apoptosis occurring in response to infection with *M. tuberculosis* was published in 1997 by Keane and colleagues. Keane used a multiplicity of infection (MOI) of between 5 and 10 bacilli per cell to infect human alveolar macrophages and showed that H37Ra caused more apoptosis than H37Rv (Keane *et al*., 1997). A later study showed that other virulent *M. tuberculosis* and *M. bovis* isolates could evade alveolar macrophage apoptosis, allowing them to grow intracellularly, whereas avirulent strains such as BCG caused host cell apoptosis that was accompanied by a decrease in bacterial viability (Keane *et al*., 2000).

There is now a large body of evidence, which shows that death of host cells by apoptosis is bactericidal (Lee *et al*., 2009). Virulent mycobacteria that prevent host cells from undergoing apoptosis are able to proliferate intracellularly. Once the number of intracellular bacteria becomes high enough the bacteria are able to induce necrotic cell death, the bacteria are released and may infect other macrophages aiding the dissemination and transmission of the mycobacteria.

Sohn and colleagues showed that an *M. tuberculosis* isolate belonging to the Beijing spoligotype clade caused less apoptosis than H37Rv and caused more necrosis, despite the strains having a similar intracellular growth rate. This suggests that virulent clinical isolates are able to prevent apoptosis, thus preventing bacterial killing

and are able to promote necrosis, which would release the bacteria from the phagocyte and allow infection of other cells. A study by Theus suggests that virulence correlates with higher intracellular growth rates (Theus *et al*., 2005). Therefore, measurement of intracellular growth rate and THP-1 cell necrosis will be used as parameters for assessment of bacterial virulence in the current study.

4.2 Development of a THP-1 cell model to compare the virulence of *M. tuberculosis* strains

The aim of this section was to develop a tissue culture assay to assess the virulence of clinical *M. tuberculosis* isolates. In order to test the model, the laboratory strain *M. tuberculosis* H37Rv was used as an example of a virulent strain and *M. bovis* BCG Danish vaccine was used as an example of an avirulent strain. Growth of bacteria within cells and death of infected cells was measured to determine virulence. The BACTEC™ MGIT™ 960 System was used to measure mycobacterial growth. The BACTEC™ MGIT™ 960 System is an indirect method of measuring the number of bacteria that are present in a sample based of the amount of time that is taken for the oxygen in a tube to be depleted by bacterial respiration, which is known as the time to positivity (TTP). The BACTEC™ MGIT™ 960 System was calibrated to allow comparisons between strains to be made.

4.2.1 Preparation of a standard bacterial inoculum

4.2.1.1 Background

MGITs™ contain 7 ml of modified Middlebrook 7H9 broth and 100 µl of tris 4,7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate which is an oxygen-quenched fluorophore. Bacteria that are inoculated into a MGIT™ deplete the oxygen as they respire until there is insufficient oxygen to quench the fluorophore, which

results in an increase in fluorescence. Tubes entered into the BACTEC™ MGIT™ 960 System are incubated at 37°C and monitored for increasing fluorescence. Once the fluorescence reaches 75 units, the tube is declared positive and described as having a positive growth index. The length of time taken for a MGIT™ to reach a positive growth index is called the TTP. Tubes that show no fluorescence for a minimum of 34 days are declared negative. MGITs™ also contain an antimicrobial mixture called PANTA™ which reduces contamination from non-mycobacterial organisms and OADC which is essential for growth of mycobacteria.

Passage number, storage conditions and phase of growth can all affect the growth rate of bacteria. Nascimento and Leite showed that three successive rounds of freezing and thawing impaired the growth of BCG Moreau strain in liquid culture (Nascimento and Leite, 2005). The inoculum was standardised by optical density to ensure that comparisons were accurate. Nascimento and Leite also showed that BCG passaged three times from actively growing cultures had impaired growth in liquid culture compared to BCG that had only been passaged twice. Andreu and Gibert found changes in the lipid composition of the cell wall of H37Rv after 20 passages (Andreu and Gibert, 2008). Molina-Torres and colleagues reported that serial subculture of four *M. tuberculosis* isolates, including H37Rv resulted in decreased virulence in a THP-1 cell model (Molina-Torres *et al*., 2010). Cardona and colleagues identified a clinical isolate that lost virulence after repeated passage when compared to frozen stock of the strain, which had not been passaged, using an immunocompromised murine model of infection (Cardona *et al*., 2006). Aranaz and

colleagues found that the spoligotype of a clinical isolate changed after eight serial passages (Aranaz *et al*., 2004).

The BACTEC™ MGIT™ 960 System has been used in the current study to equate TTP to the number of bacteria that were present in a culture. It was therefore important that all strains tested in the current study were treated in the same way so that differences in virulence could be detected, and could not be attributed to differences in passage number, storage condition or growth phase.

4.2.1.2 Hypothesis

Bacteria that have been stored at -20°C will take longer to achieve a positive growth index in a MGIT™ than bacteria that have been growing in culture.

4.2.1.3 Aims

- To compare the TTP of MGITs™ that had been inoculated from a culture in exponential growth phase to the TTP of MGITs™ inoculated from a culture which had just been removed from storage at -20°C
- To compare the TTP of MGITs™ that had been inoculated from a culture in exponential growth phase to the TTP of MGITs™ inoculated from a culture in stationary growth phase
- To prepare a stock of each mycobacterial strain for use in subsequent experiments

4.2.1.4 Materials and methods

The MRCM prepares frozen stocks of all strains that are received; briefly, a MGIT™ (BD Biosciences, Oxford, UK) is inoculated with 1 ml bacteria from either a patient

sample or a culture from the NCTC, Colindale, London, UK. *M. bovis* BCG Danish vaccine strain 1331 was obtained from the Statens Serum Institute, Copenhagen. MGITs™ are placed in a BACTEC™ MGIT™ 960 System (BD Biosciences, Oxford, UK) at 37°C until a positive growth index is detected by fluorescence. Positive cultures are confirmed by microscopy, the culture is genotyped and a 1 ml aliquot is placed in a cryovial and stored at -20°C.

During the current study, frozen mycobacterial stocks prepared by the MRCM were defrosted and 100 µl was used to inoculate a fresh MGIT™, the time taken for each MGIT™ to achieve a positive growth index was recorded. In some cases 100 µl of culture was removed from a MGIT™ with a positive growth index and was used to inoculate a fresh MGIT™, the TTP for the fresh MGIT™ was recorded. Growth rate was assessed by recording the relative fluorescent units produced by a MGIT™ culture, which was plotted against time, and then linear regression was used to calculate a doubling time. Relative fluorescent units were assessed by the BACTEC™ at hourly intervals; however, the data is not stored and has to be manually recorded.

In order to prepare a stock of mycobacteria for use in the current study a MGIT™ was inoculated from the frozen stock prepared by the MRCM and once this MGIT™ reached a positive growth index, 45 aliquots of 150 µl were prepared and stored at -20°C. All positive cultures were confirmed as pure mycobacterial cultures by microscopy. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.1.5 Results

As shown in Figure 4-3 cultures which were inoculated from frozen stock took twice as long to reach a positive growth index as cultures which were inoculated from actively dividing cultures. The mean number of hours for cultures of BCG which were inoculated with 100 µl of bacteria from frozen stock to reach a positive growth index was 116 (± 10 hours, n=5). The mean number of hours for cultures which were inoculated with 100 µl of bacteria from an actively growing culture to reach a positive growth index was 58 hours (± 5 hours n=5) (p= 0.002).

Cultures of H37Rv which were inoculated with 100 µl bacteria from frozen stock reached a positive growth index in a mean of 194 hours (± 11 hours, n=5). Cultures which were inoculated with 100 µl of bacteria from an actively growing culture reached a positive growth index in a mean of 86 hours (± 17 hours n=5) (p= 0.0008).

The variation in TTP for different cultures seen in Figure 4-3 could be caused by a difference in the number of bacteria in the inoculum; therefore, linear regression was used to calculate a bacterial doubling time based on the number of fluorescent units produced per hour. MGIT™ cultures inoculated from actively dividing cultures had a mean doubling time of 22 hours (± 1.3 hours, n=4). The mean doubling time of MGIT™ cultures inoculated with bacteria from cultures which had reached stationary phase was 2 hours longer than MGITs™ inoculated from actively dividing cultures (24 hours ± 1.1 hours, n=5). The mean doubling time for MGIT™ cultures inoculated with H37Rv directly from frozen stock was 12 hours longer than MGITs™ inoculated from actively dividing cultures (34 hours ± 1.1 hours, n=5) (p=0.03).

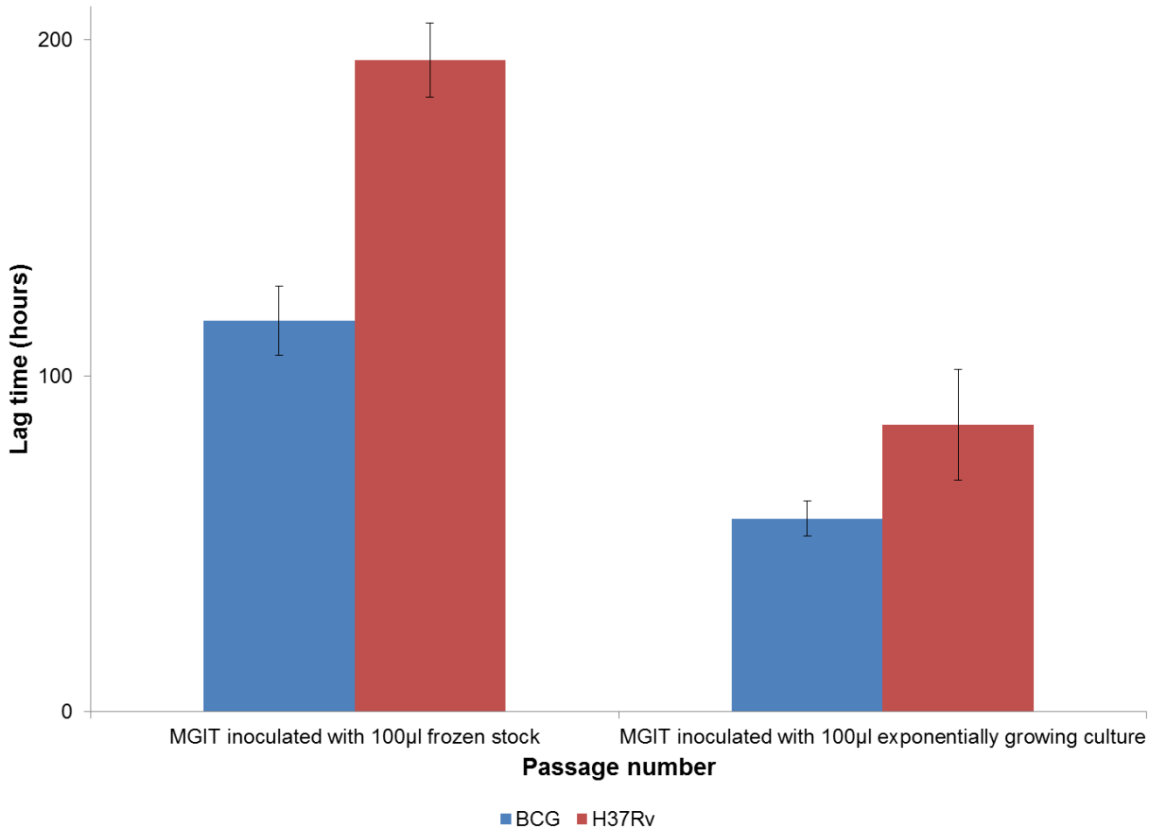


Figure 4-3 Mean TTP cultures of H37Rv and BCG, inoculated from frozen stocks or cultures in exponential growth phase

Error bars depict SEM, (n=5)

Results from experiments that calculated bacterial doubling time indicate that the growth phase of the bacterial inoculum affects the growth of the subsequent culture. In order to confirm the effect of inoculum growth phase on TTP in a MGIT™, bacteria from stationary or actively growing cultures were enumerated (for method see section 4.2.2) and used to inoculate MGITs™. The mean TTP for MGITs™ inoculated with 5×10^5 H37Rv from an exponential phase culture (113 hours ± 5 hours, n=5) was 52 hours shorter than the mean TTP for MGITs™ inoculated with 5×10^5 H37Rv from a stationary phase culture (165 hours ± 8 hours, n=5) (p= 0.02) (Figure 4-4).

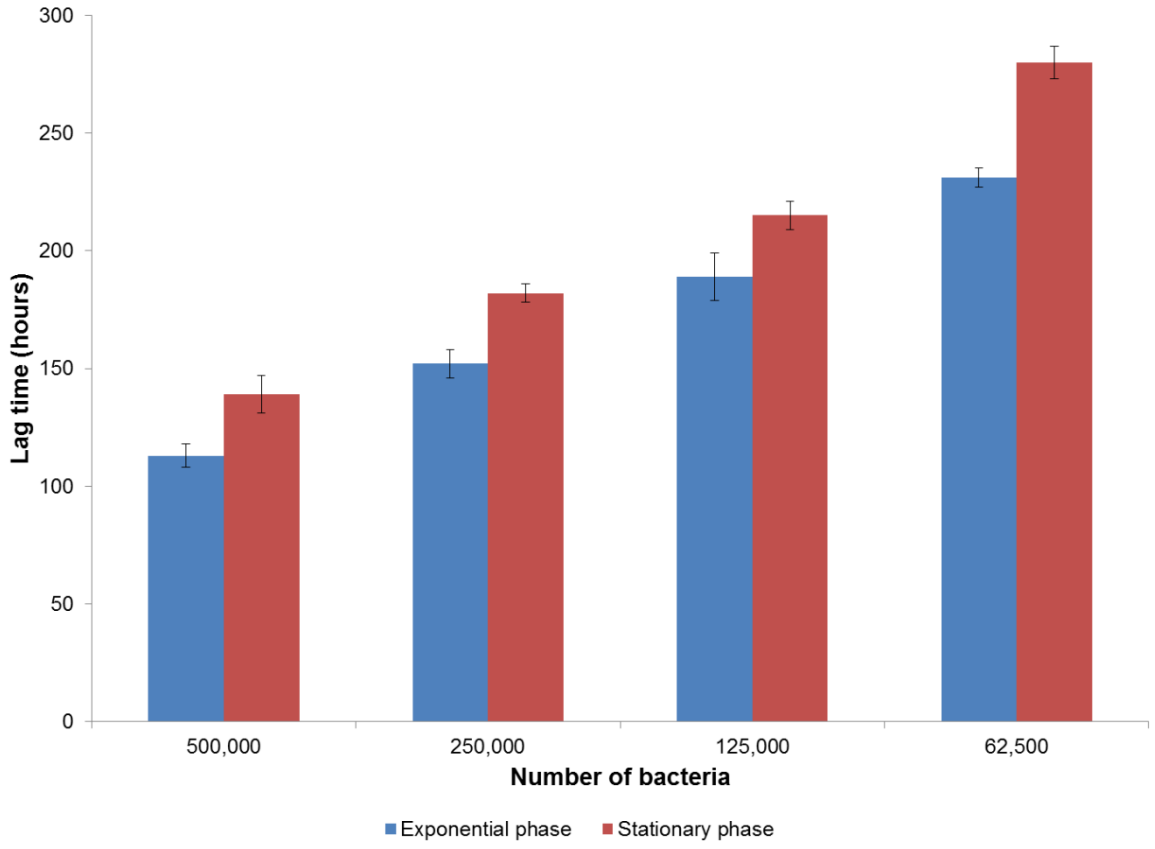


Figure 4-4 Mean TTP for MGITs™ inoculated from H37Rv cultures in exponential or stationary phase growth

Error bars depict SEM, (n=5)

4.2.1.6 Discussion

Results from the current study indicate that the TTP of a MGIT™ culture can vary significantly depending on the conditions used to store the bacterial inoculum. To reduce variability between future experiments a standard bacterial inoculum was prepared for each strain that was tested in the tissue culture model.

A standard inoculum was prepared by growing a bacterial culture until it achieved a positive growth index in a MGIT™. The positive culture was stored at -20°C in single use aliquots to avoid repeated cycles of freezing and thawing, as there are reports of a reduction in the number of colony forming units (CFU) after bacteria have been

frozen (O'Sullivan *et al*., 2007a). Each aliquot will be used to inoculate a MGIT™. Once the MGIT™ had reached a positive growth index 100 µl of the culture was used to inoculate a second MGIT™, as the current study has shown that MGITs™ inoculated with bacteria which have been frozen have a longer TTP and the bacteria have a slower doubling time than MGITs™ inoculated from actively growing cultures. This strategy is also used by O'Sullivan and colleagues (O'Sullivan *et al*., 2010) and Shorten and colleagues (Shorten *et al*., 2013). The bacteria in the second MGIT™ were harvested during exponential growth as the current study has shown that TTP and bacterial doubling time vary depending upon the growth phase of the bacterial inoculum. The harvested bacteria were enumerated and then used for further experimentation. This method was used for the preparation of all bacteria in the current study.

4.2.2 Enumeration of bacteria

4.2.2.1 Background

Mycobacteria were enumerated so that differing inoculum sizes did not cause variation between strains when they were tested in a tissue culture model. Mycobacteria can form cords, which are large clumps of bacteria that must be disrupted in order for accurate quantification to be achieved.

Addition of detergents to bacterial cultures or mechanical disruption methods such as sonication can be used to disperse clumps. These procedures may affect the bacterial phenotype. Sonication has been shown to remove bacterial cell surface lipids, which affects the rate at which bacteria are phagocytosed by macrophages (Stokes *et al*., 2004). A study by Sani and colleagues used cryo-transmission

electron microscopy to study the cell envelope of four mycobacterial strains, including BCG Copenhagen and *M. tuberculosis* strain 6020. Sani showed that when mycobacteria were grown with 0.05% tween and agitation the outer capsule-like layer was partially or completely removed (Sani *et al*., 2010). Sani also showed that removal of the capsule-like layer from BCG reduced binding of the bacteria to human MDMs and increased the production of pro-inflammatory cytokines by the macrophages. As evidence suggests that removal of cell surface lipids affects the interaction of mycobacteria with macrophages, an optimum treatment should to be defined to remove clumps whilst preserving the bacterial phenotype.

4.2.2.2 Hypothesis

Sonication and addition of Tween 80 to bacterial cultures will eliminate bacterial clumps.

4.2.2.3 Aims

- To optimise the treatment of mycobacterial cultures in order to remove clumps, whilst minimising the manipulation of the bacteria.
- To determine whether procedures to remove clumps affect bacterial growth *in vitro*

4.2.2.4 Materials and methods

Mycobacterial cultures were prepared as described in section 4.2.1.4, and purity was confirmed by microscopy using a TB ZN stain kit (Becton Dickinson) according to the manufacturer's instructions. Bacteria were washed and resuspended in 1 ml of RPMI with or without 0.05% Tween 80 (v/v) and some suspensions were subjected to two 30 second bursts of sonication in a bath sonicator. Suspensions were then left to

stand for 60 minutes to allow clumps of bacteria to fall out of suspension; the upper 500 µl was removed and added to 500 µl fresh RPMI. At this point, an aliquot of clarified bacteria was removed for auramine staining to assess the number of clumps. Staining was carried out using TB fluorescent stain kit (Becton Dickinson) according to the manufacturer's instructions. Bacteria in 10 fields of view were counted and categorised as single organisms, clumps of between 2 and 5 organisms, clumps of 6 and 20 organisms, clumps of 21 and 50 organisms and clumps of over 50 organisms. Images were captured using a Leica microscope. Samples were left to stand for a further 60 minutes and the upper 500 µl was removed. A sample was removed for auramine staining to assess the number of clumps and a further sample was taken for counting. The sample of bacteria that was taken for counting was added to an equal volume of formalin and incubated at room temperature for 30 minutes to fix the bacteria. Fixed bacteria were inoculated into MGITs™ to confirm that bacteria had been killed by the formalin treatment. The number of organisms in the fixed sample was estimated by counting using a Helber chamber and light microscopy at 50 X magnification. Counts were the average of 80 small squares. Counts were used to adjust unfixed bacterial suspensions to a density of 5×10^5 bacterial/ml using RPMI with or without 0.05% Tween 80 (v/v) and 100 µl was inoculated into a fresh MGIT™. In some cases, MGITs™ were inoculated with 100 µl of 5×10^5 bacteria/ml in RPMI with 0.5% Tween 80 (v/v).

4.2.2.5 Results

Treating a suspension of *M. tuberculosis* H37Rv with 0.05% (v/v) Tween 80 resulted in a 2 fold increase in the number of single organisms (197 ± 10 n=10) when compared to untreated bacteria (113 ± 5n=10) (p= 0.002) (Figure 4-5). When bacteria

were treated with 0.05% Tween 80 and subjected to sonication it resulted in a 5 fold increase in the number of single organisms (534 ± 21 n=10) when compared to untreated bacteria (p= 0.00004) and a 3 fold increase when compared to bacteria treated with 0.05% Tween 80 (p= 0.0001).

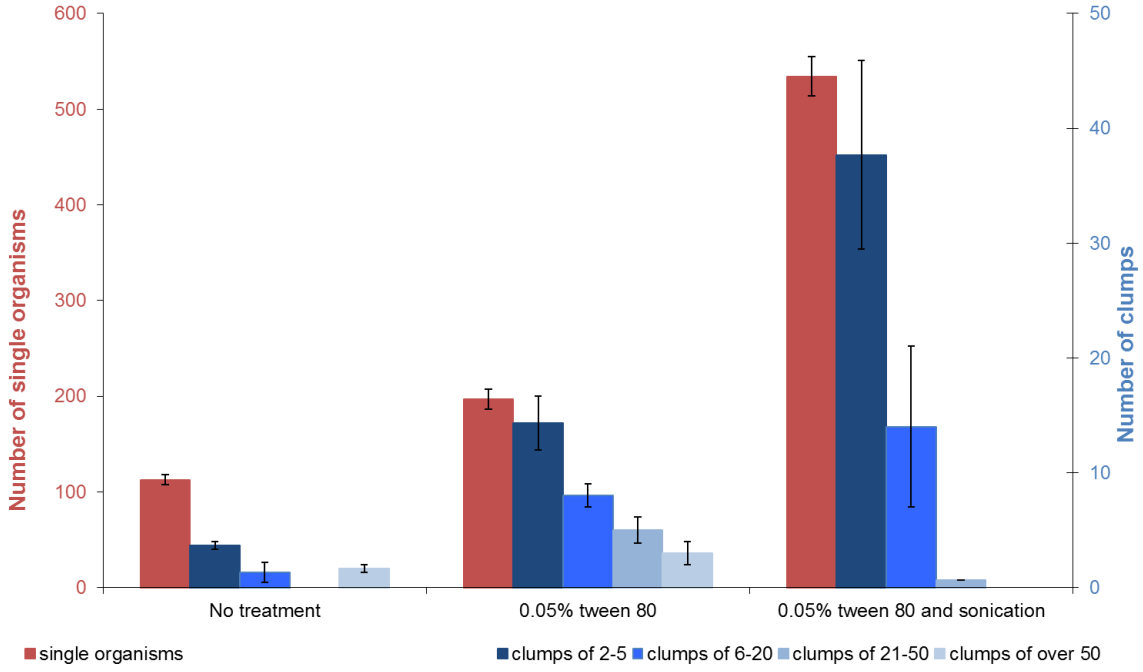


Figure 4-5 Mean number of bacteria in five subgroups after treatment to disperse clumps

Ten replicate counts were made for each treatment (± SEM).

Addition of 0.05% Tween 80 (v/v) to bacterial suspensions resulted in an increase in the number of clumps in all of the categories when compared to untreated bacteria. When bacteria were treated with 0.05% Tween 80 (v/v) and sonicated it resulted in an increase in the number of smaller clumps, but the larger clumps were eliminated. The number of clumps consisting of 2 to 5 organisms was 10 fold higher in cultures treated with sonication and 0.05% Tween 80 (v/v) (38 ± 8 n=10) compared to untreated bacteria (4 ± 0.3 n=10) (p= 0.01). The number of clumps consisting of 2 to 5 organisms was 3 fold higher in cultures treated with sonication and 0.05% Tween 80 (v/v) (38 ± 8 n=10) compared to bacteria treated with 0.05% Tween 80

(v/v) (14 ± 2 n=10) (p= 0.05). The effect of 0.05% Tween 80 (v/v) and sonication on the degree of mycobacterial clumping can be seen in Figure 4-6.

MGITs™ inoculated with 5×10^5 H37Rv, had a mean TTP of 119 hours (± 0.3 hours, n=3). Addition of 0.05% Tween 80 (v/v) to 5×10^5 H37Rv had no effect on the mean TTP (117 ± 1.2 hours, n=3) when compared to cultures with no Tween 80 (p= 0.35). Addition of 0.5% Tween 80 (v/v) to 5×10^5 H37Rv caused an increase in mean TTP of 28% (150 ± 0.9 hours, n=3) when compared to cultures which had no Tween 80 added ($p= 5 \times 10^{-6}$) (Figure 4-7). Similar results were seen for BCG (Figure 4-7).

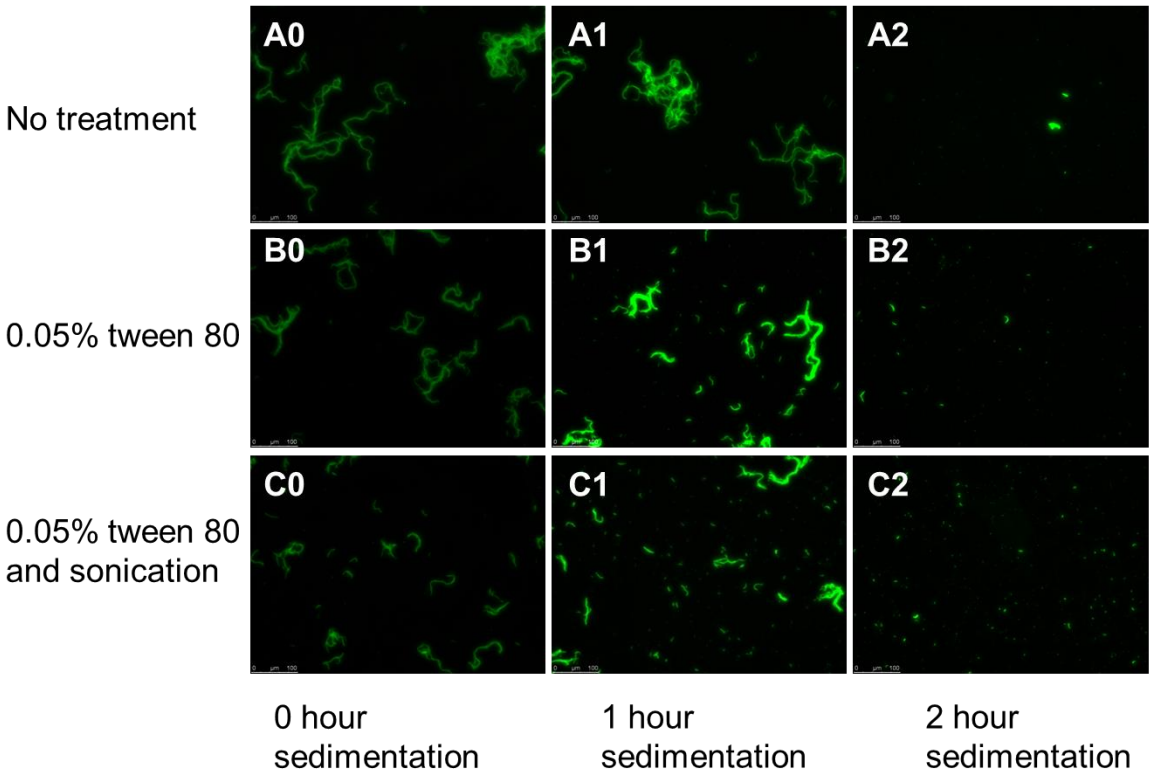


Figure 4-6 Effect of 0.05% Tween 80 with and without sonication and sedimentation on the degree of clumping in a sample of *M. tuberculosis* H37Rv as shown by auramine staining

A0, A1 and A2 were not treated, B0, B1 and B2 were treated with 0.05% Tween 80, C0, C1 and C2 were treated with 0.05% tween and sonication. Samples were removed immediately after treatment (A0, B0, C0), after cultures had settled for 1 hour (A1, B1 and C1) or 2 hours (A2, B2 and C2).

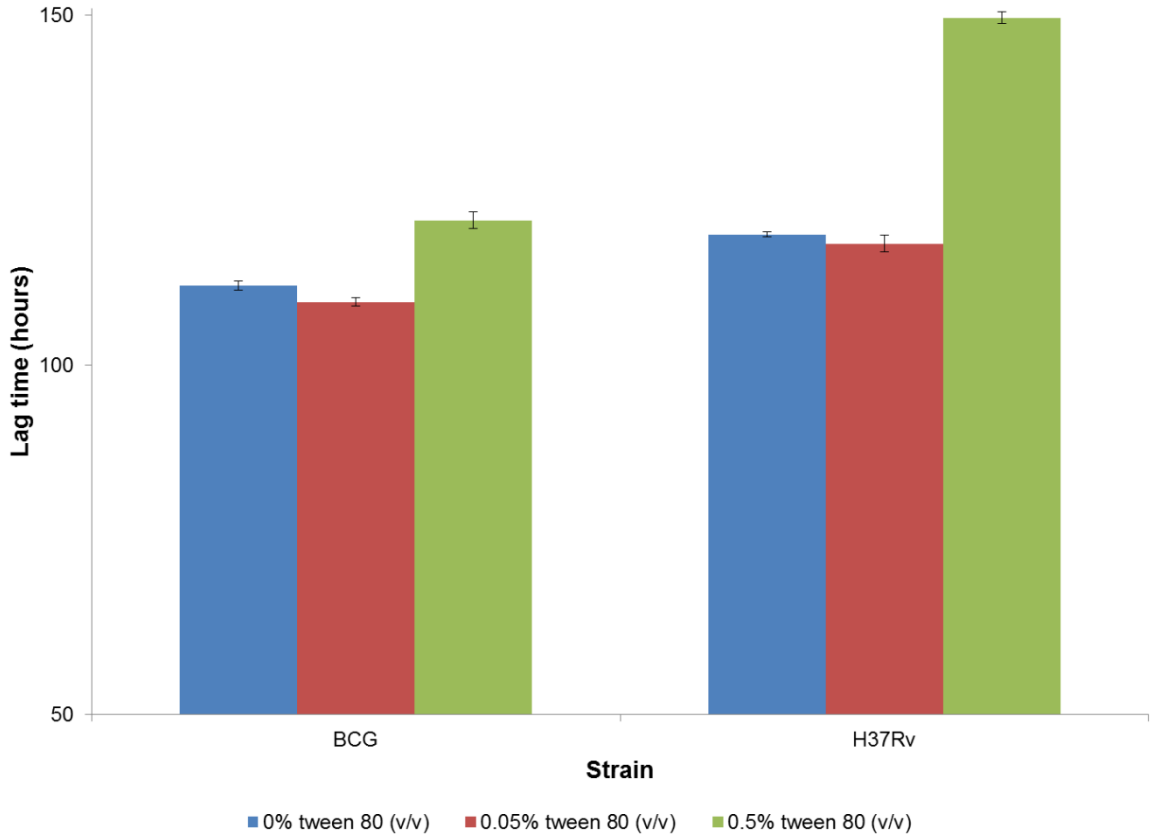


Figure 4-7 Mean TTP for cultures of H37Rv and BCG after treatment with different concentrations of Tween 80

Error bars depict SEM, (n=3)

4.2.2.6 Discussion

The study confirms the importance of sonicating bacteria in addition to treatment with detergent for removal of clumps from bacterial preparations. Both methods reduce the number of large clumps present in an inoculum and increase the number of single organisms, but sonicating the bacteria in addition to treatment with detergent destroyed all of the larger clumps and resulted in the largest increase in the number of single organisms.

Bacterial suspensions that were not sonicated or treated with detergent have a small number of clumps. This is because the cords were not disrupted and remained as

large clumps, which settled out of suspension rapidly when cultures were clarified. There were very few single organisms present in this preparation, indicating that clumps were not dispersed and the majority of the organisms were removed as clumps.

The cell envelope of *Mycobacterium* spp. has three layers, the plasma membrane, the cell wall outer membrane (mycomembrane) and the outermost layer (Zuber *et al*., 2008; Sani *et al*., 2010). There are two leaflets to the outer membrane, the inner leaflet is composed of mycolic acid residues and the outer leaflet contains large amounts of species-specific lipids. The capsule is a mixture of polysaccharides, proteins and lipids. Ortalo-Magné and colleagues showed that gentle shaking of bacterial clumps with 4 mm diameter glass beads was enough to remove this capsule (Ortalo-Magne *et al*., 1995).

Stokes and colleagues showed that three 30-second bursts of sonication broke up clumps, so that 90% of organisms were present as single organisms or clumps of two and only 1% of organisms were part of clumps that contained more than 10 organisms (Stokes *et al*., 2004). Sonicating bacteria for 5 minutes resulted in a in a loss of viability but three bursts of 30 seconds did not alter the integrity or viability of the bacterial cells. The surface hydrophobicity of *M. tuberculosis* was not altered by sonication but the appearance of the capsule was dramatically affected. Stokes describes the appearance of the capsule from bacteria that had been subjected to three 30-second bursts of sonication as loosened and bulging with an increased but uneven thickness. Sonication of *M. tuberculosis* also resulted in an increase in the

association of the bacteria with macrophages. The protocol used in the current study limited sonication to two bursts of 30 seconds and introduced two clarification steps to remove clumps whilst limiting disruption of the capsule.

Addition of 0.05% Tween to bacteria did not alter the *in vitro* growth as measured by the BACTEC™ MGIT™ 960 System, but a higher concentration of detergent resulted in a 28% increase in the mean number of hours taken for a MGIT™ to reach a positive growth index. Tween 80 is a polyethylene sorbitol ester and when it is hydrolysed, it releases traces of oleic acid, which is toxic to *M. tuberculosis* (Davis and Dubos 1948). The higher concentration of Tween 80 may have caused some of the bacteria to die, resulting in a smaller inoculum, therefore increasing the time taken for the bacteria to respire the oxygen present in the MGIT™. Tween 80 is used routinely to cultivate *M. tuberculosis* in liquid cultures. The addition of BSA to the media binds free oleic acid and removes its toxic effect (Wayne, 1994; Middlebrook and Dubos, 1948). In the current study Tween 80 was used at a concentration of 0.05% (v/v) to produce suspensions of single organisms and bacteria were suspended in RPMI with 10% fetal bovine serum (FBS) which contains BSA to complex with free oleic acid.

4.2.3 Assessment of bacterial growth

4.2.3.1 Background

The number of bacteria in a culture of *M. tuberculosis* can be estimated by performing CFU counts. In order to carry our CFU counts samples are serially diluted and put onto agar plates. Agar plates are incubated for between 14 and 30 days and the number of colonies that have formed are counted. Colonies of *M. tuberculosis*

have an irregular shape and are pale in colour, which makes them difficult to see on agar plates. Estimates of bacterial cell number that are based upon CFU counts may be subject to underestimation due to the tendency of *M. tuberculosis* to form clumps, which means that one colony may result from a clump of several organisms. Agar plates often become contaminated with other organisms that grow faster than *M. tuberculosis*, meaning that the contaminant covers the plate before the mycobacteria have had time to grow. CFU counting is time consuming and it is difficult to obtain accurate, reproducible results.

Culturing bacteria in the BACTEC™ MGIT™ 960 System or its predecessor the BACTEC™ 460 System and correlating the time taken for a positive growth index to be achieved with the number of organisms that were inoculated is an alternative method of assessing mycobacterial cell numbers. Enumeration of *M. tuberculosis* using the BACTEC™ systems has been carried out in several studies (Diacon *et al*., 2012; Bark *et al*., 2011; Pheiffer *et al*., 2008; Cheon *et al*., 2002; Wallis *et al*., 1999). Culturing bacteria in liquid media is quicker and more sensitive than culturing bacteria on solid media. A study carried out on 1,500 sputum specimens by Pfyffer and colleagues found that *M. tuberculosis* complex could be detected in the MGIT™ system in an average of 9.9 days whereas detection using solid media took an average of 20.2 days (Pfyffer *et al*., 1997). Idigoras and colleagues found that sensitivity of the BACTEC™ MGIT™ 960 System for detection of mycobacterial isolates was 75%, whereas the sensitivity of Middlebrook 7H11 agar plates was 51% and Löwenstein-Jensen agar slopes was 61% (Idigoras *et al*., 2000). The addition of antibiotics that inhibit non-mycobacterial growth prevents frequent contamination of

MGIT™ cultures. Pfyffer and colleagues reported a lower contamination rate for MGITs™ than for solid culture methods in two of the three mycobacterial reference centres that were used in their study (Pfyffer *et al*., 1997). MGITs™ contain a fluorophore that is liberated by bacterial respiration meaning the presence of clumps does not alter the estimate of bacterial number. Assessment of mycobacterial growth using the BACTEC™ MGIT™ 960 System gives good reproducibility and the automated system assesses the MGITs™ every hour.

In order to utilise the BACTEC™ MGIT™ 960 System for enumeration of bacteria it is important to know whether different strains grow at the same rate. Janulionis and colleagues assessed the growth rate of 18 clinical isolates and 5 reference strains in the BACTEC™ 460 System and found that the strains had different growth rates. Janulionis found a 2.45 fold difference between the strain with the fastest growth rate and the strain with the slowest growth rate and also noted that reference strains had faster growth rates than clinical isolates (Janulionis *et al*., 2005). Keane and colleagues reported that the growth rates for H37Ra and H37Rv in medium were similar, although it is not clear whether medium refers to broth or cell culture medium (Keane *et al*., 1997). Sohn and colleagues reported that there was no difference in the growth rate in broth of H37Rv and a clinical isolate from the Beijing clade (Sohn *et al*., 2009). Zhang and colleagues found that the growth rate of H37Ra, H37Rv, and two clinical isolates in 7H9 media were similar when assessed by CFU counts over 10 days. As there is conflicting evidence in the literature about the growth rates of clinical isolates and the reference strains H37Rv and H37Ra, the growth rates of the strains used in the current study were assessed.

4.2.3.1 Hypothesis

The number of bacteria in an inoculum will correlate with the TTP measured using the BACTEC™ MGIT™ 960 System.

4.2.3.2 Aims

- To calibrate the BACTEC™ MGIT™ 960 System so that the number of hours taken for a sample to be declared positive, or the TTP, can be used to determine the number of organisms that were present in the inoculum

4.2.3.3 Materials and methods

H37Rv was resuspended in RPMI and treated to remove clumps as described in 4.2.2.4. A sample of the bacterial suspension was fixed and counted and the unfixed portion was adjusted to 5×10^5 bacteria/ml as described in 4.2.2.4. Serial dilutions were then performed with RPMI. A MGIT™ was inoculated with 100 µl of each dilution and the relative fluorescence was recorded from the BACTEC™ MGIT™ 960 System (BD Biosciences, Oxford, UK) every 24 hours, in some cases more frequent readings were taken. It is important to note that although the BACTEC™ MGIT™ 960 System assesses the fluorescence of each MGIT™ every hour, the information is not stored and so has to be manually recorded. The experiment was performed in triplicate. The number of hours taken for each MGIT™ to achieve a positive growth index was recorded. Positive cultures were confirmed by microscopy.

A further three serial dilutions were prepared from each dilution to aid colony counting and 20 µl of each dilution was plated onto Middlebrook 7H10 agar plates (Becton Dickinson) in triplicate according to the method of Miles and Misra (Miles *et*

al., 1938). Plates were incubated for 30 days at 37°C and counts were recorded as CFU/ml.

Suspensions of clinical isolates were prepared which contained 5×10^5 bacteria/ml. Triplicate samples of 100 µl from each of these suspensions were inoculated into MGITs™ to assess the TTP of each clinical isolate. Preparation of mycobacterial suspensions was repeated on separate occasions. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.3.4 Results

Figure 4-8 shows that there is an inverse relationship between TTP and $\log_{10}$ CFU/ml for cultures of H37Rv. The correlation coefficient is -0.98. There is also an inverse relationship between TTP and $\log_{10}$ of the number of bacteria counted using a Helber chamber (Figure 4-9); the correlation coefficient is -0.98.

Figure 4-10 shows that when MGITs™ are inoculated with 100 µl of a suspension of 5×10^5 bacterial/ml the mean TTP for H37Ra (358 ± 6 hours, n=3) is 64% higher than for H37Rv (219 ± 3 hours, n=15) ($p= 3 \times 10^{-13}$). The mean TTP for BCG (227 ± 12 hours, n=15) is similar to that of H37Rv (p= 0.5). The mean TTP for MGITs™ inoculated with clinical isolates are between 12% (CE1, 193 ± 6 hours, n=18, $p= 5 \times 10^{-4}$) and 21% (AE1, 173 ± 2 hours, n=15, $p= 8.0 \times 10^{-15}$) lower than H37Rv.

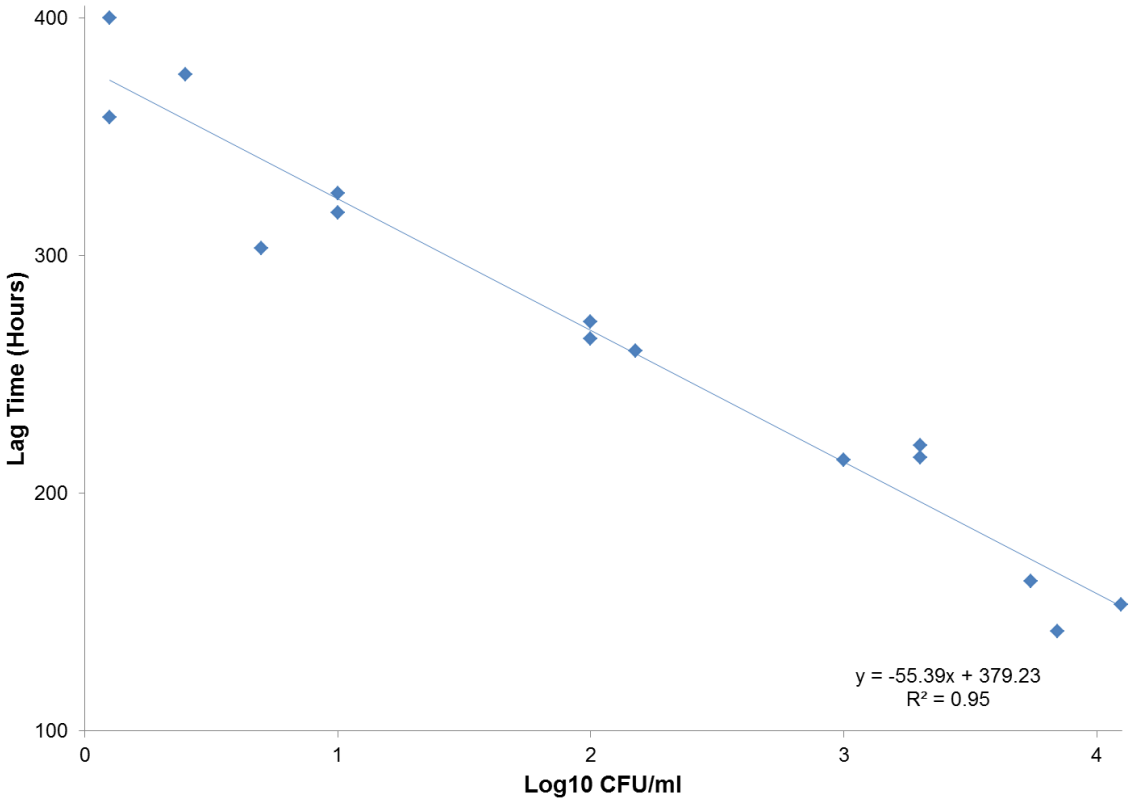


Figure 4-8 Relationship between TTP and log_{10} H37Rv CFU/ml

(n=3)

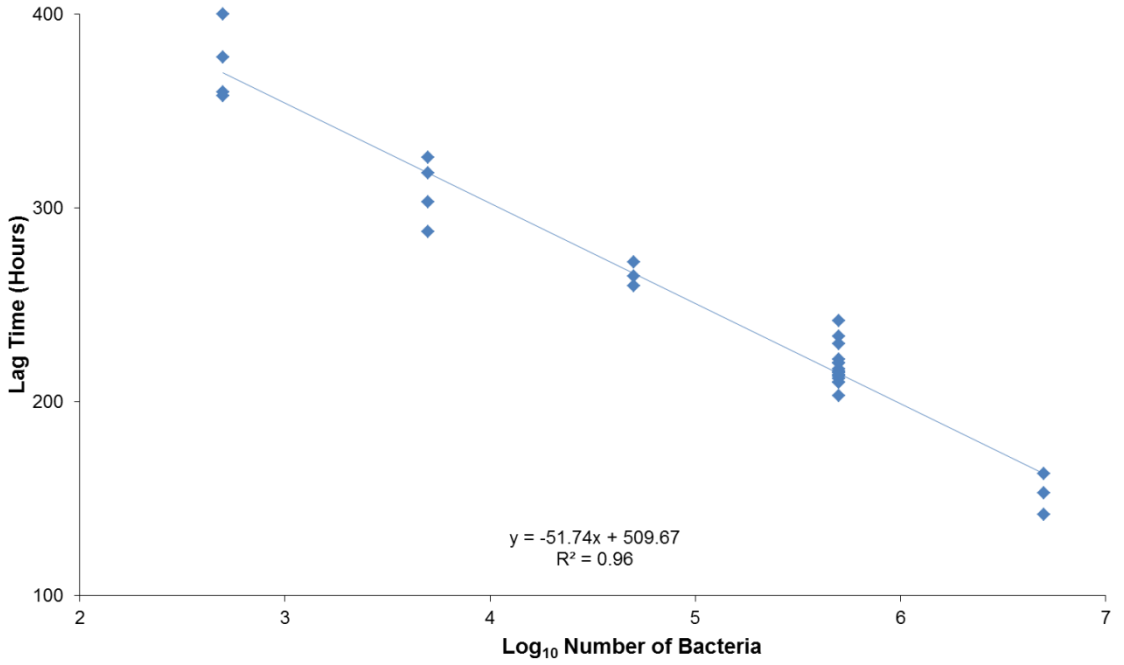


Figure 4-9 Relationship between TTP and log_{10} number of H37Rv bacteria

Bacteria were counted using a Helber chamber, n=4, except for 5×10^5 bacteria, where n=15

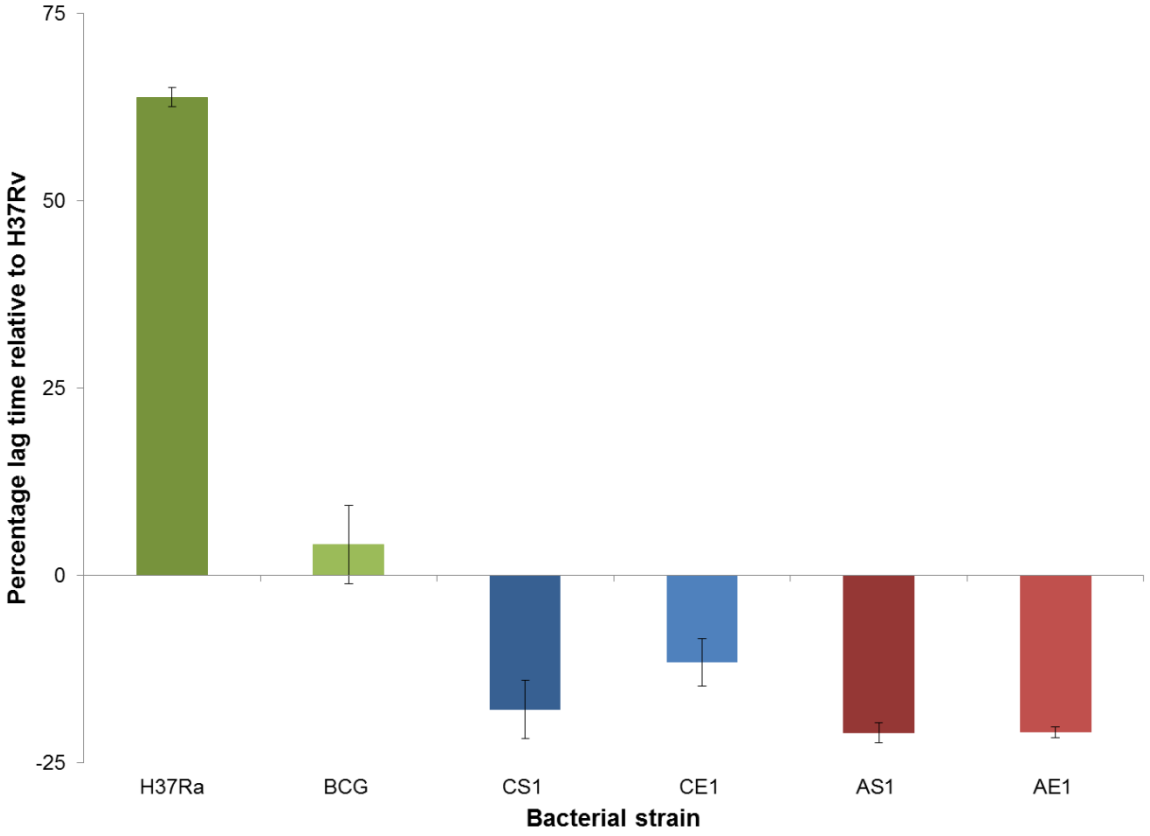


Figure 4-10 Mean TTP relative to H37Rv for 5 × 10^5 mycobacteria from six different strains

Bars above 0 indicate a shorter TTP than H37Rv, bars below 0 indicate a longer TTP than H37Rv. Error bars depict SEM, (n=15).

Table 4-8 shows that isolates from the same cluster of disease do not have the same TTP. Isolates AE7 and AE6 are from the same epidemiological cluster and have identical MIRU-VNTR profiles however the mean TTP for AE6 (225 ± 4 hours, n=3) is 25% longer than AE7 (178 ± 0.3 hours, n=3) (p= 0.0003). Figure 4-11 shows that strains belonging to the CAS clade do not have similar TTPs, although CAS strains from epidemiological clusters of disease have shorter TTPs than H37Rv and unique CAS isolates have longer TTPs than H37Rv. Figure 4-12 shows that ancient strains do not have similar TTPs. Three of the tested strains have a shorter TTP than H37Rv and four of the tested strains have a longer TTP than H37Rv.

Table 4-8 Mean TTP for *M. tuberculosis* from different clusters of disease and unique isolates

Isolate	VNTR	MIRU	Cluster of disease	Mean TTP (hours) (n=3)	Standard error of the mean
CS1	42235	2742516334	N/A	179	8.6
CS5	42235	2532516333	N/A	233	1.2
CS6	42235	2642615333	N/A	230	1.5
CE1	42235	2642517331	CAS cluster 1	193	6.4
CE2	42235	2642517331	CAS cluster 1	200	5.5
CE3	42235	2642517331	CAS cluster 1	208	1.7
AS1	614-6	2632622334	N/A	173	3.2
AE1	-4465	2422622313	Ancient cluster 1	173	1.6
AE2	61456	2432622334	Ancient cluster 2	240	1.0
AE4	61456	2432622334	Ancient cluster 2	233	0.7
AE5	61456	2432622334	Ancient cluster 2	230	1.9
AE6	-1456	2432622334	Ancient cluster 3	224	3.8
AE7	-1456	2432622334	Ancient cluster 3	178	0.3

N/A = not applicable, CS = CAS single isolate, CE = CAS epidemiological isolate, AS = Ancient single isolate, AE = Ancient epidemiological isolate

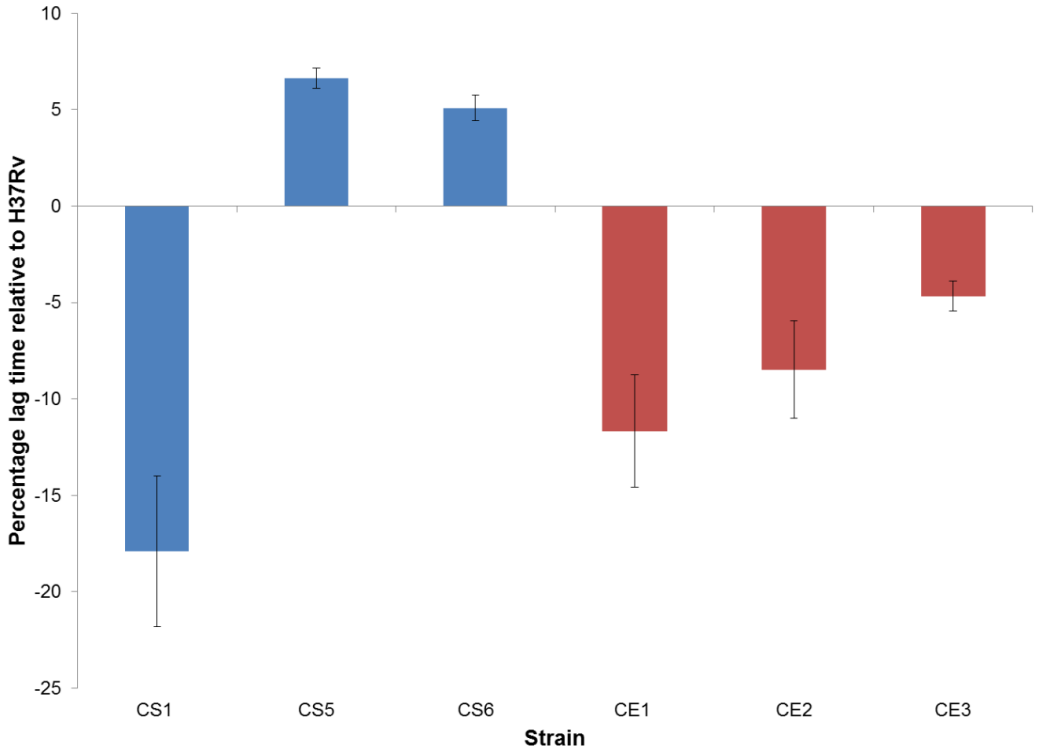


Figure 4-11 Mean TTP relative to H37Rv for 5 × 10^5 bacteria from six *M. tuberculosis* CAS strains

CS1, CS5 and CS6 are all unique isolates, only been identified in the Midlands once. CE1, CE2 and CE3 belong to the same disease cluster. Bars above 0 indicate a shorter TTP and bars below 0 indicate a longer TTP than H37Rv. Error bars depict SEM, (n=3)

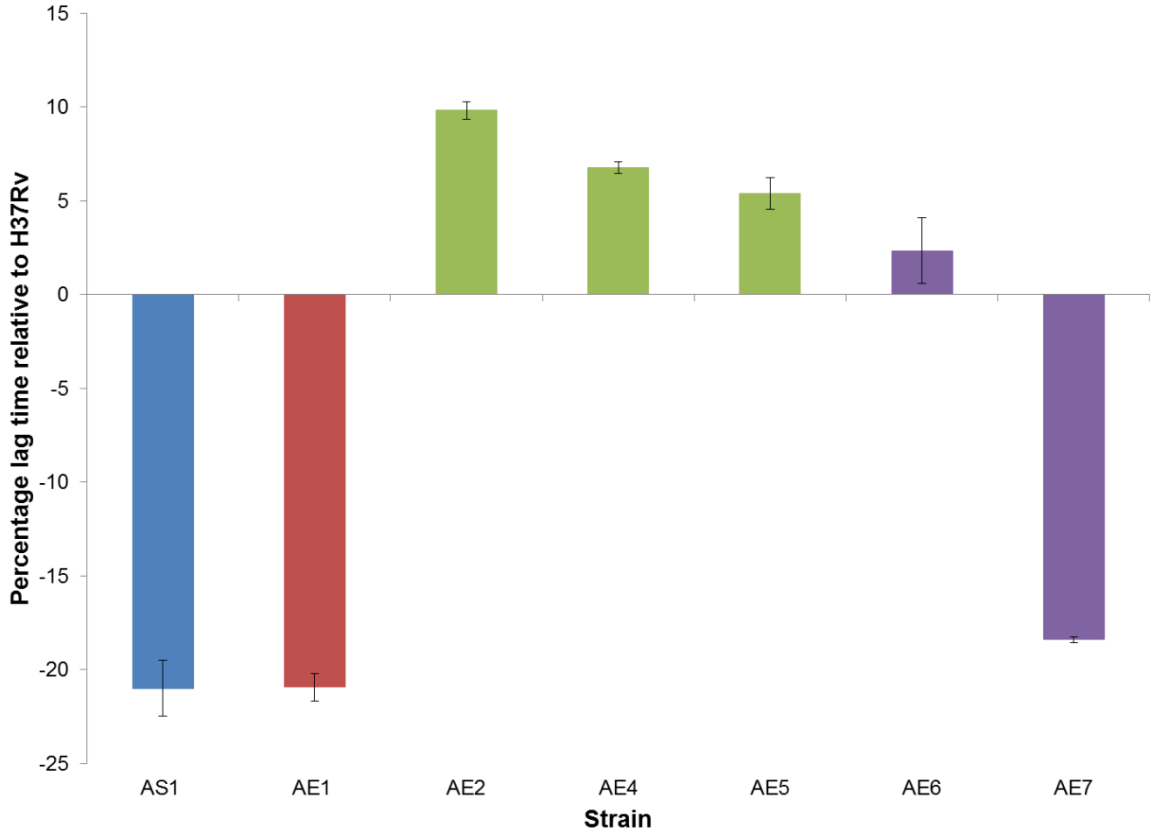


Figure 4-12 Mean TTP relative to H37Rv for 5 × 10^5 bacteria from six ancient *M. tuberculosis* strains

AS1 is a unique isolate that has been identified once in the Midlands.AE1 belongs to cluster of disease 1, AE2, AE4 and AE5 belong cluster of disease 2 and AE6 and AE7 belong to cluster of disease 3. Bars above 0 indicate a shorter TTP than H37Rv, bars below 0 indicate a longer TTP than H37Rv. Error bars depict SEM, (n=3).

4.2.3.5 Discussion

There is an inverse linear relationship between TTP and $\log_{10}$ CFU/ml, and between TTP and $\log_{10}$ of the number of bacteria counted using a Helber chamber for cultures of H37Rv. However, TTP was not constant for tested *M. tuberculosis* clinical isolates, which differed from H37Rv by between 12 and 21%. Isolates of *M. tuberculosis*, which had the same MIRU-VNTR, profile and were from the same epidemiological cluster of disease did not always have a similar TTP. The TTP of isolates with the same MIRU-VNTR profile are heterogeneous so TTP cannot be used as an indication of virulence or to identify the spoligotype clade that an isolate belongs to.

In order to enumerate mycobacteria from different strains by comparing TTP the growth rate for each strain would need to be the same. These experiments indicate that this is not the case. If the tissue culture model is to be used for high through put screening then it is not possible to construct a calibration curve for each strain to allow cell number to be determined from TTP.

In order to assess bacterial growth using the BACTEC™ MGIT™ 960 System the TTP is recorded, which is inversely proportional to the number of bacteria in the inoculum. A short TTP is therefore indicative of a high bacterial inoculum. By culturing bacteria for six days and calculating the percentage change in TTP over time for each strain the differences in growth rate between strains will be normalised. The percentage change in TTP from day one to day six shows whether bacteria are able to grow in culture over six days. A positive percentage change in TTP indicates that bacteria have increased in number and a negative percentage change in TTP indicates that bacteria have decreased in number.

4.2.4 The effect of PMA concentration on the differentiation of THP-1 cells

4.2.4.1 Background

PMA can be used to differentiate THP-1 cells, which causes them to display a macrophage like phenotype. PMA is toxic to cells and high concentrations lead to cell death. Conversely, low concentrations of PMA preclude full cell differentiation. To select an optimal amount of PMA, four concentrations were chosen which represent a range of those sited in the literature (see Table 4-6). Differentiated cells have an

adherent, non-proliferating phenotype, so cell counts can be used to determine whether full differentiation has occurred.

4.2.4.2 Hypothesis

Treatment of THP-1 cells with 8 nM PMA for 18 hours will cause the cells to differentiate and display an adherent macrophage like phenotype.

4.2.4.3 Aims

- To ascertain the optimum concentration of PMA to add to THP-1 cells in order to obtain differentiation into a macrophage-like phenotype.

4.2.4.4 Materials and methods

THP-1 cells were obtained from the Health Protection Agency (HPA) culture collection (catalogue number 88081201, lot number 071001) and cultured in RPMI 1640 with 2 mM glutamine and 10% FBS (Sigma, UK; lot number 109K3396 was used for all experiments) at 37°C in a humidified atmosphere with 5% CO_2. Cultures were maintained at between 3 and 8 x 10^4 cells/ml and passaged a maximum of 5 times. To differentiate cells into a macrophage-like phenotype THP-1 cells were seeded at 5×10^5 cells/ml and placed in 96 well tissue culture plates. Phorbol 12-myristate 13-acetate (PMA) (Sigma) was added to cells at concentrations of 8, 16, 24 and 32 nM. Cells were incubated at 37°C for 18 hours. Following incubation tissue culture plates were centrifuged at $400 \times g$ for 5 minutes, cells were washed with warm PBS and then culture medium was replaced with fresh supplemented RPMI warmed to 37°C, 24 and 72 hours after addition of PMA. To ascertain the effect of nutrient depletion on cell numbers following differentiation, culture medium was

replaced 5, 6, 7 and 8 days after PMA treatment, in addition to the usual replacement at 24 and 72 hours.

To count the number of viable cells, tissue culture plates were centrifuged at 400 × *g* prior to the removal of culture medium, adherent THP-1 cells were removed by addition of TrypLE™ Express (Gibco) for 10 minutes at 37°C. Cells were stained with trypan blue and an average of five counts was made using a haemocytometer at 20X magnification by light microscopy. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.4.5 Results

THP-1 cells treated with different concentrations of PMA appeared to have a similar morphology at all tested concentrations, as assessed by light microscopy, although the degree of confluence was reduced in samples treated with 8 and 32 nM PMA.

An 18 hour treatment of THP-1 cells with PMA resulted in a decrease in the mean number of cells from day 0 to day 1 with all concentrations that were tested (Figure 4-13). There was a dose dependant decline in mean THP-1 cell number. The lowest concentration of PMA, 8 nM, caused a 1.7 fold reduction in the mean number of cells from day 0 (522,500 ± 20,729) to day 1 (304,000 ± 15,748; $p = 1 \times 10^{-6}$). The highest concentration of PMA, 32 nM, caused a 2.7 fold decrease in the mean number of cells from day 0 (528,000 ± 14,303) to day 1 (195,000 ± 8,807; $p = .1 \times 10^{-11}$). Following the initial decline in mean cell number 1 day after PMA treatment, mean cell numbers either stabilised or increased over the subsequent 7 days.

Treatment of THP-1 cells with 8 nM PMA caused a 2.4 fold increase in the mean number of cells from day 1 (304,000 ± 15,748 cells; n=8) to day 8 (727,000 ± 48,455 cells; n=6; $p = 8 \times 10^{-7}$). Treatment of cells with 16 nM PMA results in a 1.4 fold increase in the mean cell number from day 1 (260,500 ± 13,059 cells; n=8) to day 8 (353,000 ± 62,554 cells; n=8; p=0.17). Treatment of cells with 24 nM of PMA results in a 1.1 fold decrease in the mean number of cells from day 1 (261,000 ± 13,851 cells; n=8) to day 8 (233,000 ± 10,684 cells; n=8; p= 0.13). Treatment of cells with 32 nM of PMA causes a 1.2 fold increase in the mean number of cells from day 1 (195,000± 8,807 cells; n=8) to day 8 (232,000 ± 12,939 cells; n=8; p= 0.03).

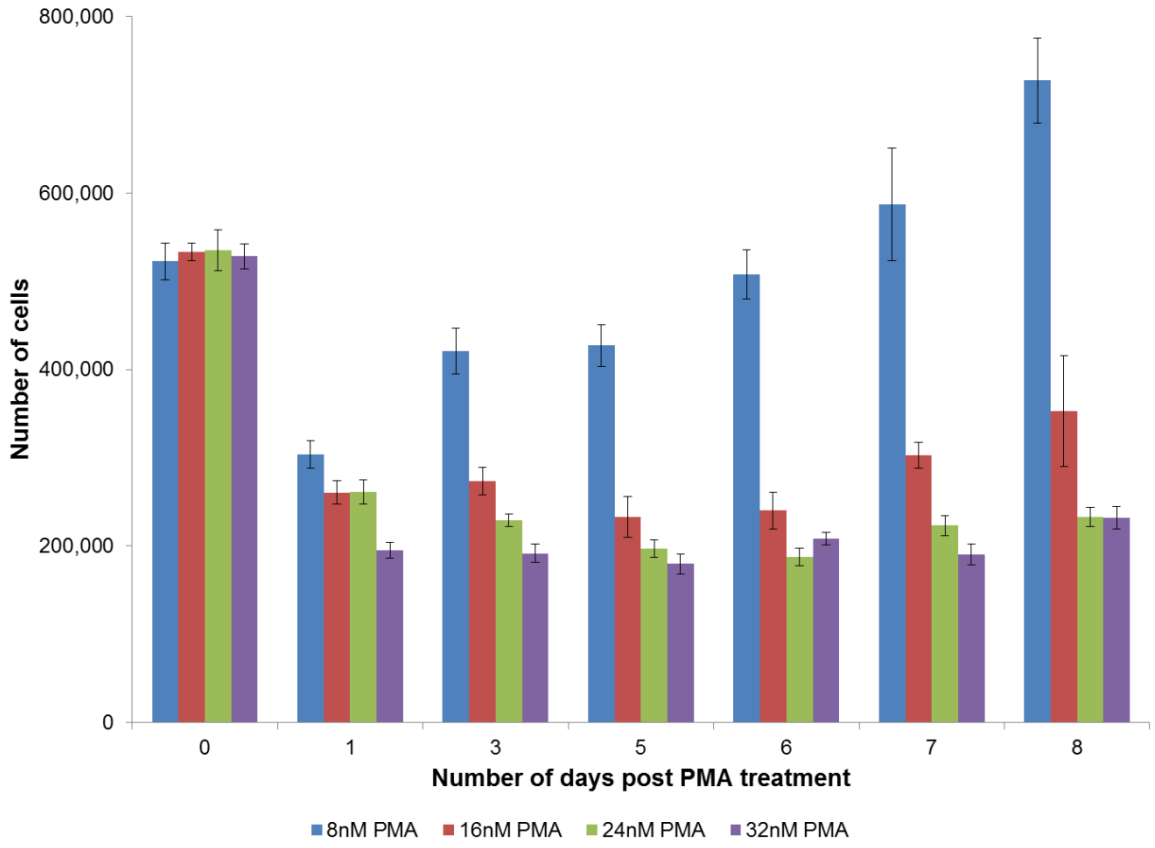


Figure 4-13 Mean number of live THP-1 cells in culture over eight days, after treatment with different concentrations of PMA

PMA was added on day 0. Error bars depict SEM, 8 replicate samples were counted on each day per concentration

The effect of nutrient depletion on cells differentiated for 3 days was tested. To prevent nutrient depletion culture medium was replaced every 24 hours, nutrient depleted cultures did not have culture medium replaced. The mean number of nutrient depleted cells 5 days after treatment with PMA (197,000 ± 9,732 cells, n=8) was 1.2 fold lower than the mean number of cells where the culture media was replaced every 24 hours (228,000 ± 18,838 cells, n=8; p= 0.17) (Figure 4-14). The mean number of nutrient depleted cells 8 days after PMA treatment (229,250 ± 9,180 cells, n=8) was 1.1 fold lower than the mean number of cells where the culture media was replaced every 24 hours (246,500 ± 16,352 cells, n=8; p= 0.4).

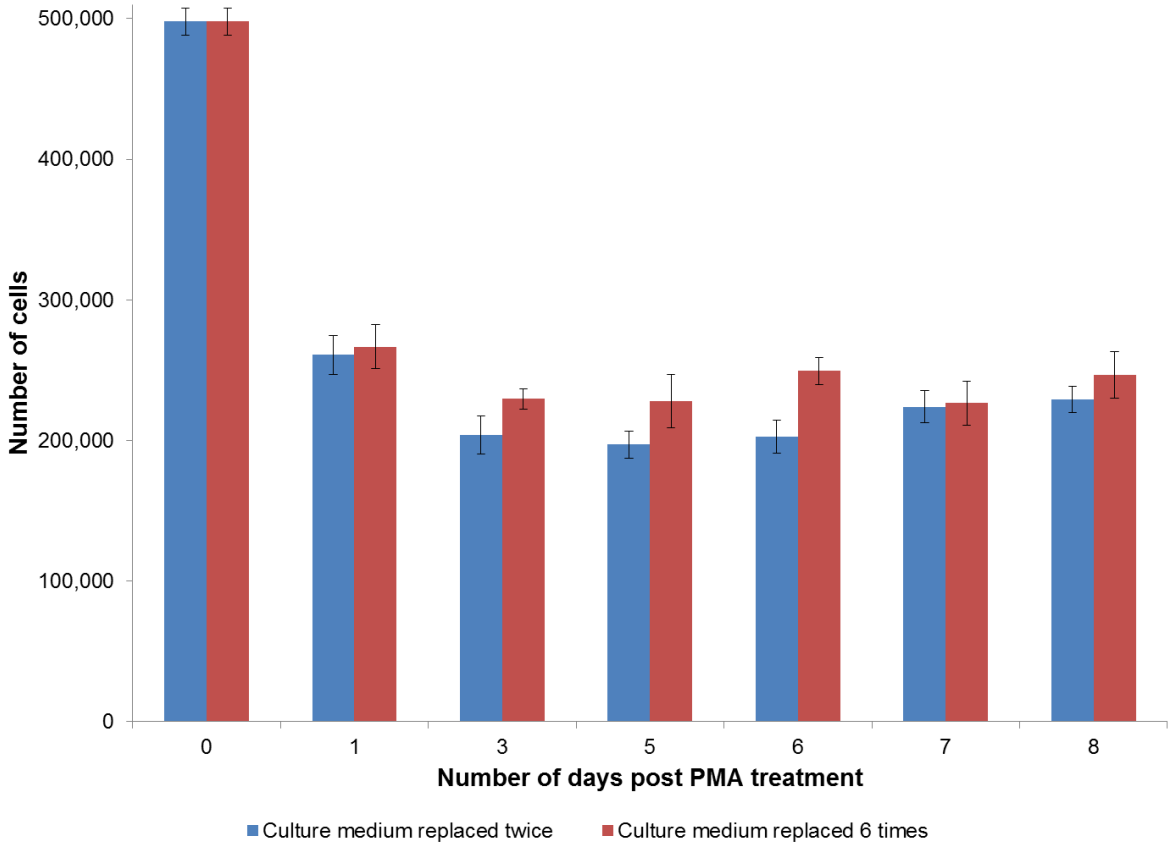


Figure 4-14 Effect of replacing culture media on mean number of live THP-1 cells in culture after 18 hour treatment with 24 nM PMA

Blue bars represent cultures where media was replaced on day 1 and day 3, red bars represent cultures where media was replaced on day 1, 3, 5, 6, 7 and 8. Error bars depict SEM, 8 replicate samples were counted on each day per concentration

4.2.4.6 Discussion

To ascertain an optimum PMA concentration for THP-1 cell differentiation, a range of concentrations was chosen. To overcome the loss of non-adherent cells when culture medium was replaced, tissue culture plates were centrifuged prior to the removal of the culture media. Tissue culture plates were also centrifuged prior to treatment of cells with trypsin, which removes adherent cells from the surface of the culture vessel to allow them to be counted. The use of 8 and 16 nM PMA resulted in an increase in THP-1 cell number over 8 days, indicating that these concentrations were not sufficient to differentiate the cells, as fully differentiated THP-1 cells are adherent and unable to undergo cell division. The use of 24 and 32 nM PMA resulted in a stable number of cells over 8 days. However, as treatment of cells with 24 nM PMA resulted in a smaller decrease in cell number from day 0 to day 1 than 32 nM PMA, 24 nM was chosen as the optimum concentration for THP-1 cell differentiation.

Replacing the culture medium every 24 hours after THP-1 cells had been differentiated did not appreciably alter the cell number compared to samples where the culture medium was not replaced. Therefore, once THP-1 cells had been differentiated the culture medium was not replaced again. Retaining the same culture medium throughout the experiment is advantageous because it reduces the amount of manipulation, which has to be carried out at containment level three, and prevents the loss of bacteria from the infection model.

4.2.5 Assessment of macrophage viability

4.2.5.1 Background

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released by cells that have lost their membrane potential, a result of necrotic cell death. Cells in culture release LDH into the culture supernatant as they die and so the background level of LDH activity in cell culture will be measured. In order to assay the amount of LDH that is released from infected cells, the cell supernatant must be sterilised to remove the risk of infection with *M. tuberculosis*. Sterilisation can be achieved by passing cell supernatant through a filter with 0.2 µm pores. Lysis of the cells prior to filtration will also be performed in order to measure total LDH activity, so that the percentage cytotoxicity caused by addition of mycobacteria can be calculated. The filtrate is then subjected to an enzyme-linked assay that converts a tetrazolium salt into a red formazan product, which can be detected by absorbance at 490 nm. The absorbance is proportional to the number of lysed cells (Singer *et al*., 1999; Miroslav, 1995).

4.2.5.2 Hypothesis

LDH can be used as a marker of necrotic cell death and total cell number.

4.2.5.3 Aims

- To test filtration of cell supernatant as a method of sterilisation
- To select a method of cell lysis
- To ascertain the number of THP-1 cells needed in order to detect LDH

4.2.5.4 Materials and methods

In order to determine whether filtration of cell supernatants would preclude the detection of LDH activity, THP-1 cells were serially diluted and then differentiated by

overnight treatment with 24 nM PMA. Tissue culture plates were centrifuged at 400 × *g* for 5 minutes then washed with warm PBS and the culture medium was replaced with fresh supplemented RPMI warmed to 37°C at 24, 96 and 120 hours after the addition of PMA. Tissue culture plates were centrifuged at 400 × *g* for 5 minutes and then the supernatant was removed and in some cases it was filtered through a 0.2 µm centrifugal filtration device (Millipore, Hertfordshire), supernatant was then assessed for LDH activity. LDH activity was assessed using a CytoTox 96® non-radioactive cytotoxicity assay according to the manufacturer's instructions (Promega) and absorbance was measured using a Triturus Automated ELISA Workstation (Biomedical Diagnostics). All data were normalised by subtraction of background absorbance.

Cell supernatants from THP-1 cells infected with H37Rv at a MOI of 1:1 were passed through a 0.2 µm centrifugal filtration device (Millipore, Hertfordshire) and then inoculated into MGITs™ to confirm that sterilisation had been achieved. Unfiltered cell supernatants from THP-1 cells infected with H37Rv at a MOI of 1:1 were also used to inoculate MGITs™ to confirm that the mycobacteria were viable. Finally, H37Rv from a positive MGIT™ culture was filtered and 100 µl of H37Rv before and after filtration was inoculated into fresh MGITs™.

A method of lysing cells is required in order to measure total LDH activity. THP-1 cells were seeded at 1.3×10^5 cells/ml and differentiated with 24 nM PMA. Differentiated cells were lysed either by addition of Triton X-100 to a final concentration of 0.05% (v/v) for 30 minutes at 37°C or by freezing at -80°C for 30

minutes followed by thawing at room temperature for 15 minutes. Monolayers were viewed by microscopy and if total lysis was not achieved then cells were incubated for a further 15 minutes. Once cells had been lysed, the supernatant was centrifuged at 400 × *g* for 5 minutes to remove cell debris, and then filtered before performing the LDH assay. It would be advantageous to store samples before the LDH assay is carried out so in some cases, samples were split in half. The first half of the sample was assayed for LDH activity immediately, the second half was frozen overnight and LDH activity was measured the following day.

To ensure that an appropriate number of cells are used for the THP-1 infection model THP-1 cells were serially diluted, differentiated with 24 nM PMA and rested for 3 days with culture media replacement at 1 and 3 days after PMA treatment. For each dilution two samples were prepared, either the supernatant was removed after centrifugation of the tissue culture plate at 400 × *g* for 5 minutes, or the cells were lysed by the addition of Triton X-100 to a final concentration of 0.05% (v/v). Each sample was passed through a 0.2 µm centrifugal filtration device (Millipore, Hertfordshire) and then assayed for LDH activity, and the experiment was performed in triplicate. Absorbance values for cell supernatant were divided by absorbance values for lysed cells so that the percentage cell death that occurs in untreated THP-1 cells could be ascertained.

4.2.5.5 Results

Filtration of THP-1 cell lysate through 0.2 µm pores caused a reduction in the average absorbance at 490 nm of less than 0.17 for all numbers of cells tested (Table 4-9).

Table 4-9 Mean absorbance at 490nm (± SEM) caused by LDH in THP-1 cell lysates before and after filtration (n=3)

Number of cells	Absorbance at 490nm		Difference (%)
	Filtered	Unfiltered	
18,750	0.4 ± 0.07	0.5 ± 0.06	0.01 (2.2)
37,500	0.7 ± 0.04	0.7 ± 0.04	0.01 (1.4)
75,000	1.1 ± 0.06	1.2 ± 0.07	0.10 (8.4)
150,000	1.8 ± 0.08	1.9 ± 0.03	0.09 (4.8)
300,000	2.7 ± 0.03	2.9 ± 0.03	0.17 (5.9)
600,000	3.1 ± 0.01	3.2 ± 0.05	0.14 (4.4)

Cell supernatants from THP-1 cells infected with H37Rv at a MOI of 1:1 were passed through 0.2 µm filters and then inoculated into MGITs™. After 60 days no growth was recorded from any of the filtered samples (n=25). The unfiltered samples achieved a positive growth index in an average of 233 hours (n=10, ± 5 hours). Filtered supernatant from actively growing H37Rv cultures also failed to grow after 60 days incubation, whilst samples of H37Rv which had not been filtered achieved a positive growth index in an average of 205 hours (n=10, ± 3 hours).

Figure 4-15 shows that lysis of 1.3×10^5 cells/ml by freeze thaw produced a 3.9 fold increase in the mean absorbance (1.032 ± 0.022, n=8) when compared to supernatant only (mean absorbance at 490 nm 0.266 ± 0.064, n=3; $p= 1 \times 10^{-7}$). Lysis with a final concentration of 0.05% Triton X-100 caused a 3.7 fold increase in the mean absorbance reading (0.987 ± 0.029, n=8) compared to supernatant only ($p= 7 \times 10^{-7}$). Freeze thaw and lysis with Triton X-100 (0.955 ± 0.005, n=3) produced a 3.6 fold increase in absorbance compared to supernatant only (p= 0.0004).

Freezing the samples at -20°C overnight before subjecting them to LDH assay resulted in a decrease in the mean absorbance for of all the conditions tested. The decrease in absorbance that occurred when samples were frozen ranged from a 2.5 fold decrease for samples that were freeze-thawed ($p= 3 \times 10^{-13}$) to a 4.2 fold decrease for samples that were lysed by freeze-thaw and addition of Triton X-100 ($p= 3 \times 10^{-8}$), compared to samples which were not frozen.

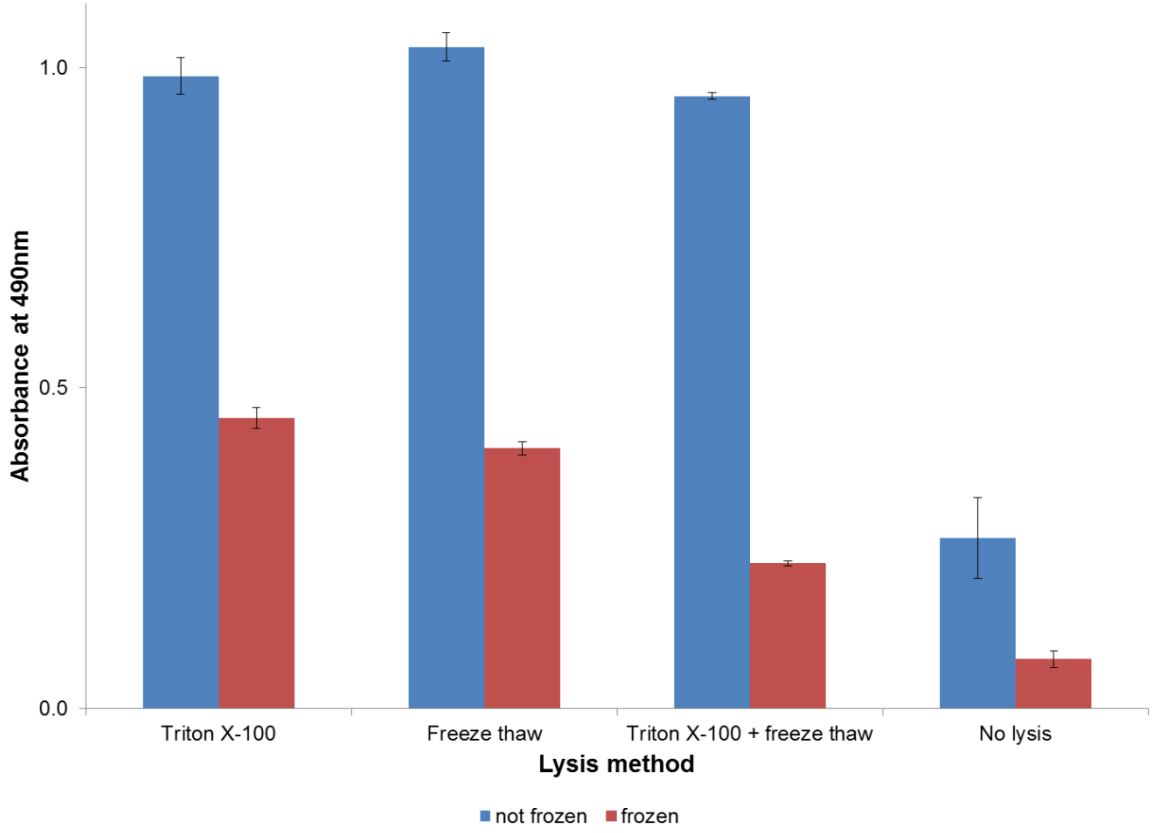


Figure 4-15 Mean absorbance at 490 nm due to LDH activity in cell lysates produced by different methods

Blue bars represent samples that were assayed for LDH activity immediately following lysis and red bars represent samples that were stored overnight -20°C before the LDH assay was performed. Error bars depict SEM, cells were seeded at 130,000 cells/ml

According to the CytoTox 96® non-radioactive cytotoxicity assay instructions the absorbance values that are measured from lysed cells should be at least two times

the level of absorbance that is recorded from media only in the absence of any cells. To establish the appropriate number of cells to use serial dilutions were prepared and the absorbance produced by LDH activity was recorded in media only, in supernatant from cells and in lysed cells.

Figure 4-16 shows that the level of absorbance caused by LDH in lysed cells was less than two times the absorbance caused by media only when 5×10^4 cells/ml or less were assayed. When 5×10^5 cells/ml were assayed the absorbance caused by LDH from lysed cells was 4.4 fold higher than the absorbance for media only ($p= 7 X 10^{-8}$), and the absorbance from supernatant was 1.7 fold higher ($p= 3 X 10^{-5}$). At 5×10^6 cells/ml the absorbance caused by LDH from lysed cells was 4.6 fold higher than the absorbance for media only ($p= 1 X 10^{-9}$), however the absorbance from the supernatant was 4.3 fold higher (Figure 4-16).

4.2.5.6 Discussion

Filtration of infected THP-1 cell supernatant was effective in sterilising the samples and no mycobacterial growth was detected in liquid culture after 60 days of incubation. Filtration of samples did not significantly reduce the level of LDH activity from THP-1 cell lysates, indicating that it is a suitable method of sterilising samples to enable them to be removed from the containment level three facility and assayed for LDH activity. All samples that are assessed for LDH activity will be filtered through 0.2 µm pores.

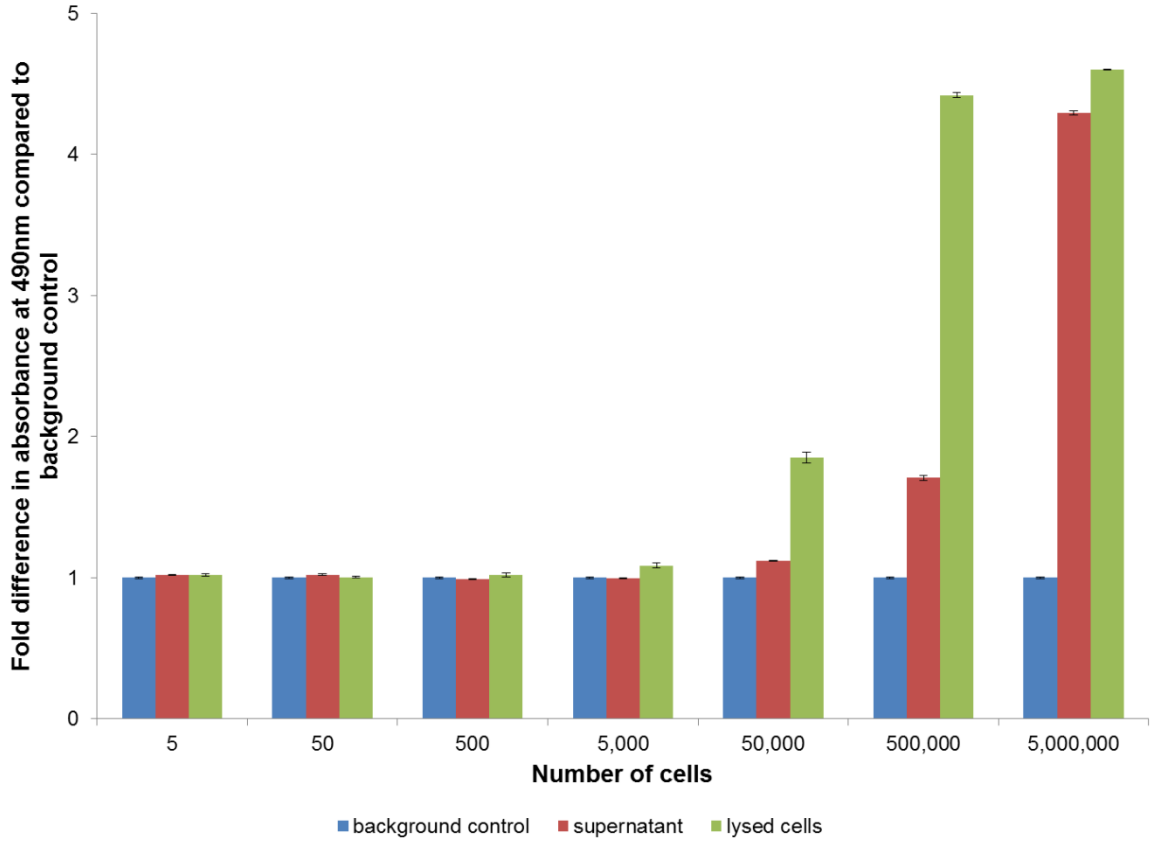


Figure 4-16 Fold difference in absorbance at 490 nm due to LDH activity in cultures of THP-1 cells seeded at different cell densities

Background control samples (blue bars) contained only cell culture media, red bars represent the level of spontaneous LDH release by cells and green bars represent maximum LDH activity of cells after lysis with Triton X-100. THP-1 cells were differentiated with PMA for 18 hours, then rested for 3 days prior to experimentation Error bars depict SEM, (n=3).

Freeze-thaw was the most effective method for lysing THP-1 cells; however it was only 5% more effective than lysis by Triton X-100 and it is more time consuming, so lysis will be carried out using Triton X-100. Freezing samples before they were assayed for LDH activity resulted in a large drop in absorbance and so samples should be assayed as soon as possible after they have been sterilised.

A cell density of 5×10^5 was chosen because the absorbance values due to LDH activity in lysed cells were more than two times the level of absorbance that was

recorded from media only in the absence of any cells. When the percentage cell death in THP-1 cultures seeded at 5×10^5 cells/ml was calculated, it showed that one day after the cells had been differentiated 22% (± 0.9%) of the cells were dead. When THP-1 cells were seeded at 5×10^6 cells/ ml the absorbance values due to LDH in lysed cells were also more than two times higher than the level of absorbance that was recorded from media only, but at a density of 5×10^6 cells/ml 92% (± 0.9%) of the cells were dead. It is likely that at high cell densities, space and nutrients become limited which leads to cell death, producing high levels of LDH in the supernatant.

4.2.6 Selection of an appropriate multiplicity of infection (MOI)

4.2.6.1 Background

Before the growth rate of BCG and H37Rv in THP-1 cells can be compared, an MOI needs to be chosen. The MOI used in THP-1 cell models reported in the literature ranges from one bacterium to ten cells (Castro-Garza *et al*., 2007) to fifty bacteria per cell (Theus *et al*., 2007b). When a high MOI has been used, investigators have incubated cells with bacteria, allowed a period for phagocytosis to occur and then cells have been washed to remove bacteria that have not been phagocytosed. It is difficult to distinguish between *M. tuberculosis* that has been phagocytosed and *M. tuberculosis* that is just bound to the surface of the cell. Initial experiments carried out in the current study with THP-1 monolayers that had been incubated with BCG, formaldehyde fixed and then acid fast stained produced obscured images because the stain bound to the formaldehyde matrix, making it difficult to see where the bacteria were in relation to the cells. If monolayers were not formaldehyde fixed then the acid fast staining destroyed the cells. This means that the length of time to allow

phagocytosis to occur could not be determined. Clinical strains of *M. tuberculosis* may have different rates of phagocytosis therefore a low MOI will be used and the cells will not be washed, so bacteria which have not been phagocytosed will remain in the model. The use of a low MOI is more likely to mimic the initial interaction of *M. tuberculosis* with alveolar macrophages in the human lung than a higher MOI.

Necrosis of THP-1 cells and growth rate of intracellular bacteria have been chosen as parameters for identification of virulent *M. tuberculosis* isolates. It is therefore important that the MOI that is chosen causes a difference in the measured parameters.

It will be necessary to lyse cells prior to inoculation of MGITs™ for assessment of bacterial number (as described in 4.2.3.3) so that the presence of cells or the presence of bacteria inside cells does not affect the rate at which oxygen in the MGIT™ is used up. The effect of the lysis reagent on bacterial growth will be ascertained.

4.2.6.2 Hypothesis

When THP-1 cells are infected with one H37Rv bacterium per cell, there will be a decrease in the TTP of the bacteria and an increase in THP-1 cell necrosis compared to bacteria and cells that have been incubated separately.

4.2.6.3 Aims

- To determine an appropriate MOI
- To assess the effect of Triton X-100 on mycobacterial growth rate

4.2.6.4 Materials and methods

To test which MOI to use, suspensions of single organisms of H37Rv and BCG were prepared as described in 4.2.2.4. Bacterial suspensions were diluted so that there was 5×10^5 bacteria/ml, 5×10^4 bacteria/ml or 5×10^3 bacteria/ml. THP-1 cells were prepared as described in 4.2.4.4 and seeded at 5×10^5 cells/ml. Once the THP-1 cells had differentiated into a macrophage-like phenotype, the culture medium was removed and replaced with 100 µl of bacterial suspension in RMPI with 5% FBS (v/v) (Sigma, UK; lot number 109K3396 was used for all experiments). Tissue culture plates were covered with AeraSeal gas-permeable sealing films (Alpha labs) and then incubated for up to six days at 37°C in a humidified atmosphere with 5% CO_2.

THP-1 cell necrosis was measured after 1, 3 and 6 days of infection. To measure necrosis caused by mycobacteria the supernatant was removed from infected cells and centrifuged at 400 × *g* for 5 minutes to eliminate whole cells and cell debris. To measure total LDH activity Triton X-100 was added to THP-1 cells which had not had the supernatant removed to a final concentration of 0.05% (v/v), which caused cell lysis. Cell lysate was centrifuged at 400 × *g* for 5 minutes to remove cell debris. Following centrifugation of cell lysate and cell supernatant; 90 µl was removed and passed through a 0.2 µm centrifugal filtration device (Millipore, Hertfordshire). Filtered cell lysate and filtered cell supernatant were assayed for LDH activity using a CytoTox 96® non-radioactive cytotoxicity assay according to the manufacturer's instructions (Promega). Percentage cytotoxicity caused by mycobacteria was calculated by dividing the absorbance caused by LDH activity in cell supernatant by

LDH activity in lysed cells, multiplied by 100 and the experiment was performed in triplicate.

To measure intracellular bacterial growth, infected cells were lysed after 1, 3 and 6 days by addition of Triton X-100 to a final concentration of 0.05% and the resulting lysate was inoculated into a MGIT™, giving a final concentration of 0.00007% Triton X-100 (v/v). The experiment was performed in triplicate.

To test the effect of Triton X-100 on bacterial growth a positive MGIT™ of H37Rv and BCG was selected and 100 µl aliquots were treated with 0.05%, 0.5% or 1% Triton X-100 (v/v) or left untreated for 30 minutes at 37°C (bacteria were not counted). Following treatment with Triton X-100, bacteria were inoculated into fresh MGITs™; the final concentration of Triton X-100 in the MGITs™ was 0%, 0.00007% or 0.007%. The TTP for each MGIT™ was recorded and the experiment was performed in triplicate. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.6.5 Results

Cellular necrosis caused by BCG and H37Rv was assessed by measuring LDH activity from 5×10^5 THP-1 cells, which had been infected with 5×10^3, 5×10^4 or 5×10^5 mycobacteria, to give MOIs of 1:1, 1:10 or 1:100 bacteria to cells respectively. LDH activity was also measured in cells that had not been infected so that the level of spontaneous necrosis in the model could be determined. Figure 4-17 shows that the amount of necrosis in THP-1 cultures increases over 6 days in both infected and uninfected cells. The level of necrosis in uninfected cells increased from

10% (± 2%) on day 1, to 27% (± 1%) on day 3 and by day 6 the level of necrosis was 49% (± 3%).

Infecting THP-1 cells with BCG at MOIs of 1:1, 1:10 or 1:100 did not significantly increase the amount of necrosis above that seen in cells that were not infected on any of the days tested (Figure 4-17A). After 6 days of infection the level of necrosis in THP-1 cells infected with BCG at an MOI of 1:1 was 1% higher than the level of necrosis in cells that were not infected (p= 0.9).

Figure 4-17B shows that the amount of necrosis caused by infecting THP-1 cells with H37Rv at an MOI of 1:1, one day after infection (21% ± 1.7%) was 7% more than the amount of spontaneous necrosis in cells that were not infected (p= 0.06). On day 6 the amount of necrosis caused by infecting THP-1 cells with H37Rv at an MOI of 1:1 (52% ± 0.6%) was 8% more than the amount of spontaneous necrosis in cells that were not infected (p= 0.01). When THP-1 cells were infected with H37Rv at MOIs of 1:10 or 1:100, the amount of necrosis was similar to or less than the amount of spontaneous necrosis.

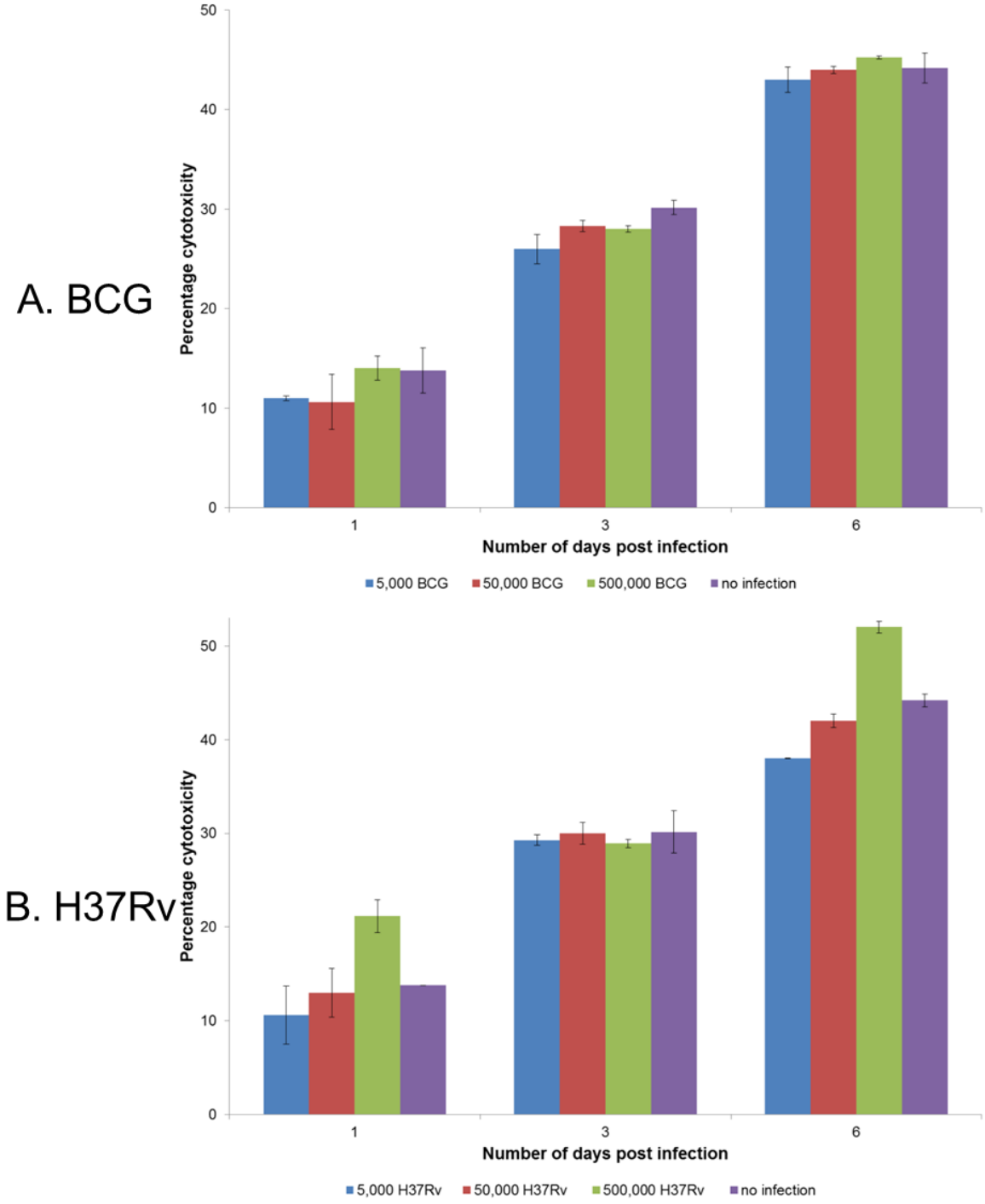


Figure 4-17 Mean percentage cytoxicity in THP-1 cells infected with different ratios of mycobacteria over six days

THP-1 cells were seeded at 5 x 10^5 cells/ml. Cytotoxicity was measured using an LDH release assay. Blue bars represent an MOI of 1 bacterium to 1 cell (1:1), red bars indicate an MOI of 1:10 and purple bars indicate an MOI of 1:100. Error bars depict SEM, n = 3.

Intracellular growth of BCG and H37Rv was assessed by lysing THP-1 cells, which had been infected at different MOIs and recording TTP from the lysate. The mean TTP for BCG from cells infected for one day at an MOI of 1:1 (175 ± 0.3 hours, n=3) was 1.2 fold lower than for BCG from cells infected at an MOI of 1:10 (217 ± 2.3 hours, n=3; p = 0.00005) (Figure 4-18). The mean TTP for BCG from cells infected for one day at an MOI of 1:100 (256 ± 2.6 hours, n=3) was 1.5 fold higher than for an MOI of 1:1 ($p = 7 \times 10^{-6}$) (Figure 4-18). Similar results were seen on subsequent days and for H37Rv (data not shown). The mean percentage change in TTP from day one to day six for BCG was 14% (± 1%) for an MOI of 1:1, 21% (± 1%) for and MOI of 1:10 and 29% (± 7%) for an MOI of 1:100.

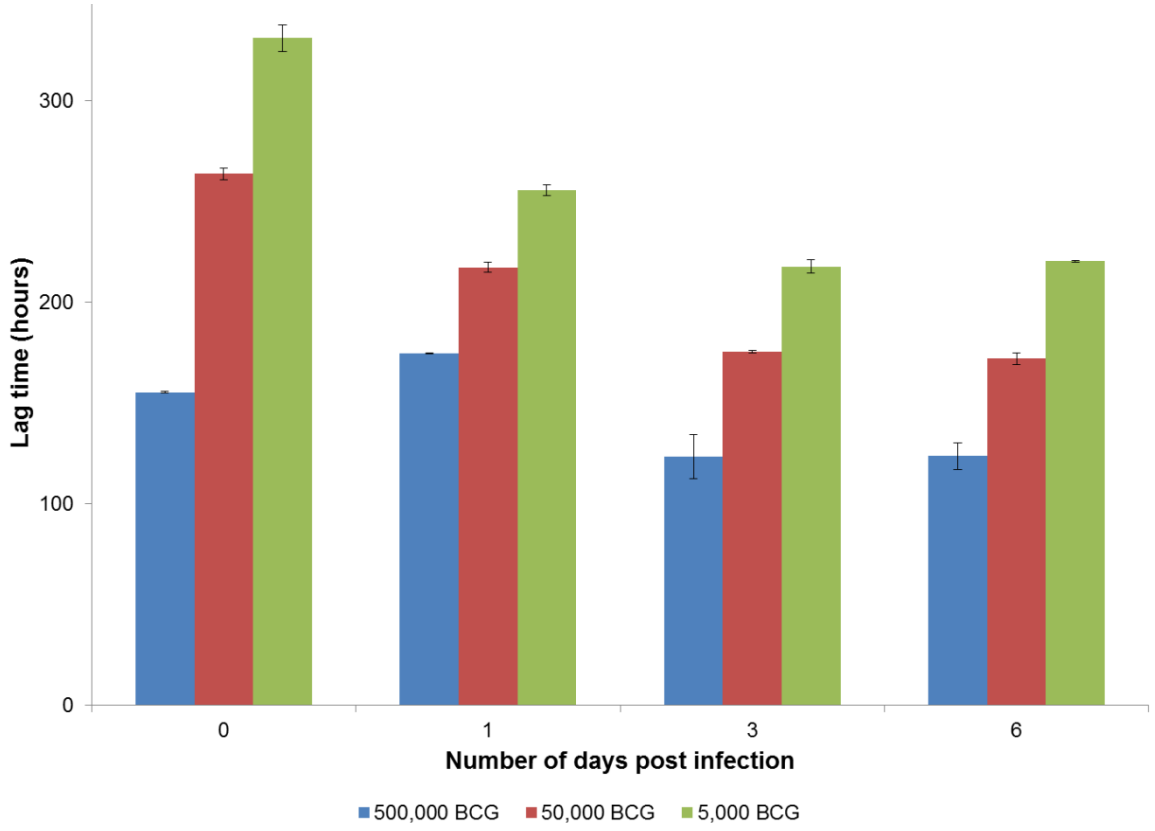


Figure 4-18 Mean TTP for MGITs™ inoculated with BCG after infection of THP-1 cells over 6 days at different MOIs

Cells were seeded at 5×10^5 and infected with BCG, blue bars represent an MOI of 1 bacterium to 1 cell (1:1), red bars indicate an MOI of 1:10 and green bars indicate an MOI of 1:100. Error bars represent SEM, n=3. A decrease in TTP corresponds with an increase in the number of bacteria.

Figure 4-19 shows that mean TTP for MGITs™ inoculated with bacteria in the absence of Triton X-100 was 113 hours for BCG (± 1 hour, n=3) and 117 hours for H37Rv (± 1 hour, n=3) (note that bacteria were not counted and so TTPs are not comparable with section 4.2.3.4). There was no difference in TTP when mycobacteria were treated with 0.05% Triton X-100 (v/v) compared to no treatment. The mean TTP for BCG treated with 0.5% Triton X-100 (141 ± 2 hours, n=3; p= 0.0003) and H37Rv treated with 0.5% Triton X-100 (142 ± 2 hours, n=3; p= 0.0005) was 80% higher than for untreated bacteria in both cases. MGITs™ that were inoculated with H37Rv or BCG that had been treated with 1% Triton X-100 (v/v) failed to reach a positive growth index.

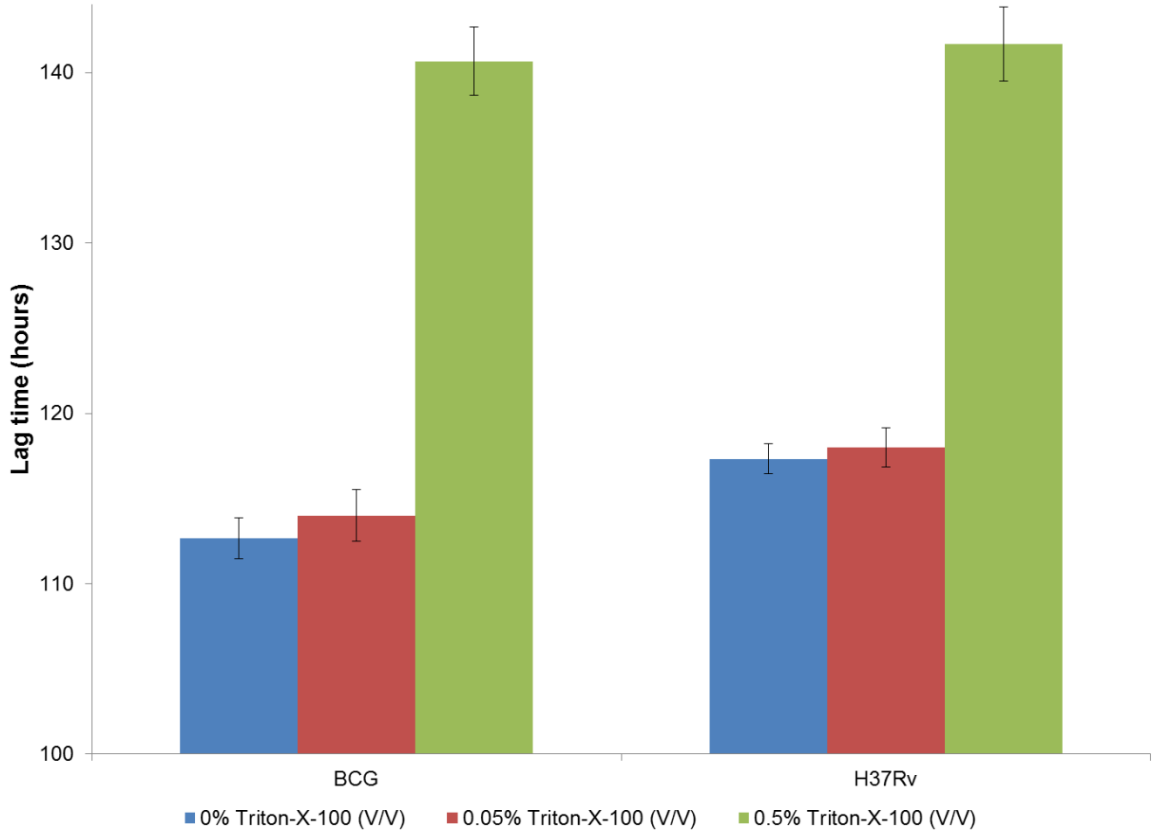


Figure 4-19 Mean TTP for H37Rv or BCG after treatment with different concentrations of Triton X-100

Error bars represent SEM, n=3.

4.2.6.6 Discussion

At an MOI of 1:10 or 1:100, the virulent *M. tuberculosis* strain H37Rv did not cause THP-1 cell necrosis. At an MOI of 1:1, the amount of THP-1 cell necrosis caused by H37Rv was greater than the amount of spontaneous necrosis that occurred in un-infected THP-1 cells. Therefore, an MOI of 1:1 was chosen for further experiments. Higher MOIs were not tested, as this would result in an excess of bacteria many of which would be extracellular. A wash step to remove extracellular bacteria could be introduced, but the quantity of bacteria that were removed would vary between samples and introduce error. An excess of bacteria is also likely to cause more cell death and so comparisons over several days could not be made.

4.2.7 Assessment of bacterial growth in tissue culture media

4.2.7.1 Background

In the current study bacteria that have not been phagocytosed by THP-1 cells will remain in the infection model. The growth rate of extracellular bacteria will be measured to ensure that it does not affect the percentage change in TTP calculated for intracellular bacteria.

Paul and colleagues reported that H37Rv and H37Ra were unable to grow in cell-free tissue culture medium (Paul *et al*., 1996). Zhang and colleagues tested H37Rv, H37Ra and four clinical isolates and reported that bacteria did not grow in RPMI medium with 10% serum, instead the number of CFU declined over 10 days (Zhang *et al*., 1999). Theus and colleagues reported that when 32 clinical isolates were tested extracellular growth did not occur (Theus *et al*., 2005). Armitige and colleagues found that heat-inactivated human AB serum does not support

M. tuberculosis H37Rv growth, nor does H37Rv grow in RPMI 1640 medium containing THP-1 cell lysate and 5% heat-inactivated AB serum (Armitige *et al*., 2000). Dobos and colleagues reported that *M. tuberculosis* Erdman, *M. tuberculosis* CDC1551 and BCG Pasteur were able to grow extracellularly (Dobos *et al*., 2000). Nozawa and Yokota reported *M. kansasii*, *M. bovis*, and *M. tuberculosis* were able to grow in F12 tissue culture medium and that growth was improved by the addition of 5% FBS and 0.05% Tween 80 (Nozawa and Yokota, 1983).

4.2.7.2 Hypothesis

BCG and H37Rv will not replicate in cell culture medium when there are no cells present.

4.2.7.3 Aims

- To determine whether different strains of mycobacteria are able to grow in cell-free tissue culture medium

4.2.7.4 Materials and methods

H37Rv and BCG in RPMI with 5% FBS were prepared at 5×10^5 bacteria/ml (as described in section 4.2.2) and then 100 µl was placed in tissue culture plates and incubated at 37°C. At days 0, 1, 2, 3 and 6 the bacteria were removed from the tissue culture plate and inoculated into MGITs™. The experiment was carried out in triplicate on five separate occasions. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.7.5 Results

Figure 4-20 shows that there was a 28% mean percentage change in TTP for H37Rv incubated in tissue culture media in the absence of THP-1 cells (extracellular) from day one (245 ± 15 hours) to day six (176 ± 10 hours) (p = 0.2). There was a 7% mean percentage change in TTP for extracellular BCG from day one (270 ± 13 hours, n=11) to day six (250 ± 9 hours, n=15) (p = 0.0006).

The mean TTP for extracellular H37Rv was 27 hours longer on day one (245 ± 15 hours, n=14) than on day zero (218 ±3 hours) (p = 0.07). Following the increase in mean TTP for extracellular H37Rv on day one, there was a decrease in mean TTP on day two and three. This indicated that the bacteria were able to grow in tissue culture media in the absence of cells. The mean TTP for extracellular H37Rv was shortest on day six (176 ± 10 hours, n=15) indicating that there were more bacteria on day six than at any other time point

The mean TTP for extracellular BCG was 37 hours longer on day one (270 ± 13 hours, n=11) than on day zero (233 ± 10 hours) (p = 0.04). The mean TTP for extracellular BCG increased on day two but by day three it had decreased. The mean TTP for extracellular BCG on day six (250 ± 9 hours, n=15) was longer than on day three but it was shorter than day one and two, indicating that bacteria had begun to grow by day 3 but could not continue growing for six days. H37Rv was able to replicate more rapidly than BCG in cell-free tissue culture medium at all time points. The mean TTP for BCG was 7% longer than H37Rv on day zero, increasing to 9%

on day two, 18% on day three and by day six the mean TTP for BCG was 30% longer than H37Rv in the absence of cells.

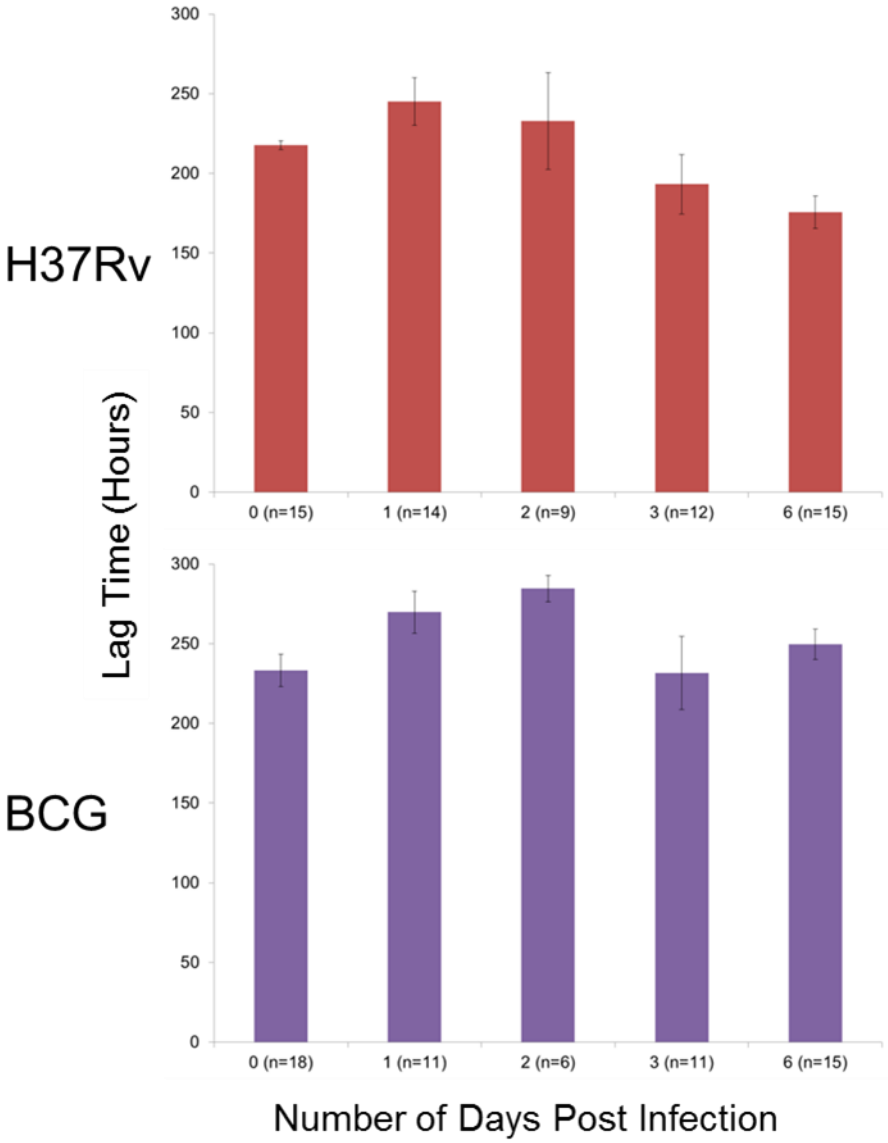


Figure 4-20 Mean TTP for MGITs™ inoculated with 5×10^5 H37Rv/ml or 5×10^5 BCG/ml after incubation in cell-free medium over six days

TTP for mycobacteria was measured by the BACTEC™ MGIT™ 960 System after incubation in tissue culture media for up to 6 days. A decrease in TTP corresponds to an increase in the number of bacteria. Error bars represent SEM.

4.2.7.6 Discussion

Results from the current study show that BCG was able to replicate in cell-free media for up to three days, and H37Rv was able to replicate in cell-free media for up to six

days. These findings are contradictory to the findings of Paul and colleagues (Paul *et al*., 1996), Zhang and colleagues (Zhang *et al*., 1999), Theus and colleagues (Theus *et al*., 2005) and Armitige and colleagues (Armitige *et al*., 2000)), all of whom used CFU counts to demonstrate that *M. tuberculosis* strains were not able to grow in cell-free tissue culture media.

Dobos and colleagues reported that *M. tuberculosis* Erdman, *M. tuberculosis* CDC1551 and BCG Pasteur were able to grow extracellularly. Dobos and colleagues used CFU counts to demonstrate the increase in the number of extracellular bacteria over time in a tissue culture model of infection. However the CFU counts recorded by Dobos were taken from cell culture medium that was removed from infected cells so the increase in the number of bacteria could be due to an increase in the release of bacteria from dead cells, rather than growth of bacteria in tissue culture media (Dobos *et al*., 2000). Nozawa and Yokota demonstrated an increase in the number of *M. kansasii*, *M. bovis*, and *M. tuberculosis* in F12 tissue culture medium over time, a finding which is in agreement with the results shown in the current study (Nozawa and Yokota, 1983). Nozawa and Yokota used turbidity measurements in liquid culture rather than CFU enumeration to show increases in mycobacterial numbers. The discrepancy in reports of extracellular growth in the current study compared to some published models of *M. tuberculosis* infection may be caused by the increased sensitivity of the BACTEC™ MGIT™ 960 System, compared to CFU counts, which were performed by Paul, Zhang, Theus and Armatige and are likely to underestimate the number of bacteria present in a sample.

Results from the current study show that there was a lag effect associated with removing bacteria from tissue culture medium and placing them into MGITs as shown by the increase in TTP from day zero to day one. This may indicate a reduction in the number of viable bacteria. It is also possible that the increase in TTP observed on day one, for H37Rv and BCG occurred because mycobacteria had adapted to the tissue culture medium and required time to readjust to the new conditions present in the MGIT or it could be an artefact introduced by cold shock, which could have occurred during handling of the samples. Venkataswamy and colleagues compared BCG that was grown in Middlebrook 7H9 broth to BCG that was grown in Sauton's broth and found that BCG grown in Sauton's broth was more persistent in macrophages. BCG that was grown in Sauton's broth could inhibit apoptosis and induce an inflammatory response more effectively than BCG grown in Middlebrook 7H9 broth (Venkataswamy *et al*., 2012). This indicates that the media that mycobacteria are grown in can affect their behaviour in macrophages and highlights the importance of standardising methods so that differences between the virulence of *M. tuberculosis* isolates are discernible.

As the BACTEC™ MGIT™ 960 System is an indirect measure of growth, changes in the rate at which the mycobacteria replicate will alter the TTP and could introduce error. However because all samples were treated in the same way and a percentage change in TTP over six days was calculated, any variation introduced by changes in growth rate should be normalised. Extracellular growth of *M. tuberculosis* in tissue culture media will need to be taken into account when THP-1 cells are infected.

4.2.8 Infection protocol

4.2.8.1 Background

A number of studies have shown that virulent strains of *M. tuberculosis* have more rapid intracellular growth rates than avirulent strains. It has also been demonstrated that avirulent strains induce macrophages apoptosis. Macrophage apoptosis results in the destruction of virus infected cells and the same mechanism may result in the removal of *M. tuberculosis* infected cells (Roulston *et al*., 1999). When virulent *M. tuberculosis* strains infect macrophages, apoptosis is not induced and instead the cells die by necrosis, releasing the intracellular bacteria which can then infect other cells. Virulent strains of *M. tuberculosis* are able to prevent phagosome-lysosome fusion within an infected macrophage whereas avirulent strains are killed within mature phagolysosomes. In order to test the THP-1 cell model and ensure that differences in virulence can be detected, H37Rv and BCG will be used as examples of virulent and avirulent mycobacteria. An LDH release assay will be used to determine cytotoxicity and the BACTEC™ MGIT™ 960 System will be used to detect changes in bacterial growth.

4.2.8.2 Hypothesis

H37Rv will replicate more rapidly than BCG in THP-1 cells, and cause more necrosis.

4.2.8.3 Aims

- To compare virulent H37Rv and avirulent BCG in the THP-1 cell model

4.2.8.4 Materials and methods

THP-1 cells were differentiated with 24 nM PMA and culture medium was replaced at 24 and 72 hours as described in 4.2.4.4. Suspensions of 5×10^5 bacteria/ml were

prepared in RPMI with 0.5% Tween 80 (v/v) as described in 4.2.3.3. Culture medium was removed from THP-1 cells 72 hours after addition of PMA and was replaced with bacterial suspensions, or fresh culture medium for non-infected controls. Day zero TTP for bacteria were measured by inoculating a fresh MGIT™ with 100 µl of the bacterial suspension which was used to infect the THP-1 cells, Triton X-100 was added to a final concentration of 0.00007%. Day one, two, three and six TTP were measured by treating each tissue culture well with 10 µl Triton X-100 for 15 minutes (as described in section 4.2.5.4) and then adding the lysate to a fresh MGIT™. A necrosis assay was performed by removing the culture supernatant, centrifuging at 400 × *g* for 5 minute to remove any intact cells, then removing 90% of the liquid which was sterilised by filtration through 0.2µm pores. Sterilised culture supernatant was then assessed for LDH activity using the Promega CytoTox 96® non-radioactive cytotoxicity assay. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.2.8.5 Results

The THP-1 model was infected with BCG and H37RV, to determine which strain has the highest intracellular growth rate and which strain causes the most necrosis. Values for TTP on day zero were recorded to ensure that the TTP of the bacteria that were used to infect the THP-1 cell model, were consistent with previous results. As shown in Figure 4-10 previous results found that the mean TTP for BCG is 4% longer than for H37Rv. The mean TTP for BCG used to infect the THP-1 model (day 0) (233 ± 10 hours, n=18) was 7% longer than for H37Rv (218 ± 3 hours, n=15; p = 0.2).

The mean TTP for H37Rv which had been cultured with THP-1 cells decreased gradually over the course of the infection, six 6 days after infection the mean TTP (123 ± 10 hours, n=15) had decreased nearly 2 fold from day zero ($p = 0.0004$). The mean TTP for BCG which had been cultured with THP-1 cells decreased on day one, two and three, but on day six the mean TTP (186 ± 11 hours, n=15) was the same as on day three (Figure 4-21). The mean TTP for intracellular H37Rv was 4% shorter than for intracellular BCG on day one and day two after infection. On day three and six after infection the mean TTP for H37Rv was shorter than for BCG by 11% and 34% respectively.

When the mean percentage change in TTP from day one to day six was calculated for H37Rv which had been cultured with THP-1 cells it showed that there was a 42% (± 2%) change in the TTP (day 1: 213 ± 11 hours, n=14; day 6: 123 ± 10, n=15; $p = 0.000002$). There was a 28% (± 3%) change in the mean TTP for H37Rv that was cultured in cell-free medium (day 1: 245 ± 15, n=14; day 6: 176 ± 10, n=15; $p = 0.0006$) (Figure 4-22).

When the mean percentage change in TTP from day one to day six was calculated for BCG which had been cultured with THP-1 cells it showed that there was a 17% (± 3%) change (day 1: 223 ± 13, n=14; day 6: 186 ± 18, n=12; $p = 0.03$). There was a 7% (± 2%) change in the mean TTP for BCG that was cultured in cell-free medium (day 1: 270 ± 13 hours, n=11; day 6: 250 ± 9 hours, n=15; $p = 0.2$) (Figure 4-22).

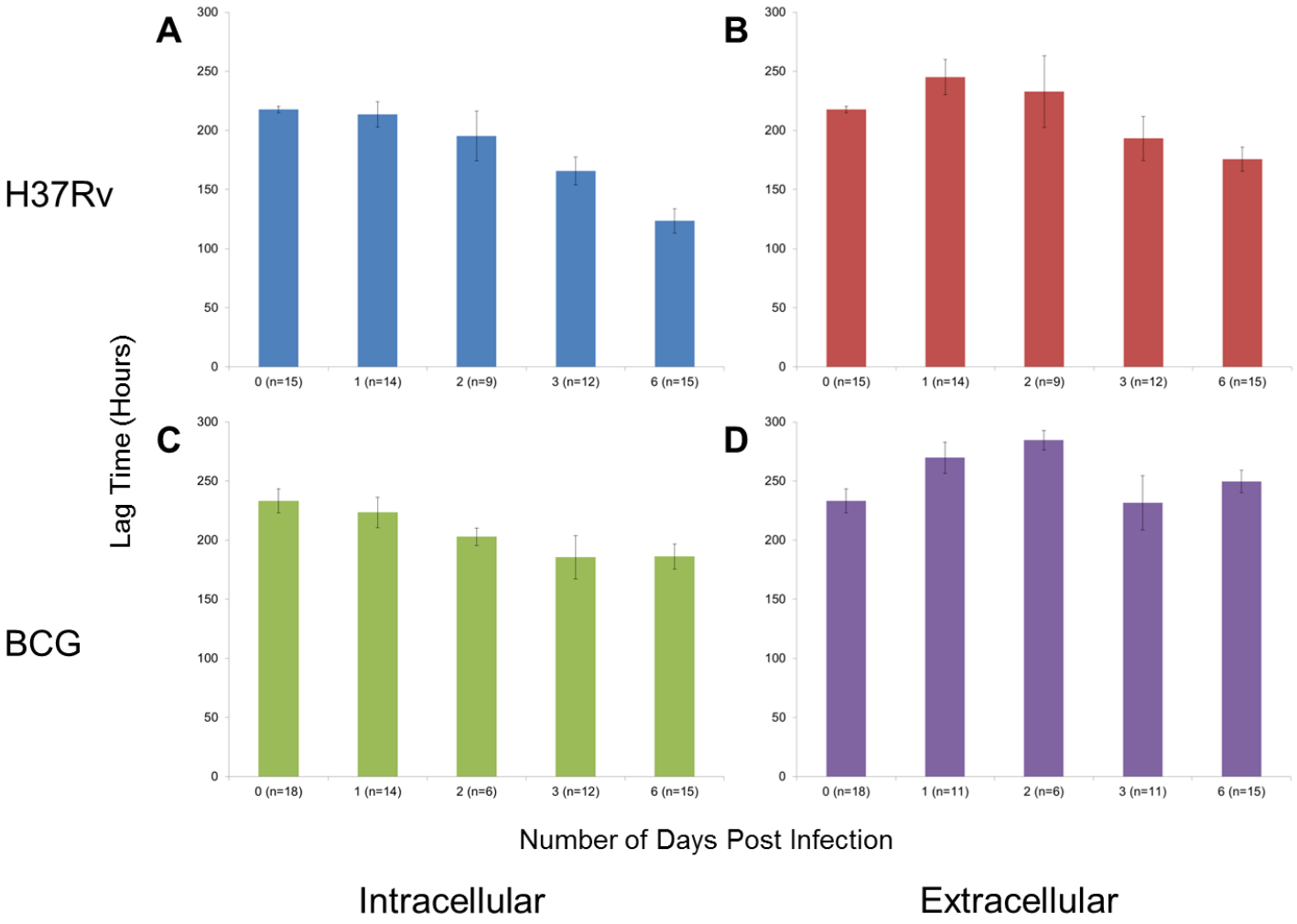


Figure 4-21 Mean TTP for bacteria cultured in cell-free medium or with THP-1 cells over six days

TTP for mycobacteria was measured by the BACTEC™ MGIT™ 960 System after incubation in tissue culture media (extracellular) or incubation with THP-1 cells (intracellular) over 6 days. A decrease in TTP corresponds to an increase in the number of bacteria. Cells were infected at an MOI of 1:1. Error bars represent SEM.

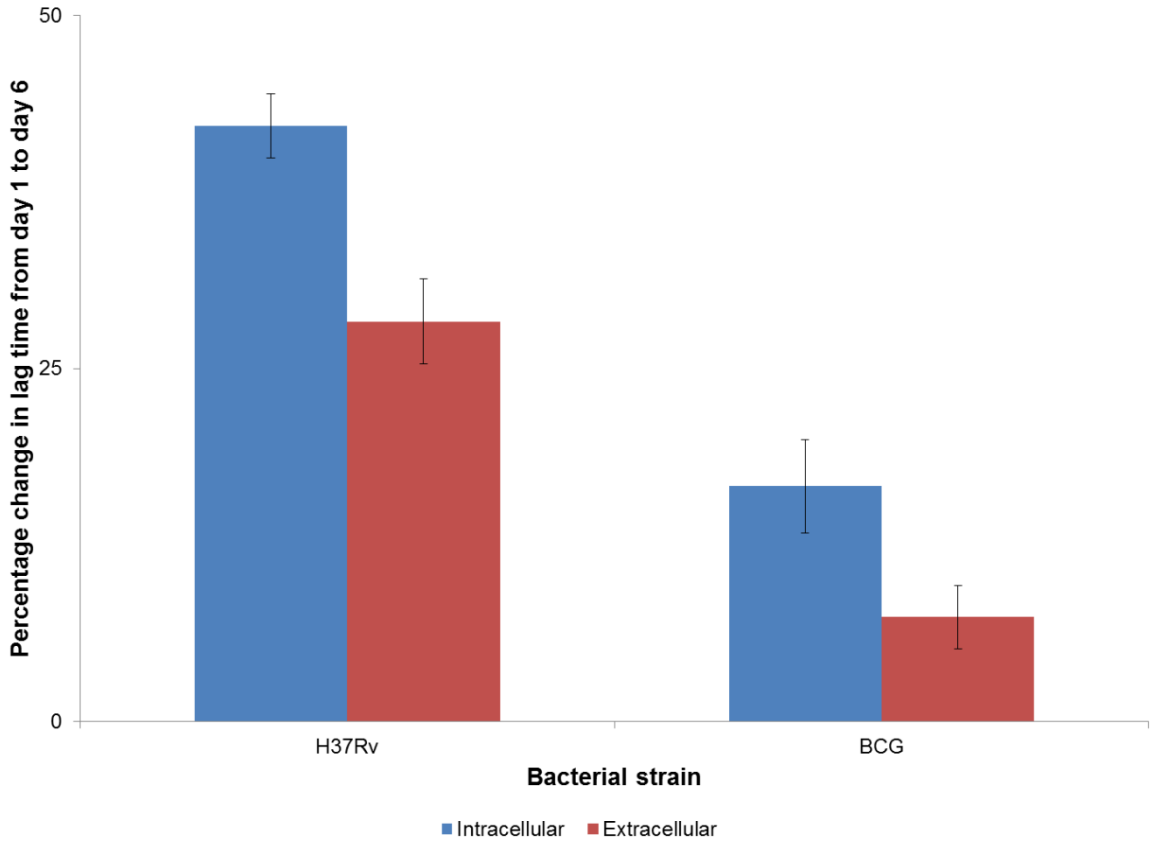


Figure 4-22 Mean percentage change in TTP over six days for H37Rv and BCG cultured in cell-free medium or with THP-1 cells

TTP for mycobacteria was measured by the BACTEC™ MGIT™ 960 System after incubation in tissue culture media (extracellular- red bars) or incubation with THP-1 cells (intracellular- blue bars). Cells were infected at an MOI of 1:1. Error bars represent SEM, n=15.

To account for bacterial growth in cell-free culture medium, the percentage change in TTP for extracellular samples was subtracted from the percentage change in TTP for intracellular samples (Figure 4-23). The normalised percentage change in TTP is 14% (± 2%) for H37Rv and 9% (± 2%) for BCG. THP-1 cells caused an increase in the growth of both H37Rv and BCG, however H37Rv had a bigger increase in growth than BCG ($p = 0.008$).

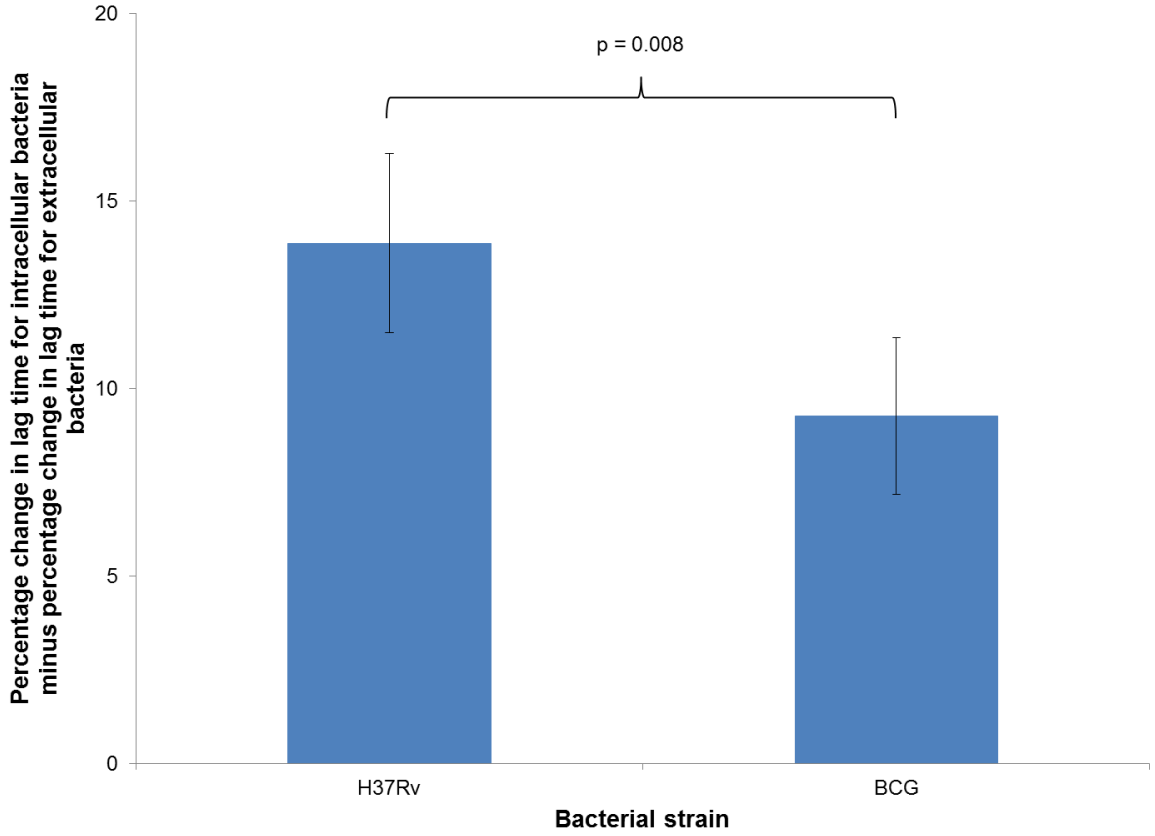


Figure 4-23 Mean percentage change in TTP over six days for H37Rv and BCG cultured with THP-1 cells normalised for extracellular bacterial growth

Error bars represent SEM, n=15.

The percentage cytotoxicity caused by infection of THP-1 cells with BCG one day after infection (22% ± 2%, n=8) was comparable to the amount of spontaneous cell death that occurred in uninfected cells (19% ± 2%, n=9; p = 0.4). The percentage cytotoxicity caused by infection of THP-1 cells with H37Rv for one day (29% ± 4%, n=7) was 1.5 fold higher than the amount of spontaneous cell death that occurred in uninfected cells (p = 0.03) (Figure 4-24).

The percentage cytotoxicity caused by infection of THP-1 cells with BCG six days after infection (47% ± 2%, n=5) was comparable to the amount of spontaneous cell death that occurred in uninfected cells (46% ± 2%, n=6; p = 0.7). The percentage

cytotoxicity caused by infection of THP-1 cells with H37Rv for six days (53% ± 2%, n=5) was 1.2 fold higher the amount of spontaneous cell death that occurred in uninfected cells (p = 0.03) (Figure 4-24).

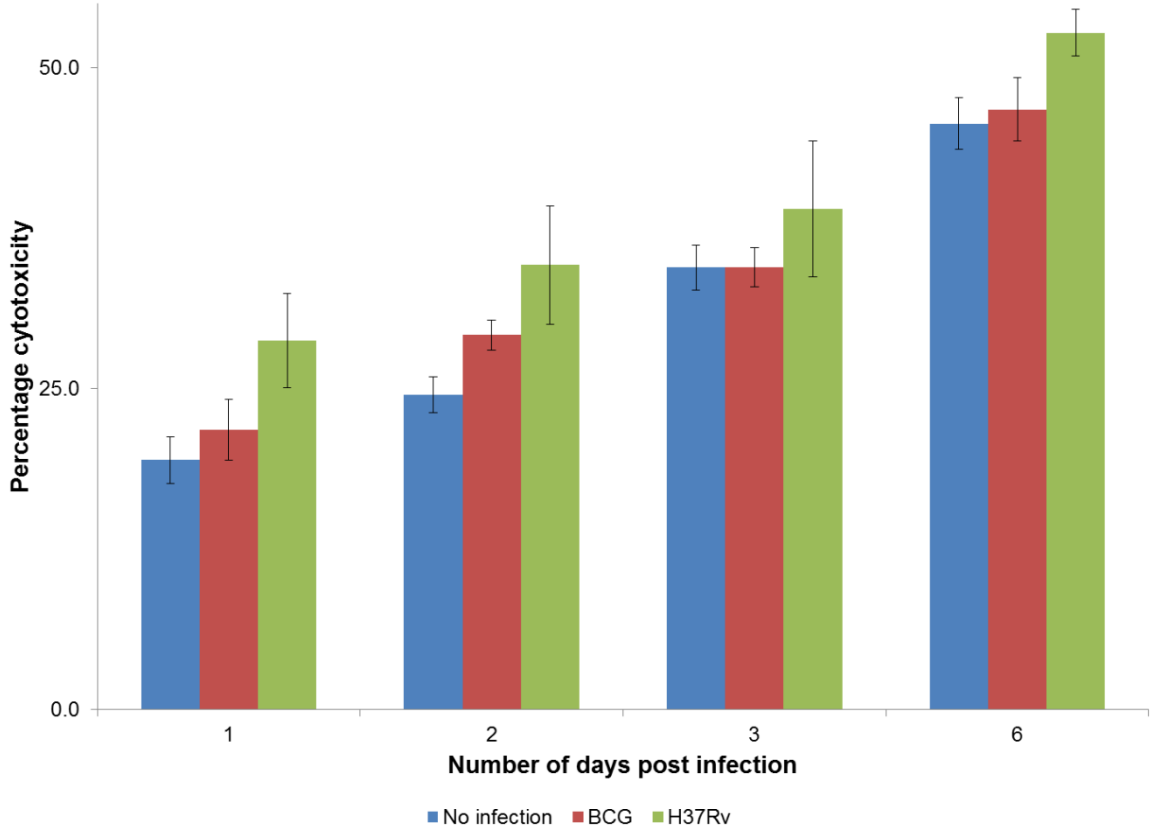


Figure 4-24 Mean percentage cytotoxicity in uninfected THP-1 cells and THP-1 cells infected with H37Rv or BCG at an MOI 1:1 over six days

Cytotoxicity was measured using an LDH release assay. Blue bars represent an
Error bars depict SEM, n = 5.

4.2.8.6 Discussion

H37Rv is a virulent strain and is able to replicate intracellularly. Rapid intracellular growth may cause the cells to burst, accounting for higher cell death when compared to BCG. BCG was able to replicate initially, but after three days, the number of bacteria began to decline. This indicates that the THP-1 cells were able to contain the infection and begin destroying the bacteria. Confirmation of this conclusion is given by the fact that necrotic cell death in BCG infected cells did not exceed

spontaneous cell death in non-infected control cells. Results from this section have validated to use of a THP-1 cell model for assessment of virulence and have shown that differences between a virulent mycobacterial strain and an avirulent mycobacterial strain can be detected.

4.2.9 Conclusions for designing a THP-1 cell model to compare the virulence of *M. tuberculosis* strains

Experiments carried out in section 4.2.1 indicate that the condition of the bacterial inoculum can affect the growth rate of subsequent cultures so stock suspensions of *M. tuberculosis* clinical isolates and laboratory reference strains were prepared for future experiments. The stock suspensions were stored at -20°C in single use aliquots. For all subsequent experiments stock suspensions of *M. tuberculosis* isolates were removed from storage and once thawed 100 µl was inoculated into a MGIT™. Once the MGIT™ had achieved a positive growth index, 100 µl was removed and used to inoculate a fresh MGIT™ to ensure that bacteria were in an exponential growth phase and that growth was not retarded by the effect of storage at -20°C. Suspensions of *M. tuberculosis* clinical isolates were prepared from the second positive MGIT™ in 1 ml of RPMI supplemented with 5% FBS (v/v) and 0.05% Tween 80 (v/v). The results obtained in section 4.2.2 show that the optimum treatment of mycobacterial suspensions to remove clumps was two 30-second pulses of bath sonication interspersed with two 60 minute periods of sedimentation, following sedimentation two thirds of the volume was removed to a fresh tube. This protocol was used to remove clumps from mycobacterial inocula in all subsequent experiments.

The optimum conditions for differentiation of THP-1 cells were determined in section 4.2.4. For all future experiments 5×10^5 THP-1 cells/ml were treated with 24 nM phorbol 12-myristate 13-acetate (PMA) for 18 hours. Cells were washed twice with PBS and then fresh RPMI supplemented with 5% FBS (v/v) was added 24 and 72 hours after addition of PMA.

Differentiated THP-1 cells were infected with mycobacteria at an MOI of one bacterium to one cell and samples were taken at zero, one and six days for assessment of bacterial growth which was carried out using the BACTEC™ MGIT™ 960 System. The percentage change in TTP over six days was used to determine whether the number of bacteria had increased or decreased, as results from section 4.2.3 indicate that different strains of *M. tuberculosis* have different TTP. Results from section 4.2.7 showed that some strains of bacteria were able to grow in cell-free culture medium and so controls for extracellular growth were included in all subsequent experiments.

For future experiments to assess the amount of cellular necrosis, THP-1 cell supernatants were removed and sterilised by filtration at one and six days after infection with mycobacteria. The amount of LDH present in filtered cell supernatants was assayed using the Promega CytoTox 96® non-radioactive cytotoxicity assay and results were shown as percentage cytotoxicity.

Virulent isolates were identified by their ability to cause THP-1 cell necrosis and their ability to replicate in THP-1 cells.

4.3 Infection of a THP-1 cell model system with *M. tuberculosis* strains from the CAS and EAI spoligotype clades

4.3.1 Background

The *M. tuberculosis* spoligotype clades CAS and EAI are both prevalent in the ISC. CAS is more widespread in the north of India and EAI in the south of India (Arora *et al*., 2009). CAS strains are also prevalent in the Midlands; nearly 40% of *M. tuberculosis* clinical strains isolated from patients in the Midlands between 2007 and 2008 belonged to the CAS spoligotype clade (Table 3-11). The prevalence of EAI in the Midlands is much lower, with only 15% of strains belonging to the EAI spoligotype clade. The prevalence of CAS strains in the Midlands compared to EAI may be due to specific importation of CAS (Arora *et al*., 2009). Alternatively, the prevalence of CAS in the Midlands could be because CAS is a more successful pathogen that is better adapted to spread amongst the population in the Midlands. To try to identify the reason for the greater prevalence of CAS strains in the Midlands compared to EAI strains, a THP-1 cell model was utilised. This model was used to assess the growth rate of different isolates from the CAS and EAI clades and the amount of cytotoxicity that they caused, to determine whether CAS strains have enhanced properties that have caused them to be more transmissible.

4.3.2 Hypothesis

M. tuberculosis isolates belonging to the CAS and EAI spoligotype clades will have different growth and cytotoxicity characteristics in THP-1 cells.

4.3.3 Aims

- To compare CAS strains to EAI strains in a THP-1 cell model to identify any differences in intracellular growth or cytotoxicity.
- To compare clinical isolates from epidemiological clusters to unique isolates that are not from epidemiological clusters to determine whether there is a difference in virulence.
- To compare isolates from the same cluster of disease to determine whether they behave in a similar way in a THP-1 cell model of infection.

4.3.4 Materials and methods

4.3.4.1 Bacterial strain selection

Clinical isolates, which were likely to belong to the CAS or EAI spoligotype clades, were selected from amongst all isolates typed by the MRCM between 2003 and 2008 (5,991). MIRU-VNTR profiles were used to infer the spoligotype of each isolate by applying a number of simple rules that were defined using information from the literature and data from section 3. Strains that were likely to belong to the CAS spoligotype clade were identified by the presence of -223- in the VNTR profile (where – represents any number), and were confirmed as modern strains by the possession of zero or one copy of MIRU 24. Strains that were likely to belong to the EAI spoligotype clade were identified by the presence of two or more copies of MIRU 24, which is indicative of ancient TbD1 positive strains. TbD1 positive isolates can belong to either the EAI clade or the AFRI clade. EAI and AFRI clades can be distinguished by species as EAI strains belong to the *M. tuberculosis* lineage and AFRI strains belong to the *M. africanum* lineage.

Two groups of CAS strains were selected. The first group was selected using epidemiological evidence that showed recent transmission had occurred, this group will be referred to as CAS epidemiological or CE. All isolates belonging to the same cluster had 100% matching MIRU-VNTR profiles. The second group, named CAS single or CS, comprised unique isolates that were only been identified once between 2003 and 2008. Unique isolates were not circulating strains and may represent reactivation disease. Culture positive isolates from smear positive sputum samples were chosen for further analysis. Two groups of EAI strains were selected using the same criteria and groups were referred to as ancient epidemiological or AE and ancient single or AS.

There were 34 CE isolates, which were split into 6 clusters, the largest cluster contained 14 isolates and the smallest cluster contained 2 isolates. There were 12 AE isolates split into 3 clusters, the largest contained 7 isolates and the smallest contained 2 isolates. There were 81 CS isolates and 7 unique AS isolates. A summary of the number of unique and clustered isolates from the CAS and ancient *M. tuberculosis* groups is shown in Figure 4-25. To develop the THP-1 cell model the most recent isolate from each of the four groups was selected and the spoligotype of these strains was determined experimentally (Table 4-10) as described in section 3.3.6. The following control organisms were used for comparison; *M. tuberculosis* H37Rv NCTC 7416 and *M. tuberculosis* H37Ra NCTC 7417 obtained from the National Collection of Type Cultures, Colindale, London, UK and *M. bovis* BCG Danish Vaccine strain 1331 obtained from the Statens Serum Institute, Copenhagen.

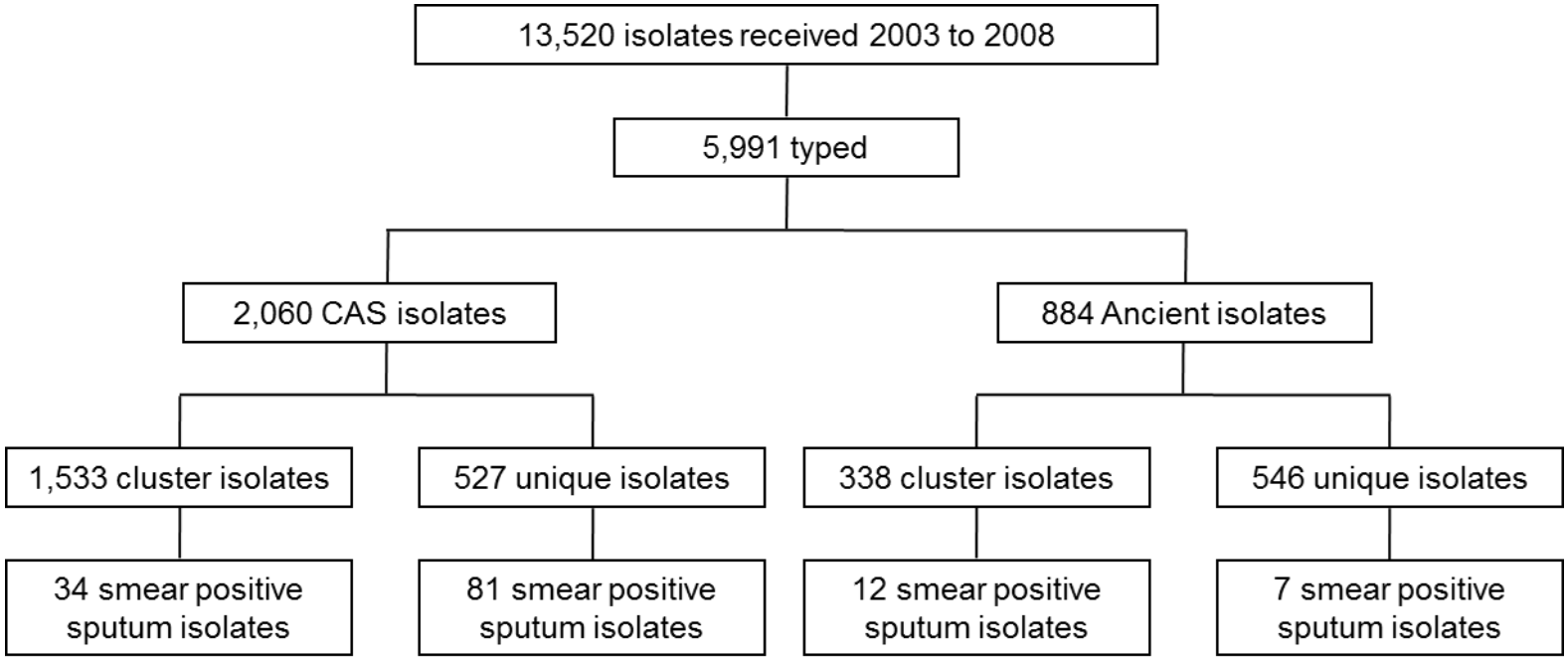


Figure 4-25 Flow chart showing numbers of CAS and ancient isolates selected for analysis in a THP-1 model of infection

4.3.4.2 Infection of the THP-1 model

Suspensions of exponentially growing *M. tuberculosis* clinical isolates and reference strains were prepared as described in section 4.2.1.4. Purity of all cultures was confirmed by microscopy using TB ZN stain kit (Becton Dickinson) according to the manufacturer's instructions. Mycobacteria were resuspended in RPMI, treated with Tween 80 and sonicated to remove clumps as described in 4.2.2.4. Suspensions of 5×10^5 bacteria/ml were prepared in RPMI with 0.5% Tween 80 (v/v) as described in 4.2.3.3.

THP-1 cells seeded at 5×10^5 cells/ml were differentiated with 24 nM PMA and culture medium was replaced at 24 and 72 hours as described in 4.2.4.4. Culture medium was removed from THP-1 cells 72 hours after addition of PMA and replaced with bacterial suspensions at an MOI of 1:1, or fresh culture medium. Samples were taken one, four and six days after infection for assessment of bacterial growth as described in section 4.2.8.4 and assessment of necrosis as described in 4.2.8.4.

4.3.4.3 Statistical analysis

Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.3.5 Results

Comparison of experimentally derived spoligotype patterns from the four selected clinical isolates (CE1, CS1, AE1 ad AS1) to SITVIT showed that both isolates that were part of epidemiological clusters (CE1 and AE1) belonged to a SIT (Table 4-10). The two unique isolates (CS1 and AS1) were not part of a SIT and these profiles

were not present in the SpolDB4 database confirming that they are orphan profiles. A newer version of the international spoligotype database (SITVITWEB) was published (Demay *et al*., 2012) and can be queried online (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Comparison of the experimentally derived spoligotypes of the four selected strains to SITVITWEB (accessed January 2013) resulted in the additional identification of AS1, which matched to an orphan profile from the EAI5 clade. Using MIRU-VNTR*plus* to identify strains that are related to CS1 and AS1 showed that CS1 was most similar to a CAS1_Dehli strain from SIT 1092 and AS1 was most similar to EAI strains from SIT 11 and SIT 138. This confirms that the rules that were used to select the isolates were accurate.

Table 4-10 Genotyping data for test strains

					Comparison with SpolDB4		Comparison with SITVITWEB	
Sample	**Year isolated**	**VNTR**	**MIRU**	**Spoligotype**	**Shared type**	**Clade**	**Shared type**	**Clade**
CE1	2008	42235	2642517331	703777400003771	485	CAS	485	CAS
CS1	2008	42235	2742516334	700777740003711				
AE1	2008	-4465	2422622313	077777777413731	6	EAI1_SOM	6	EAI1_SOM
AS1*	2008	614-6	2632622334	477777777413001			N/A	EAI5

N/A indicates that a shared type was not identified as the isolate matched to an orphan profile belonging to the EAI5 clade, which was isolated in the UK.

When growth of the four *M. tuberculosis* clinical isolates, and three reference strains was assessed the mean percentage change in TTP from day one to day six for bacteria cultured with THP-1 cells ranged from 17% for BCG to 55% for AS1 (Figure 4-26). All strains were able to replicate in cell-free media, BCG had the smallest mean percentage change in TTP (7% ± 2%) in cell-free media and AS1 had the largest mean percentage change in TTP (37% ± 2%) in cell-free media (Figure 4-26).

When the mean percentage change in TTP was normalised to take account of extracellular growth AE1 had the largest mean percentage change in TTP, an increase of 23% ± 4%, which was 9% more than H37Rv (14% ± 2) (p = 0.01) (Figure 4-27). The mean percentage change in TTP for AE1 was not significantly higher than for any of the other clinical isolates. All of the clinical isolates had a greater mean percentage change in TTP than H37Rv, although the difference was not significant (compared to H37Rv; CS1 p=0.7, CE1 p=0.1, AS1 p=0.1). H37Ra and BCG had the smallest percentage change in TTP and were both 5% less than H37Rv (compared to H37Rv; H37Ra p = 0.02 and BCG p=0.01). The normalised percentage changes in TTP for CS1 (14.6% ± 1%) and CE1 (15.3 ± 4%) were not significantly different (p = 0.2). The normalised percentage change in TTP for AS1 (17.7% ± 2%) and AE1 (23.3 ± 4%) were also not significantly different (p = 0.3).

The percentage cytotoxicity one day after infection of THP-1 cells with BCG (22% ± 2%, n=8) or H37Ra (20% ± 2%, n=3) was comparable to the amount of spontaneous cell death that occurred in uninfected cells (19% ± 2%, n=9; BCG p = 0.4, H37Ra p = 0.7, when compared to uninfected cells). The percentage cytotoxicity caused by infection of THP-1 cells with H37Rv for one day (29% ± 4%, n=7) was 1.5 fold higher than the amount of spontaneous cell death that occurred in uninfected cells (p = 0.03). The percentage cytotoxicity in THP-1 cells infected for one day with CE1, AE1 and AS1 was similar to the amount of necrosis caused by BCG, however the amount of necrosis caused by infection with CS1 was 1.4 fold higher than the amount of spontaneous cell death that occurred in uninfected cells (p = 0.07).

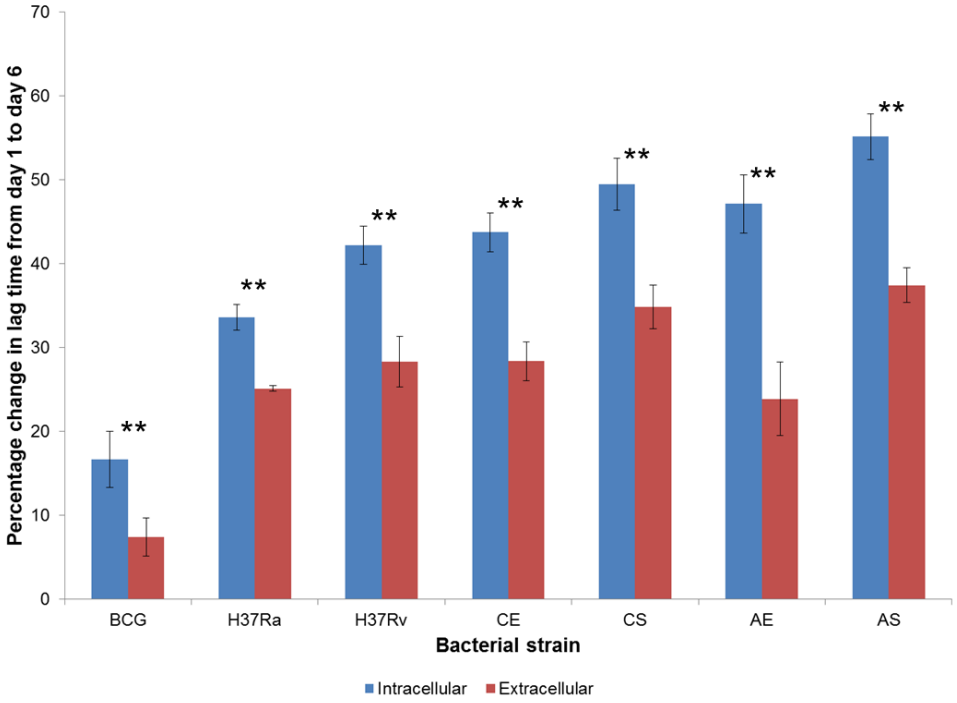


Figure 4-26 Mean percentage change in TTP for different strains of mycobacteria over six days cultured with (intracellular) and without (extracellular) THP-1 cells

** denotes p< 0.01 when comparing percentage change in TTP for intracellular and extracellular bacteria. Error bars represent SEM, n=15.

The percentage cytotoxicity six days after infection of THP-1 cells with BCG (47% ± 2%, n=5) or H37Ra (47% ± 2%, n=3) was not significantly different to the amount of spontaneous cell death that occurred in uninfected cells (46% ± 2%, n=6; BCG p = 0.7, H37Ra p = 0.9 when compared to uninfected cells) (Figure 4-28). The percentage cytotoxicity caused by infection of THP-1 cells with H37Rv for six days (53% ± 2%, n=5) was 1.2 fold higher the amount of spontaneous cell death that occurred in uninfected cells (p = 0.03). The percentage cytotoxicity in THP-1 cells infected for six days with CE1, CS1, AE1 and AS1 was similar to the amount of cytotoxicity caused by H37Rv.

These results indicate that there is no difference in the ability of CAS and ancient strains to cause cytotoxicity in THP-1 cells. The growth of CAS and ancient strains in

THP-1 cells was also similar, although the growth of the AE isolate was significantly greater than the growth of H37Rv. The growth of all of the clinical isolates was greater than the growth of the avirulent strains H37Ra and BCG.

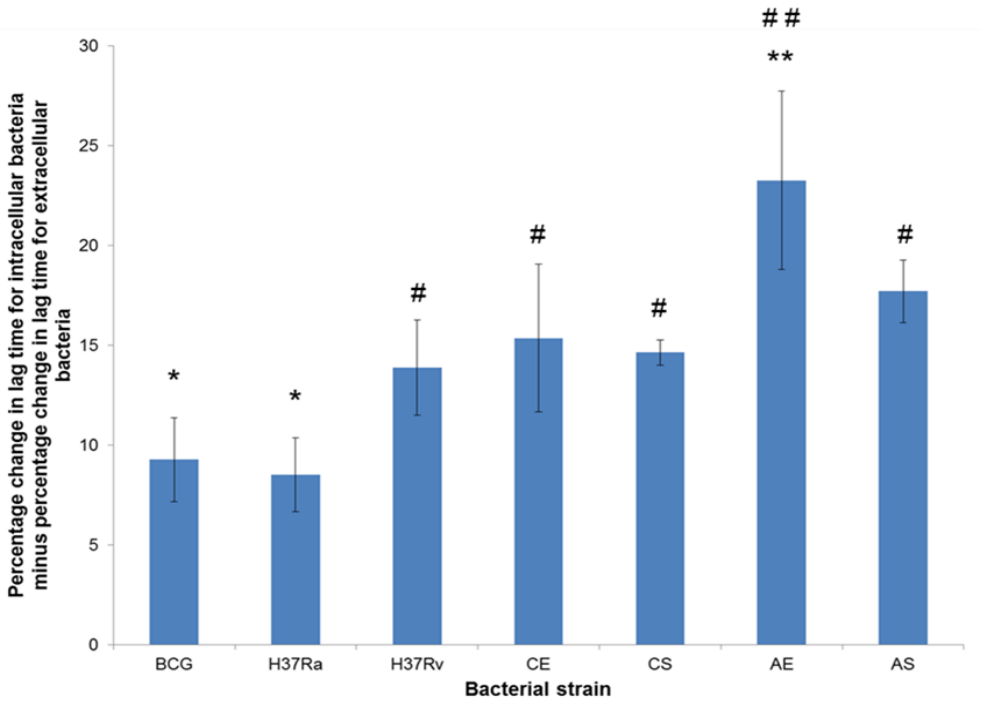


Figure 4-27 Mean percentage change in TTP for different strains of mycobacteria over six days cultured with THP-1 cells, normalised for extracellular growth

* denotes p < 0.05 and ** denotes p< 0.01 when compared to H37Rv, # denotes p < 0.05 and # # denotes p< 0.01 when compared to BCG. Error bars represent SEM, n=15.

To determine whether all isolates of *M. tuberculosis* from the CAS clade have the same virulence characteristics, six different CAS isolates were selected. Epidemiological evidence showing that recent transmission had occurred was used to select three isolates; CE1, CE2 and CE3. Three strains were selected that have been isolated once between 2003 and 2008, indicating that they are not circulating strains; CS1, CS5 and CS6. All six CAS isolates were able to replicate in cell-free media (Figure 4-29). When the mean percentage change in TTP from day one to day six was normalised for extracellular growth, CE1 and CS1 had the largest mean

percentage change in TTP. CS6 had the smallest percentage change, an increase of 7% ± 0.6% (Figure 4-30).

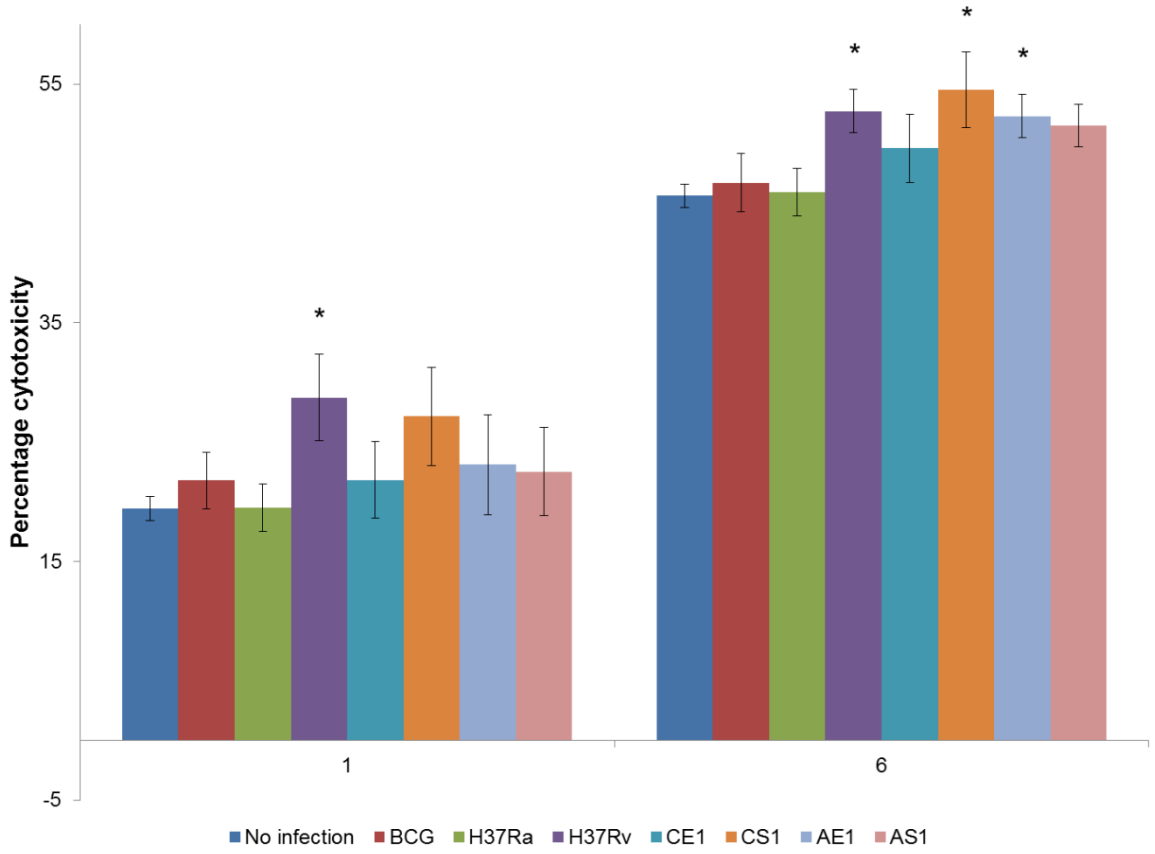


Figure 4-28 Mean percentage cytotoxicity in uninfected THP-1 cells and THP-1 cells infected with different strains of mycobacteria over six days

An MOI of 1:1 was used and cytotoxicity was measured using an LDH release assay. * denotes $p < 0.05$ when compared to no infection. Error bars represent SEM, n=15.

The growth of CE1, CE2 and CE3 in THP-1 cells (normalised for extracellular growth) was not significantly different. CE1, CE2 and CE3 all belonged to the same epidemiological cluster of disease (Figure 4-30). The growth of CS1 was not significantly different from the growth of CS5, but the growth of CS6 was significantly different from CE1 ($p=0.03$) and highly significantly different from CS1 ($p=0.0001$).

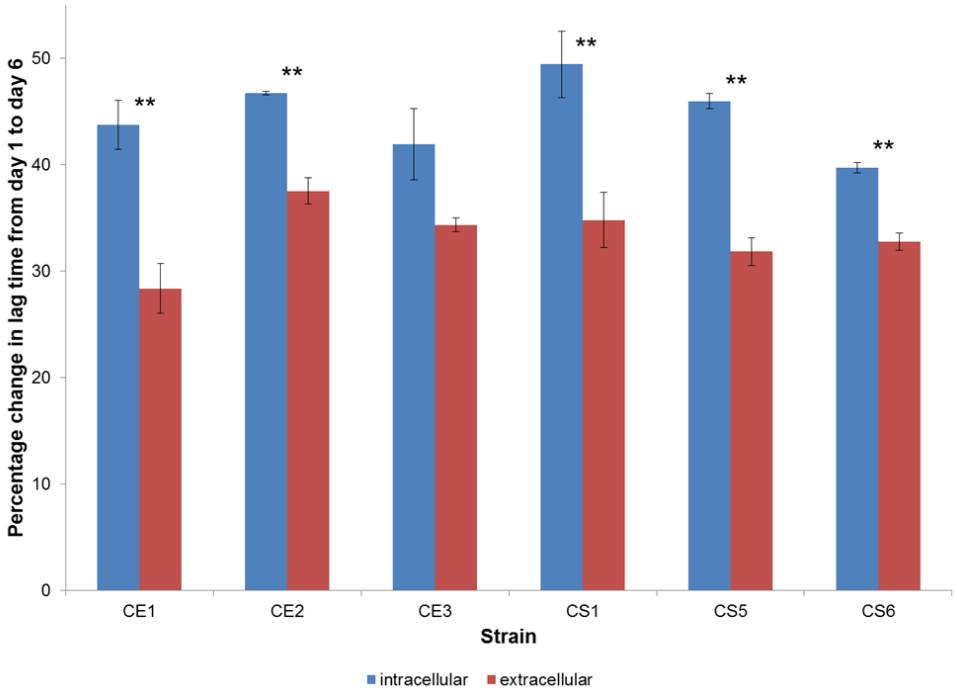


Figure 4-29 Mean percentage change in TTP for clinical isolates of *M. tuberculosis* from the CAS clade over six days cultured with (intracellular) and without (extracellular) THP-1 cells

* denotes p < 0.05 and ** denotes p< 0.01 when comparing percentage change in TTP for intracellular and extracellular bacteria. Error bars represent SEM, n=3, except for CE1 and CS1 where n=15.

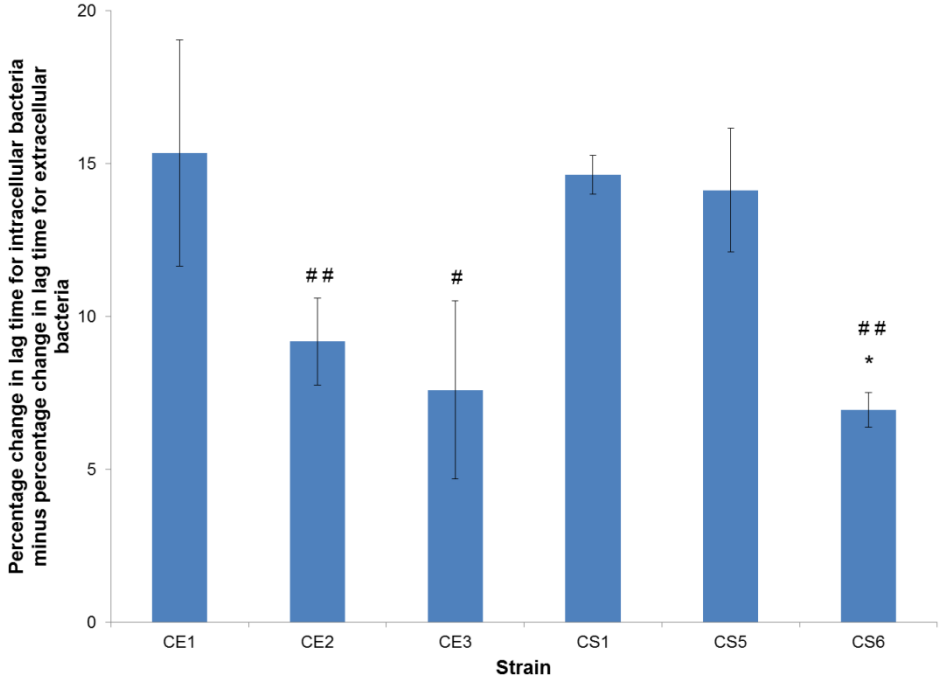


Figure 4-30 Mean percentage change in TTP for clinical isolates from the CAS clade over six days cultured with THP-1 cells, normalised for extracellular growth

* denotes p < 0.05 and ** denotes p< 0.01 when compared to CE1, # denotes p < 0.05 when compared to CS1. Error bars represent SEM, n=3, except for CE1 and CS1 where n=15.

The level of THP-1 cell cytotoxicity caused by all isolates from the CAS clade was higher than in non-infected cells after one day and six days of infection (Figure 4-31). CS5 caused the most cytotoxicity on day one (32% ± 3%, n=3, p=0.01 compared to uninfected cells) and on day six (57% ± 2%, n=3, p=0.02 compared to uninfected cells). CE1 caused the lowest percentage THP-1 cell cytotoxicity on day one (22% ± 3%, n=6, p=0.5 compared to uninfected cells) and on day six (50% ± 3%, n=6; p = 0.3 compared to uninfected cells) (Figure 4-31). When the average percentage cytotoxicity caused after six days by CAS strains from epidemiological clusters (51% ± 0.5%, n=3) was compared to the average percentage cytotoxicity for unique CAS isolates (56% ± 0.7%, n=3) there was a significant difference (p=0.05), with unique isolates causing the most cytotoxicity.

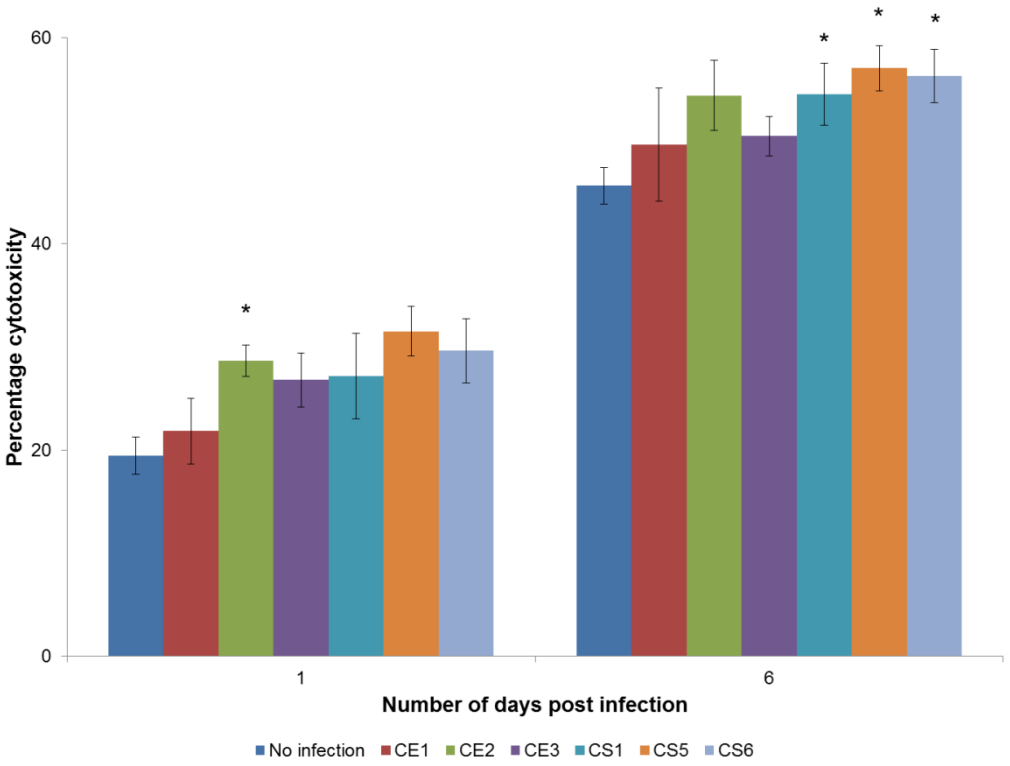


Figure 4-31 Mean percentage cytotoxicity in uninfected THP-1 cells and THP-1 cells infected with clinical isolates from the CAS clade over six days

An MOI of 1:1 was used and cytotoxicity was measured using an LDH release assay. * denotes $p < 0.05$ when compared to no infection. Error bars represent SEM, n=3. Except for CE1 and CS1 where n=6.

4.3.6 Discussion

The ancient isolate from epidemiological cluster 1 (AE1) had the highest intracellular growth of all the strains tested in the current study. Growth rate did not correlate exactly with cytotoxicity as the CAS orphan strain CS1 caused the most THP-1 cell necrosis. This chapter shows that all of the tested clinical isolates were able to grow in THP-1 cells more rapidly than the virulent reference strain H37Rv and the avirulent reference strains H37Ra and BCG. Although the AE1 isolate had the highest intracellular growth, it was not significantly higher when compared to the other clinical isolates. All the clinical isolates caused more THP-1 cell necrosis than BCG and H37Ra. After six days of infection, CS1 was the only clinical isolate that had caused more THP-1 cell necrosis than H37Rv, but the amount of necrosis caused by CS1 was not significantly greater than any of the other clinical isolates.

Previous studies have shown that high intracellular growth rates in THP-1 cells correlate with the ability of a strain to kill macrophages *in vitro*, cause disease in animals, or to spread amongst humans. It was therefore hypothesised that ancient isolates, which are not prevalent in the Midlands, would have a lower growth rate in THP-1 cells and cause less cytotoxicity than isolates belonging to the CAS spoligotype clade, which are prevalent in the Midlands and are evolutionarily modern strains. However, the results from the THP-1 infection model used in the current study did not fully confirm this hypothesis.

The intracellular growth rates of *M. tuberculosis* strains in THP-1 cells began to differ after three days, showing that THP-1 cells need to be infected for at least three days

before differences between strains can be detected. Theus also showed that intracellular growth rates for different isolates of *M. tuberculosis* began to differ after three days of incubation with THP-1 cells (Theus *et al*., 2005). Sharma and colleagues found no difference in the intracellular growth rate of H37Ra, H37Rv and a clinical isolate after infection of THP-1 cells for three days (Sharma *et al*., 2008); however, data from the current study has shown that H37Ra and H37Rv have different intracellular growth rates after three days. Sharma and colleagues suggested that the length of time that their experiments were carried out for could have prevented differences in the growth of H37Ra and H37Rv from being detected, which is consistent with data from the current study.

The current study found that differences in intracellular growth between strains occurred after three days, and differences were still discernible after six days. Kanji and colleagues assessed the growth of H37Rv and 21 clinical isolates from the CAS1_Dehli spoligotype clade (SIT26) in broth and in THP-1 cells (Kanji *et al*., 2011b). The authors found that differences in intracellular growth between strains were most evident after three days, but after five days, differences were no longer significant. Theus showed that growth of unique clinical isolates and isolates from epidemiological clusters of disease were significantly different in THP-1 cells after seven days of infection, growth of unique isolates was lowest (Theus *et al*., 2005).

The BACTEC™ MGIT™ 960 System was used to enumerate *M. tuberculosis* in the current study, whereas the studies by Kanji and colleagues and Theus and colleagues used CFU to enumerate bacteria. The BACTEC™ MGIT™ 960 System

uses liquid culture media for enumeration of *M. tuberculosis* and is highly sensitive (Diacon *et al*., 2010; Dhillon *et al*., 2004).

Dhillon and colleagues have demonstrated that there are populations of bacteria present in stationary phase, micro-aerophilic adapted cultures, which can grow in liquid culture media but are not able to grow on solid culture media (which is used for CFU enumeration). The same population of bacteria are present in the lungs and spleen of chronically infected mice, which led the authors to suggest that the growth difference between solid and liquid media could be caused by the presence of a sub-population of non-replicating or dormant bacteria (Dhillon *et al*., 2004).

The gene expression profile of *M. tuberculosis* in micro-aerophilic culture conditions had similarities to the gene expression profile of *M. tuberculosis* from the lungs of infected mice (Voskuil *et al*., 2003) and *M. tuberculosis* from murine bone marrow-derived macrophages (Schnappinger *et al*., 2003). These observations suggest that a dormant population of *M. tuberculosis* could be present when THP-1 cells are used to model infection. If dormant bacteria can only grow in liquid medium this could account for why differences in growth between CAS strains were not significant after five days in the study carried out by Kanji, where solid media was used for bacterial enumeration.

The study by Theus and colleagues also used CFU counts to enumerate bacteria. However, the THP-1 cells used in Theus's study were treated with PMA and IFN-γ, in contrast to the current study, which only used PMA. Addition of IFN-γ activates the

THP-1 causing them to destroy *M. tuberculosis* more efficiently than non-activated cells. Activation of the THP-1 cells may have prevented the bacteria from entering dormancy, accounting for the significant differences in growth rate found after six days in the study by Theus, which were not seen in the study by Kanji, despite both studies using the same method of bacterial enumeration.

The current study has shown that there was no significant difference in the intracellular growth rate of three isolates from the same epidemiological cluster of disease (CE1, CE2 and CE3) in THP-1 cells. The intracellular growth rate of two unique strains (CS1 and CS5) was not significantly different to the growth of CE1; however, growth of one of the unique isolates (CS6) was significantly lower than the growth of CE1 and CS1.

Kanji and colleagues investigated reasons for differences in intracellular growth rates of CAS1_Dehli strains in THP-1 cells by analysing the distribution of two genomic regions in 133 clinical isolates from Pakistan. They found deletion of RD149 occurred in 40% of isolates and deletion of both RD149 and RD152 occurred in 19% of isolates (Kanji *et al*., 2011a). In a subsequent study, Kanji and colleagues observed statistically significant variation in growth rates for different CAS isolates and they attempted to examine possible genetic causes for this (Kanji *et al*., 2011b). Kanji and colleagues suggested that absence of RD149 from 40% of the CAS1_Dehli clinical isolates that they studied contributed to slower growth in broth and in THP-1 cells than H37Rv; however absence of RD149 and RD152 did not produce the same effect.

The function of the RD149 and RD152 regions are not known but RD149 contains possible phage proteins (Tsolaki *et al*., 2005) and RD152 includes putative transposases and phospholipase-C gene D (*plcD*) which has been proposed as a virulence factor (Yang *et al*., 2005). Kanji and colleagues also noted that CAS1_Delhi strains with RD149 deletions that were isolated from extrapulmonary sites grew faster and induced lower levels of TNF-α and IL10 than isolates with the same deletion that were from pulmonary sites.

Theus and colleagues tested 15 clinical isolates that belonged to the Beijing spoligotype clade using a THP-1 cell model of infection and found that intracellular growth rate varied significantly amongst the isolates (Theus *et al*., 2007a). This suggests that the growth of isolates that belong to the same spoligotype clade can vary considerably in THP-1 cells.

The current study showed that isolates from the same cluster of disease had a similar growth rate, however high intracellular growth rate did not correlate with high induction of cytotoxicity. It has been reported that isolates belonging to a cluster of disease have higher intracellular growth rates and produce more cytotoxicity than single isolates that were not part of disease clusters. However, in the current study, only one of the three unique isolates that were tested had a significantly lower intracellular growth rate compared to strains that belonged to a cluster of disease, and the unique isolates caused more cytotoxicity than the isolates from disease clusters.

Theus and colleagues found that H37Rv and a clinical isolate belonging to the Beijing spoligotype clade, which was from a cluster of disease had a higher intracellular growth rate than a unique clinical isolate which had an orphan spoligotype profile (Theus *et al*., 2004). Theus followed up the work on the unique isolate and clustered isolate by testing a further 32 clinical isolates, and again found that higher intracellular growth correlated with the ability of a strain to spread in the community (Theus *et al*., 2005). However, work carried out in the current study only showed a significant difference between the intracellular growth of one unique isolate when compared to isolates that belonged to a disease cluster. The discrepancies between the current study and the study carried out by Theus may be caused by the small sample size; only three unique and three cluster isolates were tested in the current study. The differences could also have been caused by different methodology, Theus and colleagues used activated THP-1 cells infected with 50 mycobacteria per cell whereas the current study used non-activated THP-1 infected with one mycobacterium per cell.

In the current study, only the CAS orphan strain CS1 was able to cause more necrosis than H37Rv. However, after seven days in culture almost half of the THP-1 cells had died spontaneously in the absence of mycobacteria. The high degree of cell death in THP-1 cultures in the current study may have prevented subtle differences in the amount of necrosis caused by different *M. tuberculosis* isolates from being detected. Castro-Garza and colleagues (Castro-Garza *et al*., 2007) and Sohn and colleagues (Sohn *et al*., 2009) were able to show that clinical isolates of

M. tuberculosis caused more necrosis in a THP-1 cell model of infection than the reference strain H37Rv.

The high level of THP-1 cell death occurring in the absence of infection in the current study contrasts with the study carried out by Theus and colleagues which reported that the percentage of viable THP-1 cells was constant over seven days of incubation with mycobacterial isolates and did not vary between isolates. Theus and colleagues did not report the amount of cell death occurring in uninfected cells (Theus *et al*., 2005). THP-1 cells used in the current study were passaged for a maximum of five times, as it is known that the phenotype of THP-1 cells can alter after repeated passage (Tominaga *et al*., 1998). The THP-1 cells used in the study by Theus were passaged every three days and there is no mention of whether passage number was restricted.

The concentration of PMA used to differentiate THP-1 cells in the current study was four times lower that the concentration used by Theus and colleagues. The concentration of PMA chosen for THP-1 cell differentiation was optimised in section 4.2.4, and higher concentrations were shown to result in cell death. PMA is sensitive to light as well as to acid and alkali conditions and is insoluble in water. Differences in the preparation and storage of PMA in the current study and the study carried out by Theus and colleagues may have occurred.

THP-1 cell viability in the current study was assessed using a highly sensitive LDH release assay. The study by Theus and colleagues assessed cell viability using

trypan blue staining of cells that had been removed from their culture vessel using trypsin (Theus *et al*., 2005). The different techniques could account for the variation seen in cell death between the two studies.

After reviewing other published studies, a number of improvements were made so that the current study would be standardised. The passage number of the THP-1 cells was controlled, bacterial enumeration was carried out using the BACTEC™ MGIT™ 960 System rather than CFU counting and cell viability was tested using an LDH release assay. Despite these improvements, *M. tuberculosis* isolates that belong to the CAS spoligotype clade did not appear to be more virulent in a THP-1 cell model than ancient isolates from the EAI spoligotype clade. In addition, isolates that belonged to an epidemiological cluster of disease were not more virulent than isolates that did not belong to an epidemiological cluster. High *M. tuberculosis* intracellular growth rates in THP-1 cells did not absolutely correlate with increased cytotoxicity. However, the amount of spontaneous cell death that occurred in the THP-1 cell model in the absence of infection may have precluded the observation of subtle differences in cytotoxicity. The current study has provided insight into the complex relationship between macrophages and different strains of *M. tuberculosis.* Growth rates and cytotoxicity do not fully account for potential differences in disease pathogenesis. Immune evasion is likely to be an important factor in the virulence of *M. tuberculosis*, however the TB-host interaction is complex and the exact mechanism of immune evasion is yet to be fully elucidated (McEvoy *et al*., 2012; Comas *et al*., 2010). To determine whether similarity in the amount of necrosis

caused by *M. tuberculosis* strains in the current study was due to high spontaneous cell death, the strains were tested in a MDM model, described in section 4.4.

4.4 Infection of human MDMs with *M. tuberculosis* strains from the CAS and EAI spoligotype clades

4.4.1 Background

THP-1 cells differ from MDMs in their expression of surface receptors, such as the lectin-like receptors (Stokes and Doxsee, 1999). Macrophages are a heterogeneous population of cells that can exist in different states, which are referred to as M1 and M2. Although THP-1 cells can be polarised towards an M1 or an M2 phenotype, this is not commonly done as the PMA treatment that is required to convert THP-1 cells into macrophage-like cells already programs them to some extent. Therefore, most studies looking at the effects of macrophage polarisation and *M. tuberculosis* infection are conducted with MDMs. There is evidence to suggest that *M. tuberculosis* can alter the polarisation of macrophages in human lungs (Redente *et al*., 2010; Ho and Lape e Silva, 2010; Almeida *et al*., 2009; Chacon-Salinas *et al*., 2005) but the ability of polarised macrophages to control the growth of *M. tuberculosis*, especially clinical isolates has not been studied in detail. MDMs were polarised towards an M1 or an M2 phenotype in order to assess the growth of the virulent reference strain H37Rv and the avirulent reference strains H37Ra and BCG, as well as the four clinical isolates studied in section 4.3.

As discussed in section 3 there is evidence, which indicates that the global origin of a TB patient is associated with the lineage of *M. tuberculosis* that they become infected with, even when the person has immigrated to a different country. In the current

study, it was shown that 60% of the TB patients with ISC ethnicity in the Midlands, UK were infected with the CAS spoligotype clade (which is a modern TbD1 negative lineage) and 10% were infected with the EAI spoligotype clade (which is an ancient, TbD1 positive lineage), both of which are prevalent in the ISC. To investigate this association further, monocytes were obtained from Asian and Caucasian donors and used to assess whether host ethnicity affects the ability of different strains of *M. tuberculosis* to replicate within or kill macrophages.

4.4.2 Hypothesis

M. tuberculosis isolates belonging to the CAS and EAI spoligotype clades will have different growth and cytotoxicity characteristics in a human MDM model of infection.

4.4.3 Aims

- To compare an MDM model of *M. tuberculosis* infection with the THP-1 cell model used in section 4.3.
- To compare CAS strains with EAI strains in a human MDM cell model to identify any differences in virulence.
- To compare clinical isolates from epidemiological clusters to isolates that are not from epidemiological clusters to determine whether isolates responsible for a cluster of disease are more virulent than unique isolates.
- To compare the effect of M1 and M2 macrophage polarisation on the intracellular growth rate and cytotoxicity of CAS and EAI strains
- To compare the effect of blood donor ethnicity on the intracellular growth rate and cytotoxicity of CAS and EAI strains in MDMs.

4.4.4 Materials and methods

Human peripheral blood mononuclear cells (PBMCs) from eight healthy anonymous donors were isolated from buffy coats processed by the National Blood Services, Birmingham, UK. For work related to the effect of ethnicity on macrophage function fresh heparinized (10 U/ml) blood was collected from six healthy male donors who had been vaccinated with BCG (three Asian donors and three Caucasian donors). PBMCs were prepared on a Ficoll-Paque density gradient (GE Healthcare) by centrifugation (400 × *g*, 30 min at room temperature). The cells at the interphase were collected and washed four times in RPMI (Life Technologies, UK). Monocytes were purified using magnetic cell sorting with anti-CD14-MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in a miniMACS, according to the manufacturer's instructions (Hanley *et al*., 2004). Cells were seeded at a density of 5 x 10^5 cells/ml and divided into two aliquots. The first aliquot was treated with10 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and the second aliquot was treated with 50 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) to generate M1 and M2 MDMs, respectively (Verreck *et al*., 2004). Cells were incubated in 96-well tissue culture plates for 48 hours in RPMI medium supplemented with 10% FBS (Sigma, UK; lot number 109K3396 was used for all experiments) at 37°C in a humidified atmosphere with 5% CO_2. Following incubation half of the culture medium was removed and replaced with fresh media containing 20 ng/ml GM–CSF or 100 ng/ml M–CSF. Cells were incubated for a further four days, they were then washed with warm RPMI before fresh, cytokine-free media was added (RPMI with 10% FBS), cells were then used for infection studies.

Suspensions of exponentially growing *M. tuberculosis* clinical isolates and reference strains were prepared as described in section 4.2.1.4 and purity was confirmed by microscopy using a TB ZN stain kit (Becton Dickinson) according to the manufacturer's instructions. Mycobacteria were resuspended in RPMI and treated with Tween 80 and sonication to remove clumps as described in 4.2.2.4. Suspensions of 5×10^5 bacteria/ml were prepared in RPMI with 0.5% Tween 80 (v/v) as described in 4.2.3.3. Culture medium was removed from M1 and M2 MDMs and replaced with bacterial suspensions at an MOI of 1:1, or fresh culture medium. Samples were taken at one, four and six days after infection for assessment of bacterial growth and cell necrosis as described in 4.2.8.4. Measurements for each blood donor were carried out in duplicate. Where applicable, data sets were compared in Microsoft Excel using Student's two-sample t test.

4.4.5 Results

4.4.5.1 Growth of *M. tuberculosis* isolates in cell-free tissue culture medium

The reference strains H37Ra and H37Rv and four clinical *M. tuberculosis* isolates were able to grow in cell-free tissue culture media for six days (data not shown). *M. bovis* BCG was able to grow in cell-free culture media for three days but after this, the number of bacteria began to fall. The initial increase in the number of BCG followed by a decrease causes a lower percentage change in TTP from day one to day six compared to strains that were able to continue growing in cell-free culture media for six days. The growth of BCG in cell-free culture media was consistent with data obtained in section 4.2.7, as BCG had the smallest mean percentage change in TTP in cell-free media (10% ± 5%). The growth of AE1 in cell free media was the highest in the MDM model (39% ± 2%), whereas in THP-1 cells the growth of AS1

(37% ± 2%) was the highest (Figure 4-32). BCG was the only bacterial strain that could not grow in cell free tissue culture medium for six days

4.4.5.2 Growth of *M. tuberculosis* isolates in THP-1 cells compared to their growth in human MDMs

All of the strains that were tested in the THP-1 model had a higher percentage change in TTP when cultured with cells compared to cell-free media, indicating that THP-1 cells provide a more favourable environment for mycobacterial growth than media alone. However, when the strains were tested in M1 and M2 human MDMs the percentage change in TTP for some strains was lower when cultured with cells compared to cell-free media, indicating that the macrophages were inhibiting the growth of the bacteria (Figure 4-32). BCG, H37Rv, CE1 and CS1 were able to grow more in M1 cells than in cell-free media; the growth of H37Ra, AS1 and AE1 was lower in M1 cells than it was in cell-free media. The same pattern was seen in M2 cells, they supported higher growth of BGC, H37Rv, CE1 and CS1 compared to cell-free media.

The behaviour of BCG and H37Rv in M1 and M2 macrophages was similar to their behaviour in THP-1 cells. The percentage change in TTP from day one to day six for H37Rv cultured with THP-1 cells (42% ± 2%) was similar to the percentage change in M1 (40% ± 3%) and M2 cells (45% ± 5%). However, the clinical isolates behaved differently in M1 and M2 cells compared to THP-1 cells (Figure 4-32). For example the percentage change in TTP from day one to day six for AE1 cultured with THP-1 cells (47% ± 3%) was greater than the percentage change in TTP when AE1 was cultured with M1 cells (37% ± 2; $p = 0.03$) and M2 cells (33% ± 3%; $p = 0.04$). AE1

was able to grow more in THP-1 cells than in cell free culture medium, but in M1 and M2 cells AE1 grew less than it did in cell-free media.

BCG had the second lowest percentage change in TTP when it was cultured with M1 or M2 cells compared to the other six strains that were tested. However, when percentage change in TTP from day one to day six was normalised to account for extracellular growth, BCG had the largest mean percentage change in TTP in M1 cells and the second largest change in M2 cells. The percentage change in TTP from day one to day six is lower for extracellular BCG than it is for other strains, as BCG was not able to continue growing in cell-free media for 6 days. Therefore when percentage change in TTP for extracellular BCG is subtracted from percentage change in TTP for intracellular BCG the normalised value is elevated, an artefact which only occurs when bacteria are not able to continue growing in cell-free media for six days.

4.4.5.3 Growth of *M. tuberculosis* isolates in M1 and M2 macrophages

M2 MDMs provide a more favourable environment for the growth of virulent strains of *M. tuberculosis* than M1 macrophages; however, M2 macrophages inhibit the growth of avirulent strains more than M1 macrophages. When percentage change in TTP from day one to day six was normalised to account for extracellular growth, CE1 from M2 cells had the largest mean percentage change in TTP, an increase of 14% ± 4%. The normalised percentage change in TTP for CE1 in M1 cells (4% ± 4%) was 3.5 fold lower than for M2 cells. H37Rv and CS1 also grew more in M2 cells than they did in M1 cells (Figure 4-33).

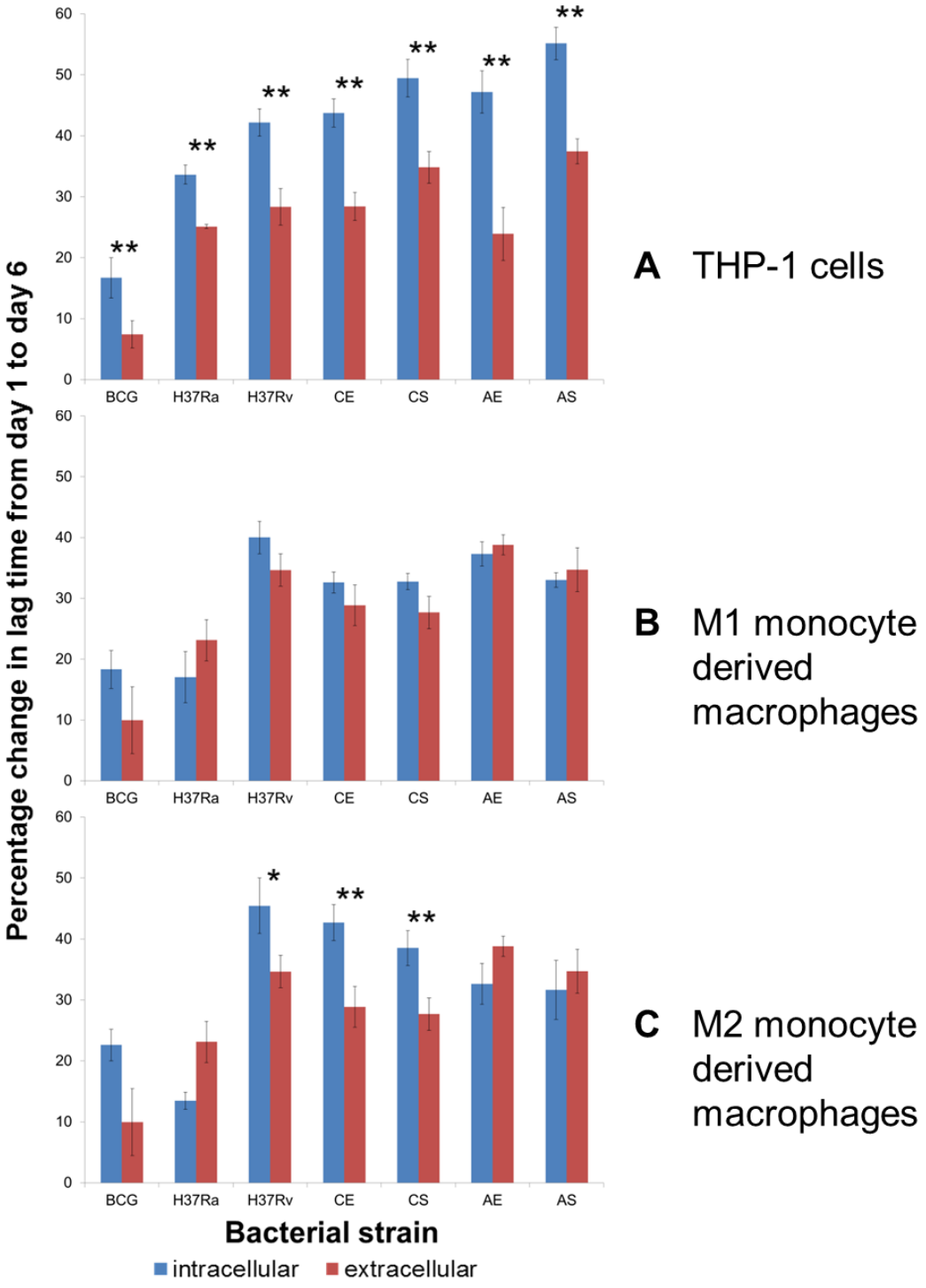


Figure 4-32 Mean percentage change in TTP for different mycobacterial strains cultured with and without different cells over six days

Mycobacteria were cultured with (intracellular) and without (extracellular) different types of cells, A) THP-1 cells (n=15), B) M1 MDMs (n=8) or C) M2 MDMs (n=8).* denotes $p < 0.05$ and ** denotes $p< 0.01$ when comparing percentage change in TTP for intracellular and extracellular bacteria. The data in panel A (THP-1 cells) is also shown in Figure 4-26. Error bars represent SEM.

H37Ra was able to grow in THP-1 cells better than in cell-free media but both M1 and M2 MDMs were able to inhibit the growth of H37Ra. When percentage change in TTP from day one to day six was normalised for extracellular growth there was a small negative change in TTP for H37Ra incubated with M1 cells (-4% ± 4%), but there was a much larger negative change in TTP for H37Ra incubated with M2 cells (-24% ± 4%). The ancient strains AE1 and AS1 grew in THP-1 cells but their growth was inhibited by M1 and M2 cells, inhibition was greatest in M2 cells.

4.4.5.4 Spontaneous cell death in the THP-1 and human MDM models of *M. tuberculosis* infection

There was a higher level of spontaneous cell death in the THP-1 cell model of *M. tuberculosis* infection than there was in human MDMs. Nearly a fifth of the cells in the THP-1 cell model had died after one day in culture in the absence of bacteria (percentage cytotoxicity 19% ± 2%, n=9); less than 1% of M1 cells had died under the same constions (percentage cytotoxicity 0.8% ± 0.2%, n=21) and the same result was seen for M2 cells (percentage cytotoxicity 0.9% ± 0.3%, n=21) (Figure 4-34). After six days in culture, nearly half of the uninfected THP-1 cells were dead (46% ± 2%), whereas only 2% (± 0.6%) of M1 cells and 3% (± 0.9%) of M2 cells had died.

After six days of infection, the percentage cytotoxicity in THP-1 cells ranged from 4 fold higher than M1 cells when infected with AS1 or CE1 to 11 fold higher when infected with H37Ra or BCG. BCG and H37Ra cause the lowest cytotoxicity in THP-1 cells, M1 cells and M2 cells; however, the amount of cytotoxicity caused by infection with the clinical isolates differed between the three cell types.

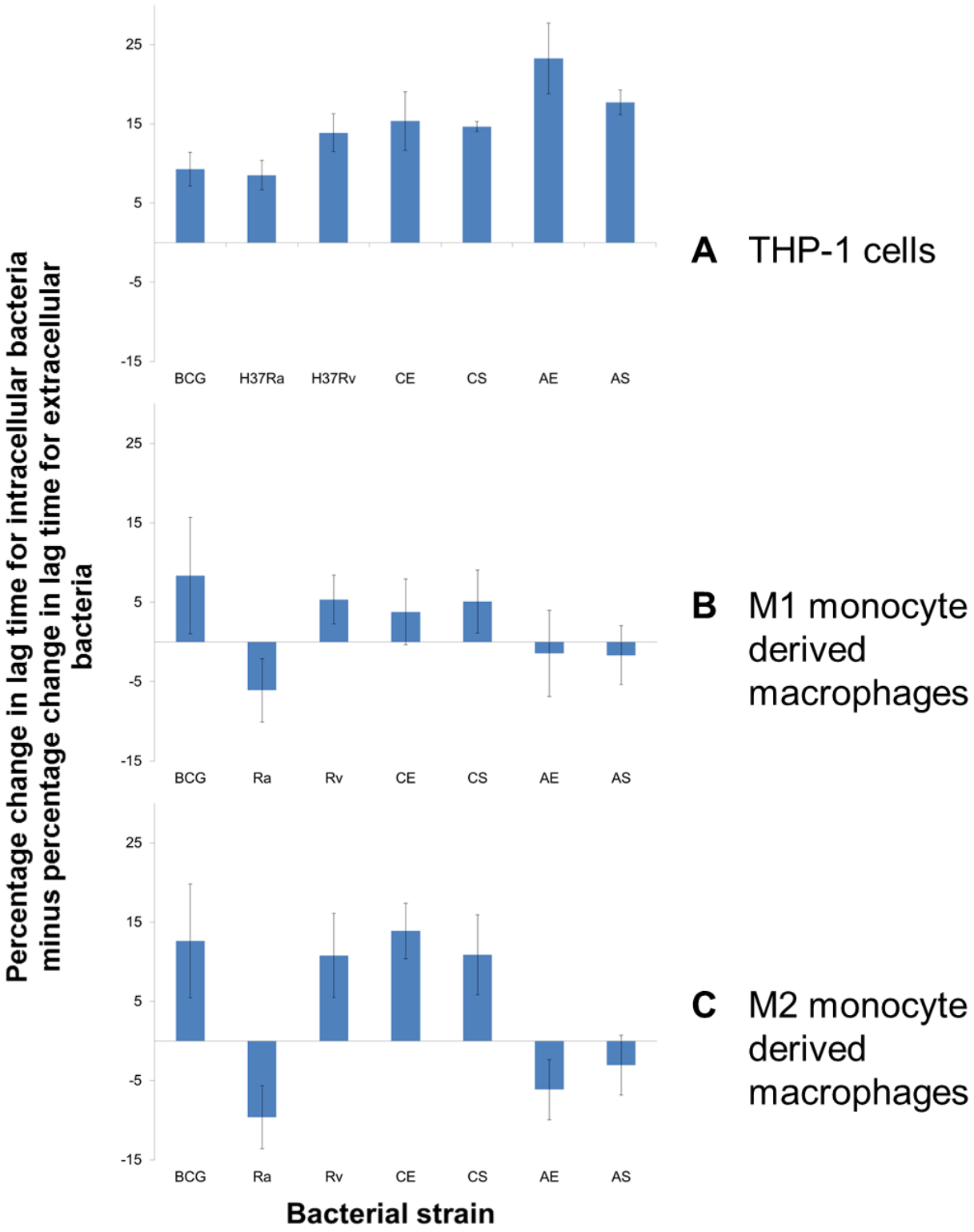


Figure 4-33 Mean percentage change in TTP for different mycobacterial strains cultured with different cells over six days normalised for extracellular growth

Mycobacteria were cultured with A) THP-1 cells (n=15), B) M1 MDMs (n=8) or C) M2 MDMs (n=8). Error bars represent SEM, n=8. The data in panel A (THP-1 cells) is also shown in Figure 4-27. Error bars represent SEM.

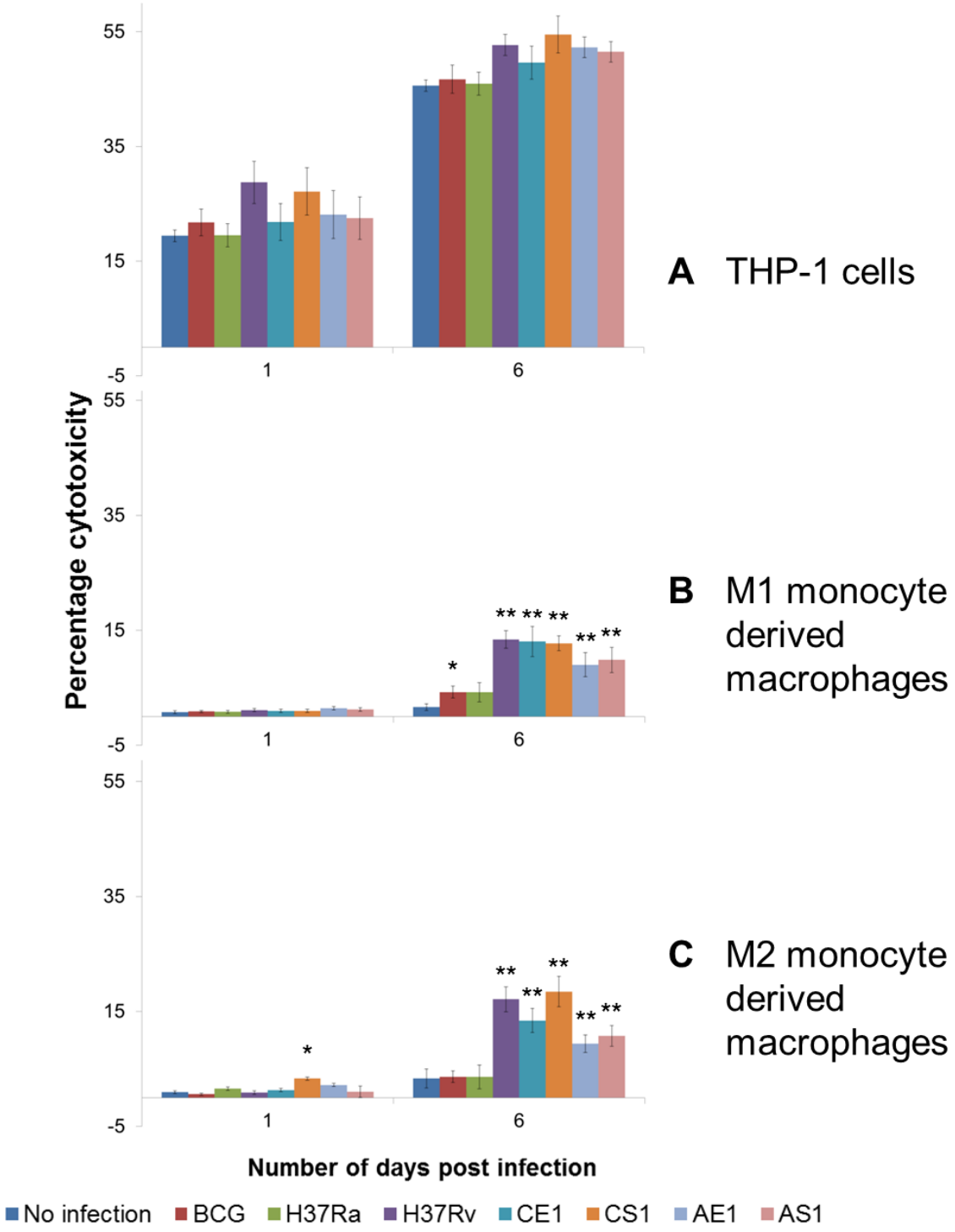


Figure 4-34 Mean percentage cytotoxicity in different cells caused by different strains of mycobacteria over six days

Mycobacteria were cultured with A) THP-1 cell, B) M1 polarised MDMs or C) M2 polarised MDMs. * denotes $p < 0.05$, ** denotes $p < 0.01$ compared to no infection. Error bars represent SEM, n=8.

Table 4-11 Mean percentage cytotoxicity in M1 and M2 cells six days after infection with mycobacterial strains

Cell phenotype	Bacterial phenotype	Mean percentage cytotoxicity	SEM	p value compared to CAS	p value compared to ancient	p value compared to virulent	p value compared to avirulent
M1	CAS	13	2	-	0.08	0.8	0.0009
	Ancient	9	1		-	0.005	0.02
	Virulent	13	1			-	0.000001
	Avirulent	5	1				-
M2	CAS	16	2	-	0.02	0.6	0.000009
	Ancient	10	1		-	0.003	0.0002
	Virulent	17	2			-	0.0000004
	Avirulent	4	1				-

The CAS strains caused the same amount of necrosis as the virulent laboratory strain H37Rv. Ancient strains caused more necrosis than the avirulent laboratory strains H37Ra and BCG, but less than the virulent strain H37Rv.

There was no significant difference in the intracellular growth or the amount of cellular necrosis that occurred when unique *M. tuberculosis* isolates (CS1 and AS1) were compared to isolates from epidemiologically linked clusters of disease (CE1 and AE1).

4.4.5.5 The effect of blood donor ethnicity on the ability of MDMs to inhibit the growth of *M. tuberculosis*

MDMs were generated from three UK-born Caucasian blood donors and three UK-born Asian blood donors to assess whether ethnicity of the blood donor affected the ability of MDMs to inhibit mycobacterial growth. Growth of H37Rv and the CAS strains (CE1 and CS1) was better in MDMs originating from Asians than it was in MDMs originating from Caucasians. Growth of H37Rv in Asian M1 MDMs was nearly

20 fold higher than in Caucasian M1 MDMs, growth of CE1 was 1.5 fold higher and growth of CS1 was 1.2 fold higher (Figure 4-35, Figure 4-36).

MDMs from Caucasians were able to suppress the growth of H37Ra more than MDMs from Asians, with M2 MDMs being more effective than M1 MDMs at inhibition of H37Ra growth. Growth of H37Ra in Caucasian M1 MDMs was 6 fold lower than in Asian M1 MDMs and 9 fold lower in M2 MDMs (Figure 4-35, Figure 4-36). The growth of the AE1 isolate did not vary according to the ethnicity of the macrophage donor, M1 MDMs from Caucasians and Asians were able to suppress the growth of AE1, but AE1 was able to grow in M2 MDMs from Caucasians and Asians.

M1 and M2 macrophages from Caucasians suppressed the growth of the AS1 isolate; growth suppression was greatest in M2 cells. Conversely, the AS1 isolate grew in M1 and M2 MDMs from Asians, highest growth occurred in M2 cells. Growth of BCG was 2.3 fold higher in M1 MDMs from Caucasians compared to Asians, but in M2 MDMs, growth of BCG was 1.5 fold higher in cells derived from Asians (Figure 4-35, Figure 4-36).

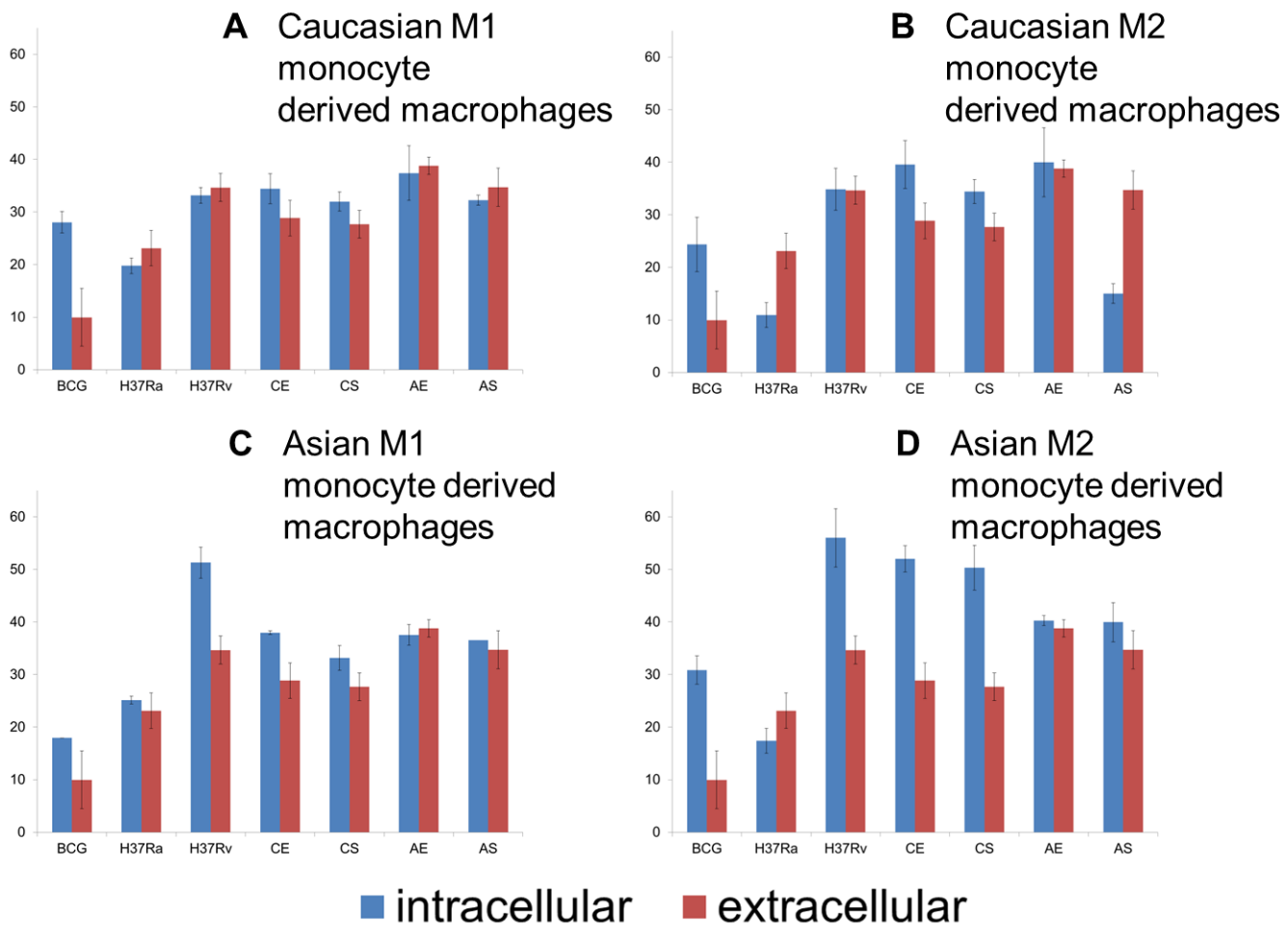


Figure 4-35 Mean percentage change in TTP for *M. tuberculosis* strains cultured for six days with MDMs generated from people of different ethnicity

Mycobacteria were cultured with (intracellular) and without (extracellular) A) M1 MDMs from three Caucasian donors, B) M2 MDMs from three Caucasian donors, C) M1 MDMs from three Asian donors or D) M2 MDMs from three Asian donors. Error bars represent SEM.

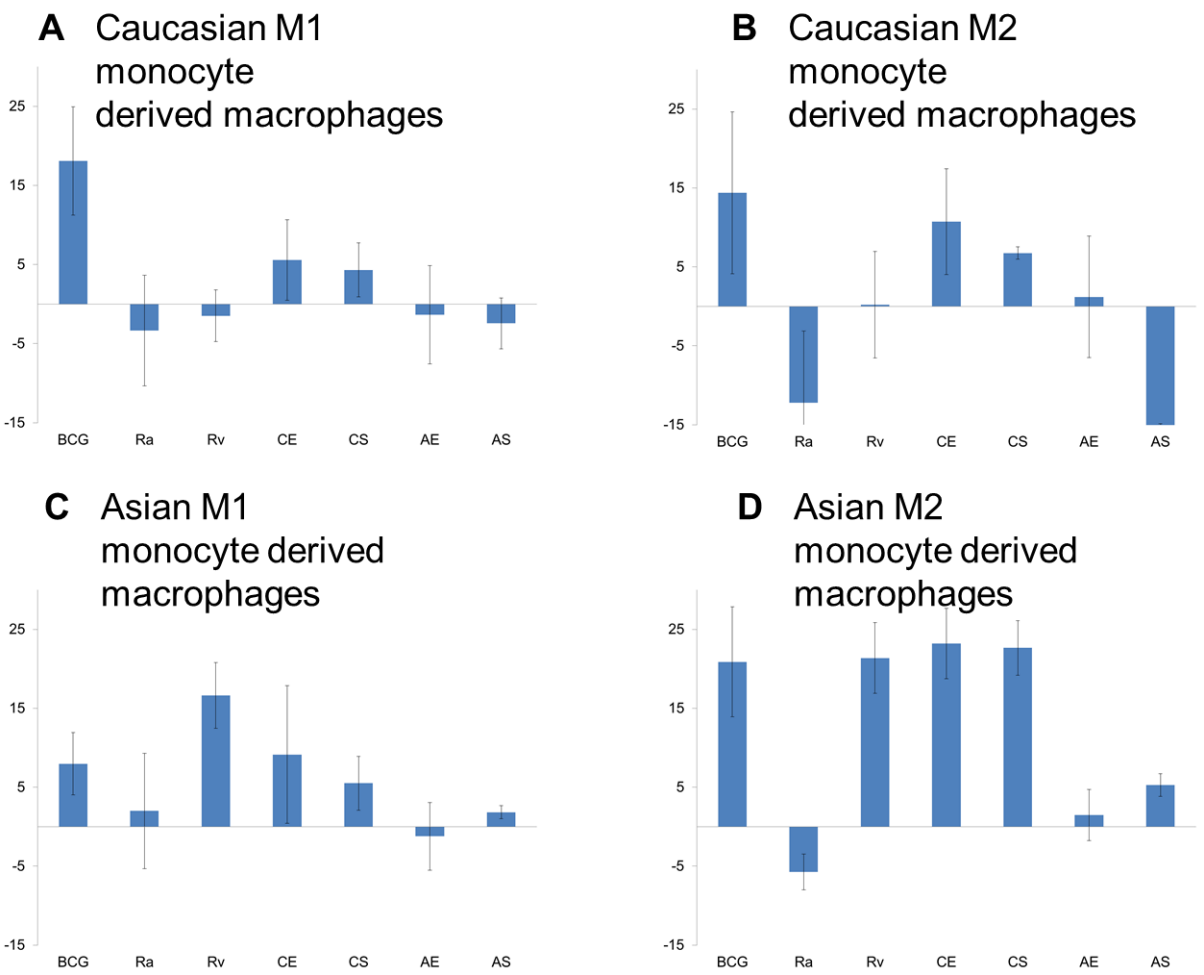


Figure 4-36 Mean percentage change in TTP for *M. tuberculosis* strains cultured for six days with MDMs generated from people of different ethnicity, normalised for extracellular growth

Mycobacteria were cultured with A) M1 MDMs from three Caucasian donors, B) M2 MDMs from three Caucasian donors, C) M1 MDMs from three Asian donors or D) M2 MDMs from three Asian donors. Error bars represent SEM.

4.4.5.6 The effect of blood donor ethnicity on the ability of *M. tuberculosis* to cause necrosis in MDMs

The amount of necrosis that occurred in MDMs infected with different mycobacterial strains did not differ significantly according to the ethnicity of the blood donor (Figure 4-37). There was a similar level of necrosis in Asian M1 cells and Caucasian M1 cells infected with the same mycobacterial strain, the same was true of M2 cells (Table 4-12).

Table 4-12 Mean percentage cytotoxicity in M1 and M2 MDMs from Asian and Caucasian blood donors six days after infection with mycobacterial strains

Cell phenotype	Bacterial phenotype	Mean percentage cytotoxicity (SEM)		p value*
		Asian	Caucasian	
M1	CAS	10 (0.8)	14 (2.3)	0.2
	Ancient	7 (0.8)	8 (0.3)	0.2
	Virulent	14 (2.3)	16 (0.5)	0.6
	Avirulent	2 (1.1)	6 (1.7)	0.1
M2	CAS	13 (3.5)	20 (2.3)	0.1
	Ancient	10 (1.6)	13 (2.2)	0.3
	Virulent	18 (0.5)	21 (1.9)	0.2
	Avirulent	3 (0.5)	4 (1.3)	0.4

* p values were calculated by comparing mean percentage cytotoxicity for Asian and Caucasian cells, there were three donors from each ethnic group and experiments were carried out in duplicate for each blood donor.

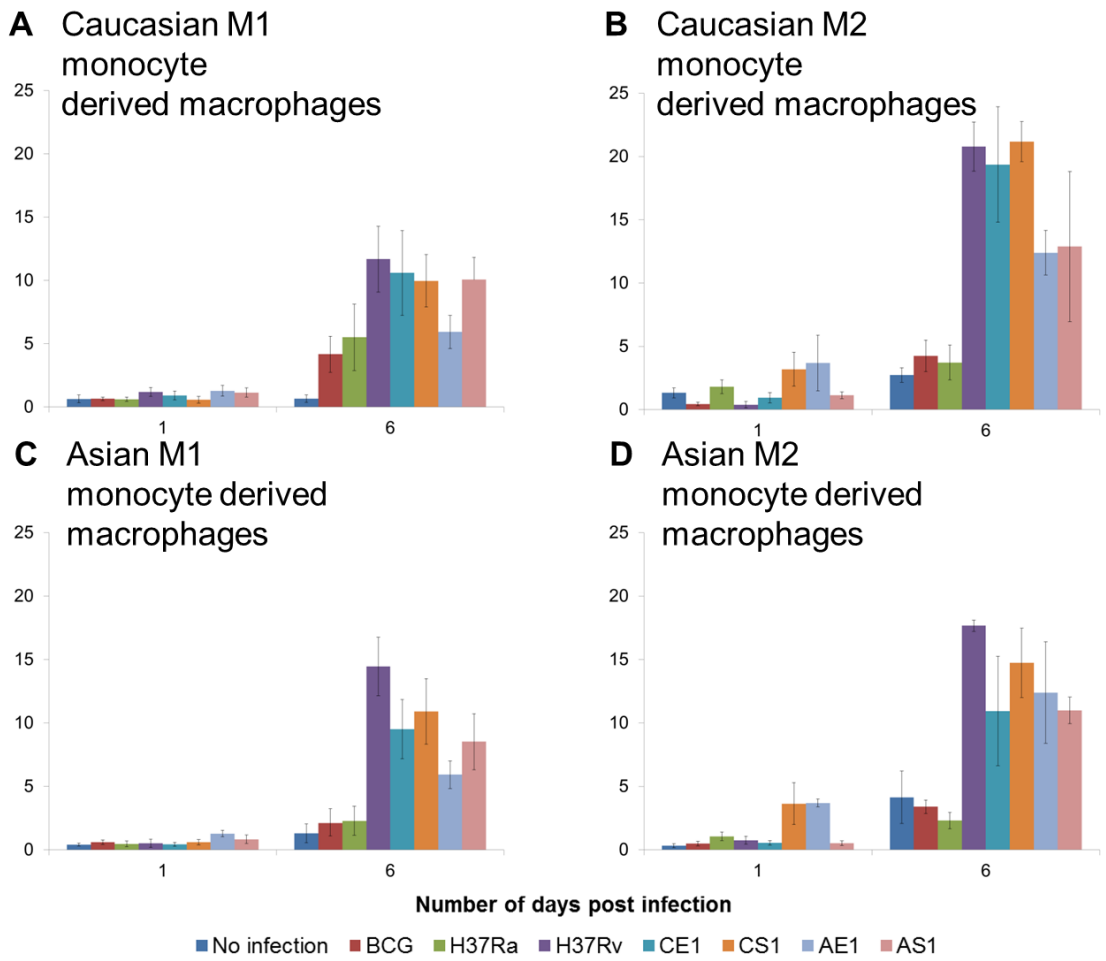


Figure 4-37 Mean percentage cytotoxicity in MDMs generated from people of different ethnicity caused by *M. tuberculosis* strains over six days

Mycobacteria were cultured with A) M1 MDMs from three Caucasian donors, B) M2 MDMs from three Caucasian donors, C) M1 MDMs from three Asian donors or D) M2 MDMs from three Asian donors. Error bars represent SEM.

4.4.6 Discussion

Results from the current study suggest that an MDM model of *M. tuberculosis* infection is more appropriate than a THP-1 cell model, especially for assessing differences in cytotoxicity caused by clinical isolates. Growth of all the *M. tuberculosis* clinical isolates and reference strains was higher in THP-1 cells than it was in MDMs. There was also a higher level of necrosis in the THP-1 cell model compared to MDMs. However, nearly 50% of THP-1 cells were dead after six days in culture in the absence of infection, whereas only 2% of MDMs died in the same conditions. The high level of spontaneous cell death in the THP-1 cell model may mask the observation of differences in the amount of cytotoxicity caused by distinct *M. tuberculosis* strains. The overall pattern of intracellular growth and cell necrosis produced by the reference strains BCG and H37Rv was similar in the THP-1 model and the MDM model but the behaviour of the clinical isolates differed between the two models. However, it is also important to note the effect of extracellular bacterial growth on the normalisation used to determine growth ratios in the current study. For example, BCG was the only strain that was not able to grow in cell-free media for six days. Therefore when percentage change in TTP for extracellular BCG was subtracted from percentage change in TTP for intracellular BCG the normalised value was elevated, an artefact which only occurs when bacteria are not able to continue growing in cell-free media for six days.

The THP-1 model has been widely used to assess the virulence of *M. tuberculosis* strains and THP-1 cells have been described as a good substitute for MDMs (Daigneault *et al*., 2010; Stokes and Doxsee, 1999; Auwerx, 1991). Riendeau and

colleagues also reported that PMA differentiated THP-1 cells undergo apoptosis in response to infection with avirulent *M. tuberculosis* in a manner that is similar to human alveolar macrophages (Riendeau and Kornfeld, 2003). Riendeau and colleagues carried out their study using four strains, wild-type *M bovis*, *M. bovis* BCG, *M. tuberculosis* H37Ra and H37Rv. However, the only direct comparison of the response of THP-1 cells and MDMs to infection with *M. tuberculosis* was published by Stokes and colleagues and it used just one mycobacterial strain; *M. tuberculosis* strain Erdman (ATCC 35801) (Stokes and Doxsee, 1999). Stokes and colleagues concluded that the capacity of both models to bind *M. tuberculosis* strain Erdman was the same and after seven days of infection. Stokes showed a 15-fold increase in the number of mycobacteria in MDMs and a 20-fold increase in the number of mycobacteria in THP-1 cells. Data from the current study shows that whilst the THP-1 cell model is a good substitute for infection of MDMs with laboratory reference strains such as H37Rv and BCG, the behaviour of clinical isolates is not the same in THP-1 cells and MDMs.

The current study shows that modern CAS strains are more virulent than ancient *M. tuberculosis* strains in an MDM model as CAS strains are able to cause more cell necrosis and to grow more than ancient strains. The differences between strains are more evident when macrophages are polarised towards an M2 phenotype. When a THP-1 cell model was used to assess virulence, it appeared that ancient strains were able to grow more than CAS strains, but the amount of cell necrosis that occurred was similar for CAS and ancient strains. In the MDM model of infection, CAS strains had similar growth characteristics to the virulent reference strain H37Rv, whereas the

growth of ancient strains resembled that of the avirulent reference strain H37Ra. In addition, CAS strains and H37Rv caused a similar level of cytotoxicity, whereas ancient strains caused less cytotoxicity than H37Rv.

The lower intracellular growth rate of ancient strains and decreased levels of necrosis in MDMs indicates that they were less virulent than the CAS strains that were tested in the current study. Portevin and colleagues showed that ancient strains of *M. tuberculosis* produced a pro-inflammatory phenotype in human M1 MDMs, whereas modern strains produced a lower inflammatory response (Portevin *et al*., 2011). If modern strains were more virulent than ancient strains it could be expected that ancient strains would not be isolated as frequently as modern strains. However, data from India suggest that ancient strains are still prevalent, for example, a study carried out in Andhra Pradesh, India showed that 40% of *M. tuberculosis* isolates belonged to the modern CAS spoligotype clade and 38% belonged to the ancient EAI clade (Thomas *et al*., 2011).

Differences in intracellular growth rate and cytokine profiles elicited by modern and ancient strains could be indications of different virulence strategies, rather than an indication of the superior virulence of modern strains (Portevin *et al*., 2011). However, it is possible that a low intracellular growth rate and the induction of a pro-inflammatory host response could have conferred a selective advantage on ancient strains in the past. Rather than evading the host immune system, until large numbers of bacteria have accumulated, ancient strains may enter a dormant state. The induction of dormancy allows the pathogen to exist for decades without killing

the host, reactivation may then occur when the host immune system weakens during old age, providing the pathogen with the opportunity to infect the next generation. Dormancy could therefore be considered as an adaptation to low host population density, which occurred during the pre-Neolithic era. Eshed and colleagues report that increased population density due to the shift from hunter-gatherer societies towards farming coincided with an increase in infectious disease during the Neolithic period (Eshed *et al*., 2010). The high intracellular growth rate of modern strains and induction of a low inflammatory response mean that modern strains are more likely to cause early, progressive TB. The rapid onset of clinical disease, which could lead to death of the host, also increases the chance of transmission. When the human population density is high and the probability of transmission is also high, the survival of the strain no longer depends on survival of an individual host so induction of primary TB could have conferred a selective advantage on modern strains, by allowing them spread to more hosts (Ahmed *et al*., 2009).

The virulent reference strain H37Rv and the modern CAS strains tested in this study caused more necrosis in an MDM model than the ancient strains. The amount of necrosis caused by avirulent H37Ra and attenuated BCG was the same as in uninfected cells. High cellular necrosis has been reported to correlate with high mycobacterial intracellular growth rates, which Park and colleagues suggested were *M. tuberculosis* virulence factors (Park *et al*., 2006). Induction of macrophage apoptosis has been described as a feature of avirulent *M. tuberculosis* strains whereas virulent strains are able to prevent macrophage apoptosis (Keane *et al*., 2000). The mode of macrophage cell death affects the fate of the infecting

mycobacteria, apoptotic cell death is bactericidal, but necrotic cell death releases the bacteria allowing them disseminate. However, the inhibition of macrophage apoptosis by virulent *M. tuberculosis* is a complex system that has not been fully elucidated.

There are contrasting reports of virulent strains of *M. tuberculosis* stimulating macrophage apoptosis and avirulent strains inhibiting macrophage apoptosis. O'Sullivan and colleagues compared the effect of infection with H37Ra and H37Rv on cell death using THP-1 cells and human MDMs. The authors showed that when cells were infected with a low MOI of 1 to 2 bacilli per cell only H37Ra induced apoptosis. When cells were infected with 10 to 20 bacilli per cell both H37Ra and H37Rv induced apoptosis, although the authors described the mode of cell death as apoptosis-like because it was caspase-independent (O'Sullivan *et al*., 2007b). Lee and colleagues showed that at MOIs of 25 bacilli per cell *M. tuberculosis* Erdman induced more apoptosis than BCG in murine bone-marrow derived macrophages. The authors also described the mode of cell death as caspase independent and showed that cell death did not cause a reduction in mycobacterial viability, which the authors explained was because apoptotic cells quickly became necrotic (Lee *et al*., 2006). Derrick and Morris showed that ESAT-6, which is a protein secreted by virulent *M. tuberculosis* strains, but not by BCG, was able to induce apoptosis in THP-1 cells. Apoptosis was also induced in a dose-dependent manner by infection of THP-1 cells with H37Rv at MOIs of 1 bacilli per cell and 10 bacilli per cell. Using the same MOIs apoptosis was shown not to occur when the RD-1 locus, which encodes nine genes including the ESAT-6 gene, was deleted from H37Rv (Derrick and Morris, 2007). In addition, alveolar macrophages isolated by bronchoalveolar lavage from TB

patients with active disease have been reported to show increased apoptosis compared to macrophages collected from healthy people (Placido *et al*., 1997; Klingler *et al*., 1997).

Butler and colleagues created two *M. tuberculosis* transposon mutants from an isolate belonging to the Beijing lineage (Butler *et al*., 2012). In contrast to the parent isolate, the mutants could not cause disease in mice or grow in macrophages and had lost the ability to prevent phagosomal maturation. Butler and colleagues showed that the virulent parent strain caused more cell death in a murine macrophage-like cell line than the avirulent mutants did. However, although the total cell death caused by the virulent parent strain exceeded the amount of cell death caused by the avirulent mutants, the mode of cell death, that is apoptosis or necrosis, was not related to the virulence of the strain. The percentage of total cell death caused by necrosis was higher for the avirulent mutants than for the parent strain, which was unexpected, as induction of necrotic cell death has been described as a feature associated with mycobacterial virulence.

The disparity between various studies describing the mode of cell death induced by virulent and avirulent *M. tuberculosis* strains may be explained by the use of different MOI. Inhibition of macrophage apoptosis has been shown to depend on MOI in a dose dependant manner as well on the virulence of the *M. tuberculosis* strain (Lee *et al*., 2006). A low MOI of one bacterium per cell was used in the current study, in order to model the initial stages of macrophage infection with *M. tuberculosis.* Butler and colleagues showed that MOI and the length of time that macrophages were

infected for both affected the induction of apoptosis (Butler *et al*., 2012). The authors concluded that isolated measurements at different time points or with different MOIs would have affected the conclusion as to whether virulent or avirulent *M. tuberculosis* induced more or less apoptosis. Butler and colleagues also pointed out that secondary necrosis can occur after the induction of apoptosis, which makes comparison between virulent and avirulent strains more complicated and emphasises the need to take carefully timed samples. Secondary necrosis occurs when apoptotic bodies are not phagocytosed by other cells.

However, Butler and colleagues were aware of the effect of time and MOI on the induction of apoptosis, so this does not explain their finding that avirulent *M. tuberculosis* mutants caused a higher percentage of necrosis than the virulent parent strain. They suggest that the mechanisms that directly stimulate necrosis are independent of apoptosis inhibition and concluded that virulence determines the level of cytotoxicity, but not the mechanism of cell death (Butler *et al*., 2012). Some viruses are able to prevent cells from undergoing apoptosis, which allows viral replication to occur. When the cell is replete and viral replication cannot continue the cell becomes necrotic providing the virus with an exit from the cell (Busca *et al*., 2009). *M. tuberculosis* may employ a similar strategy to replicate within and then escape from macrophages. This could also account for the high level of apoptosis in macrophages obtained by bronchoalveolar lavage from TB patients with active disease reported by Placido and colleagues (Placido *et al*., 1997). The mode of macrophage cell death should therefore be measured using different methods in order to make accurate comparisons between strains so that the mechanisms used

by *M. tuberculosis* to achieve intracellular growth and transmission to other cells whilst avoiding destruction by the immune system can be better understood. Several methods can be employed to differentiate between apoptotic and necrotic cell death. The morphology of the cells can be assessed using microscopy or flow cytometry. There are also numerous biochemical techniques that identify the presence of cell surface markers, fragmented DNA, protein cleavage or activation of specific enzymes such as the caspases, which are indicative of different modes of cell death (Krysko *et al*., 2008).

There is evidence to suggest that *M. tuberculosis* is able to alter macrophage polarisation (Redente *et al*., 2010; Ho and Lape e Silva, 2010; Almeida *et al*., 2009; Chacon-Salinas *et al*., 2005), however the infection of polarised macrophages with clinical isolates of *M. tuberculosis* has not been described previously. In the current study, M2 macrophages permitted higher growth of H37Rv and two clinical isolates from the modern CAS spoligotype clade compared to M1 macrophages. This is consistent with a study carried out by Denis, which found that growth of H37Rv was higher in M2 macrophages than it was in M1 macrophages (Denis, 1991). The current study also showed that M2 macrophages inhibited the growth of H37Ra and two clinical isolates from the ancient EAI spoligotype clade more than M1 macrophages. H37Ra is an avirulent strain and it was expected that M1 macrophages would be able to inhibit its growth more effectively than M2 macrophages. A recent study has shown that clearance of early apoptotic cells is more efficient in a subset of M2 macrophages (Zizzo *et al*., 2012). It has been reported that avirulent strains of *M. tuberculosis* induce macrophage apoptosis. It is

conceivable that H37Ra and the ancient clinical isolates induced macrophage apoptosis, clearance of the cells undergoing apoptosis by other macrophages in the culture may therefore explain the enhanced capacity of M2 cells to inhibit the growth of H37Ra and the ancient strains compared to M1 cells. The enhanced growth of CAS *M. tuberculosis* isolates in M1 and M2 macrophages compared to ancient isolates has provided further support for the theory that modern CAS strains are more virulent that ancient strains in an MDM model of infection. This may help to explain why the CAS strain is prevalent in the Midlands and ancient strains are not prevalent, despite both clades being prevalent in the ISC.

When MDMs from Asian and Caucasian donors were compared, it was shown that CAS isolates grew preferentially in M2 cells from Asian donors. Growth of *M. tuberculosis* isolates in M1 cells was broadly similar for Asian and Caucasian donors, although H37Rv grew better in in Asian M1 cells and AS1 was inhibited more by Caucasian cells. Despite mounting evidence describing the association of *M. tuberculosis* lineages with the geographical origin of TB patients (Rodwell *et al*., 2012; Brown *et al*., 2010; Reed *et al*., 2009; Gagneux *et al*., 2006), the mechanism behind this association has not been as well studied. Pareek and colleagues have shown that certain *M. tuberculosis* lineages are more likely to cause extrapulmonary TB than others; however, the clinical phenotype of the disease was strongly associated with the ethnicity of the patient. Pareek concluded that the ethnicity of the host was a more important determinant of clinical disease than the *M. tuberculosis* lineage (Pareek *et al*., 2013). Click and colleagues have also shown that *M. tuberculosis* lineage is associated with disease phenotype but they found that the

association between ethnicity and disease phenotype was only weak (Click *et al*., 2012). There have not been any other studies that have looked for an association between *M. tuberculosis* lineage and disease phenotype, which have taken the ethnicity of the host into account. Pareek and colleagues suggested that the difference in the effect of ethnicity on disease phenotype between their study and the study carried out by Click and colleagues may be due in part to different population structures; there was a much lower proportion of people with ISC ethnicity in the study carried out by Click and colleagues. There are a number of studies that have described human gene polymorphisms, which cause certain ethnic groups to have a higher susceptibility to *M. tuberculosis* than others (Zhang *et al*., 2012; Arji *et al*., 2012; Larcombe *et al*., 2008; Fernando *et al*., 2007; Hsu *et al*., 2006; Stead *et al*., 1990). However, the response of human MDMs from people belonging to different ethnic groups to infection with the same strains of *M. tuberculosis* has not been described. Reports that natural resistance-associated macrophage protein 1 (*Nramp*-1) gene polymorphisms cause susceptibility to mycobacterial infections indicate that innate immunity of the macrophage can influence disease outcome (Hackam *et al*., 1998). Given the co-evolution of *M. tuberculosis* with human populations that is assumed to have given rise to the distinct families of strains that are seen to be closely associated with different global regions, it is plausible that *M. tuberculosis* strains may have adapted to differences in macrophage phenotype in specific human populations (Chapman and Hill, 2012). This could explain the observation made in the current study that growth of CAS strains was higher in M2 MDMs from Asian blood donors, than it was in M2 MDMs from Caucasian blood donors.

The observation that CAS clinical isolates of *M. tuberculosis* grow preferentially in M2 MDMs from Asian donors supports the idea that *M. tuberculosis* has co-evolved with humans leading to distinct phylogeographical lineages of *M. tuberculosis*. However, the observation of macrophage polarisation in humans and the propensity for *M. tuberculosis* to induce a switch in macrophage polarisation means that identifying determinants of virulence in *M. tuberculosis* strains is further complicated. A properly powered, age matched study should be conducted in order to confirm the hypothesis that was generated in this study by assessing the response of macrophages from three Asian and three Caucasian donors to infection with different lineages of *M. tuberculosis*.

The current study showed variation in virulence between *M. tuberculosis* isolates belonging to the same spoligotype clade, which adds to the increasing evidence that suggests there is significant heterogeneity in the virulence of strains belonging to the same spoligotype clade. García de Viedma and colleagues identified three TB patients who were infected with two genotypically distinct strains of *M. tuberculosis* at the same time (Garcia de Viedma *et al*., 2006). One of the strains remained in the lungs whereas the other strain had disseminated to extrapulmonary sites. García de Viedma found that extrapulmonary strains infected macrophages more efficiently when cells were inoculated with an equal ratio of the pulmonary and extrapulmonary isolates. The authors studied the isolates from one patient in further detail, both isolates belonged to the LAM spoligotype clade, the pulmonary isolate was a LAM2 strain from SIT 17 and the extrapulmonary isolate was a LAM3 strain from SIT 33. The survival of SCID (severe combined immunodeficiency) mice was significantly

lower when infected with the extrapulmonary strain (average survival, 29 days), compared to the respiratory strain (average survival, 37 days). The authors concluded that there was a correlation between extrapulmonary dissemination and infectivity *in vivo* (Garcia de Viedma *et al*., 2005b).

Data from the current study and several others indicates that members of the same spoligotype can have different virulence properties (Kanji *et al*., 2011b; Theus *et al*., 2007a; Garcia de Viedma *et al*., 2005b). The observation that LSPs such as RD-149 deletion (discussed in section 4.3.6) in isolates from the CAS1_Dehli spoligotype clade can affect intracellular growth suggests that strain subsets exist within different spoligotype clades that have different virulence characteristics (Kanji *et al*., 2011b).

5 FUTURE WORK

Significant heterogeneity has been identified in the intracellular growth rate of CAS isolates in THP-1 cells, including differences identified between isolates from the same epidemiological cluster of disease. The study should be expanded to include a greater number of CAS and ancient isolates, which should also be tested in the MDM cell model. MDMs should be isolated from a greater number of people, who should be age matched so that putative differences in the response of MDMs from people of different ethnicities can be confirmed or refuted. *M. tuberculosis* isolates from other clades should also be included, and where possible examples of sympatric and allopatric infections should be studied in macrophage models of infection.

LSP profiling of CAS strains should be carried out to confirm whether the absence of specific RDs contributes to the virulence of subsets of CAS isolates in order to confirm the observation made by Kanji and colleagues that absence of RD149 causes slower intracellular growth rates amongst CAS isolates (Kanji *et al*., 2011b).

The mode of cell death caused by infection of macrophages with CAS strains should be assessed as Butler and colleagues have shown that the mechanisms that directly stimulate necrosis are independent of apoptosis inhibition (Butler *et al*., 2012). Commercially available apoptosis and necrosis kits are now available which use fluorescent stains to identify live and dead cells in the same sample and can also distinguish between apoptotic and necrotic cells. These assays could be used to give more information about the cause of macrophage death and could allow a time course to be constructed, so that the type of cell death induced could be accurately

correlated with intracellular bacterial numbers. This would give more information about the virulence of different members of the CAS clade and allow more accurate comparisons to be made with strains from different clades.

Some bacterial pathogens manipulate macrophage polarisation in order to down-regulate the bactericidal activity of the host cell. Kyrova and colleagues showed that *Salmonella enterica* serotype Typhimurium promoted M2 macrophage polarisation leading to enhanced intracellular growth (Kyrova *et al*., 2012). The ability of different *M. tuberculosis* strains to alter the phenotype of M1 and M2 polarised macrophages should be assessed (Chanput *et al*., 2012).

There are significant data to suggest that virulence of *M. tuberculosis* can be determined by the immune response that is elicited in the host. To that end culture supernatants from all the experiments in the current study have been filter sterilised and stored at -80°C so that multiplex cytokine assays can be performed.

This study does not prove a causal relationship for the increased incidence of strains from the CAS spoligotype clade in the Midlands. However, it does provide a rationale for future studies aimed at identifying the virulence of different *M. tuberculosis* strains.

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