DEVELOPING BACTERIOPHAGE-BASED ASSAYS FOR THE RAPID DETECTION OF MYCOBACTERIA

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ABSTRACT

Mycobacteria are a genus of bacteria that are very specialized and are the causative agents of many significant diseases across multi sectors and in a very wide range of species. Detection of these organisms can be difficult due to the slow growing nature of the majority of the pathogenic mycobacteria *in vitro*. The very close similarity between these organisms also causes cross reactions with many assay designed to detect a specific mycobacterial species.

The aim of these studies was to develop bacteriophage-based assay for the rapid detection of mycobacteria. PCR assays were designed to improve the detection of paucibacillary samples including a novel control strain of *M. smegmatis.* The novel strain could be used as a phage infection control for both the standard phage assay and the Oneday phage assay, a rapid mycobacterial detection method.

By using bacteriophage-based assays we identified in these studies MTBC bacteraemia of human patients during early LTBI stage. We also identified two separate cases of LTBI reverting to active disease months before clinical symptoms were produced by the patients.

MAP was also detected during the same double blind trial unexpectedly in a human patient with sarcoidosis. The result was produced after plaques were produced on the standard phage assay from the blood of this patient which were shown to be MTBC negative. Further PCR analysis identified the plaques as deriving from S-Type MAP.

S-Type MAP was also shown to cause a cross reaction with the tuberculin skin test in bison which were mistakenly sent to slaughter due to the false positive TST results.

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List of abbreviation

AFB – Acid-fast bacilli

Asn – asparagine

AUC – area under curve

BCG – Bacillus Calmette-Guérin

COVID-19 - coronavirus disease 2019

CRP – C-reactive protein

CSF – cerebral spinal fluid

DEFRA – Department of Environment, Food and Rural Affairs

DMSO – dimethyl sulfoxide

DNA – deoxyribose nucleic acid

ELISA – enzyme linked immunosorbent assay

FAS - ferrous ammonium sulphate

FRET – fluorescent resonance energy transfer

GFP – green fluorescent protein

Gln – glutamine

Glu – glutamic acid

HIV - human immunodeficiency virus

IAC – internal amplification control

IGRA - Interferon-γ Release Assay

IL(-1β) – interleukin(-1β)

LAMP-1 – lysosome-associated membrane protein-1

LJ – Lowenstein-Jensen

LOD – Limit of detection

LTBI – Latent tuberculosis infection

MAC – *M. avium* Complex

MAP – *M. avium* subspecies Paratuberculosis

MALDI-TOF MS - matrix associated laser desorption/ ionization – time of flight mass spectroscopy

MGB – minor groove binder

MGIT - mycobacterial growth indicator tube

MTB - *M. tuberculosis*

MTBC – *Mycobacterium tuberculosis* complex

NCBI – National centre for biotechnology information

NTM – Non-tuberculous mycobacteria

NAATs - nucleic acid amplification technologies

OTF – Officially tuberculosis free

ORFS – Officially tuberculosis free suspended

PBMC - peripheral blood mononuclear cells

PMMS - peptide mediated magnetic separation

PPD – purified protein derivative

pTB – pulmonary tuberculosis

RBC – red blood cell

REA – restriction enzyme analysis

ROC – receiver operating characteristic

RPA - Recombinase Polymerase Amplification

RT-PCR – Real Time polymerase chain reaction

SICCT - Single Intradermal Cervical Comparative Test

Subsp. – subspecies

tRNA – transfer ribose nucleic acid

Trp – tryptophan

Tyr – tyrosine

TB – Tuberculosis

TST - Tuberculin Skin Tests

QFT - QuantiFERON-TB-GOLD

qPCR – quantitative polymerase chain reaction

ZN – Ziehl-Neelsen

CHAPTER 1

1. INTRODUCTION

1.1. IMPORTANCE OF MYCOBACTERIAL DISEASE

The genus *Mycobacterium* comprises over 150 different species of mainly environmental bacteria but includes several pathogenic species (King *et al.*, 2017). Genetically, mycobacteria are grouped with the Gram-positive bacteria, however the peptidoglycan layer is characteristically enveloped by a thick, waxy cell wall that is rich in mycolic acids which resists the dyes used in the Gram stain and also cannot be decolourized with acid after staining with carbol fuschin and, as such, these organisms are termed acid-fast bacteria Maitra et al. (2019). As a member of the *Actinobacteridae* group, these organisms also have a high GC content of between 62-70 % (Saviola and Bishai, 2006), are non-motile, non-spore forming bacilli and are aerobic or microaerophilic organisms (Percival and Williams, 2014). Mycobacteria are often split into two different physiological groups: fast-growing and slow-growing mycobacteria (Arnold, 2007). The fast growing mycobacteria are generally saprophytic bacteria and produce colonies within seven days during laboratory culture and do not usually cause disease in immunocompetent hosts (Chevalier *et al.*, 2014), whereas the slow-growing mycobacteria are often pathogenic and usually require a minimum of ten days to produce visible colonies on laboratory media (Cook *et al*., 2009), however there are exceptions to both groups, such as, fast growing mycobacteria that do cause active disease and slow growing mycobacteria that are not pathogenic. The pathogenic mycobacteria typically produce severe and usually chronic diseases in a wide range of host species for example tuberculosis (in many animals), leprosy (humans) and Johne's disease (livestock) (Pierce, 2009).

The most clinically relevant group within the *Mycobacterium* genus are the closely related *M. tuberculosis* complex (MTBC) bacteria. A bacterial complex is a group of

bacterial species that are interrelated and share common traits and pathogenicity. In regard to the *M. tuberculosis* complex, the members of the group share a 99.9% genome similarity. A bacterial complex is a group of genetically very similar organisms. Bacteria within this group cause the disease tuberculosis (TB) in a large and diverse range of hosts (Sykes and Gunn-Moore, 2014). The MTBC is comprised of the following organisms (and primarily infect): *Mycobacterium tuberculosis* (humans), *Mycobacterium africanum* (humans), *Mycobacterium bovis* (cattle), *Mycobacterium microti* (rodents), *Mycobacterium canettii* (humans), *Mycobacterium caprae* (goats), *Mycobacterium pinnipedii* (seals), *Mycobacterium suricattae* (meerkats), *Mycobacterium mungi* (mongoose), *Mycobacterium dassie* (hyraxes), *Mycobacterium oryx* (bovids) (Velayati and Farnia, 2017). *M. tuberculosis* (MTB) was isolated and shown to cause TB by Koch over 100 years ago (Koch, 1882) however, despite this early discovery, TB annually still causes more fatalities worldwide than any other infectious agent although that looks set to change. In 2019 an estimated 10 million new cases of TB were reported and 1.4 million people died from the disease (WHO, 2020). In 2020, the coronavirus disease 2019 (COVID-19) pandemic claimed 1.28 million lives (Pifarré i Arolas *et al*., 2021) and with the current trends, looks set to overtake TB deaths for 2021, and as such, TB and COVID represent the two largest causes of death by infectious agents. One of the unexpected consequences of the pandemic was the reduction in global TB diagnosis and treatment which has been predicted to cause an increase in global TB deaths of 20 % over the subsequent 5 years. The lockdown measures are also expected to increase transmission rate of TB due to the increased prolonged contact between people with active pulmonary disease and non-infected individuals (Wingfield *et al*., 2021). Co-

infection of TB and coronavirus disease 2019 (COVID-19) has been suspected of resulting in increased mortality rates although the extent of the synergy between the two pathogens is not yet fully understood (Mousquer *et al*., 2021). A well-known symbiotic relationship with *M. tuberculosis* (which is especially dangerous in humans) is the co-infection with human immunodeficiency virus (HIV) since an intact cellular immune response is required to control TB infection. A symbiotic relationship is an association between two or more different organisms that confer a mutual benefit to at least one member. In this case, a HIV infection causes a decrease in CD4+ T-cells which are crucial in the control of TB (Pawlowski *et al*., 2012) this then increases the pathogenicity of *M. tuberculosis* allowing it to produce acute, disseminated disease more easily than if it infected a HIV negative human. Another co-infection with TB that is believed to increase the pathogenicity of each infecting organism is that with malaria. Malaria is caused by various different species of parasites. Both TB and malaria are believed to reduce the levels of CD4 and CD8 T-cells in the body and thus, retard a co-infection would further retard the host immune response compared to a human who is infected with only one of the two pathogens. A study in Uganda showed a prevalence of TB and malaria co-infection of 1.8 %, and a triple infection with HIV at 0.83 %, despite the large amounts of cases of all three pathogens in the country. The low incidence of co-infection could mean the opposite and that infection with TB or malaria (or HIV) reduces the likelihood of co-infection with each other, by an unknown mechanism (Baluku *et al*., 2019). There are no known reservoirs of *M. tuberculosis* other than humans, but it is known that each member of the MTBC is able to infect many different species other than its primary identified host. For example *M. tuberculosis* has been isolated from cattle (Ameni *et al*., 2011),

canine and porcine sources, amongst others, although it is noted that where *M. tuberculosis* has been isolated from non-human animals, this usually occurs when the animals have been in close and prolonged contact with humans (LoBue *et al*., 2010).

The success of *M. tuberculosis* as a human pathogen is derived from the organism's complex interaction with its host and the ability of these bacteria to adapt to, and counteract, the host immune system. After the host has become infected, one of three outcomes is seen: firstly the innate immune system can clear the infection and the host remains healthy; secondly, the innate immune system may fail to clear the infection but successfully contains the infection and the bacteria become 'dormant' and produce a latent TB infection (LTBI; a latent TB infection is an infection with TB causing organisms that produce no clinical signs of infection or dissemination due to containment of the organisms, see below); the final outcome is that the *M. tuberculosis* will overwhelm the immune system and progress to active pulmonary disease and the host will require anti-mycobacterial drug therapy to clear the infection. Although the bacteria are often described as dormant in a latent TB infection, it is believed that the organisms enter a different state of growth and although they slow down growth, the do still grow. It has also been shown that *M. tuberculosis* sheds its cell wall in granulomas and continues to divide, these cells are not observed using the Ziehl-Nielson stain and require different staining methods to visualize them (Orme and Basaraba, 2014) For the rest of this thesis, when the term dormant is used, it refers to this altered growth state. The main way these bacteria evade the immune system is by replicating within host macrophages. After *M. tuberculosis* is engulfed by the macrophage the bacteria prevents maturation of the

phagolysosome and then escapes into the macrophage cytoplasm (Queval *et al*., 2017). At this stage of the infection, the host's immune system typically produces granulomas which are dynamic groups of different immune cells that develop and evolve to contain the infection and prevent the dissemination of the bacteria and reduce disease progression. When this is successful a LTBI is developed, and the host will produce no symptoms and remain healthy. The recruitment of immune cells to the granuloma however can produce a suitable environment to allow the bacteria to proliferate within the cells, escape from the granuloma and go on to cause active pulmonary disease (Russell *et al*., 2009, Zhai *et al.*, 2019). During the development of the granuloma, macrophages infected with *M. tuberculosis*, upregulate the production of the cytokine vascular endothelial growth factor (VEGF) which induces angiogenesis and increases the growth of vascular and lymphatic vessels to the granuloma. The increase in blood vessels to the granuloma benefits the mycobacteria in two ways, firstly, it increases the amount of immune cells recruited to the granuloma, and secondly, it provides pathways of escape from the granuloma and dissemination throughout the body (Batista *et al*., 2020). In respiratory tuberculosis the lungs are the primary site of infection and in gastric tuberculosis the digestive system is the primary site of infection. Regardless of the primary site of infection, the lymph nodes are often secondary infection sites. In respiratory tuberculosis infection of the lymph nodes are likely to arise from the increased lymphatic system to the granuloma providing direct access for extracellular mycobacteria or by dendritic cells which have phagocytosed mycobacteria and are traveling to display antigens to lymphocytes within the nodes. The infected macrophages can be released from the granuloma and are then able circulate

throughout the body via the blood stream (Pai *et al*., 2016, van Leeuwen *et al*., 2018), resulting in the spread of the mycobacteria from the site of infection to different organs of the host (Loddenkemper *et al*., 2015). Haematogenous dissemination has been reported within macrophages during the early development of the granuloma (Balasubramanian *et al.*, 1994). A study in a zebrafish model showed that ESX-1 virulence factor (present within the RD1 region of MTBC) secreted by *M. marinum* (used as the model organism to produce TB and infect the zebrafish embryos) attracts macrophages and dendritic cells to the developing granuloma which are able to migrate through the granuloma structure, the macrophages are induced to become hyper mobile in an RD1-dependent manner to increase motility within the granuloma (Ramakrishnan, 2012). These phagocytes then become infected with *M marinum* or phagocytose apoptotic macrophages containing the bacilli and are then able to exit from the granuloma into the blood stream and/ or lymphatic system and disseminate to other areas, providing evidence that this may also occur with *M. tuberculosis* in humans. However, this still needs to be proved because the zebrafish model can only provide proof of concept for human tuberculosis due to the divergence of *M. marinum* from *M. tuberculosis* and that zebrafish are an imperfect model that doesn't produce an identical response to human cells even though the reactions can be similar (Davis and Ramakrishnan, 2009). This is further complicated due to the fact that fish immunology cannot be directly compared to mammalian immunology again due to the divergence and difference in the immune systems and responses. Another pair of cells that can migrate through a granuloma are mesenchymal stem cells and hematopoietic stem cells. These cells can be infected by *M. tuberculosis* and exit the granuloma and

reenter the circulatory system on their way back to the bone marrow. This means that viable MTBC could be present in any cell type when the infected stem cells differentiate (Mayito *et al*., 2019). In this thesis, the term viable refers to cells that are metabolically active and non-viable are those that are non-metabolically active and therefore not detectable by the use of phage. Another study using a guinea pig model, showed that as the granulomas' necrotic centers begin to develop and subsequently start to mineralize, a rim of highly eosinophilic debris is produced between the area of non-viable necrotic immune cells and the viable cells immune cells and is found to contain acid fast bacilli (AFB). As the necrosis develops and expands the AFB disappear, however the use of alternative staining procedures shows that the bacteria are still present but have lost their acid fastness and are actually found throughout the rim and the necrotic centre as small clumps of bacteria (Orme and Basaraba, 2014). Orme and Basaraba (2014) believe that the pressure produced within the granuloma by the mineralization process causes the necrotic centers to leak out of the granulomas and release the bacteria into the surrounding tissue where they can directly enter the circulatory system or become phagocytosed by macrophages and dendritic cells and enter the circulatory and lymphatic systems, and potentially produce a new infection site and reactivate disease. In this model, during a LTBI infection the bacteria aren't truly dormant but replicating with a different phenotype that is not detectable with the standard ZN-staining. Most humans that harbor an LTBI maintain this state for their entire lifetime and never progresses to active disease. Certain risk factors such as diabetes, HIV infection and smoking for example are implicated in the reactivation of LTBI into

active disease amongst others and approximately 5-15 % of LTBI reactivate and progresses to active disease. (Kiazyk and Ball, 2017).

Another important member of the MTBC is *M. bovis*, which is the main causative agent of TB in cattle (Allen *et al.*, 2018). It is also a known zoonotic disease and also readily infects many species and displays the largest host range of all the MTBC bacteria (O'Reilly and Daborn, 1995). A zoonotic infection is one that is naturally transmissible between humans and other animals, transmitting in either direction (Rahman *et al*., 2020). Unlike *M. tuberculosis*, the control of *M. bovis* in an ecosystem is particularly difficult due to the many reservoirs for the organism and because interspecies transmission is common in animals that share an ecosystem (Gormley and Corner, 2018). Haydon *et al*. (2002) are quoted as defining a reservoir as 'one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population'. Preventing the transmission of *M. bovis* to livestock from wild animals or from livestock to wild animals relies primarily on preventing the two from sharing the same space and constant monitoring of livestock for new infections. In terms of control of bovine tuberculosis it is just as important to stop the spread of *M. bovis* to livestock as it is from livestock to pass it on the other animals in the ecosystem.

There are many other clinically significant mycobacterial pathogens other than members of the MTBC. The group are classed as non-tuberculous mycobacteria (NTM). Within the agricultural sector, one of the most significant NTM is *Mycobacterium avium* subsp. Paratuberculosis (MAP). MAP is a member of the *M.*

avium complex (MAC) group of bacteria and causes Johne's disease in many ruminants including: cattle, sheep, deer and bison (Fawzy *et al.,* 2013). MAP was first isolated and identified as the causative agent of Johne's disease in 1895 (Johne and Frothingham, 1895). Johne's disease is a chronic wasting disease that leads to severe weight loss, reduced milk yields and eventually premature death of the animal (Orpin *et al*., 2020). Currently there is no perfect test for the diagnosis of Johne's disease (Baumgartner and Khol, 2006), compared to MTBC of which culture of the organisms is the accepted way to confirm presence of the organisms. Sub-clinical infections, which are infections that are asymptomatic and produce no apparent disease in the host (Frascella *et al*., 2020), are often missed by existing tests due to the low presence of organisms and poor sensitivity of the available testing kits (in this case for: faecal culture; see section 1.2.1., an IGRA and a ELISA; see 1.2.5 for both), especially during the early stages of detection (Magombedze *et al*., 2017). A study in Uruguay showed the incidence of MAP (44%) compared to TB (56%) is lower in cattle (Picasso-Risso *et al*., 2019). This could indicate that the rate of transmission is therefore lower when compared to TB.

Unlike MTBC, the main route of infection for MAP is through the digestive tract, although gastric tuberculosis is not uncommon in livestock. In infected animals, high cell numbers can be shed in the faeces during the disease progression which contaminates grazing land and provides the ideal mechanism for horizontal transmission by oral ingestion (Whittington *et al*., 2019). Once MAP has been ingested, it invades the host by translocating through the intestinal mucosa by the M cells present in Peyer's patches and also through enterocytes (Bermudez *et al*., 2010). During the invasion of these cells the pathogen and host interact with each

other and interleukin-1β (IL-1β) is produced by the host enterocyte which leads to the recruitment of phagocytic cells to the site of infection (Bannantine and Bermudez, 2013). Once MAP has been phagocytosed it can also survive and replicate within macrophages, as described for members of the MTBC (Abendaño *et al*., 2014, Tanaka *et al*., 2005). MAP is contained within the phagosome where it prevents its maturation into a phagolysosome by inhibiting the binding of lysosome-associated membrane protein 1 (LAMP-1) to the membrane of the phagosome which is one of the components required to allow the fusion of the lysosome (Huynh *et al*., 2007), this helps the bacteria to remain in a favourable environment protected from the antimicrobial elements of the (innate) immune system (Hostetter *et al.*, 2003, Rumsey *et al*., 2006). MAP, again, just as members of the MTBC, prevent the infected macrophage from undergoing apoptosis and preventing bacterial death by efferocytosis (phagocytosis and degradation of apoptotic cells) and increasing the 'life span' of the host macrophage to provide the longest possible time for MAP to replicate safely within the cell (Kabara and Coussens, 2012). Growth and survival within macrophage also provides the basis for mycobacteraemia and dissemination of MAP to different areas of the body (Rathnaiah *et al*., 2017). MAP can be disseminate to the mammary gland and is able to infect bovine mammary epithelial cells and persists inside vacuoles (Patel *et al*., 2006, Lamont *et al.*, 2012) and then the organisms can be shed directly into milk, providing a route for vertical transmission to calves (Sweeney, 2011). MAP being shed into milk provides concern as there is evidence that MAP may be an etiological agent of Crohn's disease in humans, a disease that shares pathological and clinical features with Johne's disease (Honap *et al*., 2020, McNees *et al*., 2015) Since it has been shown that viable MAP

can be detected in pasteurized milk (Ayele *et al*., 2005, Gerrard *et al*., 2018, Grant *et al*., 2002a, Grant *et al*., 2002b, Paolicchi *et al*., 2012) this may be a major route of exposure of humans to this potentially zoonotic agent.

Over the recent years, there has been a notable increase in the rise of NTM infections worldwide, particularly lung infections. Several studies across the globe have been performed over the past 30 years, all of which show at least a two times increase in NTM lung infections when subsequent follow-up studies have been performed. For example, a UK study showed an increase in NTM lung infections of more than three times between 1995 and 2006 from 0.9 cases/100,000 to 2.9 cases/100,000 respectively. Cases have increased in the United States of America, Canada, Europe, China, South Korea, Australia, amongst others (Ratnatunga et al., 2020). Humans and NTM cohabit many of the same areas, NTM are found in soil and on vegetation which makes it likely for humans to be exposed to these organisms (Aboagye *et al.*, 2016). However, watercourses are other frequent habitats for members of the NTM, including man-made niches such as within biofilms within plumbing, taps and other household water outlets (Honda *et al.*, 2016). Detectable levels of NTM have also been identified from aerosols produced by showerheads (Thomson *et al*., 2013), meaning that humans are at a high risk of exposure to NTM. The NTM *M. marinum,* is often isolated from home aquariums and produces a TB-like infection in fish and a granulomatous soft tissue infection in humans (Aubry *et al*., 2002). Studies have also shown that NTM have a relationship with free living amoebae in water which further complicates the rise in NTM infections. Amoebae are waterborne organisms and feed on bacteria. They can be found in two different

forms, as trophozoites, which are mobile and feeding amoebae or they develop in cysts which are a resistant form of the organism which they produce under unfavourable conditions. NTM are able to avoid digestion by amoebae and live either a symbiotic or parasitic life with its amoebae host. *M. avium* has been shown to survive in several different amoebae and in the study, every amoebae that was used, but especially in *Acanthamoeba lenticulata*, which can be found in potable water samples (Ovrutsky *et al*., 2013). *M. bovis* has also been shown to survive and persist inside amoebae and can survive for over 60 days in cysts, which further complicates the control and spread of *M. bovis* in the environment as they are able to survive in drinking water for non-human animals using amoebae as vectors (Sanchez-Hidalgo *et al*., 2017). The emergence of NTM as opportunistic pathogens, and the fact that they thrive in man-made niches, presents an ever increasing threat to our aging population in particular, since it is the elderly (Mirsaeidi *et al*., 2014), those with a compromised immune systems - such as those with HIV infections (Lapinel *et al*., 2019) or take medication that suppresses the immune system (Henkle and Winthrop, 2015) that are the more likely to be infected and suffer from a pulmonary or disseminated NTM infection

1.2. REVIEW OF EXISISTING TESTS INCLUDING LIMITATIONS

Humans have a close and long running history with TB (Barberis *et al*., 2017). We have spent over a century investigating the disease to identify successful treatment and eradication programs to control and effectively cure active cases of pulmonary TB (Daniel, 2006). Despite this, the detection and diagnosis of TB is still not an exact science and still relies on the results of a combination of tests (Loddenkemper *et al*., 2015), which are reviewed in this section.

For the identification of active pulmonary TB infections in humans, there are two primary methods of diagnosis described by the NHS in the UK, the first of which is the culture of viable MTBC from respiratory tract or other clinical samples. The second is radiographic images produced from a chest X-Ray. However, the results from the chest X-Ray are often ambiguous and further tests are needed to confirm the disease. There are ancillary tests that are also used alongside culture and chest X-ray to provide further evidence for active pulmonary TB, as they although they are the two primary tests it can take either a long time for results (culture) or the results are ambiguous (chest X-ray), so diagnosis usually relies on a battery of tests before confirmation by culture. These test are performed on sputum or branchial lavage fluid from the patient and include Ziehl-Neelsen staining for the identification of acid fast bacilli (ABF; see section 1.2.1), traditional culturing techniques for *M. tuberculosis* or the use of automated culture methods such as with the mycobacterial growth indicator tube (MGIT) culture system and PCR based methods such as the XPERT systems (Al-Ubaidi, 2018, NHSUK, 2019).

The current method for the confirmation depends on what type of infection the patient has, for instance a LTBI usually presents no symptoms and diagnosis relies on identifying an immune response to TB antigens. This can be by performing a tuberculin skin test (see section 1.2.4) and/or an Interferon-γ Release Assay (IGRA; see section 1.2.5). Due to the limitations of these methods, often cases are treated prospectively before the test results have confirmed the patient has TB, and many different assays have been developed for the rapid and sensitive detection of mycobacteria (Dinnes *et al*., 2007) to try and improve this situation.

1.2.1. AFB staining

In humans, the AFB staining and culture methods are performed on sputum or bronchial lavage fluid, usually extracted at the same time and portioned for each test. First the samples are chemically decontaminated to inactivate the commensal organisms that are present in the samples which would overgrow the slow growing mycobacteria. The mycobacteria present in the sample are relatively resistant to these chemical decontamination methods, however it does also result in a reduction in the number of viable mycobacteria remaining in the sample, and different processing methods have been shown to reduce the viability of mycobacteria more than others (Murray *et al*., 2003). In particular the standard NALC-NaOH decontamination method results in a large decrease of viable mycobacteria (Asmar and Drancourt, 2015a), which reduces the sensitivity of downstream assays. After the sample has been decontaminated, it is concentrated and is standardly stained using the Ziehl-Neelsen (Z-N) method. Briefly, Z-N stain comprises an initial staining step with carbol fuchsin-phenol solution which is then heated by steam to allow the phenolic carbol fuchsin to better penetrate the mycobacterial cell wall. The sample is then decolourized with acidified alcohol and counterstained with methylene blue before being imaged using a light microscope (Weldu *et al*., 2013). A cold version has also been adapted from the Z-N stain named the Kinyoun stain which uses a more concentrated solution of carbol fuschin and phenol to penetrate the cell-wall of the mycobacteria without heat (Kurup and Chester, 2014). Mycobacteria appear as a red rods against a blue background when present in clinical samples (Holani *et al*., 2014). The main benefit of the Z-N stain is that it is a low cost assay to perform, however it has been reported to have a low sensitivity as it requires a high bacterial load of 1

$x10^4$ to 1 $x10^5$ cfu ml^{-1} to produce a positive result (Singhal and Myneedu, 2015) and results are variable based on the staining technique and the operator (Dzodanu *et al*., 2019).

Another, more sensitive, stain is the auramine-rhodamine stain which uses a fluorescent dye but then requires the use of a fluorescent microscope and dark room. The staining method varies from laboratory-to-laboratory but generally a heat fixed smear is flooded with auramine-rhodamine reagent and incubated for 15 minutes at room temperature before the slide is then washed with purified water and decolourized with acidified alcohol solution. A counterstain of potassium permanganate solution is applied before the samples are examined with a fluorescent microscope for the presence of AFB (Annam *et al*., 2009). Mycobacteria (AFB) are detected as yellow/orange fluorescent rods against a dark background (Holani *et al*., 2014). The results using either staining technique only identify AFB and are not species-specific so further tests, such as culture based-methods or PCR identification are used to confirm the presumptive positive result gained from the staining (Kurup and Chester, 2014).

1.2.2. Culture

Solid culture methods use either Lowenstein-Jensen slopes (LJ) which is an egg based solid medium or agar based solid media such as the Middlebrook 7H10 or 7H11 agar which has been shown to produce increased growth rate of MTBC compared to LJ media (Caulfield and Wengenack, 2016). The average time to produce a positive result for *M. tuberculosis* by culture for the agar based media is between 22.5 and 32 days and solid cultures are typically kept for 8 weeks before they are classed as

negative and discarded (Rageade *et al*., 2014). For *M. bovis,* the time for positive culture is longer, a study of bovine TB extracted from bovine tissues using different solid media showed some colonies appearing after 2 weeks, the majority of the positive results were gained by 8 weeks but it took up to 15 weeks for all the positive samples to produce colonies (Corner *et al*., 2012). Culture time is even longer for other species in the MTBC such as *M. microti* which takes between 6 – 18 weeks (Boniotti *et al*., 2014). AFB-positive patients can produce a visible MTBC colony within seven to nine days due to the high bacterial load of the sample, AFB-negative patients may take up to eight weeks to produce a visible colony (Chen *et al*., 2018). If colonies are produced, the morphology is recorded and a series of biochemical or PCR-based confirmation tests are performed (Pfyffer and Wittwer, 2012), the results of which vary between the different species of MTBC and different NTM species (Ribón, 2012).

Liquid-based diagnosis offers a faster time to detection than solid media. Standard liquid culture can produce a positive result in 10-14 days when using a media such as Middlebrook 7H9 (Rageade *et al*., 2014), however these cultures are more susceptible to contamination than solid media and also it is not possible to discern whether a positive culture is a monoculture or mixed culture and further sub-culturing on solid media is required to determine whether the culture is pure (Ryu, 2015). The BACTEC 960/ MGIT (Becton Dickenson; UK) system is a fully automated system that provides a continuous detection system for slow-growing mycobacteria by producing a detectable fluorescence when O_2 levels decrease due to the aerobic respiration of the bacteria growing in the modified Middlebrook 7H9 media within the tube (Tortoli *et al*., 1999). A meta-analysis was performed to determine the

reported sensitivity and specificity of the system and produced results of 81.5 and 99.6 %, respectively (Cruciani *et al*., 2004). However, before a sample can be added to the MGIT it also needs to be chemically decontaminated to remove all other microorganisms other than mycobacteria that may be in the sample to avoid false positive results, which has the same effect on sensitivity as described above for solid culture systems (Tortoli *et al*., 1999).

New culture methods have been developed to reduce the growth time of MTB and utilize novel agar-based media which show superior growth times compared to the Middlebrook 7H media. Detection of colonies was reduced further by detecting micro colonies which are invisible to the naked eye by using microscopy to further decrease time to detection (Asmar and Drancourt, 2015b). Ghodbane *et al*. (2014) further reduced time to detection from clinical sputum by using a novel media, supplementing with ascorbic acid and using an autofluorescence detector to produce detectable colonies after an average of 4.37 days, with the quickest time to detection being three days of incubation. It is of note though, that when Robert Koch originally isolated *M. tuberculosis* he used a solidified blood serum media which typically produced colonies within ten days, and by using microscopy at x 30-40 magnification he could detect growth of micro colonies within seven days (Koch, 1882).

1.2.3. MALDI-TOF MS

Matrix associated laser desorption/ ionization – time of flight mass spectroscopy (MALDI-TOF MS) analysis of presumptive mycobacteria colonies has also been developed in order to allow rapid identification of the species of colonies of mycobacteria recovered (Saleeb *et al*., 2011, Zhang *et al*., 2014). These tests are

almost exclusively performed following culture of sputum or bronchial lavage fluid, both of which require a decontamination step to reduce the likelihood of contamination produced by the patient's microflora which are also contained in these fluids, and as described above results in a loss of sensitivity (Chatterjee *et al*., 2013, Hou *et al*., 2019). MALDI-TOF MS has been used in conjunction with the BACTEC MGIT culture system (Becton Dickenson; UK) to identify the species of mycobacteria detected in positive MGIT assays from clinical samples. The study correctly identified 100 % of the *M. tuberculosis* isolates, 85 % of the slow growing NTM and 75 % of the rapid growing NTM. This showed that the use of MALDI-TOF MS is a useful tool for the detection of *M. tuberculosis* in combination with automatic culture systems and could be used to identify the majority of NTM isolates, depending on what reference library is used to compare the MALDI-TOF MS data to (Alcolea-Medina *et al*., 2019).

1.2.4. Tuberculin Skin Tests

Tuberculin Skin Tests (TST) are used both in animals and in humans to determine whether the test subject has been exposed to a member of the MTBC. There are two TST that are used in cattle, the Caudal Fold test (Buddle *et al*., 2015) and the Single Intradermal Cervical Comparative Test (SICCT) (Awah-Ndukum *et al*., 2016, Busch *et al*., 2017), and in humans the Mantoux test (Loh, 2011). The TST is an *in vivo* test that involves the intradermal injection of antigens derived from the suspected infecting organism. An immune response, which indicates prior exposure to the organism, manifests as a skin lump which is recorded two to three days post injection (Whelan *et al*., 2010, Nayak and Acharjya, 2012). Results are often determined comparatively (with the exception of the Caudal Fold test which only uses one injection), by

comparing the level of reaction to extracts of antigens from other types of mycobacteria; in the SICCT test, bovine PPD is compared to PPD derived from *M. avium* and in the Mantoux test uses a range of NTMs (Nayak and Acharjya, 2012). For the TST tests, different cut off values can be defined as a method of increasing sensitivity at a cost of specificity or *vice versa* (Awah-Ndukum *et al*., 2016, Nayme *et al*., 2012) see section 6.2.1. The SICCT for cattle has been reported to have a sensitivity between 52 and 100 % and a specificity between 99.91 and 100 % (Karolemeas *et al*., 2012, O'Hagan *et al*., 2019). However, TSTs are susceptible to cross reacting with environmental mycobacteria, and can produce false-positive results (Barry *et al*., 2011, Broughan *et al*., 2016, Mohamed, 2017). A meta-analysis was performed by the Animal and Plant Health Agency (APHA) which looked at the sensitivity and specificity of the SICCT in cattle. The study produced results for sensitivity of 0.5 under standard interpretation, showing that the test has a 50 % chance of identifying a correct result (Nuñez-Garcia *et al*., 2018). This shows that the tests are not ideal and that further development or new tests are required to accurately identify cases of bovine TB in cattle; poor identification of positive cases hampers the efforts to control the spread of bovine TB and majorly inhibits the eradication plan. The main drawback of TST in humans is that they produce lower sensitivities in BCG-vaccinated populations and cannot distinguish between BCG vaccination and exposure to MTBC (Jones *et al*., 2017, Slogotskaya *et al*., 2018).

1.2.5. IGRA and ELISA

Another form of immunological assay that is also a rapid diagnostic method for TB and bovine TB is Interferon-γ Release Assays (IGRA). Interferon-γ Release Assays are *in vitro* tests and require a blood sample to be taken from the test subject. The assay

works by stimulating peripheral blood mononuclear cells (PBMCs) which are purified from the blood sample with antigens derived from the organism being tested for, for example; antigens extracted from *M. tuberculosis*. If the test subject has been exposed to the bacteria, then Interferon-γ is released from the PBMCs. This shows that the cells have already produced an adaptive immune response to these antigens so the test subject has already been exposed to the organism and antigens tested against. The response that is produced is often detected using a colorimetric assay (Pai *et al*., 2014), for example an Enzyme linked immune-sorbents assay (ELISA; see below). The two main commercial IGRA tests for humans that have been developed are the QuantiFERON-TB-GOLD test (QFT; QIAGEN; UK) and the T-SPOT.TB (Oxford Immunotec Ltd; UK). The benefit of IGRA compared to the TST is that it can differentiate between BCG-vaccination and exposure to MTBC. The reason for this is that the TST uses purified protein derivatives which are essentially a purified cocktail of hydrolyzed proteins, these proteins are found in members of the MTBC, the BCG-vaccination strains and some NTM. The two commercial IGRAs use different, more specific antigens which are derived from the region of difference-1 (RD-1) which is only found in members of the MTBC. The BCG-vaccination strain has lost this region due to repeated culturing in the laboratory and it is the loss of this region which renders it safe to use as a vaccine, vastly reducing its pathogenicity (Pai *et al*., 2014). However, both commercial IGRA have also been reported to produce false-positive results with NTM infections, indicating that some NTM species also contain RD-1 or a region of difference that is similar enough to produce a cross reaction with (Hermansen *et al*., 2014, Wang *et al*., 2016). In animals, the BOVIGAM IGRA has been developed and has been reported to produce a sensitivity of 95.7 % but also has a

low specificity and therefore cannot be used as a routine screening tool to detect infection. Specificities as low as 6.9 % have been reported, (Pucken *et al*., 2017) but other studies reported specificities of 97.2 and 98.6 %, depending on what cut off value was used for a positive response (Martucciello *et al*., 2020). Due to the fact that there are different cut of values that are used depending on the TB status of the farm, it makes comparisons between sensitivity and specificity more challenging. The literature also shows a large variation in specificity, indicating again that the tests are not accurate enough to be able to effectively call the correct result, as they are likely to produce a false positive result.

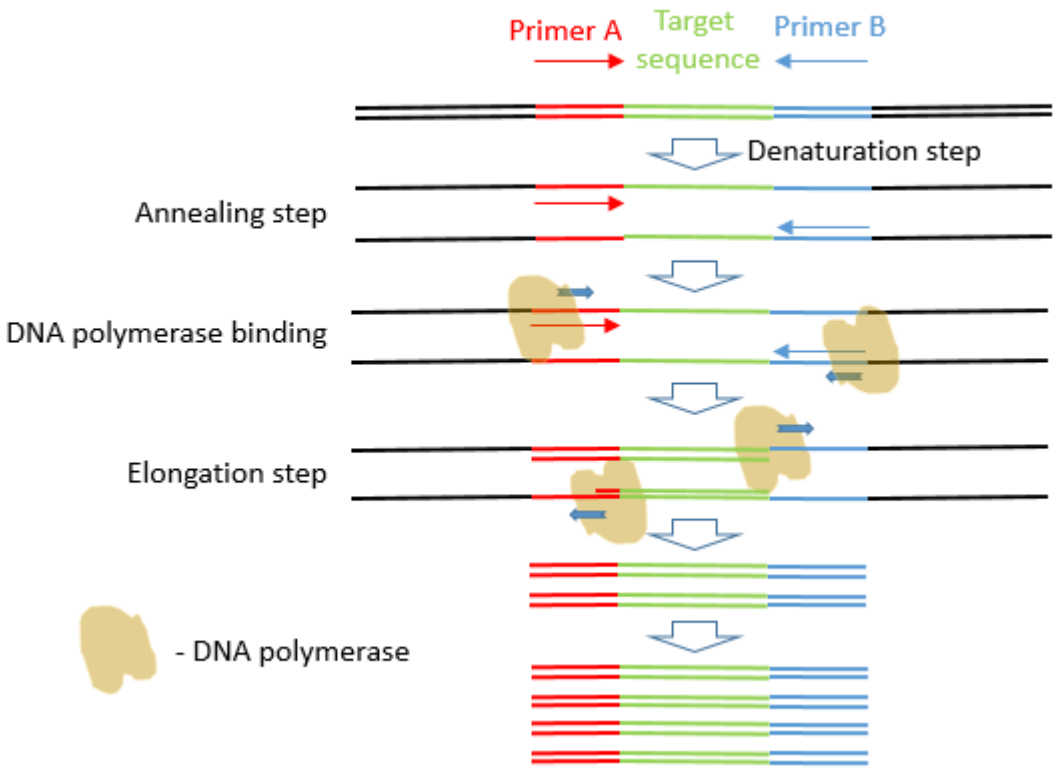
There have been many ELISA tests devised for the diagnosis of TB, and commercial ELISA for human TB have been developed that detect antibodies to *M. tuberculosis* from a subject's blood serum and can usually produce a positive result in hours. A comparison between three of these commercial ELISA designed for use with humans showed a range of sensitivities and specificities of between 5.6 to 83.3 % and 72 to 100 %, respectively. The main limitation of an ELISA in regards to TB diagnosis is that results have been inconsistent for patients co-infected with HIV, and a reduced sensitivity has been reported under those conditions (Anderson *et al*., 2008). The reason for the reduced sensitivity is that the HIV virus infects white blood cells, specifically T-cells, which it uses to produce progeny virus. Over time, this results in a reduction in T-cells due to the increased burden on the cells from the replicating virus within. Out of all of the peripheral blood mononuclear cells, T-cells produces some of the highest amounts of IFN-γ (along with natural killer cells and macrophages), so a reduction in these cells results in a reduction of IFN-γ in the ELISA and therefore a reduction in sensitivity of the assay (Ethuin *et al*., 2004). More

recently tests have been developed that detect secreted MPT64 protein produced by viable MTBC organisms in sputum (Wang *et al*., 2020).

1.2.6. Molecular assays

Molecular assays have been developed that also offer rapid methods of detecting mycobacteria. Molecular assays detect the DNA of the infecting organism and, as such, only confirm that DNA from the organism is present in a sample. The limitation here is that non-viable bacteria will still produce a positive test result (Cangelosi and Meschke, 2014). The problem with this is if the immune system clears the infecting organisms and stops an infection from taking place then the cells that have been inactivated will still produce a positive result by molecular assays and can therefore give false-positive results. The polymerase chain reaction (PCR) assay is the most used type of molecular test, and this is designed to amplify a specific unique DNA sequence known to be only present in the genome of the target organisms. The PCR assay uses oligonucleotide primers, one for the sense strand and one for the antisense strand which are designed to flank the specific DNA target sequence of interest (Fig. 1.1). The target DNA is denatured, and the primers can then anneal to the target DNA as the temperature is reduced. DNA polymerase then extends these primers to amplify the target DNA sequence between the two primers when the temperature is raised again to the optimum temperature for the thermostable DNA polymerase (72 °C for *Taq* polymerases). This three-temperature cycle is then repeated and each elongation step results in doubling the amount of the target DNA producing an exponential amplification. When a large enough quantity of DNA has been produced it can be visualized by various methods or used for other analysis or downstream assays (Coleman and Tsongalis, 2005).

Figure 1.1 – Representation of DNA amplification during a PCR assay



The diagram is a schematic representation of the PCR assay and the amplification of the target DNA during exponential amplification.

PCR based assays have the benefit that they are able to amplify different targets at the same time in multiplex PCR assays, this means that you can simultaneously test for different organisms in the same sample which provides more analytical potential when the amount of DNA available for testing is limited (Gopinath and Singh, 2009). Another benefit of a multiplex PCR is that you can test for different genes of the same target organisms, this can then increase the sensitivity and specificity. The sensitivity increases because more targets in the DNA are being detected so it is more likely that you will produce a positive result with at least one of the primer pairs, compared against a single-plex PCR which only detects one. This is not always the case though, as the cost of performing a multiplex PCR is that a compromise is often made on the parameters of the PCR so that all primer pairs used can produce a positive result. The parameters chosen may be able to produce a positive result with each of the primer pairs but may not be the optimum parameters for the individual primer pairs which will cause a decrease in sensitivity. The specificity can be increased if primer pairs targeting different genes of the same organism are used because the more of the primer pairs that produce a positive result, the more certainty can be gained that the results show the correct organism has been identified and the less likely a false positive result has been produced.

Another benefit of PCR is that you can apply it to many different sample types, from pure cultures to DNA extracted from clinical samples. When used with solid culture it is possible to produce a positive PCR assay from micro-colonies produced in culture before they reach a size visible to the naked eye, decreasing the time to produce a positive culture result (Chagas *et al*., 2010). Direct PCR from a clinical sample is also

possible and smaller numbers of bacteria are required than for AFB staining to produce a positive result due to the high sensitivity of the assay. A multiplex PCR assay described by Sevilla *et al*. (2015) designed to detect DNA from the genus of Mycobacterium (internal transcribed spacer between 16S and 23S rDNA genes), *M. avium* Complex (MAC; *IS*1311) and members of the MTBC (*devR* gene) and has been shown to detect approximately 50 fg to 5 fg of purified reference DNA depending on the species of mycobacteria tested, which equates to approximately 10-100 cells. The multiplex PCR assay was also performed on spiked clinical samples (a blend of different samples) the results showed that the detection limit of the method was 100 - 1000 cfu g^{-1} of tissue (Sevilla *et al*., 2015), which is 10 – 100-fold more sensitive than AFB staining. However, the limit of detection was less than compared to the reference DNA alone, likely due to the presence of PCR inhibitors co-extracted from the clinical samples (see below). The sensitivity of PCR assays can be improved even more by incorporating an extra amplification step, called a nested PCR (Bull *et al*., 2003); see Chapter 3 for more details). By employing this type of approach it is possible to decrease the amount of DNA needed to generate a positive signal to less than 5 fg of DNA which allows for the theoretical detection of less than one bacteria (genomic fragments) per PCR reaction (Lima *et al*., 2015).

However, PCR assays have their own draw backs and the need to extract DNA from the samples and remove inhibitors prior to starting the assay is always necessary. Clinical samples often contain many different components which act as inhibitors of DNA polymerase, preventing amplification of the DNA and leading to false-negative results (Schrader *et al*., 2012). However the ability to combine PCR reactions means that an internal amplification control can be introduced in a multiplex PCR assay (see

Chapter 3 for more details) to detect when a false-negative result is due to polymerase inhibition (Flores *et al*., 2009).

The most prominent molecular assays that have been developed for the diagnosis of active pulmonary TB in humans are the Xpert MTB/RIF and Xpert Ultra assays which are used in conjunction with the GeneXpert system (Cepheid; USA). The assays have been produced in a cartridge format that can be directly inoculated with the patient's sputum. After this, the process is fully automated and the sample is liquefied, decontaminated, and DNA extracted before the PCR assay is performed within the cartridge. The PCR assays have been designed to both detect the presence of MTBC and to determine the rifampicin resistance status of the isolate by detecting known polymorphisms in the sequence of the *rpoB* gene that result in this phenotype (Molodtsov *et al*., 2017). A review by Zifodya *et al*. (2021) showed a pooled sensitivity of the Xpert MTB/RIF assay and Xpert Ultra assay of 84.7 and 90.9 %, respectively, and a pooled specificity of 98.4 and 95.6 %, respectively. Although hugely successful, the main limitations of these assays are the cost and that they cannot differentiate between live or dead mycobacteria. Which again, means they cannot tell the difference between an infected host and a host which has successfully inactivated all the infecting organisms. The Xpert MTB/RIF assay has also been used to detect *M. bovis*-BCG spiked into blood samples. To remove the PCR inhibitors from whole blood, the red blood cells (RBC) were first lysed using a RBC lysing solution containing Triton-X. The samples were centrifuged and the supernatant discarded. The remaining pellet was then reconstituted with PBS and the Xpert MTB/RIF assay sample reagent was added to the sample. The samples were then loaded into the Xpert MTB/RIF cartridge and the standard procedure was then

performed. The results of the lysis-Xpert MTB/RIS method performed on a 1 ml blood sample spiked with *M. bovis*-BCG produced a limit of detection of 10 cfu ml^{-1} (Banada *et al*., 2013).

1.3. REVIEW OF PHAGE-BASED DETECTION TESTS

Bacteriophage (phage) are viruses that specifically infect bacteria, and infection often results in cell lysis at the end of the phage replication cycle. They have been used as a therapeutic agent for over a century (Keen, 2015) but the specificity of the viral-host interaction has also been exploited for diagnostic purposes to develop rapid methods for the detection of many different species of bacteria (Schmelcher and Loessner, 2014). When trying to use rapid DNA-based detection methods, mycobacteria are notoriously robust and difficult to lyse, as demonstrated by their resistance to chemical decontamination methods described earlier. This often reduces the sensitivity of molecular assays as the DNA remains within the intact cells after the chemical lysis step and is therefore unavailable which blocks the DNA amplification. Phage offer a revolutionary solution to this issue due to the fact that they encode lysin genes which have evolved to efficiently lyse their host cells to release the newly formed phage particles. Hence phage can be used to release the mycobacterial DNA and make it available for DNA analysis methods such as PCR assays. An additional benefit of this approach is that phage will only replicate and complete the infection cycle in viable cells, and therefore using phage as a lysis agent also differentiates between viable and non-viable cells (Stanley *et al*., 2007).

Several studies have reported using phage as a rapid detection method for mycobacteria by combining them with various nucleic acid amplification

technologies (NAATs) that are sensitive and specific. Stanley *et al*. (2007) used a commercial phage-based diagnostic assay, the *FASTPlaque*TB assay (Biotec Laboratories Ltd., UK; standard phage assay, see section 1.3.1. below), originally designed to detect MTBC from sputum samples, to detect viable MAP from milk samples by coupling the phage assay with an *IS*900 PCR assay. The assay has also been used to detect viable MAP present in purified PBMCs (see Chapter 5 for more details) from the blood of Johne's cattle, using peptide mediated magnetic separation (PMMS) beads to concentrate the MAP cells from the PBMC lysate (Swift *et al*., 2013). The PBMC faction were first lysed by osmotic shock by resuspending them in the 7H9 media used for the phage assay. In this case, a highly sensitive nested *IS*900 PCR assay (Bull *et al*., 2003) was used that could detect one MAP genome equivalent from the DNA extracted from one MAP derived plaque. However, a later study showed that the reproducibility of the combined phage-PCR method indicated that the PMMS step produced variable results in terms of recovery of MAP cells from the sample (Swift *et al*., 2016b, Swift *et al*., 2020). Swift *et al*. (2016a) provided the first evidence that viable MTBC could be detected within the PBMCs of SICCT positive cattle by using a combined phage-RPA (Recombinase Polymerase Amplification) method. The method used the standard phage assay (see section 1.3.1. below) and the DNA extracted from the plaques was amplified using RPA, which was found to be more sensitive than conventional PCR. Using this method they were able to detect as little as 1 pfu ml^{-1} of MTBC DNA from SICCT-positive cattle with non-visible legions. Swift et al. (2020) then reported a modified version of the phage assay, which has been termed the Oneday phage assay (or Actiphage® method; see section 1.3.2.). The assay can be completed and produce results on the

same day, hence why it is called the Oneday phage assay. The assay is a one tube format that does not rely on sensor cells to produce a lawn to visualize the phage infection process.

Using this novel, rapid phage method they showed that the method had increased sensitivity compared to the phage-RPA method and were able to detect MTBC in the blood of all cases of SICCT positive cattle tested that had visible legions and also detected MTBC in the blood of 93 % of the SICCT positive animals that did not have visible legions, giving a sensitivity of 95 % and a specificity of 100 %.

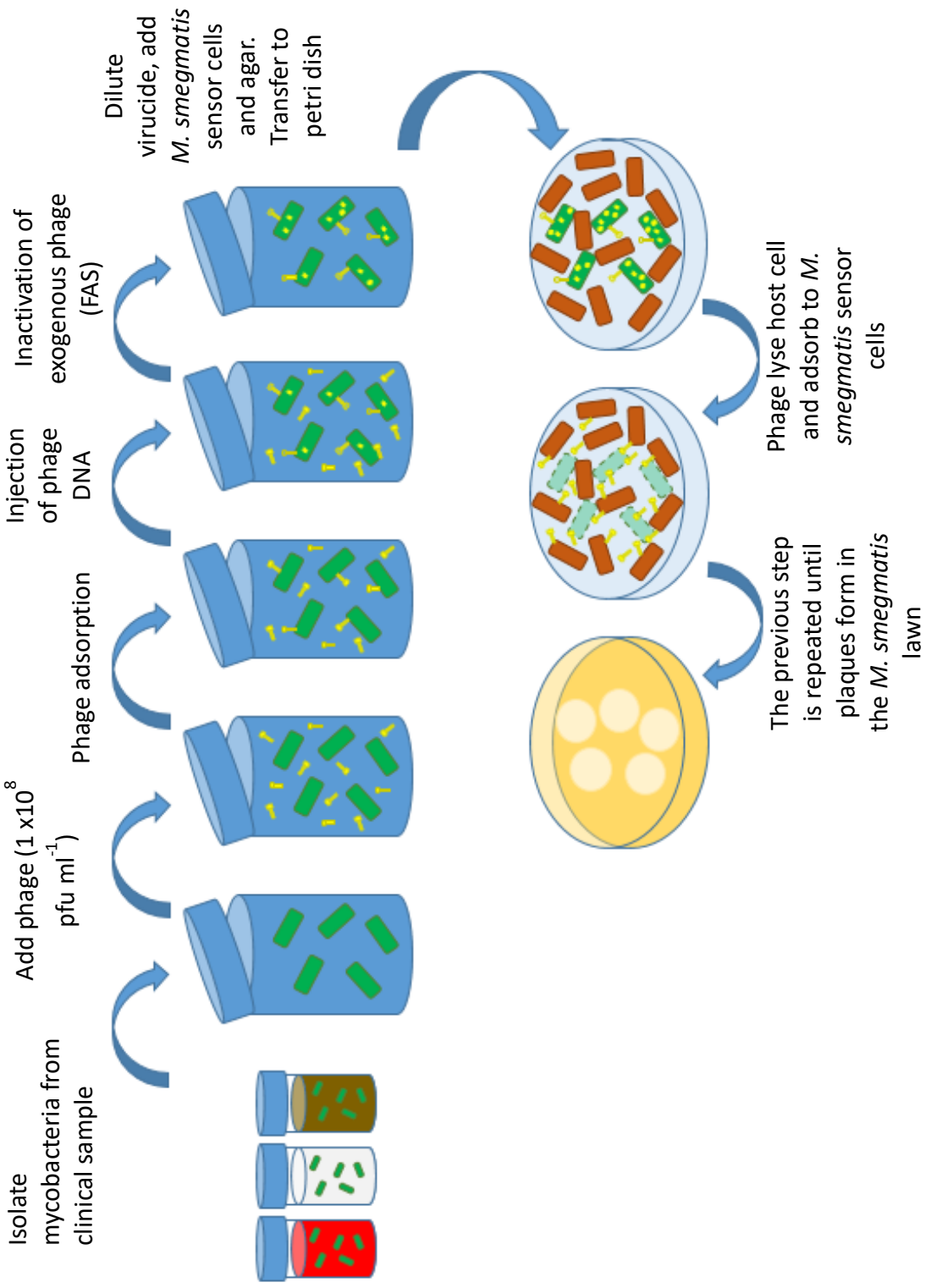
1.3.1. Standard phage assay

The standard phage assay has been used to detect both *M. bovis* and MAP from cattle sources. The assay was originally used and developed to detect *M. tuberculosis* in the sputum of human patients. The assay was commercialised as FASTPlaque-TB (Biotech Laboratories Ltd; UK) and was one the first methods developed that used phage as a diagnostic tool instead of as a therapeutic agent and it is this assay that the standard phage assay was developed from (Shenai *et al*., 2002). The standard phage assay is a simple yet effective way of detecting mycobacteria from a sample (Fig. 1.2). To perform the assay, any potential mycobacteria are first concentrated from the sample, the exact method varies depending on the type of sample being analysed. The cells are then added to Middlebrook 7H9 media supplemented with 10 % OADC and 2 mM $CaCl_2$ to facilitate phage adhesion and infection. Then mycobacteriophage such as the lytic phage D29 are then added to the mycobacteria at a concentration of 1 x10^8 pfu ml^{-1}. The mycobacteria are then incubated with the phage for an hour at 37 °C to allow the phage to adhere to the cells and infect the mycobacteria with the phage's DNA. Before the lytic cycle is completed and the

mycobacteria within the sample are lysed by the phage replicating within them, the exogenous phage that have not infected a cell are then inactivated by incubating the samples with a virucidal compound such as ferrous ammonium sulphate (FAS), ensuring that all inner surfaces of the reaction vessel are coated by the virucide. The virucide may also be toxic to the bacterial cell as FAS is to mycobacteria, to prevent the virucide from adversely affecting the mycobacteria, the sample is diluted to reduce the concentration of the virucide below a threshold that is toxic to the bacteria. The next step is to add *M. smegmatis* sensor cells to the sample. *M. smegmatis* is a rapidly growing NTM that is added to produce a lawn of phage infective bacteria. Agar is then added to the sample and the pour plating method is performed to transfer the sample to a sterile petri dish. The agar is then allowed to set, and the samples are incubated at 37 °C overnight. During this incubation step, the phage that were internalized by the mycobacteria in the sample were protected from the virucide and as such are able to complete their lytic cycle and rupture the host cell, releasing the progeny phage. These newly released phage are then able to infect the *M. smegmatis* sensor cells. This process continues until a lawn of *M. smegmatis* containing phage derived plaques are visible on the agar. The quantity of plaques produced can then be used to enumerate the amount of mycobacterial cells that were present in the original sample. The phage that is used in the phage assay has a broad host range that can infect almost all members of the mycobacterium genus, because of this, the result produced from the standard phage assay is that the sample was mycobacteria positive. The plaque contains DNA from the *M. smegmatis* lawn and phage but at the centre of the plaque remains the DNA from the original propagator cell from the sample. To identify the causative agent of the plaque,

analysis such as a PCR assay can be performed on the DNA. The DNA in the plaque is encased by the agar so before the PCR analysis can be performed it needs to be purified from the agar and other components that may be inhibitory to the PCR assay and concentrated. The most effective method for extracting the DNA from the plaque is to use a commercial kit that is designed with Spin Columns as they are a simple, quick and effective method for the recovery of the DNA from the agar. A specific PCR analysis can then be performed on the DNA depending on the suspected mycobacteria originating from the sample. This assay can be performed and produce a result within 24 hours of the sample being processed.

Figure 1.2 – Representation of the standard phage assay



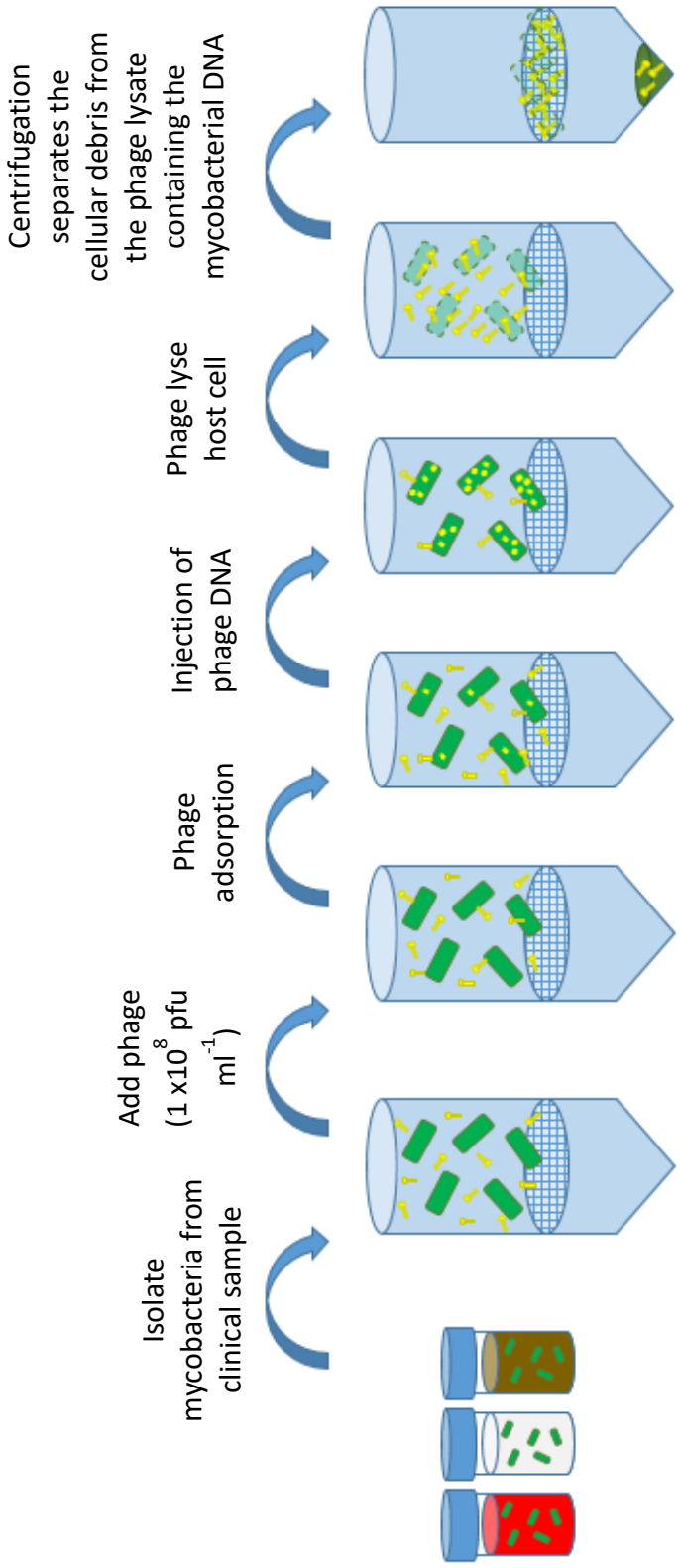
A diagrammatic representation of the steps required to perform the standard phage assay

1.3.2. Oneday phage assay

A streamlined, rapid version of the standard phage assay has been developed and commercialized (Fig. 1.3). The assay can be completed and produce results on the same day, hence why it is called the Oneday phage assay. The assay is a one tube format that does not rely on sensor cells to produce a lawn to visualize the phage infection process. Instead, the samples suspected of containing mycobacteria are processed with the same methods as in the standard phage assay to isolate and concentrate any potential mycobacteria. The bacteria are reconstituted into the same 7H9 media supplemented with 10 % OADC and 2 mM $CaCl_2$, in this assay the cells are transferred to the Actiphage reaction tube, which contains a 0.22 to 0.45 µm filter. The phage is then added to the cells at a concentration of 1 $x10^7$ pfu ml^{-1} and the sample is incubated at 37 °C for 4 h to allow the phage to complete a full lytic cycle and lyse the mycobacteria present in the sample. The sample is then centrifuged to pass the DNA through the filter and reduce the amount of cellular debris mixed with the DNA, and any bacteria (such as non-mycobacterial contaminants) are removed from the DNA sample to reduce the potential amounts of non-target DNA. The DNA in the phage lysate is then purified and concentrated. Again, the most effective method to achieve the purification are with commercial Spin Column kits. The purified DNA can then be analysed by PCR analysis as with the standard phage assay. There are several benefits to the Oneday phage assay compared to the standard phage assay. The first is that it is an even quicker detection method which can be completed within approximately 8 – 12 h. The Oneday phage assay has been shown to be a more sensitive assay compared to the standard phage assay also. The main limitation of the Oneday phage assay is that it

cannot produce presumptive positive results so if the diagnostic PCR assay produces a negative result, the sample is assumed negative. In the standard phage assay, if the diagnostic PCR is negative, then the production of the plaques shows that the sample contained a different species (or strain) of mycobacteria than was originally expected and provides further chances at identifying the unknown organism.

Figure 1.3 – Representation of the Oneday phage assay



The diagrammatic representation shows the steps required to perform the Oneday phage assay

1.3.3. Other applications of phage-based detection of mycobacteria

As previously stated, phage have been used to detect two different species of mycobacteria from the blood of cattle, however phage have also been used to detect mycobacteria from multiple dairy sources. Stanley *et al*. (2007) used the standard phage assay to detect viable MAP from the milk (individual samples) of confirmed Johne's infected cattle. It has also been shown that by using the standard phage assay MAP can be detected in bulk milk samples and that a value of 59 pfu 50 ml^{-1} of bulk milk would predict the presence of viable MAP in the sample with a sensitivity of 90 % and a specificity of 99 % (Botsaris *et al*., 2013). The standard phage assay was also used for the first time by to detect viable MAP from dried milk powder including powdered infant formula (Botsaris *et al*., 2016) and calf milk replacer (Grant *et al*., 2017). The PMMS method has also been used to improve the isolation of MAP from milk samples and was used to compare the sensitivities of the standard phage PCR assay, qPCR assay and MGIT culture. The study showed that PMMS isolation of MAP combined with the standard phage PCR assay was the most sensitive of the three methods tested (Foddai and Grant, 2017). A Oneday phage assay format has also been developed for detecting mycobacteria from milk with D29 coated magnetic beads which incorporated the MAP isolation and lysis with the same step (Foddai and Grant, 2020).

Reporter phage have also been developed by inserting cosmids containing a luciferase gene (Meile *et al*., 2020) or a fluorescent protein such as GFP into non-essential regions of the D29 and TM4 genome. Mycobacteria that are infected with these reported phage begin to produce light (Schofield *et al*., 2012). In terms of the luciferase reporter genes, production of light also indicates that the host cell is viable

as host ATP needs to be present in order to produce the luminescence (Riska *et al*., 1997). A TM4 luciferase reporter phage was developed and used to detect *M. tuberculosis* from the sputum of patients with active pulmonary TB. The results of the study showed a comparable sensitivity with the BACTEC MGIT culture technique, which is commonly used to confirm TB infections (Bardarov *et al*., 2003).

Phage growth assays have also been developed for the detection of TB in sputum samples. A phage growth assay uses the replication of phage to indirectly detect the presence of bacteria; by detecting an increase in phage particles it shows that the sample contained bacteria that the phage were able to infect and replicate in. A study by McNerney *et al*. (2004) used the phage D29 which is a broad-host range mycobacteriophage which infects the majority of the genus Mycobacterium. In the study, D29 phage were added to sputum samples and allowed to infect. Exogenous phage were then inactivated. This meant that the detection of any phage particles showed that mycobacteria were in the sample. This means that without further identification, it is impossible to know whether the positive result was caused by *M. tuberculosis* or another member of the Mycobacterium genus. The sensitivity and specificity of the assay was only of slight improvement compared to fluorescent microscopy but was not close to culture methods so the phage growth assay could not be justified for routine testing of TB.

1.4. GENERAL REVIEW OF MYCOBACTERIOPHAGE AND D29 AND TM4 BIOLOGY

The lytic lifestyle of the mycobacteriophage D29 has been exploited in bacteriophage-based assays for the detection of mycobacteria from clinical samples. Lytic phage only have one mechanism for propagation which involves attaching to a specific receptor of the host cell which is typically an essential component of the

bacterial cell wall. The phage then injects its DNA into the cytosol of the bacteria where it initiates replication of the phage DNA and production of viable progeny phage. Different genes of the phage are transcribed at different phases of the infection to produce sequential translation of the DNA to efficiently build the phage particles without compromising the viability of the host cell until the end of the replication process. At the late end of the infection the phage transcribe the genes required to lyse the host cell and release the progeny phage from the cell (Clokie *et al*., 2011). Phage that can undergo lysogenic lifestyle can undergo a second pathway when they infect a host cell. After the injection of the phage DNA into the host, the phage DNA integrates into the host genome at a specific site by homologous recombination. During this life cycle the phage does not lyse the host bacteria and is replicated with the bacterial DNA as the host grows and divides, so that the daughter cell also contains a copy of the phage in its genome. Under certain conditions such as exposure to UV light or other stress inducing events to the host, the phage can excise from the host genome and undergo a complete lytic lifestyle, producing progeny phage which is again released by lysing the host cell (Howard-Varona *et al*., 2017). The integration mechanisms used to integrate the lysogenic phage into the hosts genome have been exploited to produce plasmids that are able to integrate into the genome of a mycobacterial cell to produce stable transformants (Saviola and Bishai, 2004). The most commonly used integration system are the integrase gene and integration (*attP*) sequence from the mycobacteriophage L5, which is a temperate dsDNA phage of the *Siphoviridae* family with a GC content of 63.2 %, that can infect a broad host range of bacteria (Hatfult and Sarkis, 1993).

D29 is a close relative of the phage L5, of which D29 is known to be a deletion variant. One of the benefits of D29 is its broad host range and ability to infect multiple species of mycobacteria (Rybniker *et al.*, 2006). It is a member of the *Siphoviridae* family of viruses (Uchiyama *et al*., 2018) and contains a 49,136 bp linear dsDNA genome (Hendrix *et al*., 1999) with a GC content of 63.8 % (Tokunaga and Sellers, 1964). The genome of D29 (and L5) are split into two arms, the right arm which contains genes transcribed during the early to middle phase of infection and the left arm genes transcribed during the late phase and includes structural genes such as capsid genes (Ghosh *et al*., 2020) and it has been shown that D29 has a 3.6 kb deletion in the right arm of the L5 genome. This deletion cleaves the repressor gene of D29 (Gp71) and therefore accounts for the lytic characteristic of this particular Mycobacteriophage as it can no longer enter the lysogenic pathway. However the integrase and attachment site for the integration system are still functional and adding the L5 repressor gene restores the lysogenic lifestyle of D29 (Ford *et al*., 1998a). There are multiple binding sites throughout the D29 and L5 genome to which the repressor binds to prevent the transcription of the associated downstream gene, these repressor binding sites are termed stoperators due to their close proximity (which often overlap) with promoter sites. Both D29 and L5 share a consensus sequence for the stoperator, indicating that clinical strains of mycobacteria infected with a lysogenic L5 or L5-like phage would not be productively infected by phage D29 because the repressor will prevent D29 superinfection and would therefore not be detected by phage-based assays (Dedrick *et al*., 2017, Bhawsinghka *et al*., 2018). The reason why D29 can efficiently lyse the host cell and free the mycobacterial genome to make it available for amplification in a PCR assay is because the phage

genome contains three genes that encode lytic genes, perfectly designed to weaken the host cell wall in order to rupture it and release the progeny phage. These genes work together to produce the efficient lysis of the host mycobacteria after phage replication, these are LysA which breaks down peptidoglycan, LysB which degrades the mycolic acids in the cell wall and a holin gene which punctures the cell membrane to destabilize the cell and increase the diffusion of the two Lysin proteins to their target molecules (Bavda and Jain, 2020). There has only been one nuclease identified in the D29 genome, gp65, a 3' – 5' structure specific exonuclease that degrades the 3' strand of forked DNA. The nuclease is expressed between 15 and 20 min after the phage has injected its DNA into the host cell and is predicted to remove 3' fork junctions created during recombination events during the initial stages of phage genome assembly (Giri *et al*., 2009). Some species of phage, such as the lytic bacteriophage T4 that infects *E.coli* express nucleases that degrade the host cell genome to recycle the nucleotides for phage DNA replication (Kutter *et al*., 2018). The only nuclease identified in the D29 genome is the gp65 exonuclease and as such, the phage D29 does not degrade its host's genome, which means that the host DNA is preserved and remains detectable for PCR assays. Although the mycobacteriophage D29 does not degrade the host genome, the phage uses other mechanisms to improve phage replication. The D29 genome encodes its own DNA polymerase which has been hypothesized to improve the phage DNA replication in slow growing mycobacteria which may have a reduced rate of DNA replication machinery, in part resulting in the slow growth rate of these organisms. In line with this, the phage genome also contains five common tRNA genes ($tRNA^{asn}$, $tRNA^{gln}$, $tRNA^{glu}$, $tRNA^{trp}$ and $tRNA^{tyr}$) which are expected to improve the translation rate of

the phage DNA to reduce the likelihood of the ribosome from stalling in case of a limitation in tRNA molecules within the slow growing host (Ford *et al*., 1998a).

The mycobacteriophage TM4 is another broad host range phage that is able to infect both slow and rapid growing mycobacteria (Ford *et al*., 1998b). It is also a *Siphoviridae* and contains a dsDNA genome but is not related to the phages L5 or D29. The TM4 genome also contains a suspected deletion of integration genes which prevents a lysogenic life cycle based on TM4—like temperate phages that have been discovered that contain a fully functioning integration system (Pope *et al*., 2011). Unlike D29, TM4 does not encode its own DNA polymerase (Ford *et al*., 1998b), however it does contain a WhiB-like protein not contained in D29 which inhibits WhiB2, a gene required for appropriate septum formation and cell division and may lead to a weakened cell wall as it has been seen that the host *Mycobacterium* loses its acid fast quality during phage infection (Rybniker *et al*., 2010). The most notable difference between TM4 and D29 is that TM4 contains a peptidoglycan-hydrolyzing enzyme within the tail of the phage which allows TM4 to inject its DNA into dormant mycobacteria and produce a full lytic cycle. Mutations in the gene do not reduce infectivity in exponentially growing cells but produces a reduction of approximately 50 % in infectivity of cells in the stationary phase of growth (Piuri and Hatfull, 2006). D29 is not able to infect dormant mycobacteria which have been induced into the stationary phase by hypoxia. Swift *et al*. (2014) showed that D29 is able to adsorb onto the surface of hypoxic mycobacteria but the lytic pathway is not completed. The infectivity and lytic cycle of the D29 can be restored after the cells are returned to aerobic growth.

1.5. AIMS OF PROJECT

Mycobacteria are fundamentally one the most difficult groups of known organisms to detect and contains some of the most clinically relevant bacteria for humans, historically, currently, and potentially in the future. Phage have been shown by many reports to be an effective detection agent for mycobacteria and the recent studies showing that they can be used to detect mycobacteria from purified PBMCs from both *M. bovis* and MAP infected cattle show that blood could potentially be a suitable sample matrix for the sensitive and specific detection of other types of mycobacterial infection.

The first aim of the project is to produce an internal amplification control for the PCR assay step of the phage assays to show where samples are contaminated with inhibitors derived from the original clinical sample and as such whether a negative result is produced by the lack of template in the PCR reaction or by inhibition of the DNA polymerase.

The second aim of the project is to optimize and streamline the processing steps within the complete phage assay protocols, by trialling simpler methods of PBMC extraction and designing highly sensitive molecular assays to improve the sensitivity of the phage assays. The optimization steps were performed to translate the phage assays for use in low-technology laboratories or field laboratories to provide rapid mycobacterial diagnosis in developing countries.

The third and final aim of the study is to use the optimized phage assay protocols to detect mycobacteria from the blood of hosts with suspected mycobacterial disease.

CHAPTER 2

2. MATERIALS, METHODS AND STANDARD PROCEDURES

2.1. GENERAL MEDIA AND REAGENTS

2.1.1. Culture and growth conditions

2.1.1.1. Middlebrook 7H10 agar and 7H9 media

Middlebrook 7H10 agar and Middlebrook 7H9 media (Becton-Dickenson, UK) was prepared by dissolving 3.9 g of 7H10 agar or 0.94 g 7H9 media in 180 ml of distilled water and sterilized by autoclaving at 121 °C for 15 min. The strains of mycobacteria used in this thesis were maintained by subculture every two months on 7H10 agar supplemented with 10 percent OADC (Becton-Dickenson, UK), added to molten 7H10 agar tempered to 45 °C. All cultures were incubated at 37 °C, statically.

2.1.1.2. Miles Misra method

To perform the Miles Misra culture method first, a ten-fold serial dilution (10 dilutions) was performed on the culture. During each dilution, the culture was vortexed on full speed for 5 s then pipette mixed forcefully 10 times to disperse clumps of bacteria. Then 5 x 20 µl of each dilution was spotted onto 7H10 agar supplemented with 10 % OADC (v/v) supplemented with or without kanamycin sulphate. The spots were allowed to dry before the petri dishes were inverted and incubated at 37 °C aerobically for 7 d. The amount of colonies were counted within the countable range (3 – 30 colonies) and the cfu ml^{-1} of the culture was calculated using the equation: $Average\ colonies\ counted * \frac{1}{dilution} * \frac{1}{volume\ of\ cells\ plated} = cfu\ ml^{-1}$

2.1.1.3. Patch Plate and colony PCR method – E. coli

Transformant colonies were then patch plated by transferring a single colony with a sterile cocktail stick onto LB agar supplemented with kanamycin (50 µg ml^{-1}) with a

single stabbing motion and were incubated overnight at 37 °C. The cocktail stick used for the patch plate inoculation was then immediately used to add the remaining cells on the stick to a PCR reaction mixture for pTB-GFP, *IS*6110 PCR analysis (section 2.4.2.2.), for pIAC, *IS*6110 (section 2.4.2.2.) PCR analysis and *IS*900 (section 2.4.2.4) PCR analysis to determine whether the colonies had taken up the plasmid and were true transformants. The expected product size was 180 bp (*IS*6110) and 230 bp (*IS*900).

2.1.1.4. Patch plating method – M. smegmatis

Expected *M. smegmatis* transformant colonies were then patch plated by transferring a single colony with a sterile cocktail stick onto LB agar supplemented with kanamycin (25 µg $ml^{-}1$) with a single stabbing motion and were incubated at 37 °C for 7 d. The cocktail stick used for the patch plate inoculation was then immediately used to add the remaining cells on the stick to a PCR reaction mixture; for pTB-GFP, *IS*6110 PCR analysis was performed (section 2.4.2.2.); for pIAC, *IS*6110 PCR analysis (section 2.4.2.2.) and *IS*900 PCR analysis (section 2.4.2.4.) was performed, to determine whether the colonies had taken up the plasmid and were true transformants. The expected product size was 180 bp (*IS*6110) and 230 bp (*IS*900).

2.1.1.5. Blood culture using Stonebrink slopes

To culture MTBC from blood the remaining PBMC that were extracted using the Ficoll® extraction method (section 2.3.1.1.) or the HetaSep® extraction method (section 2.3.1.2.) from 2 ml whole blood and after the cells had been removed for the standard phage assay and Oneday phage assay were inoculated directly onto a

Stonebrink slope (EO Labs; UK). The slopes were initially incubated at 37 °C (CL3 conditions) resting at approximately 30° angle so to provide a flat surface with the cap loose (to allow air flow for aerobic conditions) for 8 weeks. After 7 d the slopes were checked for contaminating growth, any cultures found to have growth were discarded. The caps on the remaining samples were tightened and the samples were incubated standing up from this point on. The cultures were checked every week for growth and discarded after 8 week if the samples were still negative

2.1.1.6. Bespoke blood culture of M. tuberculosis

Molten and tempered Middlebrook 7H10 agar supplemented with 10 percent OADC (section 2.1.1.1.) and MP (section 2.2.1.1.) was prepared. The HetaSep® (StemCell; UK) PBMC extraction method was performed and the upper plasma layer was transferred to a sterile 1.5 ml microfuge tube. Then 9.5 ml of MP was added to a 50 ml conical bottom tube (Sarstedt; UK) along with 1 ml (5 percent) of the erythrocytes aggregated during the HetaSep® PBMC extraction method (section) and 9.5 ml of the tempered agar. The contents were then mixed by inversion until a uniform soft blood agar was produced. The agar was then poured into a sterile 90 mm petri dish and allowed to set. The upper plasma was then transferred to the blood agar produced from the same sample and allowed to absorb into the agar where it was placed. The cultures were then incubated in a 2.5 L air tight box containing a CampyGen™ 2.5 L sachet (Oxoid; UK) to generate a microaerophilic environment and incubated at 37 °C statically under CL3 conditions. The cultures were observed after 24 and 48 hours for contamination and then every week to identify slow-growing bacteria. Cultures were discarded after eight weeks if there were no signs of growth.

2.1.2. Bacterial strains

Bacteria	Strain	Ref
Mycobacterium smegmatis	*MC^2155*	Type Strain
	IAC	This project
***Mycobacterium bovis* BCG**	Pasteur	Type Strain
Mycobacterium avium	avium	Type Strain
***Mycobacterium avium* subsp. paratuberculosis**	ATCC 19851	Type Strain
	B4	Field Isolate NI
	DVL 943	National Veterinary Lab, Denmark
	K10	Type Strain
Escherichia coli	Top10 (Invitrogen)	Cloning strain
	IAC	This project

2.1.3. Electrophoresis Buffers

2.1.3.1. TAE

To prepare the TAE electrophoresis buffer first a 50x stock was produced by dissolving 242 g tris base in double-distilled H_2O and adding, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA solution (pH 8.0) and adjusting the volume to 1 L. The 50x stock was then diluted to 1x stock using double-distilled water for use in electrophoresis buffers and gels

2.1.3.2. LAB

To prepare 10 mM LAB electrophoresis buffer 1g Lithium acetate and 0.62g Boric acid were dissolved into 1L of double-distilled water.

2.1.4. Microscopy

2.1.4.1. Viable count of blood cells

To perform the viable count, cells (10 µl) were mixed with Trypan blue (Gibco; UK) at a 1:1 ratio and transferred to a haemocytometer. The clear cells were counted and viable count calculated using the equation:

$$Average\ viable\ cells * 10000(\frac{2}{quadrants\ counted})$$

2.2. Phage assays

2.2.1. Standard two day phage assay media and reagents

2.2.1.1. Media Plus

The Media Plus (MP; Middlebrook 7H9 media-based media) was prepared by supplementing 7H9 media (section 2.1.1.1.) with 10 percent OADC.

2.2.1.2. Virusol

For D29: one tablet of Virusol (ferrous ammonium sulphate) was dissolved into 4.5 ml of sterile distilled water under aseptic conditions.

For TM4: 133.5 ml of sterile distilled water was heated to boiling in a beaker, then 10 g of Gunpowder Green Tea (Whittard of Chelsea; UK) was added and boiled for 10 min. The tea was then filtered through a Whatmann 4 filter paper to remove the tea. The tea extract was then filter sterilized through a 0.22 µm filter (Sartorius; UK). To

prepare the virucide, finally 330 µl of tea was added to 700 µl FAS (one tablet dissolved in 4.5 ml sterile distilled water).

2.2.1.3. Actiphage

Freeze-dried mycobacteriophage D29 (PBD; UK) was reconstituted by adding 1.1 ml of MP (section 2.2.1.1). The phage solution was gently mixed by agitation until completely dissolved ($1x10^9$ pfu ml^{-1}).

2.2.1.4. Sensor cells

M. smegmatis – MC²155 was cultured in MP to a concentration of 1 $x10^8$ cfu ml^{-1}

2.2.2. The standard phage assay

2.2.2.1. Controls

To produce a positive control the *M. smegmatis MC²155* culture used for the sensor cells was ten-fold serially diluted into sterile MP until a final concentration of 1 $x10^3$ cfu ml^{-1} was produced. Then 100 µl of the final dilution was added to 900 µl sterile MP to produce the positive control with a final concentration of 1 $x10^2$ cfu ml^{-1}. For the negative control, 1 ml of sterile MP was used.

2.2.2.2. Standard phage assay

To perform the standard phage assay, a sample size of 1 ml was used, for sample volumes below 1 ml make up to 1 ml with MP. The samples containing mycobacteria were then transferred to a reaction vessel. Then 100 µl phage D29 (reconstituted in 1.1 ml of MP; 1 $x10^9$ pfu ml^{-1}) was added to the cells and the samples were incubated at 37 °C for 1 h. The next step was to inactivate the exogenous phage by adding 100 µl of Virusol (section 2.2.1.2.) to the samples. The reaction vessels were then inverted so that all inner surfaces were exposed to the virucide. The samples were

then rotated at room temperature for 6 min. Next 6 ml of MP was added to the samples to dilute out the virucide and 1 ml of sensor cells (section 2.2.1.4) were added. The samples were transferred to a sterile petri dish and 6 ml molten tempered (40 °C) 7H10 agar was added and the samples gently swirled to mix. The agar was allowed to set and then the petri dishes were inverted and incubated overnight at 37 °C. The following day the amount of plaques produced within the countable range (300 – 30) were counted. The plaques were picked with a 1.5 mm biopsy punch to produce standard plaque sizes. The plaques DNA was then extracted with a Zymo gel DNA recovery kit (section 2.4.1.3.).

2.2.3. Oneday phage assay

2.2.3.1. Controls

For a positive control a liquid culture of *M. bovis* BCG (for *IS*6110 PCR assay analysis) or MAP K10 (for *IS*900 PCR assay analysis) was diluted in MP to a final concentration of 1 x10^3 cfu ml^{-1}. Then 100 µl of the final dilution was added to an Actiphage reaction tube to produce the positive control. For the negative control 100 µl of sterile MP was used.

2.2.3.2. Oneday phage assay

To perform the Oneday phage assay on a liquid culture, a ten-fold serial dilution was prepared diluting the cells into sterile MP. Then 100 µl of the desired dilutions were transferred to separate Actiphage reaction tubes (0.45 µM pore size) and processed from this point the same as the PBMC extract.

To perform the Oneday phage assay the on a PBMC extract from the Ficoll® extraction method (section 2.3.1.1.) or HetaSep® extraction method (section 2.3.1.2.)

was centrifuged at 13,000 xg for 3 min. The supernatant was discarded and the cells were reconstituted in 100 µl of MP. The cells were then transferred to an Actiphage reaction tube (0.45 µm pore size). Then 10 µl of phage D29 (reconstituted in 1.1 ml of MP; 1 x10^9 pfu ml^{-1}) was added to the cells. The samples were then incubated for 4 h at 37 °C. The samples now containing the phage lysate were centrifuged at 13,000 xg for 3 min to separate the DNA from the cellular debris. The DNA extract was then purified and concentrated with a Zymo Clean & Concentrator Kit (section 2.4.1.4.).

2.3. PBMC extraction methods

2.3.1.1. Ficoll®

To extract the PBMC from 2ml of whole blood, first 3 ml of Ficoll®-Paque plus (GE healthcare Life Sciences; UK) was added to a Leucosep™ tube (Greiner BIO-ONE; UK). The tubes were then centrifuged at 1000 xg for 1 min to pass the Ficoll®-Paque plus through the insert. Next 2 ml of sterile PBS was added to the tubes. Then 2 ml of whole blood was aspirated and the mixed with the PBS by gently aspirating the PBS into the pipette with whole blood and then gently transferred back to the Leucosep™ tube. The samples were then centrifuged in a Heraeus Megafuge with a swing out rotor at 400 xg for 30 min at 19 °C with the break off. The upper plasma layer was then discarded and the buffy coat layer containing the PBMC was transferred to a 15 ml conical based tube and 6 ml of PBS was added to the PBMC. The samples were centrifuged again at 100 xg for 10 min at 19 °C. The supernatant was discarded and the pellet was resuspended in 1.5 ml of MP for the phage assays.

2.3.1.2. HetaSep®

To extract the PBMC from 2 ml of whole blood, first the blood was transferred to a 15 ml conical based tube and 400 µl of HetaSep® was added to the blood. The samples were mixed by gently shaking and allowed to incubate at room temperature for 30 min or until the plasma/ erythrocyte interface was at approximately 50 percent. Then the upper plasma layer containing the PBMC was extracted taking care not to co-extract the erythrocytes below and transferred to a 15 conical based tube. Next 6 ml of PBS was added to the PBMC and the samples were centrifuged at 100 xg for 10 min. The supernatant was removed, and the cells were reconstituted in 1.5 ml of MP for the phage assays.

2.3.1.3. HetaSep® PBMC extraction method with bison blood

The protocol used to isolate PBMCs using HetaSep® was based on information provided by the manufacturer who had developed protocols to allow their product to be used for the isolation of white blood cells from experimental mice (Elena Blanc, STEMCELL Technologies UK Ltd, personal communication). First the bison blood was diluted 1:1 with PBS and then HetaSep® added at the ratio of 1 to 1.3 blood. After thorough mixing by using gentle inversion of the tubes, the samples were then incubated at 37 °C for up to 60 min to allow the aggregation of erythrocytes to form whilst visually monitoring the formation of a clear upper phase. After 60 min no interface had formed so the blood samples were then incubated over night at room temperature. Again, separation was still low so the final step was to perform a centrifugation of the blood at 100 xg for 2 min

2.3.2. Optimized PBMC lysis

To perform the optimized PBMC lysis method 2 ml blood samples were extracted with the standard HetaSep® extraction method up to the PBS wash step (section 2.3.1.2.). After the PBS wash, the supernatant was removed and the PBMC pellet was reconstituted in 600 µl of sterile distilled water and homogenized by pipette mixing. The samples were then incubated for 20 min at room temperature. The samples were then vortexed and 750 µl 2x 7H9 media and 150 µl OADC was added to the samples to make 1.5 ml MP at the standard concentration. The samples were then incubated overnight at 37 °C. The procedure was then the same as the standard procedure for the Oneday phage assay (section 2.2.3.). After the DNA produced from the Oneday phage assay was purified the quantity and quality of the DNA was determined using a Nanodrop (ThermoFisher; UK),

2.4. Polymerase chain reactions

2.4.1. DNA extraction

2.4.1.1. Boiled lysis method

From a solid culture a single colony was emulsified in 100 µl of sterile molecular grade water in a 1.5 ml microfuge tube. For a liquid culture, 100 µl of culture (1 x 10^8 cfu ml^{-1}) was added to a 1.5 ml microfuge tube. Then the samples were heated on a heating block at 90 °C for 20 min. The samples were then allowed to cool before centrifuging at 13,000 xg for 3 min to remove cellular debris. The supernatant was then transferred to a sterile 1.5 ml microfuge tube. The samples were stored at -20 °C.

2.4.1.2. Freeze/ thaw extraction of plaque DNA

A single plaque was excised and added to 0.2 ml PCR tube. Then 10 µl of sterile molecular grade water was added to the plaque. The plaque was then heated at 95 °C for 5 minutes then immediately frozen at -20 °C for at least 15 min. The sample was finally thawed and 5 µl of the supernatant was used as DNA template per PCR assay.

2.4.1.3. Zymo Gel DNA Recovery Kit with plaques

To extract DNA with this Zymo Gel DNA Recovery Kit (ZymoResearch; Cambridge Bioscience; UK) the first step was to add 24 ml of 100 percent ethanol to the DNA Wash Buffer. First 5 plaques were picked and pooled together in a 1.5 ml microfuge tube then 300 µl of DNA ADB was added to the plaques. The plaques were then incubated in a water bath set to 55 °C for 10 minutes (or until the agar had dissolved). The solution was then added to a Zymo-Spin™ Column in a Collection tube. The samples were then centrifuged at 13,000 xg for 30 s. Then 200 µl of DNA Wash Buffer was added to the Spin-Column and centrifuged again at 13,000 xg for 30 s. This was step was repeated a second time. The Spin Column was then transferred to a sterile 1.5 ml microfuge tube and 10 µl of water (55 °C) was added to the center of the matrix and incubated at room temperature for 1 min. The samples were then centrifuged at 13,000 xg for 30 s. The spin Column was discarded and 5 µl of DNA was used as template for a PCR assay or was stored at -20 °C for future use.

2.4.1.4. Zymo DNA Clean & Concentrator-5 kit

To extract DNA with Zymo DNA Clean & Concentrator-5 kit (ZymoResearch; Cambridge Bioscience; UK) the first step was to add 24 ml of 100 percent ethanol to

the DNA Wash Buffer. DNA Binding Buffer was added at a 2:1 ratio to the DNA sample for genomic DNA extraction. The sample was then transferred to a Zymo-Spin™ Column in a Collection tube. The samples were then centrifuged at 13,000 xg for 30 s. Then 200 µl of DNA Wash Buffer was added to the Spin-Column and centrifuged again at 13,000 xg for 30 s. This was step was repeated a second time. The Spin Column was then transferred to a sterile 1.5 ml microfuge tube and 10 µl of water (55 °C) was added to the center of the matrix and incubated at room temperature for 1 min. The samples were then centrifuged at 13,000 xg for 30 s. The spin Column was discarded and 5 µl of DNA was used as template for a PCR assay or was stored at -20 °C for future use.

2.4.2. End-point PCR assays

2.4.2.1. 16s rDNA

This method used the KY18/75 primers (Table 2.2) as described by (Tevere *et al*., 1996). The PCR reaction amplifies a segment of the 16s rDNA gene specific to mycobacteria. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO 96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 15 min. This was followed by 30

cycles of: 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 5 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp.

Table 2.2 – Primers and probes used in this study

Target	Primers and probes	DNA Sequences	Tm (°C)	GC content (%)
16S	KY18	CACATGCAAGTCGAACGGAAAGG	62.43	52.17
	KY18Ex	AACACATGCAAGTCGAACGGAAAGG	62.98	48.00
	KY75	GCCCGTATCGCCCCGCACGCTCACA	71.26	70.83
IS6110	TB Complex F	CCTGCGAGCGTAGGCGTCGG	67.55	75.00
	TB Complex R	CTCGTCCAGCGCCGCTTCGG	67.55	75.00
	MTC IS6110 Probe	ACATAGGTGAGGTCTGCTACCCACA	64.62	52.00
IS900	P90	GAAGGGTGTTCGGGGCCGTCGGCCTTAGG	75.16	68.97
	P90 Short	GAAGGGTGTTCGGGGCCGTCG	67.63	71.43
	P91	GGCGTTGAGGTCGATCGCCCACGTGAC	72.56	66.67
	MAP RT F	AATGACGGTTACGGAGGTGGTT	60.25	50.00
	MAP RT R	AGCGATGAGCAAGGCGATCA	59.35	55.00
	MAP RT Probe	TGCGCGTCGTCGTTAATAACC	59.82	52.38
IS901	MK7	GTCTGGGATTGGATGTCCTG	59.35	55.00
	MK8	CACCACGTGGTTAGCAATCC	59.35	55.00
IAC GFP	IAC Probe	TCGAAAGGGCAGATTGTGTGGACA	64.62	48.00
IS1311	M119	ATGACGACCGCTTGGGAGAC	61.40	60.00
	M56	GCGTGAGGCTCTGTGGTGAA	61.40	60.00
pIAC				
Integration	pMV306 Int A F	GATACTACGTGCTCACGACACGAGAC	66.38	53.85
	pMV306 Int A R	CTCGATGAGCCGCTTCTCGCCC	67.71	68.18
	pMV306 Int B F	ACCACGGTCGTGATCTGCGACG	65.85	63.64
	pMV306 Int B R	GGTGCAACCTTGTCCCGGTC	63.45	65.00
Cloning	TBInsF - NcoI	CATGCCATGGCCTGCGAGCGTAGGCGTAGGgtatagttcatccatgccatgtg	90.30	37.74
	TBInsR - HindIII	CCCAAGCTTCTCGTCCAGCGCCGCTTCGGctgtccttttaccagacaacc	89.92	40.00
	IAC Full F	CATGCCATGGGTCTGGGATTGGATGTCCTGTATGAAGGGTGTTCGGGGCCGTCGCTTAGGTATCCTGCGAGCGTAGGCGTAGGgtat	86.81	56.32
	IAC Full R	CCCAAGCTTCACCACGTGGTTAGCAATCCTATGGCGTTGAGGTCGATCGCCCACGTGACTATCTCGTCCAGCGCCGCTTCGGctgt	87.01	56.98

2.4.2.2. IS6110

This method used the MTC F/R primers (Table 2.2) as described by Eisenach *et al*. (1990). The PCR reaction amplifies a segment of the *IS*6110 insertion element specific to MTBC bacteria and the IAC segment of the pIAC plasmid. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO 96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 123 bp for *IS*6110 of MTBC or 180 bp for the IAC.

2.4.2.3. IS6110 – OneTaq®

This method used the MTC F/R primers (Table 2.2) as described by Eisenach *et al*. (1990). The PCR reaction amplifies a segment of the *IS*6110 insertion element specific to MTBC bacteria and the IAC segment of the pIAC plasmid. DNA (1 µl) from *M. bovis* extracted with the boiled lysis method (section 2.4.1.1.) was used in each PCR reaction. First the DNA (140 ng $µl^{-1}$) was diluted 1 in 2 with sterile molecular grade

water then a tenfold serial dilution was performed from 70 ng μl^{-1} to 7 $x10^{-8}$ ng μl^{-1}. Purified pIAC plasmid (section) was diluted to 1 $x10^{5}$ ng μl^{-1}. To prepare the PCR reaction: 10 µl of One*Taq*® Quick-Load® 2x Master Mix with GC Buffer; 1 µl of each dilution was used as template; 0.5 µM of each MTC F and MTC R primers and made up to 20 µl with sterile molecular grade water. The PCR parameters used were an initial denaturation step of 94 °C for 2 min followed by 40 cycles of: 94 °C for 30 s, 66 °C for 15 s and 68 °C for 30 s; followed by a final extension step of 68 °C for 5 min. The PCR products were analysed by electrophoresis through a 2 % (w/w) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System. The expected product size for the internal control segment was 180 bp and the expected product size for the *IS*6110 element was 123 bp

2.4.2.4. IS900

This method utilized the P90/91 primers (Table 2.2) described by Sanderson *et al*. (1992). The PCR assay amplifies a segment of the *IS*900 insertion element specific to *M. avium* subsp. paratuberculosis and the IAC segment of the pIAC plasmid. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer P90 and P91 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 37

cycles of: 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 400 bp for *IS*6110 of MTBC or 238 bp for the IAC.

2.4.2.5. IS900 – OneTaq®

This method utilized the P90/91 primers (Table 2.2) described by Sanderson *et al*. (1992), the P90short primer (this study) and the P91 primer or the MAP RT F/R primers (this study). The PCR assay amplifies a segment of the *IS*900 insertion element specific to *M. avium* subsp. paratuberculosis and the IAC segment of the pIAC plasmid. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of One*Taq®* Quick-Load® 2x Master Mix (QIAGEN; UK); 0.5 µM of each primer P90 and P91 or p90short and P91 (Table 2.2) was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 94 °C for 2 min. This was followed by 37 cycles of: 94 °C for 30 s, 62 °C for 15 s, 68 °C for 45 s. This was followed by a final extension step of 68 °C for 5 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size for

the P90/91 and P90short/91 primers was 400 bp for *IS*900 of MAP or 238 bp for the IAC. The expected product size for the MAP RT F/R primers was 250 bp for MAP only.

2.4.2.6. IS1311 PCR assay

This method utilized the M56 and M119 primers (Table 2.2) described by Marsh *et al*. (1999). The PCR assay amplifies a segment of the *IS*1311 insertion element specific to MAC. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of One*Taq*® 2x Master Mix with GC Buffer (NEB; UK); 5 µM of each primer M56 and M119 (Table 2.2) to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 94 °C for 2 min. This was followed by 37 cycles of: 94 °C for 30 s, 62 °C for 30 s, 68 °C for 1 min. This was followed by a final extension step of 68 °C for 5 min. The PCR products (5 µl) were electrophoresed through a 2 percent agarose LAB (10 mM) gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 250 V for 20 min. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 600 bp. Next, the PCR products (5 µl) were further amplified so that there was enough DNA for the restriction enzyme analysis by adding the PCR products to a fresh PCR reaction mixture made as above in a 0.2 ml PCR tube. The PCR assay was performed using the same conditions as above except only 12 cycles were performed. The PCR products (5 µl) were electrophoresed through a 2 percent agarose LAB (10 mM) gel

(w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 250 V for 20 min. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). Before the restriction enzymes analysis could be performed, the PCR products were purified with a Monarch PCR & DNA Cleanup Kit as per the manufacturer's protocol (NEB; UK). First, the Wash Buffer was prepared by adding 4 parts ethanol to 1 part DNA Wash Buffer. Then, 5 µl of TE buffer was added to the PCR products to make the solution up to 20 µl as per the manufacture's recommendations. Then 100 µl of DNA Binding Buffer was mixed with the DNA sample and transferred to a Spin Column. The samples were then centrifuged at 16,000 xg for 1 min. Next 100 µl of DNA Wash Buffer was added to the columns and the samples were centrifuged again at 16,000 xg for 1 min. The previous wash step as then repeated a second time. The Spin Column was then transferred to a sterile 1.5 ml microfuge tube and 16 µl of sterile molecular grade water (55 °C) was added to the center of the matrix. The samples were then incubated at room temperature for 1 min then centrifuged at 16,000 xg for 1 min to elute the DNA. The Spin Column was then discarded.

To the purified PCR products (16 µl) from the *IS*1311 PCR assay, 2 µl CutSmart Buffer (NEB; UK) was added along with 1 µl *Mse*I and 1 µl *Hinf*III enzymes. The restriction double digests were then incubated at 37 °C for 1 h. The enzymes were then heat inactivated at 80 °C for 20 min. The restriction digest products (5 µl) were electrophoresed through a 3 percent agarose LAB (10 mM) gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 250 V for 30 min. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product sizes for: *M. avium* was 285bp, 189 bp and 34 bp; Type-S MAP was 323 bp and 285

bp; Type-C MAP was 323 bp, 285 bp, 218 bp and 67 bp; Type-B MAP was 323 bp, 285 bp and 67 bp.

2.4.2.7. Plasmid integration PCR assay

To determine whether the pTB-GFP plasmid was in a cytosolic form or an integrated state a multiplex PCR assay was designed with two primers that flanked the *attP* sequence of the plasmid and two primers that flanked the *attB* sequence on the *M. smegmatis* genome (Table 2.2). The primer sequences were designed after analysis of the tRNA-Gly gene and attB sequence in the *M. smegmatis-MC*^{2}*155* nucleotide sequence deposited in the NCBI database (Mohan *et al*., 2015) and attP sequence of the pMV306 plasmid. Depending on the state of the plasmid, different sized PCR products were designed to be produced; circular cytosolic plasmid and uninterrupted chromosome would produce PCR products of sizes: 603 bp and 251 bp respectively. Integrated plasmid would produce PCR products of sizes, 375 bp and 479 bp. DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR assay, the integration primers were used at a concentration of 0.5 µM each; with HotStarTaq Master Mix Plus DNA polymerase and buffer (QIAGEN; UK); The PCR parameters used were an initial denaturation step of 95 °C for 5 minutes, then 30 cycles of: 94 °C for 30 seconds, 68 °C for 30 seconds, 72 °C for 30 seconds, with a final extension step of 72 °C for 5 minutes.

The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK).

2.4.2.8. Temperature gradient PCR assay

This method used the KY18/75 primers as described by Eisenach *et al*. (1990) and the KY18ex as described in this study (Table 2.2). The PCR reaction amplifies a segment of the 16s rDNA gene. DNA (1 µl) extracted from cells using the boiled lysis method (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.025 µM of each primer KY18 and KY75 or KY18ex and KY75 (Table 2.2); 5 (%) DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO 96 thermocycler (VWR; UK). The PCR parameters used included a temperature gradient setting for the annealing step, the parameters used were: an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 62 – 65 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp

2.4.3. Designing a non-competitive IAC

2.4.3.1. 16s and IS6110 multiplex PCR assay

This method utilized the KY18/75 primers and the MTC F/R primers (Table 2.2) described by Tevere *et al*. (1996) and Eisenach *et al*. (1990) respectively. The PCR assay amplifies a segment of the 16s rDNA gene (KY) and *IS*6110 insertion element

specific to MTBC (MTC). DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the singleplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer KY18 and KY75 (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. For the multiplex PCR reaction mixtures: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.25 µM of each primer KY18, KY75, MTC F and MTC R (Table 2.2); 5 % DMSO; 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the *IS*6110 element.

2.4.3.2. Adapted two round multiplex

This method utilized two rounds of PCR, the first pre-amplification PCR used the MTC F/R primers (Table 2.2) described by (Eisenach *et al*., 1990). The PCR assay amplifies a segment of the *IS*6110 insertion element specific to MTBC. DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN;

UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

The PCR product (5 µl) was used as template for the 16s and *IS*6110 multiplex PCR assay (section 2.4.3.1).

2.4.3.3. Varying the primer concentrations of the adapted two round multiplex

This method utilized two rounds of PCR, the first pre-amplification PCR used the MTC F/R primers (Table 2.2) described by (Eisenach *et al*., 1990). The PCR assay amplifies a segment of the *IS*6110 insertion element specific to MTBC. DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

The PCR product (5 µl) was used as DNA template for the 16s and *IS*6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 or 0.5 µM of each MTC F and MTC R primer and 0.05 or 0.08 µM of each KY18 and KY75 primer (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the *IS*6110 element.

2.4.3.4. Decreasing the 16s rDNA primers in the adapted two round multiplex

This method was adapted from the method described above in section 2.4.4.3. The protocol was kept the same except for the PCR reaction mixture of the multiplex PCR assay. To prepare the altered multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 or 0.4 µM of each MTC F and MTC R primer and 0.04 or 0.02 µM of each KY18 and KY75 primer (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

2.4.3.5. Effect of PCR enhancers on the adapted two round multiplex PCR assay

To determine the effect of PCR enhancers on the adapted two round multiplex PCR assay

This method utilized two rounds of PCR, the first pre-amplification PCR used the MTC F/R primers (Table 2.2) described by (Eisenach *et al*., 1990). The PCR assay amplifies a segment of the *IS*6110 insertion element specific to MTBC. DNA (5 µl) extracted from plaques (section 2.2.2.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.4 µM of each primer MTC F and MTC R (Table 2.2); No PCR enhancers or 5 % DMSO (v/v) and/ or 20 % Q-Solution (v/v) (QIAGEN; UK); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

The PCR product (5 µl) was used as template for the 16s and *IS*6110 multiplex PCR assay.

To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.4 µM of each primer MTC F and MTC R and 0.05 µM of each KY18 and KY75 primer (Table 2.2); No PCR enhancers or 5 % DMSO (v/v) and/ or 20 % Q-Solution (v/v) (QIAGEN; UK); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml-1 ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the IS6110 element.

2.4.3.6. Effect of Q-Solution on the amplification of the 16s rDNA in the adapted two round multiplex PCR assay

This method utilized two rounds of PCR, the first pre-amplification PCR used the MTC F/R primers (Table 2.2) described by (Eisenach *et al*., 1990). The PCR assay amplifies a segment of the *IS*6110 insertion element specific to MTBC. DNA (5 µl) extracted from plaques (section 2.2.2.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each primer MTC F and MTC R (Table 2.2); No enhancer or 20 % Q-Solution (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

The PCR product (5 µl) was used as DNA template for the 16s and *IS*6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.02 or 0.04 µM of each KY18 and KY75 primer (Table 2.2); No enhancer or 20 % Q-Solution (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the *IS*6110 element.

2.4.3.7. Validating the adapted two round multiplex PCR assay

This method utilized two rounds of PCR, the first pre-amplification PCR used the MTC F/R primers (Table 2.2) described by (Eisenach *et al*., 1990). The PCR assay amplifies a segment of the *IS*6110 insertion element specific to MTBC. DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

The PCR product (5 µl) was used as DNA template for the 16s and *IS*6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.04 µM of each KY18 and KY75 primer (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the *IS*6110 element.

2.4.3.8. Comparison between singleplex 16s rDNA PCR and multiplex 16s rDNA and IS6110 PCR assay

This method utilized the KY18/75 primers and the MTC F/R primers (Table 2.2) described by Tevere *et al*. (1996) and Eisenach *et al*. (1990) respectively. The PCR assay amplifies a segment of the 16s rDNA gene (KY) and *IS*6110 insertion element

specific to MTBC (MTC). DNA (5 µl) extracted from plaques (section 2.2.2.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the singleplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.04 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.04 µM of each KY18 and KY75 primer (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products (15 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 530 bp for 16s rDNA gene and 123 bp for the *IS*6110 element.

2.4.4. Quantitative PCR assays

2.4.4.1. IAC qPCR assay

This method utilized the IAC probe (this project) with the MTC F/R, P90/91 and MK7/8 primers described by Eisenach *et al*. (1990), Sanderson *et al*. (1992) and Tevere *et al*. (1996) respectively. The PCR amplifies and detects the GFP segment of

the pIAC plasmid (Table 2.2). DNA (5 µl) extracted from plaques (section 2.2.2.), the Oneday phage assay (section 2.2.3.), (1 µl) cells (section 2.4.1.1.) or purified pIAC (section) was used in each qPCR reaction. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.4 µM of each primer MTC F and MTC R or P90 and P91 or MK7 and MK8 (Table 2.2); 0.2 µM of IAC probe was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a positive signal in the yellow channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.4.2. IS6110 qPCR assay

This method utilized the IAC probe and IS6110 probe (this project) with the MTC F/R primers described by Eisenach et al. (1990). The PCR amplifies and detects a segment of the IS6110 insertion element specific to MTBC and the GFP segment of the pIAC plasmid (Table 2.2). DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction.

To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 20 % Q5 GC Enhancer (v/v; NB; UK); 1 $x10^{-6}$ ng ml^{-1} purified pIAC plasmid was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a positive signal in the yellow channel and the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools. The number of plaques produced in the standard phage assay was then compared to the Cq values produced with the *IS*6110 qPCR assay on the Oneday phage assay DNA extracts.

2.4.4.3. MAP RT qPCR assay

This method utilized the MAP RT probe and IAC probe (this project) with the MAP RT F/R primers (this project) and MTC F/R primers described by Eisenach *et al*. (1990). The PCR amplifies and detects a segment of the *IS*900 insertion element specific to MAP and the GFP segment of the pIAC plasmid (Table 2.2). The IAC in this qPCR assay was amplified by the MTC F/R primers in a non-competitive format. DNA (5 µl)

extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3.) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction.

To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.5 µM of each primer MAP RT F and MAP RT R (Table 2.2); 0.25 µM MAP RT probe; 0.25 µM of IAC probe with 0.25 µM MTC F/R primers and 1 $x10^{-6}$ ng purified pIAC plasmid (where applicable) was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a positive signal in the yellow channel and the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. An extra 5 cycles was added at the end of the qPCR assay if samples were producing late positive results and the IAC showed inhibition and delayed amplification. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.4.4. BactoReal® Kit Mycobacterium tuberculosis Complex

This method utilized the commercial kit, BactoReal® Kit Mycobacterium tuberculosis Complex (Ingenetix; Austria), which detects the ITS2 region of MTBC bacteria. The diagnostic probe (FAM) was detected on the green channel and IAC (Cy5) on the red channel of the RotorGene-Q qPCR machine and companion Q-REX software

(QIAGEN; UK). The kit components (stored at -20 °C) were thawed on ice. DNA (5 µl) extracted using the Oneday phage assay (section) was used in this assay. The kit positive control (1 µl) was used, which should produce a positive result (expected Cq range of 28 – 31; Green channel) and a no template (water) negative control was also performed, which should produce a negative result. The kit IAC was also added to indicate false-negative results produced by DNA polymerase inhibition (expected Cq range of 34 – 38; Red channel). To prepare the PCR reaction: 10 µl of DNA Reaction Mix (2x); (1 µl) *Mycobacterium* MTBC Assay mix; CR Assay mix (1 µl) were added to a 0.2 ml qPCR grade PCR tube and made up to 20 µl with sterile distilled water. The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The PCR parameters used were: 50 °C for 2 min, followed by 95 °C for 20 s, followed by 45 cycles of: 95 °C for 5 s, 60 °C 1 min and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' tool.

2.4.5. Optimizing qPCR assays

2.4.5.1. Sensitivity of the IAC qPCR assay

This method utilized the IAC probe (this project) with the MTC F/R primers described by Eisenach *et al*. (1990). The PCR amplifies and detects the GFP segment of the pIAC plasmid (Table 2.2). A ten-fold serial dilution series was performed by diluting purified pIAC plasmid 1 in 10 into sterile molecular grade water. DNA (1 µl) of purified pIAC (section 2.5.3.2.) was used in each qPCR reaction. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe was added to a

qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a positive signal in the yellow channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.5.2. Sensitivity of the IS6110 qPCR assay

This method utilized the *IS*6110 probe (this project) with the MTC F/R primers described by Eisenach *et al*. (1990). The PCR amplifies and detects a segment of the *IS*6110 insertion element specific to MTBC (Table 2.2). A ten-fold serial dilution series of *M. bovis* DNA extracted with the boiled lysis method was performed by diluting 1 in 10 into sterile molecular grade water from 70 ng to 7 $x10^{-5}$ ng of DNA. DNA (1 µl) from the DNA dilutions was used in each qPCR reaction. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied

(QIAGEN; UK). The *IS*6110 probe contained the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.5.3. The effect of increasing primer concentration on the amplification of the IS6110 qPCR assay

This method utilized the IS6110 probe (this project) with the MTC F/R primers described by Eisenach et al. (1990). The PCR amplifies and detects a segment of the IS6110 insertion element specific to MTBC (Table 2.2). *M. bovis* DNA (7 x10^{-1} ng) extracted using the boiled lysis technique (section 2.4.1.1.) and diluted into sterile molecular grade water was used in each qPCR reaction. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.5 µM, 0.75 µM or 1 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; No plasmid or 1.36 x10^{-3} ng of pIAC DNA was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The *IS*6110 probe contained the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was

followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.5.4. The effect of PCR enhancers on the amplification of the IS6110 qPCR assay

This method utilized the IS6110 probe (this project) with the MTC F/R primers described by Eisenach et al. (1990). The PCR amplifies and detects a segment of the IS6110 insertion element specific to MTBC (Table 2.2). A ten-fold serial dilution series of M. bovis DNA extracted with the boiled lysis method (section 2.4.1.1.) was performed by diluting 1 in 10 into sterile molecular grade water from 70 ng ml^{-1} to 7 $x10^{-6}$ ng ml^{-1} of DNA. DNA (1 µl) from the 7 $x10^{-4}$ ng ml^{-1} to 7 $x10^{-6}$ ng ml^{-1} DNA dilutions was used for the qPCR reactions. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 2.5% DMSO (v/v; Sigma; UK), 20 % Q-Solution (v/v; QIAGENN; UK) or 20 % Q5 GC Enhancer (v/v; NEB; UK) was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The *IS*6110 probe contained the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection.

The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.4.5.5. Determining the Oneday assay detection limit of the IS6110 qPCR assay

To perform this method, first a ten-fold serial dilution of a *M. bovis* BCG liquid culture (approximately 1 x10^8 cfu ml^{-1}) was prepared to an approximate concentration of 1 cfu ml^{-1}. The Oneday phage assay (section 2.2.3.) and standard phage assay (section 2.2.2.) was then performed on 100 µl of each dilution. DNA (5 µl) extracted from the Oneday phage assay was used for the *IS*6110 qPCR assay. This assay utilized the IAC probe and *IS*6110 probe (this project) with the MTC F/R primers described by Eisenach *et al*. (1990). The PCR amplifies and detects a segment of the IS6110 insertion element specific to MTBC and the GFP segment of the pIAC plasmid (Table 2.2).

To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 1.36 x10^{-5} ng of purified pIAC plasmid was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a positive signal in the yellow channel and the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was

followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools. The number of plaques produced in the standard phage assay was then compared to the Cq values produced with the *IS*6110 qPCR assay on the Oneday phage assay DNA extracts.

2.4.5.6. Effect of PBMC DNA on IS6110 qPCR assay

This method utilized the IAC probe and IS6110 probe (this project) with the MTC F/R primers described by Eisenach et al. (1990). The PCR amplifies and detects a segment of the IS6110 insertion element specific to MTBC and the GFP segment of the pIAC plasmid (Table 2.2). A ten-fold serial dilution of a *M. bovis* BCG liquid culture (approximately 1 x10^8 cfu ml^{-1}) was prepared to produce dilutions of 1 x10^6 cfu ml^{-1} and 1 x10^4 cfu ml^{-1}. The Oneday phage assay (section 2.2.3.) was performed on 100 µl of each of the dilutions. DNA (2 µl) from the *M. bovis* BCG Oneday phage assay extracts were used for the qPCR reactions. *IS*6110 negative Oneday phage assay (Chapter 5) DNA extract containing PBMC DNA from several samples were pooled together to produce a volume of 12 µl. DNA (3 µl) from the pooled PBMC DNA was also used in the appropriate reactions. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 20 % Q5 GC Enhancer (v/v; NB; UK); 1 x10^{-6} ng ml^{-1} purified pIAC plasmid was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in a RotorGene-Q rotor and the locking ring was applied (QIAGEN; UK). The IAC probe contained the HEX fluorophore which produced a

positive signal in the yellow channel and the FAM fluorophore which produced a positive signal in the green channel with the RotorGene-Q companion software, Q-Rex (QIAGEN; UK). The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools.

2.5. General Cloning and transformation of bacteria

2.5.1. Preparation of competent cells

2.5.1.1. E. coli

The cloning strain used in this project was *E. coli* electrocompetent Top10 (Invitrogen; UK), the cells were originally stored at -80 °C. To prepare the cell, one aliquot was removed from storage and thawed on ice

2.5.1.2. M. smegmatis MC^{2}155

To prepare the *M. smegmatis* electrocompetent cells, 500 ml of MP was inoculated with a single colony of *M. smegmatis MC^{2}155* and incubated at 37 °C for 2 d with shaking. Then the culture was incubated on ice for 1.5 h. The culture was then split into two sterile 250 ml centrifuge bottles and the cells were harvested by centrifugation at 5,000 xg for 10 min at 4 °C. The supernatant was then discarded and the cells were reconstituted with ice cold 10 % glycerol (250 ml). The cells were centrifuged again at 5,000 xg for 10 min at 4 °C. The supernatant was discarded again and the pellets were reconstituted in 10 ml ice cold 10 % glycerol and combined. From this point a balance was used containing only water. The volume of the cells

was then increased to 50 ml with ice cold 10 % glycerol. The cells were then centrifuged at 2000 xg for 10 min at 4 °C and the supernatant discarded. The cells were reconstituted again with 50 ml ice cold 10 % glycerol and centrifuged at 2000 xg for 15 min at 4 °C. The supernatant was then discarded and the cells were reconstituted with 1 ml ice cold 10 % glycerol. The cells were then aliquoted (100 µl) into 1.5 ml microfuge tubes pre-chilled at -80 °C and immediately stored at -80 °C for future use.

2.5.2. Cloning plasmids

2.5.2.1. Cloning the pTB-GFP segment

To clone the pTB-GFP segment, plasmid pSB2018 was used as the template in an addition PCR assay. This plasmid contains a copy of the GFP gene (Qazi *et al*., 2001). To prepare the PCR reaction approximately 50 ng of pSB2018 plasmid was added as template; the primer concentration added for each of the addition primers TB-GFP F and TB GFP R (Table 2.2) was 0.5 µM; the buffer and DNA polymerase used was Q5® High-Fidelity 2x Master Mix; SDW was used to produce a final volume of 20 µl. The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 98 °C for 30 s. This was followed by 30 cycles of: 98 °C for 10 s, 64 °C for 20 s, 72 °C for 20 s. This was followed by a final extension step of 72 °C for 2 min. The PCR products (5 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected product size was 180 bp.

2.5.2.2. Nanodrop DNA quantification

To quantify DNA using a Nanodrop (ThermoFisher; UK), first the pedestal was cleaned with 2 µl of sterile molecular grade water and dried with a lens cloth. Then the Nanodrop software was opened and 'Nucleic Acid' selected. Then 1 µl of sterile molecular grade water was loaded onto the pedestal and a measurement taken. The pedestal was dried. Then 1 µl of the liquid used to elute the DNA into was transferred to the pedestal and blank measurement taken. The pedestal was dried. Finally, 1 µl of the DNA sample was added to the pedestal and a measurement was taken. The concentration of DNA (ng ml^{-1}) and the 260/230 and 260/280 values were recorded.

2.5.2.3. Cloning the pIAC segment

To clone the pIAC segment, the purified restriction digest of the TB-GFP segment (1 µl) was used as the template in an addition PCR assay. To prepare the PCR mixture: 10 µl Q5® High-Fidelity 2x Master Mix (NEB; UK); 0.5 µM of each of the addition primers pIAC F and pIAC R was 0.5 µM was added to a 0.2 ml PCR tube and SDW was used to make to a final volume of 20 µl. The mixture was briefly flash centrifuged to bring the contents to the bottom of the PCR tube and placed in an UNO96 thermocycler (VWR; UK). The PCR parameters used was an initial denaturation step of 98 °C for 30 s. This was followed by 30 cycles of: 98 °C for 10 s, 64 °C for 20 s, 72 °C for 20 s. This was followed by a final extension step of 72 °C for 2 min. The PCR products (5 µl) were electrophoresed through a 2 percent agarose TAE gel (w/w) containing 0.2 mg ml^{-1} ethidium bromide (Sigma Aldrich; UK) at 70 V for 1 h. The gels were visualized under UV light with a Gel Doc system (BIORAD; UK). The expected

product size was 270 bp. The plasmid insert was then prepared for purification and restriction digestion.

2.5.2.4. Restriction double digest

To prepare the insert and pMV306 plasmid for their ligation together, the TB-GFP insert (370 ng) and pMV306 vector (1 µg) or pIAC insert (268 ng) and pMV306 (1 µg) were independently digested with 1 µl of each of the restriction enzymes *Nco*I (NEB; UK) and *Hind*III (NEB; UK), 5 µl NEBuffer 2.1 (NEB; UK) and made up to 50 µl with sterile molecular grade water in a 1.5 ml microfuge tube. The double digest was then incubated at 37 °C for 20 hours. The restriction enzymes were then inactivated by heating at 80 °C for 20 min.

2.5.2.5. Monarch® PCR & DNA Cleanup Kit (5 µg)

The restriction digests were first purified using a Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB; UK) as per the manufacturer's protocol. First, the Wash Buffer was prepared by adding 4 parts ethanol to 1 part DNA Wash Buffer. Then 250 µl of DNA Binding Buffer was mixed with the digested insert and 100 µl of DNA Binding Buffer was mixed with the digested pMV306 sample and transferred to a Spin Column. The samples were then centrifuged at 16,000 xg for 1 min. Next 100 µl of DNA Wash Buffer was added to the columns and the samples were centrifuged again at 16,000 xg for 1 min. The previous wash step as then repeated a second time. The Spin Column was then transferred to a sterile 1.5 ml microfuge tube and 16 µl of sterile molecular grade water (55 °C) was added to the center of the matrix. The samples were then incubated at room temperature for 1 min then centrifuged at 16,000 xg

for 1 min to elute the DNA. The Spin Column was then discarded. The purified digests of the insert and vector were then used to prepare a ligation mixture.

2.5.2.6. Ligation Reaction

To perform the ligation, The online tool NEBioCalculator™ (http://nebiocalculator.neb.com/#!/ligation) was used to calculate the amounts required for an insert (180 bp), to vector (3995 bp) ratio of 3:1 and a vector mass of 50 ng was (as recommended by the manufacturer of the T4 DNA Ligase (NEB; UK). The final ligation mixture contained 6.76 ng of the insert, 50 ng of the pMV306 plasmid, 1 µl of T4 DNA Ligase and 2 µl T4 DNA Ligase Buffer (10X), sterile molecular grade water was used to make up to a final volume of 20 µl. The ligation mixture was then incubated at 24 °C for 20 min to allow the ligase to ligate the insert and vector together and combine the two components.

Once the pTB-GFP plasmid had been produced by the ligation of the TB-GFP segment and the pMV306 plasmid, the next step was to transform the plasmid directly into electrocompetent *E. coli*. Excess plasmid was stored at -20 °C for future use.

2.5.3. Transformations

2.5.3.1. Transformation of plasmids into E.coli

To do this, electrocompetent Top10 *E. coli* cells were prepared (section 2.5.1.1.). Freshly prepared cells were incubated on ice then mixed with 1 µl of the ligation mixture with a pipette tip. The DNA-cell mixture was then incubated on ice for 5 minutes before being added to a 0.2 cm electroporation cuvette (BIORAD; UK) and re-incubated on ice. The cells were then electroporated using a Biorad Gene Pulser (BioRad; UK) using a single pulse of: 2.5 kV, 25 µF and 250 Ω as suggested by the

manufacturer for the electroporation of *E. coli*. Immediately after the electroporation, 1 ml of LB broth pre-warmed to 37 °C was added to the electroporation cuvette and the cells were resuspended gently to avoid lysing the weakened cells. The cuvette was then incubated at 37 °C for an hour to allow the transformed cells that have acquired the plasmid to translate the kanamycin resistance gene within the plasmid. The transformed cells were plated onto LB agar plates supplemented with 50 µg ml^{-1} of kanamycin and incubated for 24 hours at 37 °C.

2.5.3.2. Plasmid DNA extraction

To extract the plasmid from the *E. coli* transformants, a single colony was inoculated into 5 ml of LB broth (Sigma Aldrich; UK) supplemented with kanamycin (50 µg ml^{-1}) in a 15 ml conical bottomed tube and incubated overnight at 37 °C with shaking. The next day the plasmid was extracted from the cells using a Monarch® Plasmid Mini-Prep Kit (NEB; UK) following the manufacturers protocol. Before using this kit 4 volumes of (> 95 %) ethanol (24 ml) was added to one volume of Monarch Plasmid Wash Buffer 2. The culture was first split equally into three separate 1.5 ml microfuge tube then centrifuged at 16,000 xg for 30 s. The supernatant was discarded, and the cell were reconstituted with 200 µl Plasmid Resuspension Buffer (B1) and pipette mixed until there were no visible clumps and the solution was fully homogenized. The cells were the lysed by adding 200 µl Plasmid Lysis Buffer (B2) and the samples were mixed by gentle inversion until the colour changed to dark pink and became clear and viscous, then the samples were incubated at room temperature for 1 min. Next 400 µl of Plasmid Neutralization Buffer (B3) was added and the sample was mixed by gentle inversion until the sample became uniformly

yellow and a precipitate formed. The samples were then incubated at room temperature for 2 min. The samples were then centrifuged at 16,000 xg for 5 min. The supernatant was then carefully transferred to a Spin Column in a collection tube taking care not to disturb and co-extract the pellet. The Spin Columns were then centrifuged at 16,000 xg for 1 min and the flow through was discarded. Then 200 µl Plasmid Wash Buffer 1 was added to the Spin Column and centrifuged at 16,000 xg for 1 min. Next, 400 µl Plasmid Wash Buffer 2 was added to the Spin Columns and centrifuged at 16,000 xg for 1 min. The spin column was then transferred to a sterile 1.5 ml microfuge tube and the collection tube discarded. Then 30 µl DNA Elution Buffer (50 °C) was added to the center of the matrix and incubated at room temperature or 1 min. The samples were then centrifuged at 16,000 xg for 1 min to extract the plasmid. The extracted plasmid was then stored at -20 °C for future use.

2.5.3.3. Transformation of plasmids into M. smegmatis-IAC - Goude and Parish (2009)

To transform the pTB-GFP plasmid into electrocompetent *M. smegmatis MC^{2}155* using the method described by Goude and Parish (2009). First the two aliquots of electrocompetent cells previously prepared (section 2.5.1.2.) were thawed on ice. To one aliquot, 466 ng of pTB-GFP plasmid (7 µl) was added and to the second aliquot, 500 ng of pMV306 plasmid (10 µl) was added. The cell-plasmid mixture was then transferred to an ice-cold 0.2 cm electroporation cuvette (BIORAD; UK) and electroporated using a BIORAD Gene Pulser using a single pulse of 2.5 kV, 25 µF and Capacitance Extender set to 1000 Ω. The cuvette was then incubated on ice for 10 min. The cells were then transferred to 5 ml MP (37 °C) and incubated with shaking for 3 h. Then 100 µl and 10 µl of cells were spread plated onto separate 7H10 agar

supplemented with 10 % OADC using an L-shaped spreader. The cells were then harvested by centrifugation at 3000 xg for 10 min, the supernatant was discarded and 10 µl of the pellet was also spread plated. The samples were then incubated at 37 °C for 2 weeks, samples without growth after 2 weeks were discarded.

2.5.3.4. Transformation of pTB-GFP into M. smegmatis-IAC – BIORAD

When performing the BIORAD transformation protocol, three plasmids were electroporated. The plasmids pMV306 and pMV261 were used as controls, pMV306 to show if the plasmid manipulation had affected the integration mechanism of the plasmid and pMV261 to show if the pTB-GFP plasmid was transforming into *M. smegmatis* but not integrating. First electrocompetent *M. smegmatis MC^{2}155* (section 2.5.1.2) was thawed on ice. Then 500 ng and 1000 ng of pMV306 and pTB-GFP and 500 ng of pMV261 was added to separate aliquots of cells. The cell-plasmid mixture was then transferred to an ice-cold 0.2 cm electroporation cuvette (BIORAD; UK) and electroporated using a BIORAD Gene Pulser using a single pulse of 2.5 kV, 25 µF and Capacitance Extender set to 1000 Ω. Immediately after electroporation, the cells were suspended in 2 ml MP. Then 1 ml of cells was transferred to a sterile 1.5 ml microfuge tube and incubated by resting on the top of ice for 10 min. The remaining 1 ml (and ice-chilled cells) were immediately incubated at 37 °C for 2 h with shaking. The cells were then centrifuged at 3,000 xg for 10 min. The supernatant was discarded and all the pellet was transferred to 7H10 agar supplemented with 10 % OADC and spread using a sterile L-shaped spreader. The samples were then incubated at 37 °C for 2 weeks, samples without growth after 2 weeks were discarded. Samples that produced growth were counted, patch plated and analyzed with *IS*6110 PCR analysis (section 2.4.2.2)

2.5.3.5. Transformation of pIAC into M. smegmatis-IAC – BIORAD

First electrocompetent *M. smegmatis MC^{2}155* (section 2.5.1.2) was thawed on ice. Then 1000 ng of pIAC DNA was mixed with the cells. The cell-plasmid mixture was then transferred to an ice-cold 0.2 cm electroporation cuvette (BIORAD; UK) and electroporated using a BIORAD Gene Pulser using a single pulse of 2.5 kV, 25 µF and Capacitance Extender set to 1000 Ω. Immediately after electroporation, the cells were suspended in 2 ml MP (37 °C). The cells were immediately incubated at 37 °C for 2 h with shaking. The cells were then centrifuged at 3,000 xg for 10 min. The supernatant was discarded and all the pellet was transferred to 7H10 agar supplemented with 10 % OADC and spread using a sterile L-shaped spreader. The samples were then incubated at 37 °C for 2 weeks. Samples that produced growth were patch plated and analyzed with *IS*6110 PCR analysis (section 2.4.2.2)

2.5.3.6. Plasmid Stability

To determine how stable the pTB-GFP plasmid integrated into the genome of *M. smegmatis*, 5 ml of 7H9 media supplemented with 10 (%) (v/v) OADC with and without 25 µg ml^{-1} kanamycin sulphate was inoculated to a density of approximately 1 x 10^4 cfu ml^{-1} by emulsifying several colonies into 1 ml of MP and adding 100 µl of cells to each assay. The comparison was repeated to produce four repeats of the experiment. The cultures were then incubated aerobically at 37 °C with shaking for 39 h until they reached stationary phase. The cultures were then sub-cultured 1 in 100 according to the conditions used in the initial culture and were incubated again aerobically at 37 °C with shaking for a further 39 hours. This process was repeated until the cultures had entered stationary phase five times. The cultures were then plated onto 7H10 agar supplemented with 25 µg ml^{-1} kanamycin sulphate using the

Miles Misra method (section 2.1.1.2.). The samples were then incubated at 37 °C for 7 d. The number of colonies that grew were counted and the average cfu ml^{-1} was calculated. A paired two tailed test was performed on Excel (Microsoft; UK) with the average cfu ml^{-1} to determine whether there was a difference between the average cfu ml^{-1} produced under the two different growth conditions.

CHAPTER 3

3. DEVELOPING A DUAL INTERNAL AMPLIFICATION CONTROL PLASMID FOR DETECTION OF MAP AND MTBC ORGANISMS

3.1. INTRODUCTION

Detecting mycobacteria in clinical samples using molecular methods presents several issues that need to be circumvented to achieve good results. First of all, blood contains several inhibitors that interfere with the activity of Taq-polymerase resulting in the possibility of false negative results (Schrader *et al*., 2012). Some examples of the inhibitors that come directly from blood include haemoglobin and lactoferrin, which are believed to release Fe ions which interfere with DNA synthesis (Al-Soud and Rådström, 2001) and immunoglobulin G, which can complex with single stranded DNA (ssDNA) and block polymerase elongation (Al-Soud *et al*., 2000). These proteins aren't the only inhibitors present in blood, and the preservatives used to prevent blood coagulation are also reported to impede the activity of Taq-polymerase. For instance, Heparin, a common anticoagulant, is also believed to complex with nucleotides due to its large negative charge (Satsangi *et al*., 1994). In contrast, EDTA chelates the Mg^{2+} ions present in DNA polymerase buffers and causes a decrease in the binding affinity of the polymerase for the template DNA (Schrader *et al.*, 2012). If any of these inhibitors are co-extracted during DNA purification, they may inhibit a PCR-based assay leading to a negative diagnostic test result (Roux *et al*., 2019). Since many different potential inhibitors are found in high levels in clinical blood samples, blood is recognised as a difficult test sample for direct PCR assays (Yang and Rothman, 2004)

In the light of these problems, to confidently assess whether a negative PCR result is a true negative, it has become necessary to include additional controls to PCR assays to prove that no inhibitors are present and that an amplification event can take place

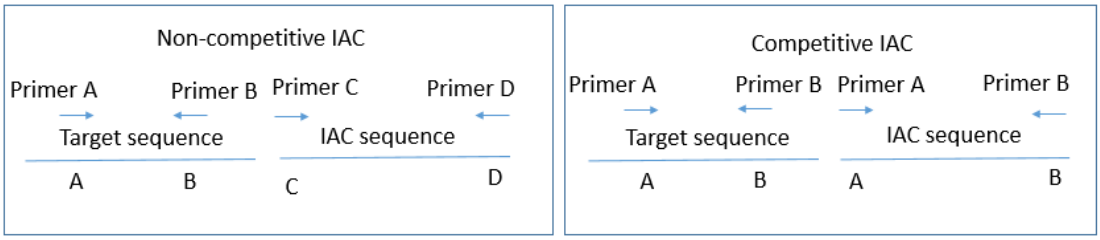
(Oikonomou *et al*., 2008). This is achieved by a positive control that can be included in the test format (Burkardt, 2000). A positive control usually takes the form of an independent PCR reaction of purified target DNA that is performed in a separate PCR tube. The function of this control is to show that the amplification of the target DNA is possible given the composition of the PCR mixture and the cycling conditions chosen (Lee *et al*., 2004). The inherent limitations with this style of positive control arise from the fact the control hasn't been through the same processing steps as the sample DNA, and therefore may not contain contaminants that originate from the sample material (Brey *et al*., 2006). Hence process controls are another integral aspect to provide assurance to the test results.

A process control is a test conducted at a critical point within an assay. One example of this is the positive control in a phage-assay, which shows that the phage used can infect mycobacteria present in the sample and carry out a full replication process leading to lysis of the infected mycobacteria. Another process control can be employed at the DNA purification step to indicate that the DNA extraction and purification process was successful. This can simply involve analysis of the purified DNA using spectrophotometry equipment such as a NanoDrop (Thermo Scientific; UK). This equipment identifies the yield of DNA extracted and co-extraction of potential inhibitory agents to PCR such as proteins and DNA extraction reagents, for example phenol (Desjardins and Conklin, 2010).

Another approach that can be employed to both detect when inhibitors are present in samples is introducing an internal amplification control (IAC) into each test PCR tube. The premise of an IAC is that the IAC product should be amplified in every

reaction, regardless of the presence of any target DNA (Akbarian *et al*., 2015). An IAC can be designed in two forms: non-competitive, which uses a multiplex PCR and two independent targets and primer pairs (Hoorfar *et al*., 2004); and competitive, which uses the same primer pair for amplification of the target and the IAC (see Fig. 3.1a) (Rosenstraus *et a*l., 1998).

Figure 3.1a Comparison of non-competitive and competitive PCR assay designs



The format for the non-competitive IAC uses four primers. Primers A and B bind to homologous DNA sequences of the target organism at sites A and B. Primers C and D bind to homologous DNA sequences on the IAC DNA. The format for a competitive IAC uses two primers. Primers A and B bind to the same homologous DNA sequences of the target organism and the IAC construct.

The IAC produced for endpoint-PCR is generally designed to produce an IAC PCR product that is of a different size to that of the target fragment, so that the two amplification events can be distinguished. In RT-PCR assays, an IAC fragment is amplified, and the signal is detected using a probe and fluorophore that is labelled with a different colour and therefore can be detected in a different channel to that used to detect the target. When an IAC is used in a PCR reaction, the IAC product should be amplified every time, therefore, PCR reactions that produce only the IAC band can then be assumed to be true negatives. Negative samples that produce neither the IAC band nor the target band indicate that inhibition has occurred, and the DNA sample being tested should be retested, or re-purified to remove inhibitors before the PCR step is repeated.

Even when causing active disease, mycobacteria have been found to be present in low numbers in blood samples (Swift *et al*., 2016a, Swift and Rees, 2013, Maggioli, 2016). Another quantitative study investigating the bacterial load of *M. tuberculosis* in both HIV positive and HIV negative humans identified these bacteria in a range of less than 1 cfu ml^{-1} to 90 cfu ml^{-1} in blood samples (Crump *et al*., 2011). An alternate study also reported a limit of *M. tuberculosis* in adult blood of 0.4 cfu ml^{-1} (Pavlinac *et al*., 2016). For this reason, it is important that any PCR assay used to detect mycobacteria in clinical blood samples is appropriately sensitive, with an ability to detect at least one genome copy of the organism. To try and increase the sensitivity of PCR-based diagnostic assays for mycobacteria, multicopy elements are often targeted instead of single copy genes to increase the amount of target sequence present in a sample. The *IS*6110 element is multi-copy and is routinely used as a PCR target for the detection of members of the MTBC, because strains of *M. tuberculosis*

may contain between zero and 27 copies within the genome, although this element is reported on rare occasions to be absent or present in low copy number of some strains of MTBC organisms (Roychowdhury *et al*., 2015). Therefore, any diagnostic PCR targeting this element would still need to be sensitive enough to detect one copy of the *IS*6110 element to ensure that all possible strains are detected.

In the literature, it is reported that the *IS*6110 PCR has a limit of detection of 100 cells when targeting clinical samples (Kolia-Diafouka *et al.*, 2018), and these levels are 10-fold less than that required to allow them to detect the levels of TB shown to be found in blood. This lack of sensitivity of the PCR assay is probably due to the difficulty lysing the mycobacteria due to the resilient nature of the cell wall of these bacteria which limits the amount of DNA released by extraction methods for detection.

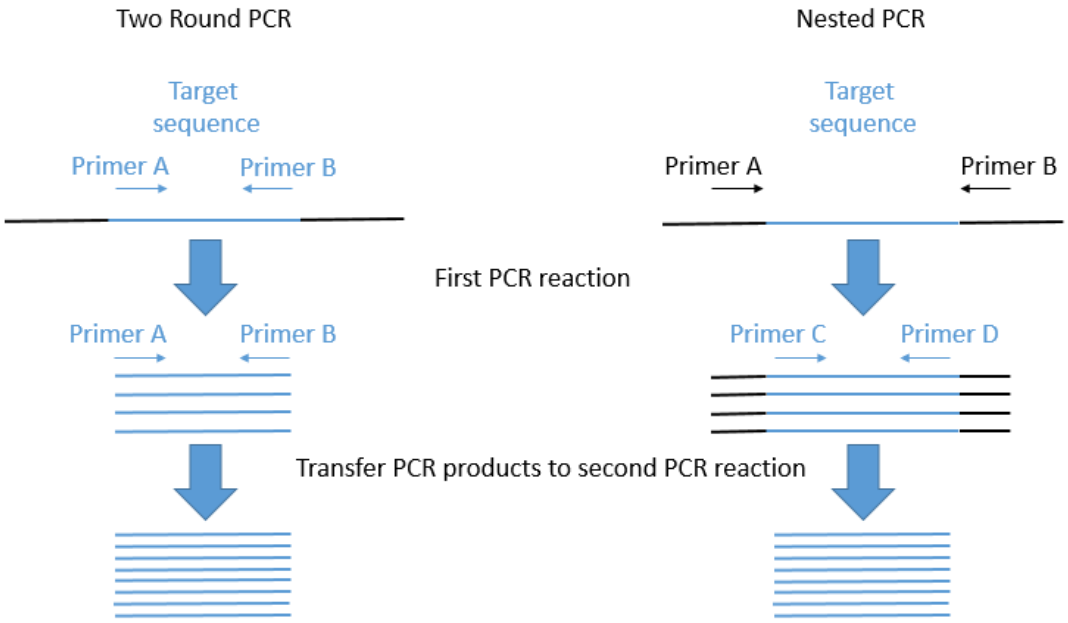
The same approach of targeting multicopy genes to improve the sensitivity of detection is also used for PCR assays that detect other mycobacteria, such as MAP. For MAP, the multi-copy *IS*900 element is a well described target for the detection of MAP by PCR amplification (Motiwala *et al*., 2006). This is chosen as a PCR target because it is primarily restricted to the MAP subspecies and there are multiple copies per genome, being between 14 and 18 copies found in MAP genome sequences (Bull *et al.*, 2000).

Most extraction methods developed for mycobacteria use complex lysis protocols to liberate the mycobacterial DNA (Käser *et al*., 2010, Manta *et al*., 2020) but are reported to produce low yields of DNA. The methods often include bead beating procedures to achieve rupture of the cells, but this can result in shearing of genomic

DNA and potential loss of PCR sensitivity if DNA targets are cleaved between the primer sites (Tell *et al*., 2003). One way to extract high quality DNA from mycobacteria for molecular analysis is to use phage as a lysis agent. Stanley *et al*. (2007), who showed that by using phage it was possible to detect one cell of MAP genomic DNA extracted from a plaque arising from phage amplification assays by targeting the multi-copy *IS*900 element by PCR. This method has also been used to detect single MAP cells recovered from milk (Botsaris *et al.*, 2013) and single *M. bovis* cells from cattle blood (Swift *et al.*, 2016a). However, in these papers, multiple plaques were combined for DNA extraction to try and increase the sensitivity of the PCR detection step.

Another method to increase the sensitivity of any PCR assay targeting low DNA concentrations is to use either two rounds of PCR amplification using the same primers or a nested PCR approach, using two rounds of PCR but with primers located within the target sequence for the second round of PCR (see Fig. 3.1b).

Figure 3.1b - Comparison between a two round PCR and nested PCR



The two round PCR format uses two rounds of PCR and two primers, primers A and B. The PCR products from the first PCR are used to spike the second PCR mixture to provide the template.

The nested-PCR format uses two rounds of PCR and four primers. In the first round, the two outermost primers, primers A and B are used. The PCR products from the first round are used to spike the second PCR mixture to provide the template. In the second PCR two different primers are used, Primers C and D, which bind between Primers A and B used in the first round.

Both modifications are designed to amplify low copy number targets by incorporating a shorter, pre-amplification round of PCR to generate more target DNA, which is then used as a template for the second round of PCR amplification. These methods work by circumventing the depletion of resources that occurs in the later cycles of PCR, which often cause high cycle number amplifications to fail to produce a detectable amount of DNA (Jansson and Hedman, 2019). The advantage of nested PCR over just using two rounds using the same PCR primers is that by using a second set of primers that have target sites within the fragment amplified in the initial PCR (*i.e.* the primer binding sites are "nested"; (Green and Sambrook, 2019), this reduces the problem of re-amplification of non-target amplicons and therefore helps increase the specificity of nested PCR assays. A nested PCR for the *IS*900 (Bull *et al*., 2003) element has also been used to detect one MAP cell derived from a phage plaque (Swift *et al*., 2013, Gerrard *et al*., 2018).

Although not as specific, a two-round PCR has the advantage of this approach over nested PCR is that only one set of reagents is required – but as mentioned above - the disadvantage is that any spurious products that accumulate in the first round, will also be amplified in the second round. Given that the nature of the amplicon is usually characterised by either product size or affinity to an internal probe sequence for RT-PCR, then this is not an issue when running a diagnostic test, but two round PCRs are often considered to be less specific.

3.2. Producing a non-competitive IAC for the *IS*6110 end-point PCR assay

A feature of the phage-PCR assay is that DNA extracted from plaques arising from phage amplification assays contain the DNA from the lysed target mycobacterial cell,

but also contain many copies of the lysed *M. smegmatis* cells that form the lawn to support plaque growth (see section 1.3.1.). This means that the 16s rRNA gene from *M. smegmatis* is always present in high copy number in the DNA extracted from the plaques. Therefore, it was decided to try and take advantage of this fact and use these as the internal control sequence for the phage-PCR assays by multiplexing the mycobacterial-specific PCR with the KY18/75 primers which target the mycobacterial 16s rRNA gene (Tevere *et al.*, 1996). The intentions of these initial experiments were to incorporate a non-competitive IAC into the plaque-PCR assay by targeting the 16s rDNA of the *M. smegmatis* that forms the lawn to support plaque formation. Another attractive aspect of targeting the *M. smegmatis* as the internal control was the fact that it would be present in all plaque assays, regardless of the species of mycobacteria being detected. Choosing the 16s rDNA from the *M. smegmatis* lawn also increased the versatility of the IAC because the PCR assay could be potentially multiplexed with any of the diagnostic Mycobacteria PCR assays, this is ideal for incorporating into a kit based diagnostic system since the same control can be used for every application of the assay.

The MTCF/R primers which target the *IS*6110 element (Eisenach *et al*., 1990) were chosen as the organism specific primers. *M. bovis* BCG was chosen for the sensitivity assessment as it contains either one or two copies of the *IS*6110 element, dependent on which strain of this attenuated *M. bovis* strain is used (Alonso *et al*., 2013). The *M. bovis* BCG strain used in this study (NCTC 5692) contains one copy of the element (Stanley *et al*., 2007), which is the absolute minimum a PCR assay should be able to practically detect.

Prior to this project starting, a previous study by Swift *et al*. (2016a) attempted to detect one *M. bovis* BCG genome using the phage-assay and the IS6110 PCR (Eisenach *et al*., 1990), however it was found that the standard PCR assay was not sensitive enough alone to produce a positive reaction, even when using phage as a lysis agent. It is pivotal that the *IS*6110 PCR assay was able to detect a single copy of the IS6110 element owing to the paucibacillary nature of *M. tuberculosis* bacteraemia and the small volume of blood available for diagnostic testing. With this in mind, a two round PCR protocol was designed using the MTCF/R primers described by Eisenach *et al.* (1990) to see whether this adaptation for low copy number PCR targets would improve the sensitivity of the PCR assay to allow a positive result to be gained from a single *M. bovis* BCG genome.

Therefore, the aims of the experiments presented in this chapter were first to incorporate an IAC into the plaque-PCR assay to improve the assurance of the results gained and secondly to investigate whether a two-round PCR could be used to improve the sensitivity of the IS6110 PCR.

3.3. RESULTS

3.3.1. Initial assessment of the 16s and IS6110 primers in a multiplex PCR for the plaque-PCR IAC.

The first experiment conducted was to determine how the MTCF/R primers and the KY18/75 primers performed when combined in a standard, one round PCR. Two different templates were used. The first was genomic DNA purified from *M. bovis* cells using a boiled lysis method (section 2.4.1.1.) and experiments were performed using this to determine how the primer pairs performed when combined in a

multiplex compared to how they performed when used on their own in PCR assays using the same template. The second DNA template used was the DNA extracted from a single *M. bovis* BCG derived plaque using the freeze/ thaw method (section 2.4.1.2.) and estimated to contain one genome equivalent. This was used to confirm whether the method developed was capable of detecting one copy of *IS*6110. For the plaque DNA, only the KY primers were tested individually, the MTCF/R primers were used only in a multiplex and not independently as it was already known that these primers under these parameters were not able to amplify MTBC DNA from a single BCG plaque (Swift *et al*., 2016).

Table 3.1 Primers used in this chapter

Primer Name	Primer Sequence	Tm (°C)	GC (%)
TB Complex F	CCTGCGAGCGTAGGCGTCGG	67.55	75
TB Complex R	CTCGTCCAGCGCCGCTTCGG	67.55	75
KY75	GCCCGTATCGCCCGCACGCTCACA	71.26	71
KY18	CACATGCAAGTCGAACGGAAAGG	61.43	52
KY18Ex	AACACATGCAAGTCGAACGGAAAGG	62.98	48
TB-GFP-F	CATGCCATGGCCTGCGAGCGTAGGCGTAGG gtatagttcatccatgccatgtg	90.30	38
TB-GFP-R	CCCAAGCTTCTCGTCCAGCGCCGCTTCGGctg tccttttaccagacaacc	89.92	40
pIAC F	CATGCCATGGGTCTGGGATTGGATGTCCTGT ATGAAGGGTGTTCGGGGCCGTCGCTTAGGT ATCCTGCGAGCGTAGGCGTAGGgtat	86.81	56
pIAC R	CCCAAGCTTCACCACGTGGTTAGCAATCCTA TGGCGTTGAGGTCGATCGCCCACGTGACTAT CTCGTCCAGCGCCGCTTCGGctgt	87.01	57

List of primer sequences and properties used in this Chapter.

Pink: NcoI and HindIII restriction sites

Lower case: GFP sequence

Mycobacteria have a genome with a very high GC content, which resists melting due to the large amount of hydrogen bonds holding the DNA strands together; when the strands do melt, they are prone to reannealing and can also produce secondary structures. The reannealing of the template inhibits the amplification of the PCR product by preventing the DNA polymerase from binding to single stranded DNA; secondary structures inhibit the polymerase by blocking the extension of the polymerase, again, due to the formation of dsDNA (Bhagya *et al*., 2013). Therefore, dimethyl sulfoxide (DMSO) was added to the PCR reactions because it improves the amplification of GC rich PCR reactions. The addition of DMSO inhibits the formation of secondary structures and the DNA from reannealing by interfering with the formation of hydrogen bonds (Assal and Lin, 2020, Jensen *et al*., 2010), which then makes the template and primers available for amplification by the DNA polymerase. DMSO also decreases the melting temperature of primers, resulting in the need to lower the temperature of the annealing step of the PCR cycles to allow the primers to anneal to the template (Obradovic *et al*., 2013).

To prepare these PCR assays, the template used was (a) approximately 25 ng of *M. bovis* boiled lysate DNA (section 2.4.1.1.) and (b) 5 µl from the 10 µl *M. bovis* BCG plaque DNA extract (section 2.2.2.); 0.5 µM of each primer was used in the single PCR reactions and 0.25 µM of each primer was used in the multiplex reactions; the buffer used was QIAGEN HotStarTaq Plus Mastermix; 5 % (v/v) DMSO was added to improve the amplification of the *IS*6110 element; 2 µl of coral loading dye was added to allow for the direct loading of the PCR product into an agarose gel. The annealing temperature selected for the PCR was altered from the temperature of 62 °C described by Tevere *et al*. (1996) for the KY18/75 primers as this was lower than the

68 °C described by Eisenach *et al*. (1990) for the MTCF/R primers. The use of the higher melting temperature would likely prevent the KY18/75 primers from annealing in a multiplex PCR. However, with the addition of DMSO to the PCR reactions, the annealing temperature used was decreased by 3 °C to compensate for the reduction in the melting temperatures of the primers, as recommended by the manufacturer. The PCR parameters used were an initial denaturation of 95 °C for 5 min, then 30 cycles of 95 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min, followed by a final extension of 72 °C for 7 min (section 2.4.3.1.).

The results of the PCR using the *M. bovis* DNA as a template are shown in Figure 3.2 (Panel A; lanes 1 – 4). A 530 bp PCR product corresponding to the amplification of the 16s rDNA when the KY primers were used alone, and a 123 bp PCR product corresponding to the amplification of the *IS*6110 element when the MTCF/R primers were used alone, were produced as expected. In addition, both the 530 bp and 123 bp amplicons were present when these primers were used in combination in the multiplex PCR, showing that there was no interference occurring between the two sets of primers.

In contrast, when plaque DNA was used as the template (Fig. 3.2, Panel A; lanes 5 – 6) low level amplification of the 530 bp 16s rDNA region when the KY primers were used alone was seen. When the multiplex was carried out with both the KY and the MTC primers, the 530 bp amplicon was again amplified weakly and there was no detectable amplification of the *IS*6110 element. This was unexpected, as there should have been ample genomic DNA from the lysed *M. smegmatis* lawn present in the sample to act as template DNA for these primers. For comparison, the 25 ng of

M. bovis boiled lysate DNA contained approximately 10-fold more copies of the 16s rDNA gene with 5.68 x 10^6 copies and this produced a strong band. The copy number was calculated by calculating the mass of a single *M. bovis* genome using the equation:

$$\frac{Genome\ size\ (Mbp)\ *\ Average\ mass\ of\ one\ bp\ (g\ mol^{-1})}{Avagadros\ constant}$$

The calculation for *M. bovis* is as follows:

$$\frac{4.35\ *\ 609.7}{6.022^{23}}$$

Which equates to 4.4 fg of DNA. As 25 ng of DNA template was used for this PCR, the calculated number of copies of genome present were:

$$\frac{25}{0.0000044}$$

Which equates to 5.68 x 10^6 genomes. The same quantity of 16s rRNA genes are also available because the *M. bovis* genome contains only one copy of the gene (*rrn*) , which is believed to be related to its slow growing phenotype (Ji *et al*., 1994). In comparison DNA extracted from a single plaque should contain a minimum of approximate 2 x 10^5 *M. smegmatis* cells (based on the initial inoculum used, and not allowing for an increase in cell number due to replication during incubation of the lawn). Each *M. smegmatis* cell contains two copies of the 16s rDNA gene (*rrnA* and *rrnB*), therefore it is predicted that there should be ~4 x 10^5 copies of the *rrn* genes per plaque. Since half of the plaque DNA is used as a template for each PCR, this means that each PCR assay should initially contain approximately 2 x 10^5 copies of 16s rDNA. The plaque-PCR IAC control band was therefore predicted to be slightly

less intense than the *M. bovis* boiled lysate IAC band. This suggested a poor extraction of the plaque DNA resulting in a low DNA yield because the high copy number IAC target amplified inefficiently and the *M. bovis* results show that the PCR conditions used are suitable to produce a strong amplification of the 16s rDNA.

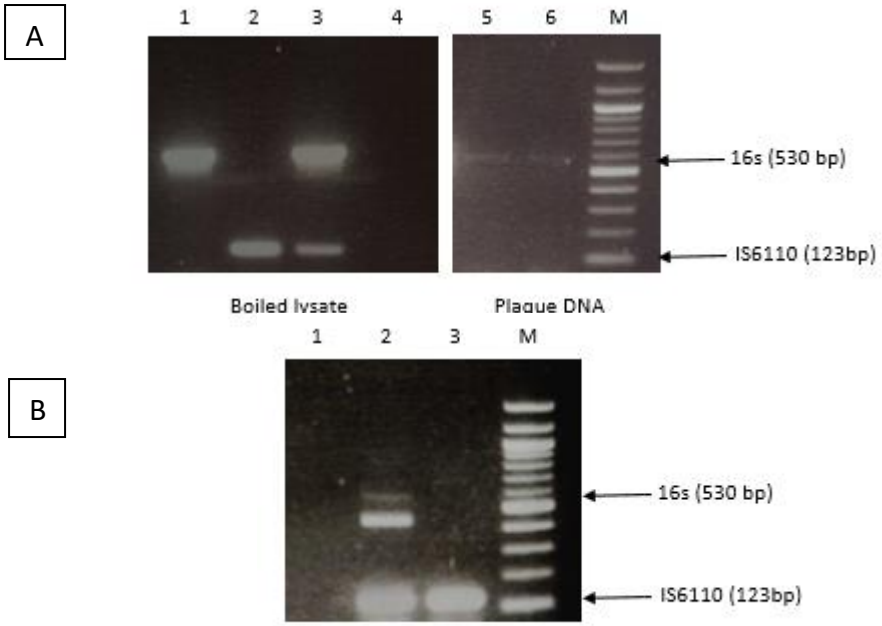
Similarly, the results of the standard one round PCR with the *M. bovis* boiled lysate DNA, which contains a high copy number of *IS*6110 elements, showed that good levels of amplification of the *IS*6110 element is possible under these PCR conditions. Again, when the plaque DNA was used as a template, the sensitivity of the one round PCR was shown to be too low to produce a detectable amount of the *IS*6110 element. This again suggested that the lack of sensitivity may have arisen from the poor extraction method used to isolate the DNA from the *M. bovis* BCG plaque.

Before opting to change the DNA extraction methods used for the plaques it was decided to try and improve the sensitivity of the *IS*6110 PCR by incorporating an initial pre-amplification PCR, using the cycling parameters: initial denaturation 95 °C for 5 min, then 12 cycles of: 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, followed by a final extension of 72 °C for 7 min (section 2.4.3.2.). Again, the annealing temperature chosen was 3 °C lower than the 68 °C previously described (Eisenach *et al*., 1990). This precursor PCR step was carried out using only the primers to amplify the *IS*6110 element and was designed to increase the amount of *IS*6110 template for the second, 30 cycle PCR step. Again, results were compared using *M. bovis* (25 ng) boiled lysate DNA (section 2.4.1.1.) which should give a positive result, and DNA extracted from a single *M. bovis* BCG plaque (section

2.4.1.2.) which would indicate whether the methods used were appropriate for the typical DNA arising from the phage-PCR assay.

The PCR products from the first PCR were used as template for the multiplex PCR containing both the *IS*6110 and 16s rDNA primers. In this case, the *M. bovis* boiled lysate showed a strong amplification of the 123 bp band corresponding to the *IS*6110 element. A 16s product of approximately 530 bp was also amplified, and in line with the expected results, the amount of product was now reduced compared to the results seen using just a one round PCR (Fig. 3.2; Panel B, lane 2). However a band was also observed at approximately 450 bp, which was believed to have arisen during the pre-amplification step. Two round PCR assays are designed to amplify low copy number templates, it is possible that this step amplified a non-specific segment of DNA which was then subsequently amplified to a detectable level in the second PCR step. Using the DNA eluted from a plaque, this time the results showed a strong amplification of the *IS*6110 element but no detectable amplification of the 16s rDNA band (Fig. 3.2; Panel B, lane 3). This may have been due to preferential amplification of the *IS*6110 element in this PCR, but this is also consistent with the weak amplification seen in the single PCR since only 25 % of the template used in that reaction was added to the second round of this PCR assay.

Figure 3.2 – Initial evaluation of the MTCF/R and the KY18/75 primers



Panel A - Lanes 1 – 3, boiled lysate M. bovis DNA used as template; Lanes 5 – 6, DNA from one BCG plaque used as template. Lane 4, no template (water) negative control.

Panel B - Lane L; 100 bp DNA Ladder (NEB; UK). Lane 1, no template (water) negative control; Lane 2, boiled lysate *M. bovis* DNA used as template; Lane 3, DNA from one BCG plaque used as template.

Panel A - Primer combinations used in each of the PCR reactions were: Lanes 1 and 5, 0.5 µM KY18/75 primers; Lane 2, 0.5 µM MTCF/R primers; Lanes 3, 4 and 6, 0.25 µM KY18/75 and MTCF/R primers. Different exposures of the two halves of the gel are shown to allow the feint bands in lanes 5 and 6 to be more clearly seen. The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on LHS of image). Lane M; 100 bp DNA Ladder (NEB; UK).

To prepare the singleplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer KY18 and KY75 (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. For the multiplex PCR reaction mixtures: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.25 µM of each primer KY18, KY75, MTC F and MTC R (Table 2.2); 5 % DMSO; 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h (section 2.4.3.1.). Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System. All primer concentrations are correct

Panel B - To prepare the first PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR product (5 µl) was used as DNA template for the second round of PCR (16s and IS6110 multiplex PCR assay). The parameters for the second round of PCR were the same as described in Panel A.

3.3.2. Improving DNA extraction method for plaques

The poor extraction of DNA from a plaque seemed to be limiting the sensitivity of the PCR analysis. The method originally used to extract DNA from the agar samples was a simple freeze/thaw method that was based on a method described for extracting DNA from agarose gels (Qian and Wilkinson, 1991, Stanley *et al*., 2007). Although simple to perform and inexpensive, this extraction method is inefficient because some of the DNA is retained by the mass of melted agarose. Swift *et al*. (2013) showed that by pooling plaques and extracting the DNA using a DNA Spin Column designed for the extraction of DNA from agarose gels, that one MAP cell could be detected in a DNA sample using the nested-PCR protocol described by Bull *et al*. (2003). This seemed to improve DNA recovery, possibly enhanced by the *M. smegmatis* DNA present in the sample that acted as carrier DNA as Spin Columns have poor binding capacity for low amounts of DNA, requiring input of extra non-target DNA to efficiently bind the DNA to the matrix (Ackerman *et al*., 2019). The carrier plaques would also increase the quantity of 16s rDNA available as template for the internal control.

To see if this method could improve the sensitivity of the two-round multiplex PCR, a Zymoclean Gel DNA extraction kit (ZymoResearch, Cambridge Bioscience; UK) was used to extract the DNA from one *M. bovis* BCG plaque and four *M. smegmatis* plaques pooled together (section 2.4.1.3.). The DNA was eluted in a final volume of 10 µl. The results were again compared to those gained using 25 ng of *M. bovis* boiled lysate DNA (section 2.4.1.1.) which should give a positive result. Half of the DNA elution from the Spin Column (5 µl) was used as the template and using QIAGEN HotStarTaq Plus mastermix. When considering the primer concentration for the pre-

amplification PCR it was decided to increase the concentration of the *IS*6110 primers from 0.5 µM to 0.8 µM, to increase the likelihood of primers annealing to the low quantity of *IS*6110 elements in the early cycles of the PCR assay so that all copies of the template are amplified in each cycle. Once the pre-amplification PCR step had been completed, 5 µl of the PCR products were used as template for the second round PCR. The primer concentrations for both the *IS*6110 element and the 16s rDNA were varied in the different PCR reactions for the second PCR assay to ascertain the optimum primer ratio required to achieve equal band intensities. An assumption was made when the five plaques were pooled together that the increase in the amount of the 16s rDNA internal control template would now be far greater than that of the pre-amplified *IS*6110 element. With this assumption in mind, when selecting the primer concentrations to be tested, the *IS*6110 primer concentrations were always the greater than the 16s rDNA primers, except in one control experiment in case the assumption was incorrect and, in this case, a standard primer concentration of 0.5 µM was again selected. Where an increased amount of *IS*6110 primers were tested, this was 0.8 µM so that the final concentration of *IS*6110 primers was 1 µM due to the 0.2 µM carryover from the pre-amplification PCR step. In addition to these reactions, PCR assays were prepared with ten-fold less 16s rDNA primers (0.05 µM and 0.08 µM) compared to *IS*6110 primers, to account for the expected increase in internal control template (section 2.4.3.3.).

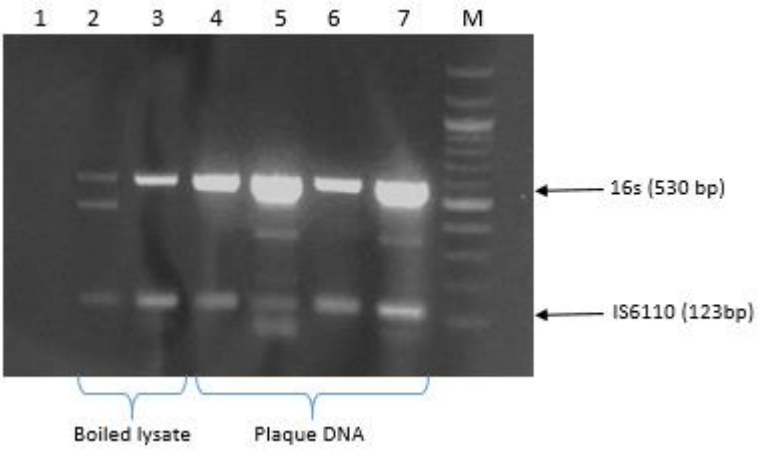
The results of the *M. bovis* DNA as template with the different primer pair ratios gave the following results. The first primer ratio tested (Fig. 3.3, lane 2) produced low level amplification of the 530 bp product corresponding to the amplification of the 16s rDNA when 0.08 µM of KY18/75 primers were used and low-level amplification of the

*IS*6110 element when 0.5 µM of MTC F/R primers were used. A non-specific band was also amplified with an approximate size of 450 bp. The second primer ratio tested (Fig. 3.3, lane 3) produced an increased level of amplification of the 530 bp rDNA amplicon when 0.5 µM of KY18/75 primers were used compared to when 0.08 µM was used. Low level amplification of the *IS*6110 element was also observed when 0.8 µM of *IS*6110 primers were used. These results were slightly unexpected in regard to the amplification of the *IS*6110 element as it was expected that the *M. bovis* boiled lysate would produce a stronger amplification of the *IS*6110 element than 16s rDNA, (especially as the pre-amplification PCR for the *IS*6110 element was performed) because the amount of *IS*6110 template was greater than the 16s rDNA template in the second PCR. Therefore, these results indicated that the PCR conditions need further optimization.

In contrast, the results for the plaque DNA gave the expected results. All PCR reactions that contained plaque DNA as the DNA template produced a strong level of amplification of the 16s rDNA and low-level amplification of the *IS*6110 element, which was in line with the expected results (Fig. 3.3, Lanes 4 – 7). The PCR reaction that contained a greater concentration of KY18/75 primers compared to *IS*6110 primers (Fig. 3.3, lane 5) showed the over-amplification of the 16s rDNA, and several non-specific bands were also amplified when using this primer concentration. When 0.5 µM of the KY18/75 primers were used (Fig. 3.3, lane 7), the same over-amplification of the 530 bp rDNA product was produced, with slightly less non-specific amplification detected. A lower level of target gene amplification was produced when the primer concentration of KY18/75 used was 0.08 µM (Fig. 3.3, lane 4) or 0.05 µM (Fig. 3.3, lane 6), with a concentration of 0.05 µM producing the

most suitable amplification. The results showed that the extraction of the plaque DNA by using the gel extraction Spin Column method and pooling one *M. bovis* BCG plaque with four *M. smegmatis* plaques, improved the amplification of the internal control 16s rDNA, by increasing the quantity of 16s rDNA template and improving the quality of the DNA extraction. This showed that the assumption made prior to the experiment that the ratio of 16s rDNA was now greater than the *IS*6110 elements was correct. It also showed that the use of a DNA Spin Column (section 2.4.1.3.) was a better extraction method than the freeze/ thaw method (section 2.4.1.2). The increase in concentration of *IS*6110 primers of 0.3 µM failed to increase the levels of amplification of the *IS*6110 element in the plaque-DNA extracts. Again, this suggested that the PCR parameters for the multiplex assay needed to be optimized to improve the amplification of the *IS*6110 element. Before the optimization was performed, the manufacturers handbook for the HotStarTaq Plus Master Mix (QIAGEN; UK) was consulted to determine the suggested primer concentration range, which was found to be 0.1 – 0.5 µM. Since some of the primer concentrations used were at the upper limit and over, a further experiment was conducted where the primer concentrations were reduced. This was to see whether the PCR reactions were saturated in primers in the multiplex PCR which was inhibiting the amplification of the *IS*6110 element.

Figure 3.3 – Assessment of the pooled, Spin Column extracted plaque DNA and primer concentrations on the two round multiplex PCR



Lane 1, no template (water) negative control. Primer combinations used in each of the PCR reactions were: Lane 2, 0.5 µM MTCF/R and 0.08 µM KY18/75 primers; Lane 3, 0.8 µM MTCF/R and 0.5 µM KY18/75 primers; Lane 4, 0.5 µM MTCF/R and 0.08 µM KY18/75 primers; Lane 5, 0.5 µM MTCF/R and 0.8 µM KY18/75 primers; Lane 6, 0.8 µM MTCF/R and 0.05 KY18/75 primers; Lane 7, 0.8 µM MTCF/R and 0.5 µM KY18/75 primers.

The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.3.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on RHS of image). Lane M; 100 bp DNA Ladder (NEB; UK). Lanes 2 and 3, 25 ng boiled lysate M. bovis DNA used as template. Lanes 4 – 7 DNA from one BCG plaque and four M. smegmatis plaques extracted with a gel Spin Column kit used as template.

To prepare the first PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR product (5 µl) was used as DNA template for the 16s and IS6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 or 0.5 µM of each MTC F and MTC R primer and 0.05 or 0.08 µM of each KY18 and KY75 primer (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

3.3.3. Improving the amplification of the IS6110 element

The poor amplification of the *IS*6110 element was hypothesized to be caused by the high concentration of the *IS*6110 primers. It is reported that increasing the primer concentrations above 0.5 µM can lead to an increased likelihood of primers annealing to themselves and produce primer dimers (Markoulatos *et al*., 2002). The primer concentrations were reduced to assess whether the high primer concentrations were inhibiting the amplification of the insertion sequences. In this instance, it was shown that reducing the primer concentration improved the amplification of the insertion sequences (see Appendix 1, section 9.1.1. for more in-depth information). The next step for improving the amplification of the IS6110 element was to extend the KY18 primer (Table 3.1) to bring the melting temperature to within 5 °C of the MTC F/R primers so that the annealing temperature could be increased to alleviate the non-specific binding. It was shown that extending the primer did not improve the amplification compared to the standard primer when the annealing temperature was increased (For further details see Appendix 1, section 9.1.2). The final step in optimizing the amplification of the *IS*6110 element was to see what effect PCR enhancers had on the reaction as DMSO was already used in the reaction mix as standard. The results showed PCR enhancers did and did not improve the amplification of the two insertion elements, but it was determined that removing PCR enhancers was the best option (for further details and figures see Appendix 1, sections 9.1.3. and 9.1.4.) as the assay is being designed as a commercial assay, so aspects such as cost reduction need to be considered.

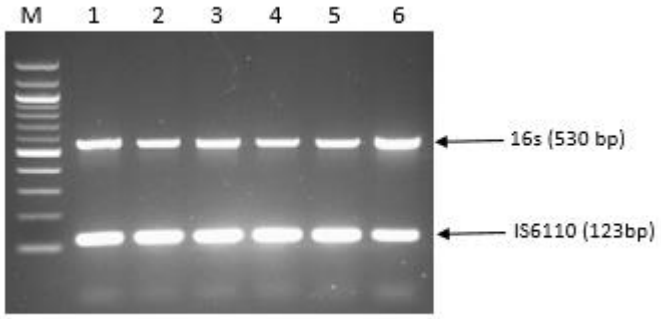
3.3.4. Validation of the two-round multiplex

The removal of the PCR enhancers was the final step in the optimization of the two round multiplex PCR assay. The next stage was the validation of the PCR assay to show that the PCR could effectively detect from one genome equivalent of *M. bovis* BCG to 5 genome equivalents and amplify an internal control product. The parameters selected for the PCR were derived from the previous optimizations (section 2.4.3.7.). The primer concentration of the *IS*6110 primers added to each round of the PCR assay was 0.6 µM resulting in a final concentration of 0.75 µM in the second round. The concentration of 16s rDNA primers used in the second round was 0.04 µM. An annealing temperature of 65 °C was used and no PCR enhancers were added to either round. To validate the assay, five plaques derived from either *M. bovis* BCG and/or *M. smegmatis* were combined and extracted with the Spin Column method (section 2.4.1.3.). The range of plaques used were from no *M. bovis* BCG plaques (i.e., 5 *M. smegmatis* plaques) to five BCG plaques (i.e., no *M. smegmatis* plaques). The plaque DNA extract was then used for the optimized two round multiplex PCR (section 2.4.3.7).

The results of the validation showed only two bands were amplified, one at 530 bp corresponding to the 16s rDNA and one at 123 bp corresponding to the *IS*6110 element. These two bands were amplified in the reactions containing one to five *M. bovis* BCG plaques (Fig. 3.4, Lanes 1 – 5). However, the sample that contained only five *M. smegmatis* plaques also produced amplification of the *IS*6110 element (Fig. 3.4, Lane 6). This was not in line with the expected results because the template used should only contain 16s rDNA, *M. smegmatis* should not contain an *IS*6110 element as it is not a member of the MTBC. This result suggested that the PCR reaction had

become contaminated with the *IS*6110 element. To confirm if this was the case, the validation was repeated using freshly extracted plaques, and new unused PCR reagents and primers. However, the results were the same and the *M. smegmatis* plaques again produced a PCR product of 123 bp as well as 530 bp. At this stage the two round multiplex PCR was not yet optimised, and the conditions used may have been resulting in the amplification of a non-specific product of approximately the same size as the expected product of the *IS*6110 element.

Figure 3.4 – Validation of the two round multiplex PCR assay



The combination of plaques extracted in the PCR assays were: Lane 1, 1 BCG and 4 M. smegmatis; Lane 2, 2 BCG and 3 M. smegmatis, Lane 3, 3 BCG and 2 M. smegmatis; Lane 4, 4 BCG and 1 M. smegmatis; Lane 5, 5 BCG; Lane 6, 5 M. smegmatis. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.7.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on RHS of image). Lane M, 100 bp DNA Ladder (NEB; UK).

To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR product (5 µl) was used as DNA template for the 16s and IS6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.04 µM of each KY18 and KY75 primer

(Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used were an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

3.3.5. Determining the cause of the false positive produced from the *M. smegmatis* plaques in the two round multiplex PCR

Due to the false positive produced by the *M. smegmatis* plaques, the cause of this needed to be investigated as the two round multiplex PCR could not be used until false-positive *IS*6110 results were not being obtained. The *M. smegmatis* DNA extracted from plaques using the spin column method (section 2.4.1.3.) was compared to the DNA extracted from the boiled lysis method (section 2.4.1.1.) using the optimized PCR parameters (section 2.4.3.7.). The results showed non-specific binding in both reactions (see Appendix 1, section 9.1.5. for figure and more details). The assay was then repeated without the addition of the pre-amplification PCR and the results this time showed there was no non-specific binding, which showed the problem was being caused by the increased sensitivity of the pre-amplification round (see Appendix 1, section 9.1.6. for figure and more information). The pre-amplification round was necessary to increase the sensitivity of the assay so that a single *IS*6110 element could be detected so it was decided this method could not be validated and that a new method of producing an IAC would be investigated

3.4. Producing a competitive IAC as an internal control

Other groups have also reported challenges in the optimization steps that are required to facilitate the efficient amplification of all the PCR products in multiplex PCRs (Randall *et al*., 2010). Due to the problems observed in the non-competitive IAC development, it was decided to take a different approach to produce an internal amplification control for the detection of mycobacteria. Due to difficulties posed by interactions between the different primer pairs, it was concluded that competitive

IAC assays were the preferred option. The premise of a competitive IAC is that the same primers that are used to detect the target organism are used to amplify the internal control band, this means that the PCR conditions do not need to be optimized as the parameters will not favour the amplification of one of the PCR products.

A common way to produce a competitive IAC is to clone the diagnostic primer sequences used to detect the target organism into a plasmid and transform this plasmid into an amplification host (*E. coli* for example) whose role is purely to amplify the internal control plasmid. The IAC plasmid is then extracted and purified from the host strain and a known quantity of the plasmid is added directly to the PCR reaction to add the internal control template (Maaroufi *et al*., 2006). Competitive internal amplification controls have already been described for *M. tuberculosis*, for instance Cortez-Herrera *et al*. (2008) and Moslemi (2013) both produced a competitive IAC for PCR assays designed on the model where the IAC plasmid is added directly to the PCR reaction. Both of these studies utilized the same primers for the *IS*6110 element, however these were different to the primers described by Eisenach *et al*. (1990) used in this study. In this study it was decided to expand upon the simple incorporation of a plasmid into a PCR reaction, and by producing a novel internal control strain of *M. smegmatis* that contained the IAC plasmid. The reason for this was it again increased the flexibility of the IAC because the strain could be used as an IAC and also as a control during critical process control points throughout the phage assays *i.e.,* it would also provide a phage infection control for the phage assay.

3.4.1. Design of the *M. smegmatis* IAC

It was decided to use an integrative-shuttle vector designed to replicate in both *E. coli* and mycobacteria to build the IAC. The advantage of this is that the IAC plasmid can be built in *E. coli* to facilitate the cloning steps and then amplified and purified to a high concentration in *E. coli*. The high concentration of plasmid is then used to transform *M. smegmatis* which requires a significant amount more DNA to successfully transform than is required for transformation of *E. coli* (Gaora, 1998). The shuttle-vectors used to transform mycobacteria come in two forms, ones that exist as cytosolic plasmids in mycobacteria and those that integrate into the mycobacterial genome. The vector chosen as the template for the IAC production was the integrative vector, pMV306 (Stover *et al.*, 1991). This plasmid was chosen for its integrative properties because this should produce a more stable transformant than a cytosolic plasmid. The benefit of this again is related to its inclusion as a reagent in a commercial kit as the integrative plasmid shouldn't need constant selective pressure to retain the IAC plasmid whereas a cytosolic will need to be grown in the presence of the appropriate antibiotic to prevent the loss of the plasmid from the cell. The pMV306 vector is designed from the L5 mycobacteriophage and contains the same integrase and the complementary *attP* sequence to the mycobacterial *attB* sequence found within the tRNA *glyV* gene where pMV306 integrates by homologous recombination (Huff *et al*., 2010). The *M. smegmatis* genome contains only one copy of the *GlyV* gene and only one plasmid can undergo homologous recombination and integrate into the mycobacteria *attB* site. This means that one *M. smegmatis* cell should contain only one copy of the plasmid, which should result in a lower amount of IAC template than diagnostic

template to reduce the likelihood of the preferential amplification of the IAC product.

3.4.2. Producing a competitive IAC plasmid in E. coli

The first step undertaken to produce the competitive IAC was to produce the internal control segment for the plasmid. The internal control segment will produce the PCR product for the internal control. To design the internal control segment a band size was first selected as the desired final size of the IAC product. In this case a size of 180 bp was chosen for the internal control of the *IS*6110 PCR assay for the detection of MTBC. The band size of 180 bp was chosen as it was approximately 50 bp longer than the *IS*6110 element which would allow the IAC to be incorporated into a Real-Time PCR assay as well as an end point PCR assay. A larger IAC product was also designed compared to the diagnostic product to increase the likelihood of the preferential amplification of the smaller diagnostic product, this should mitigate against any false negatives that may arise from the preferential amplification of the IAC product over the diagnostic product. To build the internal control segment and produce a 180 bp PCR product, a 140 bp sequence of GFP DNA was chosen as DNA to use as the template was readily available in the laboratory. No environmental strains of mycobacteria have been isolated either that produce GFP, so any probes for the IAC element that may be designed for a Real-Time PCR assay won't cross react with any potential mycobacterial genomes. The MTCF/R primers are both 20 bp in length so the final PCR product would be 180 bp. The GFP segment was then flanked with the MTC F/R primers using addition-PCR (section 2.5.2.1). The results showed a band at 180 bp (Fig 9.7) as was expected (see appendix 6 for more details on cloning and transformation into *E. coli*). After the IAC segment was produced, the PCR reaction

was cleaned up to remove contaminants from the PCR step (section 2.5.2.5.) and then cloned into the pMV306 integrative shuttle vector (section 2.5.2.6.). Once the plasmid was made, it was then transformed into *E. coli* (section 2.5.3.1.) which was being used as an amplification host to generate the large amount of plasmid required to transform *M. smegmatis* later. The transformed *E. coli* cells were plated onto LB kanamycin selective plates (due to the resistant marker on the pMV306 plasmid) and six colonies were selected at random for PCR analysis to confirm they are true transformants and had taken up the plasmid (See Appendix 2, section 9.2.1.).

3.4.3. Transforming the pTB-GFP plasmid into *M. smegmatis*

Two plasmids were initially selected to transform *M. smegmatis*: pTB-GFP, to produce the IAC *M. smegmatis* strain; and pMV306, to show that the addition of the IAC segment was not toxic to the cells. Initially to transform *M. smegmatis* the method described by Goude and Parish (2009) was used (section 2.5.3.3.). Firstly, electrocompetent *M. smegmatis* cells were prepared (section 2.5.1.2.) and 200 µl of the cells were incubated on ice. The plasmids (466 ng of pTB-GFP; 500 ng of pMV306) were then used to transform separate aliquots of electrocompetent cells (section 2.5.1.2.). However, this method did not prove successful, including problems with arcing due to the high levels of plasmid DNA required and freezing of samples during the post-electroporation incubation on ice specified by the protocol.

A second method described by the manufacturer of the electroporation equipment, was then tested (section 2.5.3.4.). To avoid the problem of arcing, more of the plasmid was purified to produce a more concentrated plasmid stock so that a greater quantity of plasmid can be added with a lower volume. An extra control was also added to this test, which was the addition of the pMV261 plasmid. The pMV261

plasmid was used because if colonies are produced with the cytosolic plasmid but no transformants were produced from the integrative vectors it shows the plasmids had been transformed into the cells, but the integrative plasmid had not integrated into the genome of the *M. smegmatis* cell. During the transformation, two variables were tested to assess their impact on the transformation efficiency. To perform the electroporation, five aliquots of electrocompetent *M. smegmatis* stored at -80 °C were thawed gently on ice, two each for pMV306 and pTB-GFP and one for pMV261. Then either 500 ng or 1000 ng of pMV306 and pTB-GFP plasmid were added to independent aliquots of the *M. smegmatis*. The final aliquot contained 500 ng of pMV261. Each of the cell-plasmid mixtures were added to independent 0.2 cm electroporation cuvettes and subjected to a single pulse of 2.5 kV, 25 µF and Capacitance Extender set to 1000 Ω. This time arcing was not observed in any of the samples. Immediately after the electroporation, 2 ml of room temperature 7H9 supplemented with 10 % (v/v) OADC was added to the electroporation cuvette and the cells dispersed into this media by gentle mixing. To investigate the effect of the ice step on the transformation efficiency, 1 ml of the cell suspension was removed and added directly to 1.5 ml of 37 °C pre-warmed 7H9 plus 10(v/v) % OADC and incubated with shaking at 37 °C for 2 h. The remaining 1 ml in the electroporation cuvette was then incubated on the top of ice (to reduce the likelihood of the suspension freezing) for 10 min. After the ice incubation, the cell suspension was removed and added to 37 °C pre-warmed 7H9 broth plus 10 % (v/v) OADC and incubated for 2 h at 37 °C with shaking. The cell suspensions were centrifuged to produce pellets which were then plated directly onto 7H10 agar supplemented with 10 (v/v) % OADC and 25 µg ml^{-1} kanamycin and incubated at 37 °C for 7 d. After the

incubation period, colonies were observed growing on several plates. The colonies were counted to calculate the transformation efficiency and to assess whether any of the variables had an impact on the transformation efficiency.

The results (Table 3.3) of the electroporation did not show whether the post ice incubation step increased or decreased transformation efficiency because it appeared to increase the efficiency of the pMV306 plasmid but not the pTB-GFP plasmid. Increasing the concentration of plasmid from 500 ng to 1000 ng doubled the quantity of colonies produced, although more colonies grew, the transformation efficiency (transformants per ng) remained the same due to also doubling the amount of plasmid used.

A patch plate and colony PCR was then performed on the colonies that were produced using the *IS*6110 PCR assay (section 2.1.1.4.). The results showed that all but one of the *M. smegmatis* colonies tested produced a 180 bp PCR product corresponding to the amplification of the IAC segment with the *IS*6110 primers (see Appendix 2, section 9.2.2.).

Table 3.3 – transformation efficiency of *M. smegmatis* electroporation

Plasmid used for transformation	DNA amount used	Incubation on Ice	No incubation on Ice
pMV306	500 ng	2	0
pMV306	1000 ng	0	0
pTB-GFP	500 ng	4	0
pTB-GFP	1000 ng	8	0
pMV261	500 ng	0	0

For the pTB-GFP samples, 10 were selected and as previously described, a patch plate was produced (section 2.1.1.3.) on 7H10 agar supplemented with 25 µg ml^{-1} kanamycin sulphate and a colony-PCR was performed using the *IS*6110 primers (section 2.4.2.2.). The results of the colony PCR showed the amplification of a 180 bp PCR product which corresponded to the amplification of the IAC segment within pTB-GFP in nine of the ten colonies tested (Fig. 3.14, Lanes 1 – 10). Again, one colony gave a negative PCR results showing it was not a true transformant (Appendix 6 - Fig. 9.9, Lane 10). The growth of the colonies on the kanamycin supplemented agar combined with the amplification of a 180 bp product with the *IS*6110 primers showed that the pTB-GFP plasmid was successfully electroporated into the *M. smegmatis* cells.

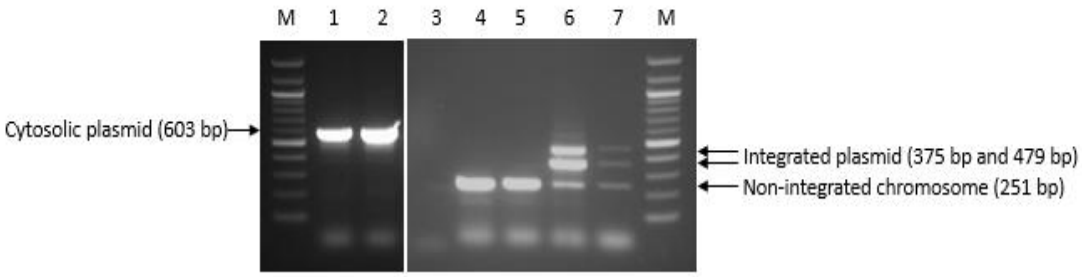
3.4.4. Determining the integration state of the pTB-GFP plasmid in M. smegmatis

The next step undertaken was to investigate whether the plasmid had successfully integrated into the genome, because although the colonies were now PCR-positive for the *IS*6110 primers, the plasmid could have been integrated into an unexpected site. In order to identify the site of integration of the plasmid, a multiplex PCR was designed with two novel primers that flanked the *attP* sequence of the plasmid and two primers that flanked the *attB* sequence of the tRNA-Gly gene of the *M. smegmatis* genome (Lee and Hatfull, 1993). Depending on the state of the plasmid, different sized PCR products were produced; circular cytosolic plasmid and uninterrupted chromosome would produce PCR products of sizes: 603 bp and 251 bp respectively. Integrated plasmid would produce PCR products of sizes, 375 bp and 479 bp (section 2.4.2.7.).

The results of the PCR assay to show the state of the pTB-GFP plasmid showed the expected results for the wild type *M. smegmatis MC*^{2}*155* and the *E. coli* pTB-GFP strain. When the *E. coli* (pTB-GFP) strain was tested (Fig. 3.5, lanes 1 and 2), a single PCR product was again produced of 603 bp which corresponded to the amplification of the circular form of the plasmid, showing no recombination of the *attP* sequence, as expected as the corresponding *attB* sequence is not present in the *E. coli* genome. When the integration primers were used with the wild type *M. smegmatis* (Fig. 3.5, lanes 4 and 5) the results showed a single PCR product of 251 bp which corresponded to the amplification of the complete uninterrupted *glyV* gene showing that nothing has integrated in between these primers. When the integration primers were used with the *M. smegmatis* (pTB-GFP) strain (Fig. 3.5 lanes 6 and 7), three PCR products were amplified: products of sizes 479 bp and 375 bp which both show that

the plasmid had integrated into the genome and that homologous recombination had occurred between the four primers. A third product was also produced, at 251 bp which again corresponded to the intact *glyV* gene, the 603 bp cytosolic plasmid band was not amplified so that shows that there was a portion of the transformant population that had lost the plasmid all together from the cell. This is line with the expected results as an insignificant loss of plasmid was observed during the plasmid stability step (see section 3.4.5. below) and this result confirms that there is indeed a heterologous population in terms of plasmid retention.

Figure 3.5 – Determining the integration state of the pTB-GFP plasmid



Lane M; 100 bp DNA Ladder (NEB; UK). The template used in the PCR assays were: Lanes 1 and 2, *E. coli*-pTB-GFP; Lanes 4 and 5, *M. smegmatis*; Lanes 6 and 7, *M. smegmatis*-pTB-GFP. Lane 3 contained the no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.7.). The expected product sized of the cytosolic plasmid was 603 bp (indicated by arrows on LHS of image). The expected product size for the non-integrated chromosome was 251 bp and the expected product sizes of the integrated plasmid were 375 bp and 479 bp (indicated by arrows on RHS of image).

To prepare the PCR assay, the integration primers were used at a concentration of 0.5 µM each; with HotStarTaq Master Mix Plus DNA polymerase and buffer (QIAGEN; UK); template DNA was approximately 30 ng of: *M. smegmatis* - pTB-GFP, *M. smegmatis* MC^2155 or *E. coli* – pTB-GFP, extracted using the boiled lysis method (section 2.x). The PCR parameters used were an initial denaturation of 95 °C for 5 min, then 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s, with a final extension of 72 °C for 5 min.

3.4.5. Determining the stability of the pTB-GFP plasmid within *M. smegmatis*

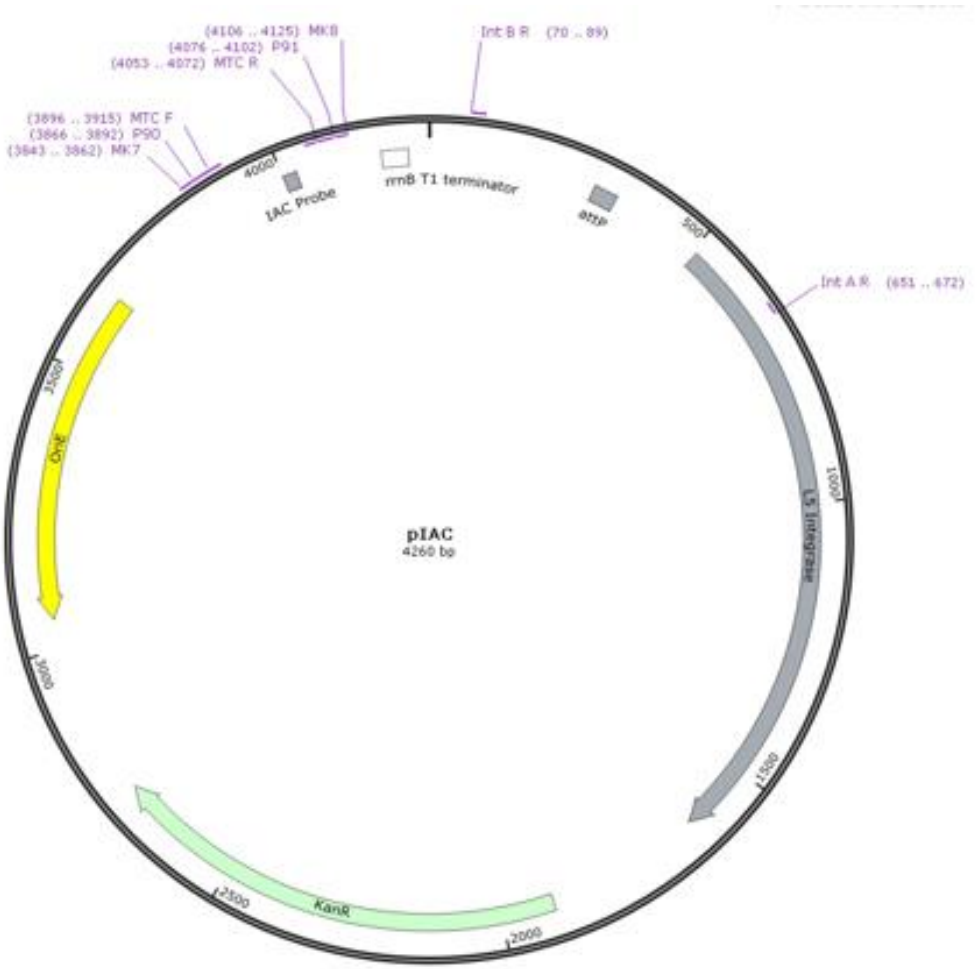
The results from the previous experiment showed that the transformation of *M. smegmatis* with the pTB-GFP was successful, but that there was evidence that the plasmid could be unstable. The pMV306 vector was initially chosen because it is an integrative vector that integrates into the genome of *M. smegmatis* through homologous recombination. The main advantage of this is that it should increase the stability of the plasmid, reducing the potential for the host cell to remove the plasmid as first it must be excised from the genome. A stable construct was desired because it meant that a reduced amount of antibiotic selection would be required to maintain the plasmid within the host strain, which would also reduce processing costs if the strain was incorporated into a commercial kit. To drive the loss of the plasmid, the transformants can be put under certain pressures. Transformant cells usually remove a plasmid when the burden of replicating and translating the plasmid is greater than the benefit that it confers. Typically, this can occur when the cells are within a nutrient depleted environment, such as when a liquid culture enters stationary phase due to high cell number, and when the antibiotic selection is no longer present (for instance if the antibiotic had degraded or was omitted). If the resistance gene is no longer required for the cell to remain viable because it is no longer in the presence of the antibiotic, then it increases the likelihood of the cell removing the plasmid in order for the cell to replicate at a quicker rate. The rate of plasmid loss of the *M. smegmatis* pTB-GFP strain was investigated by allowing the strain to enter and then be held in stationary phase several times in liquid culture without using the kanamycin sulphate selection for the presence of the pTB-GFP plasmid sequence. The results were compared with a parallel experiment that was

performed in the presence of 25 µg ml^{-1} kanamycin sulphate. A paired two-tailed test was performed on the data ($P < 0.05$) and the results showed that the plasmid had integrated in a stable manner into the *M. smegmatis* genome (for full information see Appendix 2, section 9.2.4.)

3.4.6. Increasing the primer sequences on the IAC plasmid

It was decided that the structure of the pTB-GFP should be modified to include extra primer sequences for other target organisms for the phage assays to increase the flexibility of the internal control strain so that the same IAC strain of *M. smegmatis* could be used for the internal control of different mycobacterial PCR assays. Once again, this would be beneficial for incorporating this strain into commercial kits as it helps reduce costs by maintaining a single strain compared to multiple strains for different phage assays. The diagnostic primers for two organisms were added to the pTB-GFP plasmid; specifically the P90/91 primers for detecting the *IS*900 element of MAP (Sanderson *et al.*, 1992) since the phage assay was already being used to detect this organism in cattle (Swift *et al*., 2013) and the MK7/8 primers for detecting the *IS*900 element of *M. avium* (Dohmann *et al*., 2003) because *M. avium* is also known to cause disease in cattle, birds and also humans (particularly people that are HIV positive) and was a possible further application of the phage assay.

Figure 3.6 – Plasmid map of pIAC



Plasmid map of pIAC. KanR corresponds to the kanamycin resistance gene. The plasmid also contains the origin of replication OriE and the L5 integrase. The recombination sequence corresponds to attP. The primer sequences on the plasmid are shown by pink lines with their base pair position. IAC probe corresponds to the location of the probe used in the pPCR assays. The plasmid map was produced using the software SnapGene Viewer.

To incorporate the two extra primers, two new addition primers were designed (Table 3.1 and Fig. 3.6). The plasmid was named pIAC (Fig. 3.6) and purified plasmid DNA (section 2.5.3.2.) was used to transform electrocompetent *M. smegmatis* *MC*^{2}*155* (section 2.5.1.2.) without the ice incubation step (see Appendix 6 section 9.6.2 for more information). In addition, 1 µg of pMV306 plasmid was also used to transform these cells as a control (section 2.5.3.5). After transformation, three colonies were transferred with a sterile cocktail stick to a patch plate (section 2.1.1.4.) and then transferred to separate PCR reactions to perform a colony-PCR using the *IS*6110 PCR assay (section 2.4.2.2) and *IS*900 PCR assay (section 2.4.2.4.). The results confirmed that the *M. smegmatis* cells had acquired the pMV306-IAC plasmid and the electroporation was a success (data not shown). The newly transformed strain was named *M. smegmatis*-IAC.

3.4.7. Determining the detection limit of the *M. smegmatis*-IAC in the OneDay phage assay

The next step after producing the full and expanded *M. smegmatis* IAC strains, was to test the detection limit of the strain in the Oneday phage assay when it was used as a control PCR product. A previous study for the design of a competitive IAC showed that competition between the two PCR products only produces an inhibitory effect on the lowest concentration target when there is a difference of over $1x10^4$ more copies between the two primer targets (Rosenstraus *et al.*, 1998). Therefore, it was necessary to check that the inclusion of the IAC DNA in a PCR assay did not reduce sensitivity of the PCR assay. The *M. smegmatis*-IAC strain could also serve a secondary purpose as a phage infection control to show the phage reagents from the phage-based detection kits used could produce a full lytic life cycle and breaking

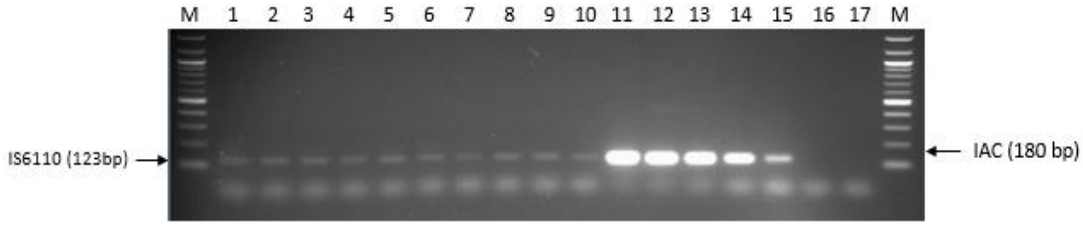
open the mycobacterial cells. This could be achieved simply by performing the Oneday phage assay using the *M. smegmatis*-IAC as the target cells and then using the primers to amplify the DNA released from the one-day phage assay. Hence to investigate this, the detection of the *M. smegmatis*-IAC strain was compared with the detection of *M. bovis* BCG cells using the Oneday phage assay because they both contain one copy of the *IS*6110 primer sequences in their genomes.

To determine the limit of detection, a liquid culture of *M. bovis* BCG with a concentration of approximately $1x10^7$ cfu ml^{-1} was ten-fold serially diluted in 7H9 media supplemented with 10 % (v/v) OADC, until a concentration of $1x10^1$ cfu ml^{-1} was achieved. Dilutions that were estimated to contain between $1x10^1$ – $1x10^5$ cfu ml^{-1} were used as samples in the Oneday phage assay (section 2.2.3.). To do this, 100 µl of the selected *M. bovis* BCG dilutions were added to an Actiphage reaction tube (0.45 µM pore size), then 10 µl of phage at a concentration of $1x10^9$ pfu ml^{-1} was added before the mixture was incubated at 37 °C for 4 h. The reaction tubes were then centrifuged at 13,000 xg for 3 min to exclude any intact cells from the supernatant and the resulting phage lysate was purified using a Zymo DNA Clean & Concentrator-5 kit (section 2.4.1.4.). This procedure was also repeated in duplicate using the *M. smegmatis*-IAC strain. The resulting DNA was used as template for PCR using the *IS*6110 primers (section 2.4.2.2.). A positive PCR control was prepared using approximately 25 ng of *M. bovis* BCG boiled lysate DNA (section 2.4.1.1.).

The results gained for the serial dilution of the *M. bovis* BCG strain were in line with the expected results. A band of 123 bp which corresponded to the amplification of the *IS*6110 element was produced for all dilutions tested (Fig. 3.7, lanes 11 – 15) and the limit of detection was determined to be approximately $1x10^1$ cfu ml^{-1} for the *M.*

bovis BCG cells. The results for the *M. smegmatis*-IAC strain were not as expected because none of the *M. smegmatis*-IAC dilutions produced a band corresponding to the IAC segment (Fig. 3.7, lanes 1 – 10). A weak *IS*6110 product was amplified in all of the *M. smegmatis*-IAC dilutions which was also unexplained because the no template (water) negative PCR control produced no amplification, showing that the PCR reagents were not contaminated. However, the positive control prepared using *M. bovis* BCG boiled lysate DNA also produced no PCR product, which was unusual considering the *M. bovis* BCG assays produced the amplification of the *IS*6110 element. The experiment was repeated (Appendix 2, section 9.2.5.) and produced the same results except the second time the *M. bovis* positive control DNA produced a positive result.

Figure 3.7 – Detection limit of *M. smegmatis*-IAC and BCG in the Oneday phage assay



The organisms used were: Lanes 1 – 10, *M. smegmatis*-IAC; Lanes 11 – 15, BCG. The concentration of cells used were: Lanes 1, 6 and 11, $1x10^5$ cfu ml^{-1}; Lanes 2, 7 and 12, $1x10^4$ cfu ml^{-1}; Lanes 3, 8 and 13, $1x10^3$ cfu ml^{-1}; Lanes 4, 9 and 14, $1x10^2$ cfu ml^{-1}; Lanes 5, 10 and 15, $1x10^1$ cfu ml^{-1}; Lane 16, *M. bovis* boiled lysate positive control; Lane 17, no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System. The expected product size for the internal control segment was 180 bp (indicated by arrows on the RHS of image) and the expected product size for the *IS*6110 element was 123 bp (indicated by arrows on the LHS of image).. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

3.4.8. Determining the detection limit of the *M. smegmatis*-IAC in the One Day phage assay using TM4 phage

To test if the stability of the integrated IAC plasmid was being affected by the Oneday assay, another experiment was prepared using a serial dilution of *M. smegmatis*-IAC cells but phage D29 was replaced with the unrelated, strictly lytic phage TM4. The same dilutions of cells and phage concentration were used as described for the last experiment (section 3.3.6). In this case the results of the PCR assay showed successful amplification of the expected 180 bp PCR product which corresponded to the amplification of the IAC segment in the samples containing 1 x 10^5 and 1 x 10^4 cfu ml^{-1}. The results showed that the detection limit was improved when the phage TM4 was used instead of D29, but still showing a lower limit of detection than was expected for the *M. smegmatis*-IAC using the Oneday phage assay (data not shown).

3.4.9. Investigating phage effect on the IAC plasmid

Due to the potential interaction between the phage D29 and the *M. smegmatis*-IAC strain, the effect of phage infection on the plasmid was investigated. There were two questions that needed to be answered, first whether the pMV306-IAC plasmid was preventing the infection of the D29 phage, and second whether phage infection was causing the loss of the plasmid. To determine the answer to the first question, the *M. smegmatis*-IAC strain was used in a standard phage assay (section 2.2.) to determine whether the phage D29 or TM4 can support productive phage infections leading to the production of plaques which would show that phage infection and host cell lysis had occurred (section 2.2.2.). The results were compared with the reference strain

M. smegmatis MC²155 which should support the production of plaques for both phage D29 and TM4 as this is the strain normally used to propagate these phage. The results of the phage assay were in line with the expected results with the *M. smegmatis MC²155* strain, which produced plaques with both D29 and TM4. The results for the *M. smegmatis*-IAC strain were comparable with the reference strain and plaques were produced when either D29 or TM4 was used as the lysis agent. This showed that both D29 and TM4 can infect and lyse the *M. smegmatis*-IAC strain with the same efficiency as the reference strain. This proved that the integration of the pMV306-IAC plasmid into the *M. smegmatis* genome was not preventing the phage D29 from producing a successful lytic infection so the first hypothesis to explain the loss of sensitivity was rejected.

The next line of investigation was to determine whether the second hypothesis for the loss of sensitivity was correct, which was that the phage infection was interfering with the pMV306-IAC plasmid. To determine whether there was a change in the plasmid state during phage infection was causing the reduction in sensitivity, the plaques produced in the previous experiment were used in the PCR assay described in section 3.3.4 to investigate the integration state of the plasmid (section 2.4.2.7.). To produce the DNA template, 5 plaques from the D29 and TM4 infections were pooled independently for both the *M. smegmatis MC²155* strain and the *M. smegmatis*-IAC strain and the DNA was extracted using the Spin Column method (section 2.4.1.3.). These DNA samples were also tested using the PCR reaction that detected the integration state of the plasmid. To test what the plasmid state was before the phage infected, the results gained were compared with DNA extracted from both strains using the boiled lysis method (section 2.4.1.1.). In addition, a

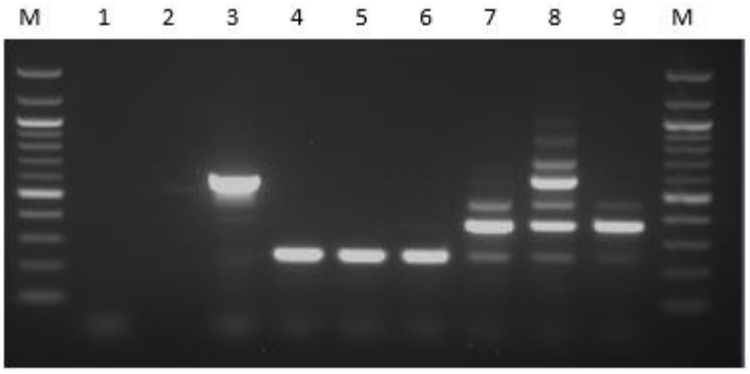
boiled lysate DNA preparation was also performed for the *E. coli*-IAC strain as a positive control for the pattern expected for a cytosolic plasmid.

The results as shown in Figure 3.9 and it was found that the results for the wild-type *M. smegmatis* were as expected for all the lysis methods tested, with a 251 bp PCR product being amplified corresponding to the amplification of the uninterrupted chromosome showing no plasmid insertion at the *attB* site even after phage infection with either D29 or TM4 (Fig. 3.8, lanes 4 – 5). The boiled lysate of the *E. coli*-IAC strain (Fig. 3.8, lane 3) produced a PCR product of 603 bp which corresponded to the amplification of the uninterrupted IAC plasmid. In contrast, the results for the *M. smegmatis*-IAC strain were variable.

When the strain was lysed through the boiled lysis method (Fig. 3.8, lane 7), three bands were produced, a 375 bp and 479 bp PCR product which indicated that the plasmid was integrated into the chromosome. However, a 251 bp PCR product was also amplified, showing that a portion of the *M. smegmatis*-IAC culture had lost the plasmid from the chromosome as seen previously. The same pattern of results was also produced when the *M. smegmatis*-IAC strain was lysed using the phage TM4 (Fig. 3.8, lane 9), showing there was no change in the state of the plasmid before and after phage infection with TM4. The results when D29 was used as the lysis agent however were different (Fig. 3.8, lane 8). The same 375 bp, 479 bp and 251 bp PCR products were amplified, but also a 603 bp product was amplified corresponding to the uninterrupted, cytosolic plasmid structure. This result showed that the infection of *M. smegmatis*-IAC with D29 was causing the replication of the plasmid which means that the copy number increased. In theory this should increase the sensitivity of the PCR by increasing the quantity of target, rather than decrease the sensitivity,

however it is also possible that these different plasmid structures were also causing competitive inhibition of exponential amplification of individual amplicons. It was also noted that following phage D29 infection, another three PCR products were also amplified of the approximate sizes of 725 bp, 850 bp and 1000 bp. This suggests that during the replication of the plasmid that DNA rearrangement was occurring with more DNA being inserted between the primer pairs.

Figure 3.8 – Phage effect on structure of *M. smegmatis*-IAC



Lane M, 100 bp DNA ladder (NEB; UK), Lane 1, no template (water) control. Lane 2 was blank. The template used for the PCR assays were: Lane 3, *E. coli*-IAC; Lanes 4 – 6, *M. smegmatis;* Lanes 7 – 9, *M. smegmatis*-IAC. The lysis agent used to extract the DNA from the cells were: Lanes 3, 4 and 7, boiled lysis method; Lanes 5 and 8, D29; Lanes 6 and 9, TM4. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.7.). The expected product size for uninterrupted chromosome was 251 bp. The expected product sizes for the integrated plasmid was 375 bp and 479 bp. The expected product size for unintegrated plasmid was 603 bp. To prepare the PCR assay, the integration primers were used at a concentration of 0.5 µM each; with HotStarTaq Master Mix Plus DNA polymerase and buffer (QIAGEN; UK); The PCR parameters used were an initial denaturation step of 95 °C for 5 minutes, then 30 cycles of: 94 °C for 30 seconds, 68 °C for 30 seconds, 72 °C for 30 seconds, with a final extension step of 72 °C for 5 minutes.

3.5. Discussion

During the development of the IAC, it was found that the competitive format of the control provided better results than the non-competitive format. The difference between the melting temperature of the primers used in the multiplex and the large difference between the quantity of control and target template resulted in a PCR reaction that produced non-specific primer binding and amplification of spurious bands that could be interpreted as a false positive result. Once it was realised that the PCR reactions were being affected by the relative concentration of template, the approach of using the 16S rDNA gene as a naturally present IAC was determined to be non-viable, as it is very difficult to closely control the level of IAC template arising from a phage-based detection event, either in the two day format which uses a bacterial lawn, or in the Oneday format which may have a range of cell numbers present, from 1 up to approximately 100 (see Swift *et al*., 2013, 2016, 2019). During the development of the competitive IAC, it was decided to incorporate the control plasmid into *M. smegmatis* genome to create a viable control strain with the added benefit that it could be used for multiple purposes rather than just as an IAC for the PCR stages of the assay. For instance, the strain could also be used in the phage assay as a phage infection control to confirm the phage-based reagents in the Actiphage kits was active. By incorporating an independent phage assay control using a pure culture of the *M. smegmatis*-IAC strain as the target organism it will be free from inhibitors that might be present in sample that are derived from a clinical sample (such as blood). So, by using this as one of the process controls, a failure to amplify the IAC segment during the PCR step of the procedure would indicate that the phage infection had failed. A second process control that the strain could be used

for is as an extraction control during the DNA spin column extraction where the DNA may be potentially lost due to extraction errors. To achieve this, a known concentration of the DNA that would produce a visible signal during the PCR step could be extracted from the *M. smegmatis*-IAC strain using the boiled lysis method and be purified as an independent process control. This should again be free from inhibitors and as the DNA has already been extracted through the boiled lysis method, the failure to amplify the IAC segment in the PCR step would indicate that the extraction had failed and that is the step that needs repeating.

The detection limit of the *M. smegmatis*-IAC strain was tested in the Oneday phage assay, a reduction in the limit was produced when the phage D29 was used as the lysis agent, when compared to the phage TM4. Both the D29 phage and the pMV306 integrative plasmid share a common ancestor, the phage L5. The phage D29 is a variant of L5 and has lost part of the right arm of the L5 genome through a 3,620 bp deletion. The deletion truncates the repressor gene rendering it non-functional, which prevents D29 from entering a lysogenic lifestyle (Ford *et al*., 1998a). Despite this, D29 still contains a functional integrase and homologous recombination sequence which are both similar to the L5 integration genes and D29 can integrate into the mycobacterial chromosome if it the L5 repressor is also added. It has also been shown that the D29-integrase can facilitate the integration of L5 and *vice versa* for the L5-integrase for D29, at a reduced efficiency to the integration of the phage with its own integration system (Peña *et al*., 1998). It is the L5 integrase and recombination sequence that is utilised in the vector pMV306 (Stover *et al*., 1991). The integrase however has also been shown to excise the plasmid from the chromosome in an excisase-independent manner (Springer *et al*., 2001). The D29

genome also contains a putative excisase gene which could also be responsible for the removal of the plasmid from the chromosome (Ribeiro *et al*., 1997). Saviola (2009) showed that integration vectors based on the L5 integration system, such as pMV306, exist in an equilibrium between an integrated and excised state because of the integrase gene. They also showed that transformation of a second L5-based integrative vector resulted in the replacement of the original integrated plasmid with the second. By removing the integrase after integration, they showed the integration became very stable. This could explain the results produced when D29 infects the *M. smegmatis*-IAC strain with the D29 acting as the second integrative vector and replacing the plasmid in the genome and causing its excision.

Hence, it was likely that the infection with D29 caused the excision of the plasmid and then lead to the vector replication during the phage amplification phase of the infection. Although in theory this should increase the amount of the pMV306-IAC plasmid and improve the detection limit of the strain by increasing the copy number of the IAC segment per cell. Apart from providing a competitive DNA target, another explanation for the loss of sensitivity is that after the phage induced replication of the pMV306-IAC plasmid, the newly generated plasmids could be packaged within phage heads along with the phage DNA, which were then subsequently removed during the purification and concentration of the DNA lysate using the Spin Column. This resulted in a purified DNA elution, free of phage and also with lowered amounts of the IAC plasmid. Hence the outcome of this work was that although the viable *M. smegmatis* IAC strain had been produced, they were still not robust enough to be used as standard controls in the Actiphage assay.

CHAPTER 4

4. DEVELOPING REAL-TIME PCR ASSAYS FOR THE DETECTION OF MYCOBACTERIA

4.1. INTRODUCTION

The phage assays effectively act as a biological lysis method to release mycobacterial DNA that can then be detected using PCR assays. The efficient nature of the infection and lysis event mean that lower numbers of mycobacteria are required to produce a positive PCR detection event compared to the limit of detection cited by most commercial PCR kits for the detection of MTBC bacteria (100 cells). Despite this, it was still difficult to detect less than five *M. bovis* cells with a standard 30 cycle end-point PCR assay since the presence of faint bands was sometimes obscured by the background accumulation of non-specific PCR amplicons, which were attributed to the presence of large amounts of competitive bovine PBMC DNA in the samples. To routinely detect between one and five *M. bovis* cells, a two round PCR was required, however this increased the likelihood of a contamination event and false positive results and is not very practical for routine applications.

Quantitative PCR (qPCR) is generally believed to be more sensitive assay than end-point PCR assays because there is less background signal from the sample, allowing low levels of the fluorescent signal to be detected more easily. There are two common variants of qPCR assays; the first uses intercalating fluorescent dyes that when bound to dsDNA produce a conformational change enabling them to become excited by the appropriate wavelength of light and therefore produce fluorescence. This method is non-specific and will produce a fluorescent signal when the chemical is bound to any form of dsDNA, including non-specific products. This feature can be used to perform a melt curve analysis of the amplified products to indicate the base composition of the main PCR product amplified during the assay. To do this, the

amplicons are subjected to a temperature gradient and when the dsDNA denatures into ssDNA the fluorescent signal of the dye diminishes, and the analysis software then compares the melt curve to that expected for the correct PCR product.

The signal from the second type of qPCR assay, commonly referred to as TaqMan assays, is sequence specific. These use a dual labelled oligonucleotide probe that is homologous to a region of the DNA between the forward and reverse primers and are labeled with a fluorophore on the 5' end and a quencher on the 3' end of the primer. The quencher extinguishes the fluorescence of the fluorophore while they are both bound to the oligonucleotide resulting in a non-fluorescent oligonucleotide. A fluorescent signal is produced when the fluorophore is cleaved from the oligonucleotide probe by the DNA polymerase during the elongation event from either the forward or reverse primer, and a detectable fluorescent signal can then be detected at the end of the extension step of the PCR cycle. (Jothikumar *et al*., 2009). The probe-based qPCR assays confer additional specificity when compared the non-specific qPCR assays and end-point PCR assays as the probe must also bind to the target DNA as well as the two primers used to amplify the sequence. Since the oligonucleotide will only anneal to homologous DNA, non-specific PCR products should not be detected.

A qPCR assay has several other benefits when compared to an end-point assay, such as being able to observe the results in real time as the intensity of the fluorescent signal is determined after each PCR cycle by the qPCR software. This also reduces the diagnosis time as the PCR products do not need to be visualized by the gel electrophoresis method after the PCR assay has been completed. Since most

standard qPCR machines can analyze fluorophores emitting up to five different wavelengths of light, it is also easy to incorporate an IAC into a probe-based qPCR by multiplexing (i.e., carrying out several different PCR reactions in one sample).

Commonly used dual labelled probe are known as fluorescent resonance energy transfer (FRET) probes. As described above, FRET probes contain the 5' fluorophore and the 3' quencher. The quencher may either be another fluorophore or a so-called dark quencher. Both effectively quench the fluorescence of the reporter dye when bound at the terminals of the oligonucleotide and therefore held in close proximity to the fluorescent label. Fluorescent quenchers such as TAMRA (5-carboxytetramethylrhodamine) work by absorbing the emissions of excited fluorescent reporter dyes preventing light emission, promoting its own excitation and photon release (fluorescence). TAMRA has a short quenching range up to an emission maximum of 560 nm; this includes FAM (6-Carboxyfluorescein), TET (Tetrachloro-fluorescein) and HEX (Hexachloro-fluorescein). These quenchers are effective in singleplex q-PCR but have an increased amount of background fluorescence due to the dual fluorescence, which decreases the sensitivity of the qPCR assay.

The other type of quenchers are dark quenchers, such as the Black Hole Quencher (BHQ) which also work by using the principles of FRET. Rather than emitting light as the final energy transfer, they transfer the electrons vibrational energy from the excited reporter dye and dissipate it as heat instead of fluorescence. This decreases the intensity of the background fluorescence and increases the sensitivity of the qPCR assay when compared to fluorescent quenchers. BHQ quenchers can also

exhibit static quenching, which again, improves quenching and thus signal to noise ratio. Static quenching is caused by the direct contact of the reporter fluorophore and the quencher. An intramolecular dimer is formed due to hydrophobic properties of the reporter and quencher which causes them to stack in the hydrophilic environment of PCR (Johansson *et al*., 2002). The flexible nature of the oligonucleotide facilitates the contact between the terminal ligands. An example of a BHQ is BHQ-1, which has an absorption range of 480 – 580 nm; the BHQ series of quenchers full absorption range is 430 – 730 nm, providing dark quenching for almost all the fluorophores commonly and uncommonly used as a reporter dye.

Various other modifications can be employed to increase sensitivity and specificity of the dual labelled probes and contribute to reducing background fluorescence of the probe, such as minor groove binders (MGB's). MGB labelled probes contain a linker on the 3' end of the oligonucleotide which binds the quencher and a binding molecule that inserts into the minor groove bend of the DNA helix which is then held in place by van der Waals forces. The function of the MGB is to improve specificity and binding of the oligonucleotide to the target DNA, the moiety itself significantly increases the annealing temperature of the probe which is one of the ways it improves specificity.

The results of a qPCR assay are visualized by software that controls the qPCR machine, the software is responsible for setting the parameters used and also for producing a graph of the raw data (fluorescence) at the end of each cycle. Once the assay has completed, the data can be normalized and smoothed by using tools inbuilt into the software and generate a threshold to dictate a positive result. A

positive result is called by the system when the levels of fluorescence cross and go above the threshold. The Cq value is recorded by the software at the point where the sample result and threshold cross, the smaller the Cq value, the more starting DNA was in the PCR assay and the more positive the sample is.

It was hypothesized that the increased sensitivity of the qPCR assay would allow successful detection of less than five *M. bovis* cells and achieve the sensitivity required to detect the minimum amount of *IS*6110 elements expected to be contained in a 2 ml blood sample. Therefore, the aim of this work was to develop the in-house IAC produced in Chapter 3 into a qPCR format so that it could be used in multiplex qPCR assays for the rapid and sensitive detection of mycobacteria. This would also allow false negative results produced by polymerase inhibition in individual samples to be more easily detected. Another benefit of a qPCR in regard to the IAC is that it can show the amount of inhibition present in a sample based on the reduction of the slope and increase in Cq value produced. The PCR assay and primers described by Eisenach *et al.* (1990) were included in the qPCR format because the primers were contained on the pIAC plasmid. The final aim of this chapter was to design a second novel qPCR to allow the detection of MAP.

4.2. RESULTS

The first aspect that was considered when expanding the *M. smegmatis*-IAC into a qPCR format was to decide whether to use the *M. smegmatis*-IAC strain as a dual phage infectivity control and IAC or use the pIAC plasmid as an IAC only. In order to produce a standardized and consistent IAC control the pIAC plasmid was chosen instead of the *M. smegmatis* strain. This was because an exact amount of plasmid

could be added to each PCR reaction to produce a consistent IAC signal and Cq value from all samples. There would be too much variation to the Cq values generated for the IAC by adding the *M. smegmatis*-IAC strain to the phage assays caused by the clumping nature of the bacteria. This meant a standard number of cells would be difficult to add to each assay and as such, a range of IAC template could be added which would then produce a large range of IAC Cq values, which is not ideal for incorporating into a commercial kit.

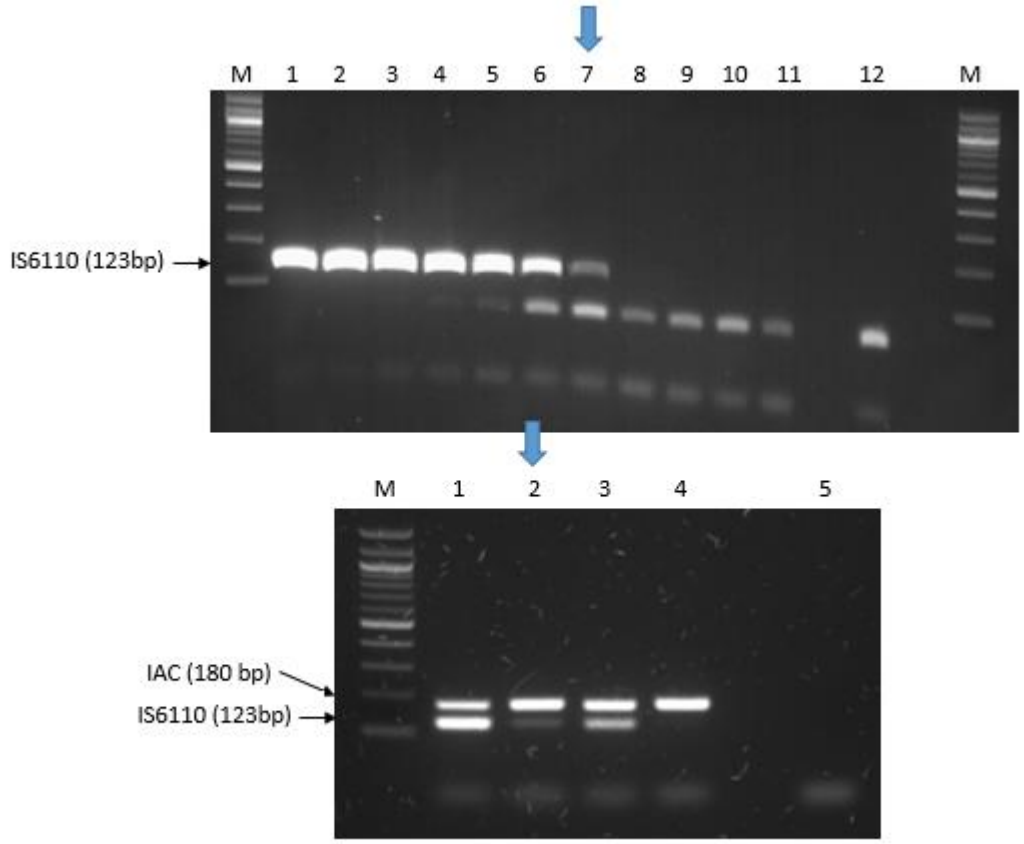
The second aspect that was considered was the design of the qPCR format. The specific, probe-based PCR assay was chosen because of the increased specificity the probe confers in the qPCR assay compared to both the non-specific version of the qPCR assay and end-point PCR assays. To design probes for the qPCR assays, FRET dual labelled probes with a dark quencher were selected because of the reduced background fluorescence they produced compared to fluorescent quenchers.

4.2.1. Determining the effect of adding the pIAC plasmid on the IS6110 amplification

The first step in producing the qPCR assay was to design the IAC step. The interaction between the phage D29 and the pIAC plasmid integrated within the M. smegmatis—IAC genome meant that a high concentration of cells would need to be added to the Oneday phage assay to produce a suitable IAC product which was not ideal. To circumvent the problem, it was decided to add the purified pIAC plasmid directly to the PCR reaction mix so the plasmid was not exposed to the phage infection. Ideally the plasmid would be added at a low concentration to produce the IAC product without reducing the amplification of the *IS*6110 element. Before the format was

tested in a qPCR format the method was proved in an end-point PCR assay first. The results showed a limit of detection of 1 x10^{-6} ng µl^{-1} of purified plasmid (data not shown). The next step was to determine the limit of detection of *M. bovis* DNA extracted with the boiled lysis method (section 2.4.1.1) with the *IS*6110 end-point PCR assayThe PCR assay produced a limit of detection result of 7 x10^{-4} ng of *M. bovis* DNA (Fig. 4.1, top panel, lane 7). The final step was to add the pIAC plasmid and the *M. bovis* DNA at their limits of detection to determine whether adding the IAC DNA would decrease the amplification of the *IS*6110 element. Originally 1.36x10^{-6} ng of plasmid was added to the assay however when combined with the *IS*6110 DNA no IAC DNA was amplified. The assay was repeated with 1.36 x10^{-5} ng pIAC plasmid DNA added to each of the *M. bovis* DNA dilutions. In this instance, a 180 bp PCR product corresponding to the amplification of the IAC DNA was produced in all PCR reactions (Fig. 4.1, bottom panel). The limit of detection for the *M. bovis* DNA was still shown to be 7 x10^{-4} ng even with the amplification of the IAC DNA in the multiplex PCR assay (Fig. 4.1, bottom panel, lane 2). The results showed that adding the pIAC plasmid to the *IS*6110 PCR assay did not decrease the amplification of the *IS*6110 element and as such adding the IAC did not decrease the sensitivity of the diagnostic PCR assay. This meant that using this format of IAC was a compatible format to use with this assay design.

Figure 4.1 – Determining the effect of the pIAC plasmid on the limit of detection of the *IS*6110 end-point PCR assay



Top panel contained only *M. bovis* DNA. Lane 1, 140 ng μl^{-1}; lane 2, 70 ng μl^{-1}; lane 3, 7 ng μl^{-1}; lane 4, 7 x10^{-1} ng μl^{-1}; lane 5, 7 x10^{-2} ng μl^{-1}; lane 6, 7 x10^{-3} ng μl^{-1}; lane 7, 7 x10^{-4} ng μl^{-1}(blue arrow); lane 8, 7 x10^{-5} ng μl^{-1}; lane 9, 7 x10^{-6} ng μl^{-1}; lane 10, 7 x10^{-7} ng μl^{-1}; lane 11, 7 x10^{-8} ng μl^{-1}; lane 12, no template (water) control.

Bottom panel contained the *M. bovis* DNA combined with 1.36 x10^{-5} ng μl^{-1} of purified pIAC plasmid. Lane 1, 7 x10^{-2} ng μl^{-1}; lane 2, 7 x10^{-4} ng μl^{-1} (blue arrow); lane 5, 7 x10^{-3} ng μl^{-1}; lane 4, 7 x10^{-5} ng μl^{-1}; lane 5, no template (water) control.

M. bovis DNA (140 ng μl^{-1}) extracted with the boiled lysis method was diluted 1 in 2 with sterile molecular grade water then a tenfold serial dilution was performed from 70 ng μl^{-1} to 7 x10^{-8} ng μl^{-1}. Purified pIAC plasmid (section 2.5.3.2.) was diluted to 1

x10^5 ng μl^{-1}. To prepare the PCR reaction: 10 µl of One*Taq*® Quick-Load® 2x Master Mix with GC Buffer; 1 µl of each dilution was used as template; 0.5 µM of each MTC F and MTC R primers and made up to 20 µl with sterile molecular grade water. The PCR parameters used were an initial denaturation step of 94 °C for 2 min followed by 40 cycles of: 94 °C for 30 s, 66 °C for 15 s and 68 °C for 30 s; followed by a final extension step of 68 °C for 5 min. The PCR products were analysed by electrophoresis through a 2 % (w/w) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.3.). The expected product size for the internal control segment was 180 bp and the expected product size for the *IS*6110 element was 123 bp (indicated by arrows on the LHS of image). The different gels were exposed under different intensities of UV light.

4.2.2. Designing in-house qPCR assays

4.2.2.1. Designing a dual-labeled probes for the detection of pIAC, IS6110 and IS900 in a qPCR assay

The first step in designing the in-house qPCR assay was to design a probe for the IAC. The position of the probe in relation to the primer sequences on the plasmid can be found in Figure 4.2. The DNA sequence that was selected for the probe and the fluorophore and quencher can be found in Table 4.1 below (for full information see Appendix 3: pIAC - section 9.3.2.; *IS*6110 – section 9.3.4.; *IS*900 – section 9.3.10.).

Figure 4.2 – Diagram of IAC segment of the pIAC plasmid

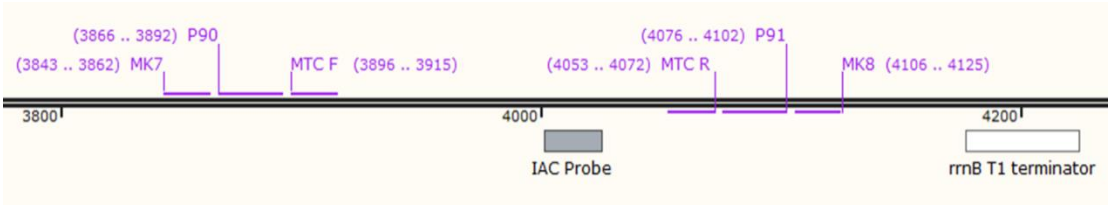


Diagram of the primer sequences (MTC F/R, P90/91 and MK7/8) cloned into the pMV306 plasmid to produce the pIAC plasmid. The placement of the IAC probe in relation to the primer sequences was also provided. The diagram was produced with the software SnapGene Viewer.

Table 4.1 - Table of primers and probes used in the in-house qPCR assays.

qPCR target	Primers and probes	Sequence (5'-3')	Tm (°C)	GC Content (%)
IAC	IAC probe	HEX-TCGAAAGGGCAGATTGTGTGGACA-BHQ1	64.62	48.00
*IS*6110 -MTBC	MTC F	CCTGCGAGCGTAGGCGTCGG	67.55	75.00
	MTC R	CTCGTCCAGCGCCGCTTCGG	67.55	75.00
	*IS*6110 probe	FAM-ACATAGGTGAGGTCTGCTACCCACA-BHQ1	64.62	52.00
*IS*900 -MAP	MAP RT F	AATGACGGTTACGGAGGTGGTT	60.25	50.00
	MAP RT R	AGCGATGAGCAAGGCGATCA	59.35	55.00
	MAP RT probe	FAM-TGCGCGTCGTCGTTAATAACC-BHQ1	59.82	52.38

Fluorophore/ Quencher	Excitation wavelength (nm)	Emission wavelength (nm)	Quenching range (nm)
FAM	495	520	N/A
HEX	535	556	N/A
BHQ-1	N/A	N/A	480 - 580

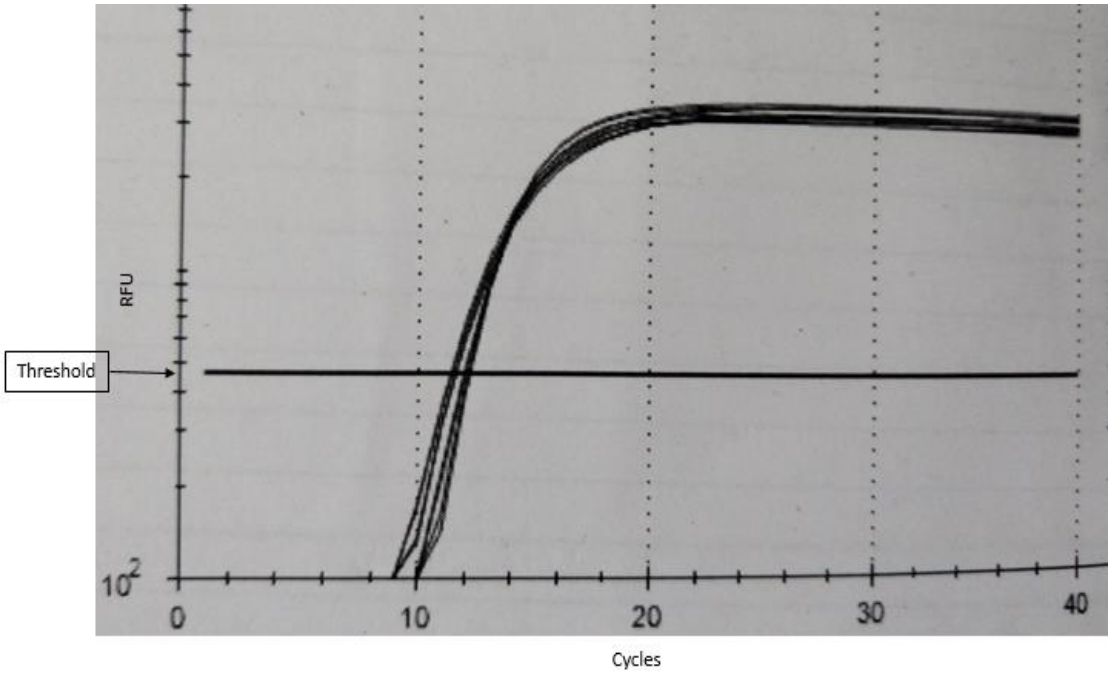
The tables show the DNA sequence, T_m and GC content of the primers and probes are shown in the top table. The excitation, emission and (where applicable) quenching range of the fluorophores and quencher are shown in the bottom table

4.2.2.2. Determining whether the IAC probe produces a positive signal with the IAC primers.

After the IAC probe was designed, an experiment was carried out to determine whether the probe efficiently bound to the pIAC plasmid and produced a detectable signal when using any of the three primer pairs that were incorporated into the plasmid (for further information see Appendix 3, section 9.3.1.).

The results of the qPCR assay were in line with the expected results. It was shown that all the primer pairs produced a successful detection with the IAC probe with each of the duplicates producing a Cq value of approximately 10.5, showing that the probe effectively binds to the pIAC segment. All the no template controls produced a negative result with no Cq value (Fig. 4.3).

Figure 4.3 – Determining whether the pIAC primer sequences produce a positive signal with the IAC probe.



Log graph of RFU. The IAC qPCR assay was performed using the standard parameters recommended for the Luna DNA polymerase (section 2.4.4.1.), on approximately 20 ng pIAC DNA extracted using the Monarch Miniprep kit (section 2.5.3.2.) and the primers MTC F/R, P90/91 or MK 7/8. Each PCR reaction was performed in duplicate. The qPCR was performed on a BIORAD C1000 Touch thermocycler with CFX9 optical unit.

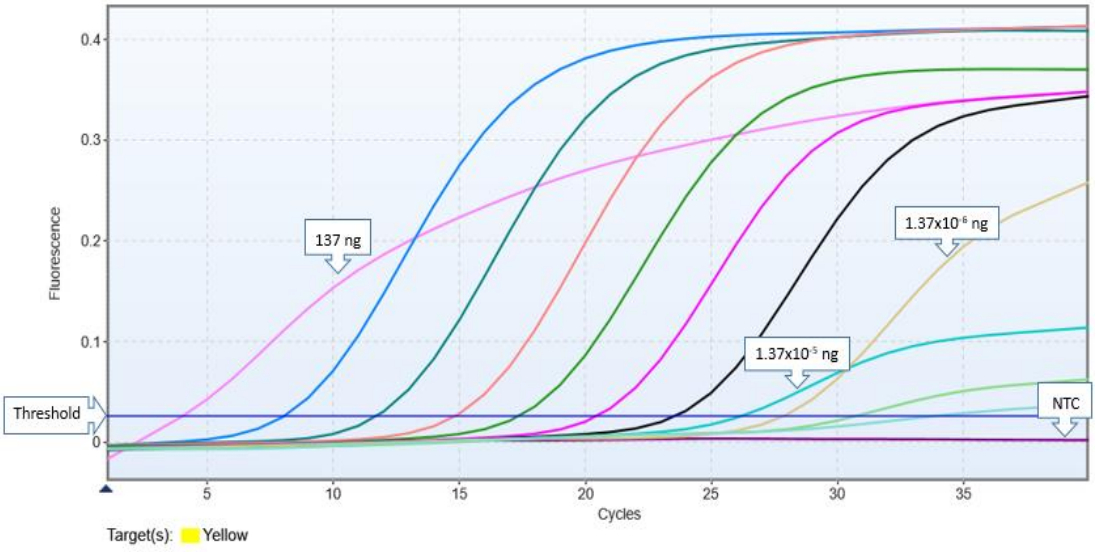
4.2.2.3. Determining the sensitivity of the IAC qPCR assay with the pIAC plasmid

The sensitivity of the IAC qPCR assay was determined next to identify the limit of detection of the qPCR assay. This information was needed to determine what the most appropriate quantity of pIAC plasmid DNA would be to add to the in-house qPCR assays to produce a Cq value between 25 and 30 cycles. This value was chosen as it was not predicted to reduce the sensitivity of the diagnostic PCR based on the results gained previously using the end-point PCR assay (section 4.2.1.) and is typical for IACs designed for other qPCR assays. Based on the previous end-point results, it was expected that the appropriate DNA quantity would be $1.37x10^{-5}$ ng or less.

The results showed that the IAC qPCR assay produced a positive signal for each of the pIAC dilutions tested (Fig. 4.4). The no template (water) control was negative and did not produce detectable amplification. Based on these results it was decided that 1.37 $x10^{-6}$ ng of pIAC DNA would be used as starting template because it was: 1) mid-way between the desired Cq range of 25-30 at 27.90 cycles; 2) was the lowest concentration of DNA added to a PCR reaction that produced a good level of amplification (Hoorfar *et al*., 2004). The Cq value was rounded up to 28 cycles and a range of ±2 cycles was chosen as an acceptable Cq range (26 - 30 cycles) to show there was no inhibition, a range was chosen to account for pipetting differences between different serial dilution preparations causing slight variations in Cq values. An efficiency graph was produced (Fig. 4.5.) to show how efficient the amplification of the qPCR reaction is. A value of 115 % was obtained which shows that the reaction is efficient but that inhibitors were present in the assay (see Appendix 3, section 9.3.3. for more information of assay results and efficiency graphs).

Figure 4.4 Determining the sensitivity of the IAC qPCR assay using the MTBC primers

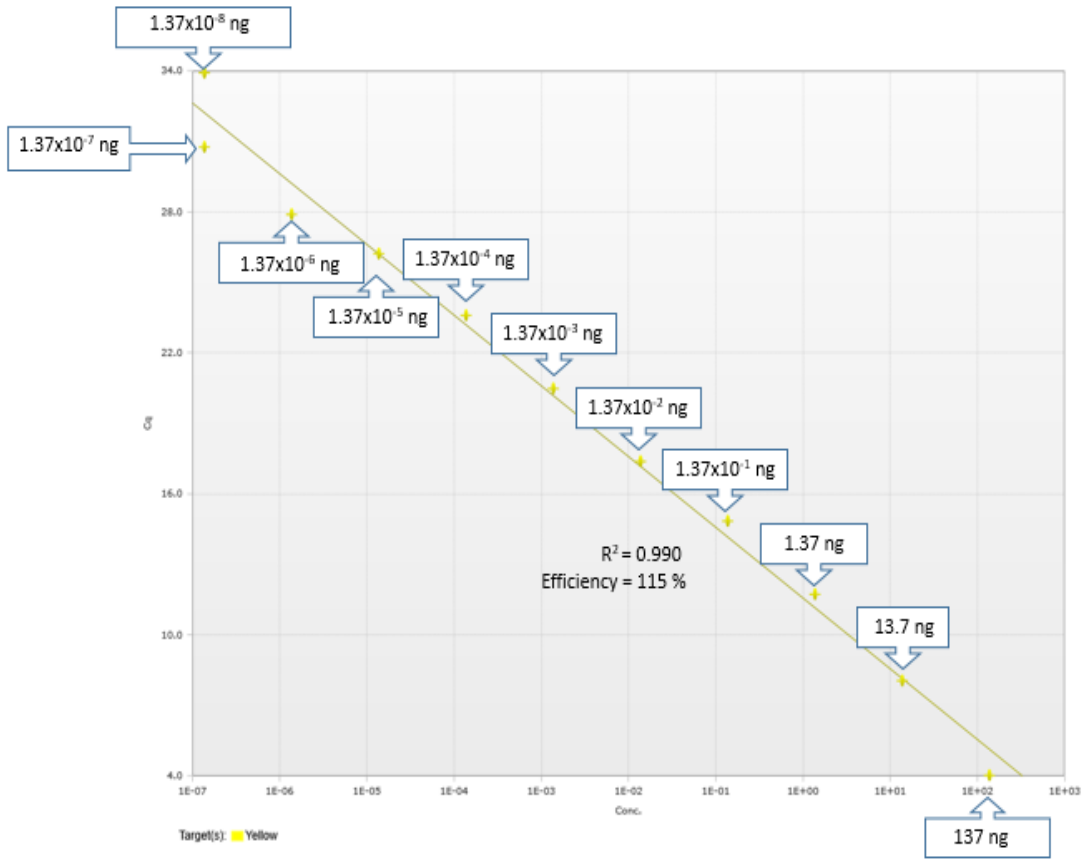
Sample	Cq values	Sample	Cq values	Sample	Cq values	Sample	Cq values
pIAC 137 ng	4.03	pIAC $1.37x10^{-1}$ ng	14.86	pIAC $1.37x10^{-4}$ ng	23.61	pIAC $1.37x10^{-7}$ ng	30.77
pIAC 13.7 ng	8.06	pIAC $1.37x10^{-2}$ ng	17.39	pIAC $1.37x10^{-5}$ ng	26.23	pIAC 1.37 $x10^{-8}$ ng	33.92
pIAC 1.37 ng	11.74	pIAC $1.37x10^{-3}$ ng	20.49	pIAC $1.37x10^{-6}$ ng	27.90	No template (water) control	ND



The IAC qPCR assay was performed using the standard parameters recommended for the Luna DNA polymerase (section 2.4.4.1.), on a ten-fold serial dilution of pIAC DNA

extracted using the Monarch Miniprep kit (section 2.5.3.2.) and the DNA concentrations used in each sample are shown in the Key. The qPCR was performed on a RotorGene-Q qPCR machine. The results were collected on the yellow channel and normalized using the tools ‘Dynamic tube’ and ’Use noise slope correction’ by the companion Q-REX software (QIAGEN; UK). The Cq value was produced where the samples crossed the threshold which was set by ‘auto threshold’ and the values obtained for each sample are shown in the table.

Figure 4.5 Efficiency graph for IAC qPCR assay



Standard curve of IAC qPCR assay using ten-fold serial dilution of purified pIAC DNA (section 2.4.4.1.). The plots have been labelled with the DNA concentration used in the corresponding qPCR assay.

4.2.3. Designing an in-house qPCR assay for the detection of MTBC

4.2.3.1. Determining the sensitivity of the in-house IS6110 PCR qPCR assay

Once the *IS*6110 qPCR probe was designed (Appendix 3, section 9.3.4.), the sensitivity of the qPCR assay was compared to the *IS*6110 end-point PCR assay described in section 4.2.1.

The results showed that the limit of detection was $7x10^{-4}$ ng μl^{-1} of DNA (Fig. 9.11). This was comparable with the end-point analysis of the *M. bovis* DNA dilutions, which also produced a limit of detection of $7 x10^{-4}$ ng of DNA, showing that the two assays are equally as sensitive. The was unexpected as qPCR assays are supposed to be more sensitive than end-point PCR assays so this showed that further optimization of the in-house *IS*6110 qPCR assay was required.

4.2.3.2. Optimizing the in-house qPCR assay for the detection of MTBC

Due to the unexpectedly low sensitivity achieved for the in-house MTBC qPCR assay an optimization process was started to try and produce an assay that was more sensitive than the end-point version of the assay. The first step in the optimization process involved varying the primer concentrations (Appendix 3, section 9.3.6.) then determining the effect of adding PCR enhancers (Appendix 3, section 9.3.7.). PCR enhancers were investigated again because a different polymerase was used compared to the enzyme used in the end-point assay.

4.2.3.3. Determining the Oneday phage assay detection limit of the IS6110 qPCR assay

One of the functions of a qPCR is the ability of the software to calculate the starting quantity of DNA added to the PCR reaction, which in terms of TB diagnostics allows the user to calculate the mycobacterial load of the original sample. This allows

clinicians to identify whether the therapeutics in use are actively decreasing the bacterial load by performing longitudinal testing. This method cannot always be directly quantifiable due to the variable number of *IS*6110 elements found in different strains of MTBC organisms with M. tuberculosis strains having up to 25 per genome, whereas *M. bovis* strains generally have a lower number of copies (Brosch *et al*., 2000). Therefore, an experiment was performed to compare the Cq value with approximate cell count of an *M. bovis* BCG (one copy of *IS*6110) culture to determine the limit of detection of the Oneday assay using the in-house *IS*6110 qPCR assay that had been developed (for more details see Appendix 9). It must be noted that to carry out this experiment, a liquid culture of BCG was used which would not contain as many inhibitors as the normal biological sample of blood which had been previously tested using end-point PCR (Swift *et al*., 2020).

To perform this experiment, a ten-fold dilution series was performed on a liquid culture of *M. bovis* BCG with an approximate density of 1 $x10^{8}$ cfu ml^{-1}, by serially diluting the culture into MP until an approximate concentration of 1 cfu ml^{-1} was achieved. The Oneday phage assay was then performed on 100 µl of each dilution and the DNA lysate produced was concentrated and purified to 10 µl for use in the in-house *IS*6110 qPCR assay (section 2.4.5.5.). A standard phage assay (section 2.2.2.) was also performed on each of the dilutions to independently measure the approximate number of phage-sensitive cells in each sample being tested. The in-house *IS*6110 qPCR assay was then performed and 1.36 $x10^{-6}$ ng of pIAC plasmid was added to each reaction to provide an internal amplification control. The Cq value generated for each sample was compared to the quantity of plaques produced using the standard phage assay (Table 4.4).

Table 4.4: Determining the limit of detection of the Oneday phage assay using the qPCR *IS*6110 assay

Dilution of *M .bovis* BCG culture (estimated cfu per sample)	Oneday Phage assay Cq value	Standard phage assay (No. Plaques per sample)	IAC Cq Value
10^{-1} (10^{6})	12.63	Complete lysis	Negative
10^{-2} (10^{5})	16.28	Complete lysis	Negative
10^{-3} (10^{4})	19.87	>300	27.50
10^{-4} (10^{3})	22.90	122	Negative
10^{-5} (10^{2})	27.24	30	26.63
10^{-6} (10)	33.02	1	27.26
10^{-7} (1)	38.04	0	25.77
MP negative process control	Negative	0	26.68
No template control	Negative	N/A	26.52

To prepare the PCR mixtures: 5 µl of the DNA extracted from each of the BCG dilutions, 10 µl of Luna Probe Mastermix, 15 µM of the MTC F/R primers, 5 µM of the *IS*6110 probe, $1.36x10^{-5}$ ng of pIAC plasmid was also added to provide an internal amplification control. The standard Luna qPCR protocol was used to amplify and detect the DNA (section) on a RotorGene-X qPCR machine (QIAGEN; UK). The data was recorded and tabulated by the companion Q-REX software provided with the machine (QIAGEN; UK).

The results of the IAC amplification showed that there were no DNA polymerase inhibitors present in the PCR reactions (IAC Cq values between 26 and 30) as expected because there are very few inhibitors from the liquid culture of *M. bovis* BCG used as the starting sample. The samples that contained large amounts of target DNA showed arrested or negative amplification of the IAC segment, however this was expected because of the competitive design of the IAC with the target DNA. Due to the fact the target DNA amplified and produced a positive signal, this showed that the PCR assay did not contain inhibitors and the IAC result can be discarded.

Regarding the DNA extracted from the Oneday phage assay of the *M. bovis* BCG cells, the first aspect that was determined was the efficiency of the qPCR assay. A standard curve was produced (R^2 = 0.987, efficiency = 73 %, mean ΔCq = 4.18) by plotting the Cq values produced against the number of cells in the assay (Fig. 9.13). This was out of the acceptable efficiency range of 80 – 120 %. Despite the low efficiency, the general pattern of results was as expected. The low efficiency and mean ΔCq of 4.18 was likely produced by the clumping nature of *M. bovis* BCG cells, which makes it difficult to transfer the cells evenly during the dilution steps creating an 'imperfect' ten-fold dilution series and would explain the variation in Cq values producing the low efficiency.

As was previously mentioned (section 4.2.3.4.) one *M. bovis* cell should produce a Cq value around 38 and the -7 dilution produced a value of 38.04, providing further evidence that one cell would produce a Cq value of 38 when there was no polymerase inhibition. The fact that the standard phage assay detected only one cell in the -6 dilution sample, and yet a positive signal in the linear range was produced using the Oneday assay, suggests that the Oneday assay is more sensitive than the

standard phage assay, as shown previously (Swift *et al*., 2020) and that incorporating a qPCR readout did not affect this sensitivity.

4.2.4. Effect of PBMC DNA on in-house IS6110 qPCR assay

The in-house *IS*6110 qPCR assay was designed to amplify viable MTBC DNA extracted from PBMCs purified from clinical blood samples, and as such would need to be able to efficiently amplify the *IS*6110 element from MTBC whilst in the presence of large quantities of human eukaryotic DNA which is co-extracted during the Oneday phage assay. It was expected that adding large amounts of non-target DNA might decrease the rate of amplification of the *IS*6110 element and reduce the sensitivity of the qPCR assay. To test whether this was true (section 2.4.5.6.), firstly an *M. bovis* BCG culture (approximately 1 x10^7 cfu ml^1 diluted to prepare 100 µl samples that contained approximately 1 x10^5 cells and 1 x10^3 cells) was prepared. The Oneday phage assay (section 2.2.3.) was then used to extract the DNA from these samples. To determine whether excess human DNA would affect the rate of the *IS*6110 amplification, this DNA was then mixed with historic DNA samples extracted from the blood of a human patients which have been shown to be *IS*6110 negative by end-point PCR (see Chapter 5). Several different *IS*6110 negative DNA samples were mixed to produce a uniform large volume so that the equivalent amount of human PBMC DNA was added to each of the appropriate PCR assays. Two sets of qPCR reactions were prepared, using both the standard 0.75 mM primer concentration and a primer concentration of 1.5 mM of primers to determine whether (a) increasing the primer concentration would relieve the expected reduction in the rate of amplification or (b) if the addition of more DNA would exacerbate the reduction as seen earlier.

The results for the IAC amplification showed that there were no inhibitors present in the samples (Table 4.5). The Cq values for the two negative controls and the PCR assays that contained 1 x10^3 cells produced Cq values between 25.56 and 27.80, showing the assays did not contain inhibitors as the expected Cq value for the IAC should be between 26 and 30 cycles. The samples that contained 1 x10^5 cells with and without the human DNA produced negative IAC results due to the strong amplification of the *IS*6110 element due to the competitive format of the IAC with the target DNA. The sample that contained 1 x10^5 cells with 1.5 mM of primers produced an IAC Cq value of 34.81, however the positive diagnostic results for these three assays showed the qPCR assay was not inhibited so the IAC results can be ignored.

Table 4.5 - Effect of non-target eukaryotic DNA on IS6110 qPCR efficiency

DNA added to sample	Primer concentration (mM)	*IS*6110 Cq value	IAC Cq value
1 x10^5 *M. bovis* BCG cells	0.75	15.17	Negative
1 x10^5 *M. bovis* BCG cells + Human DNA	0.75	15.30	Negative
1 x10^5 *M. bovis* BCG cells + Human DNA	1.5	14.70	34.81
1 x10^3 *M. bovis* BCG cells	0.75	21.99	27.69
1 x10^3 *M. bovis* BCG cells + Human DNA	0.75	21.87	25.56
1 x10^3 *M. bovis* BCG cells + Human DNA	1.5	21.65	27.04
MP negative process control	0.75	Negative	27.44
No template (water) negative control	0.75	Negative	27.80

A ten-fold serial dilution of a M. bovis BCG liquid culture (approximately 1 x10^8 cfu ml-1) was prepared to produce dilutions of 1 x10^6 cfu ml^{-1} and 1 x10^4 cfu ml^{-1}. The Oneday phage assay (section 2.2.3.) was performed on 100 µl of each of the dilutions. DNA (2 µl) from the M. bovis BCG Oneday phage assay extracts were used for the qPCR reactions along with 3 µl *IS*6110 negative PBMC DNA for the appropriate qPCR reactions. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 20 % (v/v) Q5 GC Enhancer (NEB; UK); 1 x10^{-6} ng

ml^{-1} purified pIAC plasmid was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools (section 2.4.5.6.).

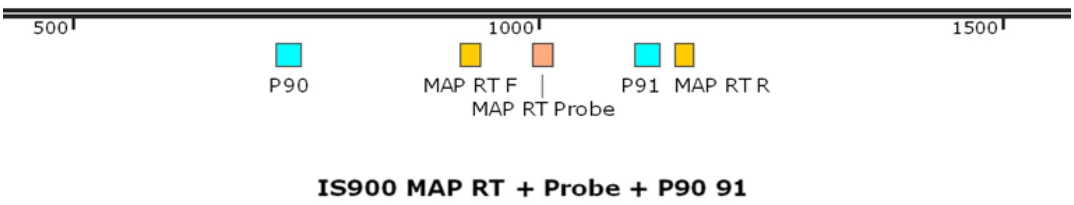
The results of adding the human DNA to the qPCR cycle were not in line with what was expected. By comparing the Cq values in the different tests, there was no obvious difference in the Cq values produced when the human DNA was added compared to the same conditions without human DNA (Table 4.5). There was a decrease in the efficiency of the amplification with the 1 $x10^5$ cells sample containing the human DNA as can be seen by the reduced angle of the slope compared to the 1 $x10^5$ cells without human DNA. This was reversed for the samples containing 1 $x10^3$ cells however, as it seemed that when the human DNA was added the efficiency of the PCR increased compared to the 1 $x10^3$ cells without human DNA. Increasing the primer concentration produced a slightly decreased Cq value for both dilutions tested, however the difference was negligible. The qPCR assays with the increased primer concentrations also produced the highest rates of PCR amplification as can be seen by the two steepest slopes produced in Figure 9.14 (Appendix 3) for both samples tested, with and without competitive human DNA. The results showed that co-extracting large quantities of eukaryotic DNA from the human blood samples with the Oneday phage assay did not produce a large difference in the rate of the amplification of the *IS*6110 qPCR assay. In this case, when competitor DNA was present, increasing the primer concentration two-fold did not inhibit the assay further as was expected but improved the efficiency of the PCR amplification slightly. Although these results were very encouraging, it was not possible to prepare duplicate samples or repeat these tests due to a limited supply of *IS*6110 negative human DNA and therefore the results gained must be considered observational.

4.2.5. Designing an in-house qPCR assay for the detection of MAP

4.2.5.1. Designing a dual-labeled probe for the detection of IS900 in a qPCR assay

Unlike the primers targeting *IS*6110, the P90/91 primer pair were located approximately 400 bp apart, which is at the upper threshold for an optimum qPCR reaction (see Fig 4.6). Therefore, a new area of the *IS*900 insertion sequence was identified that was conserved in all strains of MAP collated at the time into the NCBI DNA database that could be used to design qPCR assay with the primers located closer together. The available MAP sequences on the NCBI database were compared against each other and a consensus sequence was found in all strains. The MAP qPCR primers and probe were then designed around this region (see Appendix 3, section 9.3.10).

Figure 4.6 – MAP of *IS*900 with P90/91 primers and MAP RT primers and probe



The DNA map of the *IS*900 element and the regions of the P90/91 primer pair (blue) and the MAP RT F/R primer pair (yellow) and probe (orange) produced using SnapGene® Viewer

4.2.5.2. Detection limit of the in-house IS900 qPCR assay compared to the P90short/91 end-point PCR assay.

Once the in-house MAP primers and probe had been designed, the first stage was to determine the sensitivity of the primer probe combination. This was achieved by creating a ten-fold dilution series of MAP DNA (46.2 - 2.31x10^{-5} ng μl^{-1}). The first step in the dilution series was a 1 in 2 dilution followed on by ten-fold dilutions until an empirical concentration of 2.31 x10^{-5} ng μl^{-1} was achieved. The results of the qPCR assay (section 2.4.4.3.) were compared with two end-point PCR assays (section 2.4.2.4.), the first using the same MAP RT F/R primers and secondly to the P90short and P91 primers (P90short is a variant of the P90 primer which targets the same DNA region but has improved binding; see Chapter 6 for details).

The results of the end-point analysis with the MAP RT F/R primers showed a limit of detection of 2.31 x10^{-1} ng of MAP K10 DNA (Table 4.6) whereas the P90short/P91 primers produced a limit of detection of 2.31 x10^{-3} ng of MAP K10 DNA, showing that for an end-point PCR assay the P90short/P91 primers were more sensitive. When the end-point PCR assays were compared to the in-house MAP qPCR assay, both the end-point PCR assays were shown to be less sensitive than the qPCR assay which achieved a limit of detection of 2.31 x10^{-4} ng of MAP K10 DNA, 1000-fold more sensitive than the equivalent end-point PCR assay and 10-fold more sensitive than the end-point P90short/P91 PCR assay. The efficiency of the MAP RT qPCR assay was also identified by plotting the Cq values produced for a PCR reaction against the concentration of MAP DNA used in the qPCR assay. The standard curve was produced (R^2 = 0.995, efficiency = 96 %, mean ΔCQ = 3.42), which showed that the

qPCR was efficient as the efficiency fell between the accepted range of 80 – 120 % (Appendix 9 Fig. 9.11).

Table 4.6 comparison between the limit of detection of the MAP RT qPCR assay, MAP RT end-point PCR assay and P90short PCR assay

MAP DNA (ng)	MAP qPCR Cq Value	MAP RT end-point PCR assay result	P90short end-point PCR result
46.2	16.91	+	+
23.1	17.65	+	+
2.31	21.11	+	+
2.31×10^{-1}	25.24	+	+
2.31×10^{-2}	28.76	-	+
2.31×10^{-3}	31.94	-	+
2.31×10^{-4}	34.36	-	-
2.31×10^{-5}	ND	-	-
No template (water) control	ND	-	-

To prepare the qPCR reactions, 1 µl of the prepared dilution series of MAP DNA was added to Luna 2x Probe Mastermix, 0.5 µM of MAP RT F/R primers, 0.25 µM of MAP probe and increased to 20 µ with sterile distilled water. The cycling parameters recommended by the DNA polymerase supplier was used for the qPCR assay (section

2.4.4.3.). The qPCR assay was performed on a RotorGene-Q qPCR machine and the results were recorded and tabulated by the corresponding software Q-REX (QIAGEN; UK). The data was normalized and smoothed using the functions 'Dynamic tube' and 'Use noise slope correction' and an auto-threshold calculated. To prepare the end-point PCR assays, 1 µl of the MAP genomic dilutions were also added to OneTaq DNA Polymerase with GC buffer (NEB; UK) and 0.5 µM of either the MAP RT F/R primers or the P90short/P91 primers (section 2.4.2.5.). The cycling parameters used for both the MAP RT F/R and P90short/P91 end-point PCR assays were the same, with an initial denaturation of 94 °C for 2 min, then 40 cycles of: 94 °C for 30 s, 63 °C for 15 s, 68 °C for 45 s; followed by a final extension of 68 °C for 5 min.

4.2.5.3. Determining the specificity of the in-house IS900 qPCR assay

The next stage was to determine whether the probe and primers produced a signal with each of the MAP strains in the laboratory strain collection. To perform the in-house MAP qPCR assay firstly DNA template was extracted from each strain of MAP available in the lab (strains B4, DVL and ATCC 19851) by the boiled lysis method (section 2.4.1.1.). The quantity and quality of DNA extracted on this occasion could not be accurately determined due to a fault with the Nanodrop which prevented readings from being taken. The DNA extracts were diluted 1 in 100 with sterile molecular grade water and (1 µl) added to the PCR reactions. The quantity of DNA added was therefore calculated retrospectively using the Q-REX software by incorporating a standard into the qPCR assay of known concentrations that were used previously (section 4.2.5.2.) and thawed to room temperature (stored at -20 °C). In addition, *M. bovis* DNA extracted using the same method was used as a negative control to show that the primers and probes did not produce a detectable amplification event from all mycobacterial DNA.

The evaluation of the in-house MAP qPCR assay strain assessment produced results in line with those expected (Table 4.7). First, the *M. bovis* negative control did not produce any fluorescent signal and a Cq value could not be generated. The results for the standard were comparable to the results gained previously (section 4.2.5.2.) which showed that the DNA of the standards had not degraded by the freeze-thaw cycle and allowed the Q-REX software to calculate the starting quantity of the other three MAP DNA extracts. The calculated quantity of DNA was also similar to the expected quantity of DNA showing that the Q-REX software results were relatively accurate in regard to the empirical results and hence should also be for the MAP

samples of unknown concentration. Each of the MAP strains tested produced a positive result of varying strength and the amount of starting DNA was calculated (Table 4.7). The Q-REX software calculated starting DNA concentrations were as follows: B4, 1.06 $x10^{-3}$ ng which equated to approximately 215 cells; DVL, 6.12 $x10^{-4}$ ng which equated to approximately 120 cells and ATCC 19851, 1.71 $x10^{-5}$ ng which equated to approximately 4 cells. After considering the original 1 in 100 dilution the calculated original quantity of cells extracted with the boiled lysis method were: B4, 2.15 $x10^{4}$ cfu ml^{-1}; DVL, 2.15 $x10^{4}$ cfu ml^{-1} and ATCC 19851, 400 cfu ml^{-1}. The ATCC 19851 result could be classed as an ambiguous result due to the late positive curve that could be dismissed as noise and the fact that apparently only 400 cells were transferred to the sterile molecular grade water for the boiled lysis method, would be unusually low from a single colony.

Table 4.7 – In-house *IS*900 qPCR assay specificity

Sample name	Cq Value	Original Cq value of standards	Empirical DNA concentration (ng µl^{-1})	Q-REX calculated DNA concentration (ng µl^{-1})
MAP standard 1 (K10)	16.66	16.91	46.2	51.2
MAP standard 2 (K10)	17.75	17.65	23.1	23.7
MAP standard 3 (K10)	21.47	21.11	2.31	1.72
MAP standard 4 (K10)	24.10	25.24	0.231	0.271
B4	31.97	ND	ND	1.06 x10^{-3}
DVL	32.75	ND	ND	6.12 x10^{-4}
19851	37.83	ND	ND	1.71 x10^{-5}
M. bovis	N/A	ND	ND	ND
Negative control	N/A	ND	ND	ND

To prepare the qPCR assays, 5 µl of DNA extracted with the boil lysis method was used as the DNA template for each of the strains tested and for the standards and added to Luna 2x Probe Mastermix (NEB; UK), with 0.5 µM of each MAP RT F/R

primer, 0.4 µM of MAP RT probe and increased to 20 µl for with sterile distilled water. The parameters used during the cycling were an initial denaturation of 95 °C for 1 min followed by 40 cycles of: 95 °C for 15 s, 60 °C for 45 s and then fluorescence detection. The results were collated by the Q-REX software and Absolute Quantification Plug-in with the normalization tools 'Dynamic tools' and 'Use slope noise correction' selected to smooth the data (section 2.4.4.3.). An Auto-threshold was also calculated by the software to generate the threshold for a positive response and to define the positive Cq value.

4.3. DISCUSSION

The pIAC plasmid developed in Chapter 3 was used to produce an IAC for in-house *IS*6110 and *IS*900 qPCR assays. A low concentration of the plasmid was used in each assay to produce a late Cq value of around 27 cycles, which was shown not to decrease the sensitivity of the diagnostic qPCR assays. It was also shown that with the in-house *IS*6110 qPCR assay that one genome of *M. bovis* BCG and as such one copy of the *IS*6110 element would produce a Cq value around 38 cycles, under non-inhibited conditions. This meant that qPCR assays performed on DNA samples derived from clinical samples that contain polymerase inhibitors would likely produce a positive Cq value later than 38 cycles if the sample contained one low-copy number *IS*6110 organism. These results showed that the in-house *IS*6110 qPCR assay was sensitive enough to detect the minimum number possible of *IS*6110 containing MTBC bacteria in a clinical sample. The limit of detection of different qPCR assays are reported to be able to detect, a single genome of *M. tuberculosis* (Reed *et al*., 2016), 3 genomes (Santos *et al*., 2018) and 10 genomes (Lorente-Leal *et al*., 2019), which are in line with the results produced with the in-house *IS*6110 qPCR assay.

The *IS*6110 qPCR assay was also shown not to be inhibited by the large quantities of eukaryotic DNA co-extracted from the PBMC's from the blood with the Oneday phage assay, this showed that the assay was robust enough to be used with clinical samples and not suffer inhibition from the large quantities of non-template DNA present in the assay.

The *IS*900 qPCR assay that was developed targeted a conserved region of the *IS*900 element that avoided the 6 bp deletion present in the genomes of some MAP strains in the P90 primer sequence. The region used in the qPCR assay resides largely within the PCR product produced by the P90/91 primer pair. The in-house *IS*900 qPCR assay was shown to be more sensitive than the end-point PCR assay containing the P90short primer (section 4.2.5.2.) which contains the 6 bp 3' deletion to improve amplification with the strains containing the deletion in the element and has been shown to be more sensitive than the P90 primer. The qPCR assay also produced a positive signal with all four of the MAP strains tested. The in-house *IS*900 qPCR assay was then used to test historic DNA samples that had already been shown to be positive or negative for MAP and also ambiguous results that produced a positive signal at a late cycle and close to a late positive MP negative process control.

The in-house qPCR assay showed that all the previously negative samples were positive for MAP, it also showed that half of the ambiguous results were positive, and that half were negative. Only one sample, a previously positive sample, produced a weak positive result that could have been classed as ambiguous due to the thermo-degradation of the probe and quencher. The in-house qPCR assay showed agreement between two of the other previously positive samples but was negative for the remaining three. This either shows that in-house qPCR assay produced false negative results and there may also be clinical strains that contain polymorphism in those primers (and probe) also. It could also indicate that the previous results were false positive results however this would be difficult to distinguish based on these two tests and the two assays depleted all the DNA from the samples for further testing. The primers used in the in-house qPCR assay were

designed after the production of the pIAC plasmid and as such were not present on the plasmid. In order to use the pIAC plasmid as the IAC a non-competitive format of the multiplex was used showing that the IAC can be used interchangeable where needed in a qPCR format.

4.4. CONCLUSION

The results gained in this chapter showed that the previously developed IAC designed for an end-point PCR assay could be expanded into a qPCR assay and used successfully as both a competitive and non-competitive format. The advantage of the dual-labelled probe is that almost eliminates the possibility of spurious fragments produced in non-competitive multiplex PCR assays being detected as it is very unlikely that they will also contain the probe sequence required to produce a positive signal.

The in-house qPCR assays developed in-line with the IAC qPCR assay for MTBC was shown to be suitable for use with human clinical DNA samples and was not inhibited by large quantities of non-target eukaryotic DNA that can potentially dilute out the primers and probe. The qPCR assay developed for MAP was shown to be more sensitive that the well described P90/91 end-point PCR assay and also more sensitive as it produced a positive result with all of the MAP strains tested where the P90/91 primer pair did not.

CHAPTER 5

5. DETECTING TB FROM HUMAN BLOOD

5.1. INTRODUCTION

The detection of MTB in human patients requires complementary results from a series of different tests. Initially, cases of active pulmonary TB are diagnosed presumptively using a healthcare questionnaire and radiological images produced by chest X-Rays. Based on the results of these tests, the next step in the diagnosis is to test for acid fast bacilli. The sputum or bronchial lavage fluid is collected from the patient and then stained for acid fast bacilli using the Ziehl-Neelsen method. If acid fast bacilli are detected, then the patient is classed as smear positive. If none are detected then they are classed as smear negative, however it does not mean that there are no acid-fast bacilli present, but that the mycobacterial load is below the threshold for detection for this assay (10^4 - 10^5 cfu ml^{-1}; (Anon, 2000). Another limitation is that acid fast staining has poor specificity as non-tuberculosis mycobacteria (NTM) will also give a positive result. Regardless of whether any acid-fast bacilli are detected or not, the remaining unstained fluids are used for culture of any mycobacteria present in the sample. Due to the nature of the sample, a decontamination process is needed to kill any commensal bacterial that are also contained within the respiratory fluids to prevent the contaminating bacteria overgrowing the mycobacteria. Even though the mycobacteria are resistant to the decontamination process, the process also produces a drop in the mycobacterial viable count. In smear negative patients, this step could completely reduce the level of viable mycobacteria below the limit of detection for culture (~10–100 cfu ml^{-1}; Anon, 2000) since the number of bacteria in the initial samples is already very low. Further ancillary blood tests such as an IGRA are also performed to help identify active cases of pulmonary TB and LTBI patients.

More recently tests have been developed that using amplification of signature sequences by PCR as a rapid alternative to culture. The most common commercial test is Xpert® MTB/RIF Assay (Cepheid; Sunnyvale, CA), which is reported to have a sensitivity of 98 % for smear positive patients, however, the sensitivity for samples from smear negative patients is only 67 % (Steingart *et al*., 2014) which means patients with paucibacillary TB, such as children and people living with HIV, may be missed. Another issue is that until the disease reaches a clinical phase where sputum is being produced, patient samples cannot be tested. This is a particular issue for diagnosis in children who have difficulty producing sputum samples on demand (Parry *et al.*, 1995) and so require the use of the far more invasive bronchoalveolar lavage technique (Roya-Pabon and Perez-Velez, 2016).

At the start of this study, researchers from this group had already shown that it was possible to detect *M. bovis* circulating in the blood of cattle that have confirmed bovine TB infection by first isolating the PBMCs and then detecting mycobacteria using either the standard or the Oneday phage assays. This finding was unexpected since haematogenous dissemination of bovine TB away from the primary site of infection (the lung) in the early stages of the disease had not been commonly reported. The aim of this study was to determine whether the methods developed to detect TB in cattle could be applied for the detection of the haematogenous dissemination of *M. tuberculosis* in immunocompetent humans with active pulmonary disease and in recent contact patients who had a positive IGRA response, so that they were classified as having latent TB infection (LTBI). The use of human blood as a vector for TB diagnosis is an attractive prospect as blood is usually a sterile fluid in healthy people and so requires no decontamination step and blood samples

are already collected as part of the standard diagnostic procedure for IGRA testing. If successful this could also be a better sampling method for the detection of TB in children, as venipuncture is far less invasive compared to bronchoalveolar lavage.

For testing cattle blood, Ficoll® density gradient is used to isolate the PBMCs, however this method requires the use of expensive laboratory equipment which makes the method unsuitable for use in low technology laboratories. In this study we also attempted to simplify the sample processing steps by comparing results gained using the Ficoll® PBMC extraction method with a HetaSep® extraction method. HetaSep® is a reagent that was developed for the isolation of stem cells and promotes the formation of rouleaux by red blood cells. This then allows separation of the red blood cells from the white blood cells on the basis of gravity sedimentation. The HetaSep® separation method does not require the use of a centrifuge and as such is more transferrable to low technology settings. The final aim of the study was to determine whether the rapid, Oneday phage assay was more sensitive than the standard two-day phage assay, as had been seen when detecting bovine TB infections in cattle.

5.2. ETHICS APPROVAL

Ethical approval for the use of blood samples obtained from human patients was approved by the regional Research and Ethics Committee (REC 15/EM/0109). Upon admission to the trial, all patients provided informed written consent prior to testing.

5.3. STUDY DESIGN

5.3.1. Sample groups, patient selection and study design

The tests mentioned in this section were performed at Leicester Glenfield Hospital and the data was collected by Dr. Raman Verma as part of the study.

In order to confidently assess the sensitivity and diagnostic power of the two Actiphage assays and immune cell isolation reagents, four discrete groups of people were selected for testing. These were: people with active pulmonary TB (pTB), identified from positive Xpert-Ultra (Cepheid Inc.) or positive culture of *M. tuberculosis* from respiratory tract samples, supported by clinical symptoms and radiological graphics; Latent TB Infection/ contact, people in contact with active pulmonary group and have a positive QuantiFERON-TB Gold Plus (QFT; QIAGEN Inc.) and healthy chest X-ray; People with confirmed non-TB respiratory disease with negative *M. tuberculosis* culture and testing; healthy controls with negative QuantiFERON-TB Gold Plus, no contact with TB patients and no symptoms of disease. The total number of patients in the study was 66. All patients selected were designated adults (over 18 years of age) and human immunodeficiency virus (HIV)-seronegative and provided blood samples for testing with the Actiphage assays.

All pTB patients were sampled prior to receiving anti-TB medication. The contact group received QFT and Actiphage testing after 8 – 12 weeks, in-line with Interferon Gamma Release Assay testing. Contact group patients were accepted if the follow up QFT testing was positive and they declined prophylactic anti-TB medication.

All patients also had their blood levels of C-Reactive protein (CRP) determined. CRP levels increase due to stimulation by IL-6 mediated by infections such as active pulmonary TB. Co-infection with HIV does not change the CRP levels of a patient

infected with pulmonary TB, which can reduce the sensitivity of certain diagnostic methods (Yoon *et al.*, 2017).

The study was a double blinded trial between the clinicians and the laboratory, until the completion of the study where the patient groups were made available to the laboratory and the test results released to the clinicians.

5.3.2. Sample preparation and storage

To prepare the blood samples, 5 ml of blood was collected into two sodium heparin vacutainers (Sarstedt; UK) and stored at room temperature until processing at the collection site. Due to the limitations on the time available to collect the samples, the length of time that the samples were stored at room temperature before processing was between zero and four days.

5.3.3. Preparation of immune cells

On receipt of the blood samples, the white cells were extracted using one of two methods. The first method used Ficoll® Paque Plus (GE Healthcare; UK) with Leucosep™ tubes (Greiner Bio-One; UK) which is the gold standard of PBMC isolation in human patients, and the established method used in the laboratory at the time. The second method was HetaSep®, which is an erythrocyte aggregation agent.

5.3.3.1. Preparation of PBMC's with Ficoll® Paque Plus

To isolate the PBMC from the blood using Ficoll® Paque Plus (section 2.3.1.1.). Briefly 2 ml of blood was mixed gently with PBS in a Leucosep™ tube. The samples were then centrifuged at 400 xg for 30 min, 18 °C with no brake. The PBMC layer was recovered and washed with 6 ml PBS and finally for these experiments the cells were then reconstituted with 1.5 ml of 7H9 plus 10 % % (v/v) OADC.

5.3.3.2. Preparation of immune cells with HetaSep®

The second method that was tested was the extraction of the nucleated cells using HetaSep® (section 2.3.1.2.). To recover the white cells, 2 ml of blood was combined with HetaSep® at a ratio of 5:1. The samples were incubated at room temperature for 30 min until the interface between the erythrocytes and plasma was approximately 50 % of sample volume on visual inspection. The samples were allowed to incubate for a further 30 min (60 min total time) if the separation had not reached 50 %. The upper plasma layer was then removed and washed with 6 ml of PBS as for the Ficoll® procedure (section 2.3.1.1.). Finally, the cell pellet was reconstituted with 1.5 ml 7H9 plus 10 % % (v/v) OADC.

5.3.4. Testing for mycobacteria

Once the cells had been extracted using either Ficoll® or HetaSep® and reconstituted into 1.5 ml of 7H9 plus 10 % (v/v) OADC, they were subsequently processed in the same way. Each sample was used for the detection of *Mycobacterium* using three different methods. The first method that was used was the standard two-day phage assay, which is already a well described method for detecting mycobacteria and has been used previously to detect mycobacteria from human blood. The second method used was the Oneday version of the phage assay which is quicker and simpler to perform. This method was included to see whether it was as sensitive as the standard phage assay. The two phage methods were compared with the culture method, which is the current 'gold standard' and accepted method for the detection of Mycobacteria. To ensure that the assays were standardized, and the same concentration of cells was used in each assay, one third of the white cells recovered from each 2 ml blood sample was used in each of the three assays, and therefore

each test used white blood cells recovered from the equivalent of 0.7 ml blood. To distribute the cells for the three assays, the samples were mixed using a vortex mixer to evenly distribute the cells and break up clumps. To prepare the two-day phage assay, 500 µl of the 1.5 ml sample were transferred to a two day assay tube (section 5.3.4.1).

The remaining 1 ml of sample was then centrifuged at 13,000 xg for 3 min (room temperature), and after discarding the supernatant, the cells were resuspended in 200 µl of 7H9 plus 10 % (v/v) OADC. The cells were then pipette mixed rapidly to homogenize the sample. To prepare the Oneday phage assay, 100 µl of the reconstituted cells were then transferred to an Actiphage reaction vessel (0.45 µm filter; section 5.2.4.2). The remaining sample was then used for the detection of mycobacteria by culture method (section 5.3.4.4).

5.3.4.1. Standard phage assay

The carry out the standard phage assay (section 2.2.), 500 µl of sample extracted from the patient's blood was mixed with 500 µl of 7H9 plus 10 % (v/v) OADC was added to the standard phage assay reaction vessels to produce a final volume of 1 ml. To this 100 µl of phage D29 ($1x10^9$ pfu ml^{-1}) was added, the sample was mixed gently by inversion and then incubated at 37 °C for 1 h. To inactivate and exogenous phage, 10 mM fresh Ferrous Ammonium Sulphate (virucide) was added, and the samples well mixed using a rotating platform (PTR-60; Grant Instruments, Cambridge) at room temperature for 6 min. The FAS was then diluted out by adding 5 ml 7H9 plus 10 % (v/v) OADC to the reaction vessels and then the samples were transferred from the reaction vessels to a sterile petri dish. To form the bacterial

lawn to support plaque formation, 1 ml of *M. smegmatis* cells at a concentration of ~1x10^{7} cfu ml^{-1} was added to the petri dish and then 6 ml of molten-tempered 7H10 agar was added to the petri dish which was gently turned on the bench to fully homogenize the samples. Once the agar plates had solidified, they were incubated at 37 °C for 24 h to allow plaques to develop. Any plaques that did develop were counted and samples extracted from the center of the plaque using a 0.5 mm biopsy punch (Selles Medical) and transferred in groups of five to a sterile Eppendorf tube. Where less than five plaques were available, the remainder of the sample was made up using plaques formed using a *M. smegmatis* culture plaques to act as carrier DNA to improve the recovery of the plaque DNA produced from the patients' samples. The DNA from the plaques was then extracted using the Spin column method (section 2.4.1.3.) with the Zymoclean Gel DNA Recovery Kit (ZymoResearch; Cambridge Bioscience; UK). The DNA was eluted using a final volume of 12 µl of sterile molecular grade water for PCR analysis.

5.3.4.2. Oneday phage assay

The Oneday phage assay was prepared by adding 100 µl of the concentrated cells (500 µl equivalent) (section 2.2.3.) to an Actiphage rapid tube (0.45 µm filter) and then 10 µl of phage D29 (1x10^{9} pfu ml^{-1}) was added to the samples. These were then incubated at 37 °C for 4 h to allow the phage replication cycle to be completed and lysis of any viable mycobacteria present to occur. After the incubation, the Actiphage rapid tubes were centrifuged at 13,000 x*g* for 3 min to separate the cell debris using the integrated 0.45 µM filter. The lysate was then purified and concentrated using the Spin-column method (section 2.4.1.4.) with the DNA Clean & Concentrator-5 kit

(ZymoResearch; Cambridge Bioscience; UK). The DNA was also eluted using a final volume of 12 µl sterile molecular grade water for PCR analysis.

5.3.4.3. IS6110 PCR assay

The purified DNA extracted from plaques produced in the standard phage assay and the lysate produced in the Oneday phage assay from the human blood samples were used as template for the *IS*6110 PCR assay (Eisenach *et al*., 1990) to confirm the presence of MTBC.

The PCR reaction amplifies a segment of the *IS*6110 insertion element specific to MTBC bacteria and the IAC segment of the pIAC plasmid. DNA (5 µl) extracted from plaques (section 2.2.2.) or the Oneday phage assay (section 2.2.3) or (1 µl) cells (section 2.4.1.1.) was used in each PCR reaction. To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

5.3.4.4. Culture

The remaining 100 µl of the cells extracted from the patient's blood was used for the culture method of *M. tuberculosis* by flooding Stonebrink slopes (Becton Dickenson; UK) with the remaining suspension. No decontamination step or antibiotics were

added to the media or cells as these steps usually result in a loss of viable mycobacteria as well as contaminants. Blood samples are typically classed as sterile fluids in healthy people, so it was assumed to contain no organisms other than *M. tuberculosis* from active pulmonary TB patients. The slopes were then incubated at an angle to produce a level surface to prevent pooling of the sample at the bottom of the slope, at 37 °C for 6 – 12 weeks. The slopes were assessed for contaminating growth after one and three days and then weekly up to 5 weeks. Any samples showing contamination were discarded. The slopes were again checked weekly from 6 – 12 weeks for slow growing mycobacteria colonies. Slopes that produced no colonies after 12 weeks were classed as negative and discarded.

5.4. RESULTS

5.4.1. Determining the agreement between the combined phage assays and hospital culture techniques

One of the aims of the study was to determine the predictive power of the two phage assays when they were used together and how they compare to the current gold standard. Unfortunately, we were unable to culture any MTB from the immune cells prepared from both the Ficoll® and the HetaSep® extraction methods and therefore a direct comparison of phage-based detection with blood culture was not possible. This result was not surprising given the cited limit of detection of culture methods (10-100 cfu ml^{-1}). Therefore, for this study, the clinical markers were used as the gold standard methods to identify the rate of agreement. For diagnostic purposes, the phage tests, a positive result was given if any of the four phage assays performed (Ficoll® and HetaSep® preparations, standard and Oneday phage detection methods) on the patient produced a positive result (*i.e.,* each set of tests

was considered as the test result of one blood sample with a total volume of 2.7 ml which is the total volume used to complete the four tests). To assess how accurately the phage assays predicted the correct result compared to the gold standard, a ROC curve analysis was performed on the data and Youden's J statistic was calculated to assess the performance of the tests.

The results of the phage assays were compared to the culture techniques used to diagnose the patients within the active pulmonary TB group to determine the combined phage assays clinical diagnostic potential. This group of patients represented the clinical scenario where the phage assays could be used to confirm suspected TB cases. Each of these patients were already confirmed to have active pulmonary TB by culturing methods and should produce a positive result with both phage assays. At the baseline testing stage, this group consisted of 13 patients. The combined phage assays successfully identified nine cases of active TB by producing a positive result with at least one of the four phage assays performed, these were classed as true positive results. The results for the remaining four patients were negative by all four of the phage assays performed, these four negative patients were classed as false negative results. The number of true positive and false negative results were then used to calculate a sensitivity of 69.2 % for the combined phage assays when compared to the hospital culture techniques.

When the other clinical information for the four phage-negative patients was reviewed it was noted that the test results indicated that these patients were displaying a reduced level of disease progression compared to the nine phage positive active pulmonary TB cases in that they were all smear negative for AFB, produced "low – very low" scores using Xpert Ultra and the mean time to positive

culture was also longer (see Table 5.1), which are all indications of a lower bacterial load. In addition, they had lower median C-reactive protein values, and it has previously been reported that in active TB patients, CRP levels reflect mycobacterial load (Clark *et al*., 2014)

Table 5.1: Summary of clinical test results for Active TB patients

TB DIAGNOSTIC TEST	ACTIVE TB PHAGE-POSITIVE PATIENTS (N=9)	ACTIVE TB PHAGE-NEGATIVE PATIENTS (N=4)
AFB SMEAR TEST RESULT	positive	negative
XPERT ULTRASCORE	medium – high	low – very low
C-REACTIVE PROTEIN VALUES	63 (IQR = 36 – 65)	41 (IQR = 27 – 45.5)
TIME TO POSITIVE CULTURE FOR BRONCHIAL LAVAGE (days)	15 (IQR = 10.5 – 22)	21 (IQR = 21 – 21)

IQR; interquartile range

Test results produced at Leicester Glenfield hospital for the diagnosis of TB = in the active pulmonary TB group

At the baseline testing for the LTBI group, there were initially 20 patients within the LTBI group. These patients all had contact with a member of the active pulmonary TB group and produced a positive QuantiFERON test result but displayed normal chest X-Rays and were asymptomatic for TB. These patients were all assumed to have a LTBI and as such, they were expected to produce a negative result with the combined phage assays. When the results of the phage assays were compared to the culture techniques, it was shown that three produced a positive result with at least one of the phage assays performed producing a positive result. These were initially classed as false positive results, but the interpretation of these results changed at later points during the trial (see section 5.1.6). The combined phage assays for the remaining 17 patients produced only negative results and were classed as true negatives, due to the agreement between the combined phage assays with the other clinical information.

In the non-TB illness control group, the comparison between the combined phage assay and the patients with non-TB related respiratory illnesses were in line with the expected results. These patients were originally suspected of having active pulmonary TB but were later diagnosed with non-TB related respiratory illness by the exclusion of *M. tuberculosis* during culturing methods. All the patients produced a negative result and all four of the phage assays performed as expected.

5.4.1.1. Comparison between the combined phage assays and hospital culture techniques for the healthy group

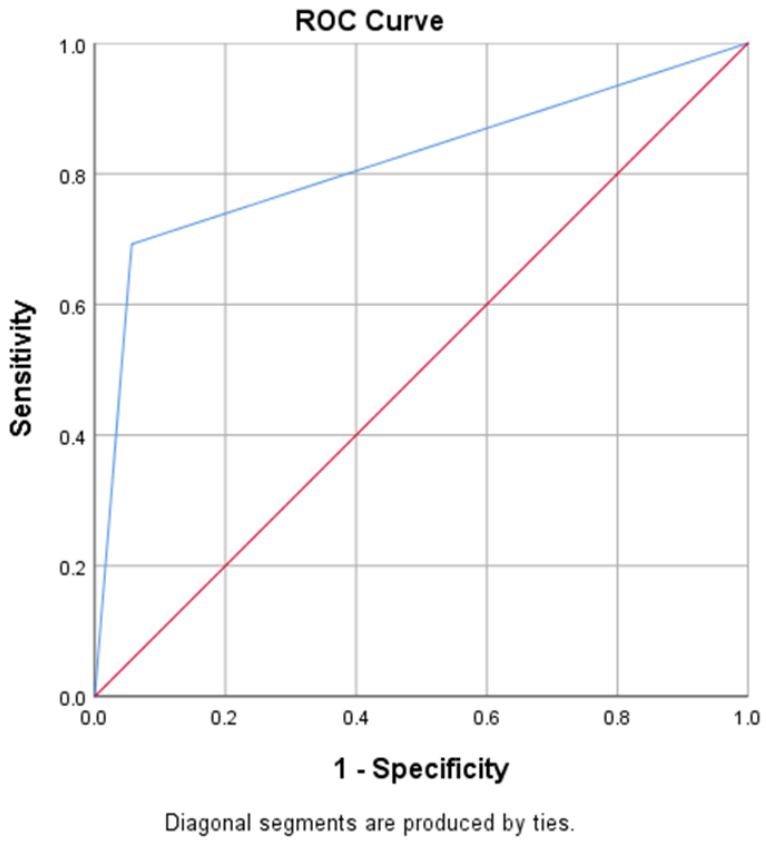
When the results for the combined phage assays were compared to that of the hospital culture techniques for the healthy group of patients, again they were in line with the expected results. All the healthy patients produced a negative result with each of the four phage assays performed. This agreed with the results produced from the culture techniques and as such they were classed as true negative results. The number of true negative and false positive results in the LTBI, non-TB illness and healthy group were analyzed, and produced a specificity value of 94.2 %.

5.4.1.2. Statistical analysis of the combined phage assays compared to the clinical diagnosis

In order to determine the performance of the combined phage assays compared to the clinical diagnosis and gold standard a receiver operating characteristic (ROC) curve analysis was performed on the data (Fig. 5.1) and the Youden's index was calculated. At this point, the sensitivity of the combined phage assays were calculated to be 69.2 %, with a specificity of 94.2 %. The area under the curve (AUC) of the ROC analysis was calculated at 0.818 with a Youden's index value of 0.635. In general, an AUC of 0.8 to 0.9 is considered to indicate that a test is producing excellent discrimination. An AUC of 0.5 means the test only predicts the correct outcome 50 % of the time and is a poor diagnostic test. The Younden's index value ranges from 0 to 1. A value of 1 means that the diagnostic test produced no false positive and false negative results and is an ideal assay. A score of 0 equates to the diagnostic assay predicting a correct positive result with the same proportion of positive results for all test groups and is a poor assay. The statistic gives equal weight to false positive and false negative results. A value of 0.635 shows the test was an

average method. The Younden's index can be used to define a threshold for a disease in combination with the ROC curve analysis when used with continuous variables however, this was not applicable with the data produced in this study and as such is not represented on the ROC analysis (Fluss *et al*., 2005).

Fig 5.1 - ROC analysis for the Oneday phage assay combined phage assays compared to the hospital culture techniques (before seroconversion)



A ROC analysis of the data from the combined phage assay compared to the gold standard of clinical diagnosis of TB, the figure was produced using the statistical package SPSS (IBM). The blue line represents the ROC curve of the combined phage assays. The red diagonal line is a representation of a poor test. AUC = 0.818

5.4.1.3. Seroconversion of TB-negative, phage-positive patients.

The three LTBI patients that produced the false positive results with the combined phage assays at the baseline testing produced negative QuantiFERON Plus results. However, at the three month follow up, another QuantiFERON Plus test was performed and at this point, two of the three patients produced a positive QuantiFERON Plus result, indicating the patients had developed an immune response to *M. tuberculosis* (see Table 5.2). The other patient produced a positive response that would be classed as borderline by the manufacturer's recommendations (Bisognin *et al*., 2020).

Table 5.2: QuantiFERON Plus test results for phage-positive patients from LTBI group

PHAGE POSITIVE, LTBI PATIENT NO.	QUANTIFERON PLUS TEST RESULT AT BASE LINE (IU $ml^{-1)}$	QUANTIFERON PLUS TEST RESULT AT 3 MONTHS (IU $ml^{-1)}$
1	0.16	0.34
2	0.18	5.5
3	0.08	2.82

IU = Results for the test sample are reported in International Units per ml (IU ml^{-1}) relative to the standard curve prepared by testing dilutions of the standard supplied with the kit. The threshold for a positive sample as directed by the manufacturer is >0.34 IU ml^{-1}, however a result <0.50 IU ml^{-1} should be classed as indeterminate (Bisognin *et al*., 2020).

At this stage these patients all displayed normal radiological results and were asymptomatic for TB except for the positive IGRA, and therefore were reclassified as LTBI and added to the LTBI group.

At the seven month follow up, two of the three phage positive LTBI patients had gone on to develop active pulmonary TB, confirmed by the culture of pulmonary *M. tuberculosis*. The time it had taken to produce a positive culture result for these two patients was lower than both subgroups in the active pulmonary group, with a median value of 26 days (IQR =23.5 – 28.5 days) indicating that they were in the early stages of active disease with a low microbial burden. At the time of TB presentation, the two patients also produced an Xpert-Ultra result of “medium”. The third phage positive LTBI patient did not progress to active pulmonary disease, showing the Oneday phage assay detecting MTBC in the blood at the early stages of infection before an adaptive immune response was produced.

For the data analysis, the two LTBI patients that developed active TB were moved to the active pulmonary TB group, reducing the LTBI sample size to 18 patients and increasing the active pulmonary TB sample size to 15 patients. This changed the classification of two of the false positive results by the combined phage assays to true positive results, and as such the sensitivity and specificity were recalculated. After the reclassification, the sensitivity of the combined phage assays was calculated to be 73.3 % and the specificity was calculated to be 98 %, when the phage results were compared to the clinical culture results, which were higher values than gained in the initial analysis of the results. A new ROC analysis was also performed using this data (Appendix 4, Fig. 9.16) and now produced an AUC of 0.875, again falling into the range of values that indicate that a test is producing excellent discrimination. The

Younden's Index was recalculated at 0.71. Which also shows an improvement and the combined phage assays would now be classed as a good diagnostic test.

The results also showed that the combined phage assays were able to detect MTB circulating in the blood of human LTBI patients before a positive result was produced with the QuantiFERON Plus IGRA. The statistical analysis performed from this point onwards in this chapter will use the data including the reclassification of these three patients.

5.4.2. Comparison between the sensitivity of the standard phage assay and the Oneday phage assay for the detection of *M. tuberculosis* in human blood

Apart from determining the performance of the phage-based test as a diagnostic, another aim of this work was to investigate the performance of the different versions of the test and to see whether the standard phage assay (section 2.2.) or Oneday phage assay (section 2.2.3.) showed the best agreement with the clinical culture techniques. It was thought that the Oneday assay would produce the best results as it has already been shown to be a more sensitive assay than the standard phage assay when testing bovine blood samples (Swift *et al*., 2020). As described above, in this study each phage assay was performed twice using the two different sample preparation methods (Ficoll®; section 2.3.1.1. and HetaSep®; section 2.3.1.2.). To compare sensitivity of the different methods, a sample was classed as positive if it produced a positive result for the *IS*6110 PCR assay with either of the two pairs of assays performed using the Oneday or standard phage assay.

As previously mentioned, the total number of patients in the active pulmonary TB group was 15 patients and were all expected to produce a positive result with the phage assays. When the results of the standard phage assays were compared with

the patients, it was determined that the standard phage assay correctly predicted six positive patients and failed to detect nine of the patients with clinically diagnosed TB (Table 5.3). The results were then compared to the Oneday phage assay which correctly diagnosed 11 of the positive patients and failed to detect four of the patients with clinical TB. The two different phage assays showed agreement between six of the positive results, with the one-day phage assay detecting five additional positive cases. The standard phage assay produced a negative result in the *IS*6110 PCR assay for all 18 members of this group, which was in line with the expected results as these patients were assumed to have a dormant, non-active MTB infection. The Oneday phage assay produced 17 negative results but also produced one positive result, which was the contact patient that seroconverted during the testing period but did not progress to active disease. This result was classified as a false positive as the patient did not develop active pulmonary TB. All of the patients in the non-TB group and Healthy group were negative by both the standard phage assay and the Oneday phage assay

Table 5.3 – Comparison of the standard phage assay and Oneday phage assay results

Patient Group	No. in group	No. positive using standard two-day assay	No. positive using Oneday assay	Agreement (positive results)		
				Standard only	Both assays	Oneday only
Active pulmonary TB	15	6	11	0	6	5
Contacts	18	0	1	0	0	1
Non-TB related disease control	5	0	0	0	0	0
Healthy control	28	0	0	0	0	0

The positive results produced with the standard phage assay (section 2.2.) and the Oneday phage assay (section 2.2.3.) from testing the PBMCs of human patients for *M. tuberculosis.*

The sensitivity of the standard phage assay for the active pulmonary TB cases was calculated at 40 % compared to a sensitivity of 73 % for the Oneday phage assay. The specificity of the standard phage assay was calculated at 100 % using the results from the LTBI, non-TB disease and healthy group. This was compared to the one-day phage assay which produced a specificity of 98 %. A ROC analysis was then performed on the data for the standard phage assay and produced an AIC of 0.688. This result does not fall within the exceptional bracket (0.8 – 0.9) but would still be considered a good test. The sensitivity and specificity of the two-day phage assay was then compared by calculating the Younden's Index, which showed a result of 0.4. The Younden's index showed that the standard phage assay was at best an average test and that there could be an increased amount of misclassified results. This was compared to the ROC analysis of the Oneday phage assays which produced an AUC of 0.875 and a Younden's Index of 0.71. The larger AUC value for the ROC analysis and Younden's index of the Oneday phage assay results compared to the standard phage assay results showed that the Oneday phage assay was more likely to produce the correct result compared to the standard phage assay because in both analyses the closer to 1 the value, the better diagnostic test. In terms of the Younden's index, the result of 0.4 indicates that there could be an increased combination of misclassifications of false positive and/ or false negative results, whereas a result of 0.71 indicates the majority of the results will be classified correctly

5.4.3. Comparison between the sensitivity of HetaSep® and Ficoll® Paque Plus for the isolation of immune cells and *M. tuberculosis* detection

To assess which of the two immune cell extraction methods provided the best agreement of the results, the PCR assays were analyzed and the assays that amplified a 123 bp PCR product corresponding to the amplification of the *IS*6110 element were classed as MTBC positive. The results of the *IS*6110 PCR assays from the standard phage assay and Oneday phage assay were used together so that each extraction method was performed twice. A positive result was classified if one out of the two phage assays performed using the same PBMC extraction method produced a positive result. The results were compared with the clinical culture techniques to determine which of the two immune cell extraction methods produced the most accurate results.

The results for the Ficoll® extraction method (section 2.3.1.1.) were compared with the HetaSep® extraction method (section 2.3.1.2.) and were showed to be comparable (Table 5.4). The most important results identified was that 6 samples (1 in the active pulmonary group, 2 in the LTBI group, 1 in the non-TB group and 2 in the healthy group) failed the HetaSep® separation process due to the degradation of the blood due to age. All of these samples were processed in parallel with Ficoll® and a buffy coat was extracted from the blood, showing that Ficoll® can be used to process older blood than HetaSep®. The two tests were in complete agreement with each other and every HetaSep® positive sample was also positive with the Ficoll® extraction, when combining the results of the two phage assays. All of the negative HetaSep® results were also negative with Ficoll®. The only difference in the results were produced from the 6 failed HetaSep® extractions.

Table 5.4 – Comparison of the results produced between the Ficoll® and HetaSep® PBMC extraction methods

Patient Group	No. in group	No. positive using Ficoll®	No. positive using HetaSep®	Agreement (positive results)		
				Ficoll® only	Both assays	HetaSep® only
Active pulmonary TB	15	11	10	1	10	0 (1 sample failed extraction)
Contacts	17	0	1	1	0	0 (2 samples failed extraction)
Non-TB related disease control	5	0	0	0	0	0 (1 sample failed extraction)
Healthy control	28	0	0	0	0	0 (2 samples failed extraction)

The positive results produced with the Ficoll® extraction method (section 2.3.1.1.) and the HetaSep® extraction method (section 2.3.1.2.).

When all the results were compared together as a whole, it was clear that there was no difference between the agreement of the results between the two extraction methods when the results of the phage assays were compared to the clinical diagnosis. This showed that both extraction methods tested were equally capable of extracting viable *M. tuberculosis* from the blood of humans for the phage assays. The manufacturer's protocol only describes an extraction method for blood samples up to 48 hours old. Blood samples that were three days old still produced some aggregation and produced comparable results to the Ficoll® extraction method, however three of the blood samples that failed the HetaSep® extraction were one day old and three were four days old at the time of processing (see appendix 1), showing some variability in extraction success. The only difference in the results was that the HetaSep® extraction method required fresher blood than the Ficoll® extraction method to produce a successful PBMC isolation, resulting in lost data for the HetaSep® extraction method. The Ficoll® extraction method was still effective with older blood as the state of the RBC does not affect the density centrifugation and so long as the PBMC's are still intact, then a buffy coat can still be produced. The results showed that as long as the blood can be processed within four days of collection, and ideally within two, that HetaSep® method provides a cheaper and less labour-intensive method for the extraction of immune cells compared to the Ficoll® extraction method. However, if a delay between the extraction of the blood sample and the processing of the sample cannot be avoided, then it was concluded that the Ficoll® extraction method would be more reliable.

5.4.4. Follow-on human study

The information gained from the previous experiments was used to design a follow-on trial to detect infections in humans. First it was decided to use only the HetaSep® method to purify the WBCs because the HetaSep® was a less labour-intensive technique and was more suitable for high throughput testing. In addition, the information gained about the effect of blood ageing (Appendix 4, sections 9.4.2 – 9.4.7.) on this method was used to determine when blood samples would be collected in the clinic. A sampling and collection program was devised based in this information so that the blood samples were never older than 72 hours old and were ideally no more than 48 hours old when they arrived at the laboratory for sample processing.

Secondly, it was also decided that only the Oneday phage assay would be used since the standard phage assay and blood culture techniques used within the first study were shown to be less sensitive than the Oneday phage assay. As only one test was being performed, it was also decided to increase the sample volume used for the Oneday phage assay which was expected to increase the sensitivity of the assay. The amount of blood sample tested in the first trial was 4 ml per patient, however only 1.34 ml of the sample was tested using the Oneday phage assays as the remainder of the sample was used for either the standard two-day phage assay or culture. Therefore, in this trial, a total of 10 ml per patient would be collected and tested but, in order to test the increased volume, five parallel Oneday assays would be performed for each patient, because 2 ml is the maximum volume of blood that can be successfully used with the Actiphage test due to the increased amounts of WBC

debris in the samples leading to the filters becoming blocked (Dr B Swift, RVC, pers. comm.). Where the blood volume from a patient exceeded 10 ml, it was also decided to try and optimize the culture techniques used in an attempt to culture viable *M. tuberculosis* from the blood of active pulmonary TB patients or LTBI patients (Appendix 5, section 9.5.1.).

Another method that can improve sensitivity of any PCR-based assay, is using qPCR rather than end-point PCR as the detection method. Again, the end-point PCR assay described by Eisenach *et al.* (1990) would be used (section 2.4.2.2.), but the results of the end-point PCR assay would be compared to those gained from an in-house *IS*6110 qPCR assay (section 2.4.4.2.) that was also based on the Eisenach *et al*. (1990) end-point PCR assay. Apart from potentially increasing sensitivity, another advantage of this qPCR assay was that it included the IAC plasmid so that any inhibition of the DNA amplification could be identified.

The aim of this work was primarily to increase the sensitivity of the Oneday phage assay so that the patients with lower bacterial load could be detected, to increase the number of patients with active pulmonary TB identified. It was shown in the pilot study that the Oneday phage assay was able to detect patients who were progressing from an LTBI to active disease however there were only a small number of patients where this was observed. Therefore, in this study, this group were again targeted to see if further evidence could be produced to support these preliminary results.

Due to the outbreak of COVID SARS-2 and the compulsory national lock down that was put in place, the second human trial had to be halted partway through the sampling and testing phase, and this had not been restarted before the submission of this thesis was due. Due to that fact, the two aims established for the study could not be completed as the clinical information had not been released for the samples where tests had been completed due to the expectations of restarting the trial when the restrictions allowed. Therefore, the data collected in the trial could only evaluate the performance of the in-house *IS*6110 qPCR assay designed in Chapter 4 and to compare against the optimization procedures tested with the excess blood samples.

5.4.4.1. Development of the sampling processing method

Initially, to standardize the concentration of MTBC present in all five samples tested, a decision was made to extract the PBMCs using the HetaSep® extraction method (section 2.3.1.2.) from five 2 ml aliquots and then pool these together at the stage of washing in PBS to create a pooled sample. The PBMCs were then lysed by resuspending in 500 µl of MP and five separate 100 µl aliquots used for the Actiphage assay (section 2.2.3.). This method was used for the first six batches of samples, but most of the results produced were negative and those that did produced a PCR product produced unexpected indeterminate/ weak positive results in the endpoint PCR assay and qPCR assay performed on the DNA extracted with the Oneday phage assay.

At this point it was concluded that as the levels of MTBC were very low, this approach to sampling could potentially be diluting the number of cells per sample below the threshold for detection using the Oneday phage assay (approximately 5

cells per sample; Swift *et al*., 2020). Accordingly, it was decided to revert to the testing procedure used in the pilot study and to simply test five independent parallel samples. This may mean that there was more variability of test results between samples from one patient but was more likely to give a positive result if only one of these samples contained sufficient MTBC cells to be detected.

Changing the protocol back to the same method used in the first study (except the equivalent of 2 ml of blood was used entirely for each Oneday phage assay) improved the amplification and the positivity of the samples. The positive samples were now producing more intense PCR products and the Cq vales had decreased for potential positive results.

5.4.4.2. Inhibition

The results of the in-house *IS*6110 qPCR assay for determining the presence of inhibitors was not in line with what was expected. The results showed that 136 out of 911 qPCR assays (14.9 %) produced heavy to complete inhibition and were classed as false negative results. Almost all samples derived from the blood of the human patients that were tested produced some partial inhibition, however it was expected that the levels of inhibition would still allow for the amplification of MTBC contained within the samples, so these were not classed as false negative results. The qPCR assays that were shown to be inhibited were repeated by increasing the volume of the remaining DNA elution (approximately 2 µl) to a final volume of 5 µl to dilute the inhibitors and increase the activity of the DNA polymerase. None of the re-tested qPCR assays produced a positive result after diluting the remaining DNA. The results comparing the endpoint PCR assays with the qPCR assays were not in line with what

was expected. Firstly, out of 337 comparable tests (tests were excluded that were processed differently at the start of the study and samples where only one assay was performed were also excluded), only 50.5 % of the parallel testing produced results that were in agreement, and only five patients produced 5 out of 5 results that were in complete agreement with each other. This was unexpected, some minor disagreement between the two PCR assays performed was expected since the qPCR assay has been shown to be more sensitive. It was possible that by increasing the volume of blood used per Oneday phage assay that the concentration of blood inhibitors co-extracted with the Oneday phage increased also. It is also possible that the extra non-target eukaryotic DNA was inhibiting the amplification of the *IS*6110 element, but without the release of the clinical information it is impossible to tell which of the tests were producing the most accurate results.

5.5. DISCUSSION

The results for the pilot human study were very promising. The results when both the assays were used in conjunction with each other and before the seroconversion events produced an initial sensitivity of 69.2 % and a specificity of 94.2 %. These values increased after the seroconversion events to produce a sensitivity of 73.3 % and a specificity of 98 %. Swift *et al*. (2020) used the Oneday phage assay for the detection of *M. bovis* in cattle and showed a sensitivity of 95 % and a specificity of 100 %. The specificity between the two studies are comparable and very similar, however the sensitivity in humans was lower than that in cattle. Although there was a difference in the sensitivities, there were similarities in the pattern of the results. The phage negative active pulmonary TB patients were all smear negative, produced "low-very low" Xpert Ultra scores, produced longer mean times to culture than the phage positive active pulmonary TB patients and had lower CRP levels. Due to the fact that all of these test values were lower suggested that the disease progression and bacterial load would also be lesser in these patients, hence the amount of cells may be below the limit of detection for the phage assays. This was also observed in cattle where a higher proportion of positive results were produced in SICCT positive cattle with lesions opposed to SICCT positive cattle without (Swift *et al*., 2016a). These results are even more interesting when they are compared to the results produced from the seroconversion events. The Oneday phage assay produced positive results that were initially regarded as false positive but were later shown to be produced: 1) in a patient who developed an LTBI; 2) in two patients with LTBIs that reverted into active pulmonary disease. These results suggest that mycobacteraemia is higher in the very early stages of infection (for both LTBI and

activation of pulmonary disease) compared later stages of infection (phage negative active TB cases) and then increases again for more advanced stages of disease (phage positive active TB). It is important to remember however that the sample size was very low as the results were acquired by chance and not by design so there may be greater variability if the study was performed on a greater number of these patients and the study was designed around detecting these events.

The fact that the Oneday phage assay detected MTBC in the PBMC of blood before the QFT could indicates that the phage assay was a more sensitive assay than the IGRA, however it more likely showed that the phage assay can detect *M. tuberculosis* at an earlier phase in the infection process than the QFT assay. The IGRA relies on the patient producing an adaptive immune response to *M. tuberculosis* antigens and as such can only produce a positive result after the activation of host CD4+ and CD8+ T cells to MTB antigens. The initial phase of the MTB infection occurs within the alveolar macrophages, a cell of the innate immune system and so may show a delayed time for the activation of the adaptive immune system. It was possible that the phage assay detected alveolar macrophages containing MTB that had migrated into the blood and was extracted using the immune cell isolation techniques. Further testing needs to be performed, however the preliminary findings showed that the combined phage assays could be a useful additional method used in the diagnosis of TB, especially considering the impact of being able to detect the activation of an LTBI into active disease, months before clinical symptoms are produced, vastly limiting the potential degree of transmission. The comparison between the standard phage assay and the Oneday phage assay showed that the Oneday phage assay was a superior diagnostic test by detecting more of the active pulmonary cases compared

to the standard phage assay and also was able to detect MTBC in the blood of the seroconversion patients whereas the standard phage assay did not. The difference in the results was likely produced due to the extra processing steps and vessels required to perform the assay compared to the Oneday assay, which could have resulted in the loss of the mycobacteria if the bacterial load was low.

The HetaSep® extraction method was showed to produce comparable results to the Ficoll® extraction method, except that HetaSep® was determined to require fresher, less degraded blood compared to Ficoll® to extract the PBMCs. The results showed that HetaSep® would make a suitable replacement for Ficoll® so long as the study design takes into account that the blood would need to be performed within 48 h of collection to produce the best results, which was shown in the HetaSep® stability trial (see Appendix 11). The main benefit of the HetaSep® extraction method is that it can also be used in low-technology laboratories, which makes it transferable to field laboratories in places like Africa, where TB is epidemic throughout the population and testing and diagnosis is difficult.

The follow-on human study was unfortunately halted due to COVID-SARS 2 and had not restarted before the completion of this thesis was due and the data could not be released due to the blind nature of the study and the expectations of restarting the study when the restrictions allow. The results gained from the study though showed that many of the samples contained PCR inhibitors even after the DNA purification step, this means that more rigorous purification procedures need to be performed to reduce the concentration of DNA polymerase inhibitors co-extracted with the DNA.

In a blood sample, infecting mycobacteria do not circulate within the plasma. They are obligatory intracellular pathogens and infect cells of the immune system (Ganbat *et al*., 2016, Swift *et al*., 2013). This means that they disseminate throughout the body within cells, potentially decreasing what is available for molecular detection, as the bacteria must be extracted from their host cells before identification can take place. It was discovered that the MP which was believed to lyse the eukaryotic cells by osmotic pressure on reconstitution did not cause the lysis of the cells even after an overnight incubation in the media. By adding a water lysis step and incubating with sterile distilled water we lysed the cells (see Appendix 11). The quantity of DNA produced from the phage assay was also increased compared to parallel samples produced without a water lysis step. It is possible though that the water lysis step was not required as positive results had been produced with previous iterations of the phage assay that relied on the MP lysing the PBMC. It has been shown that phage D29 is able to cross the membrane of macrophages and are able to cause the infection and lysis of intracellular MTBC (Jończyk-Matysiak *et al*., 2017, Xiong *et al*., 2014). This meant that even though the mycobacteria were still internalized inside viable PBMCs in the MP, that the phage used could still find and lyse the intracellular bacteria and release the DNA. The subsequent steps in the protocol then likely released the mycobacterial DNA from the PBMC through mechanical action (centrifugation through the Actiphage® filter).

A bespoke blood culture method was also trialed during the follow-on human study due to the data produced from the pilot study that showed detectable levels of *M. tuberculosis* in the blood. To try and show that the *M. tuberculosis* detected circulating within the blood was culturable a novel method was trialed that

incorporated the RBC fraction and the plasma of the blood into the agar. This was designed to create a reduced oxygen environment at the agar level and to mimic the environment the cells were extracted from to reduce any stress response they may produce going from an intracellular environment within a host to being cultured extracellularly on agar. The method required further optimization due to a small amount of bacteria cultured from the blood samples. Unfortunately, due to the pandemic and lockdown, the colonies were lost, and a definitive identification of bacteria cultured from the blood couldn't be performed. For detailed explanation of the method see Appendix 12.

5.6. CONCLUSION

In this chapter we successfully identified the haematogenous dissemination of viable MTB in the PBMC faction of human blood from patients with confirmed active pulmonary TB, by using phase as a lysis agent and coupling with an *IS*6110 PCR assay to detect the released DNA (Oneday phase assay). Further to this, using the same methods we also identified by chance, the circulation of MTB in the PBMCs of 1) a patient developing a LTBI before the QFT IGRA produced a positive result and 2) two patients that reverted from an LTBI to active pulmonary disease months before clinical symptoms manifested. Several cases of active pulmonary disease with a lower disease progression than the phage positive cases were not detected by the phage assays. A follow-on study was designed in order to improve the sensitivity of the Oneday phage assay in an attempt to detect the phage negative active pulmonary TB cases, however the study was halted due to the national lockdown implemented because of the COVID-19 pandemic.

CHAPTER 6

6. D29 IS THE SKELETON KEY FOR UNLOCKING BLOOD FOR RAPID MYCOBACTERIAL DIAGNOSTICS

6.1. INTRODUCTION

Mycobacteria are a genus of bacteria that have clinical relevance in many sectors and cause disease in a wide range of species, however detection of all these organisms comes with inherent problems. The detection of the organisms typically requires the bacteria to be cultured and slow growing clinical strains of mycobacteria can take several months to form visible colonies which results in long diagnosis times before appropriate measures can be taken to combat the infection. There have been many other detection methods that have been developed to avoid the need to culture mycobacteria and reduce the detection time, such as immunological tests. The limitation in this case is that such tests are susceptible to cross reactions with non-target organisms because of the similarity displayed between the different species of mycobacteria which may lead to false-positive results (Teixeira *et al*., 2007). The immune response is also species-specific, which may lead to false-negative results when tests developed for one host species are used to analyze samples from another host species (Thomas *et al.*, 2021). Molecular assays have also been developed, such as PCR assays that amplify signature sequences from the genomic DNA of the mycobacteria to identify the presence of the organism within the samples. There are two problems that arise with molecular assays, which are that they often have a relatively high limit of detection due to the resilient nature of the mycobacterial cell wall which resists lysis, and that standard PCR assays only identify the presence of DNA, and so it cannot distinguish between viable and dead organisms.

By using the phage lysis method coupled with diagnostic PCR assays this research group has already shown it is possible to circumvent these common issues and

produced a rapid method for the detection of mycobacteria from blood (Swift et al., 2013, Swift et al., 2016a, Swift et al., 2020), and have also showed that this test can be applied to different host species (Molenaar *et al.*, 2020, Verma *et al.*, 2020). In this chapter I explored the diagnostic power of phage D29 by using the phage detection methods to detect mycobacteria from blood and how these methods could aid our understanding of the breadth of the dissemination of mycobacteria throughout different sectors in a One Health approach. A secondary aim was to identify novel applications where phage diagnostics could be used as a replacement method or ancillary testing to improve the detection rate of clinically relevant mycobacteria.

6.2. DETECTION OF UNKNOWN MYCOBACTERIAL PATHOGENS IN BISON BLOOD

6.2.1. **Introduction**

As mentioned above, mycobacteria such as MTBC (TB) and MAP (Johne's disease), cause infections that affects many different species. Transmission of these bacteria can occur both through intra-species transmission (Palmer, 2013, Judge *et al*., 2006) and also inter-species transmission (Mbugi *et al*., 2012, Stevenson *et al*., 2009). The inter-species transmission of TB has also been documented to occur in both directions between humans and animals (Paudel and Sreevatsan, 2020). When attempting to control these diseases and prevent their potential spread, a One Health approach is important when developing specific and rapid diagnosis methods so that is the appropriate measures for the infected host (such as treatment or culling) is taken. One of the main problems with diagnosing mycobacterial infections is that, currently, the diagnosis of MTBC in animals is standardly performed with the

combination of the SICCT, which is known to have high specificity but low sensitivity, and an ancillary IGRA which is characterized by high sensitivity but low specificity (Bezos *et al*., 2014). A positive response to these tests indicates the animal tested has been exposed to a member of the MTBC, but cross-reactions are possible.

One of the main advantages of the phage assay when using the phage D29 as the lysis agent is the capacity to adapt the test to allow the detection of all members of the *Mycobacterium* genus that are susceptible to phage infection. This is reflected by the fact that the production of plaques in the standard phage assay only shows that viable mycobacterial cells were present in the sample and that the type of *Mycobacterium* detected is only determined by the diagnostic PCR assays that are then performed. This means that infections caused by unknown organisms can be identified providing the correct PCR assay is performed.

The SICCT consists of two independent comparative intradermal injections of purified protein derivatives of *M. bovis* (PPD-B) and *M. avium* (PPD-A). The immune response to these antigens (which resolves in formation of a detectable lump) are then measured and a reactor is defined as one where the PPD-B response is greater than the PPD-A response, however different limits are set depending on the interpretation criterion used. A standard interpretation is applied for Official Tuberculosis Free (OTF) and Suspended herds (OTFS). A standard interpretation for a reactor is defined by the PPD-B response measuring greater than 4 mm compared to the PPD-A response (Goodchild *et al*., 2015). A severe interpretation is applied when *M. bovis* has been cultured from an SICCT-positive reactor using the standard interpretation criteria, or when visible legions have been identified in cattle at

slaughter. For severe interpretations, an animal is classified as a reactor if it has a negative PPD-A reaction and a positive PPD-B reaction of over 1 mm. If the PPD-A response is positive, then the animal is classified as a reactor if the PPD-B response is over 2 mm larger than the PPD-A response (Coad *et al*., 2010).

In respect of this, blood samples were received from a local bison herd (*Bison bison)* that had produced a positive response to the single intradermal comparative cervical tuberculin (SICCT) test designed for the detection of *M. bovis* in cattle (O'Hagan *et al*., 2019). For this bison herd, seven produced an unexpected positive response to the skin test since there was no reports of bovine TB infection on the farm, the farmer had put in place very high levels of biosecurity to control infection and the farm was not located in a bovine TB high risk area. Therefore, although the response was positive, there was some doubt whether the results using a test developed for bovine animals was giving a specific result in bison, which are not the same species.

As discussed above, the SICCT has been reported by multiple studies to have a specificity of over 99 % (Goodchild *et al.*, 2015). Regardless of this, cattle infected with NTM have been shown to produce a cross-reaction with the PPD-B antigens (Barry *et al*., 2011). Several publications reported a reduction in the sensitivity of the SICCT test when cattle were infected with MAP (Broughan *et al*., 2016), and false positive reactions being produced in MAP-infected cattle (Barry *et al.*, 2011). The interferon-γ (IFN-γ) ancillary assay was also susceptible to a reduction in sensitivity when exposed to MAP (Álvarez *et al*., 2009). The IFN-γ assay has been shown to be more heavily influenced by a MAP infection and produced a higher rate of false reactors than the SICCT (Aranaz *et al*., 2006).

The aim of this investigation was to identify the infective microbial agent that elicited the unusual response from the bison to the SICCT test. The Oneday phage assay has already been used previously to detect both MTBC and MAP in cattle, here we have explored the Oneday phage assay as a tool to identify an unknown mycobacterial agent from the blood of an infected animal. Since MAP is known to cause false positive results for the SICCT in cattle, blood samples were taken from these animals primarily for screening for the presence of MAP using the Oneday phage test. To be complaint with the Veterinary Surgeons Act Testing and Department of Environment, Food and Rural Affairs (DEFRA) rules covering restrictions on testing animal blood samples for bovine TB, tests to detect other types of mycobacteria were not carried out until after the animals had been sent to slaughter.

6.2.2. **Results**

6.2.3. **Extraction of immune cells from bison blood**

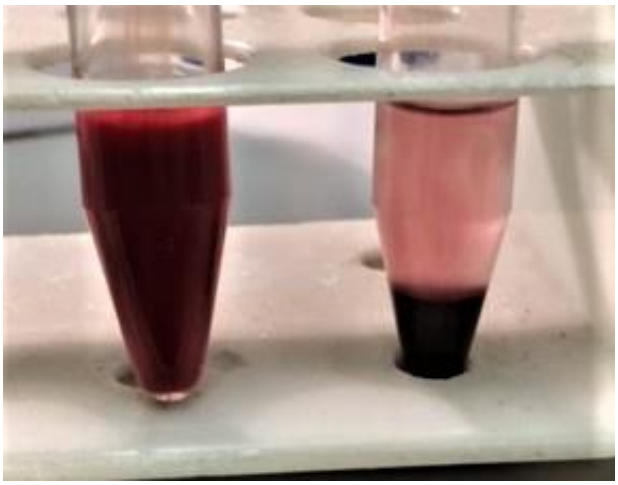
To diagnose the infecting organism, first a qualified veterinarian extracted whole blood from the bison into Na-Heparin blood containers (SARSTEADT; UK), which was subsequently brought to the laboratory by the farmer and arrived on the same day as collection. Blood from different species contain different concentrations of components which can change the way the blood reacts to certain methods and reagents. Some examples of properties that can vary are: viscosity, red blood cell and white blood cell count (Windberger *et al*., 2003, Mauricio *et al*., 2017) and as such, extraction methods designed for human blood may not be applicable to other animals without optimization. As Bison had not been tested within the laboratory before, isolation of the PBMCs from bison blood was attempted using the two PBMC

extraction methods that had previously been shown to be effective when testing bovine and human blood samples. The first method used for PBMC extraction was Ficoll® Paque-Plus, which is a density gradient medium, used in conjunction with a Leucosep™ Tube, a specialized blood separation tube (section 2.3.1.1.). The second extraction method tested was the HetaSep® extraction method, which is an erythrocyte aggregator (section 2.3.1.3.).

The protocol used to isolate PBMCs using HetaSep® was based on information provided by the manufacturer who had developed protocols to allow their product to be used for the isolation of white blood cells from experimental mice (Elena Blanc, STEMCELL Technologies UK Ltd, personal communication.). First the bison blood was diluted 1:1 with PBS and then HetaSep® added at the ratio of 1 to 1.3 blood. After thorough mixing by using gentle inversion of the tubes, the samples were then incubated at 37 °C for up to 60 min to allow the aggregation while visually monitoring the formation of a clear upper phase (see section 2.3.1.3.). Unfortunately, even after the full 60 min incubation time with HetaSep®, the bison blood exhibited minimal separation of the plasma and red blood cells. The samples were left to incubate further over night at room temperature, however this did not improve the separation, showing that HetaSep® was not suitable for use with bison blood using gravity sedimentation (Fig. 6.1). To identify whether the blood could be separate by centrifugation with HetaSep®, the two samples were pooled together and transferred to a 15 ml conical bottomed tube and centrifuged for 2 minutes as per the manufacturer's guidelines for 48 h old blood, although a setting of 100 x*g* was used instead of the recommended 90 x*g* due to limitations of the available centrifuge. After centrifugation, the expected separation of the blood was observed

(Fig. 6.1). This showed that it was possible to aggregate the erythrocytes from bison blood with HetaSep® by centrifugation, however the centrifugation is a rapid separation method and the quicker that the aggregation develops, the more white blood cells get captured within the RBC rouleaux formations, potentially reducing the sensitivity of downstream assays. Due to this reason HetaSep® blood was not used for further testing with bison.

Figure 6.1 – Comparison of methods to isolate while blood cells from bison blood



The sample on the left represents a bison blood diluted 1:1 with PBS. HetaSep® was then added to the sample at a ratio of 1:1.3 blood. The samples were incubated at 37 °C for 60 min then incubated overnight at room temperature. The sample on the right was centrifuged at 100 xg for 2 min after the overnight incubation with HetaSep® (section 2.3.1.3.).

6.2.4. **Screening of samples for mycobacterial DNA**

After the extraction of the PBMCs with the Ficoll® extraction method, the Oneday assay (section 2.2.3) was performed to extract the DNA from any mycobacteria within the PBMCs. The DNA was then analyzed by using a series of PCRs. Since the bison were still alive at this point of the investigation, it was not possible to perform tests for MTBC. So; the organisms that were tested for was firstly MAP (Appendix 6, section 9.6.1.) using the P90/91 primers (Sanderson *et al*., 1992) which were negative, and then 16s rDNA was detected (Appendix 6, section 9.6.2.) using the primers described by Tevere *et al*. (1996). A contamination check was then performed due to a positive negative process control in the 16s rDNA PCR, however the results showed the positive results gained in this PCR were true Appendix 6, section 9.6.3.). After this point the bison were culled so we performed PCR analysis for the detection of MTBC using the MTC F/R primers described by Eisenach *et al*. (1990) and then a commercial real-time quantitative PCR (Ingenetix; Austria) was performed also for the identification of MTBC (Appendix 6, section 9.6.4.). All the tests performed were negative except for the 16s rDNA PCR which showed that each of the bison were carrying mycobacteria around in the blood (for detailed information about the tests and figures see Appendix 6).

6.2.4.1. Analysis of DNA samples from bison blood for presence of the IS1311 element – detection of MAC organisms

The results of the identification at this stage showed that the bison blood did not contain the *IS*900 sequence for MAP or the *IS*6110 sequence for MTBC. The second antigen used in the SICCT is PPD-A derived from *M. avium*, and all these animals had

given a positive reaction to the PPD-A antigen as well as to the PPD-B antigen. Based on this, it was decided to test the DNA samples for the presence of signature sequences specific for the *M. avium* complex of bacteria using primers targeting the *IS*1311 element. The *IS*1311 element is not restricted to *M. avium* alone but is found to be present in all of the organisms of the MAC group of bacteria. *M. avium* itself contains two copies of the *IS*1311 element (Johansen *et al.*, 2005) and MAP contains between 7 – 10 copies (Whittington *et al*., 1998). However, the PCR products generated by amplifying the *IS*1311 sequences from these different organisms can be discriminated from each other by performing a restriction enzyme analysis (REA), which produces different sized patterns of restriction products depending on the species or strain the *IS*1311 element originates from (Marsh *et al*., 1999). Another practical reason why this PCR assay was selected, was that the quantity of DNA remaining was nearly depleted, and the PCR assay targets a group of different mycobacterial species, providing a better chance of finding any unknown mycobacterial DNA present in these samples. The PCR assays were performed as described (section 2.4.2.6.).

The PCR results for the test samples were again compared against the MP process negative control and DNA from *M. avium* and MAP K10 (20 ng each), extracted using the boiled lysis method (section 2.4.1.1.) were used as positive controls as both should give a positive result using the M56 and M119 primers.

In this instance, the *IS*1311 PCR assay did detect DNA from MAC organisms. The MAP K10 DNA (Fig 9.20, lane 1) and *M. avium* DNA (Fig. 9.20, lane 2) extracted with the boiled lysis method both amplified a 608 bp PCR product corresponding to the

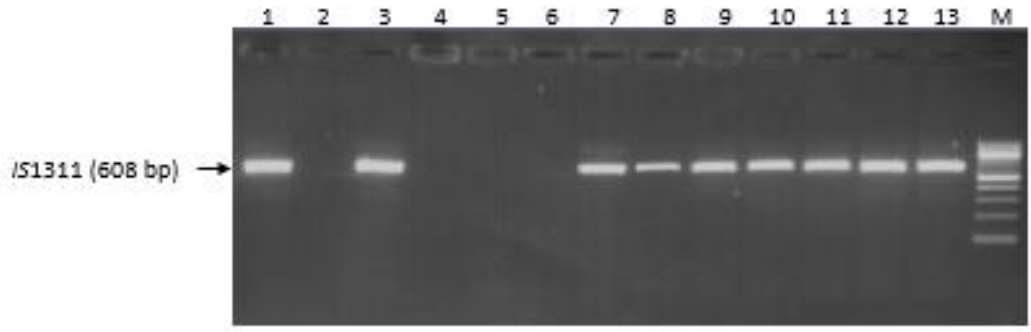
*IS*1311 element. The no template (water) control (Fig. 9.20, lane 4) and MP process control (Fig. 9.20, lane 6) were both negative, as expected, and did not amplify any PCR products. The results for bison 1 – 5 (Fig. 9.20, lanes 14 – 10 respectively) also showed the amplification of a 608 bp band corresponding to the *IS*1311 element. It appeared that bison 6 and 7 (Fig. 9.20, lanes 9 and 8 respectively) were *IS*1311-PCR negative. The results showed that five of the seven bison blood samples contained detectable levels of DNA from organisms that contained the *IS*1311 element (see Appendix 6, section 9.6.5. - Fig 9.20).

The quantity of the PCR products produced however, were too low to directly perform a successful REA. Therefore, the PCR products from each of the PCR assays, including the control reactions, were used as DNA templates for another PCR assay using the same PCR primers to re-amplify the PCR products and increase the final quantity of DNA to a suitable level for REA. The re-amplification PCR assay was performed using 5 µl of the PCR products produced in the initial *IS*1311 assay as the DNA template. The PCR assays were prepared as described above, except only twelve cycles were used due to the high levels of template DNA added to the reactions. After the amplification PCR assay, 5 µl of the PCR products were analyzed by gel electrophoresis to confirm that sufficient DNA had been produced for the REA.

The results of the re-amplification were successful, but in this, all the bison DNA samples resulted in amplification of a PCR product of 608 bp corresponding in size to that expected for the *IS*1311 element (Fig. 6.2, lanes 13 – 7 respectively). Since all the negative control samples were negative, this suggested that for the original bison 6 and 7 PCR sample did contain low levels of the *IS*1311 amplicon, but that the

amount of DNA was below the threshold to allow detection on an agarose gel stained with ethidium bromide.

Figure 6.2 – Re-amplification of the IS1311 PCR products from bison blood



Lane M, 100 bp DNA Ladder (NEB; UK). Lane 1, MAP K10 DNA (20 ng); Lane 3, *M. avium* DNA (20 ng); Lane 5, MP (negative) process control; Lane 7, bison 7; Lane 8, bison 6; Lane 9, bison 5; Lane 10, bison 4; Lane 11, bison 3; Lane 12, bison 2; Lane 13, bison 1. Lanes 2, 4 and 6 contained no PCR products. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.6.). The expected product size for the M56/119 primers *(IS*1311*)* was 608 bp (indicated by arrows on LHS of image).

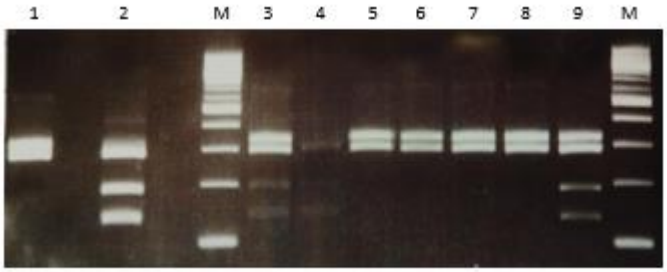
To prepare the PCR assays, 5 µl of the PCR products were used as template with the DNA polymerase and buffer Onetaq®2x Master Mix with GC Buffer and 0.5 µM of the primers M56 and M119. The PCR parameters used were: initial denaturation of 94 °C for 2 min; 12 cycles of: 94 °C for 30 s, 62 °C for 30 s, 68 °C for 1 min; followed by a final extension of 68 °C for 5 min.

The next step in the PCR-REA was to digest the remaining PCR products with the restriction enzymes *Hinf*I and *Mse*I to allow identification of which members of the MAC had been detected. The discriminatory power of the REA comes from polymorphisms within the *IS*1311 element. A conserved *Hinf*I sequence is found in all MAC members at site 285 in the *IS*1311 element. *M. avium* is identified by a unique *Mse*I sequence at site 419 bp in the *IS*1311 element that is not found within MAP strains. The PCR-REA is also able to distinguish between different strains of MAP (Table 6.1); S strains (sheep origin) contains the *Hinf*I sequence at 285 bp only, C-strains (cattle origin) contain an extra *Hinf*I sequence due to a C/T polymorphism at 223 bp in a some but not all of their *IS*1311 elements. Another strain has also been identified, the B-strain (bison origin) which contains a conserved C/T polymorphism at 223 bp in all of the *IS*1311 elements within the strains genome (Whittington *et al*., 2001).

The restriction double digest was then performed (section 2.4.2.6.). The samples were then heat inactivated at 80 °C for 20 min as recommended to prevent smearing of the DNA through the gel during the electrophoresis step. For the electrophoresis, 10 mM LAB (lithium acetate borate) buffer and 3% (w/w) LAB agarose gel were used to allow separation of the 323 bp and 285 bp PCR products with the gel being run at 250 V for 20 min. LAB buffer is designed to produce a lower electrical resistance compared to TAE when gels are run at the same voltage, which means LAB gels have lower resistance and less heat is produced compared to TAE (100 V for example will produce approximately 20 mA in 10 mM LAB buffer while TAE would produce approximately 100 mA).

The results of the REA were compared to the PCR products produced from the *M. avium* and MAP K10 PCR assays which should give a positive result and the specific band patterns. The MAP K10 REA (Fig. 6.3, lane 1) produced the expected two fragments of sizes: 323 bp and 285 bp for S-type MAP *IS*1311. The *M. avium* REA (Fig. 6.3, lane 2) produced the expected three fragments of sizes: 285 bp, 189 bp and 134 bp expected for *M. avium IS*1311. The results for the bison samples, however, were surprising. Bison samples 2 through 5 (Fig. 6.3, lanes 8 – 5) produced two fragments of sizes: 323 bp and 285 bp corresponding to the band pattern associated with S strain MAP. Bison sample 6 (Fig. 6.3, lane 4) produced three fragments of sizes: 285 bp, 189 bp and 134 bp, corresponding to the band pattern associated with *M. avium.* However, Bison samples 1 (Fig. 6.3, lane 9) and 7 (Fig. 6.3, lane 3) produced four fragments of sizes: 323 bp, 285 bp, 189 bp and 134 bp. The 323 bp and 285 bp band were of a significantly increased intensity compared to the 189 bp and 134 bp bands. The two larger fragment sizes which showed increased levels of amplification corresponded to the band patterns associated with S strain MAP and the two fragments of lower intensity corresponded to *M. avium.* This suggested that these two bison contained a dual infection with two members of the MAC. Since these results were gained by reamplification of low levels of initial PCR product, it is possible that some amplification bias occurred during the re-amplification stage and that all these animals originally contained DNA from both organisms. However, without further DNA samples to test, this question could not be resolved. As it stands, the results may explain the unexpected SICCT response produced by these animals. This is supported by the fact that post-mortem culture of tissue samples from these animals was not able to detect the presence of any MTBC organisms.

Figure 6.3 – REA of *IS*1311 PCR products from bison blood



Lane M; 100 bp DNA Ladder (NEB; UK). Lane 1, MAP K10 DNA (20 ng); Lane 2, *M. avium* DNA (20 ng); Lane 3, bison 7; Lane 4, bison 6; Lane 5, bison 5; Lane 6, bison 4; Lane 7, bison 3; Lane 8, bison 2; Lane 9, bison 1. The restriction enzyme products were analysed by electrophoresis through a 10 mM LAB 3 % (w/w) agarose gel at 250 V for 20 minutes. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.6.). The expected product size for the MAP profile was 323 bp and 285 bp (indicated by arrows on LHS of image) and the expected products for the *M. avium* profile was 285 bp, 189 bp and 134 bp (indicated by arrows in the RHS of image).

To perform the restriction digest, the PCR products were purified to remove any restriction enzymes inhibitors, using the Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB; UK) (Section 2.5.2.5). As before the volume of the sample was increased to 20 µl by addition of 5 µl of sterile TE buffer as suggested by the manufacturer. The purified DNA was eluted in a final volume of 16 µl of sterile molecular grade water and then the double digest was prepared by adding 2 µl of NEBuffer 2.1 to the purified DNA samples and 10 u of each restriction enzyme *Hinf*I and *Mse*I (NEB; UK). The samples were then incubated at 37 °C for 1 h and then the enzymes were heat inactivated at 80 °C for 20 min as recommended to prevent smearing of the DNA through the gel during the electrophoresis step.

6.2.5. **Investigation into the P90 and P91 primers**

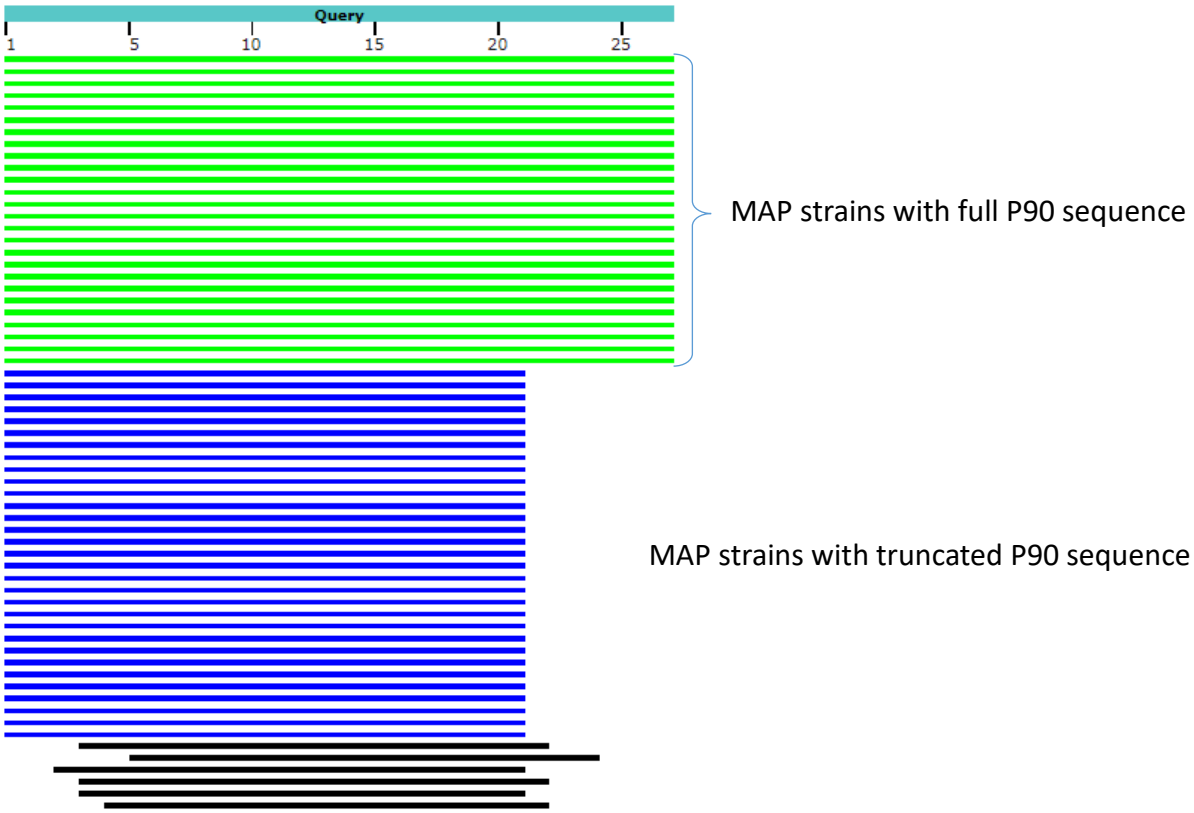
Although these results were very interesting, a contradiction remained as the original PCR assay used to detect the presence of *IS*900 (which is known to be carried by all strains of MAP isolated to date) should have produced a positive PCR assay for six out of the seven bison because they were shown to contain MAP. The false negative results produced by the *IS*900 PCR assay using the P90/91 primers were unexplained for such well described and well used primers (Sanderson *et al*., 1992), especially as MAP strains are known to contain between 15 and 20 copies of the *IS*900 element (Cunha *et al*., 2020) and therefore should be detectable even at low cell numbers. The results lead to two hypotheses: either there was a problem with primer specificity, or the strains of MAP did not contain any *IS*900 elements. To investigate the cause of the false negative result, the primer specificity was first assessed.

6.2.5.1. Homology search using the P90 and P91 primers

To determine if there were any discrepancies between the primers and MAP strains deposited after the designing of the primers, the P90 primer sequence was tested for its homology to the DNA sequences of organisms deposited in the NCBI database. The P90 primer sequence (5'- GAAGGGTGTTCGGGGCCGTCGCTTAGG-3') was used for a nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) by entering the sequence into the Query Sequence box using the following parameters: Database – Standard databases (nr etc.), Nucleotide collection (nr/nt); Organism – no selection; Exclude – no selection; Limit to – no selection; Entrez Query – no selection; Program Selection – Highly similar sequences (megablast). The results of this search revealed

a common mismatch in the homologous sequences identified (Fig. 6.4). The green lines (alignment score: 50 – 80) shown in this indicate full homology of the P90 primer sequence with approximately half of the IS900 elements of the MAP strains deposited in the NCBI database (query cover – 100 %; E-Value – $1e^{-4}$). However, the results for the matches to the remaining MAP strains in the database produced a query cover of only 77 % and an E-Value of 0.4. (Fig. 6.4; blue lines, alignment score: 40 – 50). The region of homology in these lower matching query results corresponded to just the first 21 bp of the P90 primer, although this region showed 100 % homology to the *IS*900 sequences. This meant that there was no homology in these *IS*900 sequences to the last 6 base pairs of the 3' end of the P90 primer. Since good primer pair formation at the 3' end of the primer is essential for initiating DNA synthesis (Stadhouders *et al*., 2010), it is predicted that if these sequences were present in a MAP genome, they would show a decreased sensitivity in PCR assays using the P90 primer. Since it is known that the bison blood samples only contained very low numbers of mycobacterial cells, if the strain present in the bison samples contained this mismatch, this would explain the failure of the PCR assay to detect IS900 sequences in this DNA.

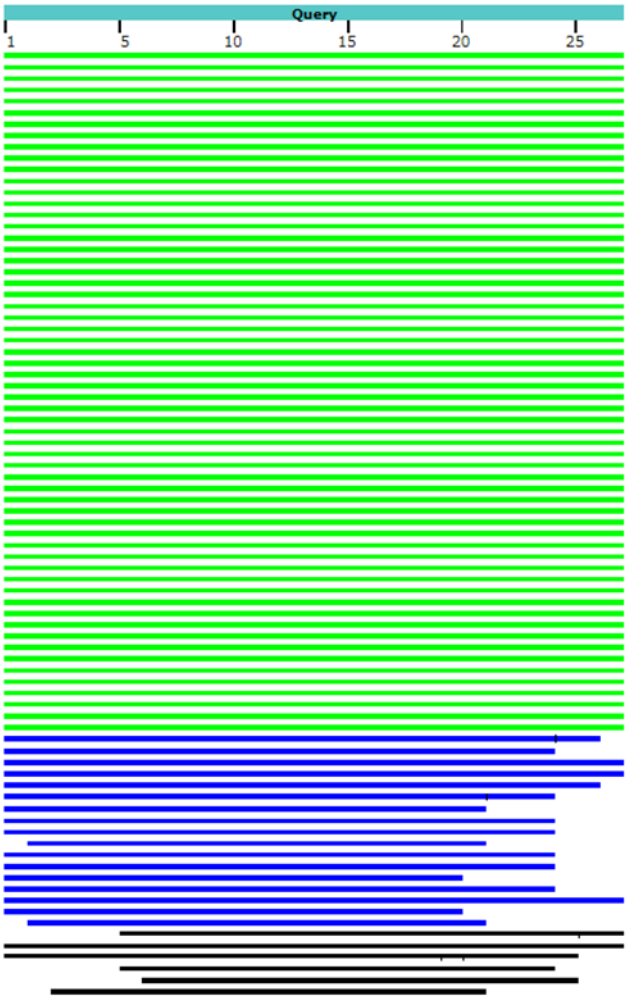
Figure 6.4 – Results of homology search using the P90 primer sequence



Nucleotide to nucleotide BLAST search of the P90 primer compared to the nucleotide sequences deposited in the NCBI database. Image represents the homology of the query sequence compared to the database sequence. Homology is represented by the presence of a line at the query site. The green lines represent an alignment score of 50 – 80 (query cover – 100 %, E-Value – $1e^{-4}$). The blue lines represent an alignment score of 40 – 50. The black lines represent an alignment score of less than 40.

The homology of the P91 primer sequence (5'-GGCGTTGAGGTCGATCGCCCACGTGAC-3') was assessed using the same procedure. In this case the P91 primer showed 100 % homology to all the *IS*900 elements present in the MAP strains held in the NCBI database (Fig. 6.8; query cover – 100 %, E-Value – $1e^{-4}$). Although some matches with lower homology scores were found, these were matches identified to different genome sequences of mycobacterial species (Fig. 6.5; blue lines) or similar organisms of the same phylum such as *Streptomyces spp* (Fig. 6.5; black lines). However, the BLAST search showed no potential issues with the P91 primer specificity when trying to amplify the *IS*900 element.

Figure 6.5 – Results of homology search using the P91 primer sequence



Nucleotide to nucleotide BLAST search of the P91 primer compared to the nucleotide sequences deposited in the NCBI database. Image represents the homology of the query sequence compared to the database sequence. Homology is represented by the presence of a line at the query site. The green lines represent an alignment score of 50 – 80 (query cover – 100 %, E-Value – $1e^{-4}$). The blue lines represent an alignment score of 40 – 50. The black lines represent an alignment score of less than 40.

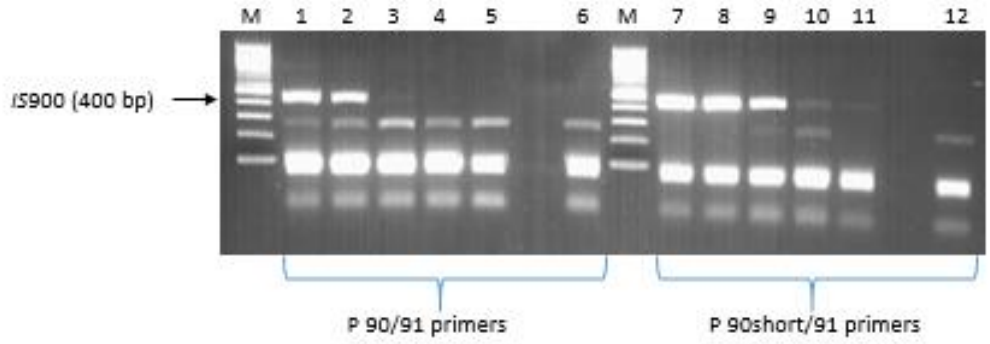
6.2.5.2. *Shortening on the P90 primer and its effect on sensitivity and specificity*

Based on the results of the homology search of the P90 primer, a shortened P90 primer was produced that did not contain the first 6 bases of the 3' end of the P90 primer (5'-CTTAGG- 3'). The primer was designated P90short and contained the sequence GAAGGGTGTTCGGGGCCGTCG (predicted T_m = 72 °C). A nucleotide-to-nucleotide BLAST search was again performed following the parameters described in section 6.3.6.1 to check that this shortened primer would not now bind to sequences in the NCBI database that had not previously been identified. The results showed 100 percent homology of the shortened primer compared to all the *IS*900 elements of MAP deposited in the NCBI database and no additional close matches to other primer sequences, suggesting that the specificity of the PCR assay would not change.

The next step was to assess the specificity and sensitivity of the P90short primer compared to the standard P90 primer. First, the sensitivity was assessed using MAP DNA extracted using the boiled lysis method (section 2.4.1.1.) and quantified as having a concentration of 46.2 ng μl^{-1} (section 2.5.2.2.). A ten-fold dilution series of was then prepared using fresh sterile molecular grade water, until an approximate DNA concentration of 4.62 $x10^{-3}$ ng μl^{-1} was achieved. PCR assays were then prepared using 1 µl of each of the dilutions of DNA. The P90/91 primers and the P90short/91 primers were used in separate PCR assays at a concentration of 0.5 µM each with the One*Taq*® Hot Start DNA polymerase and GC buffer (NEB; UK) (section 2.4.2.5.).

For both the P90 and P90short primers, amplification of the expected 400 bp PCR product was seen, indicating that shortening this primer of the *IS*900 element did not affect its ability to amplify the target sequence. In addition, amplification of a non-specific PCR product of just less than 200 bp was less prominent when using the P90short primer, indicating that this shorter primer was more specific. When comparing the sensitivity of the two different primer pairs, the endpoint detection event using the P90short primer was 100-fold lower than when the P90 primers were used (LOD = 4.62×10^{-3} ng μl^{-1}; Fig 6.6, lane 11).

Figure 6.6 – Assay to compare sensitivity of PCR assays using the P 90/91 and the P 90short/91 primers.



Lane M; 100 bp DNA Ladder (NEB; UK). The primer P90 was used in lanes 1 – 6. The primer P90short was used in Lanes 7 – 12. The concentration of MAP K10 DNA used were: Lanes 1 and 7, 46.2 ng μl^{-1}; Lanes 2 and 8, 4.62 ng μl^{-1}; Lanes 3 and 9, $4.62x10^{-1}$ ng μl^{-1}; Lanes 4 and 10, $4.62x10^{-2}$ ng μl^{-1}; Lanes 5 and 11, $4.62x10^{-3}$ ng μl^{-1}. The PCR products were analysed by electrophoresis through a 2 % (w/w) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.5.). The expected product size for the *IS*1900 element was 400 bp (indicated by arrows on LHS of image).

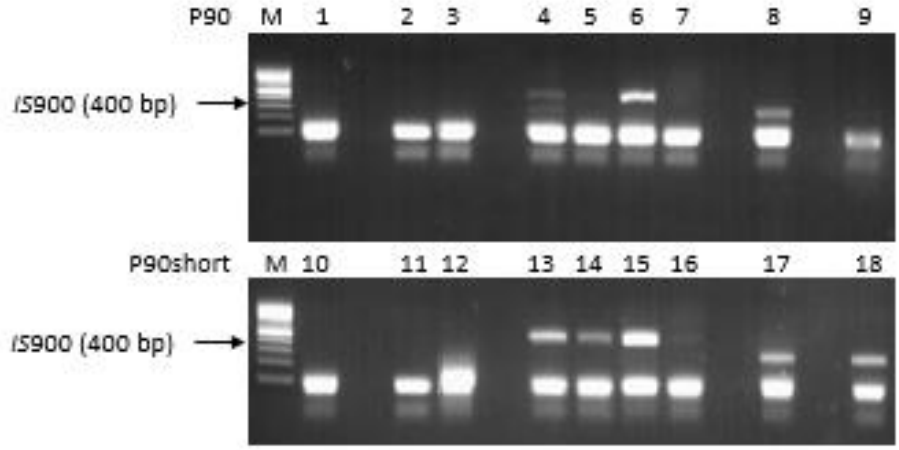
To prepare the PCR reaction mixture: 10 µl of One*Taq*® Quick-Load® 2x Master Mix (QIAGEN; UK); 0.5 µM of each primer P90 and P91 or p90short and P91 (Table 2.2) was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 94 °C for 2 min. This was followed by 37 cycles of: 94 °C for 30 s, 62 °C for 15 s, 68 °C for 45 s. This was followed by a final extension step of 68 °C for 5 min.

The next step was to further compare the specificity of the P90 primer with the P90short primers. To investigate this, four MAP strains were selected: B4, DVL, K10 and ATCC 19851, each of which should give a positive result when performing an *IS*900-specific PCR assay. In addition, DNA extracted from *M. avium*, *M. bovis, M. bovis* BCG and *E. coli* were also used, which should all give a negative *IS*900 PCR result. The DNA of all the organisms tested was extracted using the boiled lysis method (section 2.4.1.1.). The no template (water) control contained sterile molecular grade water instead of a DNA template. To prepare the PCR assays, One*Taq*® Hot Start DNA polymerase with GC buffer (NEB; UK) was again selected and 0.5 µM of each P90/91 primer or P90short/P91 with approximately 20 ng of template DNA (section 2.4.2.5.).

The results of the PCR assays containing the P90/91 primers showed there were no PCR products amplified in the no template (water) control (Fig. 6.7, lane 9) or when *M. avium* (Fig. 6.7, lane 1)*, M. bovis* (Fig. 6.7, lane 2) or *E. coli* (Fig. 6.7, lane 3) DNA was used as template. The *M. bovis*-BCG DNA (Fig. 6.7, lane 8) resulted in the amplification of a 230 bp PCR product which seems to correspond to amplification of the IAC segment when using these primers, indicating the reagents were contaminated with IAC DNA. The results of the MAP strains showed a 400 bp PCR product which corresponded to the amplification of the *IS*900 element was produced when the DNA from strains B4 (Fig. 6.7, lane 4) and K10 (Fig. 6.7, lane 6) was used as template. The MAP strains DVL (Fig. 6.7, lane 5) and ATCC 19851 (Fig. 6.7, lane 7) produced no PCR products and gave negative results. Unfortunately, the genome sequence of neither of these strains is available in public databases to determine which version of the IS900 sequence they possess.

The results gained were compared to those using the P90short/P91 primers. These again, showed there was no amplification in the negative controls when *M. avium* (Fig. 6.7, lane 10)*, M. bovis* (Fig. 6.7, lane 11), *E. coli* (Fig. 6.7, lane 12). However, on this occasion both the no template (water) control and *M. bovis*-BCG DNA produced amplification of the 230 bp PCR product which seems to correspond to amplification of the IAC segment when using these primers. The results for the DNA from MAP strains B4 (Fig. 6.7, lane 13), DVL (Fig. 6.7, lane 14) and K10 (Fig. 6.7, lane 15) produced a 400 bp PCR product which corresponded to the amplification of the *IS*900 element. This was also detected when DNA from the ATCC 19851 strain (Fig. 6.7, lane 16) was used as template, although the level of DNA amplification was much lower. These results showed that the P90short primer could produce a positive PCR detection event with more strains of MAP compared to the P90 primer. It also shows that despite their widespread use in the literature, the P90/91 primer pair are not a suitable pair of primers to use when detecting MAP from real world samples as the P90 primer can fail to anneal to the *IS*900 element of some MAP strains and produce a false negative result.

Figure 6.7 – Comparison of P90 and P90short primers using different DNA templates



Lane M; 100 bp DNA Ladder (NEB; UK). Lanes 1 and 10, *M. avium*; Lane 2 and 11, *M. bovis*; Lane 3 and 12, *E. coli*; Lanes 4 and 13, MAP B4; Lane 5 and 14, MAP DVL; Lane 6 and 15, MAP K10; Lane 7 and 16, MAP 19851; Lane 8 and 17, *M. bovis* BCG; Lane 9 and 18, no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/w) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.6.). The expected product size for the *IS*900 element was 400 bp (indicated by arrows on LHS of image). The top of the gel contained PCR assays performed with the P90 primer. The bottom of the gel contained PCR assays performed with the P90short primer.

To prepare the PCR reaction mixture: 10 µl of One*Taq*® Quick-Load® 2x Master Mix (QIAGEN; UK); 0.5 µM of each primer P90 and P91 or p90short and P91 (Table 2.2) was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 94 °C for 2 min. This was followed by 37 cycles of: 94 °C for 30 s, 62 °C for 15 s, 68 °C for 45 s. This was followed by a final extension step of 68 °C for 5 min.

6.3. *M. AVIUM* DETECTION IN SARCOIDOSIS PATIENT

6.3.1. Introduction

In Chapter 4, both formats of the phage assay were used to try and detect *Mycobacterium tuberculosis* in the blood of immunocompetent patients. During this study it was noted that several of the blood samples produced plaques using the standard phage assay, which then produced negative results when analyzed with the *IS*6110 PCR. These samples also gave a negative result when the Oneday assay was performed using primers to detect *IS*6110 as the end point PCR assay.

One reason that PCR-negative plaques can be produced in the standard phage assay is failure of the virucide to fully inactivate the exogenous phage. Therefore, a negative control sample is always prepared which contains just MP. In each case, when the plaque-positive, PCR-negative results were gained, the negative control sample produced no plaques, which showed that the Virucide had successfully inactivated the exogenous phage. This suggested that the plaques from the patient samples were due to the presence of other types of mycobacteria in the blood samples.

Therefore, an investigation was carried out to try and identify the bacterium present in the sample that gave rise to the plaques. Due to the nature of the testing performed in the human blood trial, only 5 µl of DNA remained to be tested. Since the members of the MTBC had already been excluded, it was decided to carry out the *IS*1311 PCR-REA, described in section 6.2.2.6 to identify members of the MAC. This was chosen for two reasons (a) because it detects both *M. avium* and MAP and (b) these two organisms have previously been detected in human blood samples

(Esteban *et al*., 1998, Kuenstner *et al*., 2020) and implemented in causing disease in humans. To avoid samples that may have resulted from low level break through from failure of the virucide further rule out the possibility of virucide failure, the samples that were selected for retesting with the *IS*1311 PCR-REA were chosen because they contained 20 or more plaques on either the Ficoll® Paque plus extraction (section 2.3.1.1.) or the HetaSep® extraction (Section 2.3.1.2.). In total, nine different patients that were confirmed to be MTBC negative were retested for MAC.

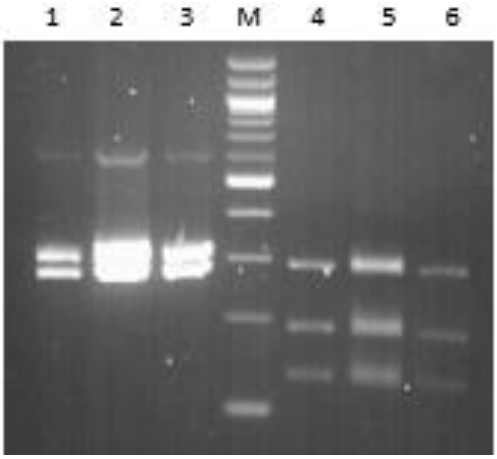
6.3.2. **Results**

The results for the positive and negative controls showed the expected results, and in addition, one of the nine patient samples tested also produced a 608 bp PCR product also corresponding to the amplification of the *IS*1311 element. As before, there was insufficient DNA in this first sample to perform REA, so the PCR products that were re-amplified as described in section 6.2.2.6. After purification of the PCR products, the REA was performed, and the restriction products analyzed by gel electrophoresis through a 10 mM LAB 3 percent (w/w) agarose gel (see section 6.2.2.6 for details of the method). When performing the electrophoresis, three separate quantities (1, 5 and 10 µl) of each human sample and the *M. avium* control sample were analyzed. The 10 µl sample was purposefully overloaded to identify more easily the 218 bp product from C-strain MAP if this was present, and the 5 µl and 1 µl sample were used to try and achieve good separation of the 323 bp and 285 bp products.

The results for the *M. avium IS*1311 REA (Fig. 6.8, lanes 4 – 6) was in line with the expected results. Three restriction products were produced: 285 bp, 189 bp and 134

bp, all of which corresponded to the restriction profile of the *M. avium IS*1311 element when digested with both *Hinf*I and *Mse*I restriction enzymes. The results for the REA of the *IS*1311 element extracted from the blood of the human patient produced two products, one of 323 bp and one of 285 bp (Fig. 6.8, lanes 1 – 3), both of which corresponded to the restriction profile of the *IS*1311 element found in S-strains of MAP. The overloaded sample did not identify the 218 bp digestion product confirming that the sample identified was an S-strain MAP. The results showed that the plaques produced in the phage assay from the blood of this patient were caused by a strain of MAP and not a member of the MTBC, even though this patient had presented to the respiratory clinic with symptoms of human TB and was IGRA positive. Interestingly, the other clinical diagnostic tests performed on this patient had identified them patient as being sarcoid, which is a term used to describe patients who present with symptoms that could be indicative of TB (i.e., formation of granulomas in the lungs and lymph nodes, difficulty breathing, night sweats, joint pain and fatigue) but no other clinical TB tests give a positive result. The results gained from this study suggest that in this case the patient may have been infected with a sheep strain of MAP. This result is in line with a small number of reports that have associated MAP infections with sarcoidosis in humans (Celler, 2018, el-Zaatari *et al*., 1996).

Figure 6.8 – REA of *IS*1311 PCR products from human sarcoid blood



The digested *IS*1311 element amplified from the human blood were Lanes 1 - 3. The digested *M. avium IS*1311 products were Lanes 4 – 6. Lanes 1 and 6 contained 2 µl digest. Lanes 2 and 5 contained 10 µl digest. Lanes 3 and 4 contained 5 µl digest. The restriction enzyme products were analysed by electrophoresis through a 10 mM LAB 3 % (w/v) agarose gel at 250 V for 20 minutes. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.6.). The expected product size for the *M. avium* profile was 285 bp, 189 bp and 134 bp (indicated by arrows in the RHS of image). Lane M; 100 bp DNA Ladder (NEB; UK). To perform the restriction digest, the PCR products were purified to remove any restriction enzymes inhibitors, using the Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB; UK) (Section 2.5.2.5.). As before the volume of the sample was increased to 20 µl by addition of 5 µl of sterile TE buffer as suggested by the manufacturer. The purified DNA was eluted in a final volume of 16 µl of sterile molecular grade water and then the double digest was prepared by adding 2 µl of NEBuffer 2.1 to the purified DNA samples and 10 u of each restriction enzyme *Hinf*I and *Mse*I (NEB; UK). The samples were then incubated at 37 °C for 1 h and then the enzymes were heat inactivated at 80 °C for 20 min as recommended to prevent smearing of the DNA through the gel during the electrophoresis step.

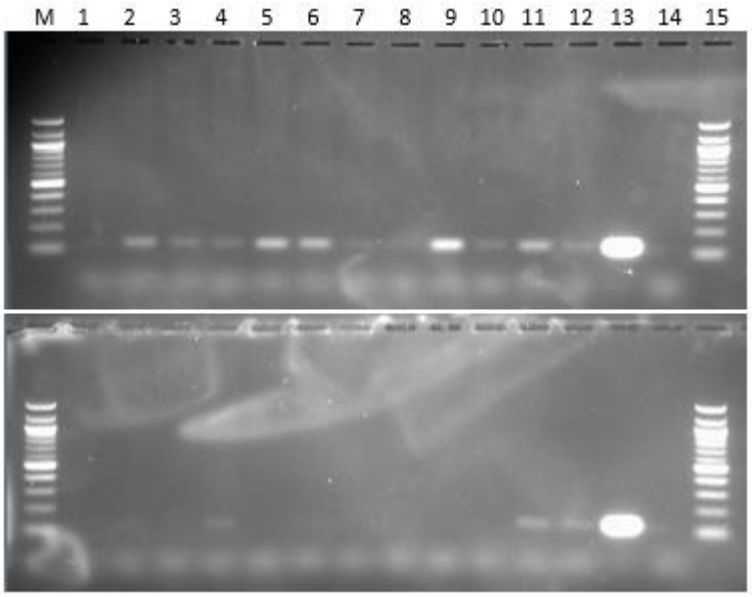
6.4. Identification of MTBC in Alpacas

A request was received from a veterinarian to see if the phage assays could be used to improve diagnostics for new world camelids, specifically farmed alpacas (*Vicugna pacos*) using blood samples from a small herd of alpacas with known MTBC infection. Again, in this study, the results gained using the standard phage assay were compared to those gained using the Oneday phage assay. A total of eight blood samples were delivered to the laboratory by overnight courier. PBMCs were prepared from 2 ml of alpaca blood using the Ficoll® extraction method (section 2.3.1.1.) and these were then stored overnight at room temperature after resuspension in 1.5 ml of MP. The following day, 500 µl of the PBMC suspension was transferred to a reaction vessel to perform the standard phage assay (section 2.2.2.). The remaining 1 ml was centrifuged at 13,000 xg for 3 min to pellet any mycobacteria in the sample, and the pellet was reconstituted with 100 µl MP before being transferred to an Oneday assay tube (section). After the standard phage assay, DNA extracted from plaques (section 2.4.1.3.) and DNA recovered from the Oneday phage assay, were tested using the *IS*6110 PCR assay (section 2.4.2.2.). In both cases, the PCR reactions were prepared with 5 µl of the purified DNA and HotStarTaq Plus Mastermix with 5 µM of MTCF/R primers in a total of 20 µl. The parameter used for the PCR assay were an initial denaturation of 95 °C for 5 min, followed by 30 cycles of: 95 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s, with a final extension of 95 °C for 7 min.

All the alpaca samples produced plaques on the standard phage assay with a range of 41 plaques to greater than 300 plaques which were out of the countable range. The plaques were shown to have originated from viable MTBC within the blood as

each PCR assay of plaque DNA produced a 123 bp PCR product corresponding to the amplification of the *IS*6110 element (Fig. 6.9). There was also complete agreement between duplicate samples tested in parallel. In contrast, only three samples produced a 123 bp corresponding to the amplification of the *IS*6110 element using the Oneday phage assay, and while two of these gave duplicate positive test results, the third sample only gave a positive test result with one of the two parallel test samples. This was not as expected as the Oneday assay has been shown to be more sensitive and produce more positive results than the standard phage assay (Swift *et al*., 2020). The results showed that the phage assays can be used to detect MTBC organisms in the blood of new world camelids, but also showed that differences in blood samples may adversely affect the Oneday phage assay due to factors that are not yet clear. Another issue when testing blood samples from exotic species is that the *IS*6110 PCR assay detects all members of the MTB complex. The MTBC group includes *M. bovis*, which does cause infection of alpacas, but also includes *M. microti*, which is also known to cause infections with very similar disease characteristics in these animals (Zanolari *et al*., 2009). Therefore, although the proof of principle results show that the phage assay can be applied to this group of animals, there are still technical questions that need to be resolved.

Figure 6.9 – *IS*6110 DNA detected from the blood of alpaca



Lane M, 100 bp DNA Ladder (NEB; UK); Lane 1, Alpaca 1; Lane 2, Alpaca 2; Lane 3, Alpaca 3; Lanes 4 and 11, Alpaca 4; Lane 5, Alpaca 5; Lanes 6 and 8, Alpaca 6; Lanes 7 and 10, Alpaca 7; Lanes 9 and 12, Alpaca 8; Lane 13, positive control (20 ng M. bovis DNA); Lane 14, no template (water control). The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml-1 ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.2.). The expected product size for the IS6110 element was 123 bp. The top panel contains plaque DNA extracted from the standard phage assay (section 2.2.) and the bottom panel contains DNA extracted from the Oneday phage assay.

6.5. DISCUSSSION

The results of the bison study confirmed that the bison that elicited the unexpected response to the SICCT test were infected with the either S strain MAP, *M. avium* or a dual infection of the two. Originally it was regarded as unusual to detect S strain MAP in bison, especially as there are distinct bison and Indian-bison strains (de Castro Campos de Souza *et al*., 2016, Sohal *et al*., 2010). It has been shown through phylogenetic classification of MAP whole genome sequence data (141 samples) that the B strain is a subgroup of the C strain. During the analysis, a Type-C strain exhibiting a S strain IS1311 PCR-REA was discovered (Bryant *et al.*, 2016), which was in concordance with our data that showed that the MAP strain K10 used in this study, also displayed an S type profile, despite being isolated from cattle and characterized as a C strain. This indicates that the C/T polymorphism(s) at site 223 bp in the strain used had reverted to the S form and was no longer digestible with *Hinf*I. It was found that although the strains are not host specific, they do show a host preference and produce a different host immune response depending on the host (Fernández *et al*., 2014). S strain MAP prefers ovine hosts for example, while cattle show a resilience to the S strain (Moloney and Whittington, 2008). Mixed infections with *M. avium* Complex bacteria are known to occur in both wild and domesticated fauna. A field study reported co-infection with *M. avium* and MAP in wild red deer in Belgium (Volpe *et al*., 2020) and Cuban hutias in a German zoo (Münster et al., 2013a). A dual infection of MAP and *M. avium* subspecies hominissuis was also discovered in Austria in red deer (Glawischnig *et al*., 2006). Evidence exists that shows there is a dominant MAP strain of ruminants within a geographic area, for example, the predominant strain isolated in India is the Indian-bison strain which has

been discovered in bison, cattle and sheep (Sonawane *et al*., 2016). It is the close contact of ruminants that appears to dictate the interspecies transmission of MAP strains, such as co-pasturing grazing stock as is practiced in New Zealand. In New Zealand a survey showed that the S strain MAP was most prevalent in sheep and beef cattle, which are commonly grazed together. The C strain was found to be dominant in dairy cattle and deer (Verdugo *et al*., 2014). It is known that the bison herd tested in this study graze next to a herd of deer, it is possible that the route of infection came from the deer, which have already been shown in other countries to harbor dual infections of *M. avium* and MAP.

As previously mentioned, MAP infections in cattle can result in false positive results for *M. bovis* in both the SICCT and IGRA. These tests have also been shown to cause false negative results when cattle are co-infected with MAP and *M. bovis*, specifically when the cattle are initially infected with MAP and then *M. bovis* (Hope *et al*., 2005). This shows, that using the SICCT in combination with the ancillary IGRA may not be the most effective tool for detecting *M. bovis,* and false negative results will facilitate the spread of the disease either locally throughout a herd or through transportation to potentially an OTF farm. This indicates that there is a need for a different testing approach to effectively control the *M. bovis* epidemic. In this instance, the phage PCR was a method that could accurately differentiate between a MAP or *M. bovis* infection in bison*,* where the standard testing procedures failed and resulted in the culling of non-tuberculous animals. The importance of correctly identifying Mycobacterial infections in the food sector and health sector is very high due to the zoonotic nature of these organisms and the ease at which they can pass between animals and humans and *vice versa*.

The Oneday phage assay is a rapid, 24-hour assay with a higher sensitivity compared to the standard phage assay. The standard phage assay, however, has an advantage over the Oneday phage assay in that it produces plaques and so indicates when there is Mycobacteria present in the sample tested. If the human study had been performed using only the Oneday assay, the nine patients that produced negative Oneday phage assay results and the *IS*6110 negative plaques, would have been classified as negative samples only, the presence of other Mycobacteria in the blood would not have been known. It was unexpected to obtain *IS*6110 negative plaques from the human blood sample, the *IS*1311 PCR-REA was used to test these DNA samples and identified that one of the nine patients was *IS*1311 positive. The subsequent restriction analysis confirmed that the positive result was derived from S-strain MAP within the blood sample. Because MAP is not a blood commensal, it indicated that the patient had a disseminated MAP infection. The group that the patient belonged to was the IGRA positive group. This meant that the patient had seroconverted and produced a positive IGRA result using the QuantiFERON-TB Gold Plus assay (QFT-Plus; QIAGEN; UK), indicating that the person had been exposed to *M. tuberculosis* and were assumed to harbor a latent TB infection. As it has been previously discussed, the IGRA test for detecting *M. bovis* in cattle is susceptible to false positive results due to cross reactions with other species of Mycobacteria. The QFT-Plus is stated to have the 'highest specificity of any test for TB infection' (QIAGEN, 2020), however the results of the *IS*6110 PCR assay and *IS*1311 PCR-REA show that the test is susceptible to cross reacting with MAP and produce false positive results. The clinicians were informed of the result gained from this patient and they reported that the patient had the inflammatory disease sarcoidosis.

Sarcoidosis is characterized by non-caseating epithelioid cell granulomas, which can present in any organ in the body (Soto-Gomez *et al*., 2016). The exact etiological agent of sarcoidosis is unknown. A number of genetic factors have been established to allow for the manifestation of the disease, such as mutations in the NOD_2/$CARD_{15}$ genes (Starshinova *et al*., 2020). A number of organisms have been implemented as the etiological agent of sarcoidosis, including: mycobacteria, *Propionibacterium acnes*, *Borellia spp.,* Epstein-barr virus, Retrovirus and others (Esteves *et al*., 2016). A review by Brownell *et al*. (2011) showed an association between mycobacteria and sarcoidosis. The review included a meta-analysis of 31 publications dated between 1980 and 2006 which showed 231 of 874 sarcoidosis patients were positive for mycobacterial DNA, supporting the link between mycobacteria and sarcoidosis (Gupta *et al*., 2007). In a recent case study, a patient displayed an episode of cardiac sarcoidosis, leading to eventual heart failure and the implantation of a pacemaker. The blood of the patient was used to isolate macrophages out of which MAP was cultured. An anti-MAP treatment was then administered, and after a year of treatment, they observed complete resolution of the patients symptoms (Celler, 2018). The results of this study support these findings as it also shows the presence of viable MAP isolated from the immune cells of a sarcoidosis patient, identified in a double-blind trial with no prior knowledge of the patient's group at the time the results were gained, further implementing MAP as a causal agent of the disease. It is also known that S-strain MAP obtained from sheep and clinical strains isolated from humans are difficult to culture, possibly indicating that the adaptations S-type MAP contains to cause disease in sheep may also make it better adapted to infecting humans. It is of interest that it was S-strain MAP that produced a false positive result

in an immune assay for both the bison and a human patient. This eludes to the fact that slow growing clinical S-strains of MAP may be phenotypically more similar to MTBC bacteria than the C-type strains and the difficulty in culturing S-type MAP means that there are relatively few strains stored in culture collections to reliable tell if these strains have the potential to cross-react with the current immunological assays (Sohal *et al*., 2009).

As part of this study, a small number of tests were performed on freshly isolated badger blood. Due to several technical reasons, many of the samples could not be processed. However, a low number of samples (3/28) did give a positive result using the standard phage assay (data not shown). Badgers have been implicated as the main reservoir and transmitter of *M. bovis* to cattle (Ní Bhuachalla *et al*., 2014). Epidemiological testing for bovine TB in badgers is challenging due to the nature of these animals and because of this, testing is performed on deceased or freshly culled animals (Murphy *et al*., 2010). The results gained in this study indicated that it is possible to detect mycobacteria from the blood of badgers and offers a humane approach for tracking the epidemiology of *M. bovis* through the badger and cattle populations. The likelihood of Mycobacteria spreading though interspecies transmission is high due to the large quantity of host that Mycobacteria are able to cause disease in. A robust and rapid detection method for these organisms is needed to determine how these organisms spread throughout an ecosystem and where reservoirs of the bacteria may lie. Since 1986 England and Wales have been going through a bovine TB epidemic which has been observed spreading from the southwest of the county to the north and east (Allen *et al*., 2018). However, before the phage assay could be applied on live wild badgers, a testing method would need

to be devised for the capture and safe collection of the blood samples due to the painful injuries these animals may cause to the phlebotomist, thanks to the aggressive demeanor these animals display when cornered, their relatively large size and the damage that they can cause with both their teeth and claws. In both the badger and alpaca studies, the standard two-day phage assay was more successful than the Oneday phage assay. As mentioned earlier, in cattle the Oneday method has proved to be more sensitive, which is not surprising as there are fewer opportunities for DNA loss during the different steps of the assay. However, when testing blood from other species there may be either phage or PCR inhibitors present in the blood sample that interfere with the test performance. In addition, when working with animal species where a wider range of mycobacterial pathogens may be causing infection, more specific PCR assays are required so that the precise identity of the organism detected can be confirmed. In this way, although the Oneday assay is simpler and more easily carried out in non-microbiological laboratories, there may be the need to use the standard two-day phage assay for blood samples from these more unusual animals until the critical parameters of the phage-based assays are better understood.

The PocketPCR provides another way to streamline the phage assays and particularly make the assay transferable to field laboratories. The PocketPCR was used with a sample of the DNA extracted from three alpacas, one that would be described as strongly positive, one weakly positive and the final one negative, to see how the minitiurized machine compares with an expensive lab thermocycler (see Appendix 7, section 9.7.1.). The fact that it produced comparable results to a standard thermocycler provides a basis for further work including adapting the machine so

that it can be used in a real time format. By doing this it further streamlines the PCR assay step by removing the need for downstream methods of analysis to visualize the results.

6.6. CONCLUSION

The results of the studies gained in this chapter showed how the use of phage PCR assays on the PBMCs extracted from blood samples identified false positive results produced from immunological assays designed for detecting whether the test subject has been exposed to a member of the MTBC. The results from the SICCT test on bison which indicated exposure to *M. bovis* and the IGRA assay on the human patient which indicated exposure to *M. tuberculosis,* were shown to have been caused by S-type MAP and *M. avium* in the bison samples and by S-type MAP in the human sample. The S-type MAP in the human trial was discovered to be from a human sarcoidosis patient which was discovered in a double-blind trial for the detection of TB and was identified through further blind testing when the blood sample yielded *IS*6110 negative plaques, indicating that Mycobacteria were present in the blood. The results indicate further evidence that MAP may be an etiological agent for the onset of sarcoidosis for a subset of sarcoidosis cases.

The use of the same phage PCR assays were also used to identify viable MTBC in the blood samples of an alpaca herd and was also used to detect viable MTBC in the heart blood of culled badgers. The final statement to conclude this chapter is that the results of this chapter indicate that the use of phage PCR assays could complement the current testing programs we have for the detection of MTBC

bacteria by identifying false positive results produced by the current testing methods and provide increased assurance and accuracy to the TB eradication program.

CHAPTER 7

7. GENERAL DISCUSSION AND FUTURE WORK

Throughout the history of man, mycobacteria have continuously presented significant health risks not only to humans, but also to the animals we've domesticated, farm and also and the wildlife we live amongst. The close genetic relationship that different members of this genus display - particularly for species within the same complex - and the robust nature of these organisms, creates the perfect storm for transmission, culminating in the ability of these closely related species of mycobacteria being able to infect and cause disease in a multitude of different host species. Other than *M. tuberculosis,* the main mycobacterial pathogens that affect man, either directly or indirectly, are *M. bovis, M. avium* and MAP. MAP has been implemented as an etiological agent in Crohn's disease (Adhikari, 2020) and *M. avium* is an opportunistic pathogen that causes infrequent infections in immunocompetent host's (Rindi and Garzelli, 2014) but can commonly co-infective HIV patients and cause disease (Karakousis *et al*., 2004). Both of these organisms are reported to have several different maintenance and spill over hosts that allow the bacteria to persist within an ecosystem and keep them circulating to potentially infect (or re-infect) domesticated stock and, potentially, humans. In contrast, *M. tuberculosis* only has one known reservoir, which are humans. However, with the high density of humans on the planet and the close proximity in which we live, it is no surprise that control of this disease has challenged humankind for centuries. The ability of mycobacterial pathogens to produce dormant latent infections in hosts, even in healthy-immunocompetent hosts, which can persist without producing further pathogenesis with the host until its natural death, or that can reactivate at any time in the hosts life and produce active disease (possibly due to the development of a comorbidity or aging), provides another dynamic to the survival of

pathogens which creates further challenges for the control of their associated diseases.

The difficulty in detecting mycobacterial pathogens is attributed to their slow-growing nature observed during *in-vitro* growth. Culture-based methods are considered to be the "gold standard" for the detection of mycobacteria, even though clinical strains of mycobacteria can take over 16 weeks to produce a visible colony and high contamination rates associated with isolating the bacteria from clinical samples often lead to culture failures. Hence many different indirect methods have been devised for the detection of mycobacteria, but all have their own limitations. For instance, immune-based assays detect a host's response to an organism as an indication of exposure but do not directly report on the current state of infection and both cross-reactions and false-positive test results have been associated with immune-based assays which hinders control efforts. Alternatively, DNA amplification-based assays have been developed that detect the presence of the organisms' DNA in the sample but generally do not differentiate between live and dead organisms, which is usually a pivotal requirement of diagnosing active disease. Another limitation of DNA amplification assays are their sensitivity to the many inhibitors of DNA polymerase that are found in most clinical samples, and co-extraction of these inhibitors along with the mycobacterial DNA produces false-negative results. Despite this limitation, a well-designed PCR assay can be very specific with minimal false positive results likely. To solve the second limitation of DNA amplification-based assays, being the false negative results produced through DNA polymerase inhibition from clinical samples, it is crucial that the DNA

amplification assay contains an IAC to indicate which samples contain inhibitors and need to be repeated.

As an alternative approach to diagnosis, broad host range of mycobacteriophages, such as D29, have been exploited to develop detection methods for mycobacteria. As phage only infect viable organisms, by combining phage-based DNA extraction with DNA amplification assays, positive results indicate the DNA detected was extracted from a live organism and solves one of the limitations of stand-alone DNA amplification-based assays.

7.1. The IAC PCR assays

As described above, although DNA amplification assays such as PCR assays have been used for many years to detect and diagnose disease, a robust PCR designed for use with clinical samples requires an IAC to indicate when DNA polymerase inhibition has occurred. The IAC produced in Chapter 4 was based on the design of IAC plasmids that have been produced by other studies (Flores *et al*., 2009, Sevilla *et al*., 2015) but in this case it was designed for use with phage-based assays. With that in mind, the IAC plasmid that was produced was built on an integrative-shuttle vector to produce a stable IAC strain of *M. smegmatis* that could also serve as a positive control for the phage-based DNA extraction step. To make this plasmid more versatile, three different pairs of primer sequences targeting signature sequences for MTBC, MAP and *M. avium* were included in the pIAC plasmid since the D29 phage can infect all three of these significant mycobacterial pathogens. The benefit of producing a viable bacterial control strain for the phage assays was that the strain itself could be added to the samples being tested, and as such could be used as a control for both the

phage-based detection event and as an IAC target for the PCR identification step. A third advantage of this control strain was that it provided a rapidly growing positive control strain that could be used in laboratories that are not registered for culture of pathogenic organism such as *M. bovis* (BS level 3 containment required), or to provide a more practical replacement for slow growing MAP cultures used as positive controls.

An unexpected limitation of the live-control strain was the interaction of phage D29 with the integrative vector. It is often forgotten that many plasmid-vectors include some phage sequences and functions, and in this case, this meant that the integration system of the plasmid and phage were the same (Ribeiro *et al*., 1997) and it appeared that infection of the control strain with D29 produced an excision event of the plasmid and may have initiated replication of the plasmid. The integrase that is responsible for the integration of the pMV306 plasmid also acts as an excisase (Springer *et al*., 2001). A previous study determined that it is possible to greatly improve the stability of the integrative mycobacteria plasmids by removing the integrase and *att* sequence from the chromosome once the plasmid has integrated (Saviola, 2009). In line with this finding, it would be advantageous to remove the integration system in an attempt to stop the interaction between D29 and the integrative plasmid. The integration system could be removed by introducing a clustered regularly interspaced short palindromic repeats (CRISPR) system into the live control strain that targets the integrase. This method has been shown to be successful in the removal of these genes from integrated plasmids in *M. smegmatis* by using CRISPR-Cas12a (Yan *et al*., 2017). By also removing the *att* sequence there

should be no other areas of homology between the plasmid and the phage genome so the phage infection should have no impact on the integrated plasmid, potentially improving the sensitivity of the live control strain in the Oneday phage assay.

Another general issue when working with mycobacteria is their clumping tendency when grown in liquid culture (Cheng *et al*., 2014, Lowe *et al.*, 2013). This adds a complication when trying to add a consistent amount of the bacterial control strain to each phage assay, especially as the sensitivity of diagnostic PCR assays can be affected if too many cells are added (Rosenstraus *et al*., 1998). This is often solved by adding detergents such as Tween which has been recognised as a method to prevent clumping of mycobacterial cells since the 1940's (Dubos and Davis, 1946). However, phage particles are sensitive to disruption by detergents and it is also known that Tween-induced changes occur in the cell wall and membrane of mycobacteria grown in the presence of Tween that affect macrophage uptake and the immune response to MTB (Sani *et al*., 2010, Wang *et al*., 2011). Although the precise nature of the receptor for phage D29 on the mycobacterial cell surface remains obscure, using reagents that alter the cell surface composition would not be recommended as a solution to the problem of clumping.

Hence a plasmid format of the IAC was also produced so that a known concentration of the pIAC plasmid could be added to each PCR reaction so a specific, consistent Ct value could be achieved so that it is easier to identify samples that contained inhibitors. Even though a section of the Oneday phage assay involves a DNA purification step, it was noticed that some of the DNA extracts contained PCR inhibitors originating from the blood sample. This is consistent with other studies

that have reported that DNA Spin Columns can co-extract DNA polymerase inhibitors from blood along with DNA, even though they are designed to remove inhibitors and requires the sample to be pre-diluted before the spin-column extraction is performed to reliably remove inhibitors from the sample (Regan *et al*., 2012). Traditional phenol-chloroform extraction of DNA was shown to more efficiently remove inhibitors (Kramvis *et al*., 1996), even from undiluted samples. Another study also noted that using DNA Spin Columns did not produce satisfactory removal of polymerase inhibitors and a precursory inhibitor removal step was required (or multiple consecutive DNA Spin Columns) to remove inhibitors and prevent false-negative PCR results (Hunter *et al*., 2019). A study by Chakravorty and Tyagi (2001) detected mycobacteria (ruptured by chemical lysis) from blood by using a novel inhibitor removing solution (consisting of guanidinium isothiocyanate) after the mycobacteria had been isolated (and prior to their lysis) to remove the inhibitors present from the blood sample in order to perform successful identification by PCR. Considering that in this method the inhibitors are removed before the mycobacteria are lysed means that the step could be implemented after the PBMCs have been isolated before the Oneday phage assay. This should mean that there would be fewer residual inhibitors remaining in the sample when the subsequent DNA Spin Column purification step is performed which should result in DNA of higher purity and less likely to cause inhibition of the DNA polymerase during the PCR assay and produce less false negative results. One thing that must be considered though is that guanidinium is a chaotrophic agent and may inactivate the phage by destabilizing or potentially denaturing the phage tail and capsid (Brockmann, 2012, Salvi *et al*., 2005). It is likely because of this that extra wash steps with MP would be needed

after the addition of the guanidinium to reduce the guanidinium concentration below the levels that are inhibitory to the phage. It would also have to be determined that the phage receptor on the mycobacteria was still functional after incubation with the chaotrophic agent. The addition of bovine serum albumin to PCR reactions has also been shown to produce a protecting effect for the DNA polymerase against co-extracted blood-derived inhibitors resulting in fewer PCR assays producing false negative results (Abu Al-Soud and Rådström, 2000, Forbes and Hicks, 1996). Introducing a second inhibitor removal step before the phage assay and adding bovine serum albumin to the PCR reaction should in theory protect the DNA polymerase from any residual inhibitors found in the DNA extract from the DNA Spin Column.

7.2. Using phage to detect mycobacteria in blood

The standard phage assay used in this thesis to detect mycobacteria in the blood of humans was adapted from a commercial phage assay, *FASTplaque*TB (Biotec Laboratories Ltd.; UK), used to detect viable MTB from the sputum of patients with active pulmonary TB. When using the assay *FASTplaque*TB on sputum samples a sensitivity of 87.5 % and a specificity of 96.9 % was achieved (Albay *et al.*, 2003). Interestingly these results are similar to the results produced when using the Oneday phage assay to detect MTBC DNA from human blood, however it is not expected to be similar and is likely due to coincidence. The *FASTplaque*TB assay requires a decontamination step which results in a loss of viable mycobacteria as well which effects the sensitivity of the assay, whereas no decontamination step is required with the Oneday phage assay. There may also be no correlation between detectable levels

of MTB in sputum and the detectable levels of MTB in blood, due to the phage negative active pulmonary TB cases that produced positive sputum culture results showing that the sputum did contain MTB when the MTB in the blood was below limit of detection of the Oneday phage assay.

The haematogenous dissemination of human mycobacterial infections, primarily of MTB (Bacha *et al*., 2004, Barr *et al*., 2020, Clark *et al*., 1991) and *M. avium* (Karakousis *et al*., 2004) has been described in patients infected with HIV. Blood culture has also been used to grow MTB from HIV infected patients and provides further evidence of viable MTB circulating in the blood of humans (Feasey *et al*., 2013, Mathuram *et al*., 2018). Despite our limited success with culturing these bacteria from blood, the use of phage was able to show that detectable levels of MTB are present in blood of immuno-competent patients, confirmed not to have a co-infection with HIV. Several other studies have also reported this including a retrospective study in China that also identified MTB circulating in the blood (by blood culture with BACTEC MGIT) of immune-competent patients using 3 – 5 ml of blood (Liu *et al*., 2016). Belay *et al.* (2021) used a digital PCR (dPCR) assay to detect MTBC DNA in CD34+ and CD34- PBMCs in humans with and without HIVV-coinfection. They also reported that they detected MTBC DNA in IGRA positive and negative patients, whereas in our study (Chapter 5) we didn't detect the presence of MTBC DNA in IGRA positive or negative LTBI patients, except in the three seroconversion patients. The disparity between the two studies could be explained for two reasons, first that Belay *et al.* (2021) used 100 ml of blood per patient, which is much larger than our 4 m per patient, and as such would isolate a greater quantity of cells and produce a more sensitive assay. A second explanation is that they used

heat inactivation to make the MTBC DNA available for dPCR analysis so they cannot determine whether the MTBC DNA was produced from viable actively growing cells and as such could also be derived from dormant cells and dead cells. The DNA extraction methods used by Belay *et al*. (2021) would isolate the DNA from these mycobacteria, regardless of their dormancy. It has been found that MTB persists in long-term repopulating pluripotent hematopoietic stem cells in both humans and mice. These cells exist in hypoxic conditions within the bone marrow but also circulate throughout the vascular system and as such can be isolated in the PBMC faction of the whole blood (Tornack *et al*., 2017). Unfortunately, because the MTB reside within a hypoxic environment they are in a dormant non-replicating state and D29 has been shown to be unable to infect hypoxic mycobacteria (Swift *et al*., 2014), so these would go undetected by the phage assays. This is possibly why we were not able to detect MTBC DNA in the PBMCs of IGRA positive LTBI patients, despite the evidence that shows that blood of LTBI patients does contain viable if dormant MTB. It would be interesting to see if performing the Oneday phage assay with phage TM4 on IGRA positive LTBI patients would produce positive results as this phage is able to 'wake-up' and infect dormant mycobacteria induced by hypoxia (Piuri and Hatfull, 2006).

The interactions between MTB and their human hosts are still not fully understood and as such the exact cause of MTB dissemination is not known. The most interesting aspect of the results gained from the pilot human study was the glimpse into the disease progression of MTB and the complex nature of the infection process. By looking at our results it seems that during the initial stage of infection, MTB is detectable in the PBMCs of the blood even when a LTBI is being established. Once a

LTBI has been established the levels of MTB in the blood drops below detectable levels with the Oneday phage assay (approximately 5 cfu ml^{-1}; (Swift et al., 2020). When a LTBI patient reactivates into pulmonary TB, the concentration of MTB in the blood then seems to increase to a detectable level as an early sign of disease progression. This strange flux in the concentration of detectable MTB in the blood of human patients is likely due to the dynamic interaction between the bacteria and the host immune response, however as discussed above, D29 cannot infect hypoxic dormant mycobacteria so the fluctuation in our results could have been caused by MTB under different metabolic rates rather than a decrease in bacterial load. Intermittent mycobacteraemia has also been described with *M. avium* infections in patients with HIV co-infection (Kemper *et al*., 1994) and also with MAP in a breeding Simmental bull (cow), which also showed the intermittent presence of MAP in faeces and semen (Münster et al., 2013b). Fluctuations in antibody titre against MAP has also been described in the serum of cattle has also been seen in a yearlong longitudinal study testing the same MAP infected cows (Faruk *et al*., 2020). Throughout the course of this thesis, we identified mycobacteria circulating in blood derived PBMCs in several mammals including humans, bison, llamas and badgers. Other studies have also shown that MTBC disseminate in the blood of cattle (Srivastava *et al*., 2008), which suggests that the intermittent dissemination of mycobacteria may be a characteristic of mycobacterial infections, although it must be said that we only detected a small number of different mycobacterial species so the assumption cannot be made to the entire genus of pathogenic mycobacteria.

The results gained when studying humans were in slight contrast compared to when the Oneday phage assay was used on SICCT positive cattle. MTBC was detected in the

blood of all SICCT positive cattle including those with non-visible legions (Swift *et al*., 2020), however failed to detect human LTBI patients with positive IGRAs, except the three seroconverting patients. Although a direct comparison between the two cannot be made as the TST is less sensitive than the QFT IGRA (and they are designed for different purposes), the difference in the results may indicate the disease progression between *M. bovis* in cattle and MTB in humans are not the same. There is limited evidence of *M. bovis* producing latent infections in cattle and tends to produce persistent active disease (Sabio y García *et al*., 2020). If this is the case and *M. bovis* isn't induced into a hypoxic dormancy in cattle it would explain why the MTBC DNA was detected in the Oneday phage assay of the SICCT cattle, and not in the IGRA positive LTBI patients. To make a better comparison, the results should be compared to IGRA positive cattle also tested with the Oneday phage assay, to determine whether there are a portion of IGRA positive, phage negative cattle that may indicate a latent infection. The tests should be repeated at several different intervals because of the intermittent haematogenous dissemination that may occur.

A subgroup of the human active pulmonary TB cases were also phage negative by the Oneday phage assay and these were shown to have a lower microbial burden of sputum compared to the phage positive cases.

Blood acquired a reputation as being a barren diagnostic sample for the detection of MTB in humans before viable MTB was readily cultured from the blood of HIV patients, while blood culture is still a challenge with immunocompetent patients which suggests that the bacterial load is below the threshold of detection of detection for blood culture with a competent immune system; then it stands to

reason that human LTBI patients may contain a low grade mycobacteraemia that is below the threshold of detection of the Oneday phage assay.

With this in mind, the next step in the optimization of the Oneday phage assay would be to increase the sensitivity of the assay in order to determine whether human LTBI patients do have a low grade mycobacteraemia. One of the limitations of the Oneday phage assay is that a maximum volume of 2 ml of blood can be used with each of the Actiphage reaction tubes used to perform the assay in due to the in-built filter blocking due to cellular debris and preventing the complete passage of the liquid phase through the filter resulting in residual DNA being retained in the upper section. So the next logical step in improving the sensitivity of the assay would be to adapt the processing steps of the protocol to reduce the cellular debris carried over into the Oneday phage assay so that a larger starting volume of blood can be used per test. MTB has been described at a blood concentration of 0.4 cfu ml^{-1} (Pavlinac *et al*., 2016) which would mean that a 2 ml blood sample would not consistently contain MTB, let alone be consistently detectable, and this is with what we saw as not all parallel samples from the same patient produced positive results. An optimum volume of blood tested for each Oneday phage assay would be 10 ml, 5-fold more than the maximum limit. This could potentially be solved by introducing concurrent PBMC lysis and centrifugation steps to concentrate the released mycobacteria and separating them from the cellular debris which should remain in the supernatant. By improving the sensitivity of the Oneday phage assay, it may be possible to differentiate QFT-positive patients that harbour a LTBI and QFT-positive patients that have successfully cleared the bacteria. A secondary benefit of improving the sensitivity of the Oneday phage assay could be its use to further our understanding

of the disease progression and the haematogenous dissemination of mycobacteria by tracking the mycobacterial load during the different stages of the infection process and development of milliary and extrapulmonary TB.

7.3. The similarity of mycobacteria can confound diagnostic testing methods

Another important finding was that the current immunological based assays for the detection of an exposure to MTBC are susceptible to cross reacting with NTM, in our case with members of the MAC. We identified the presence of MAP circulating in the blood of a sarcoid patient that produced a positive QFT IGRA result for MTB. In this vein, we also identified MAP and *M. avium* circulating in the blood of bison that produced a positive SICCT response. Immunological-based assays for the detection of an immune response to specific mycobacterial pathogens have been shown to produce false positive reactions and cross react with non-target mycobacteria, including bacteria outside of the target organisms bacterial complex and most commonly with NTM (Jenkins *et al*., 2018). Several different NTM have been implemented in causing false positive results in immunological-based assays, including MAP (Barry *et al*., 2011), *M. simiae, M. marinum* (Gcebe and Hlokwe, 2017), *M. kansasii* (Vordermeier *et al*., 2007) and *M. fortuitum* (Michel *et al*., 2011). These false-positive tests hamper the One Health approach to TB eradication and result in unnecessary economical costs, both in terms of administering the inappropriate treatment plans and by culling non-tuberculous animals.

Exposure to NTM has also been hypothesized to provide protection against tuberculous mycobacteria (MTBC bacteria) due to cross-reactions occurring between the hosts acquired immune response against the NTM bacteria; upregulating with

subsequent infections with MTBC bacteria. However, exposure to NTM is believed to reduce the efficacy of a subsequent immunization with the BCG vaccine (Shah *et al*., 2019). The fact that potential pre-exposure to NTM could reduce the efficacy of the BCG vaccine through a dampened immune response could also mean that pre-exposure to NTM would also cause a decreased immune response to subsequent wild type MTBC infections, allowing the MTBC bacteria to produce a more significant infection and more significant disease. This shows that further research is required to identify what relationship there is between immune responses and subsequent NTM and MTBC infections. Research into this area could also identify more accurately why immunological assays produce cross reactions with other mycobacteria but the fact that the immunological cross reactions do occur provide evidence that the same happens *in vivo* during the immune response. Considering the fact that NTM bacteria are ubiquitous with the environment, and that all species that are susceptible to TB are also readily exposed to environmental mycobacteria; previous exposure to NTM could potentially illicit negative responses in immunological assays and immune responses to vaccination strains. These antagonistic reactions undermine the effectiveness of the assays and the vaccination programmes designed to detect and protect against TB, again, hampering the TB eradication program. Separation of our domestic stock from environmental mycobacteria unfortunately isn't a feasible option. For instance, MAP has been isolated from free-living amoebae in water sources in cattle fields of farms that are positive for Johne's disease, suggesting that free-living amoebae may be an environmental maintenance organism for mycobacteria (Samba-Louaka *et al.*, 2018). The fact that MAP can survive in this manner in water sources makes it difficult to prevent free-range grazing animals

from ingesting the organisms and becoming immunologically exposed. *M. bovis* has also been found to be shed in faeces of cattle with pulmonary disease and found to survive in faeces for up to two months in the summer and up to six months in the winter months and could potentially be an environmental route of transmission (Allen *et al*., 2021). Another intriguing potential source of environmental contamination is from the urine of badgers infected with *M. bovis*. *M. bovis* can be cultured directly from the urine and can contain a mycobacterial load of up to 2.5 $x10^5$ cfu ml^{-1}, however in urine the mycobacteria only survived on pastureland for approximately three days in summer and two weeks in winter (Gallagher and Clifton-Hadley, 2000). The shedding of these organisms on to pasture land could potentially lead to infection of healthy cattle through ingestion. Excretion of *M. bovis* containing urine onto dry dusty earth could result in infection through inhalation as cattle disturb the dust as they walk then inhale the contaminated dust. It is possible that *M. bovis* shed onto the dry pasture land through badger urine could infect soil-associated amoebae where they would be able to persist for an extended period of time. This would increase the chance of livestock ingesting the *M. bovis* infected amoebae during grazing (or also through inhalation), allowing the bacteria to then go on and produce disease in their new host.

Even man-made water sources are susceptible to NTM and another potential source for exposure comes from man-made water outlets and plumbing (Waak *et al*., 2019). NTM have been shown to be resistant to the water treatment methods used to make water potable and can be isolated from chlorinated tap water (Fernandes *et al*., 2020, Haig *et al*., 2018). So, watering livestock with treated water could still lead to

exposure to NTM which could then go on to produce false-positive results in immunological assays.

One course of action would be to improve the immunological assays by isolating more specific antigens for the target organism that won't cross react with non-target mycobacteria. Recent modifications to the bovine TB IGRAs and TSTs have been developed that rather than using purified protein derivatives (PPD), which in simple terms is a crude cell lysate, instead use a cocktail of defined antigens to differentiate between *M. bovis* infected cattle and BCG- and MAP-vaccinated cattle (Middleton *et al*., 2021, Srinivasan *et al*., 2019, Srinivasan *et al.*, 2020). Although these novel immunological assays have been developed to improve specificity against BCG or MAP vaccination, the fact that they don't rely on PPD should also mean they have increased specificity and produce less false-positive reactions with NTMs as there will be a far less likelihood of the defined antigens being shared between different mycobacteria compared to the amount shared with the large quantity of antigens in current PPD-based assays.

7.4. Future work

Here we have shown that the Oneday phage assay was able to identify the infecting organism where the standardly used immunological assay produced a false positive result. Unfortunately, the sample size was small so the results are only observational, as such, it would be good to do further testing of SICCT positive cattle to determine the rate of agreement between the Oneday phage assay and the immunological assays to determine whether the Oneday phage assay could accurately and reliable identify false positive reactors. If this was the case the Oneday phage assay could

provide as an ancillary diagnostic test to the immunological assays to identify false-positive results as a secondary measure to provide assurance of the immunological result.

In Chapter 6 an open-source miniaturized thermocycler (PocketPCR; Gaudi labs, Switzerland) was used with historic DNA samples and showed that the levels of amplification were comparable to original results produced on a standard thermocycler. This was exciting as the PocketPCR is ideal for use within the field as not only is it small, lightweight and robust, but can be powered by any 2 A power outlet, making the device completely mobile. A limitation with the device though is the fact that it can only run five PCR assays at a single time due to the small size of the heating block. Increasing the size of the block however is not exactly feasible as the increase in energy required to rapidly heat and cool a larger object will likely prevent it from being able to be powered by a 2 A power outlet, one of its main attributes. The device also requires the use of gel electrophoresis analysis after the PCR step also, to visualize the DNA and results, which obviously requires extra equipment and resources which may not be available in a field laboratory. With these two limitations in mind, it would be ideal to develop a real time PCR assay that could be performed on the PocketPCR without the need for further analysis steps such as gel electrophoresis, for use as a field assay. In an ideal would a fluorescent-based PCR assay such as qPCR could be developed, however incorporating a miniaturized fluorescence detector to the PocketPCR within the power constraints may prove a challenge. One method that should be applicable under the current design would be to develop a colorimetric real time loop-mediated isothermal amplification (RT-LAMP) assay. These assay have been developed of for a number of

viruses including influenza and COVID-SARS-2 (Ahn *et al*., 2019). The RT-LAMP assay mixture contains phenol red which turns yellow as the pH of the assay mixture decreases during DNA amplification to show a positive result (Dao Thi *et al*., 2020). RT-LAMP assays use strand displacement DNA polymerases and four primers, two internal primers which initiate amplification and two outer primers which generate the full loop structure for exponential amplification. Each of the primers contain a secondary complementary sequence on the opposite side of the primer to the target DNA so that when the strand is amplified a loop structure is formed at the end of the structure which is used to initiate the next round of amplification when the outer primer binds (Becherer *et al*., 2020). By using this type of assay it should be possible to detect MTBC DNA at a point of care setting/ field laboratory. The second aspect of the PocketPCR that would need to be addressed would be to increase the quantity of tests that can be performed on the device. This could be by performed simply by designing a heating block of the same size for use with thinner 0.1 ml PCR tube strips, which would fit more tubes in. Another method would be to develop a microfluidic PCR device which uses convection kinetics to produce highly efficient and rapid DNA amplification with very small reaction mixture volumes (Ahrberg *et al*., 2016, Kulkarni and Goel, 2020, Li *et al*., 2019), however this is a more complicated method that will require more extensive design and testing to upgrade the PocketPCR for microfluidic PCR assays.

As the follow-on human study was halted due to the outbreak of COVID-SARS 2 the immediate future work involves further optimization of the Oneday phage assay to improve the sensitivity to detect the phage-negative active pulmonary cases that

were shown to have a lower bacterial load compared to the phage positive active pulmonary patients. I also attempted to culture MTBC from the PBMC fraction of the blood however we were unsuccessful with the culture methods we used and trialled creating bespoke agar from the blood samples so that the mycobacteria weren't shocked by a sudden and complete environment change, however this work was again halted due to the Covid closures. Ideally, I would expand on this method further as it has been shown that *in vitro* slow-growing mycobacteria can grow to very high concentrations *in vivo*, in particular, the ovine strains of MAP despite the fact that these strains are notoriously difficult to culture. One of the areas we would like to investigate is whether MAP strains are primed by the digestive process of the host organism to promote growth rate and pathogenicity in the host and if by replicating these conditions we can increase the growth rate of MAP *in vitro.*

Another future area of work would be to optimize the phage assays for the detection of MTBC in TB meningitis from cerebral spinal fluid (CSF). Diagnosis of this form of TB current relies on health questionnaires and culture of MTB from CSF. Culture unfortunately offers a low sensitivity and usually by the time colonies are produced and confirmed to be MTB, it is too late to effectively treat the patient (Foppiano Palacios and Saleeb, 2020). The fact that it is difficult and slow culture methods are currently the gold standard for diagnosis provides an attractive new niche to use the phage assays in where they could make an immediate impact on people's health. The first step would be to determine whether components of CSF inhibit the phage assay and if so, the location of MTB within the fluid (extracellular or intracellular) would need to be determined so the most appropriate processing steps could be performed

to remove the inhibitors but retain the bacteria. This would then allow the phage assay to be performed and so that the MTB DNA could be detected by PCR.

7.5. Conclusion

Mycobacteria continue to be thorn in the side of humanity and are a constant threat to the health of humans and many other animal species we share the planet with. Using phage to detect these introverted and obstinate bacteria uses natural evolution and biology to solve the majority of the problems when it comes to detecting mycobacterial DNA. Effective and successful surveillance and eradication programs could be complemented and improved if they used rapid phage-based detection methods for the early detection of mycobacteria.

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9. APPENDICES

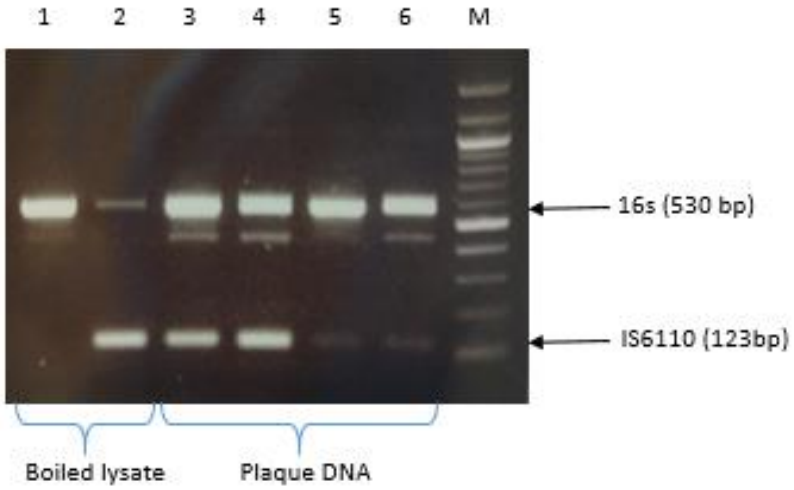
9.1. Appendix 1

9.1.1. Effect of primer concentration on sensitivity of non-competitive IAC

The poor amplification of the *IS*6110 element was hypothesized to be caused by the high concentration of the *IS*6110 primers. It is reported that increasing the primer concentrations above 0.5 µM can lead to an increased likelihood of primers annealing to themselves and produce primer dimers (Markoulatos *et al*., 2002). When primer dimers are formed, this reduces the concentration of primers available to anneal to the template which in turn, can reduce the sensitivity of the assay. One characteristic of primer dimer formation is the development of DNA smears on electrophoresis gels which usually migrate below the 100 bp DNA marker. However, these were not observed in the previous experiments, despite the high concentration of primers used. To determine whether the high *IS*6110 primer concentration in the second PCR was inhibiting the amplification of the *IS*6110 element, two lower concentrations of *IS*6110 primers were tested in the multiplex PCR assay. The concentration of 16s rDNA primers used in the multiplex PCR assay were also decreased, in line with the previous results, to reduce the amount of 16s rDNA product amplified (section 2.4.3.4). Using the DNA extracted from the plaques as a template, the results showed a strong amplification of the 530 bp product corresponding to the 16s rDNA in all the PCR assays regardless of whether 0.04 µM or 0.02 µM was used (Fig 9.1; Lanes 3 – 6). The results for the amplification of the *IS*6110 element this time showed a low-level amplification of the 123 bp product corresponding to the *IS*6110 element when a final *IS*6110 primer concentration of

0.6 µM was used (Fig 9.1; Lanes 5 and 6). An increased level of amplification of the 123 bp product was observed when a final *IS*6110 primer concentration of 0.6 µM was used (Fig 9.1; lanes 3 and 4) showing that a high concentration of *IS*6110 primers were still needed when the multiplex PCR was carried out on a low copy number of *IS*6110 elements. A non-specific band of 450 bp was again produced in all of the plaque DNA samples and also with the *M. bovis* boiled lysate DNA when the 16s rDNA primers were used alone. This suggested that although the high *IS*6110 primer concentrations used previously were inhibiting the amplification of the *IS*6110 element, and that the stringency of the PCR was an issue as previously predicted. In this instance, reducing the *IS*6110 primer concentration for the *M. bovis* lysate DNA to 0.6 µM from 0.8 µM, improved the amplification showing that the high concentration of primers were inhibiting the assay previously (see Appendix 1 f or further information, sections 9.1.1 and 9.1.2). A non-specific band of 450 bp was again produced in all of the plaque DNA samples and also with the *M. bovis* boiled lysate DNA when the 16s rDNA primers were used alone. This suggested that although the high *IS*6110 primer concentrations used previously were inhibiting the amplification of the *IS*6110 element, and that the stringency of the PCR was an issue as previously predicted.

Fig 9.1 - Assessment of the effect of lowering the primer concentrations on the amplification of the IS6110 -elements and 16s rDNA



Primer combinations used in each of the PCR reactions were: Lane 1, 0.04 µM for KY18/75; Lane 2, 0.04 µM for KY18/75 and 0.4 µM for MTCF/R; Lane 3, 0.04 µM for KY18/75 and 0.6 µM for MTCF/R; Lane 4, 0.02 µM for KY18/75 and 0.6 µM for MTCF/R; Lane 5, 0.04 µM for KY18/75 and 0.4 µM for MTCF/R; Lane 6, 0.02 µM for KY18/75 and 0.4 µM for MTCF/R. Lane M; 100 bp DNA Ladder (NEB; UK). The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.4.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on RHS of image). Lanes 1 and 2, 25 ng boiled lysate M. bovis DNA used as template. Lanes 3 – 6 DNA from one BCG plaque and four M. smegmatis plaques extracted with a gel Spin Column kit used as template.

To prepare the first PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.8 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR product (5 µl) was used as DNA template for the 16s and IS6110 multiplex PCR assay. To prepare the altered multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 or 0.4 µM of each MTC F and MTC R primer and 0.04 or 0.02 µM of each KY18 and KY75 primer (Table 2.2); 5 % DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

9.1.2. **Effect of primer T_m on stringency of the non-competitive IAC**

Typically, to increase the stringency of a PCR assay it is necessary to increase the temperature of the annealing step of the PCR. Increasing the annealing temperature increases the specificity of the primers as it reduces the likelihood of the primer from annealing to non-complementary DNA sequences as the hybrids formed between the template and the primers are not stable.

Before the PCR parameters were adjusted, the melting temperature and GC content of each of the primers in the multiplex were calculated to identify any obvious differences in the primer pairs that would prevent raising the annealing temperature. The melting temperatures of the primers were calculated using the NCBI Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The results of the analysis showed a disparity between the melting temperature and GC content of the KY18 primer, when compared to the other three primers used in the multiplex (Table 3.1). The KY18 primer was calculated to have a melting temperature of 62.7 °C and a GC content of 52 %. These values are much lower than that of the other primers which have melting temperatures and a GC content of: 73.4 °C and 71% for KY75; 67.9 °C and 75% for MTCF; 68.4 °C and 75% for MTCR. Ideally, the melting temperature of primer pairs used in a PCR mixture should be within 5 °C of each other, this is to reduce the possibility of the primer with the highest melting temperature binding to the template non-specifically. In regard to this multiplex PCR, all the primers are outside of this range when compared to KY18. Therefore, it was decided to see whether the KY18 primer could be extended to increase its melting temperature. Before the primer was extended, the original primer sequence was searched using BLAST against the *M. smegmatis* MC^2155 reference strain. It was

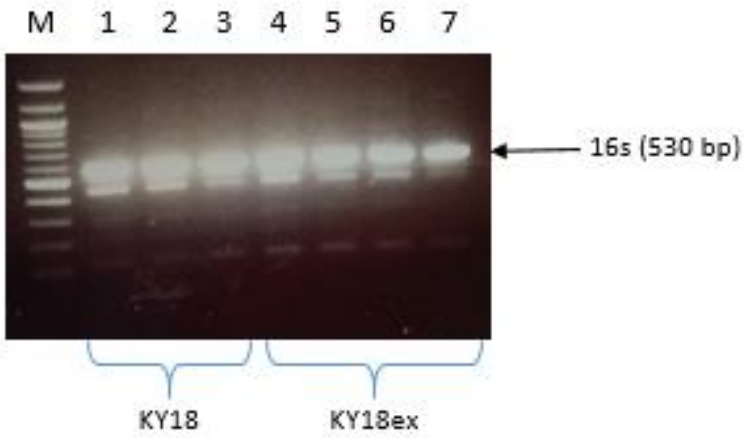
found that the maximum length the primer could be extended by was 2 bp in the 5' direction before the specificity of the primer began to increase. Based on this result, the primer was extended 1 bp in each direction and the primer sequence re-analysed using BLAST to ensure the primer still retained the ability to anneal to all the species of Mycobacteria that the standard KY18 primer could bind to. It was important to retain the ability to detect a wide range of Mycobacteria because this provided the IAC with flexibility for use as an internal control for assays using different types of Mycobacteria. The extension of the KY18 primer theoretically increased the melting temperature by 1.7 °C, which brought the melting temperature of the primer within 5 °C of the MTCF/R primer, but it was still 9 °C below the KY75 primer. The new extended primer was named as KY18ex, to denote the extension.

Once the primer had been extended, the next step was to determine how the extension had practically affected the annealing of the primer. To do this, a temperature gradient was performed comparing the results with the original KY18 primer (section 2.4.2.8). The temperature range was determined by starting at 62 °C, which was the original annealing temperature described by Tevere *et al*. (1996), and increasing the temperature in 1 °C increments until 65 °C was reached, as this was the temperature used for the amplification of the *IS*6110 element in the pre-amplification PCR.

The results of the temperature gradient showed that there was very little difference between the levels of non-specific binding produced at the temperatures 62 °C - 64 °C for both the KY18 and the KY18ex primers (Appendix 2). There was a minor improvement in the level of non-specific priming when an annealing temperature of 65 °C was used with the KY18ex primer. Increasing the annealing temperature did

little to affect the non-specific binding produced in the multiplex PCR assay, showing that the stringency of the PCR was still an issue. Increasing the length of the KY18 primer seemed to have little effect on its melting temperature, which was expected due to the small increase in melting temperature achieved. Interestingly, the original KY18 primer was shown to amplify the 16s rDNA at 64 °C which is above the theoretical melting temperature of the primer. Because of this, the use of the original KY18 primer was continued in the subsequent optimization steps.

Figure 9.2 – Temperature gradient of KY18 and KY18ex



Lane M; 100 bp DNA Ladder (NEB; UK). The KY18 primer was used in Lanes 1-3 and the KY18ex primer in Lanes 4 – 7. The annealing temperature used for each PCR reaction were: Lanes 1 and 4, 62 °C; Lanes 2 and 5, 63 °C; Lanes 3 and 6, 64 °C; Lane 7, 65 °C. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.8.).

The expected product size for the KY18/75 primers (16s rDNA) was 530 bp (indicated by arrows on RHS of image).

To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.025 µM of each primer KY18 and KY75 or KY18ex and KY75 (Table 2.2); 5 (%) DMSO (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water.

The PCR parameters used included a temperature gradient setting for the annealing step, the parameters used were: an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 62 – 65 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

9.1.3. **Determining the effect that PCR enhancer have on stringency**

The non-specific binding was still being produced even at elevated annealing temperatures, which showed that increasing the temperature did not improve the stringency of the multiplex to satisfactory levels. The next step taken in the optimization of the multiplex PCR was to investigate PCR enhancers. PCR enhancers were investigated because they can be used to amplify challenging PCR targets, particularly those that are high in their GC content, which is the case with the 16s rDNA and *IS*6110 multiplex assay. The PCR enhancer DMSO was already being used in the PCR reactions to improve the amplification of the *IS*6110 element, due to the high GC content of both the template and the MTCF/R primers. Some examples of other PCR enhancers are betaine, β-mercaptoethanol and trehalose (Spiess *et al*., 2004, Ralser *et al*., 2006). These also work by modifying secondary structure of single stranded DNA and/or reducing the melting temperature of the DNA. Along with these standardly used PCR enhancers, most polymerases are supplied with their manufacturers own PCR enhancers they have developed to use with their own polymerases and buffers. Typically, the recipe of these commercial enhancers are trade secrets and are known by their trade name.

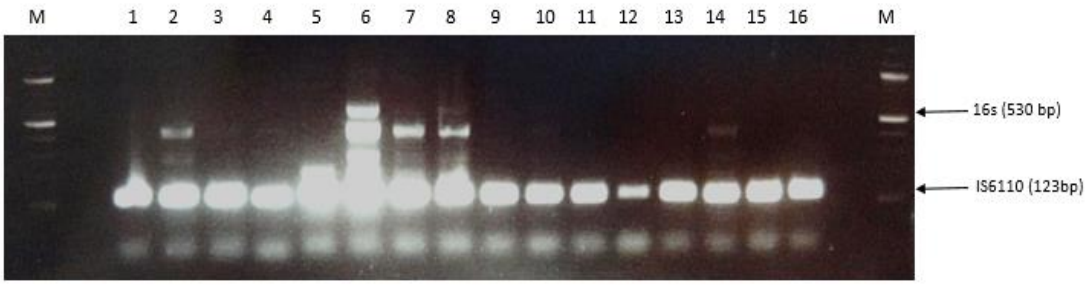
In order to investigate what effect PCR enhancers have on the two round multiplex PCR assay, two enhancers were selected, DMSO and Q-Solution, the commercial PCR enhancer supplied with HotStarTaq Plus Mastermix used in the PCR assays (QIAGEN; UK). DMSO was added at the concentration of 2.5 % (v/v) used previously. Q-Solution was added at a standard volume of 20 % (v/v) as advised by the manufacturer. The same annealing temperature as the pre-amplification PCR (65 °C) was used for the multiplex PCR in the following assay (section 2.4.3.5.). Initially, the two round

multiplex PCR was performed without any PCR enhancers added to either round, this was then compared to DMSO and Q-Solution being added independently either to the first, second or both rounds. Finally, the two enhancers were tested together in the first, second or both rounds. In this instance, the template used was the DNA from five *M. bovis* BCG plaques pooled and extracted with the Spin Column method (section 2.4.1.3.), so that an estimated 5 genome equivalents were used. This was to evaluate whether the non-specific binding was being produced from the *M. smegmatis* DNA or the *M. bovis* BCG DNA, although the *M. smegmatis* DNA was still present in the plaque, the increase in *M. bovis* BCG DNA should increase the levels of non-specific binding if the *M. bovis* BCG DNA is the template for the non-specific binding.

The analysis of the results were not in line with what was expected. The PCR assay that didn't contain enhancers in either round produced strong amplification of the 123 bp corresponding to the *IS*6110 element but no detectable amplification of the 16s rDNA (Fig. 9.3, lane 1). The fact that the 16s rDNA wasn't amplified could again have been caused by the preferential amplification of the *IS*6110 element due to the five-fold increase in starting template. Only when DMSO was used in both rounds of amplification was the expected pattern produced of a band of 530 bp corresponding to the 16s rDNA and the 123 bp *IS*6110 element (Fig. 9.3, Lane 6), which replicated the conditions used in the previous PCR assays. However in this case large amounts of non-specific binding was also produced. Non-specific binding was also produced in the other PCR assays that contained DMSO in the first round (Fig. 9.3, Lanes 5, 7 and 8), but the addition of Q-Solution to the second round reduced the non-specific binding caused by the DMSO (Fig. 9.3, lanes 7 and 8). When Q-Solution was used

independently in the first round, non-specific binding was not produced in any of the PCR reactions (Fig. 9.3, Lanes 9 – 12) but this also reduced the level of amplification of the *IS*6110 element (Fig. 9.3, Lane 12), showing that a higher concentration of Q-Solution than recommended may have an inhibitory effect on the PCR assay. These results ultimately showed that DMSO was the cause of the non-specific binding, and that Q-Solution can be used to improve the stringency of the PCR assay. The results also showed an increased level of non-specific binding occurred with the increase in *M. bovis* template (Fig. 9.3, Lane 6), which suggested that the non-specific binding was originating from the *M. bovis* BCG genome.

Figure 9.3 – Effect of PCR enhancers on PCR stringency



Lane M; 100 bp DNA Ladder (NEB; UK). The PCR enhancers added to the PCR reaction were: Lane 1, No enhancers; Lane 2, DMSO (2nd round); Lane 3, Q-Solution (2nd round); Lane 4, DMSO and Q-Solution (2nd round); Lane 5, DMSO (1st round); Lane 6, DMSO (both rounds); Lane 7, DMSO (1st round) Q-Solution (2nd round); Lane 8, DMSO (1st round) DMSO and Q-Solution (2nd round); Lane 9, Q-Solution (1st round); Lane 10, Q-Solution (1st round) DMSO (2nd round); Lane 11, Q-Solution (both rounds); Lane 12, Q-Solution (1st round) DMSO and Q-Solution (2nd round); Lane 13, DMSO and Q-Solution (1st round); Lane 14, DMSO and Q-Solution (1st round) DMSO (2nd round); Lane 15, DMSO and Q-Solution (1st round) Q-Solution (2nd round); Lane 16, DMSO and Q-Solution (both rounds). The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.5.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on RHS of image). To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.4 µM of each primer MTC F and MTC R (Table 2.2); No PCR enhancers or 5 % DMSO (v/v) and/ or 20 % Q-Solution (v/v) (QIAGEN; UK); 2 µl of Coral Load Dye was

added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min. The PCR product (5 µl) was used as template for the 16s and IS6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.4 µM of each primer MTC F and MTC R and 0.05 µM of each KY18 and KY75 primer (Table 2.2); No PCR enhancers or 5 % DMSO (v/v) and/ or 20 % Q-Solution (v/v) (QIAGEN; UK); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

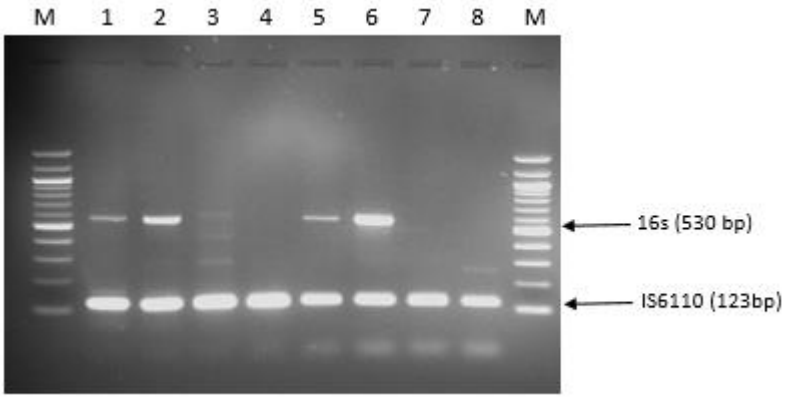
9.1.4. **Assessing the effect of Q-solution on the amplification of the 16s rDNA**

Since DMSO was shown to be facilitating the amplification of the non-specific products in the PCR assays it was removed from the subsequent assays. The next step in the optimization of the multiplex PCR investigated whether Q-Solution was a better alternative as a PCR enhancer. This was also used to see whether the 16s rDNA could be amplified in the presence of Q-Solution, as no 16s rDNA product was produced when Q-Solution was used in the previous assay. In this experiment, one *M. bovis* BCG plaque was again pooled with four *M. smegmatis* plaques and used as the DNA template for the PCR reactions (section 2.4.1.3.). This was chosen in case the failure to amplify the 16s rDNA was because of the over-amplification of the *IS*6110 element. To assess the effect of Q-Solution on the 16s rDNA primers, two concentrations of 16s rDNA primers were tested, 0.02 µM and 0.04 µM. Based on the previous tests performed, the concentration of *IS*6110 primers used was 0.6 µM. An annealing temperature of 65 °C was also used in the second round based on previous results, to reduce any potential non-specific binding that may be produced. The PCR assays containing Q-Solution were again compared with PCR assays without the addition of the PCR enhancer (section 2.4.3.6.).

The results (Fig. 9.4) showed that when PCR enhancers were not added to either round of the PCR assay that the two expected PCR products were amplified, which were a product of 530 bp corresponding the 16s rDNA and a 123 bp product corresponding to the *IS*6110 element. When 0.02 µM of primers were used a low level amplification of the 16s rDNA was produced (Fig. 9.4, lane 1), but an improved level of amplification was produced when 0.04 µM of primers were used (Fig. 9.4, lane 2) and in these cases the assays also didn't produce any non-specific binding.

The intensity of the band produced with 0.04 µM of 16s rDNA primers was ideal for use as an internal control band under these parameters. When Q-solution was added to the first round of the PCR assay, again, the two expected PCR products were amplified, the 16s rDNA and the *IS*6110 element (Fig. 9.4, lanes 5 and 6) and the amplification of non-specific PCR targets was again not seen. The level of amplification of the 16s rDNA was increased when Q-Solution was added to the first round compared with no enhancers being used in either rounds, which showed that a low concentration of Q-Solution is also beneficial for the amplification of the 16s rDNA as well as inhibiting the non-specific binding. The PCR reactions that contained Q-Solution in the second round (Fig. 9.4, lanes 3 and 4) and Q-solution in both rounds (Fig. 9.4, lanes 7 and 8) failed to amplify the 16s rDNA but produced a strong amplification of the *IS*6110 element. This shows that although a low concentration of Q-Solution allows for the amplification of the 16s rDNA, the standard concentration or higher resulted in the inhibition of the amplification. This is likely because the Q-solution has adapted the melting profile of the KY18 primer with its low melting temperature and GC content so that it can no longer anneal to the template at the annealing temperature of 65 °C. There were low levels of non-specific binding produced in the reaction with 0.02 µM of 16s rDNA primers and contained Q-Solution in the second round only (Fig. 9.4, lane 3).

Figure 9.4 – Effect of Q-solution and KY primer concentration on the TB/KY multiplex PCR



Lane M, 100 bp DNA Ladder (NEB; UK). The PCR enhancers used were: Lanes 1 and 2, no enhancers; Lanes 3 and 4, Q-Solution (2nd round); Lanes 5 and 6, Q-Solution (1st round); Lanes 7 and 8, Q-Solution (both rounds). The concentration of KY18/75 primers used were: Lanes 1, 3, 5 and 7, 0.02 µM; Lanes 2, 4, 6 and 8, 0.04 µM. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.6.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (IS6110 element) was 123 bp (indicated by arrows on RHS of image).

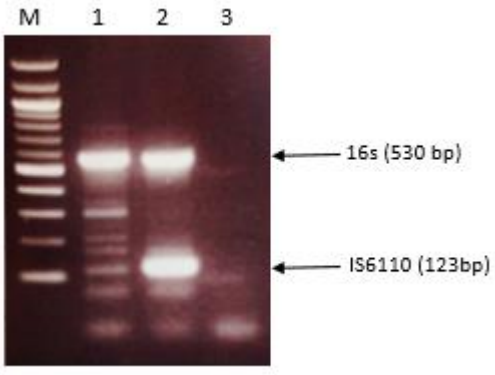
To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each primer MTC F and MTC R (Table 2.2); No enhancer or 20 % Q-Solution (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 12 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for

7 min. The PCR product (5 µl) was used as DNA template for the 16s and IS6110 multiplex PCR assay. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.02 or 0.04 µM of each KY18 and KY75 primer (Table 2.2); No enhancer or 20 % Q-Solution (v/v); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used were an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

9.1.5. **Determining the cause of the false positive *IS*6110 results in *M. smegmatis***

Due to the false positive produced by the *M. smegmatis* plaques, the cause of this needed to be investigated as the two round multiplex PCR could not be used until false-positive *IS*6110 results were not being obtained. The five *M. smegmatis* plaques extracted with the Spin Column method (section 2.4.1.3) was compared with approximately 20 ng of DNA extracted from *M. smegmatis* using the boiled lysis method (section 2.4.1.1.). The optimized PCR parameters were again used (section 2.4.3.7). In this instance the results of the *M. smegmatis* boiled lysate produced a PCR product of 530 bp which corresponded to the amplification of the 16s rDNA (Fig. 9.5, Lane 1). This experiment also detected low levels of non-specific binding, with a band of approximately 123 bp also again being amplified at low levels, however it cannot be said for certain whether this product was derived from an *IS*6110 element or if it is a spurious product of the same size. The use of five *M. smegmatis* plaques as DNA template again produced the amplification of both the 16s rDNA and the falsely positive *IS*6110 element (Fig. 9.5, lane 2). In contrast, the no template (water) negative control sample did not result in amplification of any PCR products (Fig. 9.5, lane 3), which shows that the reagents were not contaminated and that the positive *IS*6110 band in the plaque DNA extract was not produced due to a contamination event.

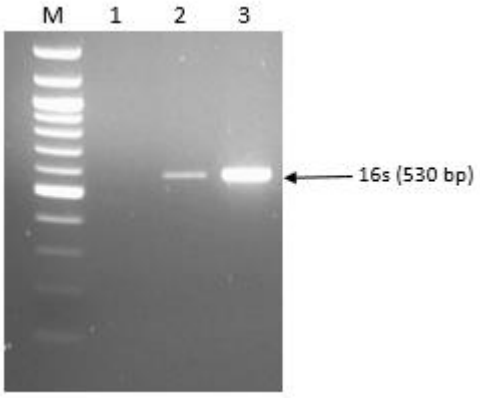
Figure 9.5 – Assessment of the two round multiplex using the *M. smegmatis* genome as template



Lane M, 100 bp DNA Ladder (NEB; UK). The DNA template used was: Lane 1, *M. smegmatis* boiled lysate; Lane 2, 5 *M. smegmatis* plaques; Lane 3; no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.8.). To prepare the singleplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.04 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.04 µM of each KY18 and KY75 primer (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

After this result, the next stage of investigating the false positive *IS*6110 band was to determine whether the false positive was being amplified because of the pre-amplification PCR and if the high sensitivity of the assay was causing the unexpected result. During the investigation, the *M. smegmatis* plaque DNA extract from 5 pooled plaques (section 2.4.1.3.) was tested with the *IS*6110 and 16s rDNA primers independently and as a multiplex using only the parameters for the second round and omitting the pre-amplification PCR (section 2.4.3.8.). The PCR assay that contained only the *IS*6110 primers did not amplify any PCR products and produced a negative result (Fig. 9.6, Lane 1) as expected because *M. smegmatis* should not contain the *IS*6110 element. The PCR assay that contained only the 16s rDNA primers and the multiplex with both primers only produced a PCR product of 530 bp corresponding to the amplification of the 16s rDNA (Fig. 9.6, Lanes 2 and 3). An unexpected observation was that the multiplex PCR showed an increased level of amplification of the 16s rDNA compared to the PCR assay that used the 16s rDNA primer pair independently, despite the same concentration of 16s rDNA primers being added to both the PCR assays. This shows that an unexplained interaction was occurring between the primer pairs. The challenges posed by this two round multiplex assay was likely due to the large difference in thermodynamics and profile of the KY18 primer compared to the other three primers used in the multiplex. There was little that could be done to adjust the KY18 primer so that its properties were within the same range as the other primers, which indicates the difficulties of trying to combine primer pairs produced independently and that when designing a multiplex assay it may be beneficial to design in-house primers which all contain similar melting temperatures and GC content.

Figure 9.6 – Assessment of using only the second round of the multiplex PCR using *M. smegmatis* DNA template



Lane M, 100 bp DNA Ladder (NEB; UK). The primers used in the PCR assays were: Lane 1, *IS*6110 primers; Lane 2, KY18/75 primers; Lane 3, KY18/75 and *IS*6110 primers. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h (section 2.x). Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.3.8.). The expected product size for the KY18/75 primers (16s rDNA) was 530 bp and the expected product size of the MTCF/R primers (*IS*6110 element) was 123 bp (indicated by arrows on RHS of image). To prepare the singleplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.04 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. To prepare the multiplex PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.6 µM of each MTC F and MTC R primer and 0.04 µM of each KY18 and KY75 primer (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s,

65 °C for 30 s, 72 °C for 1 min. This was followed by a final extension step of 72 °C for 7 min.

9.2. Appendix 2

9.2.1. Cloning the pTB-GFP plasmid and transformation into bacteria

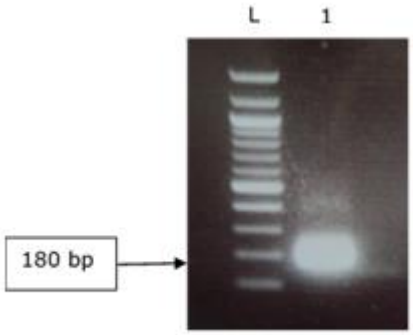
To flank the segment of GFP with the MTCF/R primer sequences, addition-PCR primers were designed. The add the MTCF primers to the GFP DNA chosen, a primer was designed to a 23 bp sequence of the GFP protein and the MTCF primer sequence was then added to the 5' end of the GFP sequence. A restriction enzyme sequence (*NcoI*) was added to the 5' side of the MTCF sequence to facilitate the ligation of the segment into the pMV306 plasmid. Similarly to add the MTCR primer to the GFP sequence, a 21 bp primer complementary to the sequence of GFP located approximately 140 bp upstream of the first primer site was selected and the MTCR primer sequence and restriction sequence for *HindIII* added to the 5' end of the GFP sequence. The restriction sequences were selected to allow directional cloning of the segment into the plasmid pMV306 (see Table 3.1)

To create the IAC sequence that contained both the primer sites and the GFP sequence, these primers were used in a PCR reaction using plasmid pSB2018 as the template (section 2.5.2.1.). This plasmid contains a copy of the GFP gene (Qazi *et al.*, 2001) including the 140 bp sequence required for the production of the internal control segment. To prepare the addition PCR reaction the high-fidelity DNA polymerase Q5 (NEB; UK) was used instead of the HotStarTaq (QIAGEN; UK) used for routine PCR assays because of its ultra-low error rate which was critical to ensure the *IS*6110 primer sequences and the restriction sequences used in the addition primers were amplified precisely so that there were no changes in the sequences. Any errors produced in the primer sequences of the IAC segment may inhibit the annealing of the *IS*6110 primers to the segment. Errors in the restriction sequences may result in

the loss of the restriction site and the inability to cut and ligate the segment into the pMV306 plasmid.

The PCR product was analysed by gel electrophoresis analysis and the results of the addition PCR were as expected, in that a PCR product of approximately 180 bp was amplified (Fig. 9.7, lane 1).

Figure 9.7 – Production of the internal control segment



Lane M, 100 bp DNA Ladder (NEB; UK). Lane 1, internal control segment. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.5.2.1.). The expected product size for the internal control segment was 180 bp (indicated by arrows on the LHS of image). Approximately 50 ng of pSB2018 plasmid was added as template; the primer concentration added for each of the addition primers was 0.5 µM; the buffer and DNA polymerase used was Q5® High-Fidelity 2x Master Mix; SDW was used to produce a final volume of 20 µl. The PCR parameters used was an initial denaturation step of 98 °C for 30 s. This was followed by 30 cycles of: 98 °C for 10 s, 64 °C for 20 s, 72 °C for 20 s. This was followed by a final extension step of 72 °C for 2 min.

The next stage in the development of a competitive IAC was to clone the IAC segment amplified in the addition PCR into the pMV306 plasmid. To do this, the PCR product was diluted with 5 µl of TE buffer and then was purified using a Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB; UK). A volume of 20 µl is the minimum volume usable with the purification kit (section 2.5.2.5.). The concentration of the purified PCR product was 37 ng µl-1 (Nanodrop; section 2.5.2.2) which was sufficient for cloning into the pMV306 shuttle vector.

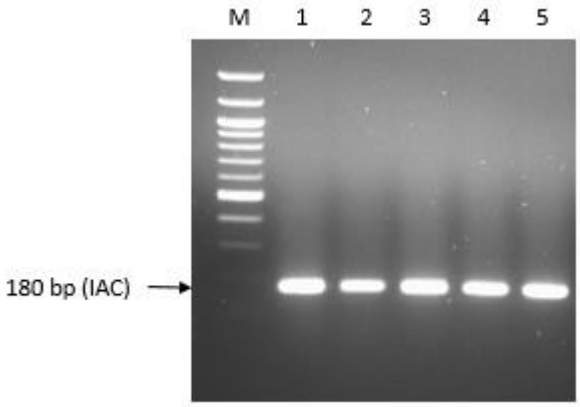
To prepare the IAC segment and pMV306 plasmid for their ligation together, the insert (370 ng) and vector (1 µg) were both independently digested with the restriction enzymes NcoI (NEB; UK) and HindIII (NEB; UK) using NEBuffer 2.1 (NEB; UK) in a double digest for 20 hours. The restriction enzymes were heat inactivated by heating at 80 °C for 20 min (section 2.5.2.4.) and the restriction fragments purified again using Monarch® PCR & DNA Cleanup Kit (5 µg) (NEB; UK) (section 2.5.2.5.). The purified digests of the insert and vector were then used to prepare a ligation mixture. The online tool NEBioCalculator™ (http://nebiocalculator.neb.com/#!/ligation) was used to calculate the amounts required for an insert (180 bp), to vector (3995 bp) ratio of 3:1 and a vector mass of 50 ng (as recommended by the manufacturer of the T4 DNA Ligase (NEB; UK). The final ligation mixture contained 6.76 ng of the IAC segment, 50 ng of the pMV306 plasmid, 1 µl of T4 DNA Ligase and 2 µl T4 DNA Ligase Buffer (10X), SDW to a final volume of 20 µl. The ligation mixture was then incubated at 24 °C for 20 min (section 2.5.2.6.) and the ligated DNA was used to transform the plasmid into electrocompetent E. coli. The transformed cells were plated onto LB agar plates

supplemented with 50 µg ml-1 of kanamycin and incubated for 24 hours at 37 °C (section 2.5.3.1.).

9.2.2. **PCR analysis of pTB-GFP patch plate**

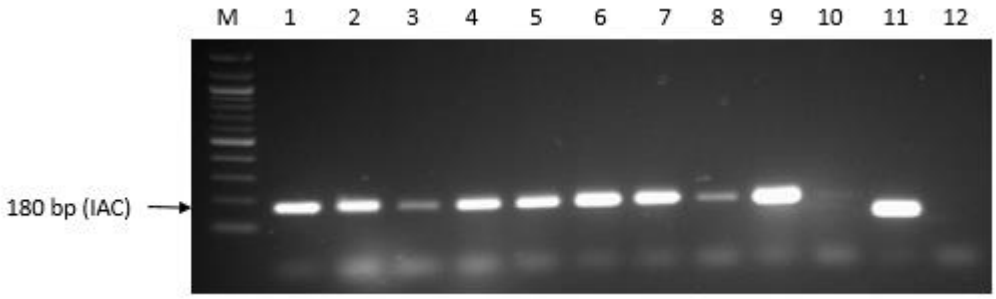
Six transformants were then patch plated using a sterile cocktail stick onto LB agar supplemented with kanamycin (50 µg ml-1; section 2.5.3.2.) and the cocktail stick used for inoculation was then immediately used to add cells to a PCR reaction mixture for colony PCR analysis using the IS6110 PCR assay (section 2.4.2.2.). The results of the PCR assay showed five colonies amplified a PCR product of 180 bp which corresponded to amplification of the IAC segment (Appendix 6; Fig. 9.8, Lanes 1 – 5) and as such showed that these colonies had taken up the plasmid during the electroporation. Once the pTB-GFP plasmid had been successfully built and transformed into *E. coli*, the transformants were inoculated into 5 ml of LB broth supplemented with 50 µg ml^{-1} of kanamycin and incubated overnight with shaking at 37 °C to produce a high cell density culture. DNA was then prepared using a Monarch® Plasmid Miniprep Kit (NEB, UK) to concentrate and purify the plasmid (section 2.5.3.2.) to produce pure DNA with a concentration of 46.6 ng $µl^{-1}$ that was suitable for transformation of *M. smegmatis.* Interestingly, after the transformants were cultured overnight in preparation for a mini-prep, all the transformants lost their plasmid and no longer produced a band on the *IS*6110 PCR. A further ten transformants were picked and new overnight liquid cultures were produced. This time nine out of the ten transformants kept their plasmid and still produced a positive result after the overnight incubation (Fig 9.9)

Figure 9.8 – Colony PCR analysis of pTB-GFP transformed into *E. coli*



Lane M, 100 bp DNA Ladder (NEB; UK). Lanes 1 – 5, *E. coli*-pTB-GFP transformant colonies. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.5.3.1.). The expected product size for the internal control segment was 180 bp (indicated by arrows on the LHS of image). To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

Figure 9.9 – Colony PCR analysis of *M. smegmatis* transformed with pTB-GFP



Lane M, 100 bp DNA Ladder (NEB; UK). Lanes 1 – 10, *M. smegmatis*-pTB-GFP transformant colonies; Lane 11, *M. bovis* boiled lysate positive control; Lane 12, no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.1.1.3.). The expected product size for the internal control segment was 180 bp (indicated by arrows on the LHS of image). To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

9.2.3. **Extending the IAC to incorporate sequences for MAP and *M. avium***

The first primer was designed so that the MK8 primer sequence was on the 5' end, the P91 primer sequence in the middle, joined the MTC F primer sequence and GFP segment on the 3' end. The second primer was designed with the MK7 primer sequence on the 5' end, the P90 primer sequence in the middle, joined to the MTC R primer sequence and GFP segment on the 3' end. A *Hind*III and *Nco*I restriction sequence were added to the 5' end of each addition primer respectively, to facilitate cloning into the pMV306 plasmid.

PCR reactions were prepared using 0.5 µM of each extended addition primer; the previously produce TB-GFP segment was used as DNA template and again Q5 Hi-Fidelity DNA Polymerase 2x Master Mix (NEB: UK) was used to reduce the likelihood of errors being produced in the DNA amplification (section 2.5.2.3.). The results showed a PCR product of 270 bp was produced which corresponded to the amplification of the new full size of the IAC segment (data not shown). As before, the PCR product was diluted with 5 µl of TE to increase the volume to 20 µl and then purified using a Monarch PCR & DNA Cleanup kit (5 µg) (section 2.5.2.5.). The purified PCR product (1 µg) was restricted the enzymes *Hind*III and *Nco*I. After heat inactivated at 80 °C for 20 min (section 2.5.2.4.) the PCR product was ligated into pMV306 as described earlier (section 2.5.2.6.) using 54.6 ng of the vector pMV306 and 11 ng of the digested insert at a 3:1 vector to insert ratio. A sample (1 µl) of the ligation mixture transformed into electrocompetent *E. coli* Top10 cells (section 2.5.1.1.) and transformants plated onto LB agar supplemented with 50 µg ml^{-1} of kanamycin. Five colonies were picked with sterile cocktail sticks for patch plating and colony-PCR (section 2.1.1.3.).

The colony PCR was performed with 0.5 µM of MTCF/R primers or P90/91 primers with HotStarTaq Plus Master mix and the results showed a that a PCR product of 180 bp was produced using the MTCF/R primers and a product at 220 bp was produced when the P90/91 primers were used, both of which corresponded to the expected size of amplicon from the extended internal control plasmid.

The colony PCR was performed with 0.5 µM of MTCF/R primers or P90/91 primers and the results again were as expected, with PCR products of sizes 180 bp and 220 bp which corresponds to the expected amplicons from the integrated IAC plasmid when using the MTCF/R and P90/91 primers, respectively.

9.2.4. **Stability of the pTB-GFP plasmid**

Before the experiment was carried out, the generation time of *M. smegmatis* was used to calculate the time required for the cultures to enter stationary phase from the amount of cells used as the initial inoculum. An initial inoculum of 2 x 10^4 cfu ml^{-1} was chosen since *M. smegmatis* has a generation time of approximately 3 h when grown at 37 °C, therefore it was calculated that it would require approximately 39 h for the 13 generations that would be required to reach a stationary phase cell density of 1 x 10^8 cfu ml^{-1} . To initiate the experiment, 5 ml of 7H9 media supplemented with 10 % (v/v) OADC with and without 25 µg ml^{-1} kanamycin sulphate was inoculated to a cell density of approximately 2 x 10^4 cfu ml^{-1}. The cultures were then incubated with shaking at 37 °C for 39 h to allow the cells to enter stationary phase. After the cells were grown to stationary phase for the first time, the cultures were sub-cultured 1 in 100 into fresh 7H9 media supplemented with 10 % OADC (v/v), again with or without 25 µg ml^{-1} kanamycin sulphate according to the

conditions used in the initial culture. The cultures were then incubated with shaking at 37 °C for a further 39 h to allow the cells to reach stationary phase again, and this process was repeated a further three times until the cultures had entered stationary phase five times. The cells were incubated for 39 hours so that when the cells entered stationary phase they resided within the nutrient depleted and stressed state for several hours, which would provide a prolonged environment for the cells that would encourage the loss of the plasmid. To identify any differences in the percentage of cells that had retained the pTB-GFP plasmid in the two culture conditions, after the cultures had reached stationary phase for the fifth time, the viable count was determined in the absence (total viable cells) and presence (plasmid-containing cells) of 25 µg ml^{-1} kanamycin (section 2.5.3.6.). The experiment was conducted four times to produce an average plasmid loss for a comparison between the two growth conditions. A paired two tailed t-test was performed between the two sets of results to determine whether there was a significant difference between the results. The results of the plasmid stability test are shown in Table 9.1. and showed that there was no significant difference between the rate of plasmid loss when the M. smegmatis (pTB-GFP) strain was grown with or without kanamycin selection (P value of $P = 0.14$, was greater than the limit set of $P < 0.05$). This showed that the plasmid was integrated stably into the genome and that even after prolonged growth in nutrient depleted conditions, there was minimal plasmid loss.

Table 9.1 – Stability of the pTB-GFP plasmid in *M. smegmatis*

	With Kanmycin (25 µg ml)						
	Colony count						
Culture	1	2	3	4	5	Average	cfu/ml
1	18	14	16	15	13	15.2	2.90E+08
2	12	12	11	14	19	13.6	6.80E+08
3	9	9	9	8	5	8	4.00E+08
4	17	11	14	8	22	14.4	7.20E+08

	Without Kanamycin						
	Colony count						
Culture	1	2	3	4	5	Average	cfu/ml
1	7	8	3	8	3	5.8	2.90E+08
2	7	8	8	6	4	6.6	3.30E+08
3	7	5	4	9	7	6.4	3.20E+08
4	11	12	11	8	14	11.2	5.60E+08

M. smegmatis-IAC was cultured in 5 ml of 7H9 media supplemented with 10 (%) (v/v) OADC with and without 25 µg ml-1 kanamycin sulphate was inoculated to a density of approximately 1 x 104 cfu ml-1 by emulsifying several colonies into 1 ml of MP and adding 100 µl of cells to each assay. The comparison was repeated to produce four repeats of the experiment. The cultures were then incubated aerobically at 37 °C with shaking for 39 h until they reached stationary phase. The cultures were then sub-cultured 1 in 100 according to the conditions used in the initial culture and were incubated again aerobically at 37 °C with shaking for a further 39 hours. This process was repeated until the cultures had entered stationary phase five times (section 2.5.3.6.). The cultures were then plated onto 7H10 agar supplemented with 25 µg ml-1 kanamycin sulphate using the Miles Misra method (section 2.1.1.2.). The samples were then incubated at 37 °C for 7 d.

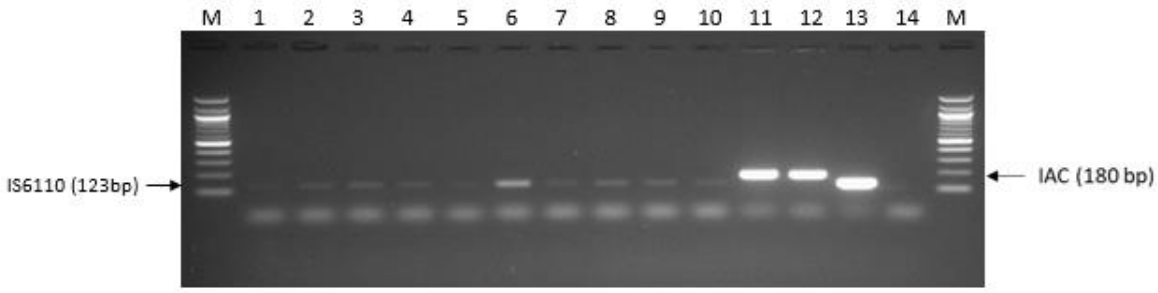
9.2.5. *M. smegmatis*-IAC Oneday assay limit of detection repeat

To determine whether there had been a problem during the cycling conditions, the PCR was repeated with the remaining DNA from the *M. smegmatis*-IAC Oneday phage assay extracts (each Oneday phage assay produces sufficient DNA for 2 PCR assays). The *M. bovis* BCG dilutions were not tested again. In addition, the boiled lysate method (section 2.4.1.1.), was used to prepare DNA from the two *M. smegmatis*-IAC liquid cultures used for the Oneday assays and 25 ng of this was used as an extra DNA template control to confirm that the initial *M. smegmatis*-IAC cultures did contain the pIAC plasmid sequence integrated into its genome.

This time the results for the boiled lysate *M. bovis* positive control were as expected with the amplification of the 123 bp PCR product corresponding to the *IS*6110 element (Fig. 3.18, lane 13). The results for the *M. smegmatis*-IAC DNA extracted using the Oneday assay remained the same (Fig. 3.18, lanes 1 – 10), showing that the expected IAC segment from the integrated plasmid was again not amplified, and instead a weak amplification of a 123 bp product, potentially corresponding to the amplification of an *IS*6110 element which should not be present in this strain was detected. In contrast, the boiled lysate DNA for both of the *M. smegmatis*-IAC cultures amplified a PCR product of 180 bp corresponding to the amplification of the IAC segment (Fig. 3.18, lanes 11 and 12). This showed that the cultures originally contained the integrated pMV306-IAC plasmid before the Oneday phage assay was performed and suggested that the plasmid may have been lost during the Oneday assay and subsequence phage infection, which in turn caused a reduction in the limit of detection to over $1x10^5$ cfu ml^{-1}. One possible explanation for this was that infection with phage D29 may be driving the loss of the IAC plasmid from the

chromosome. The IAC plasmid contains the integrase and *attP* sequence from the temperate phage L5, which was used as the parent phage for the production of the lytic D29 phage used in the Oneday assay. It was thought that the D29 phage infection may have either caused the loss of the plasmid, or that the plasmid was preventing the infection of the D29 phage, resulting in no replication and lysis of the cell to release the DNA.

Figure 9.10 – Repeat of Detection limit of *M. smegmatis*-IAC and BCG in the Oneday phage assay



Lanes 1 – 5, culture 1; Lanes 6 – 10, culture 2. The cfu ml^{-1} of the cells used in the Oneday phage assay were: Lanes 1 and 6, $1x10^5$; Lanes 2 and 7, $1x10^4$; Lanes 3 and 8, $1x10^3$; Lanes 4 and 9, $1x10^2$; Lanes 5 and 10, $1x10^1$. Lane 11, culture 1 positive control. Lane 12, culture 2 positive control. Lane 13, *M. bovis* boiled lysate positive control. Lane 14, no template (water) control. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System. The expected product size for the internal control segment was 180 bp (indicated by arrows on the RHS of image) and the expected product size for the *IS*6110 element was 123 bp (indicated by arrows on the LHS of image). To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer MTC F and MTC R (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 5 min. This was followed by 30 cycles of: 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 7 min.

9.3. Appendix 3

9.3.1. Developing Real-Time PCR assays

The detection limit of the pIAC plasmid was determined by producing a ten-fold serial dilution of purified pIAC plasmid into sterile molecular grade water from 136 ng μl^{-1} to 1.36 $x10^{-8}$ ng μl^{-1}. The DNA polymerase One*Taq®* Quick-Load 2x Master Mix (NEB; UK) was used instead of the HotStarTaq DNA polymerase used in Chapter 3 as it was shown to produce comparable results (data not shown) and already contains the loading dye which reduces processing steps and the buffer was designed to amplify high GC targets, such as mycobacteria (section 2.4.2.3.). *M. bovis* DNA (140 ng μl^{-1}) was diluted 1 in 2 with sterile molecular grade water then a tenfold serial dilution was performed from 70 ng μl^{-1} to 7 $x10^{-8}$ ng μl^{-1} and the DNA used as template for the PCR assay (section 2.4.2.3.). The DNA from the same dilution series were used but only 7 $x10^{-2}$ ng μl^{-1} to 7 $x10^{-5}$ ng μl^{-1} of *M. bovis* DNA and to each of these PCR reactions 1.36 $x10^{-6}$ ng of pIAC plasmid was added and the *IS*6110 PCR assay was performed (section 2.4.2.3.). The results showed that the limit of detection of the *M. bovis* DNA had not been affected and was still 7 $x10^{-4}$ ng even with the addition of the pIAC plasmid, however there was no amplification corresponding the IAC DNA (data not shown). The results showed that the *IS*6110 element decreased the amplification of the IAC DNA, which was likely because the *IS*6110 PCR product is smaller than the IAC PCR product, which caused preferential amplification of the diagnostic DNA.

9.3.2. **Designing a probe for the pIAC plasmid**

The sequence of the GFP segment inserted into the pMV306 plasmid to produce the IAC was entered into PrimerQuest™, to identify the optimum DNA sequence for the IAC probe. A 28 bp sequence was selected which was located 50 bp upstream of the last nucleotide of the MTC F primer with a Tm of 64.62 °C (Figure 4.2; Table 4.1). A BLAST search of the probe sequence was carried out to confirm it wasn't homologous to any DNA sequence in any other species of Mycobacterium, and the results did not find any significant homology to any sequences present in the NCBI database. The fluorophore HEX was chosen as the 5' reporter dye for this probe because the excitation and emission spectra of this fluorophore do not overlap with FAM which was used as the fluorophore for the diagnostic probe. The 3' quencher selected was BHQ-1, since its absorption range completely encompasses the emission range of HEX and provide efficient quenching (Table 4.1).

9.3.3. **Limit of detection of pIAC in a Real-Time qPCR**

The sensitivity of the IAC qPCR assay was determined next to identify the limit of detection of the qPCR assay. This information was needed to determine what the most appropriate quantity of pIAC plasmid DNA would be to add to the in-house qPCR assays to produce a Cq value between 25 and 30 cycles. This value was chosen as it was not predicted to reduce the sensitivity of the diagnostic PCR based on the results gained previously using the end-point PCR assay (section 4.2.1.) and is typical for IACs designed for other qPCR assays. Based on the previous end-point results, it was expected that the appropriate DNA quantity would be 1.37×10^{-5} ng or less. To determine the limit of detection, a ten-fold serial dilution of pIAC plasmid DNA

extracted with the Monarch Miniprep Kit (section 2.5.3.2.) was produced starting at 137 ng μl^{-1} of plasmid DNA and ended at $1.37x10^{-8}$ ng μl^{-1}. To prepare the qPCR assay, 0.5 mM of MTC F and R primers were used with 0.25 mM of IAC probe and 1 µl of each DNA dilution with the Luna Probe Mastermix (NEB; UK). The manufacturers recommended parameters for the Luna polymerase were used (section 2.4.4.1.).

The results showed that the IAC qPCR assay produced a positive signal for each of the pIAC dilutions tested (Fig. 4.4). The no template (water) control was negative and did not produce detectable amplification. The qPCR assay that contained 137 ng of DNA produced a very early positive signal with a Cq value of 3.45, however the reaction contained a saturating amount of DNA template which then produced a less efficient amplification as can be seen with the decreased angle of the slope of the amplification curve compared to that produced for the sample containing 13 ng of template. The reactions that contained from 13.7 ng to $1.37 x10^{-4}$ ng all showed exponential amplification. The remaining PCR reactions with lower quantities of DNA did not produce a full exponential amplification. The lowest amount of pIAC plasmid tested was $1.37 x10^{-8}$ ng which produced a Cq value of 33.92, but again the rate of amplification was low and only produced a small non-sigmoidal curve.

A ten-fold dilution series of DNA for an efficient qPCR assay should produce a ΔCq of -3.3 for each subsequent ten-fold dilution. To determine the efficiency a standard curve was produced, plotting the PCR assay DNA concentration against the Cq value produced (R^2 = 0.990, efficiency = 115 %; Fig. 4.5). The range of acceptable efficiencies are 80 – 120 % (Zhang *et al.*, 2015). This meant that the efficiency of this qPCR assay was within the acceptable range and was an efficient qPCR assay. An

efficiency over 100 % usually means that inhibitors of DNA polymerase are present in the sample, which would explain why the 1.37 x10^{-5} ng sample (Fig. 4.4) showed an arrested amplification after it crossed the threshold. Based on these results it was decided that 1.37 x10^{-6} ng of pIAC DNA would be used as starting template because it was: 1) mid-way between the desired Cq range of 25-30 at 27.90 cycles; 2) was the lowest concentration of DNA added to a PCR reaction that produced a good level of amplification (Hoorfar *et al*., 2004). The Cq value was rounded up to 28 cycles and a range of ±2 cycles was chosen as an acceptable Cq range (26 - 30 cycles) to show there was no inhibition, a range was chosen to account for pipetting differences between different serial dilution preparations causing slight variations in Cq values.

9.3.4. **Designing a dual-labeled probe for the detection of IS6110 in a qPCR assay**

The next step to expand the end-point *IS*6110 PCR assay into a qPCR format was to design a dual labelled probe homologous to a region of *IS*6110 between the binding sites of the MTC F and R primers. To do this the qPCR design program PrimerQuest™ (https://eu.idtdna.com/Primerquest/Home/Index) was again used with the sequence of the *M. tuberculosis IS*6110 element chosen as target sequence. From the different options identified by the program, a 20 bp sequence was chosen with a T_m of 64.62 °C, which was 3 °C below that of the two MTC primers, which is not ideal, but due to the high GC content of these primers this was the highest T_m of the options identified, and was still within the effective range of the MTC F primer (Table 3.1). The specificity of the probe sequence for a 25 bp section that is conserved throughout all members of the MTBC was determined by performing a BLAST search of the NCBI database. The results showed that the probe sequence selected was

present in all members of the MTBC except for *M. pinnipedii*, a member of the MTBC group that is primarily associated with infections in seals, which did not contain this sequence, but this was not seen as a limitation as the intended use of this assay was for livestock and human infections. FAM was selected as the 5' fluorophore and 3' quencher chosen was again BHQ-1 which produces effective quenching of FAM when bound to the probe as FAM's emission range is completely within the absorption range of this quencher.

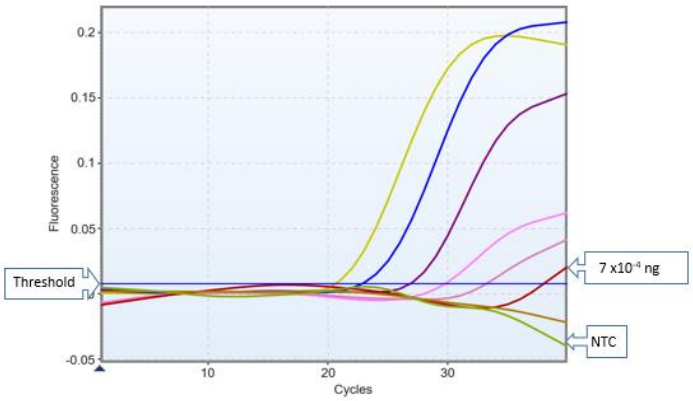
Since it was not possible to achieve a melting temperature of the probe above that of the MTC F/R primers, this could result in amplification events occurring before the probe annealed to the target DNA, resulting in a reduce the efficiency of the qPCR detection event.

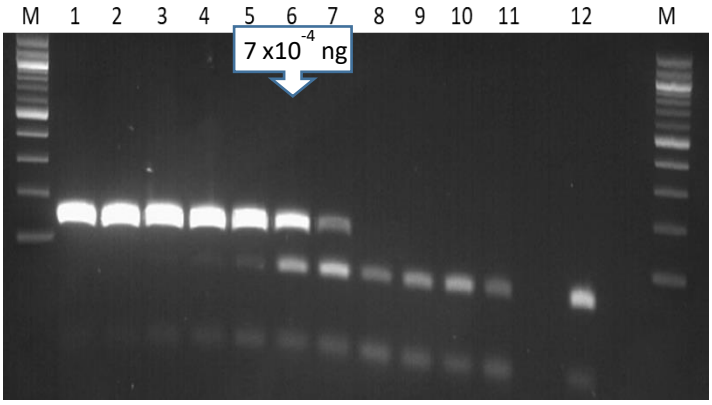
9.3.5. **Sensitivity of *IS*6110 probe**

To assess the sensitivity a ten-fold serial dilution of *M. bovis* DNA extracted with the boiled lysis method (section 2.4.1.1.) was produced with a range of concentrations from 70 ng μl^{-1} to 7 $x10^{-5}$ ng μl^{-1}. The in-house *IS*6110 qPCR assay was then performed by preparing PCR mixtures with 1 µl of each ten-fold serial dilution, 10 µl of Luna Probe Mastermix, 15 µM of the MTC F/R primers, and 5 µM of the *IS*6110 probe. The standard Luna qPCR protocol was used to amplify and detect the DNA (section 2.4.5.2.). The results showed a sensitivity of $7x10^{-4}$ ng of DNA (Fig. 9.11) The efficiency of the in-house *IS*6110 qPCR assay was then calculated by plotting a standard curve (R^2 = 0.990, efficiency = 96 %; Fig. 4.7). The efficiency falls within the acceptable range of 80 % to 120 % and as such shows the qPCR reaction was efficient (Fig. 9.12).

Figure 9.11 - Determining the sensitivity of the in-house *IS*6110 qPCR assay

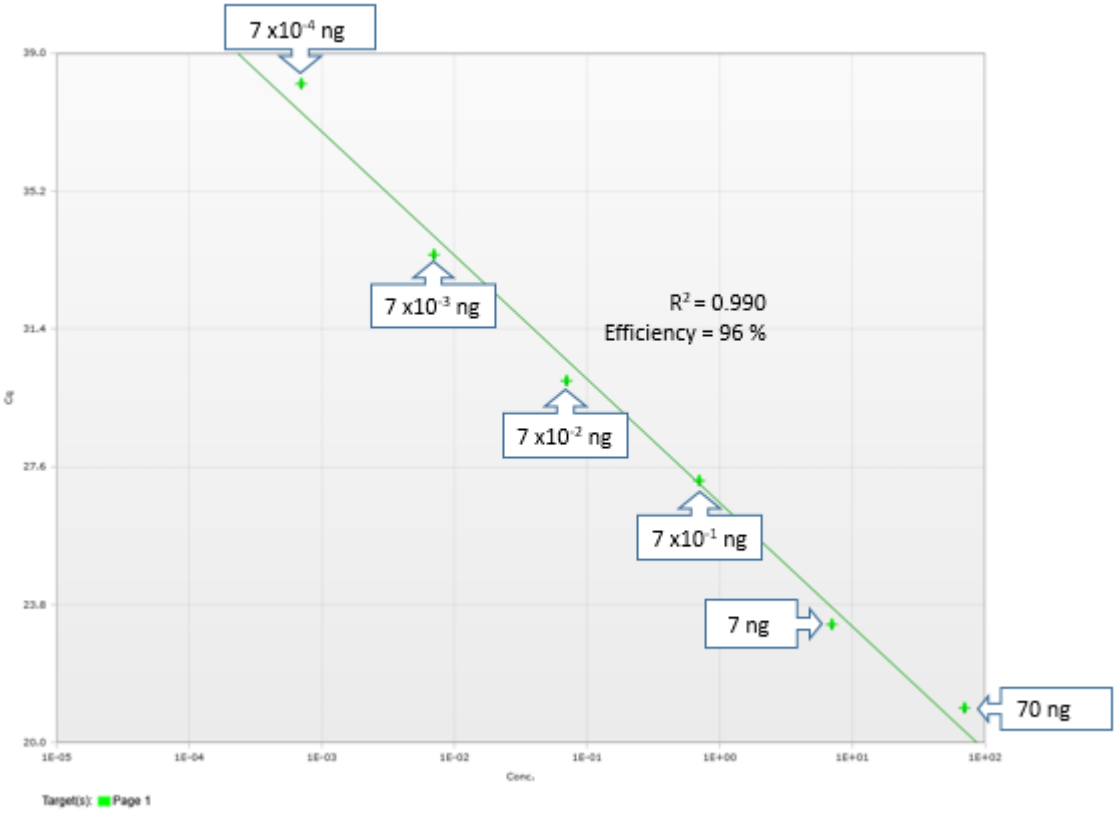
Lane	Key	Cq value
2	M. bovis DNA 70 ng	20.66
3	M. bovis DNA 7 ng	22.86
4	M. bovis DNA $7x10^{-1}$ ng	26.89
5	M. bovis DNA $7x10^{-2}$ ng	29.79
6	M. bovis DNA $7x10^{-3}$ ng	33.22
	M. bovis DNA $7x10^{-4}$ ng	38.00
8	M. bovis DNA $7x10^{-5}$ ng	ND
12	No template control	ND





The in-house *IS*6110 qPCR assay was performed using the standard parameters recommended for the Luna DNA polymerase (section 2.4.5.2.), on a ten-fold serial dilution of *M. bovis* DNA extracted using the boiled lysis method (section 2.4.1.1.) and the DNA concentrations used in each sample are shown in the key. The qPCR was performed on a RotorGene-Q qPCR machine. The results were collected and normalized by the companion Q-REX software (QIAGEN; UK) and the tools 'Dynamic tube' and 'Use noise slope correction'. The Cq value was produced where the samples crossed the threshold. The Cq values can be found in the table along with the comparable lane in the end-point PCR analysis.

Figure 9.12 – Efficiency graph for the in-house *IS*6110 qPCR assay



Standard curve of IAC qPCR assay using ten-fold serial dilution of purified pIAC DNA (section 2.5.3.2.) produced with the Q-REX software (QIAGEN; UK). The plots have been labelled with the DNA concentration used in the corresponding qPCR assays.

9.3.6. **Determining the effect of increasing the primer concentration on the Cq values produced during the in-house IS6110 qPCR assay**

The IAC incorporated into the qPCR assay was designed as a competitive IAC, in that the same primers were used to amplify the diagnostic target as the IAC. Since both amplicons require the same primers, it was assumed that the incorporation of the IAC into the in-house *IS*6110 qPCR assay would result in a delayed Cq value for the diagnostic target compared to the assay without the IAC, because of resource competition between the two reactions. In the end-point assay, it was shown that the addition of the pIAC plasmid did not reduce the sensitivity of the IS6110 detection. However, qPCR assays are supposed to be more sensitive assays compared to end-point assays. In this case it was found that the qPCR assay developed was equally as sensitive as the end-point so optimization steps were undertaken to achieve a more sensitive qPCR assay. It was also assumed that by increasing the primer concentrations in the reactions that also incorporated the IAC, that the delay in the diagnostic Cq value could be reversed so that the Cq values were comparable to the qPCR assay without the IAC. To do this, different concentration of primers were used and the limit of detection compared to the results gained when only the *M. bovis* DNA was present as a target. To prepare the qPCR assays, 7 $x10^{-1}$ ng of *M. bovis* DNA was added to each qPCR reaction, and where appropriate the pIAC plasmid (1.37$x10^{-3}$ ng) was added. The final concentration of primers used in the different reactions were 0.5 mM (the same as the *M. bovis* only reaction), 0.75 mM (1.5-fold higher), and 1 mM of primers (2-fold higher). Each of the test conditions were prepared in triplicate (section 2.4.5.3).

The results of the experiment were not in line with what was expected (Table 9.1). The *M. bovis* only qPCR assays produced a mean Cq value of 28.71 ± 0.32 whereas the mean Cq value for the qPCR assay which also contained the competitive pIAC plasmid DNA was 28.11 ± 0.42. A one-way ANOVA test ($P<0.05$) was performed on the data to test if the difference observed was significant and produced a P-value of 0.291 which showed that the difference in mean Cq value were not significant. With that level of variance these two values are not statistically different from each other (i.e. 28.71-0.32 = 28.39; 28.11+0.42 = 28.53 – the overlap means you can't tell them apart (unless based on hundreds of measurements). Therefore, the incorporation of the IAC into the qPCR assay did not delay the amplification of the diagnostic amplicon. When the primer concentration was increased, the mean Cq values decreased (Table 9.1) and a two-way ANOVA test showed that the difference in Cq value when a concentration of 0.75 mM primer was used was significant ($P = 0.0482$). Although the Cq value when using a concentration of 1 mM primers was also lower than when the standard primer concentration was used, the variation in the results gained for the three replicates meant that the difference in Cq value was not significant ($P = 0.0502$). This variation may have been due to pipetting errors, although the results for the other experiments would not indicate that this was a general problem. It was also possible that this high primer concentration was resulting in large amounts of primer dimer formation which was then interfering with the amplification. Therefore, in further testing it was decided to increase the concentration of primers to 0.75 mM which gave reproducible results without evidence of any significant template competition.

Table 9.1 - Effect of increasing primer concentration on detection of *IS*6110 target

Target DNA	Primer concentration		
	0.5 mM (Cq value)[a]	**0.75 mM (Cq value)[a]**	**01 mM (Cq value)[a]**
M. bovis	28.71 ± 0.32[b]	ND	ND
M. bovis and pIAC	28.11 ± 0.42[b]	27.17 ±0.575 [b]	25.99 ±2.56 [b]

[a]n = 3; [b]SD values

M. bovis DNA (7 x10^{-1} ng) extracted using the boiled lysis technique (section 2.4.1.1.) and diluted into sterile molecular grade water was used in each qPCR reaction. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.5 µM, 0.75 µM or 1 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; No plasmid or 1.36 x10^{-3} ng of pIAC DNA was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection. The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools (section 2.4.5.3.).

9.3.7. **Determining the effect of PCR enhancers on the Cq values produced during the in-house IS6110 qPCR assay**

The next step was to determine whether the addition of PCR enhancers could improve the sensitivity of the in-house *IS*6110 qPCR assay because the high GC content of the target bacteria and primers could possibly result in a reduced amplification efficiency, which could inhibit qPCR assays with low starting template or bacterial load. In order to determine whether the sensitivity of the *IS*6110 qPCR assay could be improved, three different PCR enhancers were tested and compared to a qPCR reaction without any PCR enhancers. The first enhancer that was tested was DMSO (2.5 % [v/v]), the second was Q-Solution (20 % (v/v); QIAGEN, UK) and the third was Q5 GC enhancer (20 % (v/v); NEB, UK). To carry out the comparison, a ten-fold serial dilution of *M. bovis* DNA was prepared with concentrations ranging from 7 $x10^{-4}$ ng μl^{-1} to 7 $x10^{-6}$ ng μl^{-1}. These concentrations were chosen because the lowest limit of detection produced previously was 7 $x10^{-4}$ ng of DNA (section 4.2.3.2.) and 7 $x10^{-6}$ ng of DNA equates to approximately one MAP cell, the practical minimum limit of the in-house *IS*6110 qPCR assay. The qPCR assays were prepared with Luna Probe Mastermix (NEB, UK) and 1 µl of the prepared *M. bovis* DNA dilutions and the PCR enhancers mentioned above (section 2.4.5.4.). The qPCR assay was then performed on the three dilutions of *M. bovis* DNA.

The results are shown in Table 9.2, and it was found that including PCR enhancers did improve the amplification of the *IS*6110 element by reducing Cq values. The results showed that without PCR enhancers the lowest limit of detection was 7 $x10^{-5}$ ng of DNA which was ten-fold more sensitive that in the initial sensitivity test (section 4.2.3.2.) due to the increased primer concentration used. The PCR enhancer that

improved the rate of amplification the most was Q-Solution as it produced the lowest Cq values for the 7 $x10^{-4}$ ng assay and $7x10^{-5}$ ng assay compared to the other PCR enhancers and without PCR enhancers. Q-solution however did not amplify the 7 $x10^{-6}$ ng assay and also cannot be purchased separately from the supplier which would make it unfeasible to add it to the in-house *IS*6110 qPCR assay. When DMSO was used as the PCR enhancer an amplification was produced for the 7 $x10^{-6}$ ng assay, however a small ΔCq value of 0.8 was produced between the 7 $x10^{-5}$ ng sample and the 7 $x10^{-6}$ ng sample which was likely an anomalous result because the ten-fold dilution should have produced ΔCq of around 3.3. This meant that the Q5 GC enhancer produced the best results as it was the only qPCR assay which convincingly amplified the 7 $x10^{-6}$ ng sample which equated to approximately 1 bacterial cell. The enhancer can also be purchased from the supplier who produces both the Luna Probes and this enhance or can be provided as a bespoke Mastermix by this supplier.

Table 9.2: Effect of PCR Enhancers amplification of the *IS*6110

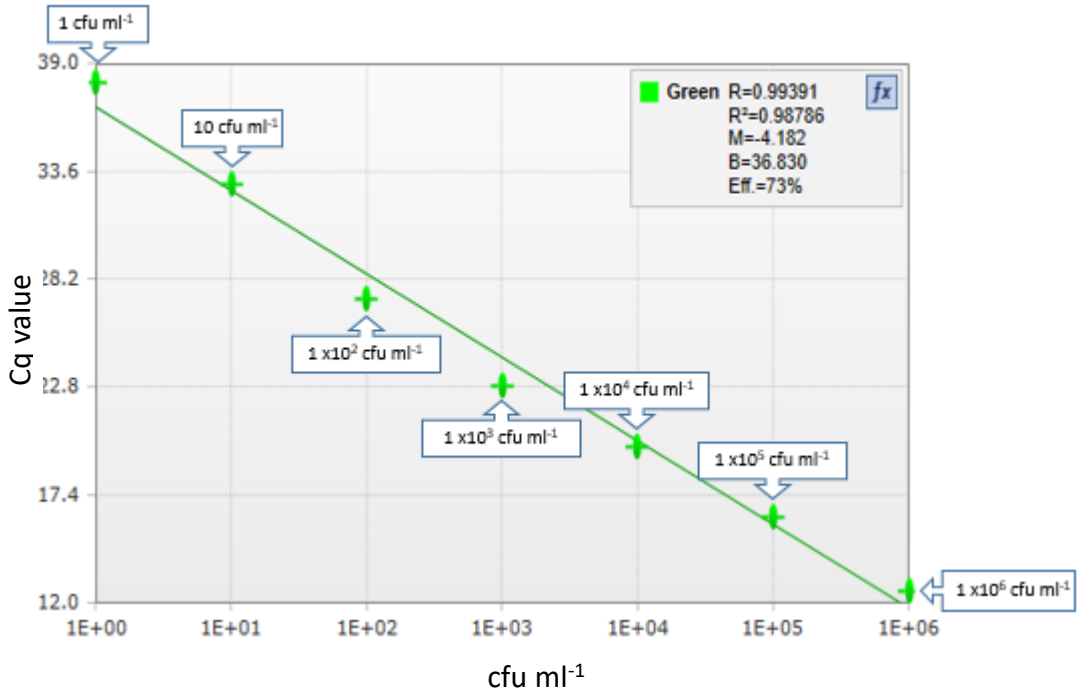
Starting concentration of *M. bovis* DNA (ng)	Enhancer used			
	No enhancer (Cq)	DMSO (2.5 %) (Cq)	Q-Solution (20 %) (Cq)	Q5 GC enhancer (20 %) (Cq)
$7x10^{-4}$	33.34	32.33	31.43	32.03
$7x10^{-5}$	35.13	35.11	34.39	35.43
$7x10^{-6}$	ND[a]	35.91	ND	38.46.

[a]ND = not detected

PCR reactions were prepared as follows; 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 4.2); 0.25 µM of IAC probe; 2.5% DMSO (v/v; Sigma; UK), 20 % Q-Solution (v/v; QIAGENN; UK) or 20 % Q5 GC Enhancer (v/v; NEB; UK) was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The standard parameters recommended for the Luna DNA polymerase were used on a RotorGene-Q qPCR machine (section 2.4.5.4) and the results were collected and normalized by the companion Q-REX software (QIAGEN; UK) and the tools 'Dynamic tube' and 'Use noise slope correction'. The Cq value was produced where the samples crossed the threshold.

9.3.8. **Efficiency graph of the M. bovis BCG Oneday assay dilutions**

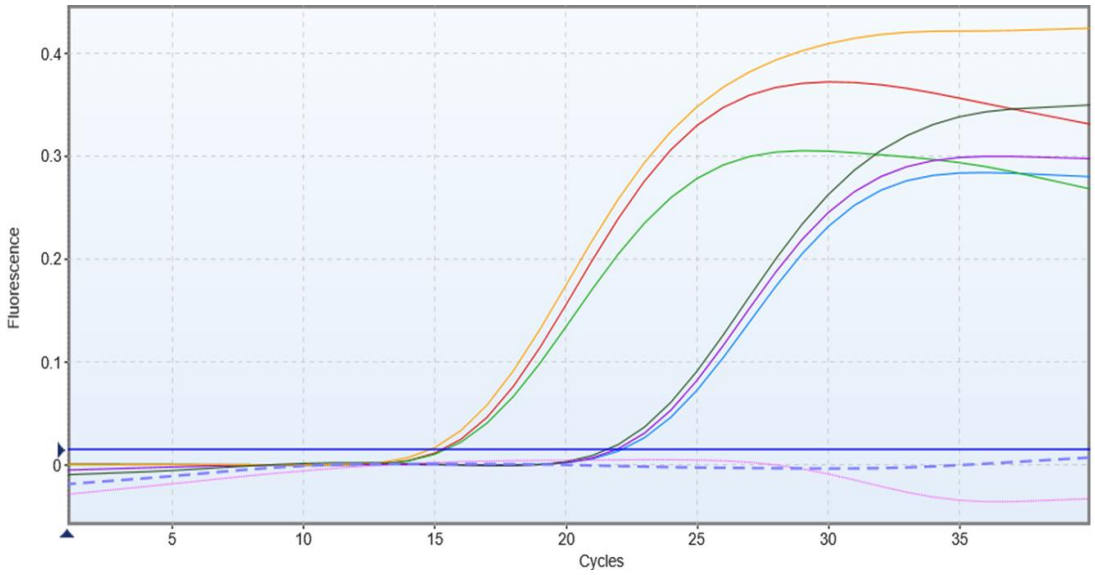
Figure 9.13 – Efficiency graph of the *M. bovis* BCG Oneday assay dilutions



The in-house *IS*6110 qPCR assay with IAC was performed (section 2.4.4.2.) on the DNA extracted from the Oneday phage assay (section 2.2.3.) of ten-fold serial dilutions of *M. bovis* BCG cells. A standard curve was produced from the Cq data using the Q-REX Software (QIAGEN; UK).

9.3.9. **Effect of non-target eukaryotic DNA on IS6110 qPCR amplification plot**

Figure 9.14 Effect of non-target eukaryotic DNA on IS6110 qPCR amplification plot



Key: DNA Sample (primer concentaition)	
1 x10^5 *M. bovis* BCG cells (0.75 mM primers)	1 x10^3 *M. bovis* BCG cells (0.75 mM primers)
1 x10^5 *M. bovis* BCG cells + Human DNA (0.75 mM primers)	1 x10^3 *M. bovis* BCG cells + Human DNA (0.75 mM primers)
1 x10^5 *M. bovis* BCG cells + Human DNA (1.5mM primers)	1 x10^3 *M. bovis* BCG cells + Human DNA (1.5 mM primers)
MP negative process control (-----)	No template (water) negative control

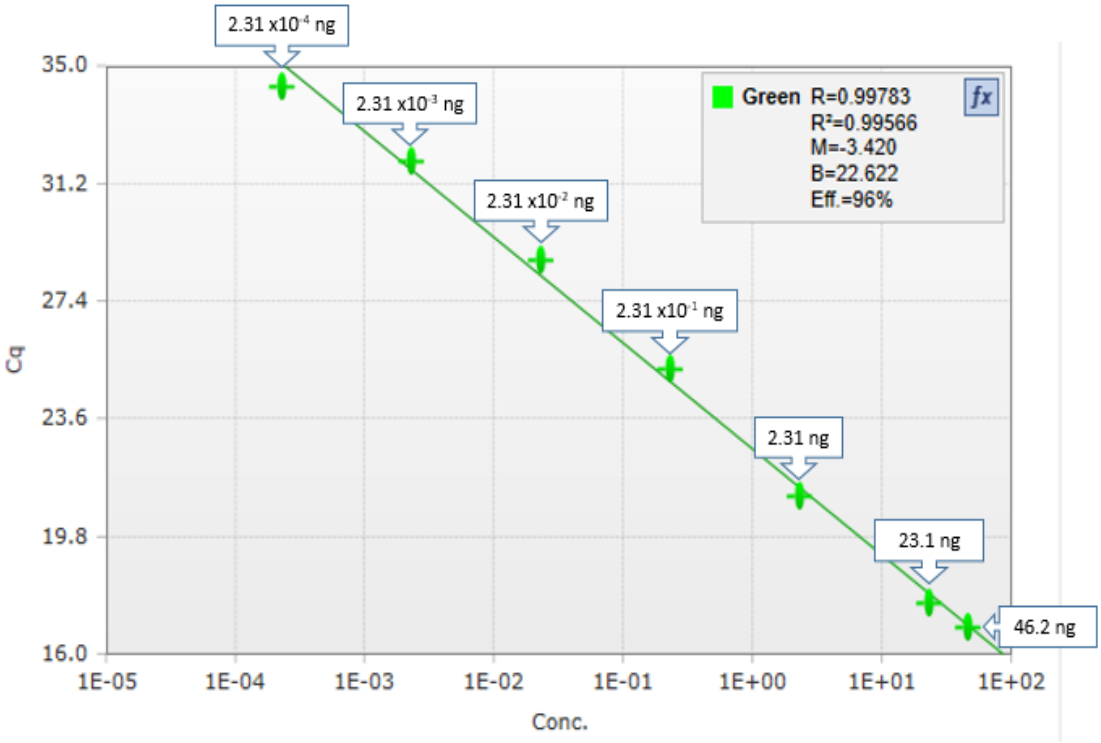
A ten-fold serial dilution of a M. bovis BCG liquid culture (approximately 1 x10^8 cfu ml-1) was prepared to produce dilutions of 1 x10^6 cfu ml^{-1} and 1 x10^4 cfu ml^{-1}. The Oneday phage assay (section 2.2.3.) was performed on 100 µl of each of the

dilutions. DNA (2 µl) from the M. bovis BCG Oneday phage assay extracts were used for the qPCR reactions along with 3 µl *IS*6110 negative PBMC DNA for the appropriate qPCR reactions. To prepare the qPCR reaction mixture: 10 µl of Luna® Universal Probe Master Mix (NEB; UK); 0.75 µM of each primer MTC F and MTC R (Table 2.2); 0.25 µM of IAC probe; 20 % (v/v) Q5 GC Enhancer (NEB; UK); 1 $x10^{-6}$ ng ml^{-1} purified pIAC plasmid was added to a qPCR grade 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used were recommended by the manufacturer and included: an initial denaturation step of 95 °C for 1 min. This was followed by 40 cycles of: 95 °C for 15 s, 60 °C for 30 s and fluorescence detection (section 2.4.5.6.). The data was normalized and smoothed with the 'Dynamic tube' and 'Use noise slope detection' tools with the Q-REX software (QIAGEN; UK).

9.3.10. **Designing MAP real-time qPCR probe**

The PrimerQuest qPCR Assay design tool (IDTDNA, UK) was again used to generate appropriate primer and probe combinations and the optimum pairings were chosen. This resulted in a 21 bp probe with a T_m of 59.82 °C, a forward primer of 22 bp with a T_m of 60.25 °C and a 20 bp reverse primer with a T_m of 59.35 °C. Again, the 5' reporter dye selected was FAM and the 3' quencher selected was BHQ-1. The primers were designated MAP RT F/R and the probe MAP RT Probe (Table 4.1).

Figure 9.15 – Standard curve of MAP RT qPCR assay



The MAP RT qPCR assay was performed (section 2.4.4.3.) on a ten-fold serial dilution of MAP K10 DNA extracted with the boiled lysis technique (section 2.4.1.1.). A standard curve was produced from the Cq data using the Q-REX Software (QIAGEN; UK).

9.3.11. **Performing the MAP RT qPCR assay on historic DNA samples.**

The in-house *IS900* qPCR assay developed for the detection of MAP has been shown to be more sensitive than the well described P90/91 primers and also produces a positive signal from MAP strains that contain the deletion in the region targeted by the P90 primer (see section 6.2.3). Later in this thesis it has also been shown that the P90/91 primers produced false negative results when testing Bison blood samples for the presence of MAP since a subsequent *IS*1311 PCR REA showed that low levels of MAP were present (section 6.2.2.6.). There are also commercial kits available for the detection of the *IS*900 element of MAP which have been used previously in this laboratory. The primer and probe sequences of the commercial kits are trade secrets and as such are not available for analysis. In case the commercial kits were based on the P90/91 primers it was decided to test a selection of historic DNA samples that had been prepared using the Oneday assay previously in the laboratory from bovine blood samples with the in-house *IS*900 qPCR assay to see if any of the previous negative results for MAP gained using a commercial qPCR assay were positive with the in-house assay. Firstly, six of each randomly picked positive, negative and samples scored as “ambiguous” were chosen to be tested. Due to the fact that the MAP RT primer sequences are not found on the pIAC plasmid, it was also an opportunity to test the qPCR assay in a non-competitive format and the MTC F/R primers were used to provide the amplification of the IAC segment as they wouldn’t cross react with the MAP DNA (section 2.4.4.3.).

The results of the in-house qPCR assay were not line with what was expected (Fig. 4.9). Firstly, the results for the IAC step of the qPCR assay showed three of the

Oneday assay DNA samples (IAC Cq values 29 – 30) produced an IAC Cq value within the acceptable range (26 – 30 cycles), showing that there wasn't any inhibitors present. The remaining samples showed that inhibitors were present and produced a range of Cq values between 31 and 34 cycles. The results for the in-house *IS*900 qPCR assay on the samples that were confirmed to be positive in the original analysis showed two positive results in agreement with the original analysis, one sample that produced an ambiguous result and three were negative. Out of the originally ambiguous results, three were confirmed to be positive by the in-house qPCR assay and three confirmed to be negative. The results for the five samples that previously tested negative produced a positive result with the in-house *IS*900 qPCR assay and as such were originally false negative results. The MP negative process control sample was negative for both qPCR assays. The results show that there was a difference between the results produced between the original commercial qPCR assay and the in-house *IS*900 qPCR assay. Both assays target the *IS*900 element present in MAP species, the primer and probe sequences of the commercial kit are trade secrets and unknown however the in-house primers target a small, conserved region within the *IS*900 element that avoids the P90 sequence deletion in some MAP strains.

Table 9.3 – Comparison between the results of the in-house *IS*900 qPCR assay and the original qPCR

Sample ID	Original qPCR result	In-house *IS*900 qPCR assay result
51	+	Ambiguous
56	+	-
57	+	-
58	+	-
60	+	+
62	+	+
30	Ambiguous	+
39	Ambiguous	-
42	Ambiguous	-
46	Ambiguous	+
53	Ambiguous	-
54	Ambiguous	+
13	-	+
31	-	+

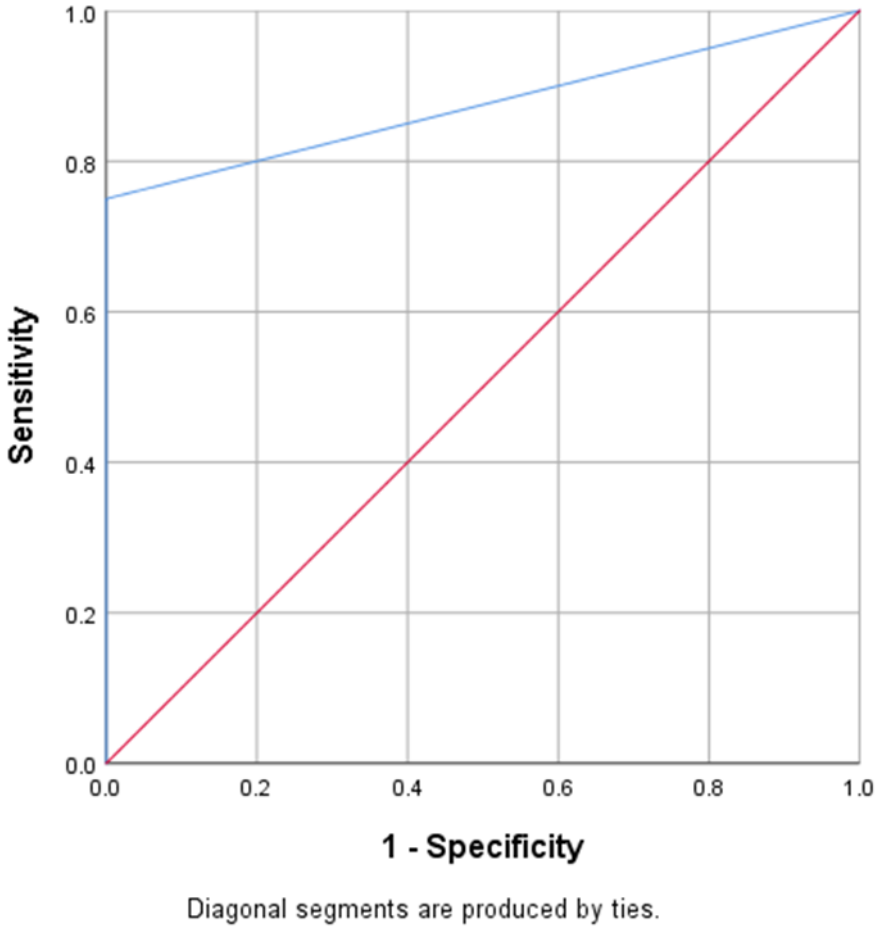
37	-	+
44	-	+
75	-	+
MP	-	-

The in-house *IS900* qPCR assay was performed using the standard parameters recommended for the Luna DNA polymerase (section 2.4.4.3.), on historic DNA samples extracted from bovine blood with the Oneday phage assay previously tested for *IS*900, six previously positive, six samples previously scored as ambiguous and six previously negative sample (including the MP negative process control). A no template (water) control was also included. The qPCR was performed on a RotorGene-Q qPCR machine. The results were collected on the green channel and normalized using the tools 'Dynamic tube' and 'Use noise slope correction' (section) by the companion Q-REX software (QIAGEN; UK), the graph was produced on Microsoft Excel (Microsoft; USA) The Cq value was produced where the samples crossed the threshold which was set by 'auto threshold'.

9.4. Appendix 4

9.4.1. ROC analysis for the combined phage assays compared to the clinical culture techniques after seroconversion

Figure 9.16 - ROC analysis for the combined phage assays compared to the clinical culture techniques (after seroconversion)



A ROC analysis of the data from the combined phage assay compared to the gold standard of clinical diagnosis of TB, the figure was produced using the statistical package SPSS (IBM). The blue line represents the ROC curve of the combined phage assays. The red diagonal line is a representation of a poor test. AUC = 0.875

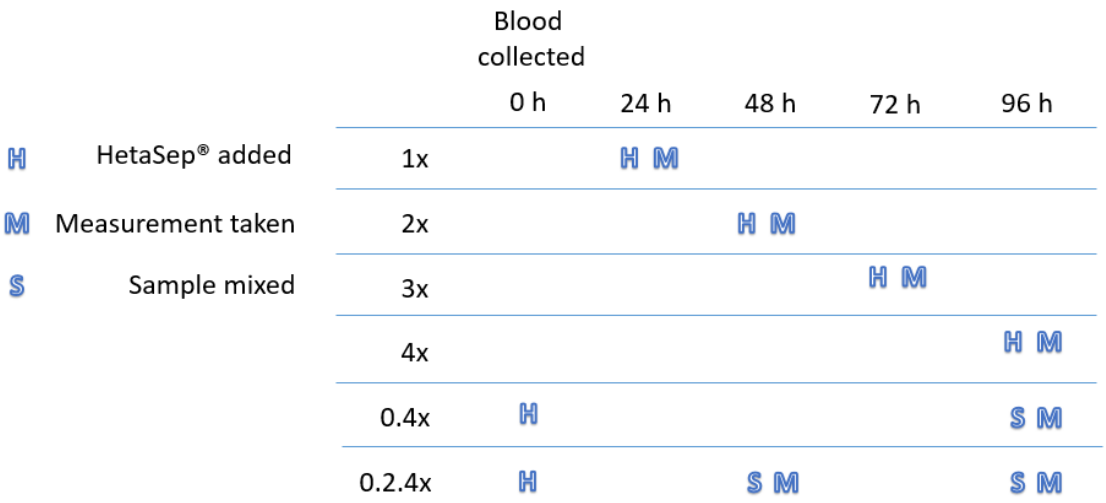
9.4.2. **Evaluation of HetaSep® stability and aged blood**

Based on the results of the first human trial, a second, follow on study was planned to see if the sensitivity of the phage assay for the detection of MTB in human blood could be increased, and also to see if the sample processing methods could be refined. Due to the agreement seen between the HetaSep® and Ficoll® extraction methods, it was decided that in this study, HetaSep® would be the only extraction method used as the reagents were less expensive and also made the process quicker and less labour intensive. The first question to be addressed was the impact of the age of blood samples on the efficiency of the HetaSep® method to recover WBCs. This was important because it was not always possible for the blood samples to be delivered on the day they were taken due to the distance between the hospital where the samples were taken and the laboratory in Nottingham University. The manufacturer's protocol for the HetaSep® extraction method gives guidance for blood up to 48 hours old, however, in the initial study the blood samples that failed the HetaSep® extraction method were approximately one or four days old, whereas three-day old blood still produced aggregation and comparable results to the Ficoll® extraction method. Therefore, experiments were designed to determine how long could be allowed between collection of the blood samples from patients and processing of samples, so that results were not affected due to RBC degradation.

Specifically, the questions to be asked were (1) how much does the age of blood affect the gravity sedimentation with HetaSep®? (2) Can the addition of HetaSep® at the time of blood extraction preserve the blood samples sedimentation rate? (3) If HetaSep® is added at the time of sample collection, will mixing the two layers back

together help to preserve gravity sedimentation rate? The blood sample used for this experiment was donated from a healthy individual and approximately 25 ml was collected. To answer these questions, two parameters were recorded: the rate of gravity sedimentation measured by the distance the interface descended (mm) and the PBMC viability was determined by Trypan blue staining (section 2.1.4.1.). The sedimentation was measured 30 min and 60 min after HetaSep® addition to the blood using a ruler immobilized at the beginning of the study to provide a consistent guide. At the two time points, the height of the erythrocyte aggregation was subtracted from the total height of the sample to calculate the amount of plasma produced. All tests were carried out in duplicate. AN overview of the study design can be seen in figure 5.3.

Figure 9.17 Overview of study design



The table denotes when HetaSep® was added to the samples, when measurements were taken and when the samples mixed to homogenize the two distinct layers during the HetaSep® stability trial.

9.4.3. **How does the age of human blood effect separation with HetaSep®?**

The first set of tests looked at the gravity sedimentation rate and viable count of the blood when HetaSep® was added on consecutive days from extraction. On day one, 24 h after the blood sample was extracted, 400 µl of HetaSep® was added to two of the aliquots and mixed, then after 30 and 60 min the amount of plasma produced was measured (section 2.3.1.2.). The six other aliquots were incubated at room temperature and HetaSep® was then added to duplicate samples at 48 h, 72 h and 96 h after the blood was extracted (Table 9.4). When HetaSep® was added 24 h after extraction, the size of the plasma layer measured 12 mm after both 30 and 60 min, indicating that the separation process reached maximum level within 30 min. When the HetaSep® was added on 48 h after collection, both duplicate samples produced a plasma layer of 12 mm after 30 min, but after 60 min, one of the samples remained at 12 mm, while the other sample increased to 13 mm, so some variation in the separation process was noted. When the HetaSep® was added 72 h post-collection, after 30 min one of the duplicate samples produced a plasma layer of 9 mm and the other sample had a layer of 12 mm. After 60 min incubation, the plasma layers had increased to 11 mm and 13 mm, respectively, again revealing sample-to-sample variation in the process. Finally, when HetaSep® was added after 92 h, no separation was visible in either sample after 30 min and after 60 min incubation, only small plasma layers were formed one (2 mm and 4 mm). These results confirmed what was observed in the first trial, that HetaSep® will provide consistent aggregation with human blood 48 h after collection, but after this the separation process becomes more and more compromised with the age of the blood. Therefore, for any follow on study, the samples would need to be processed ideally no later than two days after

extraction, although even after three days the separation could also be acceptable providing there is no earlier option for processing.

Table 9.4 – Addition of HetaSep® on consecutive days

Sample	Size of plasma layer (mm)	
	30 min incubation with HetaSep®	60 min incubation with HetaSep®
1a	12	12
1b	12	12
2a	12	13
2b	12	12
3a	12	13
3b	9	11
4a	0	2
4b	0	4

HetaSep® was added to 2 ml of blood at a 1:5 ratio and incubated at room temperature for 30 min and the size of the plasma layer was recorded, the samples were then incubated for further 30 min (60 min total) and a final measurement taken. HetaSep® was added to the samples 24 h (1x), 48 h (2x), 72 h (3x) and 96 h (4x) after blood collection.

9.4.4. **Viability of PBMCs in aged human blood**

When the reduction in the sedimentation rate was observed as the blood aged and degraded it was decided to determine whether the reduction was associated with the cell viability of the blood. The viable count of the WBCs in the plasma layer was also determined immediately after the 60 min incubation using Trypan blue staining (40x magnification on an inverted microscope with a haemocytometer).

The results showed that the viability of the PBMC do not decrease between 48 h and 96 h after collection (Table 9.5). Although no reduction in PBMC viability was observed between the samples, the volume of plasma produced when HetaSep® was added 96 h after collection was far too small to extract without also extracting copious amounts of erythrocytes and introducing too many PCR inhibitors. The concentration of PBMCs in the blood samples were also approximately 10-fold lower than the range of 4.5 – 11 $x10^6$ cfu ml^{-1} which has been described previously for the WBC count of whole human blood (Dean, 2005). It is also lower than the concentration of PBMCs extracted from human blood by other studies which reported concentration of 6.2 $x10^6$ cfu ml^{-1} (Crosley *et al*., 2009) and 0.9 – 1.9 $x10^6$ cfu ml^{-1} (Chen *et al*., 2020). These studies used density gradients to extract the PBMCs which may extract more of the cells compared to the HetaSep® method as a portion of the PBMCs will get captured by the aggregating RBCs and become trapped in the erythrocyte layer.

Table 9.5 – Viability of PBMCs from the plasma fraction of whole blood 48 to 96 h after collection

	48 h after collection	72 h after collection	96 h after collection
Viable cells (cfu ml^{-1})	2.53E+05	2.22E+05	3.04E+05

The human heparinized whole blood was first treated with HetaSep® (section 2.3.1.2.) to aggregate and remove the RBC from the plasma, 10 µl of the upper plasma layer was then removed and mixed at a 1:1 ration with Trypan blue stain (Sigma Aldrich; UK). The solution was then transferred to a haemocytometer, the cells were then visualized with an inverted microscope at 40x magnification and the clear cells that had not taken up the stain were counted (section 2.1.4.1.).

9.4.5. **Can the addition of HetaSep® at the time of blood extraction preserve the blood samples sedimentation rate?**

The next test performed was to see if adding HetaSep® on the day of the blood extraction and leaving the sample separated for the full 96 h would improve the gravity sedimentation rate when it would then be mixed for processing (samples 0.4x). These samples were prepared again by adding 400 µl of HetaSep® to the 2ml aliquots and then incubating at room temperature for 96 hours.

The results of the gravity sedimentation showed that there was no sedimentation produced in one of the duplicates even after 60 minutes. The second duplicate produced very minor separation (<1 mm); however, it was not possible to measure as the size was below the scale of the ruler used in the study. This showed that adding HetaSep® on the day of the blood extraction and mixing at the laboratory would not be a suitable method to increase gravity sedimentation of aged blood and appears to decrease the sedimentation rate compared to adding 96 h after collection (Table 5.7).

Table 9.6 – Rate of RBC aggregation of the different samples in the HetaSep® stability trial

	Size of plasma layer (mm)	
Samples	30 min incubation with HetaSep®	60 min incubation with HetaSep®
2a	12	13
2b	12	12
0.2a	13	13
0.2b	10	11
0.2.4a	0	6
0.2.4b	7	7
4a	0	2
4b	0	4
0.4a	0	0
0.4b	0	<1

HetaSep® was added to 2 ml of blood at a 1:5 ratio and incubated at room temperature for 30 min and the size of the plasma layer was recorded, the samples were then incubated for further 30 min (60 min total) and a final measurement taken.

9.4.6. **If HetaSep® is added at the time of sample collection, will mixing the two layers back together help to preserve gravity sedimentation rate?**

The next question that was addressed was to determine if adding HetaSep® on the day of the blood extraction and mixing the samples at the halfway point (48 h) would improve the gravity sedimentation after 96 h, compared to adding HetaSep® after 96 h of storage. On the day that the blood was extracted (day 0), 400 µl of HetaSep® was added to two 2 ml aliquots of blood. The sample was then mixed and incubated at room temperature for 48 h. After 48 h (day 2) the blood samples were shaken gently to homogenize the two layers and allowed to re-sediment at room temperature. The distance the interface descended was measured (samples 0.2x) after 30 and 60 min post mixing and the PBMC viability was assessed after the 60 min incubation. The samples were then incubated at room temperature for 24 h. Then 72 h after HetaSep® addition, the samples were removed to test PBMC viability but the two layers were left undisturbed. Finally, 96 h after HetaSep® addition, 10 µl of the upper phase was removed for PBMC viability testing, before the samples were gently mixed. The samples were then allowed to sediment and the interface depth was measured again after 30 and 60 min. A final PBMC viability assessment was performed 60 min after mixing the two interfaces.

The results for this set of testing showed that when the blood samples were mixed 48 h after HetaSep® was added on the day of blood collection; that the amount of separation was comparable to the amount of separation produced when HetaSep® was added to blood 48 h old after 60 min separation time (Table 9.6). After the samples were allowed to stand for another 48 h (96 h after collection and HetaSep®

addition) they were mixed again a final time. After the full 60 min separation time, the first duplicate produced a plasma layer of 6 mm, and the second duplicate of 7 mm, again, still showing a minor degree of variation between duplicate samples. The results produced 96 h after adding HetaSep® on the day of the blood collection and mixing after 48 h (samples 0.2.4x) were then compared to the results gained from adding HetaSep® 96 h after collection (samples 4x). Both the duplicates showed an increased rate of gravity sedimentation when HetaSep® was added on the day of blood extraction then mixed after 96 h (6 and 7 mm) compared to adding HetaSep® 96 h after collection (2 and 4 mm).

It was noticed that during the prolonged incubation of blood with HetaSep® that a white/ grey layer was produced on top of the erythrocytes. This was expected to be the PBMCs also sediment out of the upper plasma layer over time. If this was correct, then it would decrease the sensitivity of the downstream phage assay if the samples were not combined and re-separated (so keeping the samples separated without mixing before extraction wouldn't be an option). To determine if this was true, the viable cell count was determined before the samples were mixed to identify if there had been a decrease in cell concentration and then again after they had been mixed to see if the cell concentration increased back to the expected levels. The duplicates that had HetaSep® added on the day of extraction were used for this testing (0.2.4x). A viable count was performed 72 h after the HetaSep® was added to the blood sample and then a second viable count was taken 96 h after the HetaSep® was added before the samples were mixed and then a final time after the samples were mixed. The results showed that there was a reduction in viable cell count (cfu ml^{-1}) for both of the duplicates between 72 h and 96 h. After the samples had been combined

again and allowed to sediment one of the duplicates failed to separate so no viable count of the PBMC's could be performed due to heavy erythrocyte contamination. The second duplicate produced <1 mm plasma separation and showed that the viable cell count did increase back to normal levels Table 9.7). The results showed that the PBMCs did start to sediment after prolonged incubation with HetaSep®, but that mixing reintroduced them back into solution. The sample size tested was small and there may be greater variation in the results produced if more samples were tested so these results are an indication of the expected pattern of results. Despite the fact it was possible to reintroduce the cells back to the upper plasma layer, the amount of separation produced 96 h after HetaSep® added was too low to make the technique effective.

Table 9.7 – The concentration of PBMCs in the plasma layer of HetaSep® aggregated blood between 48 and 96 h after HetaSep® addition

Sample	Viable cells (cfu ml^{-1})		
	72 h after HetaSep® addition	96 h after HetaSep® addition	96 h after HetaSep® addition - mixed
A	$2.96x10^5$	$1.56x10^5$	ND
B	$9.6x10^4$	$2.00x10^4$	$3.28x10^5$

The human heparinized whole blood was first treated with HetaSep® (section 2.3.1.2.) to aggregate and remove the RBC from the plasma, 10 µl of the upper plasma layer was then removed and mixed at a 1:1 ration with Trypan blue stain (Sigma Aldrich; UK). The solution was then transferred to a haemocytometer, the cells were then visualized with an inverted microscope at 40x magnification and the clear cells that had not taken up the stain were counted (section 2.1.4.1.).

The results of the trial showed that to perform a successful immune cell extraction with HetaSep®, the blood samples would need to be processed within 2 days (48 h) of the extraction. Sedimentation could still be achieved after 72 h however the amount of sedimentation was reduced. Adding HetaSep® on the day of extraction and mixing the sample on subsequent days before processing produced improved separation on day four but the separation was still too low and the small volume of plasma and PBMC's produced would have reduced the sensitivity of the downstream phage assays.

9.4.7. **Evaluating eukaryotic lysis and the effect on DNA concentration**

During these experiments it was observed that all the PBMCs were not lysing as they were expected to do when they were reconstituted with MP. This meant that the intracellular *M. tuberculosis* would not be released into the media so that they could be infected by phage D29 during the Oneday phage assay, potentially reducing the sensitivity of the assay. As a result, experiments were performed to optimize the WBC lysis by resuspending them in distilled water rather than MP to induce a more severe osmotic shock.

The first step was to identify how long it would take for the sterile distilled water to cause the complete lysis of the PBMC's. To determine this the PBMC's were extracted from 2ml of whole blood using the HetaSep® extraction method. The PBMC's were reconstituted into 1 ml of MP and incubated overnight at room temperature. A sample was then taken and combined at a 1:1 ratio with Trypan blue to determine the viability of the cells; a 100 µl sample of the PBMC's in the MP was then removed to determine whether the subsequent centrifugation step would

cause the lysis of the PBMCs through the centrifugal force (centrifugation control). Both samples were then centrifuged at 13,000 xg for 3 min. The centrifugation control was then mixed at a 1:1 ratio with Trypan blue for viability testing. The supernatant was then removed and the PBMC pellet was reconstituted with 1 ml of sterile distilled water. A sample was taken immediately for viability testing (T=0) and then every 10 min for 30 min then every 15 min until a total time of 60 min was achieved (section 2.1.4.1.).

The results were not in line with what was expected (Table 9.8). The results for the viability of the PBMC's after being reconstituted in MP showed that the majority of the PBMC were still viable with intact membranes (91.7 % viability). The centrifugation step did not promote the lysis of the PBMCs but did remove a portion of the non-viable cells (97.4 % viability). When the PBMC's were reconstituted with sterile distilled water at T=0 the viability of the cells was calculated at 73.2 %. After ten min incubation (T=10) the results showed that the viability of the PBMC's had decreased to 36.3 %. After 20 min incubation with sterile distilled water none of the PBMC's remained viable (0 %). After this time point the following viability samples (T=30, 40, 50 and 60) all showed that the percentage viability was 0 % and all the eukaryotic cells were no longer viable. The results showed that incubating the PBMC's overnight in MP resulted in a percentage of viable cells of 91.7 % compared to 0 % (complete lysis) when the PBMC's were incubated with sterile distilled water for 20 minutes.

Table 9.8 Effect of lysis method on viability of PBMCs

	MP overnight	Post-centrifugation	T=0	T=10	T=20	T=30	T=45	T=60
Viable cells (cells ml^{-1})	2.22×10^6	2.24×10^6	1.86×10^6	5.8×10^5	0	0	0	0
Non-viable cells (cells ml^{-1})	2.00×10^5	6×10^4	6.8×10^5	1.02×10^6	2.60×10^6	9.2×10^5	1.72×10^6	2.08×10^6
Viability (%)	91.7	97.4	73.2	36.3	0	0	0	0

The PBMCs were first extracted with the HetaSep® technique (section 2.3.1.2.) and then tested for viability by combining at a 1:1 ratio with Trypan Blue viability stain. The cells were then transferred to a haemocytometer and visualized under an inverted microscope at 40x magnification. The clear, viable cells (that had not taken up the stain) were counted along with the blue stained, non-viable cells and the percentage viability was calculated.

The water lysis procedure that had been developed, was then tested using blood samples from six patients. To perform the optimization procedure (section 2.3.2.), the samples were extracted with the standard HetaSep® extraction method up to the PBS wash step (section 2.3.1.2.). After the PBS wash, the supernatant was removed and the PBMC pellet was reconstituted in 600 µl of sterile distilled water and homogenized by pipette mixing. The samples were then incubated for 20 min at room temperature. The samples were then vortexed and 750 µl 2x 7H9 media and 150 µl OADC was added to the samples to make 1.5 ml MP at the standard concentration. The samples were then incubated overnight at 37 °C. The procedure was then the same as the standard procedure for the Oneday phage assay (section 2.2.3.). After the DNA produced from the Oneday phage assay was purified, one sample from the standard testing procedure was selected from each 6 different patients and the quantity and quality of the DNA was determined using a Nanodrop (ThermoFisher; UK), the results were compared to the DNA extracted from the optimized PBMC lysis method.

When the new lysis method was used, five out of the six samples tested produced a greater concentration of DNA from the optimized PBMC lysis procedure, and this was also found to be of better purity compared to the standard lysis procedure (Table 9.9). For the remaining sample, the standard lysis procedure had a greater concentration than the new lysis method, but the purity of the two samples were comparable. The results showed that by adding the water lysis step, the final quantity of DNA produced from the Oneday phage assay was greater than the standard testing procedure, showing that more PBMC's were lysed with the water lysis step than compared to the standard method.

Table 9.9 – Determining the viability of PBMC's in MP compared to sterile distilled water

	Standard testing procedure			Optimized PBMC lysis procedure		
Test ID	DNA ($ng\ ul^{-1}$)	260/280 ratio	260/230 ratio	DNA ($ng\ ul^{-1}$)	260/280 ratio	260/230 ratio
1	13.1	1.24	0.36	93.2	1.79	1.50
2	28.7	1.38	0.71	32.0	1.41	0.75
3	14.6	1.15	0.49	83.6	1.09	0.71
4	9.5	1.50	0.47	110.2	1.79	1.51
5	35.6	1.47	0.44	82.6	1.15	0.71
6	70.9	1.70	1.11	38.6	1.60	1.23

The PBMCs were extracted with the HetaSep® method (section 2.3.1.2.) and lysed using the optimized PBMC lysis protocol (section 2.3.2.). The DNA concentration and purity was then determined using a NanoDrop™ (ThermoFisher; UK)

9.5. Appendix 5

9.5.1. Optimizing blood culture methodology

In the pilot study an attempt to culture viable MTBC from the PBMCs extracted from the blood of human patients with active pulmonary disease did not produce positive MTBC colonies. To try and better simulate the blood environment the MTBC would be isolated from, a bespoke agar was produced using the waste erythrocytes from the HetaSep® extraction method and an in-house semi-solid agar. Semi solid agars have been shown to improve the growth rate of slow growing mycobacteria (Knox *et al*., 1956). A microaerophilic environment was also used for incubation to better simulate the conditions of the blood and lungs compared to the normal atmospheric conditions.

To prepare the agar for each patient sample, 2 ml of whole blood was aggregated with the HetaSep® extraction method (section 2.3.1.2.). The upper plasma layer was removed (approximately 1 ml) and set aside to inoculate the agar once it had been prepared. The separated erythrocytes (approximately 1 ml) were then mixed thoroughly with equal volumes (9.5 ml) of molten and tempered 7H10 agar supplemented with 10 % (v/v) OADC and 7H9 media supplemented with 10 % (v/v) OADC was added to and mixed by thoroughly by gentle inversion. The semi-solid blood agar was then poured into a petri dish and allowed to set (section 2.1.1.6.). The upper plasma layer was then gently spread over the surface of the blood agar and using a sterile spreader. The blood cultures were then incubated in an air tight box with a CampyGen pack (OXOID; UK) to generate a microaerophilic environment before being incubated at 37 °C for up to eight weeks. Samples that showed no growth were discarded at this point.

In contrast to the first trial, five samples produced presumptive *M. tuberculosis* colonies (one to three colonies per sample) based on colony morphology (i.e. growth was only evident after one week of incubation and colonies were characteristically small, rough and either pale white or non-pigmented). The colonies were produced from the blood of three separate patients, two patients produced colonies on two consecutive samples whilst one patient produced colonies on a single sample. The growth results were compared to the end-point and qPCR results to determine whether there was a correlation between the results. All the end-point results were negative. The qPCR results were variable. Patient 570 produced one positive result out of five, then no positive results in the second sample. Patient 575 produced one positive result out of five for both samples. Patient 584 produced four out of five positive results. By comparing the results it would be expected that only patient 584 may have MTBC circulating within the blood based on the four positive qPCR results, however there was also only one small colony that was cultured, which would indicate that if these colonies were MTBC that the culture method still needs refining because the bacterial load of the blood would be greater than 0.5 cfu ml^{-1} as this is below the limit of detection of the Oneday phage assay combined with the in-house *IS*6110 qPCR assay. The colonies were sub-cultured onto 7H10 agar supplemented with 10 % OADC to ensure purity and prior to DNA extraction. However, these had not grown sufficiently when the laboratory closed due to the national lockdown. Unfortunately, over the course of the lock down all the samples were lost due to the overgrowth of white mould and therefore had to be discarded so further identification by PCR could not be carried out.

Table 9.10 – comparison between the amounts of colonies produced using the bespoke blood culture method and PCR results

Samples (test ID)	Colonies	End-point positives	qPCR positives
1a (570)	1	0	1
1b (570)	1	0	0
2a (575)	2	0	1
2b (575)	3	0	1
3 (584)	1	0	4

The bespoke blood culture method described in section 2.1.1.6. was used with human blood to try and culture viable *M. tuberculosis*. PCR identification could not be performed on the colonies so they were compared to the amount of positive end-point and qPCR assay results (up to 5 tests from each PCR assay) to indicate the likelihood of the colony being MTBC.

9.6. Appendix 6

9.6.1. Screening of bison samples for the presence of the IS900 element – detection of MAP

A MAP diagnostic PCR was carried out using the P90/91 primer pair targeting the IS900 element conserved with MAP. To prepare the PCR assays, 5 µl of the eluted DNA was used as the template for the PCR assay and added to HotStarTaq-Plus Mastermix DNA polymerase (QIAGEN; UK) and 0.5 µM of each P90/91 primers. The PCR parameters used were described by Sanderson *et al.* (1992). The results were compared with the DNA extracted from the Oneday phage assay positive control, which should give a positive result (section 2.4.2.4.). The results for the MAP K10 cells produced the amplification of a 400 bp PCR product which corresponded to the amplification of the IS900 element with the P90/91 primers. The MP negative control sample gave a negative test result.

In this case, the results for all seven animals were negative, showing that no MAP DNA had been detected in these samples using this method (data not shown). The remaining DNA from these bison blood samples was then stored at -20 °C until the farmer confirmed that the animals has been sent to slaughter, in accordance with the rules of the UK bovine TB control program.

9.6.2. Screening of samples for the presence of mycobacterial 16s rDNA – detection of Mycobacterium species

The question remained whether these animals were infected with another type of mycobacteria, as suggested by the positive SICCT to determine whether the infecting organism was a member of the *Mycobacterium* genus by using the pan-mycobacteria

primers (KY18/75) to detect the presence of mycobacterial 16s rDNA sequences. Of the seven bison tested, a result was only gained for six animals, as one PCR tube perished during the PCR assay, and the assay was not repeated due to the limited amount of DNA available. To prepare the PCR assays, the primers were used at a concentration of 0.5 µM with HotStarTaq Plus Master Mix (QIAGEN; UK), 5 µl of DNA elute was used as template (section 6.2.2.2). The PCR parameters used were those described by Tevere *et al*. (1996). The results were compared with a no template (water) control where 5 µl of molecular grade water replaced the DNA elution as the template and the MP negative process control from the Oneday assay which should both produce a negative result. A positive control sample of approximately 25 ng of *M. smegmatis* DNA extracted using the boiled lysis method (section 2.4.1.1.) was also included which should produce a positive result.

The results of the PCR assay gained (Fig. 9.18) were in line with the expected results. The *M. smegmatis* DNA (Fig. 9.18, lane 2) produced a PCR product of size 530 bp which corresponded to the amplification of the 16s rDNA and the no template control (Fig. 9.18, lane 4) was negative.

All six of the bison tested produced a band of 530 bp corresponding to the amplification of the 16s rDNA of mycobacteria (Fig. 9.18, lanes 8 – 13). The intensity of the PCR product generated for the bison 1, 2 and 3 samples (Fig. 9.18, lanes 13 – 11) was noticeably higher than that produced from the bison 4, 5 and 6 samples (Fig. 9.18, lanes 10 – 8). Although this PCR is not quantitative, this suggested that the latter three samples had a lower bacterial load compared to the first three. However,

the MP negative process control (Fig. 9.18, lane 6) also produced a PCR product of 530 bp, at a higher intensity than all the bison samples.

Figure 9.18 – Analysis of DNA samples from bison blood using a pan-mycobacterial 16s rDNA PCR assay

Lanes 1, 3, 5 and 7 contained no PCR products. Lane 2, M. smegmatis DNA (25 ng); Lane 4, no template (water) control; Lane 6, MP negative process control; Lane 8, Bison 6; Lane 9, Bison 5; Lane 10, Bison 4; Lane 11, Bison 3; Lane 12, Bison 2; Lane 13, Bison 1. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.1.). The expected size of the PCR product produced by the KY18/75 (16s rDNA) primers was 530 bp (indicated by arrows on the LHS of image). Lane M, 100 bp DNA Ladder (NEB; UK).

To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 15 min. This was followed by 30 cycles of: 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 5 min.

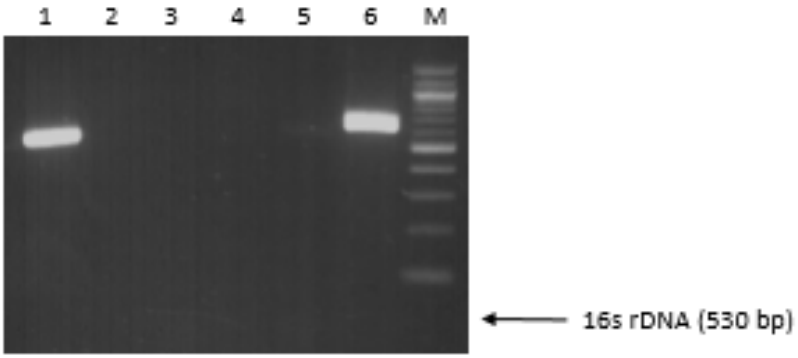
9.6.3. **Investigation of source of mycobacterial rDNA contamination**

Due to the issue of the unexpected result gained from the MP control sample, an experiment was then performed to identify the source of the contamination. It was hypothesized that either the batch of MP was contaminated by mycobacterial cells or that the mycobacterial DNA originated from the D29 phage stock which is propagated on *M. smegmatis*. To resolve this question, Oneday assays were prepared using 100 µl of the same bottle of MP used for the Oneday phage assay but with no addition of phage and, to replicate the sample that had given the positive result in the 16s rDNA PCR assay, a second tube was prepared with 100 µl of the same MP but with 10 µl of the same phage ($1x10^9$ pfu ml^{-1}) used in the Oneday phage assays. The two tubes were then immediately centrifuged at 13,000 x*g* for 3 min at room temperature (as normal after the incubation of Oneday assays), and then the DNA was purified and concentrated using a Clean and Concentrator – 5 kit (ZymoResearch; UK), again eluting the DNA in a final volume of 10 µl (section 2.4.1.4.). PCR assays were prepared and carried out using the parameter described above (section 6.2.2.5) using 5 µl of the DNA as template. The results were again compared with a no template (water) control and also a positive control of approximately 20 ng of *M. smegmatis* DNA extracted using the boiled lysis method (section 2.4.1.1.).

The results of the PCR assay showed that the MP sample without phage (Fig. 9.19, lane 5) and the no template control (Fig. 9.19, lane 3) were negative, which showed that the media used for phage assays of the bison samples was not contaminated with mycobacteria. The MP sample to which D29 phage had been added (Fig. 9.19,

lane 6) however produced a PCR product of 530 bp corresponding to the amplification of the 16s *rrn* gene sequence of *Mycobacterium*. This result showed that the D29 phage samples still contained low levels of extracellular DNA produced during the lysis of *M. smegmatis* during the phage propagation that was carried through the phage purification process. This result meant that the results for the bison samples which had been screened using the pan-mycobacterial primers were inconclusive.

Figure 9.19 – Investigation of source of mycobacterial rDNA contamination



Lane M, 100 bp DNA Ladder (NEB; UK). Lane 1, *M. smegmatis* (20 ng) DNA; Lane 3, no template (water) control; Lane 5, MP: Lane 6, MP and D29. Lanes 2 and 4 contained no PCR products. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.1.). The expected size of the PCR product produced by the KY18/75 (16s rDNA) primers was 530 bp (indicated by arrows on the RHS of image).

To prepare the PCR reaction mixture: 10 µl of HotStarTaq Plus Master Mix (QIAGEN; UK); 0.5 µM of each primer KY18 and KY75 (Table 2.2); 2 µl of Coral Load Dye was added to a 0.2 ml PCR tube and made up to 20 µl with sterile molecular grade water. The PCR parameters used was an initial denaturation step of 95 °C for 15 min. This was followed by 30 cycles of: 94 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s. This was followed by a final extension step of 72 °C for 5 min.

9.6.4. **Screening of the bison samples for the *IS*6110 element**

The bison were originally tested for MAP to see if this was the causative agent of the unexpected SICCT reaction, however since the P90/91 primers failed to identify any MAP DNA, the next question was whether the Oneday assay could be used to identify any other types of mycobacteria present in the blood samples. As the animals had been culled, their blood samples could now legally be tested using PCR to detect the presence of MTBC DNA, which would be the obvious cause of the positive SICCT result.

In this case the MTC F/R primers (section 2.4.2.2.) were used which target the *IS*6110 element of MTBC bacteria. PCR reactions were prepared using 5 µl of template DNA template, 0.5 µM of each primer and HotStarTaq Plus Master Mix (QIAGEN; UK). The PCR parameters used were described by Eisenach *et al.* (1990) and the results were compared with a MP process negative control and as a positive control approximately 20 ng of *M. bovis* BCG DNA extracted using the boiled lysate method was used. For this experiment, the BCG boiled lysate DNA produced the expected PCR product of 123 bp corresponding to the amplification of the IS6110 element and the two negative control samples (MP and water) did not amplify any PCR products. In this case the results for the bison samples were also PCR-negative (data not shown), indicating that *M. bovis* DNA was not detected in the blood of these animals. Swift *et al*. (2016, 2020) have shown that SICCT-positive cattle have a very high change of having detectable levels of *M. bovis* in their blood. However, it must be noted that bison are a different species, and therefore disease progression may

differ. However, based on this result, it suggested that these animals were not infected with *M. bovis.*

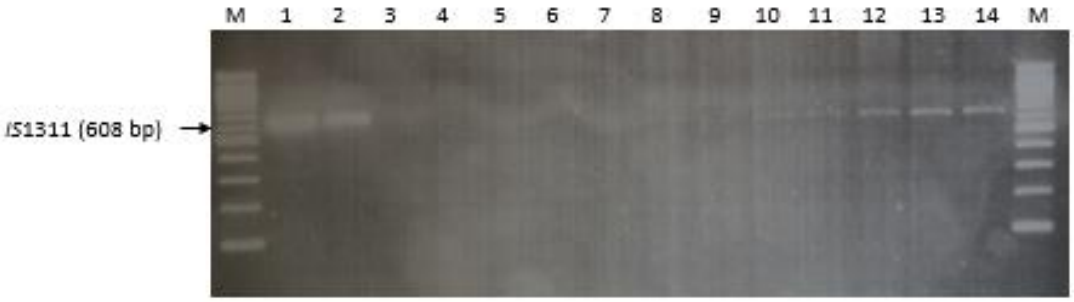
To confirm whether these results were true negatives, a commercial Real-Time quantitative PCR-assay (Ingenetix; Austria) was used that also detects the *IS*6110 element of MTBC. Quantitative PCR is a more sensitive assay than end point PCR and should be able to detect even a single copy of the *IS*6110 element if it was present in the blood. The PCR assays were prepared as to the manufacturer's protocol (section 2.4.4.4.) and the assay was performed on a RotorGene Q Real-Time PCR machine (QIAGEN; UK) and the results were recorded and analyzed by the companion software, Q-REX (QIAGEN; UK). The positive control supplied with the kit was used which should give a positive result and a no template (water) negative control, which should give a negative result. The kit IAC control was also used in each PCR reaction.

The results for the IAC channel firstly showed the PCR reactions were not inhibited and produced a Cq values of 28-30 although this was under the expected 34 – 38 range described in the kit. The results of the assay were in agreement with the end-point *IS*6110 PCR assay. The kit positive control DNA crossed the cycle threshold and produced a positive result at a Cq value of 26.5, again this was below the expected value of 28 – 31 expected by the manufacturer. The no template (water) negative control did not produce a signal and was negative as expected, however a gentle increase in fluorescence was produced indicative of probe break down. The results for the seven bison samples were again all negative and showed the same pattern as the negative control. This confirmed that the bison blood samples did not contain

detectable levels of the *IS*6110 element and as such there was no indication that they were infected with *M. bovis* or another member of the MTBC (data not shown).

9.6.5. **Screening of the bison samples for the *IS*1311 element**

Figure 9.20 – Analysis of DNA samples from bison blood using the S1311 PCR assay



Lane M, 100 bp DNA Ladder (NEB; UK). Lane 1, MAP K10 DNA (20 ng); Lane 2, *M. avium* DNA (20 ng); Lane 4, no template (water) control; Lane 6, MP process control; Lane 8, bison 7; Lane 9, bison 6; Lane 10, bison 5; Lane 11, bison 4; Lane 12, bison 3; Lane 13, bison 2; Lane 14, bison 1. Lanes 3, 5 and 7, contained no PCR products. The PCR results for the test samples were again compared against the MP process negative control and DNA from *M. avium* and MAP K10 (20 ng each), extracted using the boiled lysis method (section 2.4.1.1.) were used as positive controls as both should give a positive results using the M56 and M119 primers. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml $^{-1}$ ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.6.). The expected product size for the M56/119 primers *(IS*1311*)* was 608 bp (indicated by arrows on LHS of image).

To prepare the PCR assays, 5 µl of the DNA elute was used as template with the DNA polymerase and buffer Onetaq®2x Master Mix with GC Buffer and 0.5 µM of the primers M56 and M119. The PCR parameters used were: initial denaturation of 94 °C

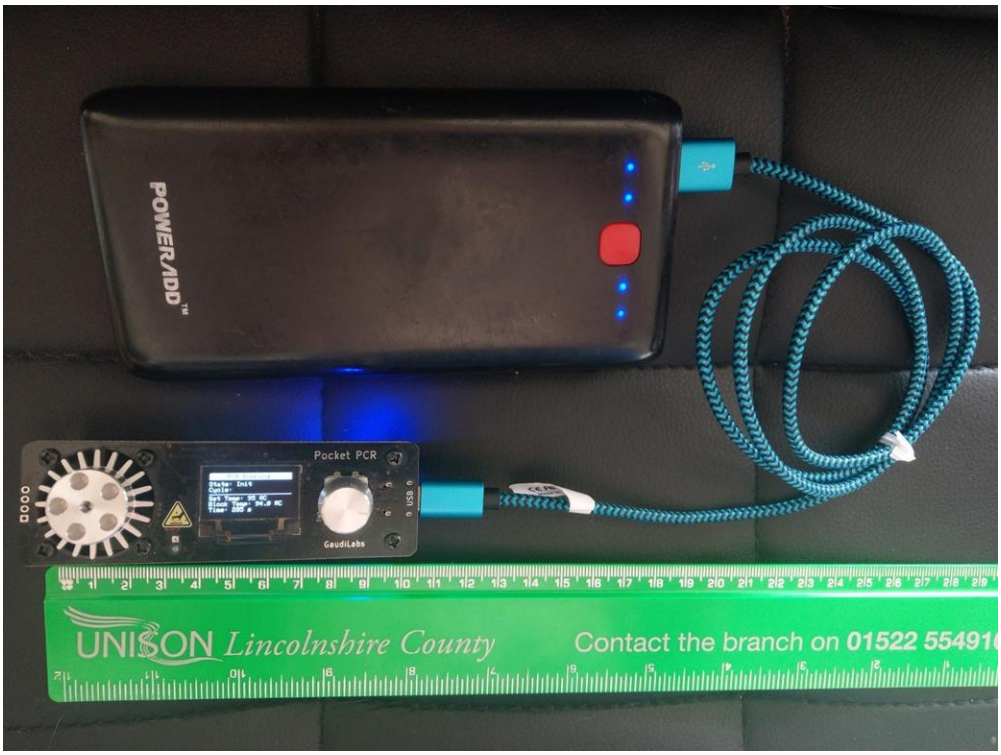
for 2 min; 37 cycles of: 94 °C for 30 s, 62 °C for 30 s, 68 °C for 1 min; followed by a final extension of 68 °C for 5 min.

9.7. Appendix 7

9.7.1. PocketPCR – taking the laboratory out of the laboratory

One of the main aspects of this thesis was to simplify the technology required in order to perform the Oneday phage assay in low-technology laboratories. There are two major pieces of equipment that prevented the transfer of the phage assay to low-technology or field laboratories: the first was the centrifuge requiring a swing-out rotor in the Ficoll® PBMC extracton method; the second was the PCR machine itself. PCR machines are generally a high technology piece of equipment that requires a large power input to achieve quick ramping rates and efficient PCR reactions. They are also delicate, and transport of the equipment must be performed with care so as not to damage the internal components. These aspects limit the applications where a PCR machine could be used. An open source piece of equipment called the PocketPCR has been developed (GaudiLabs, 2020), which is a miniaturized PCR machine that fits in the palm of your hand and is capable of running from any standard 2 A power output, such as a computer/ laptop or portable power pack (Fig 9.21).

Figure 9.21 – PocketPCR powered by a standard mobile power pack



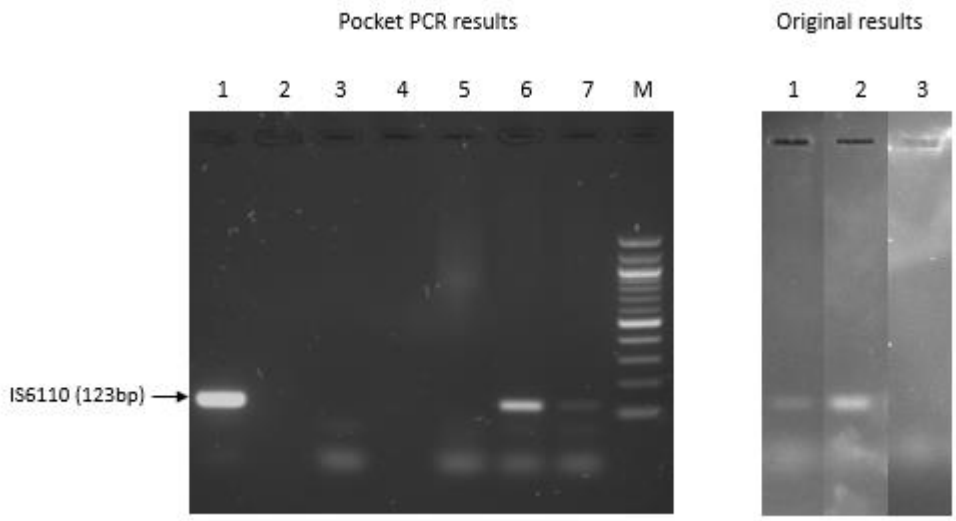
A mock PCR assay was performed to show the low power requirement and size of the PocketPCR machine.

The PocketPCR machine is also robust and will not be damaged by transportation, no matter how careless the carrier is, which would make the device ideal for using in a low-technology or field laboratory. The operator can set the cycling conditions directly on the device and does not require the use of a computer or laptop to define or run the PCR assay parameters. Due to the fact that this device could solve the issue of requiring a standard PCR machine to perform the Oneday phage assay it was used to test historic MTBC samples already confirmed to contain MTBC DNA by the amplification of a PCR product corresponding to the *IS*6110 element using a standard PCR machine. The PocketPCR results were then compared to the original results. The PCR assays were prepared the same as during the original analysis of the samples as were the PCR parameters used, both were described in Section 2.4.2.2. The only difference to the method was that mineral oil was layered on top of the PCR mixture in the PCR tube as the PocketPCR did not contain a heated lid and as such the oil was necessary to prevent evaporation of the PCR reaction during the cycling conditions. Currently, the PocketPCR only contains five wells for a 0.2 ml PCR tube, because of this, the standard positive and negative PCR assay controls were prepared and three DNA samples were selected for retesting with the PocketPCR. A new positive control consisting of approximately 20 ng of *M. bovis* DNA extracted using the boiled lysis method (section 2.4.1.1.) was prepared, which was expected to give a positive result with the *IS*6110 PCR assay. A new no template negative control was also produced which was expected to produce a negative reaction. The three historic DNA samples that were selected for retesting comprised the following characteristics: a sample shown to be negative by the *IS*6110 PCR assay; a sample shown to be strongly positive by the *IS*6110 PCR assay; and a sample shown to be weakly positive by the

*IS*6110 PCR assay. These three samples were selected as they provided the broadest range of amplification produced from the same sampling set of DNA which were originally processed and tested in tandem with each other. It was expected that the PocketPCR would be less efficient at amplifying the DNA compared to a standard thermocycler and produce lower levels of amplification meaning that the weakly positive sample in the original results was expected to be negative by the PocketPCR results.

The results produced from the PocketPCR were not in line with what was expected. The results showed that all three of the samples tested produced comparable results to the results produced by the standard PCR machine. The positive control amplified a 123 bp PCR product corresponding to the amplification of the *IS*6110 element (Fig. 9.22, lane 1) and the negative control produced negative reaction (Fig. 9.22, lane 3), as expected. The negative historic sample in the original results was negative with the PocketPCR (Fig. 9.22, lane 5); the strong positive sample in the original results also achieved strong amplification of the 123 bp PCR product corresponding to the amplification of the *IS*6110 element with the PocketPCR (Fig. 9.22, lane 6); the weak positive sample in the original results also produced low levels of amplification of the *IS*6110 element with the PocketPCR (Fig. 9.22, lane 7), which was in line with the original results. The levels of amplification were slightly reduced in the PocketPCR results however this could be attributed to the age of the DNA and the repeated freeze/ thaw cycles due to power cuts and freezer malfunctions they have experienced since their extraction and storage.

Figure 9.22 - comparison of the PocketPCR results to the original results



Lane M, 100 bp DNA Ladder (NEB; UK); Lane 1, positive control *M bovis* DNA (20ng); Lane 3, no template (water) control; Lane 5, negative result from the original results; Lane 6, strong positive in the original results; Lane 7, weak positive in the original results. Lanes 2 and 4 contained no PCR products. The PCR products were analysed by electrophoresis through a 2 % (w/v) TAE agarose gel at 80 V for 1 h. Gels were stained with 0.2 µg ml^{-1} ethidium bromide and visualized under UV illumination using a Biorad Gel-Doc System (section 2.4.2.2.). The expected product size for the *IS*6110 element was 123 bp (indicated by arrows on the LHS of image). The images of the original results were cut and pasted next to each other for easier comparison between the PocketPCR results and the results gained from a standard thermocycler.

9.8. Appendix 8

9.8.1. Results of the initial human trial

Table 9.23 - Results of the initial human trial

				standard phage assay				Oneday phage assay	
				Plaque number	Plaque number	Ficoll®	HetaSep®	Ficoll®	HetaSep®
PHAGE ID	Disease Type	Date Taken	Date Collected	Ficoll®	HetaSep®	Ficoll®2	HetaSep® 3	Ficoll®4	HetaSep® 5
395	Pulmonary TB	19/07/2017	19/07/2017	0	1	neg	neg	pos	pos
400	Pulmonary TB	26/07/2017	27/07/2017	45	60	pos	pos	pos	pos
432	Pulmonary TB	22/08/2017	23/08/2017	30	76	pos	pos	pos	pos

443	Pulmonary TB	30/08/2017	31/08/2017	12	14	pos	pos	pos	pos
461	Pulmonary TB	13/10/2017	13/10/2017	9	2	pos	pos	pos	pos
462	Pulmonary TB	13/10/2017	13/10/2017	29	1	pos	pos	pos	pos
467	Pulmonary TB	18/10/2017	19/10/2017	0	0	neg	neg	neg	neg
469	Pulmonary TB	18/10/2017	19/10/2017	0	0	neg	neg	neg	neg
470	Pulmonary TB	18/10/2017	19/10/2017	0	0	neg	neg	neg	neg
490	Pulmonary TB	16/01/2018	17/01/2018	1	3	Neg	Neg	Pos	Pos

505	Pulmonary TB	31/01/2018	01/02/2018	20	12	Neg	pos	pos	Neg
513	Pulmonary TB	19/02/2018	20/02/2018	0	0	Neg	Neg	Neg	Neg
518	Pulmonary TB	22/02/2018	21/02/2018	1	0	Neg	Neg	pos	pos
455	IGRA Positive	18/01/2018	19/01/2018	0	1	Neg	Neg	Pos	Pos
493	IGRA Positive	18/01/2018	19/01/2018	0	N/A	Neg	N/A	Pos	N/A
494	IGRA Positive	18/01/2018	19/01/2018	0	N/A	Neg	N/A	Pos	N/A
498	IGRA Positive	24/01/2018	25/01/2018	0	0	Neg	Neg	Neg	Neg

082	IGRA Positive	12/02/2018	13/02/2018	0	0	Neg	Neg	Neg	Neg
261	IGRA Positive	13/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
262	IGRA Positive	13/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
512	IGRA Positive	14/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
510	IGRA Positive	14/02/2018	15/02/2018	0	1	Neg	Neg	Neg	Neg
267	IGRA Positive	15/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
422	IGRA Positive	15/02/2018	15/02/2018	0	1	Neg	Neg	Neg	Neg

472	IGRA Positive	15/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
279	IGRA Positive	19/02/2018	20/02/2018	0	0	Neg	Neg	Neg	Neg
356	IGRA Positive	19/02/2018	20/02/2018	1	0	Neg	Neg	Neg	Neg
249	IGRA Positive	05/03/2018	06/03/2018	37	47	Neg	Neg	Neg	Neg
523	IGRA Positive	06/03/2018	09/03/2018	2	N/A	Neg	N/A	Neg	N/A
445	IGRA Positive	08/03/2018	09/03/2018	4	20	Neg	Neg	Neg	Neg
525	IGRA Positive	13/03/2018	13/03/2018	29	24	Neg	Neg	Neg	Neg

527	IGRA Positive	14/03/2018	15/03/2018	1	0	Neg	Neg	Neg	Neg
530	IGRA Positive	15/03/2018	15/03/2018	0	0	Neg	Neg	Neg	Neg
456	IGRA Negative	18/01/2018	19/01/2018	0	1	Neg	Neg	Neg	Neg
070	IGRA Negative	06/02/2018	08/02/2018	0	1	Neg	Neg	Neg	Neg
081	IGRA Negative	12/02/2018	13/02/2018	0	0	Neg	Neg	Neg	Neg
062	IGRA Negative	13/02/2018	13/02/2018	0	0	Neg	Neg	Neg	Neg
477	IGRA Negative	13/02/2018	15/02/2018	1	0	Neg	Neg	Neg	Neg

478	IGRA Negative	13/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
479	IGRA Negative	13/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
480	IGRA Negative	13/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
336	IGRA Negative	14/02/2018	15/02/2018	1	0	Neg	Neg	Neg	Neg
055	IGRA Negative	19/02/2018	20/02/2018	0	0	Neg	Neg	Neg	Neg
484	IGRA Negative	20/02/2018	21/02/2018	0	0	Neg	Neg	Neg	Neg
485	IGRA Negative	20/02/2018	21/02/2018	0	0	Neg	Neg	Neg	Neg

486	IGRA Negative	20/02/2018	21/02/2018	0	N/A	Neg	N/A	Neg	N/A
116	IGRA Negative	21/02/2018	21/02/2018	0	0	Neg	Neg	Neg	Neg
483	IGRA Negative	21/02/2018	21/02/2018	0	1	Neg	Neg	Neg	Neg
122	IGRA Negative	05/03/2018	06/03/2018	0	15	Neg	Neg	Neg	Neg
072	IGRA Negative	05/03/2018	06/03/2018	22	53	Neg	Neg	Neg	Neg
126	IGRA Negative	06/03/2018	06/03/2018	13	30	Neg	Neg	Neg	Neg
524	IGRA Negative	06/03/2018	09/03/2018	1	N/A	Neg	N/A	Neg	N/A

482	IGRA Negative	12/03/2018	13/03/2018	16	33	Neg	Neg	Neg	Neg
125	IGRA Negative	13/03/2018	13/03/2018	51	41	Neg	Neg	Neg	Neg
526	IGRA Negative	13/03/2018	13/03/2018	5	3	Neg	Neg	Neg	Neg
474	IGRA Negative	14/03/2018	15/03/2018	0	0	Neg	Neg	Neg	Neg
528	IGRA Negative	15/03/2018	15/03/2018	2	1	Neg	Neg	Neg	Neg
529	IGRA Negative	15/03/2018	15/03/2018	0	0	Neg	Neg	Neg	Neg
531	IGRA Negative	15/03/2018	15/03/2018	0	0	Neg	Neg	Neg	Neg

533	IGRA Negative	19/03/2018	20/03/2018	0	9	Neg	Neg	Neg	Neg
534	IGRA Negative	19/03/2018	20/03/2018	60	30	Neg	Neg	Neg	Neg
499	NOT TB	24/01/2018	25/01/2018	0	0	Neg	Neg	Neg	Neg
503	NOT TB	31/01/2018	01/02/2018	2	5	Neg	Neg	Neg	Neg
509	NOT TB	14/02/2018	15/02/2018	0	0	Neg	Neg	Neg	Neg
515	NOT TB	20/02/2018	21/02/2018	0	0	Neg	Neg	Neg	Neg
522	NOT TB	06/03/2018	09/03/2018	0	N/A	Neg	N/A	Neg	N/A

The compilation of the results gained from the first human trial comparing the standard phage assay (section 2.2.) with the Oneday phage assay (section 2.2.3.) and the Ficoll® PBMC extraction method (2.3.1.1.) with the HetaSep® extraction method (section 2.3.1.2.).