NUI MAYNOOTH

Ollscoil na hÉireann Má Nuad

Evaluation of Different Methods for the Detection of *Mycobacterium bovis* in Lymph Node Tissue

Gillian Madigan

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Supervisor

Dr. Kevin Kavanagh

Department of Biology

NUI Maynooth

Maynooth

Co. Kildare

Head of Department

Prof. Kay Ohlendieck

Department of Biology

NUI Maynooth

Maynooth

Co. Kildare

Declaration

I declare that I am the sole author of this thesis and that it has not previously been submitted as an exercise for a degree at the National University of Ireland, or any other university.

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Gillian Madigan Date

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Presentations

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Abbreviations

Ab	antibody
Ab-Ag	antibody-antigen
AccuP	AccuProbe
Ag	Antigen
AFB	acid-fast bacilli
AIDS	Acquired Immune Deficiency Syndrome
BB	Bead Buffer
BCG	*Bacillus Calmette-Guérin*
BD	Becton Dickinson
BIN	Binding Buffer
BSA	Bovine Serum Albumin
BSC	Biological Safety Cabinet
CC	Conjugate Control
CDC	Centre for Disease Control and Prevention
cDNA	complementary DNA
cfu	colony forming units
CMC	Critical Micellar Concentration
Cntl	Control
CPC	Cetylpyridinium chloride
CVRL	Central Veterinary Research Laboratory
DNA	Deoxyribonucleic acid
DR	Direct Repeats
ECDC	European Centre for Disease Prevention and Control
EML	Enzyme Mix Lyophilisate
GI	Growth Index
GP	GenProbe
GTMD	GenoType Mycobacteria Direct
H&E	Hematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HL	Heat Lysis
HP kit	High Pure PCR Template Preparation kit
HYB	Hybridisation Buffer
ICR	Internal Control RNA
IMS	Immunomagnetic Separation
LAM	Lipoarbinomannan
LB	Lysis Buffer
LJ	Lowenstein-Jensen media
LJG	Lowenstein-Jensen with glycerol media
LJP	Lowenstein-Jensen with pyruvate media
LN	Lymph Node

MAC	*Mycobacterium avium* complex
MB	Magnetic Beads
MDR-TB	Multidrug resistant TB
MGIT	Mycobacterial Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat
MOTT	Mycobacteria other that Tuberculosis
MSP	Mycobacteria Species Primer
MTBC	*Mycobacterium tuberculosis* complex
Myco.	Mycobacteria
NALC-NaOH	N-acetyl-L-cysteine sodium hydroxide
NASBA	Nucleic Acid Sequence Based Amplification
Neg	Negative
NTM	Non-Tuberculosis Mycobacteria
OA	Oxalic Acid
OADC	Olecate-Albumin-Dextrose-Catalase
PANTA	antibiotic mixture of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin
PAS	para-aminosalicylic acid
PBS	Phosphate Buffered Saline
PBS-Tw	Phosphate Buffered Saline with Tween
PCR	Polymerase Chain Reaction
PGRS	polymorphic GC-rich sequences
PNB	Paranitrobenzoic acid
PNM	Primer Nucleotide Mix
POES	Polyoxyethlene sterate
PPB	Purified Protein Derivative
PPE	Personal Protective Equipment
Q-Solution	PCR buffer that alters the melting behaviour of DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
RLU	Relative Light Units
RNA	Ribonucleic acid
rRNA	ribosomal RNA
sbsp.	sub-species
SICTT	Single Intradermal Comparative Tuberculin Test
SQCap	Sequence Capture PCR
TES	100mM Tris-HCl (pH 7.4), 50mM EDTA, 150mM NaCl
TB	Tuberculosis
TZN	Terminal ZN
UC	Universal Control

VCAT	antibiotic mixture of Vancomycin, Colistin, Anisomycin and Trimethoprim
WHO	World Health Organisation
XDR-TB	Extensively-drug Resistant TB
X-RIN	Rinse Solution
X-STR	Stringent Wash Buffer
ZN	Ziehl-Neelsen

List of Figures and Tables

Abstract

The MGIT 960 a high capacity, automated, non-invasive liquid culture system was proposed as an alternative to the BACTEC 460, a semi-automated, radiometric liquid culture system, for the growth of mycobacteria from bovine lymph node (LN) tissues. A limit of detection study for *Mycobacterium bovis*, spiked into negative LN tissue, showed that both liquid culture systems supported growth down to a concentration of 2.5×10^2 cfu/ml.

A total of 867 routine LN tissues mainly from bovines but including some badger, deer and sheep tissues, were cultured on both liquid culture systems and on solid culture systems (Stonebrinks and LJP). For MTBC (*Mycobacterium tuberculosis* complex) isolates 92%, 93.2% and 73.9% grew on BACTEC 460, MGIT 960 and solid culture systems respectively. MGIT 960 detected growth of MTBC isolates slightly faster than the BACTEC 460 system (14.6 days compared to 15.2 days respectively). For *Rhodococcus equi* isolates 42%, 96.6% and 42% grew on BACTEC 460, MGIT 960 and solid culture systems respectively. For atypical mycobacteria isolates 89.5%, 43.4% and 10.5% grew on BACTEC 460, MGIT 960 and solid culture systems respectively. For *Mycobacterium avium* isolates 33.3%, 100% and 33.3% grew on BACTEC 460, MGIT 960 and Solid culture systems respectively. Contamination rate was 3.1%, 8.2% and 10.9% for BACTEC 460, MGIT 960 and solid culture systems respectively. For growth of MTBC, *M. avium* and *R. equi* isolates MGIT 960 system gave a similar or better detection rate compared to BACTEC 460 but gave a poorer isolation rate for the growth of atypical mycobacteria. Even with the higher contamination rate the MGIT 960 system was a suitable alternative to the BACTEC 460 system.

A decontamination study, comparing 5% oxalic acid and 0.75% CPC (cetylpyridinium chloride), was carried out to determine if CPC could be used with the MGIT 960 system. CPC is not a suitable decontamination chemical for use with MGIT 960 system since only 47.4% of MTBC isolates were identified with CPC compared to 100% with oxalic acid decontamination.

A study to compare five extraction methods to detect mycobacterial DNA directly from decontaminated LN tissue to determine which if any could be used, with culture, for rapid diagnosis of high priority samples such as singleton reactors and samples with potential human involvement. This study showed that heat lysis and the Roche High Pure Template Preparation kit were not specific or sensitive enough for

this purpose. Immunomagnetic separation (IMS) gave a limit of detection result for *M. bovis* of 2.3 x 10^2 cfu/ml when spiked into PBS but this increased to 2.3 x 10^3 cfu/ml when spiked into negative LN tissue. IMS is not sensitive enough for the detection of mycobacterial DNA directly from decontaminated LN tissue.

GenoType Mycobacteria Direct kit, with the addition of an overnight Proteinase K digestion, correctly identified 63.3% of the decontaminated LN tissues with 16.7% false negatives, the remainder of the samples failed. A persistent problem with the test was the failure of the positive control sample, supplied with the kit, to be correctly identified.

Sequence Capture PCR, a known method for the detection of mycobacterial DNA in LN tissue, correctly identified 73.3% of the samples and the remaining 26.7% samples gave false negative results.

Both the GenoType Mycobacteria Direct kit and Sequence Capture PCR could be suitable for the direct detection of mycobacterial DNA from decontaminated LN tissues with further work required to reduce the number sample failures and improve the sensitivity of both tests.

Chapter 1.

Literature Review.

1.1 Introduction

Tuberculosis (TB) has been an important disease of man and animals since antiquity. It has been detected using molecular biology methods, from the bones of 1000-year-old mummified remains in South America and from 3000-year-old remains in Egypt. The oldest isolated tuberculosis sequence has been identified from 17,000-year-old fossilized bison remains (Zink *et al*., 2002; Chadwick, 1982).

The causative agent of tuberculosis was isolated in 1882 by Robert Koch when he identified *Mycobacterium tuberculosis* in infected tissue and announced his discovery to the Physiological Society of Berlin (Chadwick, 1982). He also developed staining techniques for *M. tuberculosis*, a culture medium that would grow the organism and proved that the bacterium he identified was the causative agent of TB (Chadwick, 1982; Collins, 1997; Schluger, 2005).

The World Health Organisation (WHO) estimates that a third of the world's population is infected with TB. Active TB develops in 8 - 10 million people annually and causes an estimated 3 million deaths. Even with this high infection rate only 10% of the non-immunocompromised individuals infected will ever become symptomatic. People that have a dual infection with TB and Human Immunodeficiency Virus (HIV) have a 50% chance of developing the disease and there is evidence to suggest that having a latent TB infection may accelerate the progression of HIV/AIDS (Collins *et al*., 1997; Schluger, 2005; Mathema *et al*., 2006).

In Ireland the current TB notification rate, as of 2009, stands at 10.9 per 100,000 of population (472 new TB cases). This has dropped from 230 cases per 100,000 of population in 1952, when the first national survey of TB in Ireland took place. This drop was due to the development of a suitable treatment protocol and the success of the bovine TB eradication scheme. The notification rate for TB continued to drop annually until 2001 after which it stabilised, with minor fluctuations in the annual figures (National TB Advisory Committee, 2010; ECDC, 2011).

1.1.1 TB Infection

The symptoms of a TB infection are non-specific and patients typically present with coughing, expectoration (coughing up sputum), haemoptysis (coughing up blood), chest pain and general symptoms, such as fever, sweating and weight loss, that have continued for several weeks. The organ most frequently infected by TB is the lung but the disease often occurs in the lymph nodes, kidneys, central nervous system,

intestine, bones and reproductive system with no part of the body exempt from infection (González-Martín, 2010).

TB is most commonly spread by inhalation of small droplets that contain a few bacilli, from the lungs of a TB patient. The bacilli enter the lungs and are phagocytosed by the alveolar macrophages. This starts the host cellular immune response, involving large amounts of cytokines and chemokines, which limit the infection to the site of invasion and the local draining lymph nodes. This is known as a "Ghon Complex" (Collins *et al.*, 1997; Mathema *et al.*, 2006).

The macrophages also activate a cell-mediated immune response, which takes about 10 days to develop. This response leads to the formation of a granuloma, which halts and contains the infection for approximately 95% of infected individuals. Most of the bacilli are eliminated but some remain in a dormant state within the granuloma and this is a latent TB infection. In the remaining 5% of individuals the infection is not contained and the bacilli continue to replicate leading to the development of disease symptoms and associated pathology, including cavitation and tissue necrosis (Collins *et al.*, 1997; Mathema *et al.*, 2006).

In a further 5% of individuals, within whom the TB infection was contained by the granuloma formation, the disease becomes reactivated and symptoms develop some time after the initial infection. It is not fully understood why this happens but it may possibly be due to a weakening in the patient's immune system for several reasons such as age, uncontrolled diabetes mellitus or chemotherapy (Collins *et al.*, 1997; Mathema *et al.*, 2006).

1.1.2 TB Treatment

Once the causative agent of TB had been discovered attempts were made to find a successful treatment and a vaccine against the disease. Koch made an error in 1890 when he declared that he had discovered a compound that would cure the disease and provide protection against it. What he had identified was a glycerine extract from the mycobacterium, which provides no protection against the disease, and does not improve symptoms (Thoen and Steele, 1995). However, it did lead to the development of a useful diagnostic tool for TB, the tuberculin skin test (Chadwick, 1982).

For many years after the isolation of mycobacteria, care for TB infection remained palliative, providing good nutrition, fresh-air, exercise and rest, with

patients being isolated in hospitals or sanatoria away from the community to limit the spread of the disease. With the discovery of effective anti-tuberculosis drugs, beginning in the 1940s with streptomycin and para-aminosalicylic acid (PAS), treatment of the infection became possible (Chadwick, 1982; Palomino *et al*., 2007).

Early drug trials indicated that a combination of both streptomycin and PAS was more effective against TB than either drug alone, and so long courses of multi-drug anti-tuberculosis treatment began (Palomino *et al*., 2007). Continued research lead to the discovery of more anti-tuberculosis drugs, which improved the treatment efficacy and shorted its duration, these included isoniazid in 1952, ethambutol, as a replacement for PAS, in 1960, rifampicin in the 1970s and pyrazinamide in 1980 (Palomino *et al*., 2007).

The aim of anti-tuberculosis treatment is to rapidly kill metabolically active bacilli within the lung tissue, to prevent further transmission of the infection in sputum, and to achieve complete sterilization and elimination of any semi-dormant TB bacilli within closed lesions in the lungs or in host tissues throughout the body. The current TB treatment regime, recommended by the WHO, is a minimum of six months long and includes an intensive two month, daily treatment with a combination of isoniazid, ethambutol, rifampicin and pyrazinamide, followed by a four month, daily treatment with isoniazid and rifampicin. These four drugs, along with streptomycin, are the first-line TB drugs (Palomino *et al*., 2007; WHO, 2009).

A human infection with *Mycobacterium bovis* can be cured with the recommended TB treatment regime since wild-type *M. bovis* is sensitive to most anti-TB drugs, except pyrazinamide to which it has a natural resistance. Partly for this reason, but also due to limited laboratory resources, more efforts are not made in countries with a high TB burden to diagnose the type of TB infection (Michel *et al.*, 2010). In animals, treatment for a TB infection depends on the specific reactions of individual species to the anti-TB drugs, the logistics and cost of administering multiple drugs over several months. It has been carried out for some rare animal species in captivity but it would not be viable for herd animals. In the UK, drug therapy for bovine TB is illegal since its use has been shown to compromise the tuberculin skin test (Michel *et al.*, 2010; Dean *et al.*, 2008).

Drug resistance develops as a result of TB treatment failure due to numerous reasons such as presence of cavitation, with TB positive sputum, or co-infection with HIV or non-adherence to the required treatment regime, either due to cost and

availability of the drugs, length of treatment or adverse drug reactions (Palomino *et al*., 2007; Cole and Riccardi, 2011; Ahmad and Mokaddas, 2009). Where possible, drug susceptibility testing on TB isolates should be carried out and if resistant strains are identified the TB treatment regime can be modified. First-line drugs can be replaced with second-line drugs such fluoroquinolones, aminoglycosides, ethionamide and D-cycloserine, however these are less effective, lengthen the treatment regime and cause more adverse drug reactions (Palomino *et al*., 2007; Cole and Riccardi, 2011; González-Martín *et al.*, 2010).

Multidrug resistant TB (MDR-TB) refers to *M. tuberculosis* isolates that are resistant to both isoniazid and rifampicin. Among the total worldwide TB cases an estimated 5.3% are MDR-TB. This form of TB infection is problematic to treat and often leads to relapse and treatment failure (Cole and Riccardi, 2011). It is also a risk factor for the emergence of XDR-TB (extensively-drug resistant TB) strains, which are MDR-TB strains that have acquired resistance to the second-line TB drugs, fluoroquinolones and aminoglycosides. Over 50 countries have reported one or more incidence of a XDR-TB infection with up to 10% of all MDR-TB cases in former Soviet Union countries and 5.6% of MDR-TB cases in South Africa identified as XDR-TB. XDR-TB strains are difficult to treat in developed countries and nearly untreatable in developing countries (Cole and Riccardi, 2011; Ahmad and Mokaddas, 2009).

The search for new drugs to treat TB continues and with the emergence of XDR-TB strains there is urgent requirement for new treatment regimes. The difficulty is that any new TB drug needs to be effective not only against metabolically active bacilli but also against the latent or non-replicating bacilli spread throughout the host and, due to a high TB co-infection with HIV, it must be compatible with anti-retroviral therapy. Even so, some drugs are starting clinical trials such as newly developed fluoroquinolones, gatifloxacin and moxifloxacin, and rifapentine, a derivative of rifampicin that has been shown to shorten TB treatment in mice when used in conjunction with moxifloxacin (Cole and Riccardi, 2011; Ahmad and Mokaddas, 2009).

1.1.3 TB Vaccine

Calmette and Guérin in the Pasteur Institute in Lille, France developed a vaccine for TB in 1921. Based on an erroneous statement by Koch that *M. bovis* was not infectious to humans they chose an *M. bovis*, Laid Nocard strain, to use in the vaccine development. It was attenuated to a non-virulent form by sub-culture of the bacterium on a glycerol-potato-bile medium every three weeks for thirteen years (230 subcultures). Due to the loss of its pathogenic potential it is considered to be a separate strain from *M. bovis* and is known as *Mycobacterium bovis* sbsp. *Bacillus Calmette-Guerin (M. bovis BCG)* (Chadwick, 1982; Gheorghiu, 1990).

It was chosen as a suitable vaccine due to its retention of its immunogenicity and its ability to generate a delayed hypersensitivity to tuberculin and provide protection against virulent *M. bovis*. The vaccine was first approved for use in 1928 and to date is the only vaccine approved for use against TB. It provides a variable degree of protection against the disease ranging from 0-80% depending on the study, with most studies indicating that it provides good protection against severe forms of TB, such as meningitis and miliary TB, particularly in young children. This variation could be due to prior exposure to environmental mycobacteria, age at vaccination and/or genetic factors (Chadwick, 1982; Gheorghiu, 1990; Thoen and Steele, 1995; Chilima *et al.*, 2006; Hope and Villarreal-Ramos, 2008).

Alternatives to BCG vaccination are currently being developed with the aim of preventing a TB infection, boosting the immune system of individuals that have a latent TB infection to prevent the progression of the disease and utilising a vaccine that can boost the effectiveness of anti-TB drugs, especially in cases of MDR-TB, with the latter proving most difficult to achieve (Girard *et al*., 2005). Several different strategies are being used such as genetically modifying the *M. bovis BCG* strain to induce a greater protective immunity against TB and developing a new live vaccine using attenuated auxotrophic *M. tuberculosis* strains (Collins, 2000; Girard *et al*., 2005). Other strategies include the use of attenuated Salmonella vectors or virus vectors that express mycobacterial antigens to boost the effectiveness of the BCG vaccination such as attenuated vaccinia virus MVA strain expressing Ag85A, an antigen secreted by *M. tuberculosis* (Vordermeier *et al*., 2009; Girard *et al*., 2005).

1.2 Genus Mycobacterium

1.2.1 Mycobacteria

TB is caused by members of the genus *Mycobacterium* that contains approximately eighty-five different species. Mycobacteria are mostly aerobic, though some can be microaerophilic, non-chromogenic, rod-shaped and non-motile bacteria. The acid-fast staining reaction is the primary test for identifying mycobacteria since the cells are resistant to decolourisation with a dilute mineral acid (or acid-alcohol solution) after staining with an arylmethane dye, such as fuchsin (Ziehl-Neelsen stain) (Collins *et al.*, 1997; Grange, 1996; European Food Safety Authority, 2003; Rastogi *et al*., 2001).

The majority of mycobacteria are free living and are found in water or watery environments such as mud, soil and as biofilms in water pipes. They tend to cause "opportunistic" infections in humans and animals such as Buruli ulcers that are caused by *Mycobacterium ulcerans*, which enter the body through spikes on vegetation. Another opportunistic pathogen is the "*Mycobacterium avium* complex" (MAC) that causes pulmonary disease and can spread to other organs in the body. It affects people who are immunosuppressed such as transplant recipients, AIDS and cancer patients. MAC includes *M. avium* and *Mycobacterium intracellulare* that are closely related slow growing mycobacteria (Collins *et al.*, 1997; Grange, 1996; Rastogi *et al*., 2001).

The second group of mycobacteria are obligate pathogens; this group includes the members of the "*Mycobacterium tuberculosis* complex" (MTBC) and *Mycobacterium leprae*, which causes leprosy. *M. leprae* does not have a wide host spectrum and is predominantly a human pathogen but it does occur in some animals such as the nine-banded armadillo. Although it cannot be cultured *in vitro* yet, it does grow in the footpads of mice (Collins *et al.*, 1997).

"*Mycobacterium tuberculosis* complex" (MTBC) has seven approved members and these are *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis BCG*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium tuberculosis sbsp. canetti* and *Mycobacterium bovis sbsp*. *caprae* (Haddad *et al.*, 2004; Collins, 2011). Four members of this group cause human tuberculosis i.e. *M. bovis, M. tuberculosis, M*. *africanum* and *M canetti* (Collins *et al.*, 1997; Collins, 2011). Members of the MTBC are extremely similar genetically

having at least 99.9% similarity on the nucleotide level and an identical 16rRNA sequence (Brosch *et al.*, 2002).

1.2.2 Mycobacteria Structure

Mycobacteria are similar to other bacteria in many respects except for a unique cell wall that is made up of four parts, Figure 1.1. The first part is a peptidoglycan layer that is similar to that found on other bacterial species. The second layer contains arabinogalactan, which is made up of branched macromolecules of arabinose and galactose (Grange, 1996).

The third layer, which contributes to the thickness of mycobacterial cell walls, is made of mycolic acids that are long branched chains of fatty acids with differing 50 and 30 carbon atom lengths. The mycolic acids are responsible for the acid fast staining reaction of mycobacteria cells (Grange, 1996; Thoen and Steele, 1995).

The fourth, outer, layer consists of a mixture of lipids and related compounds such as trehalose-containing glycolipids and peptidoglycolipids called mycosides. Trehalose-containing glycolipids include a "cord-factor" compound that was previously believed to be responsible for the "serpentine cords" of virulent MTBC but this was later proved to be inaccurate, Figure 1.2 (Grange, 1996).

The mycosides on the outer layer of the cell wall have similar functions as the capsule in capsulated bacteria and allow mycobacterial cells to survive in less than ideal conditions. *M. bovis* may survive depending on the environment, specifically exposure to direct sunlight, for period of time ranging from several days to months in soil, on pastures or in faeces leading to a source of infection (O'Reilly and Daborn, 1995). The mycosides also allow for certain mycobacteria, such as those in the MAC complex, to be subdivided due to an agglutination reaction to specific antisera. The thick layer of mycosides on some species of mycobacteria gives them a smooth colony when grown on solid media and in the case of MAC the colonial morphology can be correlated to virulence with transparent and rough colony forming strains being more virulent than opaque and intermediate colony forming strains (Kuze and Uchihira, 1984; European Food Safety Authority, 2003).

Another benefit of the presence of mycosides is that they may provide protection to intracellular organisms against the 'cidal effects of intermediate oxygen metabolites' (Grange, 1996).

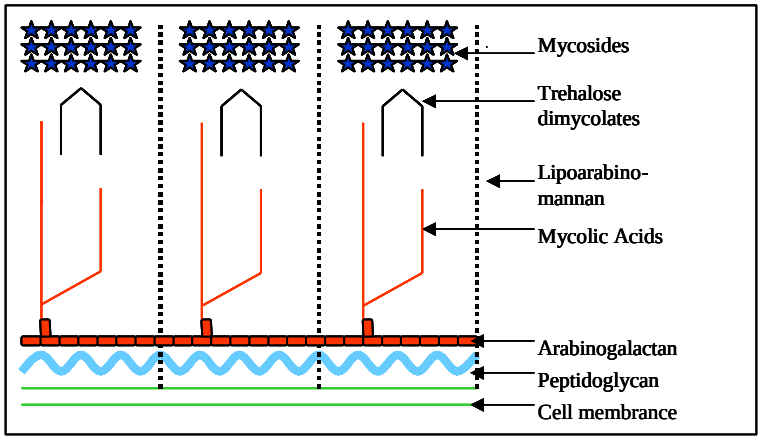

Figure 1.1: Schematic diagram of the mycobacterial cell wall. Adapted from Grange, 1996.

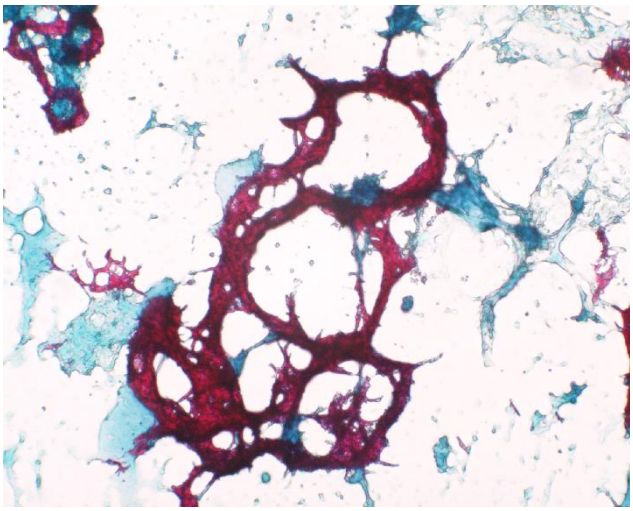

Figure 1.2: Slide showing "Serpentine cords" of *M. bovis* made up of small, non-motile rods 1-4µm long (Costello, 2004).

An important component of the cell wall is lipoarabinomannan (LAM), which stretches from the cell membrane up though the cell wall and is probably responsible for keeping the layers of the cell wall together and binding it to the cell membrane. LAM induces the production of different cytokines, such as tumour necrosis factor when exposed to macrophages in a host organism and since it differs structurally between virulent and attenuated stains of mycobacteria it may contribute to the pathogenicity of a particular stain (Grange, 1996; Thoen and Steele, 1995).

1.3 Bovine TB

1.3.1 *M. bovis* in Cattle

M. bovis has a broad host range that includes both wild and domesticated animals and is the main causative agent of TB in animals. *M. bovis* was differentiated from *M. tuberculosis* by Theobald Smith, in 1896, by small but constant culture differences (Chadwick, 1982). Inhalation of *M. bovis* bacilli is the most common route of infection with only a small number of mycobacteria required to cause an infection and spread of the infection can happen between animals when that are confined together in the same air space, such as during housing over the winter period. A secondary source of infection is the ingestion of contaminated milk or contaminated pasture and water, though environmental contamination is not believed to be a significant source of infection for bovine TB. Infection of the reproductive system can lead to genital transmission of the bacilli but this is a particularly rare event as is congenital infection (Thoen *et al*., 2006; Neill *et al*., 2005; Haddad *et al*., 2004; Neill *et al*., 2001; Menzies and Neill, 2005).

Clinical diagnosis of a TB infection, in cattle, is difficult due to the chronic nature of the disease and the wide variety of symptoms depending on the location of the infection. In cattle, infection with *M. bovis* is a progressive, two stage, disease. The initial phase involves TB granuloma formation, which occurs at the site of infection and the local lymph node. This phase is usually asymptomatic and further progression of the disease varies considerably in rate and route (Blood *et al.*, 1979; Thoen and Steele, 1995; Budka *et al*., 2003).

Healthy, TB free, adult cattle are shown in Figure 1.3 to compare to Figure 1.4 which shows a 9 month old animal with miliary TB. Cattle with miliary TB, in which the infection is spread throughout the animal, are clinically normal but suffer from progressive emaciation, large appetites and fluctuating temperature. The animal,

Figure 1.3 Healthy, TB free, adult cattle from Abbotstown Farm (Courtesy of J. McGuirk).

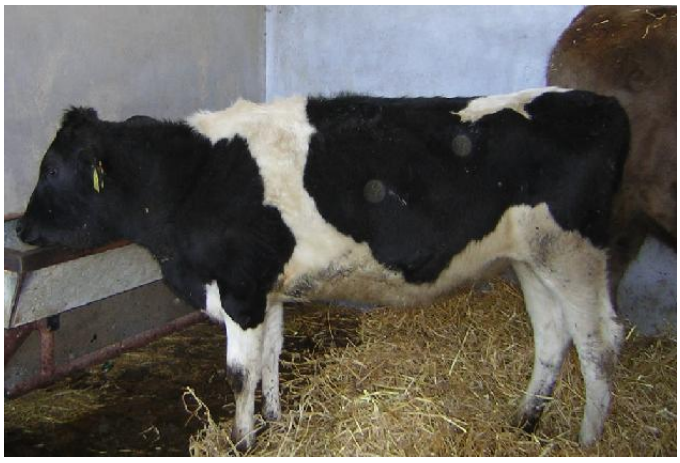

Figure 1.4: A 9 month old animal with miliary TB. It was visible different to other animals of the age with weight loss, a rougher coat and signs of respiratory distress (Costello, 2004).

shown in Figure 1.4, was visibly different to other animals of the same age. It was smaller with a rougher coat, was losing weight and was showing signs of respiratory distress. The exact route of infection for the animal is unknown but was probably due to drinking infected milk as a calf (Blood *et al.*, 1979; Budka *et al.*, 2003; Costello, 2004).

Pulmonary TB is characterised by a chronic, low, suppressed and moist cough due to bronchopneumonia. The symptoms of alimentary TB, dysphagia (difficulty swallowing) and noisy breathing are due to pressure of swollen lymph nodes on the surrounding organs (Blood *et al.*, 1979; Budka *et al*., 2003).

TB mastitis is a significant danger to public health and a route for transmission of the infection to calves. This is due to the possibility that TB bacilli may be present in milk before clinical signs of a TB infection are obvious. It is also difficult to differentiate from other forms of mastitis with the most characteristic symptoms being the development of hard nodules in the upper, rear portion of the udder and enlarged supramammary lymph nodes. Initially milk production is reduced but physically normal, even with the presence of bacilli, followed by milk which contains fine floccules that settle out to leave a clear amber fluid. Advanced clinical cases of the disease still occur but are uncommon due to the bovine eradication scheme (Blood *et al.*, 1979; Thoen and Steele, 1995; Budka *et al*., 2003).

1.3.2 *M. bovis* in Humans

Due to poor experimental controls, *M. bovis* was initially believed not to be a disease of man but this was later proved to be inaccurate (Thoen and Steele, 1995). The consumption of unpasteurised milk from infected cattle is the primary route of *M. bovis* infection in man and is associated with nonpulmonary TB, particularly in children. Cervical lymphadentitis and lupus vulgaris (chronic skin TB) are the most common presentations of a nonpulmonary *M. bovis* infection. Pulmonary TB due to *M. bovis* is clinically, radiologically and pathologically identical to one caused by *M. tuberculosis* but it is uncommon and usually associated with animal handlers and abattoir workers (Thoen and Steel, 1995; Thoen *et al*., 2006; Neill *et al*., 2005).

Human TB caused by *M. bovis* is unusual in countries in the developed world, due to the implementation of eradication programs for domesticated animals, accounting for <1% of TB infections (Neill *et al*., 2005). There are no figures available to show how many TB cases are caused by *M. bovis* in Ireland. However, a

project in Southwest Ireland (Cork and Kerry), the region that reports the highest annual TB cases, showed that *M. bovis* accounted for 3% of human TB cases in the years 1998-2006 (Ojo *et al*., 2008; National TB Advisory Committee, 2010).

In the developing world, *M. bovis* is responsible for 5-10% of human TB cases but this varies between countries (Haddad *et al*., 2004). Limited laboratory facilities, in most developing countries, means that bacteriological diagnosis of a TB infection tends to be carried out by acid-fast bacillus smear examination only, so under-diagnosis of *M. bovis* infection may be occurring (Thoen *et al*., 2004). Due to the effectiveness of the WHO recommended TB treatment regime against human *M. bovis* infections, an argument can be made that diagnosis is not necessary from a case management and therapy viewpoint (Michel *et al*., 2010).

1.3.3 *M. bovis* in Wildlife

In Ireland the main wildlife reservoir of *M. bovis* is the Badger (*Meles meles*), which is a protected species. There is a high density of badgers in Ireland with an estimated population number of 200,000 animals. TB is a chronic infection in badgers and in a naturally infected population disease severity varies widely; with the infection most commonly seen in a latent subclinical form with no visible lesions (NVL) on post mortem examination (Thoen and Steele, 1995; Philips *et al.*, 2003; Wilson *et al*., 2011). The prevalence of *M. bovis* in badgers varies depending on the source of samples, though samples are collected from wide geographical areas. Badgers sourced from culling operations associated with TB herd breakdowns had a *M. bovis* prevalence rate of 36.3% (78 *M. bovis* culture positive animals from 215 badgers in total) (Murphy *et al*., 2010). Badgers culled from areas with a low TB prevalence in cattle, Greenfield sites, had a lower *M. bovis* prevalence of 14.9% (13 *M. bovis* culture positive animals from 87 badgers in total) (Murphy *et al*., 2011).

The degree to which badgers contribute to TB in cattle is unclear but it is estimated that they are responsible for about 50% of TB infections within an experimental area (Jenkins *et al*., 2008). The exact route of transmission of *M. bovis* from badgers to cattle is not known but may occur during direct contact with badgers or their excretory products. Badgers tend to form setts in hedgerows, close to domesticated animals, they forage for food in fields (earthworms are a primary component of their diet) and in farm buildings. When their infection reaches an

advanced stage they can excrete bacilli in aerosols, faeces and urine (Thoen and Steele, 1995; Philips *et al.*, 2003; Wilson *et al*., 2011).

The methods used to reduce the transmission of *M. bovis* between badgers and cattle include culling, vaccination and bio-security measures to prevent contact between badgers and cattle. Reactive culling takes place in Ireland where badgers are removed under license from a 1-2km area around a farm which has suffered a herd breakdown. This has been shown in studies, particularly the Four Areas Project, to reduce the TB prevalence in cattle herds (Griffin *et al*., 2005). Field trials have begun on the use of a BCG vaccine in badgers but development work still needs to be carried out to determine an effective dose in wild badgers, the optimum delivery method and environmental impact so a reduction of TB infection rates will take time (Wilson *et al*., 2011; Gormley and Corner, 2011). Bio-security measures, such as badger-proof gates and electric fences, are slow to be implemented by farm owners due to the expense and the unknown usefulness of such measures in preventing a TB herd breakdown (Wilson *et al*., 2011).

Other wildlife reservoirs of the disease include deer with a TB prevalence of approx 5% of the population (Clifton-Hadley and Wilesmith, 2005). Deer are not believed to be a significant source of *M. bovis* in cattle in Ireland however they can be a source of infection in other countries such as with the red deer (*Cervus elaphus*) in Spain and the white tailed deer (*Odocoileus virginianus*) in Michigan, particularly when herds of wild deer are maintained on large estates for hunting and regularly come into contact with farmed animals (Castillo *et al*., 2011; Miller *et al*., 2005). Improved fencing to prevent farmed cattle from entering woodland areas and to deter wild deer from cattle pastures is the currently the main strategy for prevention disease transmission since control of TB in wild deer is impractical at the current time (Clifton-Hadley and Wilesmith, 2005). In New Zealand, where the brush-tailed possum is the main wildlife reservoir of *M. bovis*, wild deer are believed to act as a vector for tuberculosis, contracting the disease when they travel though areas occupied by infected possums and as they move on spreading the infection to non-infected possum groups (O'Reilly and Daborn, 1995).

A wildlife survey in England confirmed *M. bovis* infection in a wide variety of wild mammals including grey squirrel, fox and wood mouse (Delahay *et al*., 2007).

1.3.4 Bovine TB Eradication Scheme

The *M. bovis* eradication scheme began in Ireland, on a voluntary basis, in 1954 and became compulsory in 1957. *M. bovis* infectious status of Irish cattle herds was estimated to be 17% at that time in Ireland.

The eradication scheme involved monitoring the TB status of cattle herds, on an annual basis, using the Single Intradermal Comparative Tuberculin Test (SICTT). The SICTT works by the generation of a measurable response to purified protein derivative (PPD) following intradermal injection. Both bovine and avian tuberculin PPDs are used in combination. Some animals, due to their own immune systems, do not respond to the test regardless of their infection status. Other animals, that are infected with *M. bovis* become anergic with suppression of the cellular immune response in the blood and at the site of the infection and do not show any response to the skin test. Herds are deemed to be experiencing a TB breakdown if an animal responds to the SICTT i.e. are identified as a 'reactor'. Positive reactor animals were removed from the herd and slaughtered with the owner being paid compensation for the loss of the animal (Daly, 2002; Thoen and Steele, 1995; Good and Duignan, 2011).

Another element of the eradication scheme was the diagnosis of TB in slaughtered cattle, provided as part of a meat hygiene and inspection service by the Department of Agriculture and Food. As part of a meat inspection, visual examinations of pleura, peritoneum and the split vertebral column are carried out to detect TB granulomas, see Figure 1.5. Incisions are made into the carcass lymph nodes, in particular the bronchial and mediastinal lymph nodes, for the same purpose. Any suspect TB lesions detected are removed and sent to the Central Veterinary Research Laboratory (CVRL) for confirmatory testing (Budka *et al*., 2003).

Meat Inspections are an important part of the scheme, since, in any single year, during 1993-2001, between 27% and 46% of all new herd TB breakdowns were detected by meat inspectors (More and Good, 2006). A study carried out during meat inspections for reactor animals during 1982-1990, showed the majority of TB visible lesions detected were associated with a respiratory route of infection, 57% of cattle, averaged annually, had lesions confined to bronchial and/or mediastinal lymph nodes, Figure 1.6, with a further 23% associated with head lymph nodes (retropharyngeal and submaxillary) (Neill *et al*., 1994). It should be noted that a significant percentage of animals that give a positive reaction to the SICTT have no visible lesions detected

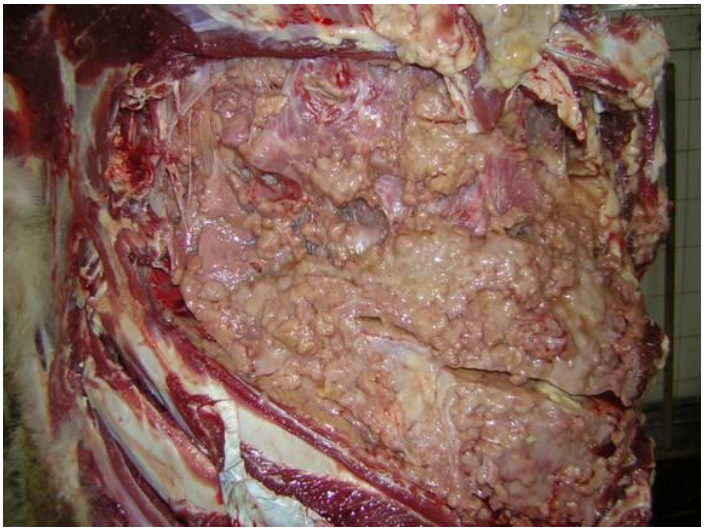

Figure 1.5: Tuberculosis pleurisy and lung lesion (from the animal shown in Figure 1.4) (Costello, 2004).

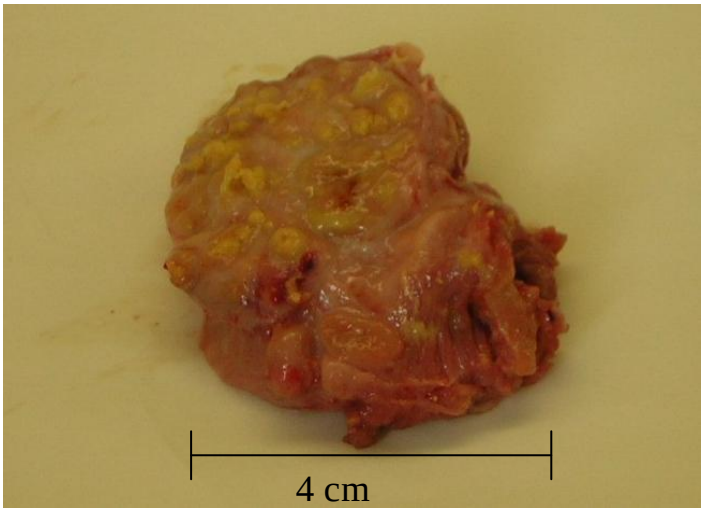

Figure 1.6: Tuberculosis lesion in a lymph node tissue sample (Costello, 2004).

during an abattoir meat inspection, between 8-10% in Ireland and the United Kingdom. This may be due to a non-specific reaction of the SICTT, as a result of exposure of the animal to environmental mycobacteria, or the presence of very small TB lesions in the lung or other tissue that cannot be detected except by a more detailed examination than would routinely take place during a meat inspection (Neill *et al*., 2001; Monaghan *et al*., 2005).

The combination of SICTT use for TB screening, abattoir meat inspections, laboratory monitoring of suspect tissue samples and improved animal husbandry methods lead to an improvement in animal health. In 1965 the national herd was declared free of *M. bovis* infection due to a drop in the level of TB infection in herds to 3%. To date, the eradication scheme has failed to reduce the incidence of TB below this level, with the percentage of TB infected herds in Ireland standing at 5.17% in 2009, for a number of reasons. These include the limitations of the tuberculin skin test and the presence of wildlife reservoirs of infection, such as badgers, which provides a means of re-infection of a TB free herd (European Food Safety Authority, 2011; Thoen and Steele, 1995).

1.4 Laboratory Detection of *M. bovis* infection

1.4.1 Direct Smears

The fastest and simplest method of confirming a mycobacterial infection is a direct acid-fast bacilli (AFB) smear examination from a suspect sample. A Ziehl-Neelsen (ZN) stain or auramine stain using fluorescent microscopy can be used to determine this. Both stains work on the simple principle that any mycobacteria present in the smear retain an arylmethane dye, such as carbol fuchsin or a fluorescent analogue, within the cell giving an acid-fast staining reaction, following treatment with a weak acid-alcohol solution. This is due to the mycolic acids in the bacterium's thick cell wall (Grange, 1996; Collins *et al*., 1997).

This procedure is best used to diagnose pulmonary TB from sputum, in areas with high TB infection and limited laboratory resources. Diagnosis of TB infection from tissues, faeces or other biological material with this technique is poor due to low numbers of mycobacteria and sample contamination with other acid-fast bacteria. Direct staining does not provide any information on the species of mycobacteria causing the infection or differentiate between viable and non-viable cells (Thoen and Steele, 1995; Collins *et al*., 1997).

1.4.2 Histology

Mycobacterial infection can be diagnosed with histopathological examination of tissue samples. A TB infection such as *M. bovis* in cattle causes characteristic cellular changes to the tissues which can be identified with experience and training. The most efficient stain for this purpose is a hematoxylin and eosin (H&E) stain (Thoen and Steele, 1995). An example of a tuberculosis granuloma, which has caseous necrosis in the centre surrounded by an area of epithelioid cells, lymphocytes and some granulocytes with multinucleated giant cells near the necrotic area, from a lymph node tissue is shown in Figure 1.7. A correlation of 94% was found between the use of histopathology and culture to diagnose *M. bovis* infection in cattle (Costello, 2004). A weakness with this method is that other bacteria or mycobacteria can induce similar lesions to those caused by bovine tuberculosis and that a *M. bovis* infection in other animals may not cause the same cellular changes as those seen in cattle making diagnosis more difficult (Thoen and Steele, 1995).

Rhodococcus equi, an environmental microorganism found in soil, is an opportunistic pathogen that can cause a granuloma similar, on visual examination, to a TB granuloma in the lymph nodes of cattle. It is possible to distinguish a *R. equi* granuloma from a TB granuloma by histopathological examination of the tissue however a definitive diagnosis requires bacteriological examination. *R. equi* is able to resist decontamination with 5% oxalic acid, due to the presence of mycolic acids in the cell wall similar to that of the genus *Mycobacterium*, though the number of viable bacteria will be reduced. As a result of its cell wall structure, *R. equi* is isolated during TB culture, Figure 1.8 shows *R. equi* growing on blood agar (Flynn *et al.*, 2001; Rahman *et al.*, 2003).

1.4.3 Culture

Culture of mycobacteria can provide more information on the mycobacterial infection than a direct smear or histopathology. Most mycobacteria grow on media that provides a carbon and nitrogen source and mineral salts that supply phosphorus, sodium, potassium, sulphur, iron and magnesium. Impurities in the media provide a supply of any trace elements required for growth. Most mycobacteria require atmospheric oxygen for growth but some such as *M. bovis* are microaerophilic (Grange, 1996; European Food Safety Authority, 2003).

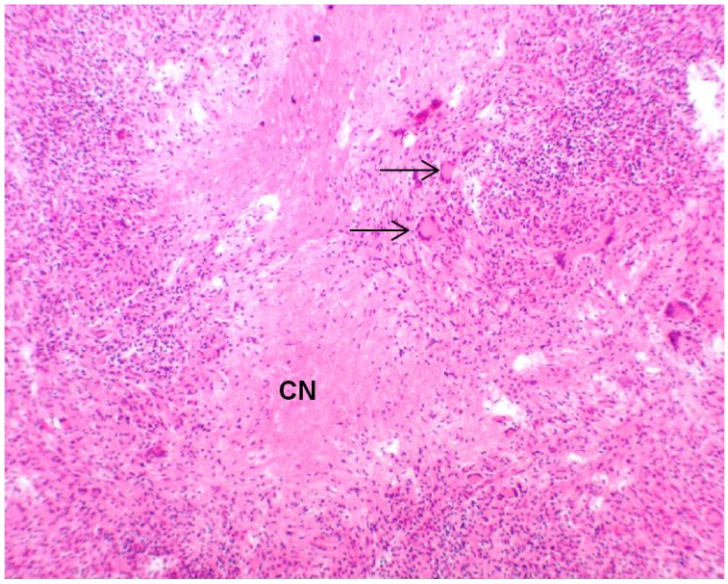

Figure 1.7: Tuberculosis granuloma, showing caseous necrosis (CN) and multinucleated giant cells formed due to macrophage fusion (arrows), from lymph node tissue stained with H&E stain (Costello, 2004).

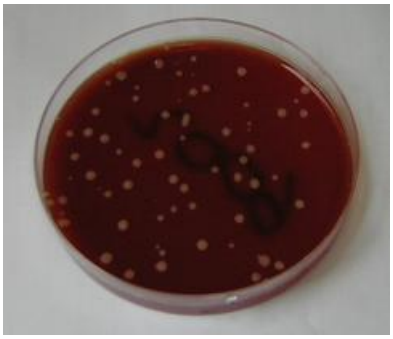

Figure 1.8: *R. equi* growth on a blood agar plate (Costello, 2004).

Due to the slow growth rate of some mycobacteria, *M. bovis* can take 14-21 days of culture prior to growth detection; the culture media can become overgrown with contaminating bacteria and fungi if present in the inoculum (Corner and Trajstman, 1988). To prevent contamination the specimen for culture must be decontaminated prior to inoculation. The mycobacteria cell wall allows the bacterium to be relatively resistant to biocides used in decontamination procedures (Chilima *et al*., 2006).

The choice and method of decontamination is critical with the ideal one having no effect on the viability of the mycobacteria but an ability to kill all contaminating micro-organisms (Corner *et al.,* 1995). However no reagent or procedure is capable of fulfilling this requirement and a compromise must be made to ensure enough mycobacteria survive to propagate in culture even if some cultures become contaminated. A contamination rate of between 2-10% is acceptable depending on the type of sample, age of sample prior to culture and transport conditions (Collins *et al*., 1997).

Different decontamination protocols have been evaluated for the pre-treatment of tissue samples prior to culture for MTBC. These include 2% sodium hydroxide (NaOH), 5% oxalic acid (OA) and 0.075% and 0.75% cetylpyridinium chloride (CPC). The use of 2% NaOH, which is recommended by the Centres for Disease Control and Prevention (CDC) for use on tissue samples, is an effective method but it requires the addition of a neutralization step prior to inoculation onto culture media which increases the decontamination time (Collins *et al*., 1997).

A study carried out that compared several decontamination reagents concluded that CPC, at different concentrations depending on the risk of contamination in the sample, was the most suitable method for use on tissues for isolation of *M. bovis* using solid culture media (Corner *et al*., 1995). Further work, carried out by the Veterinary Laboratories Agency in England, showed that CPC, when used to decontaminate tissues during transport to the laboratory, was toxic to *M. bovis*, when grown on solid culture media, at concentrations below 0.32%, which is its Critical Micellar Concentration (CMC) (Brown *et al*., 2005). CPC has been shown to inhibit growth of *M. bovis* on the BACTEC 460 liquid culture system when compared to 5% oxalic acid (McLernon *et al*., 2004).

1.4.4 Culture Media

Since no single medium can isolate every mycobacterium within a sample, culture is normally carried out using a combination of liquid and solid culture media. Solid media used can be egg-based, Lowenstein-Jensen (LJ) and Stonebrinks, an example of *M. bovis* growth is shown in Figure 1.9, or agar based, Middlebrook 7H10 and Middlebrook 7H11, an example of *M. bovis* growth is shown in Figure 1.10.

Culture on solid media has an added advantage in that it can also provide information about the species of mycobacterial growth without carrying out any biochemical or molecular tests. MTBC grows only when incubated at 37^{o}C +/- 1^{o}C, whereas other mycobacteria can grow at different temperatures. Addition of pyruvate to LJ medium (LJP) will support the growth of *M. bovis* while addition of glycerol to LJ medium (LJG) will support the growth of *M. tuberculosis* but inhibit the growth of *M. bovis* (Collins *et al*., 1997).

Liquid culture can be carried out on Kirchner media and Middlebrook 7H9 broth, which can be modified by the addition of supplements such as antibiotic mixtures and growth promoters to improve mycobacterial growth (Collins *et al*., 1997). Liquid culture systems have many advantages, several studies have shown that they have a shorter time to detection and have a higher recovery rate of mycobacteria when compared to solid culture. This difference may be due to the added enrichment of the liquid culture media or the ability of bacteria within a liquid medium to spread though the media and access all the nutrients whereas with solid media, bacteria are limited to the nutrients in the vicinity of the colony. The disadvantages of liquid culture systems are the high cost and the requirement for specialised equipment to monitor the cultures. The use of solid media with liquid culture does increase the recovery rate of mycobacteria when compared to liquid culture alone. The actual improvement varies between studies from 1% - 8% depending on which culture systems are being compared (Zuhre Badak *et al*., 1996; Kanchana *et al.,* 2000; Yearsley *et al*., 1998; Stager *et al*., 1991; Scarparo *et al*., 2002; Hines *et al*., 2006).

Liquid culture systems include the BACTEC 460, Figure 1.11, and BACTEC Mycobacterial Growth Indicator Tube 960 (MGIT 960), Figure 1.12, both of which are based on enriched Middlebrook 7H9 broth and supplemented with antibiotics and growth promoters, such as OADC (Olecate-Albumin-Dextrose-Catalase) and POES (Polyoxyethlene sterate). The BACTEC 460 system is a semi-automated liquid culture system, in which growth is indicated by the detection of radioactive CO_2 that

Figure 1.9: *M. bovis* growth on Stonebrinks media (Costello, 2004).

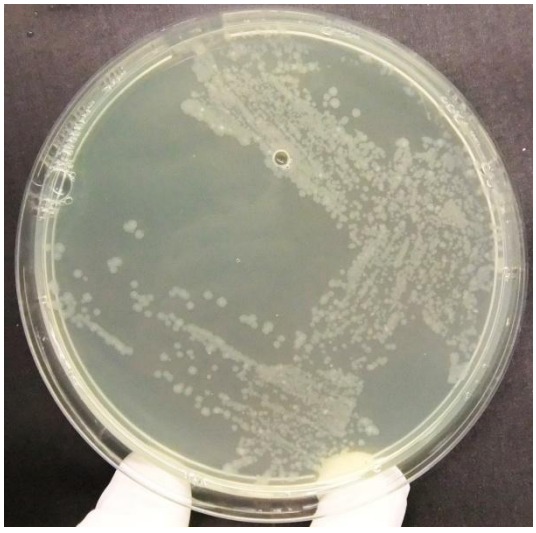

Figure 1.10: *M. bovis* growth on 7H11 (courtesy of J. McGuirk).

Figure 1.11: BACTEC 460 Instrument (Costello, 2004).

Figure 1.12 BACTEC MGIT 960 Instrument (Courtesy of J. McGuirk)

is produced when C^{14} labelled palmitic acid, incorporated in the medium, is metabolised (Yearsley *et al*., 1998). The MGIT 960 is a fully automated culture system that monitors microbial growth by the use of an O_2 quenching fluorescent sensor, embedded in the base of the culture tube, Figure 1.13 (Scarparo *et al*., 2002).

1.4.5 Culture Isolate Identification

When a liquid culture system signals positive a ZN stain is carried out, to detect if any AFB are present, and a blood agar plate is inoculated, to determine if the positive signal is caused by *R. equi* infection or contamination with other bacteria or fungi. The morphology of any AFB present is recorded. Within the CVRL, when AFB are identified on a ZN stain the liquid media is sub-cultured, as described below, and tested with the GenProbe AccuProbe system.

Colonies growing on solid media are examined for typical *M. bovis* morphology, as shown in Figure 1.9, or that of *R. equi*, as shown in Figure 1.14., or contamination. A ZN stain is carried out when examination of the colony morphology is inconclusive. All suspect mycobacteria from solid media are sent for testing with the GenProbe AccuProbe kit.

For sub-culture, the liquid media is inoculated onto 4 solid media; 3 Stonebrinks or LJP media, one each incubated at either 25^{o}C, 37^{o}C or 45^{o}C, and 1 Lowenstein-Jensen with 500µg/Ml paranitrobenzoic acid (PNB) media, incubated at 37^{o}C. MTBC grows only when incubated at 37^{o}C +/- 1^{o}C so any growth on the 25^{o}C or 45^{o}C subculture indicates a non-MTBC organism in the culture (Collins *et al*., 1997). This is also indicated by growth on the PNB media since paranitrobenzoic acid inhibits growth of the MTBC but allows other mycobacteria to grow (Gross and Hawkins, 1985).

The GenProbe AccuProbe system utilises species-specific DNA probes to hybridise with ribosomal RNA (rRNA) released from bacterial cultures. The probes are labelled with a chemiluminescent label; acridinium ester, and any labelled DNA: RNA hybrids are measured with a luminometer. The AccuProbe kits are widely used, easy to perform, fast since the test can be completed in 2 hr, highly sensitive and specific (Neonakis *et al*., 2008; Soini and Musser, 2001).

Depending on the results of the sub-cultures and GenProbe AccuProbe system mycobacteria isolates are reported as; MTBC, *M. avium* or atypical

Figure 1.13 BACTEC MGIT 960 culture media tube (Costello, 2004).

Figure 1.14 *R. equi* growth on Stonebrinks media (Courtesy of J. McGuirk).

mycobacteria isolates. Due to the limited resources and the need to maintain the sample turnaround time within the CVRL, strain typing is not routinely carried out.

When contamination is detected on solid media, the media tube is discarded. When contamination is identified, on a liquid culture system, within the first 3 weeks of culture the sample is decontaminated for a second time or treated with antibiotics, (VCAT (Vancomycin, Colistin, Anisomycin and Trimethoprim), Becton-Dickinson, Cat No.: 212404) depending on the liquid culture method used. If contamination is identified after 3 weeks, the culture is removed from routine monitoring, incubated at 37^{o}C and a terminal ZN (TZN) stain is carried out at the end of the 7 week culture period.

If *R. equi* growth is detected on solid media the result is recorded and the solid continues to be routinely monitored for any additional colony types. When *R. equi* is isolated, from a liquid culture system, the culture is removed from routine monitoring, incubated at 37^{o}C and a TZN stain is carried out at the end of the 7 week culture period.

If no AFB are present in the TZN stain the sample is reported as a *R. equi* isolate or as a 'no isolate' in the case of contamination. When AFB are identified in the TZN stain, the sample is sub-cultured and tested with the GenProbe AccuProbe system. Mixed isolates in liquid culture systems are not unusual due to the slow growth rate of some mycobacteria. A summary of the handling of suspect TB tissues is shown in Figure 1.15.

1.4.6 Molecular Methods

As stated previously, strain typing is not routinely carried out within the CVRL. Strain typing would be done on isolates required for epidemiological studies to attempt to trace the source of the infection, in particular to determine if a wildlife reservoir was the source or if cattle-to-cattle transmission had occurred. Several techniques have been developed for this purpose including Restriction Fragment Length Polymorphism (RFLP), spoligotyping and mycobacterial interspersed repetitive unit-variable-number tandem repeats (MIRU-VNTR).

RFLP involves a restriction enzyme digest of the genomic DNA, the digested products are separated on an agarose gel, transferred onto a filter and hybridised with different probes. A high level of discrimination between different strains of *M. bovis* has been shown with RFLP when each isolate is analysed with a combination probes

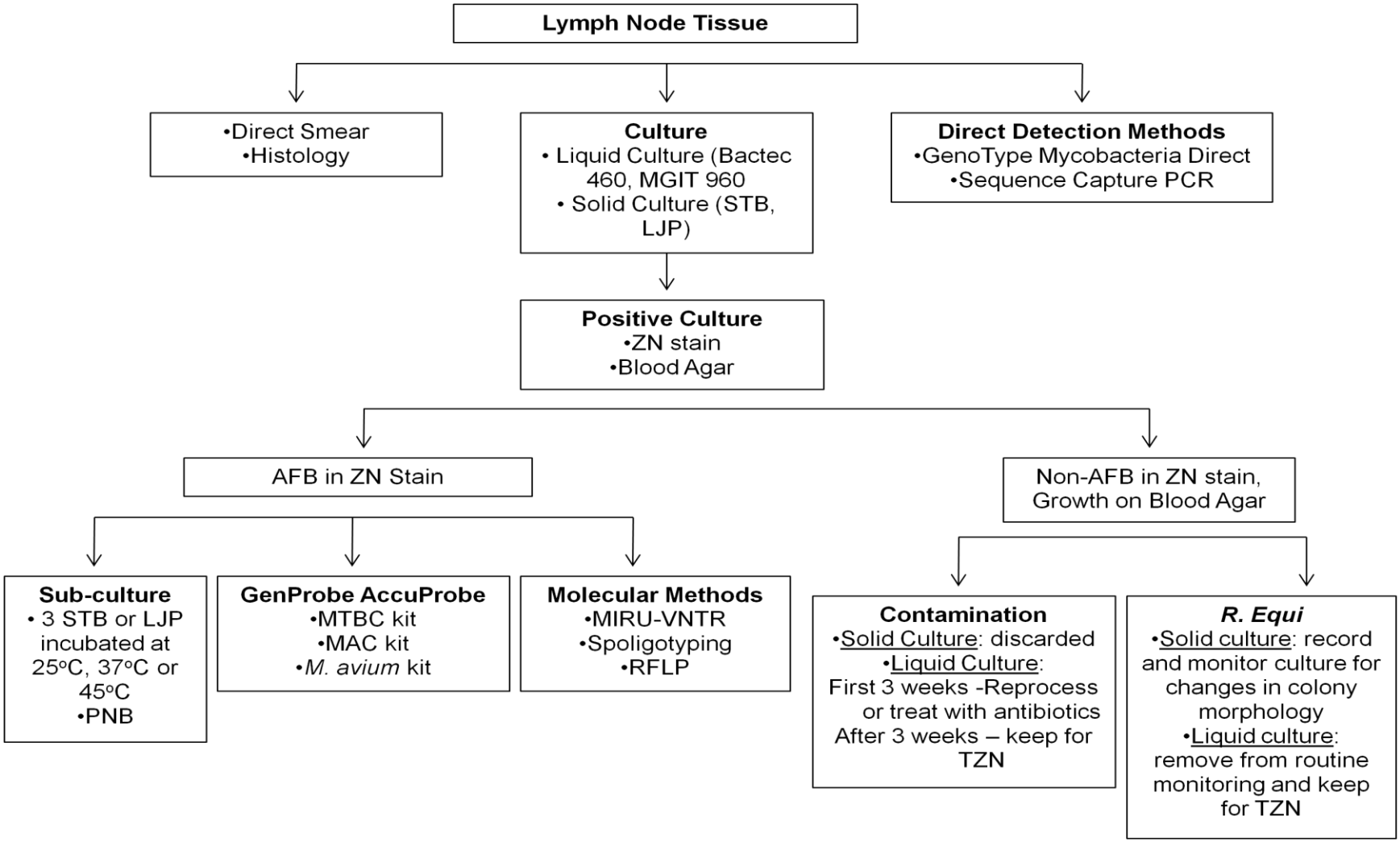

Figure 1.15 Summary of the handling of suspect TB positives tissues

to the insertion sequence IS6110 and the repetitive DNA elements including the polymorphic GC-rich sequences (PGRS) and the direct repeat (DR) region. This technique requires a large amount of DNA, is technically demanding and time consuming with the production of complex banding patterns that make interpretation and inter-laboratory comparisons difficult (Collins, 2011; Durr *et al*., 2000; Kanduma *et al*., 2003; McLernon *et al*., 2010).

Spoligotyping involves PCR amplification of the DR (Direct Repeat) region, hybridization of the resulting product onto a set of immobilised oligonucleotides, that correspond to one of 43 unique DNA spacer sequences within the DR region, and detection of the hybridized DNA by a chemiluminescent system. The test is rapid, easy to perform and the results can be expressed in a digital format. However it does not have the same discriminatory ability for *M. bovis* as RFLP (Restriction Fragment Length Polymorphism) and tends to be used as a screening technique (Collins, 2011; Durr *et al*., 2000; Kanduma *et al*., 2003; McLernon *et al*., 2010).

MIRU-VNTR involves the amplification of different variable number tandem repeat sequences, there have been 41 of these regions identified for *M. tuberculosis* and termed the mycobacterial interspersed repetitive units (MIRU), and the size of the product is determined by gel electrophoresis. This technique is a useful alternative to RFLP analysis for *M. bovis* strain differentiation since it is easy to perform and, similar to spoligotyping, provides results that can be expressed in a digital format making inter-laboratory comparisons easier. The technique can differentiate between *M. bovis* strains with an analysis of as few as 6 loci but analysis of 13 loci have been shown to give optimum discrimination (Collins, 2011; Durr *et al*., 2000; Kanduma *et al*., 2003; McLernon *et al*., 2010).

The use of these techniques for direct detection of *M. bovis* from clinical lymph nodes is limited without a method for the extraction of the organism or the MTBC DNA from the tissue. This is required since excess DNA inhibits a PCR reaction. Lymph node tissue contains a source of host contaminating DNA and could also be a source of PCR inhibitors. Since bovine tuberculous tissues commonly contain low numbers of acid-fast bacilli, diluting the sample to reduce host contaminating DNA and PCR inhibitors will also dilute the target mycobacterial DNA (Mangiapan *et al*., 1996; Roring *et al*., 2002). Any extraction method used will be hampered by lymph node tissue, which shows strong fibrosis and calcification,

limiting access to any mycobacterial cells within the tissue (Liébana *et al*., 1995; Butcher *et al*., 1996).

Direct detection methods, for *M. bovis* diagnosis, have been developed for use on samples with a moderate to high mycobacteria load and in cases were rapid TB diagnosis is important. They have been shown to be unreliable when used on tissues with low numbers of organisms. This unreliability has also been shown for direct detection *M. tuberculosis* diagnosis from clinical samples, so much so that clinical guidelines advise that direct detection methods that give a negative result cannot exclude the possibility of a tuberculosis infection. Advances continue on improving DNA extraction methods and increasing PCR sensitivity however replacing culture, as the gold standard of TB diagnosis, remains a long-term goal (Neonakis *et al*., 2008; Collins, 2011).

Aims of Study

The overall aim of this study was to evaluate new culture and molecular methods for the detection of MTBC, ideally *M. bovis*, in lymph node tissue within the Central Veterinary Research Laboratory (CVRL). The objectives were:

1) To determine if the MGIT 960 liquid culture system can support the growth of *M. bovis* when spiked into bovine lymph node tissue.
2) To determine if the MGIT 960 system, for MTBC culture from LN (lymph node) tissues, would be a suitable replacement for the BACTEC 460 liquid culture system based on the ability to support growth of mycobacteria, specifically MTBC, labour requirements and safety concerns.
3) If the MGIT 960 system is a suitable replacement for the BACTEC 460 system for MTBC culture from LN tissue, to determine if CPC decontamination is an appropriate method to use with this culture system.
4) To evaluate several different methods for the extraction and detection of mycobacterial DNA from LN tissues following decontamination for use with high priority samples, that would benefit from a faster turnaround time, such as singleton reactors and from animals with suspected human TB involvement. The techniques to be evaluated were:
    a) Heat Lysis
    b) Roche High Pure PCR Template Preparation Kit
    c) Immunomagnetic Separation (IMS)
    d) HAIN GenoType Mycobacteria Direct kit
    e) Sequence Capture PCR

Chapter 2.

Materials and Methods.

All work with suspect positive tuberculosis tissues and cultures was carried out in a Class 1 Biological Safety Cabinet (BSC) (Medical Air Technology Ltd., TriMAT Class 1 Safety Cabinet). Suitable PPE (Personal Protective Equipment) was worn at all times when handling any biological material.

2.1 Decontamination of Lymph Node (LN) Tissue with 5% Oxalic Acid

LN tissue samples were removed from -20^{o}C freezer and allowed to defrost overnight at room temperature. The samples were transferred to a filter lined stomacher bag (Grade Plastics Ltd., Cat No.: Grade Sep 400) and approximately 30 ml of 5% OA (Oxalic Acid), made by dissolving 500 g of oxalic acid dihydrate (Merck, Cat No.: CE495.0500) in 10 L of distilled water, was added to each bag. Two bags at a time were placed in a stomacher (Seward Stomacher 80 Lab Systems) and homogenized for 2 min. The homogenate was decanted into a 20 ml sterile tube (Sparks Lab Supplies, Cat No.: SLS7518). Sample tubes were transferred to a 37^{o}C incubator and left for 20 min.

After incubation the samples were centrifuged (Hettich Rotanta 460R Centrifuge) for 15 min at 1970 g. The supernatant was discarded and each pellet was resuspended in 20 ml of sterile saline (Oxoid, Cat No.: BR0053). The tubes were centrifuged for 25 min at 1970 g and the supernatant discarded.

A sterile swab (Sparks Lab Supplies, Cat No.: SW001) was used to inoculate part of the pellet onto one Stonebrinks (Media for Mycobacteria) and one Lowenstein Jensen Medium with Pyruvate (LJP - Media for Mycobacteria, Cat No.: NaP2) medium, which were incubated at 37^{o}C and visually checked for growth once a week. The remainder of the pellet was resuspended in 2 ml of sterile PBS (Oxoid, Cat No.: BR0014) and vortexed for 15 sec.

Just prior to inoculation 0.8 ml of MGIT PANTA (antibiotic mixture of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin - Becton-Dickinson, Cat No. BD245124), lyophilized PANTA mixture reconstituted with OADC (Olecate-Albumin-Dextrose-Catalase), was added to a MGIT tube (Becton-Dickinson, Cat No.: 245122) and 0.1 ml of PANTA (Becton-Dickinson, Cat No.: BD444764), lyophilized PANTA mixture reconstituted with POES (Polyoxyethlene sterate), was added to a BACTEC 460 bottle (Becton-Dickinson, Cat No.: 442004).

A 1 ml luer-lock syringe (Fannin Healthcare, Cat No.: FB309628.30128) was used to add 0.2 ml of the decontaminated pellet solution to a BACTEC 460 bottle and

the bottle was transferred to a 37°C incubator. A 3.5 ml transfer pipette (Sarsteadt, Cat No.: 86.1172.001) was used to add 0.5 ml of the pellet solution to a MGIT tube and the tube was scanned into the MGIT 960 Instrument (BD Diagnostic Systems, Cat No.: 445870) and monitored continuously by the instrument. The BACTEC 460 bottle was monitored in the BACTEC 460 machine (BD Diagnostic Systems) twice a week for the first 3 weeks and once a fortnight for the remaining 4 weeks. The remainder of the pellet was stored at -20°C.

2.2 Decontamination of LN Tissue with 10% OA

LN tissue samples were removed from -20°C freezer and allowed to defrost overnight at room temperature. The samples were transferred to a filter lined stomacher bag and PBS was added to each bag. Two bags at a time were placed in a stomacher and homogenized for 2 min. The resulting solution, 10 ml, was decanted into a 20 ml sterile tube and 10 ml of 10% OA, made by dissolving 100 g of OA dihydrate in 1 L of distilled water, was added to each tube to give a final concentration of 5% OA. The culture procedure was carried out from this point as per section 2.1.

2.3 Preparing a smear from a suspect positive MGIT tube

The MGIT 960 Instrument indicated that a tube has become positive, caused by the detection of fluoresce at the base of the sample tube due to oxygen depletion within the media, by an alarm and a red light on the front panel of a drawer. The flagging tube was scanned out of the machine and transferred to a BSC (Biological Safety Cabinet). Samples monitored with the MGIT 960 Manual Reader (BD Diagnostic Systems) indicated a positive tube by a reading of 14 or higher on the scale on the front of the reader (no units given).

A portion of the pellet was removed from the base of the tube by means of a transfer pipette. A few drops were placed on a glass microscope slide and the remainder was inoculated onto a blood agar plate. Blood agar plates were prepared by dissolving 37 g of blood agar base (Lab M, Cat No.: Lab 28) in 1 L of deionised water, autoclaving at 121°C for 15 min, after the media had cooled to 48°C, 5-7% defibrinated horse blood (TCS Biosciences, Cat No.: HB034) was added, the plates were poured, allowed to set at room temperature for at least 3 hr and stored at 4°C until ready for use. The blood agar plate was incubated at 37°C and checked for

bacterial growth after 24 hr and 48 hr of incubation. The slide was heated to 80^{o}C for 90 min on a slide heater placed in the BSC and a ZN stain was then carried out.

2.4 Preparing a smear from a suspect positive BACTEC bottle

When the growth index (GI), measured by the BACTEC 460 machine, gave a reading above 20 a positive signal was recorded, however a smear for a ZN stain was not prepared until the GI reading was 50 or higher. A 1 ml syringe was used to remove 0.5 ml of the liquid from a BACTEC bottle in a BSC. The liquid was centrifuged, in a 1.5 ml screw-cap tube (Sarsteadt, Cat No.: 72.692.005) at 15200 g for 10 min. Most of the liquid was discarded and the remainder was used to resuspend the pellet. A smear and a blood agar plate were prepared as per section 2.3 above.

2.5 ZN Stain

The slide was flooded with concentrated carbol fuchsin (Difco, Cat No.: 212511) and heated by means of an alcohol soaked taper until steaming. Care must be taken during this process that the carbol fuchsin does not boil on the slide. The stain was left for 10 min and washed with water. An acid-alcohol solution, prepared by the addition of 20% sulphuric acid, 50ml, (Sigma, Cat No.: 320501, diluted with distilled water) to 950 ml of 85% ethanol (Sigma, Cat No.: E7023, diluted with distilled water), was placed on the slide and allowed to decolourise for 5 min. The slide was washed with water and counterstained with a 0.5% malachite green solution (Merck, Cat No.: 15942) for 2 min. The slide was washed with water and allowed to dry.

2.6 Serial Dilution of *M. bovis* stock

M. bovis stock solution was removed from the –80^{o}C freezer and allowed to defrost at 4^{o}C. The stock had been stored in a mixture of glycerol and PBS (50:50). When fully defrosted the tube was centrifuged at 1970 g for 25 min and the supernatant discarded. The pellet was resuspended in an equal volume of PBS-Tw (approx. 2.5 ml). The PBS-Tw was prepared by the addition of 1 ml of Tween 80 (Sigma, P-1754) to 1 L of PBS and autoclaved at 121^{o}C for 15 min. An aliquot of the *M. bovis* stock solution, 1 ml, was inoculated into 9 ml of PBS-Tw and vortexed for 30 sec. A serial 1/10 dilution was carried out down as far as 10^{-10} dilution.

2.7 Limits of Detection of *M. bovis*

Approximately 35 g of negative LN tissue, split into two aliquots, was stomached in PBS as described in section 2.2. The homogenized LN tissue solution was mixed to form an even suspension that was split into eleven 9 ml aliquots. Each aliquot was spiked with 1 ml of the *M. bovis* stock serial dilution (section 2.6) and 1 ml of PBS-Tw was added to the final tube to act as a negative control. A 10% Oxalic acid solution, 10 ml, was added to each tube and the decontamination procedure was carried out as in section 2.2.

After decontamination the pellets were resuspended in 6 ml of PBS-Tw and vortexed for 30 sec. The pellet solution, 0.2 ml, was added to three 7H11 plates that were then sealed with parafilm and incubated at 37^{o}C for 28 days. After incubation the colonies on each plate, were counted and this was used to determine the cfu/ml as per equation below.

Cfu/ml = average of plate colony count x 5 x 10 x dilution factor.

The 7H11 plates were prepared by the addition of 18.9 g Middlebrook 7H11 powder (Difco, Cat No.: 283810), 2 g of Bacto Agar (BD, Cat No.: 214010) and 1 ml of 0.05% malachite green solution to 789 ml of deionised H_2O. The medium was autoclaved at 121^{o}C for 15 min, allowed to cool to 50^{o}C before the addition of 100 ml of denatured Adult Bovine Serum (Sigma, Cat No.: B-9433, heated to 60^{o}C for 60 min) and dissolved Krichner Mycobacteria Selectabs (Mast Diagnostics, Cat No.: MS24, 2 tablets dissolved in 10 ml of sterile H_2O). The plates were poured, allowed to set at room temperature for at least 3 hr and stored at 4^{o}C until ready for use.

A total of six MGIT 960 tubes were also inoculated with 0.5 ml of the pellet solution and 0.2 ml of the pellet solution was added to six BACTEC 460 bottles.

2.8 Subculture of Suspect Mycobacteria

When AFB were seen on examination of a ZN stained slide, from a liquid culture sample, a few drops of the liquid culture medium was inoculated onto 3 tubes (either Stonebrinks or LJP) and 1 Paranitrobenzoic Acid Medium (PNB – Media for Mycobacteria). Each Stonebrinks or LJP was incubated at three temperatures, 25^{o}C, 37^{o}C and 45^{o}C, and the PNB medium was incubated at 37^{o}C.

2.9 GenProbe (GP) AccuProbe (AccuP) MTBC, MAC and *M. avium* Testing Kits (bioMérieux, Cat No.: MTBC kit - 39000, MAC kit – 39001, *M. avium* kit – 39305).

All isolates with suspect mycobacterial growth on solid media or with AFB present on a ZN stain are tested with the MTBC AccuP kit. Samples that have non-corded or diffuse AFB are also tested with a MAC AccuP kit and a positive result on the MAC AccuP kit prompts the use of the *M. avium* AccuP kit.

All of the liquid, except approximately 0.5 ml, was removed from the MGIT 960 tube or BACTEC 460 bottle to be tested and centrifuged at 1970 g for 30 min. The supernatant was discarded and the pellet was re-suspended in 100 µl of Reagent 1 (Lysis Reagent, GP Culture Identification kit - bioMérieux, Cat No.: 39305) and 100 µl of Reagent 2 (Hybridization Buffer, GP Culture Identification kit). The solution was transferred, using a 3.5 ml transfer pipette, to the Lysing Reagent Tube (GP AccuP kit). For use of the kit with solid culture media, a loopful of culture from a suspect positive solid was transferred to the Lysing Reagent Tube to which Reagents 1 and 2 had previously been added.

The Lysing Reagent Tube was briefly vortexed and transferred to a water bath sonicator (VWR, Model No.: USC300D), in which the water had been degassed thoroughly before use, and sonicated for 15 min. After sonication, the tube was transferred to heating block (Test Tube Heater SHTD, Stuart Scientific) and heated for 10 min at $95^{o}C \pm 5^{o}C$.

The solution, 100 µl, from the Lysing reagent tube was transferred to the Probe Reagent Tube (GP AccuP kit) and incubated at $60^{o}C \pm 1^{o}C$ for 15 min in a heating block. Reagent 3 (Selection Reagent, GP Culture Identification kit), 300 µl, was added to the Probe Reagent Tube and vortexed. The tube was then incubated, in a heating block, at $60^{o}C \pm 1^{o}C$ for 5 min, when using the MAC and *M. avium* kits, or for 10 min, when using the MTBC kit. The Probe Reagent Tube was removed from the heating block and allowed to cool at room temperature for at least 5 min.

The GP Leader 50i Luminometer (GenProbe, Cat No.: 103100i-02) was primed for use with Detection Reagents I and II (GP Detection Reagent kit - bioMérieux, Cat No.: 39300). After the Probe Reagent Tube had been allowed to cool, it was inserted into the Luminometer and analysed. Any sample which produced a signal equal to or greater than the cut-off value of 30,000 RLU (Relative Light Units) was considered positive.

All isolates with suspect mycobacterial growth on solid media or with corded AFB present on a ZN stain are tested with the MTBC AccuProbe kit (bioMérieux, Cat No.: 39000).

Non corded or diffuse AFB are tested with a MAC AccuProbe kit (bioMérieux, Cat No.: 39001)

A positive result on the MAC AccuProbe kit prompts the use of the *M. avium* AccuProbe kit (bioMérieux, Cat No.: 39305).

2.10 Decontamination of LN tissue with 0.75% CPC

LN tissue samples were removed from -20^{o}C freezer and allowed to defrost overnight at room temperature. The samples were transferred to a filter lined stomacher bag and PBS was added to each bag. Two bags at a time were placed in a stomacher and homogenized for 2 min. The resulting solution, 10 ml, was decanted into a 20 ml sterile tube. An equal volume of 1.5% CPC, prepared by dissolving 15 g of CPC (Sigma, Cat No.: C-9002) in 1 L of distilled water, autoclaving for 15 min at 121^{o}C and storing the solution at room temperature, was added to each tube to give a final concentration of 0.75% CPC. The samples were incubated at room temperature for 30 min and the remainder of the decontamination procedure was carried out as per section 2.1.

2.11 Preparation of Decontaminated Tissue Samples

Thirty lymph node tissues were decontaminated with 5% oxalic acid and cultured as per section 2.1, with 6 ml of PBS being used to resuspend the tissue pellet following the wash step. Following culture, the remainder of the decontaminated tissue homogenates were divided into 0.5 ml aliquots and stored at –20^{o}C. Prior to dividing them a few drops from each homogenate was placed on a slide, heat killed at 80^{o}C for 90 min on a slide dryer and ZN stained as per section 2.5. The slides were examined for the presence of AFB and the results recorded. These results were used to classify the positive MTBC cultures into Low positive (no AFB visible on the slide), Medium positive (up to 2 fields with at least 1 AFB in each) and High positive (3 or more fields with at least 1 AFB in each). There were 27 MTBC positive samples and 3 culture negative samples.

2.12 Dilution of Decontaminated Tissue Samples

Tissue pellets were removed from the –20°C freezer and allowed to defrost overnight at 4°C. A rough estimate of the size of the settled pellet was made using the graduation markings on the side of a 1.5 ml tube. Each sample was diluted, in PBS, to a final pellet concentration of 50 µl of pellet per 500 µl of PBS.

2.13 Heat Killing Procedure

A water bath was preheated to 80°C and the sample e.g. tissue pellet or culture isolate, in a 1.5 ml tube, was submerged in the water for 60 min. The tube was removed from the water and centrifuged at 15200 g for 10 min. The supernatant was discarded and the pellet resuspended in 500 µl of PBS. The sample was centrifuged at 15200 g for 10 min and the supernatant discarded. The pellet was frozen at –20°C until required for use. The sample is now safe to be handled outside the category 3 laboratory.

2.14 Heat Lysis (HL) Procedure

The heat-killed pellet, produced in section 2.13, was removed from the –20°C freezer and transferred to the PCR extraction lab. The defrosted pellet was resuspended in 400 µl of UltraPure distilled water (Gibco, Cat #: 10977-035). The sample was placed in a heating block, preheated to 100°C, and incubated for 15 min with periodic vortexing. The sample was centrifuged at 5900 g for 1 min and the supernatant was transferred into a fresh 1.5 ml tube. The pellet was discarded. The supernatant collected was amplified as per section 2.23 and detected as per section 2.25.

2.15 Pre-treatment and Procedure for use of High Pure PCR Template Preparation Kit (HP kit)

The samples were heat-killed, as described in section 2.13, and transferred to the PCR extraction lab. The pellets were resuspended with 100 ml of 10 mg/ml lysozyme (Sigma, Cat #: L-7651), and incubated at 37°C for 2 hr with periodic mixing. The tubes were centrifuged at 15200 g for 10 min and the supernatant was discarded. Lysis Buffer, 200 µl, (10mM Tris-HCl, 10mM EDTA, 1M GITC, 0.5M

NaCl, 2% Trition X-100, pH 8.5) was added to each sample and incubated, in a shaking waterbath heated to 37°C, overnight.

Binding buffer (HP kit), 200 µl, and 40 µl of 20 mg/ml Proteinase K (Sigma, Cat #: P-2308) was added to each sample and incubated, in a shaking waterbath, for 30 min at 72°C. Isopropanol (Sigma, Cat #: I-9516), 100µl, was added to each tube and mixed well by pipette. All the liquid mixture was transferred to the filter column in a filter tube/collection tube assembly and centrifuged at 5900 g for 60 sec. The flow-through and the collection tube were discarded.

The filter column was transferred to a fresh collection tube and 500 µl of Inhibitor Removal Buffer (HP kit) was added to the filter column. The sample was centrifuged at 5900 g for 60 sec and the filter column was transferred to a fresh collection tube. Wash Buffer (HP kit), 500 µl, was added to the column and the sample was centrifuged at 5900 g for 60 sec. The filter column was transferred to a fresh collection tube and the Wash Buffer step was repeated twice.

After the third wash buffer step the filter column was transferred to a fresh collection tube and centrifuged at 5900 g for 3 min to remove any residual wash buffer. The filter column was transferred to a 1.5 ml micro-tube and 100 µl of Elution Buffer (HP kit), that had been pre-warmed to 70°C, was added to the filter column. The sample was centrifuged at 5900 g for 60 sec and the filter column was discarded. The collected liquid was amplified as per section 2.23 and detected as per section 2.25.

2.16 Pre-treatment and Modified Procedure for the High Pure PCR Template Preparation Kit

The samples were heat-killed as described in section 2.13 and transferred to the PCR extraction lab. The pellets were resuspended with 100 ml of 10 mg/ml lysozyme, and incubated at 37°C for 2 hr with periodic mixing. The tubes were centrifuged at 15200 g for 10 min and the supernatant was discarded. Each pellet was resuspended in 300 µl of Tissue Lysis Buffer (HP kit) and 60 µl of 20mg/ml Proteinase K and mixed well. The samples were incubated at 55°C for 2 hr with periodic mixing. Binding Buffer (HP kit), 300 µl, was added to each tube and vortexed for 20 sec. The samples were incubated at 72°C for 30 min with vortexing of samples every 10 min. The tubes were centrifuged at 5900 g for 5 min. The

supernatant, 540 µl, was transferred to a fresh tube and 100 µl of Isopropanol added. The samples were vortexed for 20 sec and the solution was transferred to the filter column in a filter tube/collection tube assembly. The remainder of the DNA purification procedure was carried out as per section 2.15.

2.17 Immunomagnetic Capture (IMS)

A 0.1% Bovine Serum Albumin Buffer (BSA Buffer) was prepared by dissolving 0.5 g of Bovine Serum Albumin (Sigma, Cat No.: A7030) in 500 ml of sterile PBS and adjusting the pH to 7.4. A volume of 25 µl of Dynabeads® Sheep-anti Mouse IgG (Dynal, Cat #: 110-31) was used per sample tested.

The required amount of Dynabeads was washed with BSA Buffer to remove preservatives, and resuspended, in the original volume, with BSA Buffer. *M. tuberculosis* Mouse Monoclonal Antibody (Ab) (Vision Biosystems (Europe) Ltd.**,** Cat #: NCL-MT), the antibody cross-reacts with all members of the MTBC, was added to the Dynabeads, 1 µl of Ab per 25 µl of Dynabeads, and incubated on a rotator (Stuart SB3 Variable Speed, Timed Rotator) for 24 hr at 2-8^{o}C.

The beads were washed after incubation to remove any unbound Ab from the beads. The tube was placed in a magnetic separator for 1min and the supernatant was discarded. The tube was removed from the magnetic separator and the pellet resuspended in 1.5 ml of BSA Buffer. The wash step was repeated for a total of three times and after the final wash the pellet was resuspended in the original volume of beads used.

The decontaminated pellets were removed from the –20^{o}C freezer, defrosted and diluted as described in section 2.12. After dilution the sample was centrifuged at 15200 g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of BSA Buffer. Antibody bound beads, 25 µl, were added to each sample and the tubes were incubated on the rotator for 30 min at 2-8^{o}C. BSA Buffer, 0.5 ml, was added to each sample and then the tubes were placed on the magnetic separator for 2 min. The supernatant was discarded and the pellet was resuspended in 1 ml of BSA Buffer. A total of 3 washes were carried out and after the final wash the pellet was resuspended in 0.5 ml of PBS. A schematic diagram of this procedure is shown in Figure 2.1 (Method adapted from Grant *et al*., 1998). The samples were heat-killed, as per section 2.13, and heat-lysed, as per section 2.14. The samples were amplified as per section 2.23 and detected as per section 2.25.

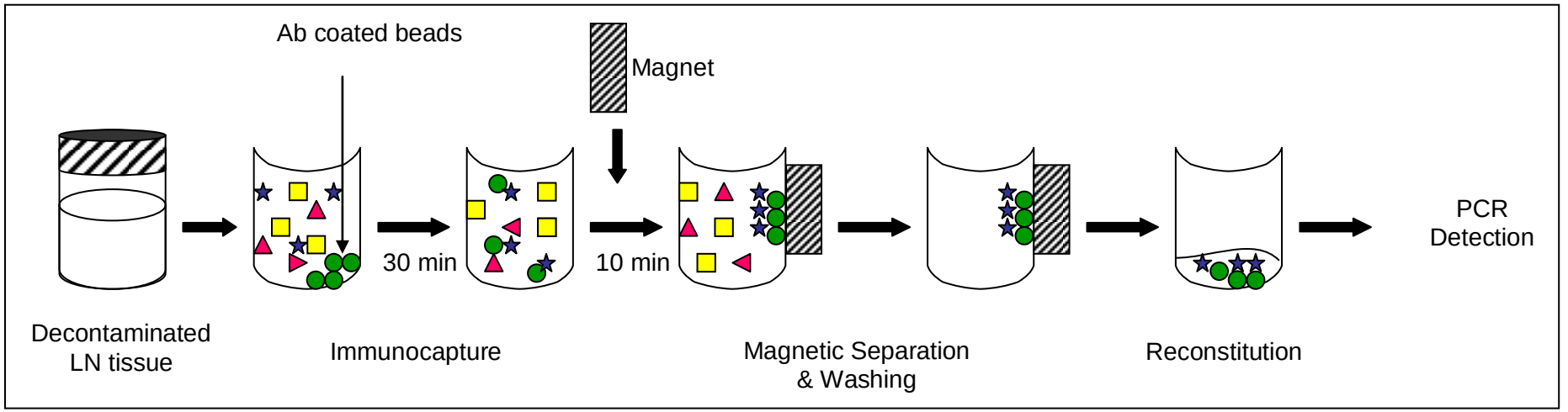

Figure 2.1: Schematic Diagram of a Direct IMS procedure. Adapted from Grant *et al*., 1998.

2.18 Bead Beating with a Hybaid Ribolyser Homogenizer

Approximately 0.5 ml of 212-300 µm glass beads (Sigma, Cat #: G1277) were added to each decontaminated tissue sample. The samples were placed in the Ribolyser, at speed 5, for 10 sec (A), 20 sec (B), 30 sec (C) and 40 sec (D). After the bead-beating step the samples were transferred to fresh 1.5 ml tubes and care was taken to minimise the transfer of any glass beads.

2.19 Limits of Detection of *M. bovis* on previously decontaminated tissue samples

A *M. bovis* stock solution was prepared as per section 2.6, using PBS. To determine the cfu/ml count, 200 µl of each dilution was inoculated onto three 7H11 plates. Each plate was sealed with parafilm and incubated at 37^{o}C for 28 days. After incubation the colonies on each plate were counted and this was used to determine the cfu/ml as per equation below.

Cfu/ml = average of plate colony count x 5 x 10 x dilution factor.

A culture negative decontaminated lymph node sample, decontaminated as per section 2.11, was diluted as per section 2.12. Aliquots, 0.5 ml, of the diluted culture negative sample were centrifuged at 15200 g for 10 min and the supernatant was discarded. The pellets were resuspended in 0.5 ml of a dilution of the *M. bovis* stock.

2.20 HAIN GenoType Mycobacterium Direct

The kit version being evaluated was GenoType Mycobacteria Direct 4.0 (Hain Lifesciences, Cat #: H4010). The test was carried out as per manufacturer's instruction and summarised below.

The magnetic beads (MB - Hain kit) were mixed with the Binding Buffer (BIN – Hain kit) and added to the decontaminated tissue sample. The sample was allowed to incubate at room temperature for 15 min. Using a magnetic separator, the supernatant was discarded, and the MB resuspended in Lysis Buffer (LB – Hain kit) and incubated for 5 min at room temperature. Absolute ethanol was added to each sample and incubated at room temperature for 5 min. The sample was placed on the magnetic separator and the supernatant discarded. The beads were resuspended in 70% ethanol and vortexed. The supernatant was separated from the beads by the use of the magnetic separator. The beads were resuspended in a Bead Buffer (BB – Hain kit) that had the Internal Control RNA (ICR – Hain kit) added to it. The sample was incubated at 85^{o}C for 20 min and the supernatant was transferred to a fresh tube using

the magnetic separator. The supernatant was mixed with the Primer Nucleotide Mix (PNM – Hain kit) and the Enzyme Mix Lyophilisate (EML – Hain kit), both pre-prepared in a clean room, and a NASBA (Nucleic Acid Sequence Based Amplification) was carried out on the Twincubator® with amplification block (Hain Lifesciences, Cat #: 11620).

The product from the NASBA reaction was bound to a membrane strip (Hain kit), which had specific probes bound to its surface, and detected with a colorimetric reaction. The amplified product was denatured with a denaturation solution (X-DEN – Hain kit), in a well of a plastic tray (Hain kit). Hybridisation buffer (HYB – Hain kit) was added to the sample and a membrane strip placed in the well. The tray was incubated in the Twincubator® with hybridisation block (Hain Lifesciences, Cat #: 11621) at 50^{o}C for 30 min. After incubation the buffer was discarded. Stringent Wash Buffer (X-STR – Hain kit) was added to the strip and incubated for 15 min at 50^{o}C. The X-STR was discarded. Conjugate buffer was added to the well and incubated for 30 min at room temperature. After removing the buffer, the membrane strip was washed with 1 ml of Rinse Solution (X-RIN – Hain kit). A second wash step with distilled water was carried out. Substrate buffer was added to the membrane and incubated at room temperature until a banding pattern was seen on the strip or for a maximum incubation of 15 min. The banding pattern was compared to the one supplied with the kit to determine the result.

2.21 HAIN GenoType Mycobacterium Direct with enzymatic pre-treatment

The decontaminated tissue samples were diluted as per section 2.12. The samples where then centrifuged at 15200 g for 10 min and the supernatant was discarded. The pellets were resuspended in 200 µl of BIN buffer (Hain kit) and 40 µl of Proteinase K (10 mg/ml) was added to each tube. The samples were incubated overnight at 58^{o}C in a heating block. The solution, 220 µl, was added to 30 µl of the MB/BIN mix. The kit procedure was then carried out as described in section 2.17 with the following modifications.

The decontaminated tissue samples were diluted as per section 2.12. To ensure that no magnetic beads remained in the sample tube after last magnetic bead separation each tube was centrifuged at 5900 g for 1 min. The supernatant was transferred to a fresh tube and used in the amplification step and the pellet was discarded. The PNM (primer nucleotide mix – Hain kit) was made up approximately

15 min prior to use within the TB Laboratory. This was carried out in a BSC that was not used for any other molecular biology work. The cabinet was suitably cleaned and treated with UV light prior to use. Care was taken to ensure the EML (Enzyme Mix Lyophilisate - Hain kit) was prepared with gentle mixing only.

2.22 Sequence Capture PCR method (adapted from Skuce *et al*., 2003)

In a 1.5 ml tube, 0.5 ml of decontaminated tissue homogenate was added to 0.5 ml of zirconia beads (Biospec Products, Inc, Cat No: 11079101z). The tubes were spun down at 1520 0g for 10 min and the supernatant was discarded. The beads and pellet were resuspended in 530 µl of TES buffer (100 mM Tris-HCl (pH7.4), 50 mM EDTA and 150 mM NaCl) and shaken in the bead beater for 45 sec at 6 m/s. Proteinase K, 20 µl at a concentration of 100 mg/ml, was added to each tube and incubated overnight at 55^{o}C.

The samples were shaken in a bead beater for 45 sec at 6 m/s. The supernatant was transferred to a fresh tube and made up to 550 µl with TES buffer. The samples were denatured by heating to 100^{o}C for 7 min and cooling on ice for 5 min.

A capture probe solution (2.5 pmol of each capture probe in 3.75 M NaCl), 200 µl, was added to each sample and incubated at 42^{o}C for 3 hr with gentle agitation. The magnetic beads (MB), Dynabeads® M-280 Streptavidin (Dynal, Cat No.: 112.05D), were prepared as per manufacturer's instructions. After capture probe incubation, 10 µl of the MB (5 mg/ml concentration) was added to each sample and incubated at 36^{o}C for 2 hr with gentle agitation.

After the second incubation the samples were placed on a MB separator for 3 min and the supernatant was discarded. The samples were removed from the MB separator and the beads were resuspended in 750 µl of wash buffer (10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA). The samples were placed back on the MB separator for 3 min and the supernatant was discarded. The wash step was repeated twice more. After the finally wash, the beads were resuspended in 25 µl of UltraPure distilled water and stored at -20^{o}C. A schematic diagram for this procedure is shown in Figure 2.2.

Capture Probes (Biotinylated) (Skuce *et al.*, 2003)

CapDR (5'biotin-AAA AAG GTT TTG GGT CTG ACG AC-3')

CapDF (5'biotin-AAA AAC CGA GAG GGG ACG GAA AC-3')

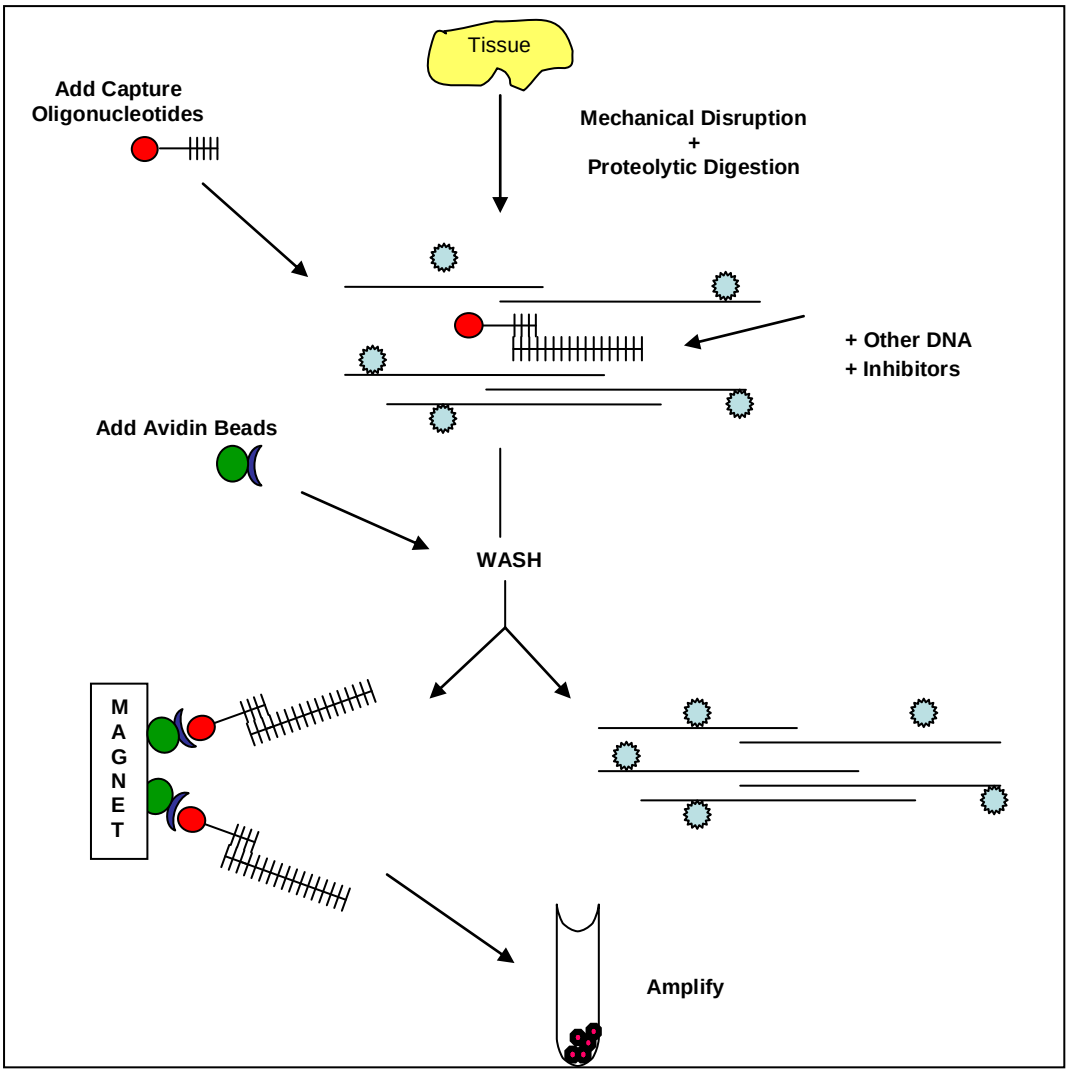

Figure 2.2: Schematic representation of a sequence capture PCR. Adapted from Mangiapan *et al.*, 1996.

The capture probes are specific for the DR region of MTBC. The DR region is specific to the MTBC and consists of a variable number of two types of short sequences, DR sequences, which are identical and spacers, which are all unique. Different isolates can be identified by the presence / absence of one of more spacers (Butcher, 1996; Haddad *et al*., 2004).

2.23 DNA amplification: Primer MSP 1/2

A PCR master mix, 40 µl, was made up in a clean room as follows: 5 µl of 10 x PCR Buffer, 2.5 µl of 25 mM $MgCl_2$, 2.5 µl of 10 mM dNTPs (Sigma, Cat #: DNTP-100), 0.25 µl of Jumpstart TAQ DNA Polymerase (Sigma, Cat #: D4184), 0.2 µl of MSP 1/2 (Mycobacteria Species Primer) primer mix and 29.55 µl of UltraPure Distilled Water.

The extracted DNA, 10 µl, was added to each 40 µl PCR mix in the PCR extraction laboratory, with each sample tested in duplicate. The mix was transferred to the PCR detection laboratory and amplified on thermocycler (Techne Flexigene) with heated lid, preheated to 105^{o}C. The program used was 1 cycle of 95^{o}C for 1 min, 35 cycles of 95^{o}C for 30 sec, 50^{o}C for 40 sec and 72^{o}C for 40 sec, 1 cycle of 72^{o}C for 10 min and hold at 4^{o}C.

MSP 1/2 Primer (Roth *et al*., 2000)

Primer 1 (5'-ACC TCC TTT CTA AGG AGC ACC-3')

Primer 2 (5'-GAT GCT CGC AAC CAC TAT CCA-3')

This primer targets part of the 16S-23S ribosomal DNA (rDNA) internal transcribed spacer and is specific to mycobacteria.

2.24 DNA Amplification: Primer Cap F/R

A PCR master mix, 45 µl, with Q solution, was made up in a clean room as follows: 5 µl of 10 x PCR buffer (containing 15 mM $MgCl_2$) 1µl of 10 mM dNTP, 10 µl of Q solution, 0.25 µl Hotstar TAQ DNA Polymerase (Qiagen, Cat #: 203205), 0.4 µl of CAP F/R primer mix and 28.35 µl of UltraPure Distilled Water.

A PCR master mix, 45 µl, without Q solution was made up in a clean room as follows: 5 µl of 10 x PCR buffer (containing 15mM $MgCl_2$) 1 µl of 10mM dNTP, 10 µl of Q solution, 0.25 µl Hotstar TAQ DNA Polymerase, 0.4 µl of CAP F/R primer mix and 38.35 µl of UltraPure Distilled Water.

The extracted DNA, 5 µl, was added to each 45 µl PCR mix in the PCR extraction laboratory, each sample was tested in duplicate. The mix was transferred to the PCR detection laboratory and amplified on thermocycler with heated lid, preheated to 105^{o}C. The program used was 1 cycle of 95^{o}C for 15 min, 35 cycles of 94^{o}C for 40 sec, 68^{o}C for 60 sec and 72^{o}c for 60 sec and 1 cycle of 72^{o}C for 7 min.

Cap F/R Primer (Skuce *et al.*, 2003)

Cap F (5'-AAA AAC GAA CGG CTG ATG ACC AAA CTC-3')

Cap R (5'-AAA AAG GAG GTG GCC ATC GTG GAA G-3')

2.25 Analysis of PCR Product

An agarose gel was made by dissolving 1.5 g of agarose (Sigma, Cat #.:A9539) and 10 µl of SYBR Safe DNA gel stain (Invitrogen, Cat #: S33102) per 100 ml of 1 x TAE (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) Buffer (Sigma, Cat #:T8280). The amplified sample was prepared for loading onto the gel by mixing 10 µl of the amplified sample with 2 µl of gel loading buffer (Sigma, Cat #: G7654). A 50 bp DNA ladder (BioLabs, Cat #: N3236S) was prepared by mixing 1 µl of DNA ladder with 9 µl of water and 2 µl of gel loading buffer. Each well on the gel was loaded with 10 µl of sample mixture. The gel was run at 100 volts for 2 hr with a Power pack (Bio Rad Power Pack Basic). The gel was photographed, under UV light, by a FluorChem SP (Alpha Innotech).

2.26 Statistical Analysis

Statistical analysis, using the chi square test, was done to perform a pair-wise comparison of the isolate recovery rates, contamination rates and time to detection for the different culture systems. Comparisons with a P value of <0.01 were considered significant. This was carried out using an online program available at http://graphpad.com/quickcalcs/chisquared1.cfm.

Chapter 3.

Development of Culture Methods for the Detection of *M. bovis* in Lymph Node Tissue.

3.1 Introduction

The BACTEC 460 is a semi–automated liquid culture system that has evolved to become the "gold standard" to which other systems are compared (Zuhre Badak *et al*., 1996). The system has many limitations including the handling, storage and disposal of radioactive material and labour requirements related to the loading and unloading of sample bottles from the incubator to a semi-automated instrument, Figure 1.10, for growth monitoring. Other drawbacks include the risk of needle stick injury, needles are used to inoculate and remove material from the culture bottles, Figure 3.1, and an incompatibility with modern data handling systems (Zuhre Badak *et al*., 1996; Scarparo *et al.*, 2002).

The MGIT 960 is a liquid culture system that addresses these limitations. It is a fully automated system, Figure 1.11, which continuously monitors growth in the tubes, has a high capacity, is non-radiometric and non-invasive (Kanchana *et al*., 2000). The MGIT 960 liquid culture system has been shown, in previous studies, to perform well for the isolation of MTBC, from human clinical samples, when compared to BACTEC 460 and solid media (Kanchana *et al*., 2000; Zuhre Badak *et al.*, 1996; Scarparo *et al*., 2002). It was of limited use in veterinary laboratories due to its poor growth of MTBC from bovine lymph node tissue samples (Yearsley *et al*., 2000). The manufacturer altered the media formulation to improve this and a study in Iowa has shown that the MGIT 960 system can now support the growth of *M. bovis* from bovine lymph node tissues (Hines *et al*., 2006).

Decontamination with OA is the preferred method, within the CVRL, for preparation of lymph node tissue for MTBC culture. CPC is not recommended by the manufacture for use with the MGIT 960 liquid culture system (Siddiqi and Rüsch-Gerdes, 2006) but it is used to decontaminate badger tissues for MTBC culture as part of the Badger Vaccine Project. This is a research project run by UCD, with funding supplied by the Department of Agriculture and Food, with the aim of developing a vaccine to control TB in badgers and remove a potential source of TB transmission to cattle (Gormley and Corner, 2011). A comparison was carried out between OA and CPC to determine if any difference between the performances of the two decontamination protocols, using MGIT 960 as the liquid culture system, could be noted.

Figure 3.1 BACTEC 460 bottle showing rubber septum used to inoculate the bottle (Costello, 2004).

3.2 Results

3.2.1 Limits of Detection for *M. bovis* in Liquid Culture

The primary aim of this work was to determine if the MGIT 960 liquid culture system could support *M. bovis* growth and to determine if the limits of detection for culture of *M. bovis*, with the MGIT 960, are similar to those for the BACTEC 460. The procedure was set up as described in section 2.7.

The negative lymph node tissue needed to be decontaminated prior to culture, to prevent growth of contaminating bacteria, but the *M. bovis* stock could have been added after this step. The decision was made to spike the *M. bovis* dilutions into the tissues prior to decontamination to keep the conditions as similar as possible to that of routine culture.

The growth on the 7H11 plates was recorded after 28 days of incubation when the colonies were counted. The colonies of *M. bovis* are circular with an undulating periphery, white/cream in colour and elevated in the middle, Figure 1.9. The only dilution that gave a useable count was the 1×10^{-3} dilution. The average colony count on the 3 plates was 50.33 colonies. Using the equation to determine the cfu/ml, described in section 2.7, the starting concentration of *M. bovis* was determined to be 2.5×10^{6} cfu/ml.

M. bovis growth was indicated in both MGIT 960 tubes and BACTEC 460 bottles down to the 1×10^{-4} dilution with cording, as shown in Figure 1.2, being seen in each ZN stain. Cording was detected in two BACTEC 460 bottles and three MGIT 960 tubes at the 1×10^{-5} dilution while only one MGIT 960 tube showed cording at the 1×10^{-6} dilution. No growth was detected in the remaining dilutions or the negative control after 7 weeks of incubation.

From Figure 3.2 it can be seen that both liquid culture systems showed similar results and were able to detect growth in all tubes down to 2.5×10^{2} cfu/ml. The time to detection of growth for the first four dilutions ($1 \times 10^{-1} - 1 \times 10^{-4}$) was 7.42 days for BACTEC and 7.36 days for MGIT. The dilutions lower than 2.5×10^{2} cfu/ml were excluded from the calculation of the average time to growth detection since not all media tubes showed growth.

These results show that the MGIT 960 system is capable of supporting *M. bovis* growth.

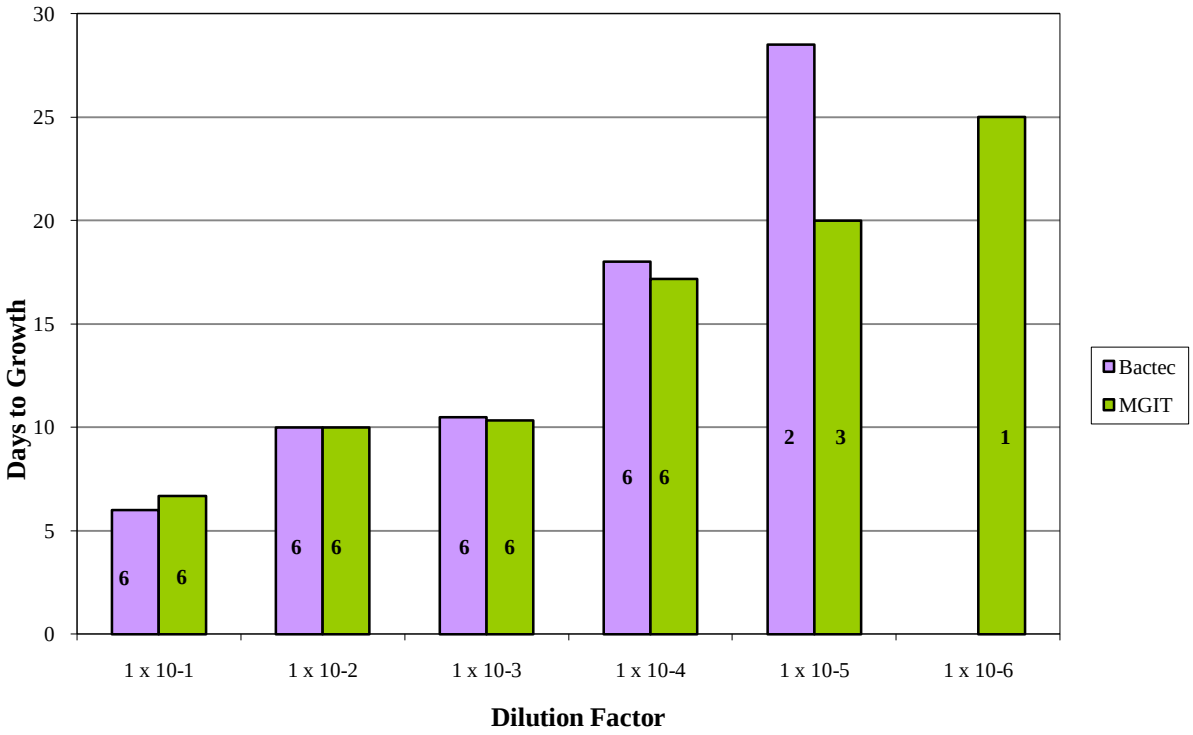

Figure 3.2: Time to detection of cording at each dilution by the BACTEC 460 (BACTEC) and MGIT 960 (MGIT) liquid culture systems. The number in each column indicates the number of media, for each system, that showed cording.

3.2.2 Comparison of Different Culture Systems for MTBC Growth from LN Tissues

Having determined that the MGIT 960 system can support the growth of *M. bovis*, the two liquid culture systems, MGIT 960 and BACTEC 460, and solid culture media (1 LJP and 1 Stonebrinks medium) were compared for the detection of Mycobacteria in routine lymph node (LN) samples.

Decontamination of 867 routine LN samples, submitted to the CVRL for TB culture, from bovine and a small number of samples other species, specifically 51 badgers, 10 cervine, 19 ovine and miscellaneous animal species (4 Alpaca, 2 Porcine, 1 Canine and 1 Caprine), was carried out as described in section 2.1. After a liquid culture media flagged positive and had AFB been seen on a ZN stain, section 2.5, the sample was sub-cultured, section 2.8, and identified using GenProbe Accuprobe kits, section 2.9.

From the 867 lymph node tissues cultured the number of isolates identified is shown in Table 3.1.

Both liquid culture systems performed better than or as well as solid culture system over all isolates identified. Figure 3.3, shows a breakdown of the number of isolates identified by the two liquid culture systems, MGIT 960 and BACTEC 460 and by the solid culture system (LJP and Stonebrinks Mediums). Both liquid culture systems gave similar results for the isolation of MTBC but neither identified all MTBC positive tissues (92% for BACTEC and 93.2% for MGIT). A pair-wise comparison, section 2.26, of the MTBC recovery rates between each of the different culture system is shown in Table 3.2. There is no statistically significant difference between the two liquid culture systems however there is a statistically significant difference between each of the liquid cultures systems and solid media.

There appears to be a difference for the isolation of atypical mycobacteria between the liquid culture systems (Figure 3.3) but due to the low number of isolates detected, 19 isolates, it was not possible to carry out statistical analysis. The MGIT 960 system isolated only 9 of the atypical mycobacteria and the BACTEC 460 system isolated 17 isolates. This agrees with the findings of previously published reports (Kanchana *et al*., 2000; Zuhre Badak *et al.,* 1996; Scarparo, *et al*., 2002). No further work was carried out to identify which isolates the MGIT media supported and which were unable to grow. Each culture system should be able to support the growth of as

Table 3.1: Total number of Tissue Samples from which isolates were identified. Atypical mycobacteria are mycobacteria that are not part of the MTBC group or *M. avium*.

Total Samples Cultured	867
MTBC Isolates	176
R. equi Isolates	88
Atypical Myco. Isolates	19
M. avium Isolates	6

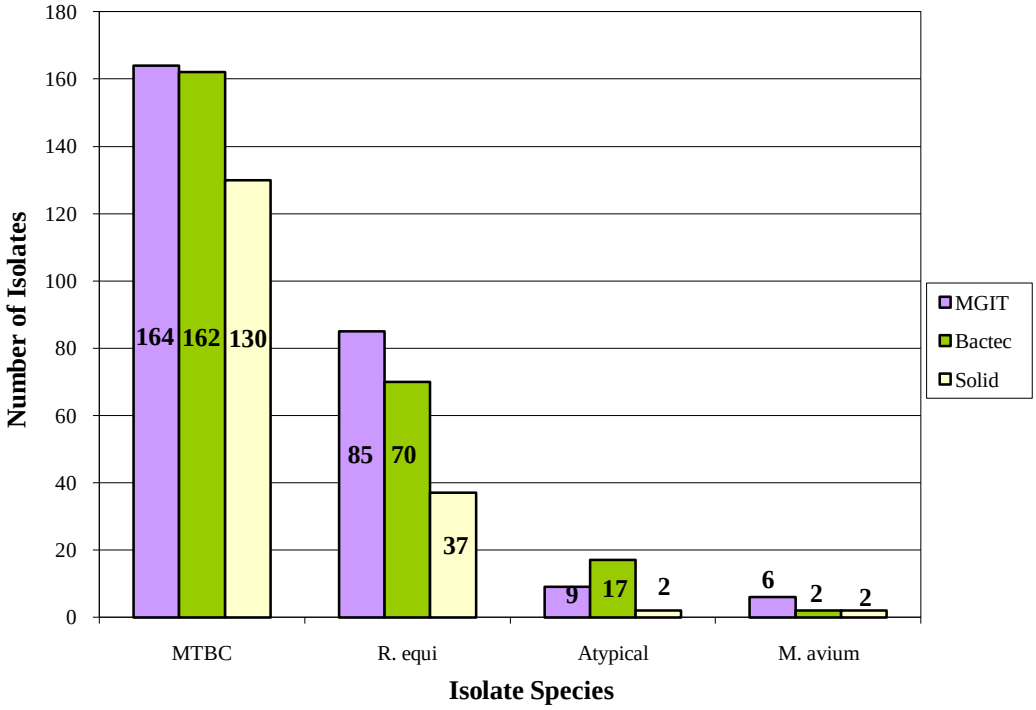

Figure 3.3: The difference in the number of isolates with each of the different culture systems.

Table 3.2: Pair-wise comparison of the 3 culture systems for the difference in the recovery rate of MTBC

Systems compared	Percent Difference	P-Value
MGIT versus BACTEC	1.1	>0.58
MGIT versus Solid Media	18.8	<0.01*
BACTEC versus Solid Media	17.7	<0.01*

* Statistical significant difference (P<0.01) between the 2 media systems

many species of mycobacteria as possible but atypical mycobacteria have no clinical significance to the eradication of bovine TB.

The number of *M. avium* isolates was very low at a total of 6 isolates. The MGIT system identified all of these and the other culture systems identified only 2 isolates each.

Due to the cell wall structure of *Rhodococcus equi*, it is resistant to decontamination with 5% oxalic acid and is isolated during TB culture. *R. equi* is distinguished from mycobacteria by the presence of coccobacilli, which are usually non-acid-fast, on a ZN stain, the growth of salmon pink colonies, Figure 1.8, on a blood agar plate and the colony morphology on solid media, Figure 1.14. The liquid medium used in the MGIT system seems better able to support the growth of *R. equi* than either BACTEC or solids (Percentage of *R. equi* isolated: 96.6% with MGIT, 79.5% with BACTEC and 42% with solids). Statistical analysis, section 2.26, shows that there is a significant difference between all three culture systems for the isolation of *R. equi*, Table 3.3. Use of the MGIT system routinely will increase the number of *R. equi* isolates from samples.

No individual culture system can identify all isolates and to increase the effectiveness of the culture procedure a combination of solid and liquid culture is used. When each liquid culture system was combined with solid culture, Figure 3.4, the isolation of MTBC (96.6% for BACTEC and solids and 98.3% for MGIT and solids) and other isolates was improved.

Most of the MTBC isolates are identified in the first 3 weeks of culture with both liquid culture systems (81.1% on BACTEC and 78.9% on MGIT). The MGIT 960 system detects growth of MTBC, on average, slightly faster than the BACTEC 460 system (14.5 days (range 1-49 days) on MGIT to 15.2 days (range 2-49 days) on BACTEC), shown in Figure 3.5, but both liquid culture systems detect MTBC growth faster than solid culture systems which take an average of 25.6 days (range 10–49 days). A pair-wise comparison of the time to detection for MTBC growth, section 2.26, indicates that there is no statistically significant difference between the liquid culture systems but there is a difference between each of the liquid culture systems and solid culture, Table 3.4. MGIT 960 also shows faster growth for atypical mycobacteria and *R. equi* isolates. This is probably due to the continuous monitoring of the tubes in the MGIT 960 instrument whereas the BACTEC 460 bottles are monitored on a schedule.

Table 3.3: Pair-wise comparison of the 3 culture systems for the difference in the recovery rate of R. equi

Systems compared	Percent Difference	P-Value
MGIT versus BACTEC	17.1	<0.01*
MGIT versus Solid Media	54.6	<0.01*
BACTEC versus Solid Media	37.5	<0.01*

* Statistical significant difference (P<0.01) between the 2 media systems

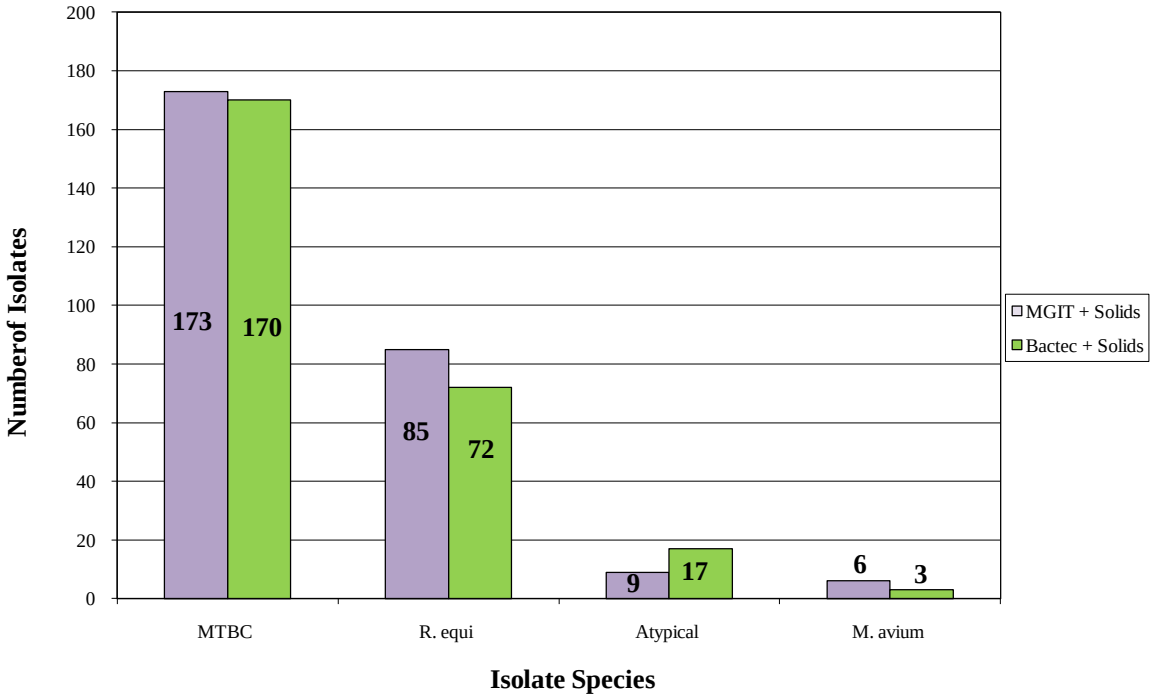

Figure 3.4: The number of isolates for each of the liquid culture systems when combined with solid culture.

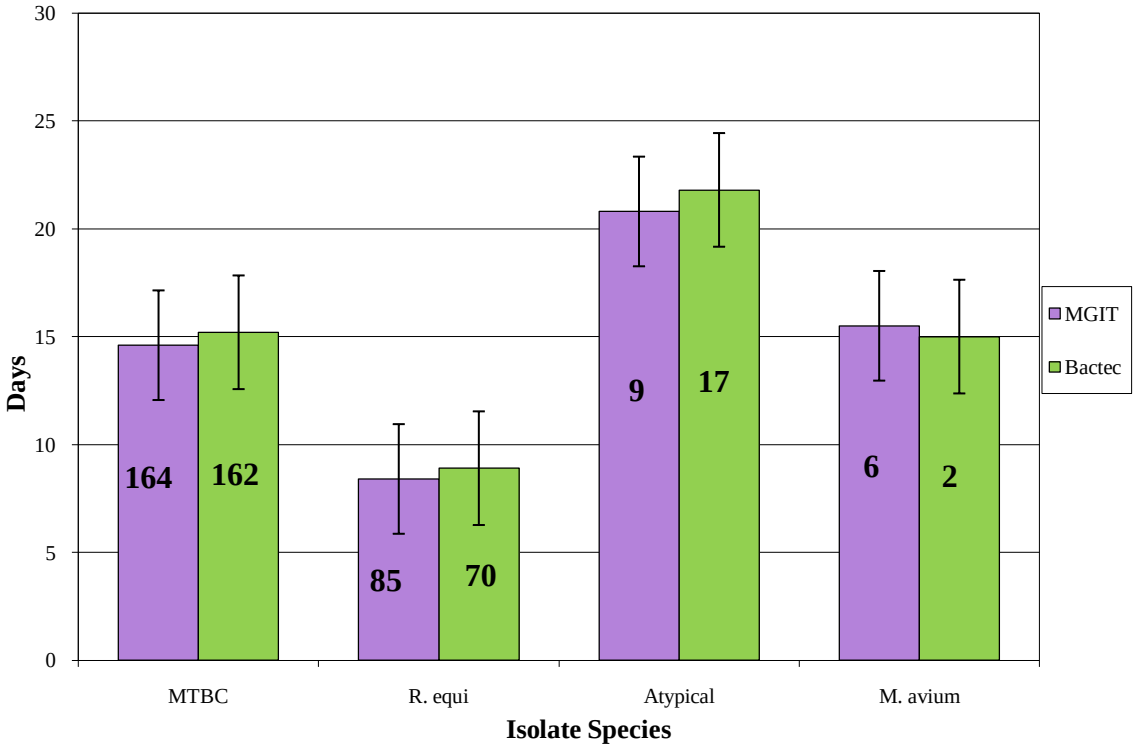

Figure 3.5: The average length of time to growth detection in both the MGIT 960 and BACTEC 460 liquid culture systems. The numbers in the columns indicate the number of samples the average is taken over.

Table 3.4: Pair-wise comparison of the time to detection of MTBC between the different culture systems

Systems compared	Difference in Days	P-Value
MGIT < BACTEC	0.6	>0.79
MGIT < Solid Media	11	<0.01*
BACTEC < Solid Media	10.4	<0.01*

* Statistical significant difference (P<0.01) between the 2 media systems

The contamination rate for the MGIT 960 system was close to three times greater than the BACTEC 460 system, at 8.2% for the MGIT system and 3.1% for the BACTEC system. While not ideal, this is within the acceptable contamination rate of between 2-10% (Collins *et al.*, 1997). Routine use of the MGIT system may cause an increase in the number of samples that have contamination and will require reprocessing. For solid culture systems the contamination rate was higher than both liquid culture systems and outside the acceptable range, at 10.9%. The solid culture systems do not benefit from the addition of the PANTA antibiotic mixture, prior to sample inoculation, like both liquid culture systems. Table 3.5 shows that there is a statistically significant difference between the contamination rates in all three culture systems when pair-wise comparison, section 2.26, was carried out.

3.2.3 Decontamination Study

Having shown the ability of the MGIT 960 liquid culture system to support the growth of *M. bovis*, from routine lymph node tissues, a comparison was carried between two different decontamination protocols. The comparison was carried out with 5% OA, the preferred decontamination method within the CVRL, and with 0.75% CPC, the decontamination method used as part of the badger project, for decontamination of lymph node tissues prior to culture.

Decontamination of 144 routine bovine lymph node samples was carried out with OA, section 2.2, and CPC, section 2.10. After a tube flagged positive and had AFB seen on a ZN stain, section 2.5, the sample was sub-cultured, section 2.8, and identified using GenProbe Accuprobe kits, section 2.9.

Table 3.6, shows the total number of isolates identified with a combination of liquid and solid culture for both decontamination protocols. The two atypical mycobacteria isolated grew on the CPC decontaminated solid media one of which was also isolated from an OA decontaminated MGIT tube.

The breakdown on the number of MTBC and *R. equi* isolates on solid and liquid culture, with each decontamination protocol, is summarised in the following series of Tables 3.7 and 3.8 and Figures 3.6 and 3.7. The difference between the two decontamination methods is more pronounced in the MGIT 960 liquid culture system than with solid culture. Statistical analysis could not be carried out due to the small number of isolates. All MTBC isolates are identified with OA decontamination but this method identified only 8 of a total of 13 *R. equi* isolates.

Table 3.5: Pair-wise comparison of the contamination rates for the 3 culture systems

Systems compared	Percent Difference	P-Value
MGIT > BACTEC	5.1	<0.01*
MGIT < Solid Media	2.7	<0.01*
BACTEC < Solid Media	7.8	<0.01*

* Statistical significant difference (P<0.01) between the 2 media systems

Table 3.6: Total numbers for each isolate on a combination of liquid and solid culture with each decontamination method.

Liquid and Solid Media Combined			
	OA	CPC	Total
MTBC	19	9	19
Atypical	1	2	2
R.equi	8	11	13

Table 3.7: The number of MTBC isolates with each decontamination method.

MTBC Isolates (total = 19)			
	MGIT	Solid	Total # Isolated
5% OA	17	12	19
0.75% CPC	1	9	9

Table 3.8: The number of *R. equi* isolates with each decontamination method.

***R. equi* Isolates (total = 13)**			
	MGIT	Solid	Total # Isolated
5% OA	8	2	8
0.75% CPC	0	11	11

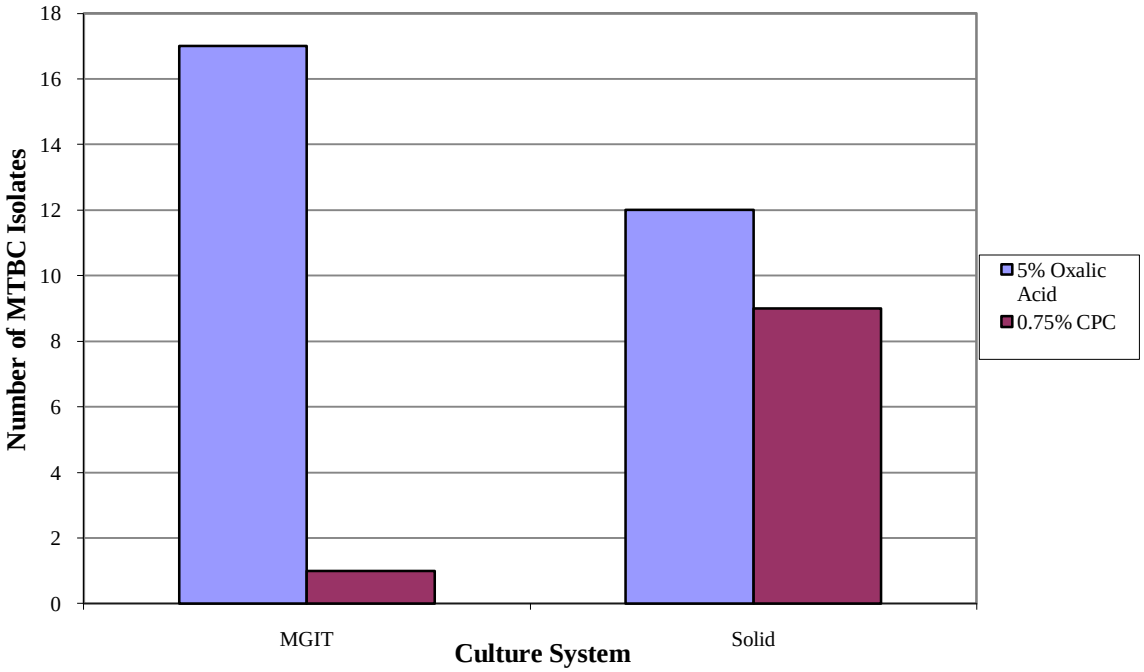

Figure 3.6: A comparison of the number of MTBC isolates, on MGIT 960 liquid culture system and solid culture system, with each decontamination method.

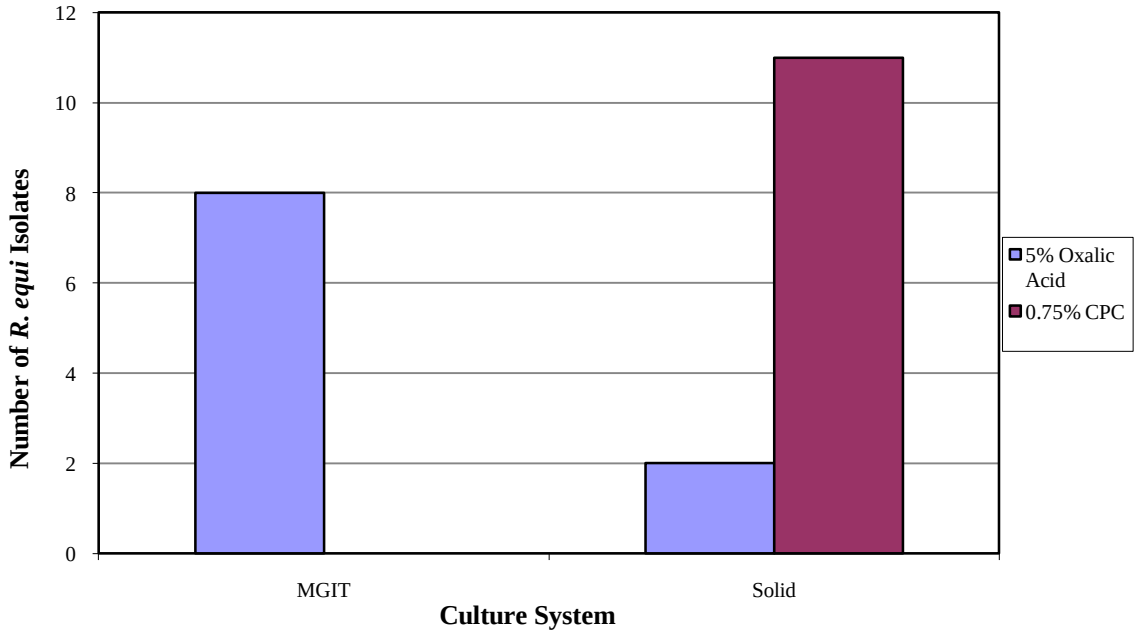

Figure 3.7: A comparison of the number of *R. equi* isolates with each decontamination method.

CPC decontamination has clearly been shown to be not suitable for use with MGIT 960 liquid culture system, Tables 3.3 and 3.4 and Figures 3.7 and 3.8. However, this decontamination method performed much better than OA decontamination for the detection of *R. equi* infection and identified both atypical mycobacteria isolates with solid culture, Table 3.4 and Figure 3.8. Neither of these isolate types are vital results since the main aim of culture was to identify a MTBC infection. However, if further study was ever carried out on either of these isolate types the possibility of using CPC decontamination, on solid culture only, should be borne in mind.

In this study, the contamination rate was 4.86% for Oxalic acid and 1.39% for CPC in MGIT liquid culture. In the solid culture system the rate of contamination was much higher with 10.42% for oxalic acid and 18.75% for CPC. As stated previously, a contamination rate of between 2 and 10% is acceptable depending on several conditions such as sample type, age and storage conditions (Collins *et al.*, 1997). A difference in contamination rates between liquid and solid culture has to be expected since the MGIT liquid culture media is supplemented with an antibiotic mix, PANTA, prior to inoculation with the decontaminated samples. This mix would aid in the suppression of growth of contaminating bacteria. The solid media have no such antibiotic mix added and are dependent upon the effectiveness of the decontamination protocol to ensure the level of contamination is kept to a minimum.

For OA decontamination the contamination rate is close to the acceptable range, with 4.86% for MGIT media and 10.42% for solid media. The MGIT media contamination rate is lower than that shown above, in the comparison between the BACTEC 460 and MGIT 960 liquid culture systems, of 8.2% for MGIT 960. This difference is probably due to the sample conditions.

For CPC decontamination the contamination rate is outside the acceptable range, with a rate of 18.75% for solid media, and 1.39% for MGIT media. This extreme difference between the solid and liquid culture may be caused by the dilution of any remaining CPC in the sample, following the decontamination procedure, in the liquid culture media. For CPC the Critical Micellar Concentration (CMC) is 0.32%. At this concentration the molecules of the detergent form small monomers which appear to be toxic since they are small enough to pass though the bacterial cell wall. At a concentration above 0.32% the molecules form micelles which are too large to pass though the bacterial cell wall (Brown *et. al.*, 2005). This would not affect solid

media since any excess decontaminant would be absorbed by the media before the CMC would be reached.

3.3 Discussion

Over time it had become necessary to find a replacement for the BACTEC 460 liquid culture system due to its safety concerns and lack of data handling system. The MGIT 960 liquid culture system had many advantages over the BACTEC 460 system however a previously published report by Yearsley *et al*. had indicated the MGIT 960 system could not support the growth of *M. bovis* from bovine LN tissue (Yearsley *et al.*, 2000). The MGIT media had been modified, since that work was carried out, to allow the culture system to support *M. bovis* growth. The success of the change to the media formulation was shown by the results of limits of detection study for *M. bovis*, after being spiked into TB negative LN tissue, since both MGIT 960 and BACTEC 460 liquid culture systems gave the same result at 2.5 x 10^2cfu/ml, section 3.2.1.

While this result was promising it was not sufficient to allow the MGIT 960 system to replace the BACTEC 460 system, the current "gold-standard" for TB culture (Zuhre Badak *et al*., 1996). A total of 867 routine lymph node tissues were cultured on both liquid culture systems and solid culture system. For the detection of MTBC isolates the results from the MGIT 960 system were comparable to that of the BACTEC 460 system, 93.1% on MGIT and 92% on BACTEC, and both liquid culture systems preformed better that solid culture system, which detected 73.9% of MTBC isolates. The MGIT 960 system detected growth of MTBC isolates slightly faster than the BACTEC 460 system with average days to detection of 14.6 days for MGIT compared to 15.2 days for BACTEC which was probably due to the continuous monitoring of the cultures in the MGIT 960 instrument compared the schedule used to monitor the BACTEC bottles.

Previously published work that compared both BACTEC 460 and MGIT 960 for the culture of mycobacteria from humans all showed broadly similar results to this study for MTBC detection, even though differing decontamination protocols were used (Chew *et al*., 1998; Scarparo *et al*., 2002; Kanchana *et al*., 2000; Hanna *et al.*, 1999; Somoskövi *et al.*, 2000; Tortoli *et al*., 1999; Zuhre Badak *et al*., 1996). These studies had been carried out primarily on respiratory samples with most including less than 10% of extra-pulmonary sample types. A single article, Chew *et al*. included

over a third of the samples from LN tissues but it was not stated how many of the LN samples were positive for mycobacterial growth (Chew *et al*., 1998).

A study from Iowa compared the MGIT 960, BACTEC 460 and solid media (7H10 and 7H11) for TB culture of bovine LN tissues, using the N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) decontamination method (Hines *et al*., 2006). They reported a *M. bovis* detection rate of 94.6% for the MGIT 960 system and 74.4% for the solid culture system which, even though a different decontamination method and different solid culture systems were used, was similar to the MTBC detection rate in this study of 93.1% for MGIT 960 and 73.9% for solid culture systems. A difference in the results was seen for the BACTEC 460 system which detected only 79.1% of *M. bovis* isolates in Hines *et al.* compared to a MTBC detection rate of 92% here. This was an unusual result since the results from human studies had put the MTBC detection rate for BACTEC 460 system very similar to MGIT 960 system (Chew *et al*., 1998; Scarparo *et al*., 2002; Kanchana *et al*., 2000; Hanna *et al.*, 1999; Somoskövi *et al.*, 2000; Tortoli *et al*., 1999; Zuhre Badak *et al*., 1996). The days to detection was also much longer in Hines *et al*., 2006 report for the BACTEC 460 system at 28.2 days with 15.8 days reported for the MGIT 960 system.

The decontamination method is unlikely to be the reason for the differing results with BACTEC 460 system since the results from the MGIT 960 system were so similar. A possible explanation may that in this study the result is based on growth of MTBC isolates compared to *M. bovis* isolates in the Hines *et al*., 2006 study since due to limited resources further testing was not done on the routine culture MTBC isolates to identify the isolate species. Another explanation is the differing criteria when the time to detection result was recorded in the Hines *et al.*, 2006 study the time to detection was noted when the BACTEC bottle gave a GI reading of 300 compared to a GI reading of 50 in the work reported here. Culture procedure was altered for BACTEC 460 system in Hines *et al.*, 2006 compared to this study, with erythromycin, at a concentration of 32 µg/ml per BACTEC bottle, being added to the PANTA antibiotic mixture. It was not known how this could affect the *M. bovis* detection rate with the BACTEC 460 system since this antibiotic had been shown to have no inhibitory effect on *M. bovis* but it may have reduced the concentration of growth promoter, POES, in the BACTEC bottle (Demoulin *et al*., 1983).

For the detection of atypical mycobacteria or *M. avium* isolates it is difficult to draw conclusions from the results since the total number of isolates, 25 non-

tuberculosis mycobacteria (NTM), identified by the different culture systems was small, Figure 3.5. For *M. avium* the MGIT 960 system, which identified a total of 6 isolates, performed better than both the BACTEC 460 system and solid culture systems, which only identified 2 isolates each. However, for the detection of atypical mycobacteria the MGIT 960 system, which identified a total of 9 isolates, had a poorer performance that the BACTEC 460 system, which identified 17 isolates. Solid culture systems only identified 2 atypical mycobacteria isolates. No work was carried out to identify which NTM could be supported by the MGIT 960 system and which NTM species failed to grow. The lower detection rate for NTM with the MGIT 960 system does agree with the finding of some previously published reports on human clinical samples (Zuhre Badak *et al*., 1996; Kanchana *et al*., 2000; Scarparo *et al*., 2002). With human TB cases NTM are of clinical significance, particularly in immune-compromised patients, however diagnosis of these infections, which could provide information to improve animal health and husbandry, are not critical for the Bovine TB Eradication Scheme.

The contamination rate for the MGIT 960 system was nearly three times great than the BACTEC 460 system, at 8.2% for the MGIT system compared to 3.1% for the BACTEC system, but both had a lower contamination rate that the 10.9% for solid culture systems. The MGIT media is more nutrient rich than the BACTEC media. The BACTEC media only provides C^{14}-labelled palmitic acid and most bacteria cannot utilize this fatty acid so will not grow under these conditions (Hines *et al*., 2006). The higher contamination rate with the solid culture systems was probably due to its dependence on the effectiveness of the decontamination protocol since both liquid culture systems benefited from addition of an antibiotic cocktail prior to sample inoculation. The higher contamination rate with the MGIT 960 system, compared to the BACTEC 460 system, had been reported previously though the rate of contamination differs depending on the sample type and decontamination method used (Scarparo *et al*., 2002; Kanchana *et al*., 2000; Hanna *et al.,* 1999; Somoskövi *et al.,* 2000; Hines *et al.,* 2006)

The MGIT 960 system supports the growth of *R. equi* much better that BACTEC 460 and solid culture systems, 96.6% for the MGIT system compared to 78.4% for BACTEC system and 40.9% for solid culture systems. There nothing in the published literature about the isolation of *R. equi* during TB culture from veterinary samples and it is likely that this bacterium is classed as contaminant in

other studies. *R. equi* diagnosis is an important result, since a *R. equi* infection causes a granuloma in lymph nodes tissue that would be visually similar to a TB granuloma and detected during abattoir meat inspections.

This study also evaluated the suitability of 0.75% CPC as a decontaminant for the culture of MTBC from LN tissues for use with the MGIT 960 and has shown that it is not a suitable chemical for this purpose, Figure 3.8 and Table 3.3. This result agrees with the manufacturer's recommendations and with Brown *et al*. and McLernon *et al*. had both shown that CPC decontamination was not suitable for use with BACTEC 460 liquid media (McLernon *et al.*, 2004; Brown *et al.*, 2005; Siddiqi and Rüsch-Gerdes, 2006). Corner *et al*. had shown that on balance CPC was most suitable chemical for the decontamination of tissues prior to *M. bovis* culture on solid media (Corner *et al.* 1995). The results from this study disagree with that, since even though solid culture systems identified more MTBC isolates than the MGIT 960 system with CPC decontamination, OA decontamination detected more MTBC isolates on both liquid and solid culture systems, Figure 3.8.

For the isolation of *R. equi*, use of CPC decontamination coupled with solid culture systems only, would enhance the number of isolates detected, Figure 3.9. This could be useful if further research was undertaken on *R. equi* infections in cattle. The low number of atypical mycobacteria identified in this study, 2 isolates, means that it is difficult to draw any conclusions from this result.

3.4 Conclusion

This study has provided more evidence on the suitability of the MGIT 960 system as a replacement for the BACTEC 460 system for the culture of MTBC from veterinary LN tissue. No single culture system identified all isolates and even when each liquid culture system was combined with solid culture system (LJP and Stonebrinks) not all isolates were identified, Figure 3.6. The use of all three systems, MGIT 960, BACTEC 460 and solid culture, together continually, would allow for the maximum number of isolates but would increase the workload and the cost, since the liquid culture systems are expensive to maintain. A compromise has to be made between the maximum number of isolates, cost, ease of use and safety concerns. Identification and confirmation of MTBC infected tissues is of the primary significance for the Bovine TB Eradication Scheme. While detection of atypical

mycobacterium and *M. avium* infections are important they are not critical for the reduction and potential elimination of *M. bovis* from the bovine population.

This study had shown that both the liquid culture systems performed equally well for the detection of MTBC infected tissues and that the MGIT 960 system is a suitable replacement for the BACTEC 460 system. The use of the MGIT 960 system in place of the BACTEC 460 system will involve compromise. These include the reduction in the detection of atypical mycobacteria infections, increased number of *R. equi* isolates being grown in culture and a possible increase in the number of cultures that require treatment for contamination. These are balanced out by a reduction in safety concerns, due to the elimination of needles and radioactive material, C^{14}, from the culture procedure, a reduction in labour requirements and a reduced time to detection of MTBC, due to the automated monitoring system. On balance, the advantages offered by the MGIT 960 system over the BACTEC 460 system outweigh the disadvantages.

As recommended, the use of CPC does not appear to be a suitable decontamination method for tissues prior to MTBC culture when coupled with the use of the MGIT 960 liquid culture system. It should be considered if further study was to be carried out on *R. equi* infections in cattle but only for use with solid media.

Chapter 4.

Evaluation of Different Molecular Methods for the Direct Detection of *M. bovis* in Lymph Node Tissue.

4.1 Introduction

For the majority of suspect Bovine tissue lesions received in the TB Laboratory, a diagnosis can be made based on histopathological examination within one week. However, it is not possible to make a diagnosis based on histopathology in all cases and these samples must be cultured.

Culture of tissue samples for isolation of MTBC can take up to 7 weeks or longer, during which time the TB status of the herd is undetermined. For the majority of herd owners this delay is not problematic but for a small number, such as Singleton Reactor herds, it may cause significant inconvenience. Annual testing to determine the TB status of cattle herds is done using the Single Intradermal Comparative Tuberculin Test (SICTT). Approximately a third of the herds, which disclose reactors to this test, have only one animal giving a positive reaction. Because the tuberculin test is not 100% specific it is possible that some of these are false positive reactions. If epidemiological criteria suggest this possibility and no visible lesions are detected during a post slaughter examination of the carcass, then, LN tissue samples are obtained for culture. These samples are submitted to the TB Laboratory as Singleton Reactors. Samples from approximately 600 Singleton Reactors are examined at the CVRL each year, most of which are culture negative (Good and Duignan, 2011).

A direct detection test, which could provide a result in a few days, would be of great benefit both for singleton reactors and samples from animals that are linked to a suspected human TB infection i.e. pets. PCR based direct detection tests are used for the detection of mycobacteria in human sputum samples but so far have had only limited application for the detection of mycobacteria in animal tissue samples. There have been difficulties in regard to samples with low numbers of organisms, problems in extracting and harvesting DNA or RNA from mycobacteria and also problems with PCR inhibitors within tissues (Mangiapan *et al.*, 1996; Roring *et al.*, 2002).

For this study five different methods were used to isolate and detect MTBC DNA from decontaminated lymph node (LN) tissue samples. These were heat lysis, High Pure PCR Template Preparation Kit (Roche Applied Science, Cat #: 11796828001), Immunomagnetic Separation (IMS), GenoType Mycobacteria Direct 4.0 (GTMD) (Hain Lifesciences, GMBH, Nehren Germany, Cat #: H4010) and Sequence Capture PCR.

A total of 30 samples were used for each method, made up of 27 MTBC culture positive and 3 culture negative tissues. The MTBC positive tissues were

classed as high, medium to low positives, based on a direct ZN examination of the decontaminated tissue homogenate prior to inoculation into the culture media and storage (this is described in more detail in section 2.11).

4.1.1 Heat Lysis

The first method evaluated was heat lysis (HL), of a heat killed decontaminated tissue sample. This was selected since it is a simple and effective method for releasing DNA from cells and it was the method used to extract the *M. bovis* DNA, used as the PCR positive control, from a culture. Centrifugation of the sample, after heat lysis, is the only method of purification of the released DNA from the cell debris and any other contaminating molecules within the sample.

4.1.2 High Pure PCR Template Preparation Kit (High Pure Kit)

The second method evaluated was the High Pure PCR Template Preparation Kit (Roche Applied Science, Cat #: 11796828001) which is designed to purify nucleic acids from other contaminating molecules. To do this, the kit uses a combination of chemical and enzymatic digestion to lyse cells and inactivate any nucleases present in a sample. Absorption chromatography is then used for nucleic acid purification.

Lysozyme and a tissue lysis buffer weaken the cell walls. A Proteinase K digestion breaks open the cell membranes and chaotropic salt (guanidine HCl), in the binding buffer, inactivates all nucleases. The extract is loaded onto a filtration column, which contains glass fibres that any nucleic acids present in the extract will selectively bind to. Washes with the different buffers remove any contamination molecules from the column. A low salt elution buffer releases the purified nucleic acids from the glass fibres and the extract can then be used in a PCR reaction (Galli, 2006; Roche Diagnostics Ltd.).

The kit is used routinely, in the CVRL, to extract DNA from cultures of *M. avium* subsp *paratuberculosis* (*M. paratuberculosis*), which has a much tougher cell wall than *M. bovis*.

4.1.3 Immunomagnetic Separation

The third method evaluated was Immunomagnetic Separation (IMS). This is a technique used for the extraction of a desired cell from a heterogeneous cell suspension (Grant *et al*., 1998). IMS has been successfully applied to the detection of *M. paratuberculosis* from spiked and natural infected milk samples (Grant *et al*., 1998; Djønne *et al*., 2003) and from faecal samples (Khare *et al*., 2004). The technique has been used to isolate *M. avium* from stool samples from AIDS patients (Li *et al*., 1996) and *M. tuberculosis* in cerebrospinal fluid (Mazurek *et al*., 1996). *M. bovis* was detected, using IMS, from spiked milk samples (Antognoli *et al*., 2001). The successful adaptation of IMS to isolate mycobacteria from other sample types indicates the effectiveness of this technique.

IMS relies on the interaction between cell surface antigens and antibodies bound to paramagnetic beads. The application of a strong magnetic field allows the target cells to be separated from the suspension. After IMS, the bead bound cells can be resuspended in a smaller volume, which allows for concentration of the target cells. IMS is a powerful yet gentle technique for concentration of a target cell since no potentially harmful chemicals are used during the procedure. Consequently the physiological state of the cells is not affected (Grant *et al*., 1998).

4.1.4 GenoType Mycobacteria Direct 4.0

The fourth method evaluated was the propriety detection method GenoType Mycobacteria Direct 4.0 (GTMD, Hain Lifesciences). GTMD, version 4.0, is an assay based on Nucleic Acid Sequence Based Amplification (NASBA) and DNA strip technology. It allows for the direct detection of MTBC, *M. avium*, *Mycobacterium malmoenese*, *Mycobacterium intracellulare* and *Mycobacterium kansasii*, by amplification of 23S rRNA, from decontaminated clinical samples (Franco-Álvarez de Luna *et al*., 2006). This kit has been used to successfully detect mycobacteria directly from decontaminated sputum samples and a limited number of other sample types including tissue biopsies, cerebrospinal fluid and pleural fluids (Seagar *et al*., 2008; Franco-Álvarez de Luna *et al*., 2006; Fakrin *et al*., 2007; Kiraz *et al*., 2010; Neonakis *et al*., 2009).

The assay is divided into three parts. The initial part is a magnetic bead capture and RNA extraction procedure.

The second stage in the process is a NASBA process, which is an isothermic cyclical series of reactions, shown in Figure 4.1. The reaction mixture contains nucleoside triphosphates, buffer components, two oligonucleotide primers and three enzymes, T7 RNA polymerase, reverse transcriptase and RNaseH, which work together to amplify sequences from a single stranded RNA template. The first primer binds to the RNA, which allows the reverse transcriptase to synthesise a complementary DNA (cDNA) strand. The RNaseH digests away the RNA template. A second primer binds to the cDNA and reverse transcriptase forms a double-stranded cDNA. The double stranded cDNA then acts as a mini "gene" which T7 RNA polymerase uses to produce RNA transcripts. These RNA transcripts then cycle though the reaction (Cook, 2003).

The amplified RNA product is then reverse hybridised onto a thin strip, which has immobilised gene probes on its surface. A Streptavidin-conjugated alkaline phosphate is used to carry out a colorimetric detection on the hybridised samples with an appropriate substrate. Different banding patterns correspond to the isolate identification. An internal RNA control is included in each sample, during the NASBA step, to indicate if any inhibitory substances are present in the sample (Seagar *et al.*, 2008).

4.1.5 Sequence Capture PCR

The fifth method evaluated was sequence capture PCR. This consists of a process that provides enrichment of a specific target sequence and removal of PCR inhibitory substances. This method was included with this study since it has been successful for the direct detection of *M. bovis* in lymph node tissue and decontaminated tissue homogenates (Roring *et al.*, 2000; Mangiapan *et al.*, 1996; Parra *et al.*, 2008). The technique has also been used for the detection of *M. paratuberculosis* from faeces (Marsh *et al.*, 2000; Marsh and Whittington, 2001; Halldórsdóttir *et al.*, 2002) and *M. ulcerans* from water and plant material (Stinear *et al.*, 2000).

Samples are mechanically disrupted and enzymatically digested. Biotinylated capture oligonucleotides are added to the crude extract and allowed to hybridise. Streptavidin coated paramagnetic beads are added to the sample and bind to the hybridised biotinylated oligonucleotides. A strong magnetic force is applied and the target sequences are retained, with the beads, and the remainder of the sample is

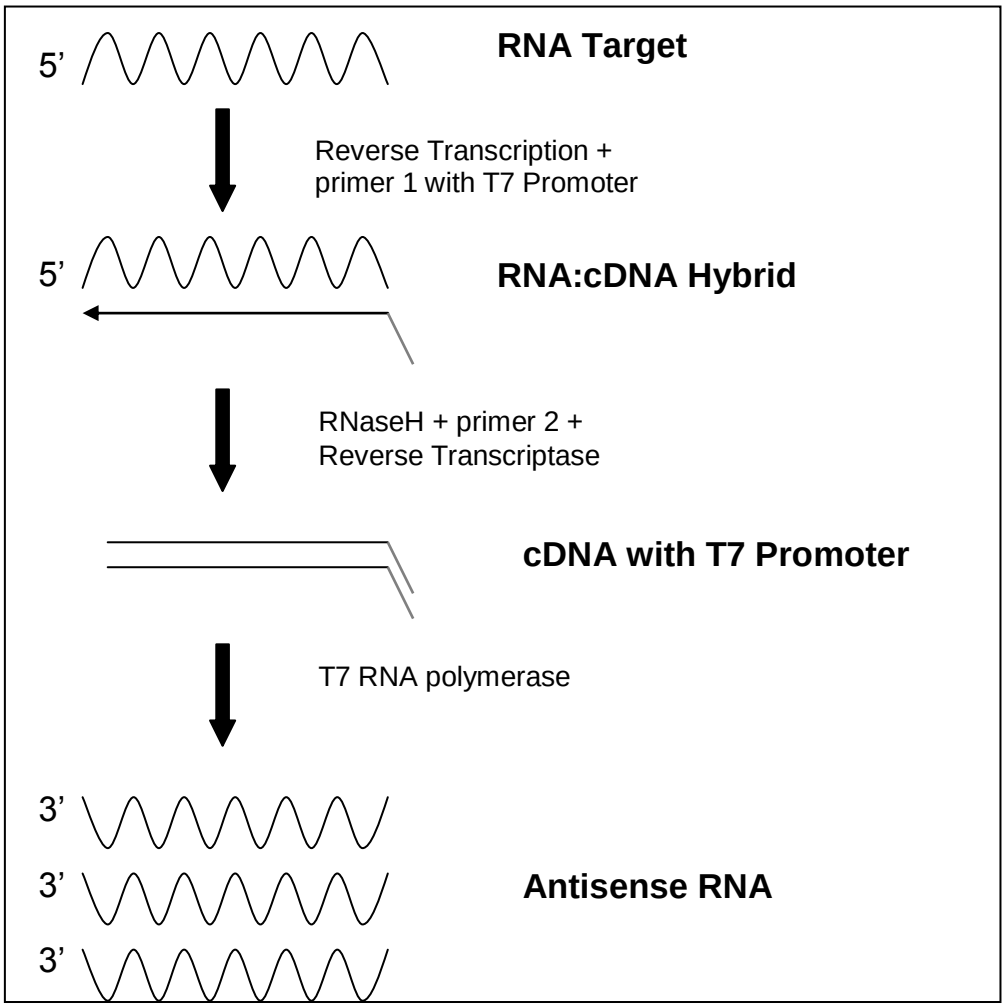

Figure 4.1: Schematic Diagram of a NASBA reaction. Adapted from Monis *et al*., 2005.

discarded. The samples can be used for PCR immediately or the captured sequences can be released from the magnetic beads by a heat treatment step (Roring *et al*., 1998; Roring *et al*., 2000; Mangiapan *et al*., 1996; Parra *et al*., 2008).

Each of the methods evaluated as part of this study have been used to prepare mycobacterial DNA for PCR but only Sequence Capture PCR has been successfully used to detect *M. bovis* directly from lymph node tissue.

4.2 Results

A total of 30 lymph node tissue samples were cultured, as per section 2.1, and classified as described in section 2.11. The remainder of the decontaminated culture material, following inoculation of the culture media, was divided into 0.5 ml aliquots and stored at -20^{o}C. From the 30 tissue samples, there were 27 MTBC culture positive tissues and 3 culture negative tissues. Due to the limited amount of decontaminated culture material available not all samples were used on each procedure described below.

4.2.1 Heat Lysis

To determine if a heat lysis extraction procedure could be used to extract MTBC DNA from decontaminated tissue samples, all of the samples were diluted as described in section 2.12, heat killed as per section 2.13 and a heat lysis extraction was carried out as per section 2.14. The samples were split into three groups, for convenience, and each group was heat killed with MTBC positive solid culture and a PBS blank, 500 µl of PBS, included as controls. All three sample groups were heat killed separately, on different days, and heat lysed on the same day at different times. All 30 decontaminated samples and controls were amplified and detected together.

Heat lysis is a simple and efficient method of extracting nucleic acids from cells but it failed to extract a detectable amount MTBC DNA from the 30 decontaminated tissues, shown in Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5. This was probably due to the low number of MTBC cells in the sample and DNA from non-MTBC cells, such as contaminating bacteria and cells from the lymph node tissue. The presence of PCR inhibitors in the final extract since the centrifugation step, after heat lysis, will remove the heavier cell debris but not the smaller contaminating molecules may also have contributed to the failure of this method.

DNA was extracted from each of the MTBC positive cultures, Figure 4.2, Lane 16-17, Figure 4.3, Lane 18-19 and Figure 4.4, Lane 6-7. The PCR blank control, which tests for contaminating DNA in the clean room when the PCR mix was prepared, and the PCR water control, which tests for contaminating DNA when the extracted DNA is inoculated into the PCR mix, were both negative, Figure 4.5. The PCR *M. bovis* DNA control did give a band on the gel, which is shown in Figure 4.5.

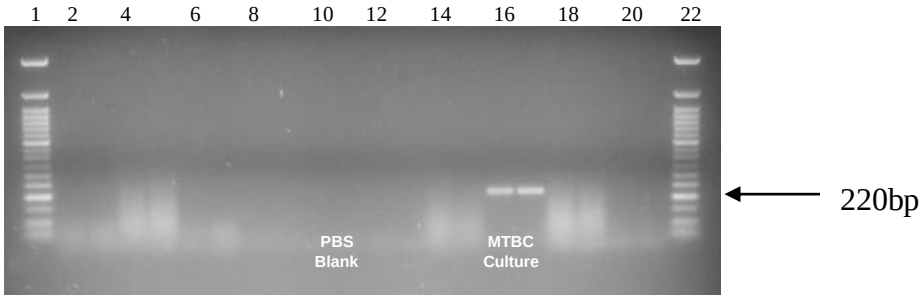

Figure 4.2: Gel showing PCR product of samples extracted with a heat lysis procedure. Lanes 1 & 22 50bp ladder, lanes 2-9, 12-15 and 18-21 decontaminated tissue samples, lanes 10-11 PBS blank and lanes 16-17 MTBC culture.

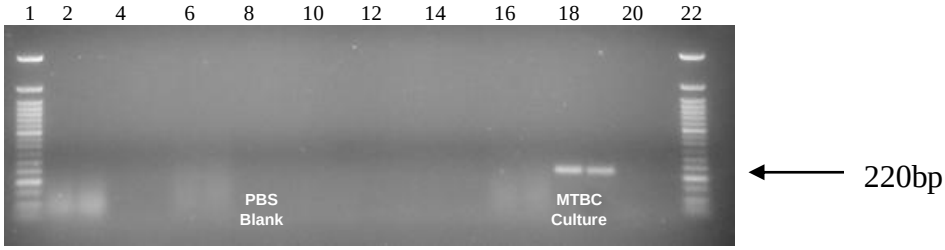

Figure 4.3: Gel showing PCR product of samples extracted with a heat lysis procedure. Lanes 1 & 22 50bp ladder, lanes 2-7, 10-17 and 20-21 decontaminated tissue samples, lanes 8-9 PBS blank and lanes 18-19 MTBC culture.

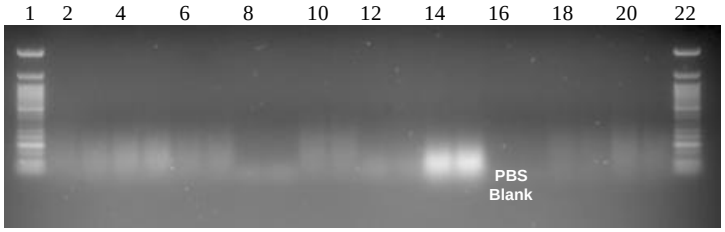

Figure 4.4: Gel showing PCR product of samples extracted with a heat lysis procedure. Lanes 1 & 22 50bp ladder, lanes 2-15 and 18-21 decontaminated tissue samples and lanes 16-17 PBS blank.

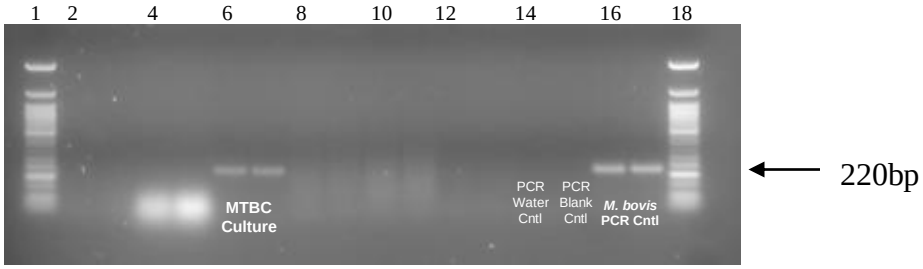

Figure 4.5: Gel showing PCR product of samples extracted with a heat lysis procedure. Lanes 1 & 18 50bp ladder, lanes 2-5 and 8-13 decontaminated tissues samples, lane 6-7 MTBC culture, lane 14 PCR water control, lane 15 PCR blank and lane 16-17 PCR *M. bovis* DNA control.

4.2.2 Roche High Pure PCR Template Preparation Kit

To determine if the Roche High Pure PCR Template Preparation Kit (High Pure Kit) would be able to extract MTBC DNA from decontaminated tissue samples.

Samples 1-10 of the decontaminated tissue samples were removed from the –20^{o}C freezer, heat killed as described in section 2.13 and treated by High Pure Kit as described in section 2.15. All the tissue material in these samples had not been completely broken down by the lysis buffer and Proteinase K digestion when placed in the filter column. The columns became clogged with the excess tissue material and only some liquid was able to pass though. The samples were discarded since the extraction was unable to proceed. To prevent wastage of the limited amount of decontaminated tissue for each sample, the initial 10 tissues were not retested with the pre-dilution step.

Samples 11-30 of the decontaminated tissue pellets were removed from the -20^{o}C freezer, diluted as per section 2.12, heat killed as per section 2.13 and treated by High Pure Kit as described in section 2.15 with the addition of an extra step. After the incubation of the sample with Binding Buffer (BB) and Proteinase K any undigested tissue within the sample were discarded by centrifugation, at 5900 g for 1 min. The supernatant was transferred to the filter column and pellet was discarded. An MTBC positive solid culture was used as a positive control and PBS was used as a Blank. All the 20 decontaminated tissue samples and controls were heat killed, extracted with the High Pure kit, amplified and detected, on 3 separate gels due to space constraints, at the same time.

No DNA was detected from the PBS blank and any of the decontaminated tissue samples, Figure 4.6, Figure 4.7 and Figure 4.8. DNA was extracted from the MTBC positive solid culture, shown in Figure 4.8, Lane 8-9.

Pre-dilution of each sample, to an equal settled tissue volume, before testing, as described in section 2.12, was selected over a set dilution, such as 1 in 2 dilution, since each of the decontaminated tissues had a different settled tissue volume. A sample with the larger volume of tissue may have been at a disadvantage, since some MTBC cells may have remained in the undigested tissue, for extraction of MTBC DNA compared to a sample with a smaller tissue volume. The practical effect of this was that some samples were diluted up to 1 in 5 while others required no dilution at all. The amount of tissue that required digestion was similar for each sample but the load of any MTBC cells for the diluted samples was reduced.

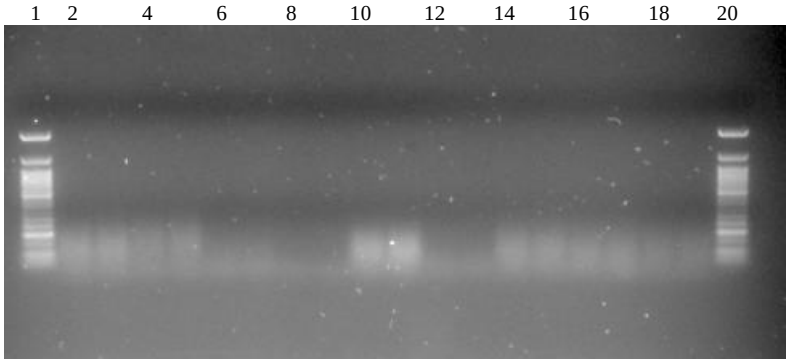

Figure 4.6: Gel showing PCR product from tissue samples after use of High Pure Kit, Lane 1 & 20 50bp ladder, Lane 2-19 decontaminated tissue samples.

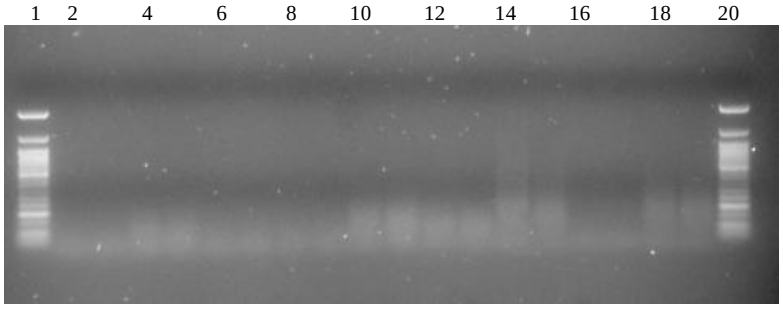

Figure 4.7: Gel **s**howing PCR product from tissue samples after use of High Pure Kit, lane 1 & 20 50bp ladder, Lane 2-19 decontaminated tissue samples.

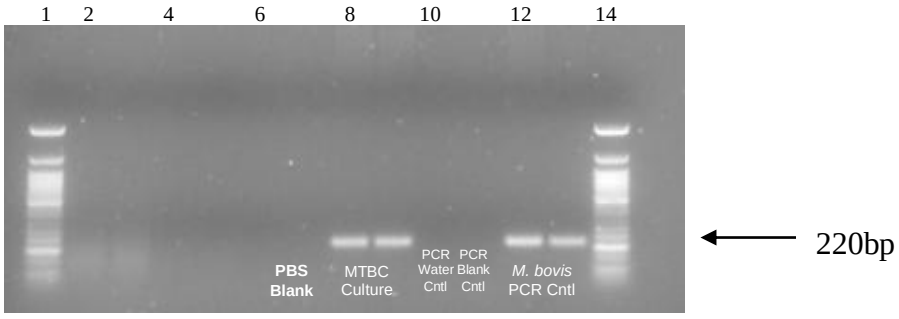

Figure 4.8: Gel showing PCR product from tissue samples after use of High Pure Kit. Lane 1 & 14 50bp ladder, Lanes 2-5 decontaminated tissue samples, Lane 6-7 PBS blank, Lane 8-9 MTBC culture, Lane 10 PCR water control, Lane 11 PCR blank control and Lane 12-13 PCR *M. bovis* DNA control.

Even with pre-dilution of the samples, the tissue was not fully digested prior to addition of the sample to the filter column and a centrifugation step was required. The failure to digest all the tissue present meant that it was probable that some MTBC cells would be discarded with the undigested tissue which would reduce the yield of any MTBC DNA from the column.

Modifications were made to the pre-treatment of the decontaminated tissue samples prior to use of High Pure Kit, section 2.16, to determine if these changes would allow the extraction MTBC DNA from the tissues.

To avoid wasting reagents and sample material only 12 of the 30 decontaminated tissue samples, 2 culture negative and 10 culture positive tissues, were tested using this modified procedure. The decontaminated tissue samples were diluted as per section 2.12, heat killed with controls, 2 MTBC positive solid cultures and a PBS blank, as per section 2.13 and treated by High Pure Kit as described in section 2.16. The samples were amplified and detected as described in section 2.23 and 2.25, using a 100bp DNA ladder (BioLabs, Cat #: N3231S).

The modifications to the testing procedure did ensure that all the tissue was digested prior to addition of the sample to the column but no MTBC DNA was detected in any of the decontaminated tissue samples, Figure 4.9 and 4.10. Both MTBC positive controls were extracted and detected on the gel, Figure 4.10, lane 6-9, and no DNA was detected from the PBS blank.

Several of the decontaminated tissue samples, Figure 4.9 and 4.10, had smears on the gel which could be caused by excess DNA in the sample or the formation of primer dimers during PCR amplification. Each sample was diluted 1 in 10, with UltraPure Distilled Water, to ensure that excess DNA was not the cause of the failure to detect MTBC DNA, amplified as per section 2.23 and detected as per section 2.25. The kit would have removed most, if not all of the PCR inhibitors with the samples but the extract would contain nucleic acids from non-MTBC cells. Many of the non-MTBC cells would have been destroyed during the decontamination process but due to the low MTBC load within a given tissue they still would be a significant source of contaminating nucleic acids. Any MTBC DNA present would also have been diluted. If primer dimers are the cause of smears on the gel then dilution of the sample should have no effect on their formation and the PCR conditions, $MgCl_2$ concentration, reaction temperatures, nucleotide concentration etc., would need to be altered to minimise their formation.

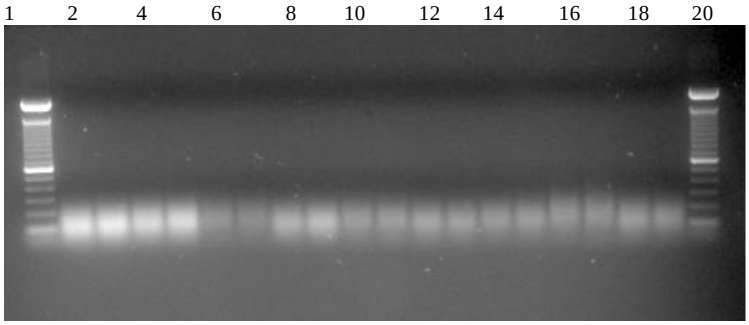

Figure 4.9: Gel showing PCR product from a modified procedure for High Pure Kit. Lane 1 & 20 100bp ladder, Lane 2-19 decontaminated tissue samples.

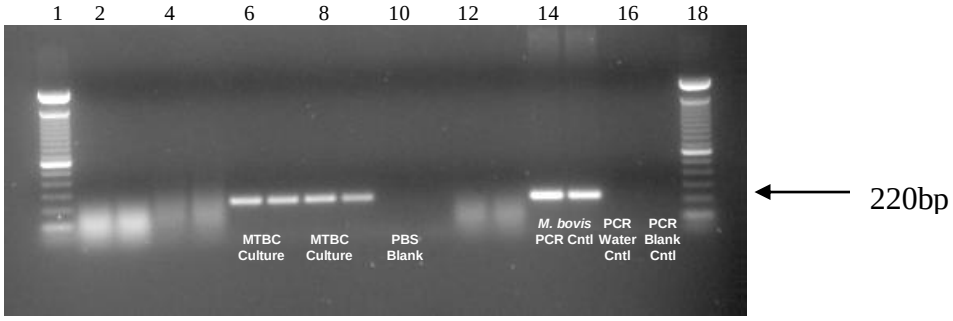

Figure 4.10: Gel showing PCR product from a modified procedure for the High Pure Kit. Lane 1 & 18 100bp ladder, Lane 2-5 & 12-13 decontaminated tissue samples, lane 6-9 MTBC cultures, Lane 10-11 PBS Blank, Lane 14-15 PCR *M. bovis* DNA control, Lane 16 PCR water and Lane 17 PCR blank.

The gels, shown in Figure 4.11 and 4.12, indicate that excess DNA in the extracted sample is not the explanation for the absence of bands on the gels. Neither is the formation of primer dimers since the smears on the gel are much reduced and the PCR conditions were not altered.

The High Pure Kit is designed to purify nucleic acids from other contaminating molecules but it cannot select for nucleic acids from a particular cell type within a mixture of cells making it unsuitable for extraction of MTBC DNA from decontaminated tissues.

4.2.3 Immunomagnetic Separation

To determine if IMS coupled with a Heat Lysis Extraction can be used to extract MTBC cells from decontaminated tissue samples, 30 decontaminated tissue samples were diluted as per section 2.12 and tested, with a MTBC positive solid culture and PBS blank, on the same day, as per section 2.17. The product was amplified and detected as per section 2.23 and 2.25.

IMS did not isolate any MTBC cells from the decontaminated tissues, Figure 4.13 and 4.14. Possible explanations for this include the presence of molecules, such as immunoglobulins, in the lymph node tissue that could interact with the variable regions *M. tuberculosis* Mouse Monoclonal Ab or with the surface antigens (Ag) on the MTBC cells thus preventing the Antibody-Antigen binding reaction from taking place. Proteases, that may be present in the LN tissue, could non-specifically digest the *M. tuberculosis* Mouse Monoclonal Ab before it had a chance to bind to the antigens on the surface of the MTBC cells (Fu *et al.*, 2005).

IMS did detect MTBC cells in the positive control sample tested, Figure 4.14 Lane18-19. Care was taken to ensure that only MTBC cells bound to the magnetic beads remained in the extract that was heat killed. The wash step was repeated a total of 3 times and all the supernatant, following application of the magnetic field, was carefully removed from each sample.

Dilution of the decontaminated tissue samples would reduce the concentration of any molecules that could interfere with the IMS. To determine if the amount of tissue in the sample is inhibiting the IMS of MTBC cells, 9 decontaminated tissue samples were diluted as described in section 2.12. Each sample was further diluted by 1/10 with PBS, a MTBC positive solid culture was also diluted 1/10 with PBS, and the IMS procedure continued as described in section 2.17.

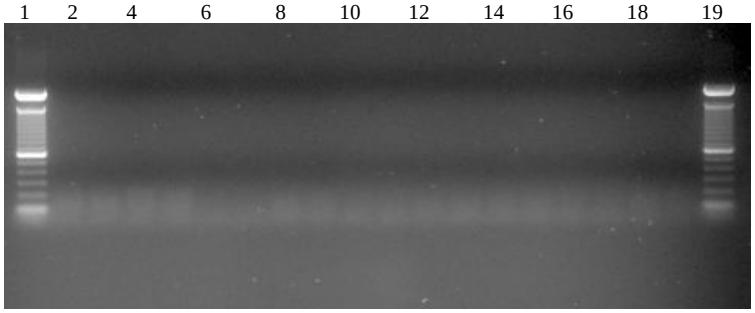

Figure 4.11: Gel showing 1/10 dilution of PCR product from a modified procedure for the High Pure Kit. Lane 1 & 20 100bp ladders, Lane 2-19 decontaminated tissue samples.

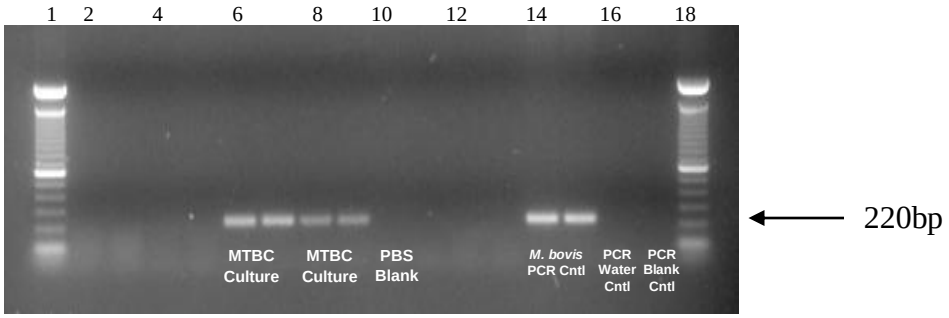

Figure 4.12: Gel showing 1/10 dilution of PCR product from a modified procedure for the High Pure Kit. Lane 1 & 18 100bp ladder, lane 2-5 & 12-13 decontaminated tissue samples, Lane 6-9 MTBC cultures, Lane 10-11 PBS blank, Lane 14-15 PCR *M. bovis* DNA control, Lane 16 PCR water and Lane 17 PCR blank.

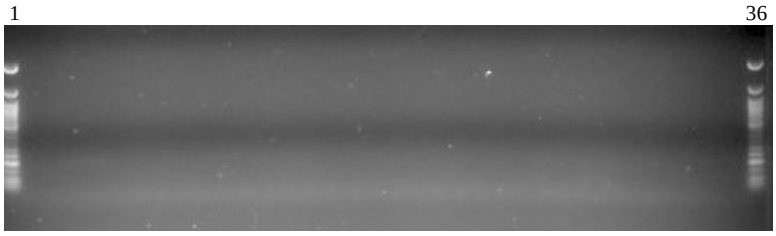

Figure 4.13: Gel showing PCR product from IMS and HL. Lane 1 & 36 50bp Ladder, Lane 2-35 decontaminated tissue samples.

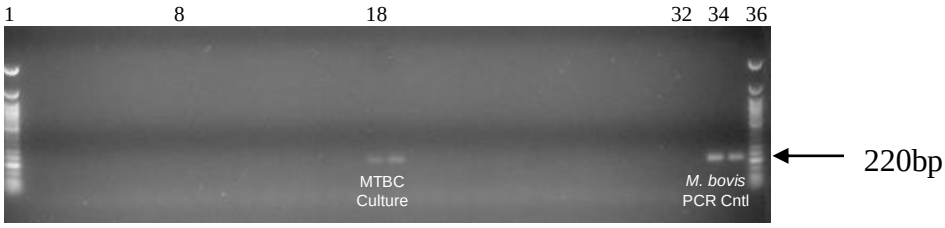

Figure 4.14: Gel showing PCR product from IMS and HL. Lane 1 & 36 50bp ladder, Lane 2-7, 10-17 and 20-30 decontaminated tissue samples, Lane 8-9 PBS blank, Lane 18-19 MTBC culture, Lane 32 PCR water control, Lane 33 PCR blank, Lane 34-35 PCR *M. bovis* DNA control.

A 1 in 10 dilution of the samples, some of which had already been diluted as per section 2.12, did not improve the detection of MTBC cells with IMS, Figure 4.15 and 4.16. Dilution may reduce the amount of contaminating molecules and cells in the sample but it would have also reduced the number of MTBC cells by the same amount.

Pre-treatment of the samples prior to IMS is required to remove or destroy the remaining LN tissue, which may hamper the movement of the bound magnetic beads though the sample, and any contaminating molecules. The choice of pre-treatment is limited to centrifugation or mechanical disruption since any enzymatic and chemical treatment could adversely affect the antibody-antigen binding reaction. This is due to the difficulty with complete removal of any lysis buffer or enzyme, used to digest the tissue, from the target cells. Even a small amount of lysis buffer or enzyme remaining could easily destroy the antibodies bound to the magnetic beads leading to false negative results.

Due to the small sample size and the existing centrifugation step, a mechanical disruption, in the form of bead beating of the samples, was selected for a pre-treatment prior to IMS. This method was selected since it is suitable for use on a small sample volume, it was carried out in an enclosed tube, limiting the risk of cross-contamination, and different sized glass beads could be selected to prevent damage to any MTBC cells. It should be noted that none of the decontaminated LN tissues samples contained any noticeable pieces of tissue.

To determine if addition of a bead-beating pre-treatment would allow IMS to extract MTBC cells from decontaminated tissue samples, 9 tissue samples were diluted as per section 2.12. A bead-beating step was added as per section 2.18 and the samples then processed for IMS as per section 2.17. Three MTBC positive solid cultures were used as controls, one was not pre-treated, and the other two were treated as per section 2.18 with, 0.5 ml of 212-300 μm beads used on one MTBC culture and in the second 0.5 ml of 710-1180 μm beads (Sigma, Cat #: G1152) were used.

The addition of this step did not improve the isolation of MTBC cells from the tissue, as in Figure 4.17 and 4.18, which shows the results after 30 sec of bead-beating. Only the results of 30 sec of bead-beating are shown since all the results were identical. IMS was able to detect all the MTBC positive culture controls regardless of the length of time the samples were in the Ribolyser or the size of beads

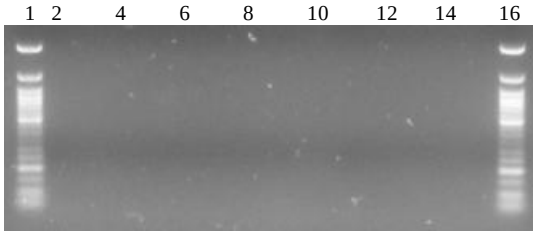

Figure 4.15: Gel showing PCR product from IMS and HL after 1/10 dilution of the decontaminated tissue sample. Lane 1 & 16 50bp ladder, Lane 2-7 & 10-15 decontaminated tissue samples, Lane 8-9 PBS blank.

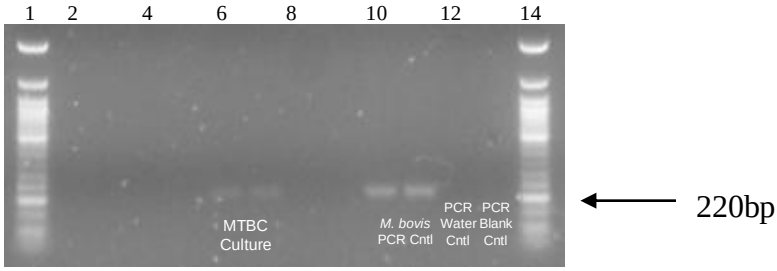

Figure 4.16: Gel showing PCR product from IMS and HL after 1/10 dilution of the decontaminated tissue sample. Lane 1 & 14 50bp Ladder, Lane 2-5 & 8-9 decontaminated tissue samples, Lane 6-7 1/10 dilution of MTBC culture, Lane 10-11 PCR *M. bovis* DNA control, Lane 12 PCR water control, Lane 13 PCR blank control.

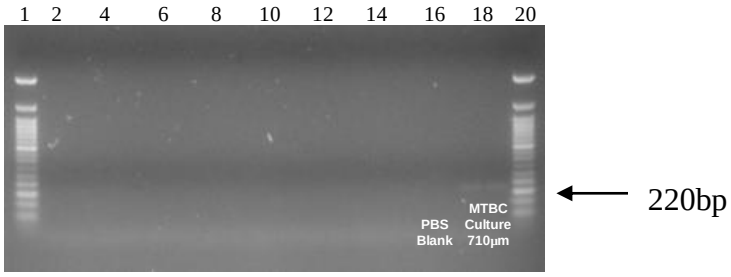

Figure 4.17 Gel showing PCR product from IMS and HL after bead-beating for 30sec (C). Lane 1 & 20 50bp ladder, Lane 2-9, 12-17 decontaminated tissue samples, Lane 10-11 PBS blank, Lane 18-19 MTBC culture (710-1180µm beads).

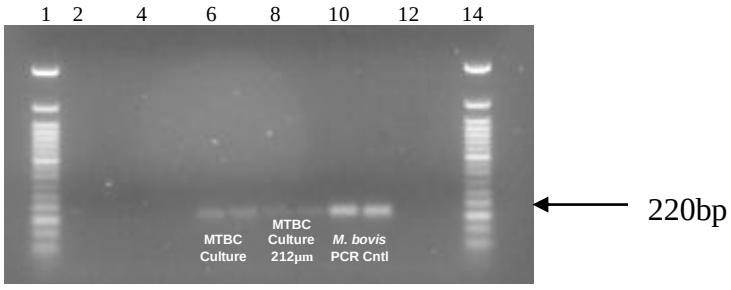

Figure 4.18: Gel showing PCR product from IMS and HL after bead-beating for 30sec (C). Lane 1 & 14 50bp ladder, Lane 2-5 decontaminated tissue samples, Lane 6-7 MTBC culture (no bead-beating), Lane 8-9 MTBC culture (212-300µm beads), Lane 10-11 PCR *M. bovis* DNA control, Lane 12 PCR water control, Lane 13 PCR blank control.

used, Figure 4.17 Lane 18-19 and Figure 4.18 Lane 6-9. This indicates that the MTBC cells were still intact after bead-beating with the different sized beads.

The effectiveness of the bead-beating step could have been improved by the use of smaller glass beads such as <106 μm in size. The risk with using the smaller beads is that any MTBC cells in the sample could be damaged and they need to be intact for IMS to be successful. Increasing the length of the bead-beating step, past 40sec, may increase the effectiveness of this technique but again runs the risk of destroying any MTBC cells in the sample. The technique is also limited in that it cannot remove any of the small molecules in the sample, which could interfere with the Antibody-Antigen binding reaction.

There is a possibility that IMS failed previously due to antibody bound beads not having enough time to locate and bind to the low number of MTBC cells within the sample. To determine if increasing the time the antibody bound beads are allowed to interact with the decontaminated tissue sample would allow IMS to extract MTBC cells from the samples, 9 tissue samples were diluted as per section 2.12. The IMS procedure was carried out as per section 2.17 with the antibody bound beads allowed to interact with the decontaminated tissue samples for 30 min (W), 60 min (X), 120 min (Y) and 180 min (Z). A MTBC positive solid culture and PBS blank were included with each set of decontaminated tissue samples.

Increasing the incubation time of the antibody bound beads with the decontaminated tissue sample had no effect on the detection of MTBC cells using IMS, Figure 4.19 and 4.20 showing the result after 180 min (Z). All the MTBC positive cultures were identified and no DNA was found in the PBS samples. IMS technique may not be sensitive enough to detect the low numbers of MTBC cells that would be present in the decontaminated tissues.

To determine the sensitivity of IMS, a comparison was made between the limits of detection with a serial dilution of a *M. bovis* culture spiked into PBS and into a culture negative decontaminated lymph node tissue, described in section 2.19. A single negative decontaminated tissue pellet was resuspended in the PBS which was used as a negative control for the dilution series. A second set of each *M. bovis* stock dilution was tested just in PBS. The IMS procedure was carried as per section 2.17 and the product was amplified and detected as per section 2.23 and 2.25.

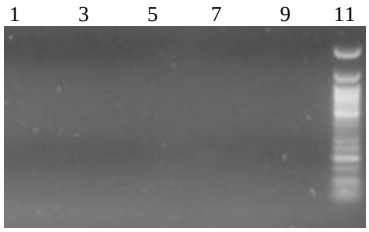

Figure 4.19: Gel showing PCR product from IMS and HL after incubation with antibody bound beads for 180 min (Z). Lane 1-10 decontaminated tissue samples, Lane 11 50bp ladder.

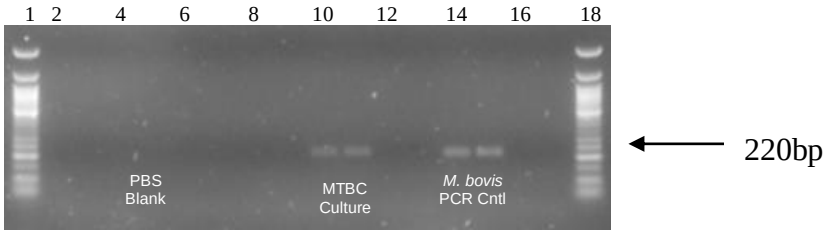

Figure 4.20: Gel showing PCR product from IMS and HL after incubation with antibody bound beads for 180 min (Z). Lane 1 & 18 50bp Ladder, Lane 2-3, 6-9 & 12-13 decontaminated tissue samples, Lane 4-5 PBS blank, Lane 10-11 MTBC culture, Lane 14-15 PCR M. bovis DNA control, Lane 16 PCR water control and Lane 17 PCR blank control.

IMS was able to detect *M. bovis*, in PBS, down as far as 1 x 10^{-2} dilution, Figure 4.21 and 4.22, and this corresponds to 2.3 x 10^{2} cfu/ml of *M. bovis*. In PBS, which should contain no molecules to interfere with IMS, the limits of detection for *M. bovis* was similar to that of culture, shown section 3.2, of 2.5 x 10^{2} cfu/ml. However, when the serial dilution of *M. bovis* was spiked into culture negative decontaminated lymph node tissue the sensitivity dropped by a factor of 10 to 2.3 x 10^{3} cfu/ml of *M. bovis*, Figure 4.23 and 4.24. It is clear from the above results the difficulty of using IMS to isolate MTBC cells from decontaminated tissue samples. As stated previously, there is no way to remove molecules, such as immunoglobulins and proteases, which could interfere with IMS without inhibiting with the Ab-Ag binding reaction. All the control gave the expected results.

Another possible explanation for the increased limit of detection of *M. bovis* with IMS in decontaminated tissues is the difficulty of a magnetic bead, bound to a MTBC cell, moving through the tissue sample after application of the magnetic field. A MTBC cell bound to a single magnetic bead would move slower through the tissue than MTBC cell bound to multiple beads. Due to the size of the 4.5 micron magnetic bead in relation to the size of a MTBC cell, steric hindrance may limit the number of magnetic beads that could attach to any given MTBC cell. Using a smaller magnetic bead of 2.8 μm may allow more beads to bind to a single MTBC cell and may modify the limits of detection.

The limits of detection of *M. bovis* with IMS in decontaminated tissue samples and PBS, described above, was repeated using smaller magnetic bead for the separation step. IMS was carried out as described in section 2.17 with the use of Dynabeads® M-280 Sheep Anti-Mouse IgG (Dynal, Cat No.: 112-01D), which have a diameter of 2.8 μm, instead of the Dynabeads® Sheep-anti Mouse IgG (Dynal, Cat No.: 110-31), which have a diameter of 4.5 μm.

Reducing the diameter of the beads used with IMS, from 4.5 μm to 2.8 μm, had no effect on the limits of detection of *M. bovis* spiked into either PBS or decontaminated tissue. IMS on the serial dilution of *M. bovis* in PBS was able to detect down to 1 x 10^{-2}, which corresponds to 2.3 x 10^{2} cfu/ml of *M. bovis*, Figure 4.25 and 4.26. When the *M. bovis* dilutions were spiked into culture negative decontaminated lymph node tissues the limits of detection of IMS fell to 1 x 10^{-1}, or

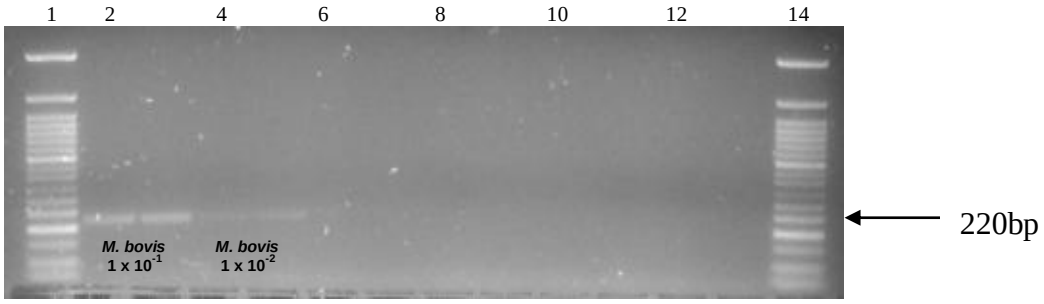

Figure 4.21: Gel showing product from IMS and HL on serial dilution of *M. bovis* spiked into PBS. Lane 1 & 14 50bp Ladder, Lane 2-12 serial dilution of *M. bovis*, $1x10^{-1} – 1x10^{-6}$.

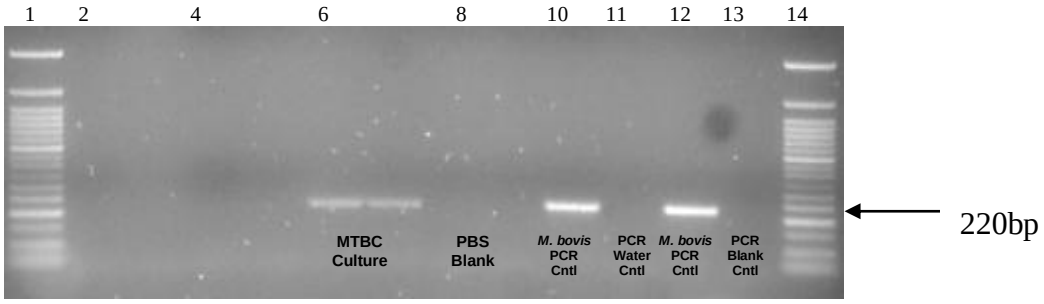

Figure 4.22: Gel showing product from IMS and HL on serial dilution of *M. bovis* spiked into PBS. Lane 1 & 14 50bp ladder, Lane 2-5 serial dilution of *M. bovis*, $1x10^{-7} – 1x10^{-8}$, Lane 6-7 MTBC culture, Lane 8-9 PBS blank, Lane 10 & 12 PCR *M. bovis* DNA control, Lane 11 PCR water control and Lane 13 PCR blank control.

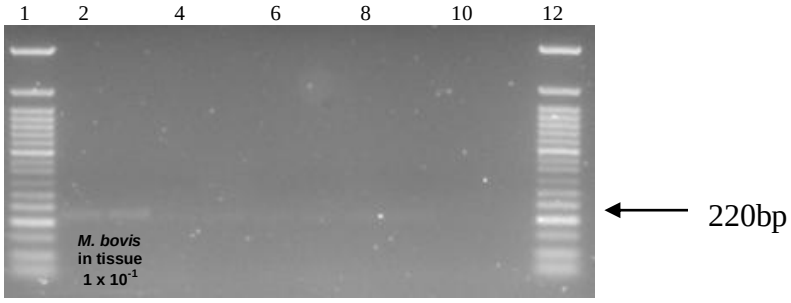

Figure 4.23: Gel showing product from IMS and HL on serial dilution of *M. bovis* spiked into negative decontaminated tissue sample. Lane 1 & 12 50bp Ladder, Lane 2-11 serial dilution of *M. bovis*, $1x10^{-1} – 1x10^{-5}$.

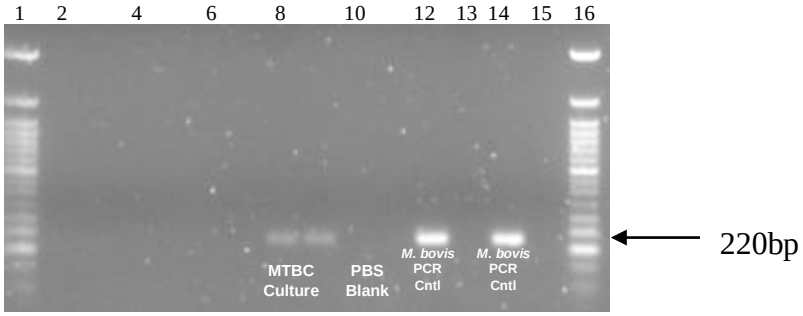

Figure 4.24: Gel showing product from IMS and HL on serial dilution of *M. bovis* spiked into negative decontaminated LN sample. Lane 1 & 16 50bp ladder, Lane 2-7 serial dilution of *M. bovis*, $1 \times 10^{-6} – 1 \times 10^{-8}$, Lane 8-9 MTBC culture, Lane 10-11 PBS blank, Lane 12 and 14 PCR *M. bovis* DNA control, Lane 13 PCR water control and Lane 15 PCR blank control.

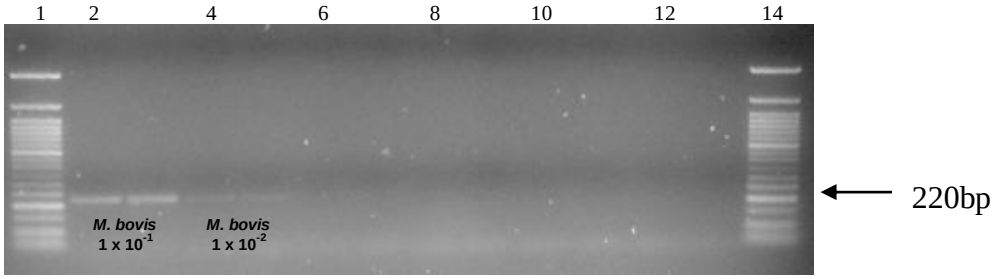

Figure 4.25 Gel showing product from IMS and HL on serial dilution of *M. bovis*, in PBS, using M-280 beads (2.8 μm beads). Lane 1 & 14 50bp Ladder, Lane 2-12 serial dilution of *M. bovis*, $1x10^{-1} – 1x10^{-6}$.

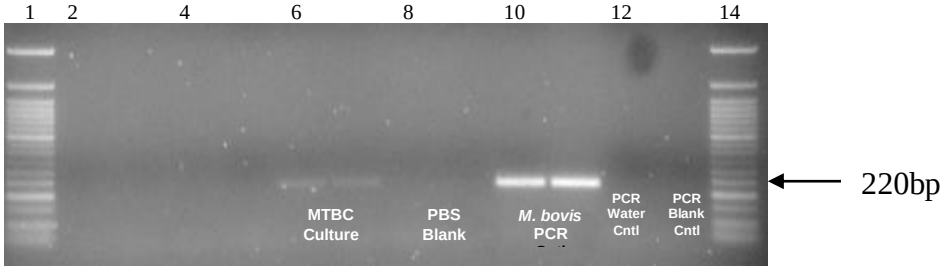

Figure 4.26: Gel showing product from IMS and HL on serial dilution of *M.* bovis, in PBS, using M-280 beads (2.8 μm beads). Lane 1 & 14 50bp ladder, Lane 2-5 serial dilution of *M. bovis*, $1 x 10^{-7} – 1 x 10^{-8}$, Lane 6-7 MTBC culture, Lane 8-9 PBS blank, Lane 10-11 PCR *M. bovis* DNA control, Lane 12 PCR water control and Lane 13 PCR blank control.

2.3 x 10^3 cfu/ml, Figure 4.27 and 4.28, the band is faint but it is present. All the controls gave the expected results.

IMS has been used to extract mycobacteria from a variety of different sample types, as described section 4.1.3. The results shown here clearly indicate that IMS is not a suitable technique for the direct detection of MTBC cells in decontaminated lymph node tissue.

4.2.4 GenoType Mycobacteria Direct 4.0

To determine if the GenoType Mycobacteria Direct Kit can be used to detect mycobacteria in decontaminated tissue samples, the GTMD kit was carried out as per section 2.20. Twelve samples at a time could be processed by the Twincubator® with hybridization block. Each run included the kit positive control RNA and up to 11 decontaminated tissue samples. One run included a PBS blank and a MTBC positive solid culture.

The GTMD did not perform well for the direct detection of MTBC cells from decontaminated tissues, only correctly identifying 6 out of 30 decontaminated tissue samples and giving two false negative results, Table 4.1 and sample result sheet Figure 4.29. The false negative results are not problematic but failure of the remaining 22 samples was due to inhibition of the NASBA reaction. This was indicated by the development of the CC (conjugate control) band, first band, on the strip only. Each sample had an Internal Control RNA added to it during the extraction procedure which should be amplified during the NASBA reaction. This amplification may be inhibited by the presence of nucleic acids from the sample. As a result of this, every band on the strip, except the CC, must be stronger than the UC (universal control) band, second band on the strip, to be considered relevant.

Many factors could have led to failure of the NASBA reaction including the presence of contaminating molecules in the extract following the MB separation. This may be due to failure to remove all of the supernatant after each magnetic bead wash step or magnetic beads still remaining in the sample following the final separation. The problem may be with the NASBA reaction mix such as the PMN mix may have been left standing too long before being used and the EML may have been mixed too much prior to use. Another issue may be the presence of excess tissue in some samples since pre-dilution, as per section 2.12, was not carried out.

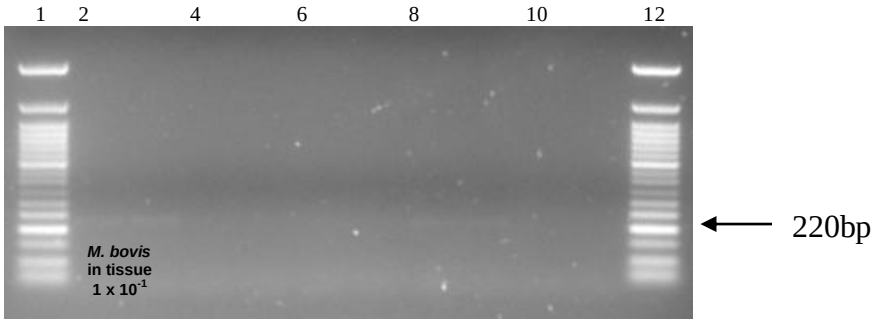

Figure 4.27: Gel showing product from IMS and HL, using M-280 beads (2.8 µm beads), on serial dilution of *M. bovis* spiked into negative decontaminated tissue. Lane 1 & 12 50bp Ladder, Lane 2-11 serial dilution of *M. bovis*, $1x10^{-1}$ – $1x10^{-5}$.

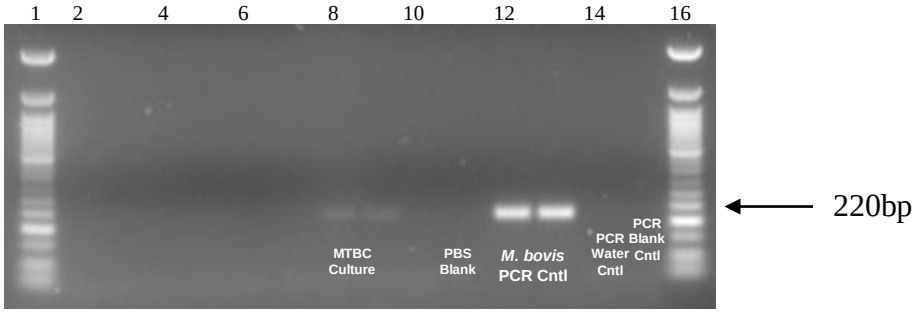

Figure 4.28: Gel showing product from IMS and HL, using M-280 beads (2.8 µm beads), on serial dilution of *M. bovis* spiked into negative decontaminated tissue sample. Lane 1 & 16 50bp ladder, Lane 2-7 serial dilution of *M. bovis*, 1×10^{-6} – 1×10^{-8}, Lane 8-9 MTBC culture, Lane 10-11 PBS blank, Lane 12-13 PCR *M. bovis* DNA control, Lane 14 PCR water control and Lane 15 PCR blank control.

Table 4.1: The results of the GenoType Mycobacteria Direct kit testing 27 MTBC positive and 3 culture negative decontaminated tissue samples.

	MTBC	**Neg**	**Fail**
High (7)	4		3
Medium (9)		1	8
Low (11)	2	1	8
Total (27)	**6**	**2**	**19**
Negative (3)			3

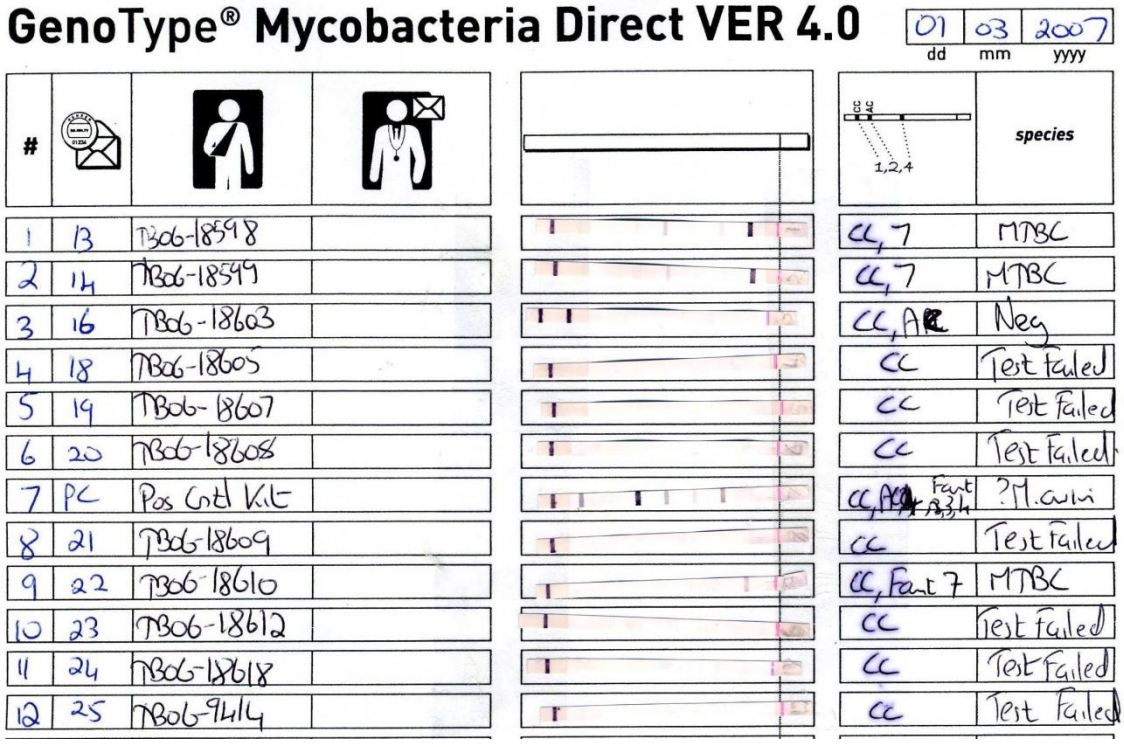

Figure 4.29: Sample result sheet showing the results of 11 tissue samples and the positive control RNA supplied with the kit.

It should be noted that the Positive Control RNA, supplied with the kit, failed to give a conclusive banding pattern, e.g. Sample 7 in Figure 4.29. The control was prepared and tested as per kit instructions. As soon as bands appeared on its test strip the reaction was stopped with distilled water but a clear banding pattern was not seen. This may have been due to problems with the NASBA reaction such as the delay between the time the PNM was made up and its use or excess agitation of the EML before utilization

The GTMD kit was repeated with modifications to the sample preparation and care was taken to ensure that all liquid following each magnetic bead separation was carefully removed, all NASBA reagents were made up correctly and as close to the time of use as possible. The GTMD kit was carried out as per section 2.21 and each decontaminated tissue sample was diluted as per section 2.12. To ensure that no magnetic beads remained in the sample tube after last magnetic bead separation each tube was centrifuged at 5900 g for 1 min. The supernatant was transferred to a fresh tube and used in the amplification step and the pellet was discarded. Instead of the PNM being made up in the clean room about an hour before it was required, it was made up immediately prior to use within the TB Laboratory, approximately 15 min prior to use. This was carried out in a BSC (biological safety cabinet) that was not used for any other molecular biology work. The cabinet was suitably cleaned and treated with UV light prior to use. Care was taken to ensure the EML was prepared with gentle mixing only.

The minor changes made to the test procedure did not improve the effectiveness of the GTMD kit, Table 4.2 and sample result sheet Figure 4.30. From the above table it can be seen that the kit failed for 23 decontaminated tissue samples and only correctly identified 4 MTBC positive decontaminated tissue samples and gave 3 false negative results.

Again the positive control RNA supplied with the kit failed to give a clear banding pattern e.g. sample labelled PK on Figure 4.30, but this may be due to the problems causing the failure of the NASBA reaction with the decontaminated tissue samples. A MTBC positive solid culture was processed with the tissue samples and the kit correctly identified this sample, Sample labelled Pos on Figure 4.30

A pre-treatment of the decontaminated tissues, prior to testing with the GTMD kit, appears to be necessary. No pre-treatment information was supplied with the kit

and since not enough information was provided with the kit to identify a suitable pre-treatment the manufacturer was contacted for assistance.

The manufacturer supplied the pre-treatment protocol, involving an enzymatic digestion, which was carried out as per section 2.21. The GTMD kit correctly identified 19 out of the 30 decontaminated tissue samples and had 5 false negative results, Table 4.3 and sample result sheet Figure 4.31. The false negative results may be due to the low number of MTBC cells in the samples, particularly following the dilution of the samples, as per section 2.12, prior to carrying out the GTMD kit.

The GTMD kit gave no false positive results, however this is not a definitive conclusion since it is based only 3 MTBC culture negative samples included in the study, 2 of which failed. A total of 6 samples failed, giving only a CC band on the test strip, Table 4.3 and sample result sheet Figure 4.31. The reason for this failure is unknown but these tissues may contain NASBA reaction inhibitors that were not fully removed by the magnetic bead extraction. These results were an improvement on the samples tested with the kit without the enzymatic digestion.

A persistent problem with this kit was the failure of the positive control supplied with the kit to give a clear banding pattern. There was no clear reason for this failure. All reagents supplied with the kit were used in date, correctly stored and the directions for use of the positive control were followed carefully. Different kit lot numbers have been used over the repeated testing of the decontaminated tissue samples with the GTMD kit. To ensure that the kit was performing correctly two MTBC positive solid culture controls were included with each test run to act as positive controls. One aliquot of the positive control was pre-treated with an enzymatic digestion with the tissue samples, Figure 4.31 Sample 5, and the second was tested without the digestion, Figure 4.31 Sample 12. Both controls gave an MTBC banding pattern.

Since the GTMD kit, with the addition of an enzymatic pre-treatment has been shown to detect MTBC cells in decontaminated tissue samples, a limit of detection study was carried out with a *M. bovis* stock spiked into culture negative decontaminated tissue samples as described in section 2.19. One negative decontaminated tissue pellet was resuspended in the PBS that was used as a negative control for the dilution series. Two samples of the neat *M. bovis* stock was tested without spiking into the decontaminated tissue, a PBS sample and the positive control supplied with the kit were also tested.

Table 4.2: The results of the GenoType Mycobacteria Direct kit testing 27 MTBC positive and 3 negative decontaminated tissues.

	MTBC	**Negative**	**Fail**
High (7)	1	1	5
Medium (9)	1	1	7
Low (11)	2	1	8
Total (27)	**4**	**3**	**20**
Negative (3)			3

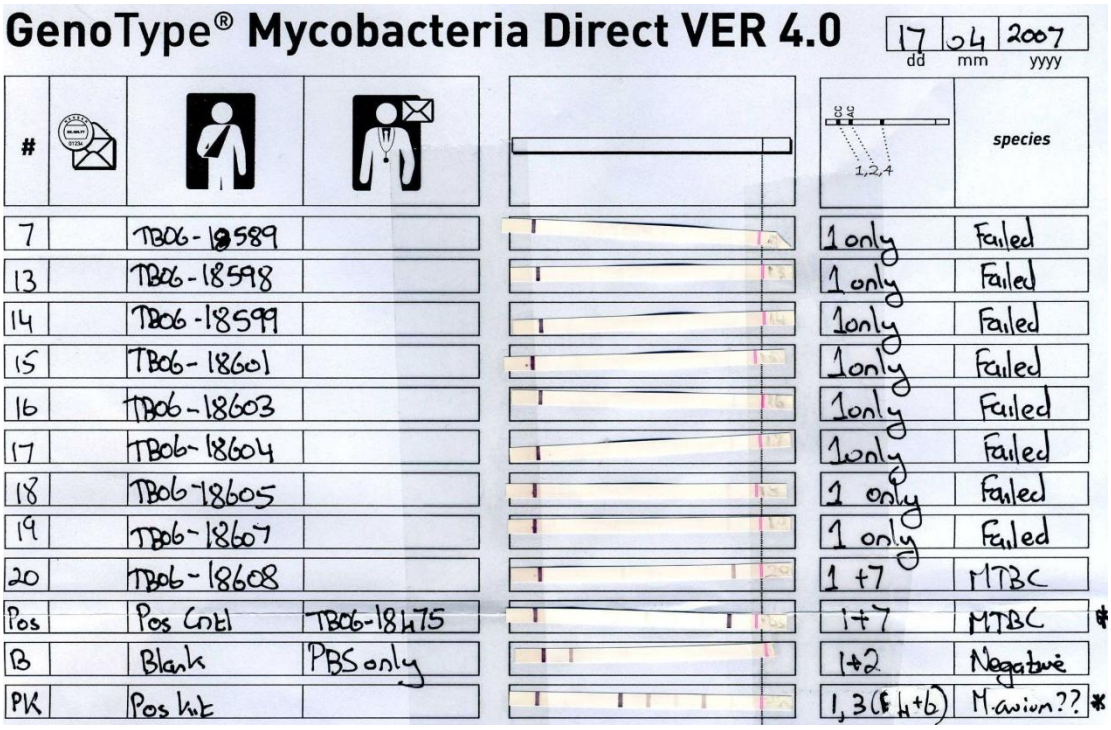

Figure 4.30: Sample result sheet showing the results of 9 tissue samples and three controls, a MTBC culture, PBS blank and the positive control RNA supplied with the kit.

Table 4.3: The results from the GenoType Mycobacteria Direct kit with addition of an overnight Proteinase K digestion.

	MTBC	**Negative**	**Fail**
High (7)	4	2	1
Medium (9)	7		2
Low (11)	7	3	1
Total (27)	**18**	**5**	**4**
Negative (3)		1	2

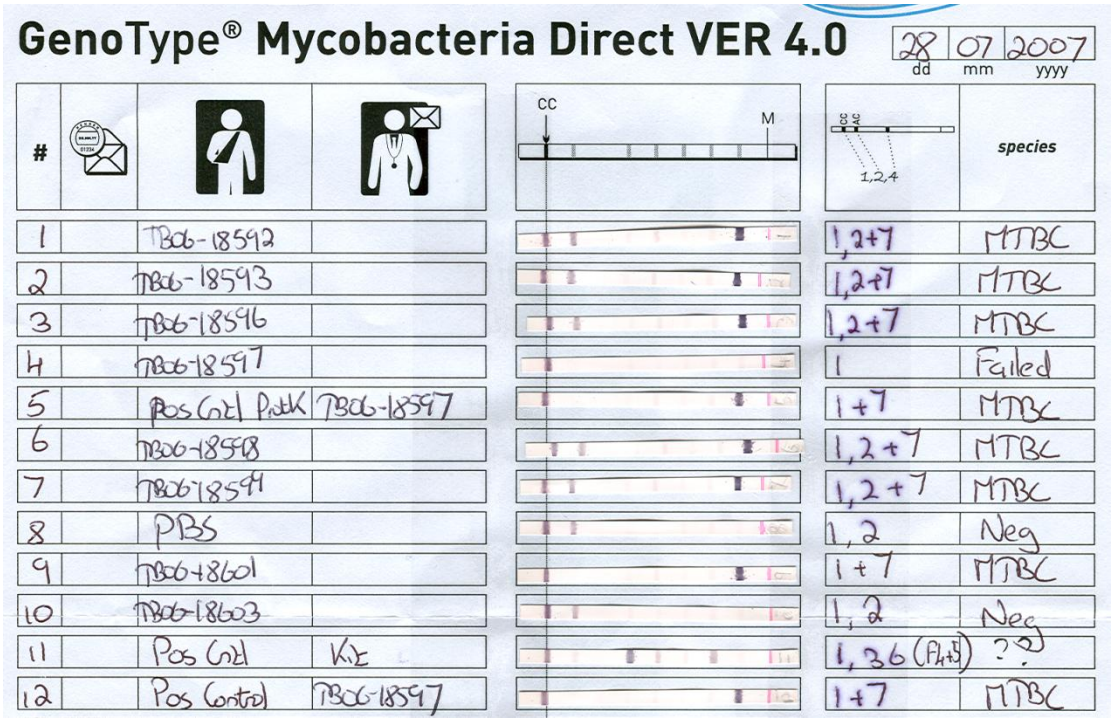

Figure 4.31: Sample result sheet showing the results of 8 tissue samples and four controls with the addition of an overnight Proteinase K digestion. These include two MTBC cultures, the positive control supplied with the kit and a blank of PBS.

All the samples were tested with the GTMD as described in section 2.20, excluding the positive control supplied with the kit and one neat *M. bovis* stock sample which were not enzymatically pre-treated.

As can be seen from, Figure 4.32, the kit was able to detect the *M. bovis* spiked into the decontaminated tissue sample down to a dilution of 1 x 10^{-2}. This corresponds to a concentration of *M. bovis* of 2.3 x 10^2 cfu/ml, which is a similar to the sensitivity of culture of 2.5 x 10^2 cfu/ml, see section 3.2.

The neat *M. bovis* samples, both digested and undigested, gave a MTBC band on the strip. Both PBS samples gave a negative result. The positive control supplied with the kit again showed an inconclusive banding pattern.

This kit allows for a rapid turnaround of suspect TB positive sample. The enzymatic digestion of the decontaminated tissue samples can be started after inoculation of the culture media. The GTMD kit procedure takes a single day, meaning that a preliminary result could be available to be reported at the end of the following working day after culture. For a definitive result the culture procedure would have to be allowed to run to completion, due to the presence of false negative results, described previously. For the majority of suspect TB tissues received, which cannot be diagnosed by histopathology, there is no necessity for such a rapid turnaround of results. For samples received from singleton reactor animals and tissue samples received from animals where there is a risk of infection to humans, such as household pets, this kit could be of great benefit as long as it was used in tandem with TB culture, rather than as replacement of it, due to false negative results.

4.2.5 Sequence Capture PCR

Sequence Capture PCR was the only one of the five methods evaluated that had previously been shown to detect *M. bovis* in decontaminated tissue samples (Roring *et al.*, 1998; Roring *et al.*, 2000; Mangiapan *et al.*, 1996; Parra *et al.*, 2008). The aim of this section was to determine if Sequence Capture PCR can detect MTBC cells in the 30 decontaminated tissue samples. The tissue samples were diluted as per section 2.12 and the sequence capture PCR was carried out as described in section 2.22.

The decontaminated tissue samples were split into four groups for testing. With each group a MTBC positive solid culture and PBS blank were included for the complete extraction procedure. At the sequence capture step 5 µl of *M. bovis* DNA

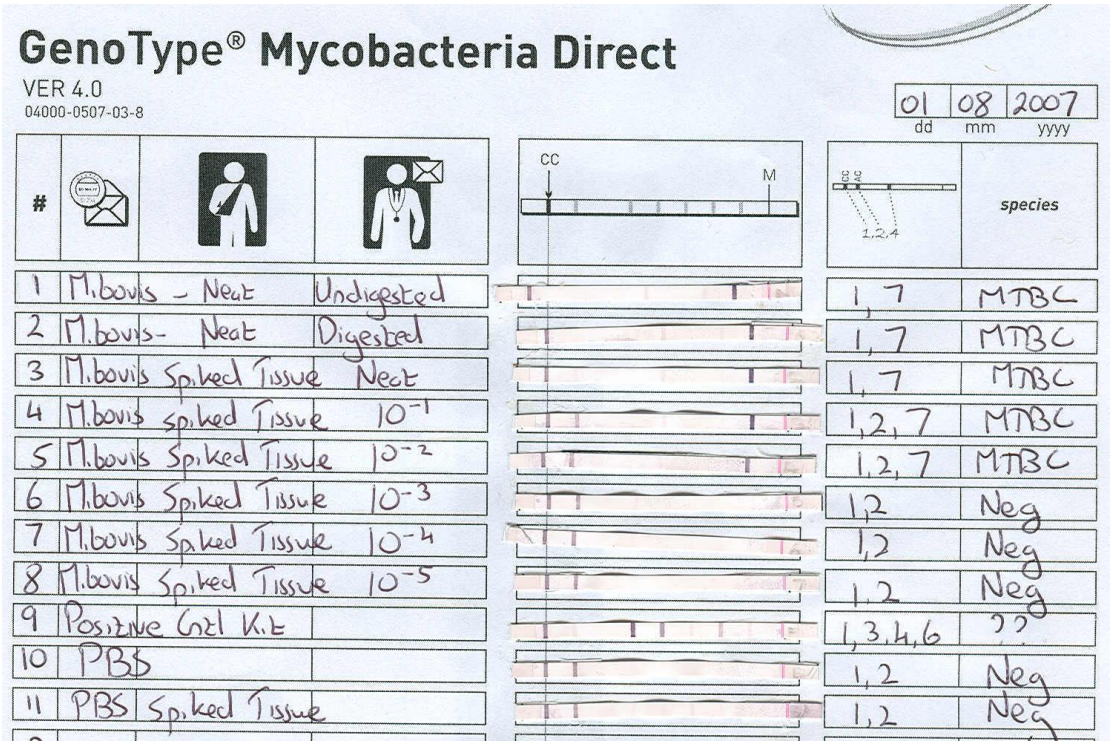

Figure 4.32: Sample result sheet showing the results of limit of detection of GTMD kit with *M. bovis* spiked into decontaminated tissue samples.

was added to 545 µl of TES buffer to act as a positive control. The product was amplified in a PCR mix containing Q solution and in a PCR mix in which no Q solution was used, as per section 2.24. Q solution alters the melting behaviour of DNA and can improve a suboptimal PCR caused by templates high GC-content, like *M. bovis* (Qiagen, 2010). The amplified PCR product from both mixes was detected as per section 2.25.

The Tables 4.4 and 4.5 show that sequence capture was suitable for the detection of MTBC cells in decontaminated tissue samples. The MTBC positive solid culture and *M. bovis* DNA controls gave a band of approximately 270 bp when amplified both with and without Q solution in the PCR mix. No bands were detected with the PBS blank. There was a difference in the results when sequence capture product was amplified with the use of Q solution, Table 4.4, and without Q solution, Table 4.5. The percentage of false negatives with the Q solution was 56.67%, or 17 out of 30 samples, and the percentage of false negative without the Q solution was 26.67%, or 8 out of 30 samples. An example of the difference in the detection of amplified product with and without the use of Q solution in the PCR mix is shown in Figure 4.33 and 4.34 for the same decontaminated tissue samples.

The false negative results may be due to the low number of MTBC cells present in some tissues, since some samples were diluted, as per section 2.12, prior to testing. Another possibility is the presence of molecules remaining in the sample after the mechanical and enzymatic pre-treatment, which could have prevented the binding of the oligonucleotides to the released DNA.

Since sequence capture PCR, without the use of Q solution in the PCR mix, has been shown to be an effective method to detect MTBC cells in decontaminated tissue samples a limit of detection study for a *M. bovis* stock solution spiked into negative decontaminated tissue was carried out, as described in section 2.19. A single negative decontaminated tissue pellet was resuspended in the PBS solution that was used in the dilution series. A sequence capture PCR was carried out as described in section 2.22 and the product was amplified, without Q solution in the PCR mix, and detected as per section 2.24 and 2.25.

Sequence Capture PCR was able to detect *M. bovis* DNA, in the spiked decontaminated, culture negative, tissue samples, down as far as the 1×10^{-3} dilution, though the bands for this dilution are faint on the gel, as shown in Figure 4.35, 4.36 and 4.37. This corresponds to 2.3×10^{1} cfu/ml of *M. bovis*, which is 10 times more

Table 4.4: Results of sequence capture PCR of 27 MTBC positive and 3 negative decontaminated tissue samples. The DNA was amplified in a PCR mix containing Q solution.

SQCap with Q	**MTBC**	**Neg**
High (7)	4	3
Medium (9)	5	4
Low (11)	1	10
Total (27)	**10**	**17**
Negative (3)		3

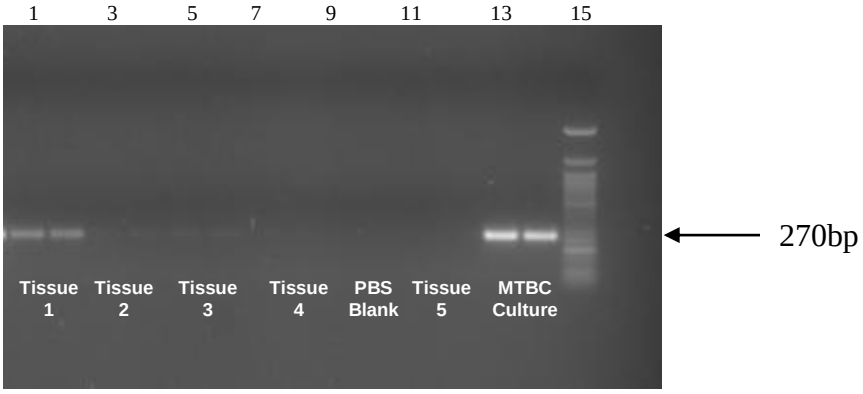

Figure 4.33: Gel showing products of sequence capture PCR, amplified with Q solution. Lane 1-8 & 11-12 decontaminated tissue, Lane 9-10 PBS blank, Lane 13-14 MTBC culture and Lane 15 50bp ladder.

Table 4.5: Results of sequence capture PCR of 27 MTBC positive and 3 negative decontaminated tissue samples. The DNA was amplified in a PCR mix without Q solution.

SQCap no Q	**MTBC**	**Neg**
High (7)	5	2
Medium (9)	7	2
Low (11)	7	4
Total (27)	**19**	**8**
Negative (3)		3

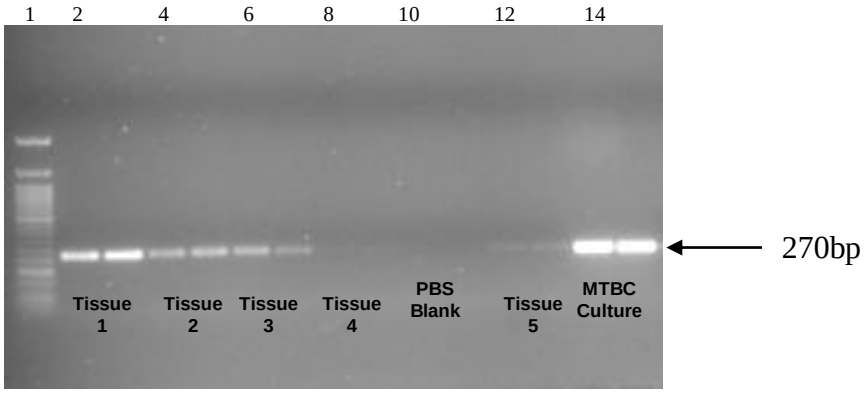

Figure 4.34: Gel showing products of sequence capture PCR, amplified without Q solution. Lane 1 50bp ladder, Lane 2-9 & 12-13 decontaminated tissue samples, Lane 10-11 PBS blank and Lane 14-15 MTBC culture.

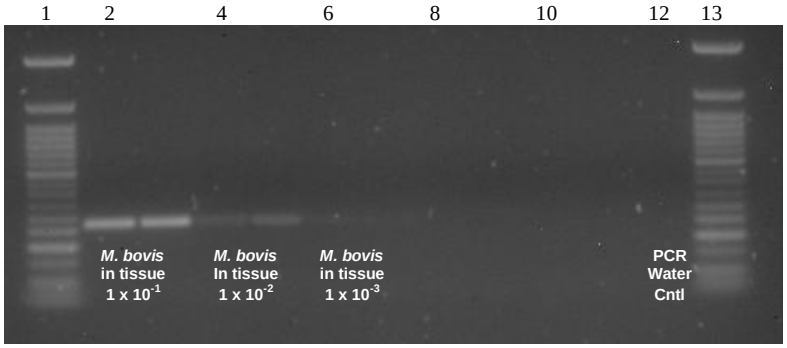

Figure 4.35: Gel showing products of sequence capture PCR of a serial dilution of *M. bovis* spiked into negative decontaminated tissue. Lane 1 & 13 50bp ladder, Lane 2-11 serial dilution of *M. bovis,* $1x10^{-1} – 1x10^{-5}$ and Lane 12 PCR water control.

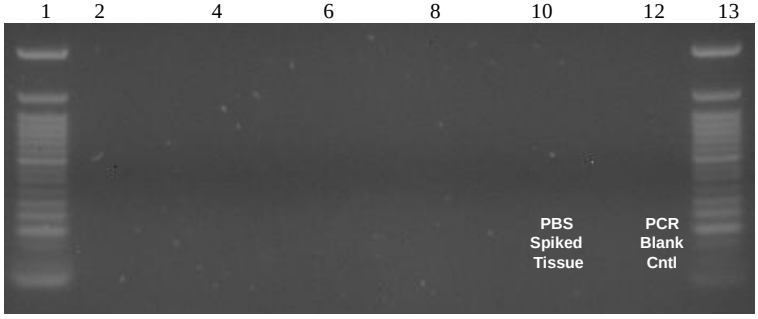

Figure 4.36: Gel showing products of sequence capture PCR of a serial dilution of *M. bovis* spiked into negative decontaminated tissue. Lane 1 & 13 50bp ladder, Lane 2-9 serial dilution of *M. bovis,* $1x10^{-6} – 1x10^{-9}$, Lane 10-11 PBS spiked negative tissue and lane 12 PCR blank control.

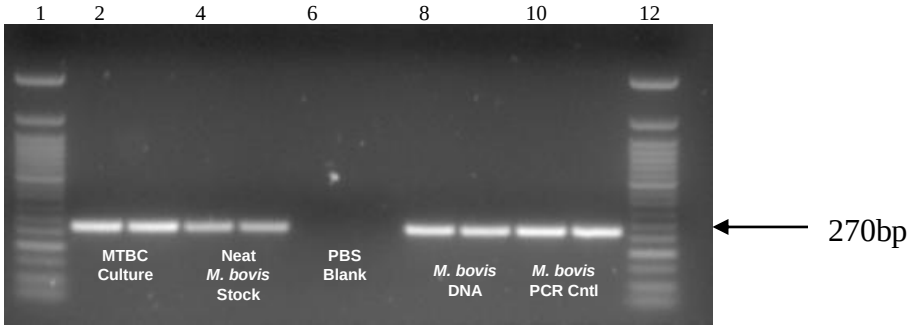

Figure 4.37: Gel showing products of sequence capture PCR of a serial dilution of *M. bovis* spiked into negative decontaminated tissue. Lane 1 & 12 50bp ladder, Lane 2-3 MTBC culture, Lane 4-5 neat *M. bovis* stock, Lane 6-7 PBS blank Lane 8-9 *M. bovis* DNA, Lane 10-11 *M. bovis* PCR positive control.

sensitive than the culture result of 2.5 x 10^2 cfu/ml, shown in section 3.2. All the controls gave the expected results.

Sequence Capture PCR is extremely sensitive and could potentially detect a MTBC infection that was missed by culture. The increase sensitivity may be due to the fact that culture can only detect the viable *M. bovis* cells remaining in the tissue following storage of the sample at -20^{o}C prior to culture and decontamination. Sequence capture may be able to pull *M. bovis* DNA from cells that are no longer viable but still remain in the tissue.

4.3 Discussion

The purpose of this study was to evaluate several different methods for the extraction of mycobacterial DNA from decontaminated LN tissue for use with high priority samples. Each of the five methods used, heat lysis, Roche High Pure Template Preparation kit, Immunomagnetic Separation (IMS), GenoType Mycobacteria Direct kit and Sequence Capture PCR, had previously been employed to extract mycobacterial DNA from a wide variety of sample types including milk, sputum, tissues and mycobacterial culture isolates.

Heat Lysis was included in this study, since it was a simple and effective technique for releasing DNA from a sample. It had no DNA purification capability and was shown not to be sensitive or specific enough to extract mycobacterial DNA from decontaminated LN tissue, section 4.2.1. The Roche High Pure Template Preparation kit isolated the total nucleic acids, separating them from other contaminating molecules, within the sample using absorption chromatography. Due to the sample type, lymph node tissue, nucleic acids from the tissue itself and any contaminating bacteria would be purified along with nucleic acids from any mycobacterial cells present. This meant that the technique was not specific enough to extract mycobacterial DNA from decontaminated LN tissue, section 4.2.2.

IMS was included in this study since it was a technique designed to extract a target cell type from a heterogeneous cell suspension. An advantage of IMS, over the other methods evaluated, was that it could provide information on the viability of any mycobacteria within the tissue and could also be adapted for use as an enrichment step prior to culture. IMS had been used in the isolation of mycobacterial cells from a wide variety of sample types (milk, faeces and cerebrospinal fluid), including *M. bovis* from milk (Li *et al*., 1996; Mazurek *et al*., 1996; Grant *et al*., 1998; Antognoli *et*

al., 2001; Djønne *et al.*, 2003; Khare *et al*., 2004). Fu *et al*., had shown the suitability of IMS for the isolation of *E. coli* 0157:H7 cells from ground beef for direct detection with a real-time PCR method (Fu *et al*., 2005). The matrix used in that study, ground beef, differs from LN tissue but would have a greater similarity to it than milk, faeces or cerebrospinal fluid.

IMS was not sensitive enough to detect the low numbers of MTBC cells found in the decontaminated samples, section 4.2.3. A pre-treatment step was required to breakdown the tissue before the MTBC cells could be isolated. The technique relies on the specificity of antibodies, bound to magnetic beads, to isolate the target cells meaning a chemical or enzymatic pre-treatment step could not be used, since it would be impossible to ensure than all molecules of the chemical or enzyme used in the pre-treatment were removed from the sample prior to addition of the antibody bound beads. This could lead to a source of false negative results for IMS. This limited the pre-treatment to physical disruption, bead-beating was select, and this was shown not to be successful. Increasing the length of time to antibody bound beads were allowed to interact with the sample also failed to improve the results. Fu *et al.*, 2005 suggested that reducing the size of the magnetic bead used to bind to the antibodies, might improve cell capture, however this was ineffective.

When a serial dilution of *M. bovis*, spiked into both PBS and negative LN tissue, was extracted using IMS, the technique was shown to have a similar sensitivity to that of culture, 2.3×10^2 cfu/ml, when used with PBS but the sensitivity dropped by a factor of 10, 2.3×10^3 cfu/ml, when used with LN tissue. This figure is similar to the lowest detection limit of IMS when used on *E. coli* 0157:H7 cells in ground beef of 1.6×10^3 cells/ml (Fu *et al*., 2005).

The primer, MSP 1/2, used to amplify the extraction products from heat lysis, Roche High Pure Template Preparation kit and IMS was specific to mycobacteria not MTBC cells (Roth *et al*., 2000). If any mycobacterial DNA had been detected by PCR in the decontaminated tissue cells further testing would have been required to confirm the presence of MTBC DNA. This primer was selected since it would allow the detection of non-tuberculosis mycobacteria (NTM) from the decontaminated tissues. These mycobacteria are not critical to the bovine eradication scheme but they can provide information to improve animal health and husbandry.

The GenoType Mycobacteria Direct kit was shown in this study, after the addition of an overnight Proteinase K digestion, to be able to correctly identify 63.3%

(19 out of 30) of the decontaminated tissue samples, section 4.2.4. The previously published evaluations of this kit for the direct detection of mycobacteria from clinical samples are based primarily on respiratory samples, with only a small number of extra-pulmonary samples tested, and none of which include a pre-treatment step report a sensitivity for mycobacteria of approximately 90%, depending on the report (Kiraz *et al*., 2010; Neonakis *et al*., 2009; Seagar *et al*., 2008; Franco-Álvarez de Luna *et al*., 2006; Fakrin *et al*., 2007).

This difference may be due to the pre-treatment with Proteinase K but this is unlikely. The more probable reason is the nature of the sample type evaluated with LN tissue potentially containing more PCR inhibitors than respiratory sample. Work carried out by Ben Kahla *et al*. comparing the sensitivity of a IS6110 PCR for direct detection of *M. tuberculosis* in pulmonary samples (sputum, bronchial wash and gastric lavage) and extra-pulmonary samples (pleural fluid, cerebrospinal fluid, tissue biopsy, urine, sperm, peritoneal fluid and joint fluid) noted a drop in sensitivity of the PCR from 93.8% for pulmonary samples to 63.6% for extra-pulmonary samples which was put down to the presence of PCR inhibitors or loss of *M. tuberculosis* cells during the DNA extraction process (Ben Kahla *et al*., 2011).

The remainder of the 30 decontaminated tissues samples were split into false negative results, 16.7% (5 out of 30), and test failures, 20% (6 out of 30). The false negative results were probably due to the low number of MTBC cells in the decontaminated tissue, reduced even further by the pre-dilution of some of the samples, and the test failure, indicated by the absence of a UC (universal control) band on the strip, were due lack of amplification the internal control RNA during the NASBA reaction which was probably caused by the presence of PCR inhibitors within the tissues. It is possible that the addition of extra washing steps to the decontaminated tissue pellets, with PBS or saline, may help remove any PCR inhibitors present in the tissue, as done by Ben Kahla *et al*., however this may cause the loss of some MTBC cells within the tissue leading to a false negative result (Ben Kahla *et al*., 2011). It should be noted that 2 of the 3 culture negative decontaminated tissue samples failed when tested with the kit after addition of the Proteinase K digestion, so it was not possible to draw a conclusion from the lack of false positive results.

Using a serial dilution of *M. bovis* spiked into negative LN tissue and pre-treated with the Proteinase K the limits of detection for the kit were shown to be 2.3 x

10^2 cfu/ml, Figure 4.32, which is similar to the limits of detection of *M. bovis* for liquid culture shown in section 1.2.l. This indicates the potential of the kit for use with high priority samples but at the current time, due to the false negative results and the lack of conclusive information about false positive results in this study, it could only be used as an information tool within the laboratory and not as a basis of reporting the TB infection status of an animal. The ability of kit to detect more mycobacterial species than MTBC, including *M. avium*, *M. intracellulare*, *M. malmonense* and *M. kansasii*, which have all previously been detected during TB culture in the CVRL (unpublished data), is also a advantage for future use of this kit.

A major obstacle against the future use of this kit was the failure of the supplied positive control to give a clear banding pattern and no reason can be found for this error. Two different kit lot numbers were used during this project, all reagents were prepared correctly and used in date and all equipment, except pipettes and plastic tubes, used in the amplification and detection steps were supplied by the manufacturer. Further work also needs to be carried out to determine if the number of test failures and false negative results can be reduced and to determine if any false positive results are detected with this kit. The tissue samples that had been pre-diluted before being tested on the kit could not be repeated due to the lack of sample material.

Sequence Capture PCR was a known method for the direct detection of MTBC cells from decontaminated LN tissue. It was included in this study to prove that mycobacterial DNA could be recovered from the 30 decontaminated tissues used. The method correctly identified the infection status of 73.3% of the decontaminated tissues, this was lower than the sensitivity seen in Roring *et al*., which has 97% when a sequence capture extraction method was coupled with spoligotyping to detect *M. bovis* in LN tissues, but similar to that seen in other studies, Parra *et al.* average sensitivity of 73.87%, and Mangiapan *et al.* sensitivity of 76.5%, for the detection of MTBC from tissues (Roring *et al*., 2000; Mangiapan *et al*., 1996; Parra *et al*., 2008). This was probably due to the low number of MTBC cells in some decontaminated tissues, which was not helped by the pre-dilution step for some samples. Unfortunately, there was not enough material remaining to re-test all 30 samples without the pre-dilution step to determine if this would improve the sensitivity of the technique. As with the GTMD kit, the level of false negative results identified in this study means this test could be used to provide information on the TB infection status

of high priority samples, such as Singleton Reactor animals or pets, for the laboratory use only and not as a definitive diagnostic tool.

A limit of detection study was carried out with *M. bovis* spiked into negative LN tissue and this showed that sequence capture PCR was able to detect *M. bovis* down to a concentration of 2.3×10^1 cfu/ml, Figure 4.35 to 4.37. This indicated that it could detect lower concentrations of *M. bovis* than liquid culture systems that had a limit of detection of 2.5×10^2 cfu/ml. This points to the value of the technique since it could be used to detect bovine TB in tissues which have too few MTBC cells or cells that are not viable for culture.

4.4 Conclusion

The MTBC direct detection methods evaluated in this study have been used to extract mycobacterial DNA from a wide variety of sample types. Heat lysis, a simple and effective method for extraction of DNA from pure cultures is not sensitive or specific enough for use with lymph node tissue. The Roche High Pure Template Preparation Kit removes the total DNA from a given sample, but it does not have the ability to select mycobacterial DNA from any other contaminating DNA within the sample.

The use of IMS to remove the MTBC cells from the decontaminated tissues prior to DNA extraction was unsuccessful. The method was not sensitive enough to remove the low number of MTBC cells, typically found in TB infected tissues, from the tissue sample.

The GTMD Kit Version 4.0, with the addition of the Proteinase K incubation overnight, was able to detect MTBC cells in the decontaminated tissue samples and had a similar sensitivity to that of culture. The GTMD kit can be started immediately after culture and completed the following day. It can be easily integrated into the TB laboratory workflow, since the MB extraction and NASBA reaction can be carried out within the Category 3 Laboratory. With bio-security concerns, removal of an amplified product is preferable to removal of potentially viable MTBC cells. The continued failure of the positive control supplied with the kit is an issue, but a MTBC positive solid culture was always correctly identified.

The sequence capture PCR was shown by this study to be a very effective and rapid method for the diagnosis of an *M. bovis* infection in lymph node tissues. A result could be available for preliminary reporting 3 days after culture. Similar to the

GTMD kit, the enzymatic digestion of the decontaminated tissues can be started soon after completion of the culture procedure. The remainder of the extraction procedure, mechanical disruption and binding of the released DNA to the capture oligonucleotides and MB, could be carried out the following day. Amplification of the extracted DNA could be carried out overnight and detected, on a gel, on day 3 after culture. Modifications to the amplification and detection procedure for use with Real-Time PCR would speed up this part of the test.

The MTBC positive tissues, 27 in total, were classed as high, medium to low positives based on a direct ZN examination of the decontaminated tissue homogenate prior to inoculation into the culture media, as per section 2.11. This was done on the theory that samples that had a high MTBC load may be more likely to be identified by direct detection methods. This was found not to be the case, for the GTMD kit the percentage number of positive decontaminated LN tissue samples correctly identified was, 57.1% of high positives, 77.8% of medium positive and 63.7% of low positives. For Sequence Capture PCR, without Q solution in the PCR mix, the percentage of the number of samples correctly identified was 71.4% of high positives, 77.8% of medium positive and 63.6% of low positives.

For this study, following enzymatic and mechanical disruption, the samples for sequence capture PCR were removed from the Category 3 Laboratory due to lack of the necessary equipment for the capture oligonucleotides and MB binding steps. With modifications to the allocation of resources both these steps could be carried out in the Category 3 Laboratory with only the amplification and detection work needing to be carried outside of the TB laboratory.

This study has found that both the GTMD kit and sequence capture PCR are suitable for use in detection of MTBC cells from decontaminated lymph node tissue with sequence capture PCR having an increased sensitivity over the GTMD kit but both need further work to determine if the sensitivity can be improved. The choice between both methods would probably be made on a cost per test basis and the ease of adaption of either method into the existing TB workflow. Ease of integration of any direct detection method into the existing TB workflow is important since the turnaround time for samples must be maintained and only a limited number of staff have the necessary training for working with mycobacteria.

Chapter 5.

General Discussion.

This study was concerned with the detection of MTBC, specifically *M. bovis*, from LN tissue. The project looked at the possibility of developing improved MTBC culture methods and molecular methods for the direct detection of MTBC from decontaminated LN tissue.

As part of this study a comparison was carried out between two liquid culture systems to determine if MGIT 960 system would be a suitable replacement for the BACTEC 460 system. Previously published work had shown that the MGIT 960 compared favourably to the BACTEC 460 and solid culture systems for the culture of MTBC from human clinical samples but was of only limited use for MTBC culture from bovine tissue samples (Chew *et al*., 1998; Scarparo *et al*., 2002; Kanchana *et al*., 2000; Hanna *et al.,* 1999; Somosközi *et al.,* 2000; Tortoli et al 1999; Zuhre Badak et al., 1996; Yearsley *et al*., 2000). The manufacturer altered the media formulation to improve this and a study published in 2006 showed that the MGIT 960 system was capable of supporting *M. bovis* growth however it showed a much lower detection rate for *M. bovis* in the BACTEC 460 system (Hines *et al*., 2006).

For the culture of MTBC from lymph node tissue the BACTEC 460 is a well-established culture system that has evolved to become the "gold-standard" by which other culture systems are measured (Zuhre Badak *et al.*, 1996). It is a semi-automated liquid culture system that detects growth by the release of radioactive CO_2 from the use of C^{14} labelled palmitic acid by bacteria in the culture media (Yearsley *et al.*, 1998). MGIT 960 is a fully automated, continuous monitoring, high capacity, non-radiometric, non-invasive instrument (Kanchana *et al.*, 2000). It uses an O_2 quenching fluorescent sensor, imbedded at the base of the tube, to detect microbial growth with the culture vials (Scarparo *et al.*, 2002).

The results from the limit of detection study, shown in section 3.2.1, showed that MGIT 960 liquid culture system was now capable of supporting the growth of MTBC, specifically *M. bovis*, and had a similar limit of detection for *M. bovis* to that of the BACTEC 460 liquid culture system.

In the comparison between the two liquid culture systems, each combined with solid culture systems (LJP and Stonebrinks), section 3.2.2, MGIT 960 detected 97.7% of the MTBC infected tissues compared to BACTEC 460 detecting 96.6% of the MTBC infected tissues, using routine lymph node tissue samples. The MGIT 960 detected the growth of MTBC approximately slight faster than the BACTEC 460 (14.6 days for MGIT 960 and 15.2 days for BACTEC 460).

Due to the limited number of non-tuberculosis mycobacteria (NTM) isolated during this study it is difficult to draw firm conclusions. From the limited data available it would seem that the MGIT 960 system does not support as many NTM isolates as the BACTEC 460 system. Further work would need to be undertaken to determine which NTM the MGIT 960 system can support and if alterations to the culture conditions or the solid culture systems utilized could improve the number of NTM isolates identified from LN tissue.

The MGIT 960 liquid culture system does have added labour requirement for reprocessing of contaminated sample tubes, with contamination rate of 8.2% for MGIT 960 compared to a rate of 3.1% for BACTEC 460. Also the increased isolation of *R. equi* with the MGIT 960 system, 96.6% with MGIT 960 compared to 78.4% with BACTEC 460, will increase the number of samples that require handling. This would be offset by the reduced number of atypical mycobacteria detected by the MGIT 960 liquid culture system and the automated monitoring of culture in the MGIT 960 instrument is an automated system and once the MGIT tubes are entered into the MGIT 960 instrument (see Figure 1.9) they are continuously monitored. The MGIT tubes are read every 60 min and a LED indicates which tubes have flagged as positive.

The MGIT 960 culture monitoring has a much reduced labour requirement compared to the BACTEC 460 system. The BACTEC 460 media bottles need to be removed from the 37^{o}C incubator, loaded onto sample boats, monitored by the BACTEC 460 instrument, unloaded and returned to the incubator. The BACTEC 460 instrument converts any detected radioactive CO_2 into a Growth Index (GI) number and this is printed on a strip of paper along with the sample boat and position. This information then has to be transcribed to the sample number in that sample boat position adding a risk of handling errors to the process. There is no computerised data handling system compatible with the BACTEC 460 instrument. Even with the increased numbers of samples that will require handling due to contamination or growth of *R. equi*, the MGIT 960 liquid culture system has an overall reduced labour requirement when compared to the BACTEC 460.

When safety concerns are looked at the MGIT 960 system is a non-radiometric and non-invasive culture system and is much safer to operate than the BACTEC 460 system. This is due to the necessity of handling, storage and disposal of radioactive

material, C^{14}, and the potential of needle stick injuries during vial inoculation and sampling procedures with the BACTEC 460.

The manual supplied by the manufacturer stated that CPC decontamination was not suitable for use with the MGIT 960 liquid culture system (Siddiqi and Rüsch-Gerdes, 2006). CPC decontamination of tissues was used as part of a long-running badger project within the CVRL, which included MTBC culture on BACTEC 460 liquid culture system a part of the protocol. Confirmation was required as to how unsuitable this method was for culture of MTBC from lymph node tissue. Bovine LN tissues were used instead of badger LN tissue due to a more readily available source.

It was shown, section 3.2.3, that CPC decontamination reduced the isolation of MTBC on MGIT media tubes, from 89.5% with OA to 5.3% with CPC, and on solid culture systems, from 63.2% with OA to 47.4% with CPC. The contamination rate between the two culture methods also indicated that CPC was not suitable for decontamination of LN tissue for MTBC culture, in MGIT media CPC contamination rate was much lower, at 1.32%, than OA, at 4.86%. The contamination rate for solid culture systems, with CPC decontamination, was much higher, at 18.75%, than for OA decontamination, at 10.42%. A surprising result was how much more *R. equi* was isolated, when grown on solid culture systems, with CPC decontamination, 100%, than with OA decontamination, 72.7%. If further studies take place on *R. equi* isolation the use of CPC decontamination, with solid culture systems only, should be considered.

This study has shown that the MGIT 960 liquid culture system can support the growth of MTBC, specifically *M. bovis* and is a suitable replacement for the BACTEC 460 system. It agrees with the findings of the work on human clinical samples in that both liquid culture systems give similar MTBC detection rates unlike the Hines *et al*. study which showed a much lower *M. bovis* detection rate with the BACTEC 460 system compared to the MGIT 960 system (Chew *et al*., 1998; Scarparo *et al*., 2002; Kanchana *et al*., 2000; Hanna *et al.,* 1999; Somoskövi *et al.*, 2000; Tortoli et al 1999; Zuhre Badak et al., 1996; Hines *et al*., 2006). The difference can't really be explained by the addition of erythromycin to the BACTEC 460 bottle in the Hines *et al*. report so it is unknown at this time what it could be the cause of this differing result.

It would not be possible, on a safety and cost basis, to continue limited use of the BACTEC 460 system for a project and the MGIT 960 system has been shown to

be unsuitable for use with CPC decontamination. The Badger Vaccine Project, described in brief in section 3.1, could increase the number of solid culture systems (LJP, Stonebrinks and 7H11 plates) included in the culture protocol to compensate for the removal of the BACTEC 460 system and the unsuitability of MGIT 960 system for use with CPC decontamination protocol used in the project. The CPC decontamination protocol is used with the Badger Vaccine Project since it allows for the alterations to the concentration of decontaminant, depending on the source and condition of the tissue samples for culture, potentially increasing the number of MTBC isolates detected (Corner *et al*., 1995).

A method to diagnose a MTBC infection directly from LN tissue would be a useful technique to have available in cases were a rapid diagnosis of MTBC infection is required. These would include samples where there is a suspected human involvement i.e. family pet or where unpasteurised milk or milk products had been consumed from an animal with a suspect TB infection, and samples submitted from Singleton Reactor animals. The five methods evaluated for the extraction and detection of MTBC cells, Heat Lysis, Roche High Pure PCR Template Preparation Kit, Immunomagnetic Separation (IMS), GenoType Mycobacteria Direct kit (GTMD) and Sequence Capture, shown in Chapter 4, were all used to extract or detect mycobacteria from a wide variety of samples. Sequence Capture PCR was the only method that had already been shown to detect *M. bovis* in decontaminated LN tissue (Roring *et al*., 2000; Mangiapan *et al*., 1996; Parra *et al*., 2008).

Heat Lysis was a simple and effective method for releasing DNA from cells but it was shown, section 4.2, not to be sensitive or specific enough for use with decontaminated LN tissue. Roche High Pure PCR Template Preparation Kit isolated the total DNA from the decontaminated sample, but it does not have the ability to select mycobacterial DNA from any other contaminating DNA.

IMS was selected for evaluation since along with being used to diagnose a MTBC infection; it could also provide information on the viability of the infection. IMS had already been used to detect mycobacterial infections, including *M. bovis*, in a wide variety of samples such as milk, faecal samples and cerebrospinal fluid (Grant *et al*., 1998; Djønne *et al*., 2003; Khare *et al*., 2004; Li *et al*., 1996; Mazurek *et al*., 1996; Antognoli *et al*., 2001). In this study IMS was shown not to be powerful enough to concentrate MTBC cells from decontaminated LN tissue, section 4.2.3. This may have been due to a wide variety of reasons including the low number of

MTBC cells typically found in decontaminated tissue samples and the presence of molecules, such as immunoglobulins and proteases that may interfere the antibody-antigen binding reaction.

GTMD had already been shown to detect MTBC infection in a wide variety of samples but it has yet to be shown suitable for detection of a *M. bovis* infection in LN tissue (Seagar *et al*., 2008; Franco-Álvarez de Luna *et al*., 2006; Fakrin *et al*., 2007; Kiraz *et al*., 2010; Neonakis *et al*., 2009). The advantage of this kit is that it can be used to diagnose some NTM infections along with the MTBC. In section 4.2.4, after the addition of an overnight enzyme digestion, the GTMD had a sensitivity for *M. bovis* similar to that of culture, at 2.3 x 10^2cfu/ml, when spiked into culture negative decontaminated LN tissue. However, when tested on routine culture decontaminated tissue samples it only correctly identified 63.3% of the MTBC positive samples. There were no false positive results detected with this kit but this is not a definitive conclusion due to the small sample size, 3 samples, of MTBC culture negative tissues included in the study and the test failure of 2 of the 3 samples.

As stated previously, Sequence Capture PCR was the only method evaluated that had already been shown to detect *M. bovis* in decontaminated LN tissue. In this study, section 4.2.5, it was shown to have sensitivity, for *M. bovis*, 10 times greater than culture, at 2.3 x 10^1cfu/ml, when spiked into decontaminated LN tissue while only correctly identifying 73.3% of the decontaminated tissue samples. A similar sensitivity level for detection of MTBC isolates directly from bovine LN tissues had been reported previously (Mangiapan *et al*., 1996; Parra *et al*., 2008)

The choice between GTMD or sequence capture for direct detection of an MTBC infection in LN tissue would depend on a number of factors including cost, ease of use within the current TB workflow, requirement of the downstream processes and increasing the sensitivity of both techniques when compared to culture. The product of sequence capture PCR can be adapted for a wide variety of molecular tests while the GTMD can diagnose several mycobacterial infections within a single test.

Further evaluations need to be carried out to determine if the sensitivity of either of these tests could be improved, perhaps by removing the pre-dilution step for the decontaminated tissue samples included in this study. It may be that increased familiarity within either test might also improve the sensitivity results. This work was carried out on only 30 decontaminated tissue samples and comparing both tests on a wider sample pool may indicate which test would be more suitable for use on

veterinary samples. Both tests remove MTBC DNA from LN tissue material and the possibility of coupling either to another extraction procedure to determine if the sensitivity can be improved would be remote, though perhaps increasing the number of washing steps, as suggested in Ben Kahla *et al*., could be considered (Ben Kahla *et al*., 2011). For sequence capture PCR the option exists of varying the capture oligonucleotides to target a different section of the *M. bovis* genome, this may increase the sensitivity of the technique.

In conclusion, this study has shown that MGIT 960 system is a suitable liquid culture system to replace the use of the BACTEC 460 system for the culture of MTBC from LN tissues. It was also shown that the MGIT 960 liquid culture system is not suitable for use with CPC decontamination. For the extraction and detection of MTBC cells from decontaminated LN tissue the GTMD kit and Sequence Capture PCR are both potential methods and the choice between either techniques is based on cost, ease of adaptation to the current TB workflow within the Central Veterinary Research Laboratory (CVRL) and improved the sensitivity for MTBC. It is unlikely that either direct detection test will replace culture for the diagnosis of MTBC infection in the near future, though sequence capture PCR coupled with a real-time PCR has been proposed as a screening tool for *M. bovis* as part of the bovine tuberculosis abattoir surveillance program (Parra *et al*., 2008)

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