2013

An Evaluation of MIRU-VNTR Analysis and Spoligotyping for Genotyping of M. Bovis Isolates and a Comparison on RFLP Typing

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An evaluation of MIRU-VNTR analysis and spoligotyping for genotyping of *M. bovis* isolates and a comparison with RFLP typing.

By

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For award of M.Phil
August 2013

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Abstract

Common strain typing methods for differentiation of *Mycobacterium bovis* isolates include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, spoligotyping and more recently, mycobacterial interspersed repetitive unit-variable-number tandem repeat (MIRU-VNTR) typing.

MIRU-VNTR and spoligotyping were evaluated in this study and these typing methods were compared with RFLP typing. A total of 386 *M. bovis* isolates from cattle, badgers and deer in the Republic of Ireland that had previously been typed by IS*6110*, polymorphic GC-rich sequence (PGRS) and direct repeat (DR) RFLP were included in the study. An initial panel of VNTR loci was established to analyse the first 60 isolates to determine that six VNTR loci (QUB 11a, QUB 11b, ETR A, 4052, MIRU 26 and 1895) displayed the greatest degree of discrimination between strains.

Analysis of these six VNTR loci and spoligotyping was performed on all 386 isolates. RFLP was the method that gave the greatest differentiation of strains with a Hunter Gaston discriminatory index (HGDI) of 0.927, the HGDI recorded for MIRU-VNTR was marginally lower at 0.918 and spoligotyping was the least discriminatory method with a HGDI of 0.7. The HGDI for VNTR (6 loci) and spoligotyping was 0.933, however when 4 VNTR loci were used a comparable HGDI of 0.93 was recorded. Spoligotype SB0140 represented approximately 50% of the isolates. Within the group of isolates represented by SB0140 there was a high level of diversity between RFLP and MIRU-VNTR typescompared to groups represented by other spoligotypes. The most common MIRU-VNTR types within the SB0140 group were widely distributed geographically which limited their usefulness for tracing geographic spread of infection. However, the less common MIRU-VNTR types with the SB0140 group were largely

concentrated in defined geographic areas. A combination of spoligotyping and MIRU-VNTR typing offered advantages over MIRU-VNTR typing alone. In a combined spoligotyping and MIRU-VNTR typing protocol the number of VNTR loci could be reduced to four (QUB 11a, QUB 11b, ETR A and 4052) while maintaining a high level of strain differentiation.

Declaration

I certify that this thesis which I now submit for examination for the award of _____, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any other third level institution.

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Acknowledgements

I would like to thank the Department of Agriculture, Food and Marine especially the Central Veterinary Research Laboratory for giving me the opportunity to complete this study.

I appreciate all the support and advice that I received from Eamon Costello throughout the duration of the study; even though retired he has continued to show me tremendous support

A big thanks you to DIT especially Fergus Ryan for all the assistance that he has given me throughout the research and the thesis write up.

For my family and friends, a huge thank you for all the love and encouragement that you have all given me. For those you were a great help with the babysitting duties throughout the write up, viva and corrections, thank you.

To my husband Eamon and my children Eoin and Maria, thank you for being my rock through the hard times.

To my late Dad and Mum without you both I would not be where I am today and I much appreciate all the love and support that you gave me over the years.

Abbreviations

AFB	Acid Fast Bacilli
Bp	Base Pair
CFU	Colony Forming Units
Ctrl	Control
CVRL	Central Veterinary Research Laboratory
DNA	Deoxyribonucleic Acid
DR	Direct Repeat
DVR	Direct Variable Repeat
G&C	Guanine and Cytosine
GIS	Geographical Information System
H&E	Hematoxylin and Eosin
HG	Hazard Group
IS	Insertion Sequence
LJG	Löwenstein-Jensen with Glycerol
LJP	Löwenstein-Jensen with Pyruvate
M. bovis	*Mycobacterium bovis*

MIRUs	Mycobacterial Interspersed Repetitive Units
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats
PFGE	Pulsed Field Gel Electrophoresis
PGRS	Polymorphic Guanine and Cytosine Rich Repeat Sequences
RDs	Regions of Difference
RD9	Difference Number 9
REA	Restriction Endonuclease Analysis
RFLP	Restriction Fragment Length Polymorphism
STRs	Short Tandem Repeats
SICTT	Single Intradermal Comparative Tuberculin Test
Spacers	Non-Repetitive Spacer Sequences
TB	Tuberculosis
UV	Ultraviolet
VNTR	Variable Number Tandem Repeats
ZN	Ziehl-Neelsen

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Chapter one

Introduction

1.1 *Mycobacterium bovis*

Mycobacterium bovis (*M. bovis*), is a slow-growing acid-fast bacillus and is a member of the *Mycobacterium tuberculosis* complex. There are seven species and subspecies within the *Mycobacterium tuberculosis* complex, these are; *M. tuberculosis*, *Mycobacterium canettii*, *Mycobacterium africanum*, *Mycobacterium pinnipedii*, *Mycobacterium microti*, *Mycobacterium caprae* and *Mycobacterium bovis*. *M. bovis* has a wide host range that includes wild and domesticated animals and humans. It is the most common cause of tuberculosis (TB) in animals, whereas *M. tuberculosis* is the most common cause of tuberculosis in humans (Thoen and Steele, 1995; Corner *et al.*, 2011).

The tubercle bacillus was firstly observed and cultured by Robert Koch in 1882 (Collins and Grange, 1983, Daniel, 2006). Lehmann & Neumann then assigned the species to the genus *Mycobacterium* in 1896 (Collins and Grange, 1983; Collins *et al*., 1997). At first Koch believed that there was only one type of tubercle bacillus that affected both man and animals, however he then discovered small but distinct differences between the human and bovine strains. Bovine strains were shorter with human strains tending to be longer bacilli. Human strains were able to grow for longer periods *in vitro* which was not the case with bovine strains.(Collins and Grange, 1983; Thoen and Steele., 1995; Smith 1898).

The Sanger Institute completed the sequence of the genome of *M. bovis* in 2002 (Sanger.ac.uk 2002). The genome is a double stranded circular deoxyribonucleic acid (DNA) molecule approximately 4.4 million base pairs (bp) long with a guanine and cytosine (G & C) content of approximately 65% (Durr *et al*., 2000a). Genetically all the members of the *M. tuberculosis* complex are similar, having 99.9% similarity at

nucleotide level and have identical 16s rRNA sequences (Haddad *et al*., 2004). Given this level of similarity, it has been suggested that the members of the complex 'might be best described as a series of host adapted ecotypes, each with a different host preference representing different niches' (Hewinson *et al*., 2006). The evolutionary lineage of the complex has been described by Brosch *et al*., (2002). Regions of difference (RDs) between the genomes of the various members of the complex were identified. For the most part these RDs consisted of deletions of DNA sequences. Since recombination is not known to occur in this group, a lost DNA sequence cannot be restored, and the deletion will characterise the lineage forever (Brosch *et al*., 2002). Strains host adapted to animals from a nested lineage are marked by the absence of the specific Region of Difference number 9 (RD9) (Smith *et al*., 2006b). This group of lineages includes *M. bovis, M. africanum* and *M. microti* and they are distinguished from *M. tuberculosis* by the RD9 deletion (**Figure 1.1**, reproduced from Brosch *et al*., 2002). 'Classical' *M. bovis* and *M. bovis* BCG are distinguished from other closely related lineages such as the goat lineage (*M. caprae*) and the seal lineage (*M. pinnipedii*) by the RD4 deletion (**Figure 1.1**).

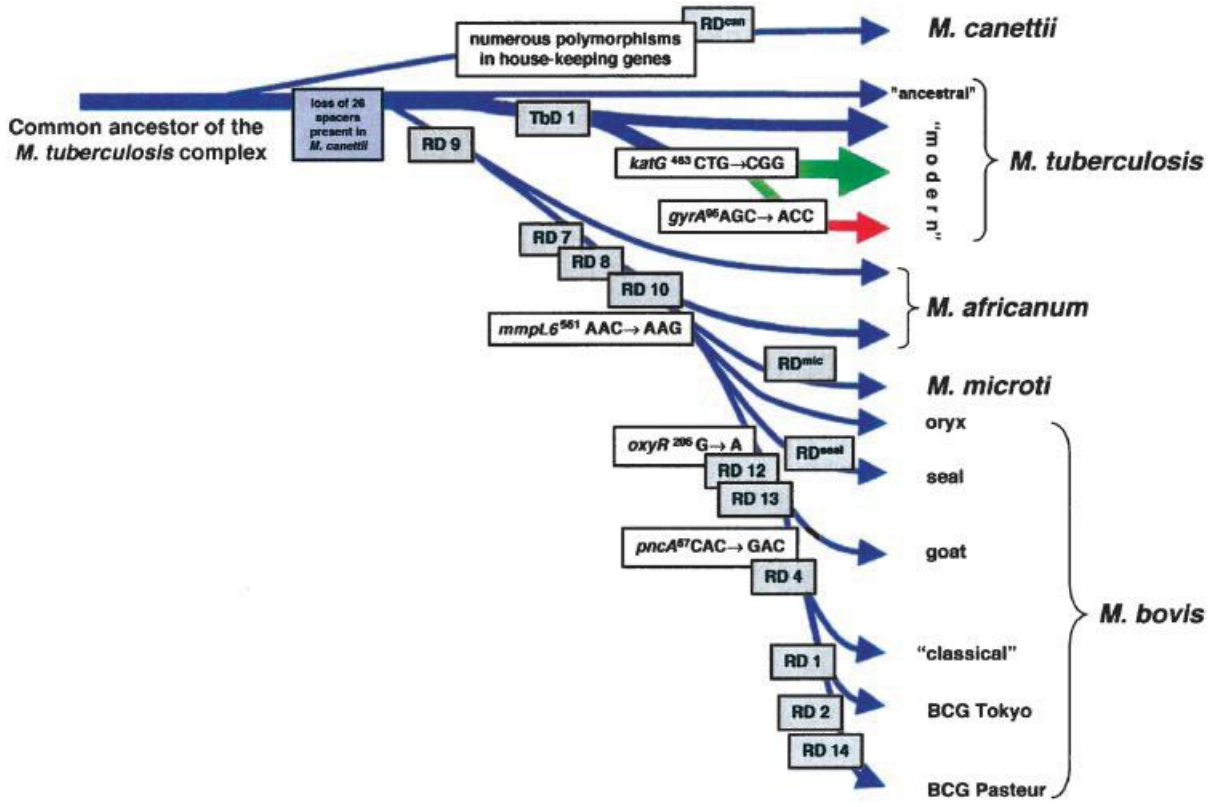


Figure 1.1 Scheme of the proposed evolutionary pathway of the *M. Tuberculosis* complex illustrating successive loss of DNA in certain lineages (gray boxes). The scheme is based on the presence or absence of conserved deleted regions and on sequence polymorphisms in five selected genes. This illustration is reproduced from Brosch *et al*., 2002.

The *M. bovis* genome is rich in gene regulatory circuits, consistent with the need to selectively activate genes in order to survive for prolonged periods within the host's macrophages as well as in the outside environment during the transmission stages of TB. A large portion of the genome is devoted to enzymes for the metabolism of lipids such as mycolic acids, which are an essential ingredient of the distinctive hydrophobic waxy cell wall of mycobacteria (Durr *et al.,* 2000a).

The cell wall is organised into four layers which overlay the membrane as shown in **Figure 1.2, (**reproduced from Mc Lean *et al*., 2007**).** The membrane consists of carotenoids which differ in structure from species to species. The presence of the carotenoids in sufficient quantities passes the bright yellow colour to the bacteria when grown in culture. The peptidoglycan layer is the first layer of the cell wall and can be found in most other bacteria. The second layer is composed of arabinogalactan. The Arabinogalactan side chains link in the third layer, mycolic acids. Mycolic acid is the major component of the cell wall contributing to its thickness. It is this thickness that is responsible for the characteristic 'acid fast' staining of the mycobacteria i.e. when the cells are stained with an arylmethane dye such as fuchsin they tend to retain the colour even after decolourisation with acid alcohol. The fourth layer consists of various lipids and related compounds such as glycosides, glycolipids and pepidoglycolipids called mycosides (Grange, 1996).

TB is still the single greatest cause of human morbidity and mortality in many developing countries (Thoen and Steele, 1995). However, it is not generally regarded as a significant public health risk in the developed regions of the world, but bovine TB still

poses a major economic problem for agriculture trade and industry (Pollock and O' Neill., 2002).

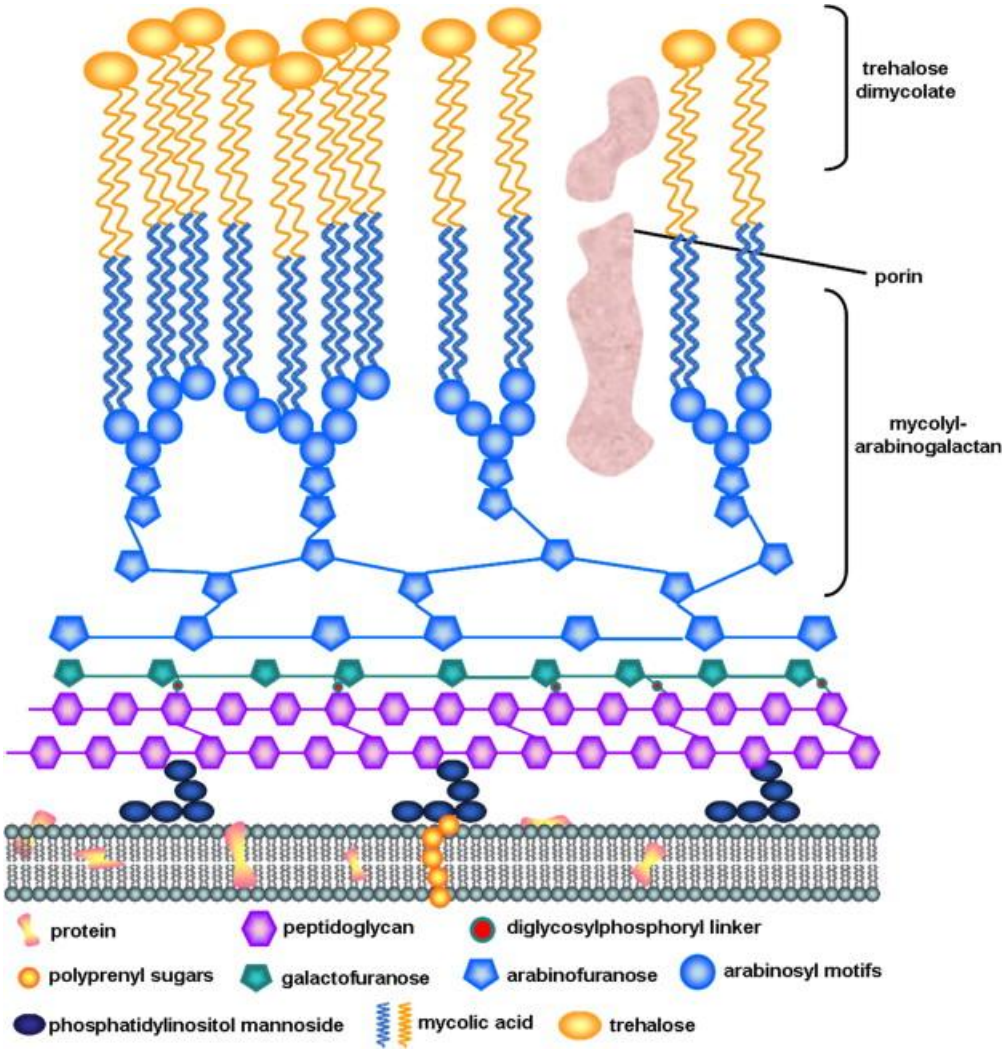


Figure 1.2 Schematic diagram of the mycobacterial cell wall (Mc Lean *et al*., 2007).

1.2 TB in Cattle

Tuberculosis is a significant disease of cattle in many countries, including Ireland and the UK. It leads to economic losses, disruption to trade and farming practices and has the potential for zoonotic spread (O'Reilly and Daborn, 1995). It is usually a slow progressive disease and it can take a number of years for clinical signs to become apparent (Francis, 1947).

The principal mode of transmission in cattle is by the respiratory route. The infective dose is thought to be as low as one organism, when delivered to the surface of pulmonary alveoli, whereas it can take up to several million to establish infection via the oral route (Neill *et al.*, 2001; Pollock and O'Neill, 2002; Philips *et al.*, 2003). TB lesions are generally found in the lungs and the lymph tissues of the thoracic cavity, usually the bronchial and/or mediastinal lymph nodes. The lymph nodes in the head region are the second most frequent site for TB lesions (Neill *et al.*, 2001). In advanced disease multiple organs and tissues may be affected (generalised tuberculosis). If disease spreads to the udder there is a high risk of zoonotic infection if unpasteurised milk is consumed (Francis, 1947). Infection can also be transmitted to calves or domestic pets via infected milk. The development of the disease depends on the ability of the tubercle bacillus (*M. bovis*) to multiply within cells and to induce a host's immune response. A typical 'cell-mediated immune' response can take up to 10 days, after which the activated macrophages and lymphocytes form a compact aggregate around the bacilli creating the histological structure known as the granuloma as shown in **Figure 1.3**. The 'cell-mediated immune' response will then produce a positive skin reaction to the intra-dermal tuberculin test (Thoen and Steele., 1995; Collins *et al.*, 1997; Pollock *et al.*, 2001).

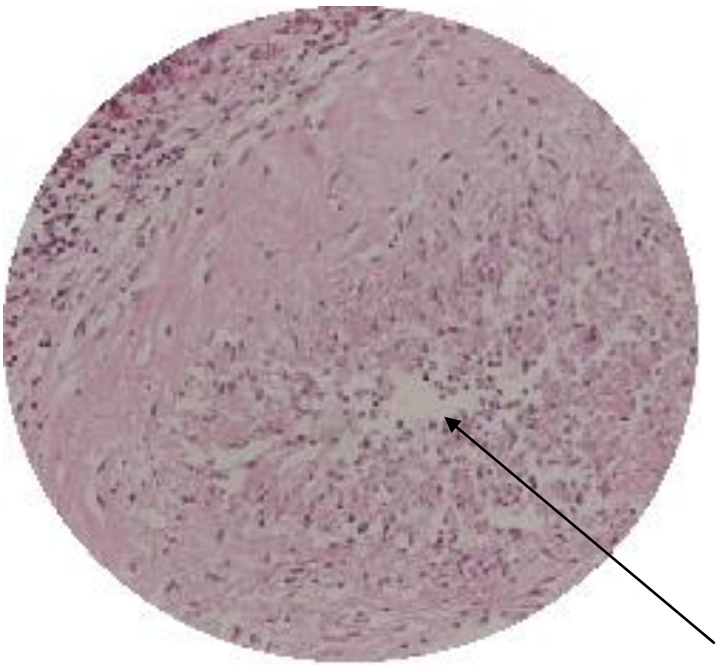


Figure 1.3 Chronic avascular granuloma consisting of 3 regions; central zone of large multinucleated giant cells containing mycobacteria; a mid-zone of radially arranged pale epithelioid cells and a peripheral zone of fibroblasts, lymphocytes and monocytes (www.microrao.com).

In cattle, the signs of TB infection are non-specific. The majority of infected animals do not show signs of any abnormalities in the early stages of infection. However cattle with advanced TB present with progressive emaciation, respiratory signs, changeable appetite, a fluctuating fever, and, in some cases, a chronic mastitis (Francis, 1947). It is suggested that the respiratory transmission of TB from cattle to cattle is commonly associated with housing and is less likely at pasture. The high numbers of cattle and the high humidity create an idyllic environment for TB transmission (Neill *et al.*, 1994; Pollock and O'Neill., 2002; Philips *et al.*, 2003). Ingestion of *M. bovis* directly from

infected animals or from contaminated pastures, water or fomites can also cause of TB infection (Pollock and O'Neill., 2002).

1.3 Wildlife Reservoirs

In Ireland the incidence of TB in cattle remains at approximately 0.5% due to some extent to the re-infection of TB free herds by wildlife reservoirs such as the badger and deer (Sheridan, 2011). The main wildlife reservoir in Ireland is the badger (*Meles meles*). They were first suspected to be a source of infection for cattle in the early 1970's. They are a protected species in Ireland, however licenced culling has been employed since 1973 as a means of removing infected badgers. The culling programme has complemented the eradication programme in controlling the spread of the disease (Wilson *et al.*, 2011).

The badger is a member of the *Mustelidae* family, a family of carnivorous mammals that include otters and stoats. They are largely nocturnal and live in social groups of mixed ages and sexes in underground setts. Setts are usually complicated structures of multiple interconnecting tunnels that can have numerous openings many metres apart. Setts are used for resting, breeding, protection from predators and as shelter from harsh weather. In Ireland social groups of badgers tend to be small comprising groups of 2-3 badgers. Social groups are territorial and they mark their territorial boundaries with latrines that are deposited with urine and faeces (Rogers *et al.*, 2003; Corner *et al.*, 2011).

The badger is an ideal host for TB infection as they can excrete *M. bovis* in exhaled air, sputum, urine, faeces and pus (Philips *et al.*, 2003). The distribution of lesions in badgers at post mortem examination and the isolation of *M. bovis* from sputum during live sampling suggests that the respiratory route may be the principal mode of transmission from badger to badger. The transmission from badger to cattle can occur either by the respiratory route through direct contact with a badger or by their excretory products (Wilson *et al.*, 2011) with large quantities of bacteria being excreted in the urine. The badger urinates whilst walking and can produce a trail of urine of more than 0.5m long (Philips *et al.*, 2003).

Badgers can live longer than rodents, hedgehogs and most other farmland mammals allowing the disease to develop to an advanced and highly infectious phase. A sett with infected badgers found within one kilometre of a cattle herd can increase the risk of TB infection within that herd. Cattle and badgers tend to avoid each other. However, infected badgers tend to display more deviant behaviour that brings them into direct contact with cattle in cowsheds, in the field and through the use of feeding troughs. Cattle, particularly the more dominant animals, may investigate infected and moribund badgers by sniffing them, thus exposing themselves to the risk of respiratory infection. The pus from infected bite wounds of badgers can also be a potential source of spreading TB and may contaminate cattle through direct contact (Philips *et al.*, 2003). Badgers infected with TB from a bite wound tend to have a shorter survival rate as the disease progresses more rapidly than if infected through the respiratory route with the minimum infective dose being a single bacillus (Corner *et al.*, 2011).

Infected deer are another possible source for the spread of tuberculosis to cattle. TB has been found sporadically in wild deer and more frequently in farmed deer in Ireland

(Dodd, 1984; Quigley *et al*., 1997). Fallow deer (*Dama dama*), sika deer (*Cervus nippon*) and red deer (*Cervus eleaphus*) have been affected. Wild deer can harbour large numbers of *M. bovis* and show no signs of the disease. However, when the infected deer starts to show clinical evidence of tuberculosis, death of the animal may occur quickly, sometimes within 1-2 weeks (Griffin and Buchan., 1994). The distribution of lesions indicates that, as in cattle, the principal route of infection is the respiratory tract (Clifton-Hadley and Wilesmith., 1991).

1.4 Eradication of bovine TB in Ireland

The bovine TB eradication programme began in Ireland in 1954. It first began on a pilot basis and later became compulsory in 1962. Significant progress was made in the early years with the initial incidence level of 17% in cows down to 0.44% by 1965. However the incidence level continues to be approximately 0.5%, with the majority of infected animals being identified by the tuberculin test before they develop clinical signs (Sheridan, 2011).

New cases of bovine tuberculosis are identified using a combination of field and abattoir examinations. Field examination involves cattle being tested using the single intra-dermal comparative tuberculin test (SICTT), the primary diagnostic tool used in the TB eradication programme. The SICTT uses both bovine and avium tuberculins to measure and compare skin responses within 72 hours of the intradermal injection. In Ireland, it is compulsory for all herds to have an annual tuberculin test. Diseased herds require follow up testing. Compensation is issued for positive ‘reactor’ animals and a range of movement controls are placed on the herd (Thoen and Steele, 1995; Clegg *et*

al., 2011; Good and Duignan, 2011).

Abattoir examination involves the inspection of the split carcass, organs and associated lymph nodes, with inspection of the broncho-mediastinal and retropharyngeal lymph nodes in particular, to detect TB lesions. In the case of a carcass having suspect TB lesions the carcass is detained pending a more detailed examination before it is either released for human consumption or discarded. The carcass is deemed unfit for human consumption when either generalised TB has been diagnosed or the animal had a positive or inconclusive reaction to the SICTT test and inspection has exposed localised TB lesions in a number of organs or in a number of regions in the carcass. When a TB lesion has been detected in a single organ or part of the carcass or its associated lymph nodes, only the affected organ or part of the carcass are declared unfit for human consumption (EFSA Journal, 2003).

1.5. Laboratory diagnosis of *M. bovis* infection

1.5.1 Histological diagnosis

A definitive diagnosis of *M. bovis* infection can only be made by isolation of the organism on culture. However, because *M. bovis* grows very slowly on culture media, up to seven weeks may be required for isolation. Histological examination can be used to fast track the diagnosis of TB infection as a result can be obtained within three days (www.agriculture.gov.ie/Costello presentation). In areas of high TB prevalence histological diagnosis is acceptable but it cannot be relied on in all circumstances (Corner, 1994).

In the TB section of the Central Veterinary Research Laboratory (CVRL), lymph nodes are inspected macroscopically for the presence or absence of suspect TB lesions **(Figure 1.4)**. Suspect TB lesions are cut into approximately 3 mm segments and placed into a cassette which is then immersed in 10% buffered formalin. A portion of the lymph node is also kept for TB culture if required. An electronically programmed unit then prepares the segments into a paraffin block and using a microtone, 6 µm sections can be cut which are then stained with a hematoxylin and eosin (H&E) stain. In a typical TB lesion there is a granulomatous reaction comprising macrophages, epithelioid macrophages and multinucleated Langhans giant cells, with variable numbers of neutrophils surrounding an area of caseous necrosis. A typical TB lesion is shown in **Figure 1.3** (Thoen and Steel, 1995). In a proportion of lesions mineral deposits may be laid down in the caseous centre. This gives the lesion a hard gritty texture when cut. Typically, in the CVRL 85% of TB lesions are diagnosed based on histological examination (Costello, Agriculture presentation online). Diagnosis is based on examination of H & E stained sections. If the typical granulomatous reaction comprising macrophages, epithelioid macrophages and multinucleated Langhans giant cells and necrosis is present then the lesion is classified as tuberculous. Histological examination has limitations for the diagnosis of *M. bovis* infection in some species, for example, in farmed deer and pigs a high proportion of tuberculous lesions may be due to infection by *M. avium* species (Quigley *et al*., 1997; Matlova *et al*., 2003).

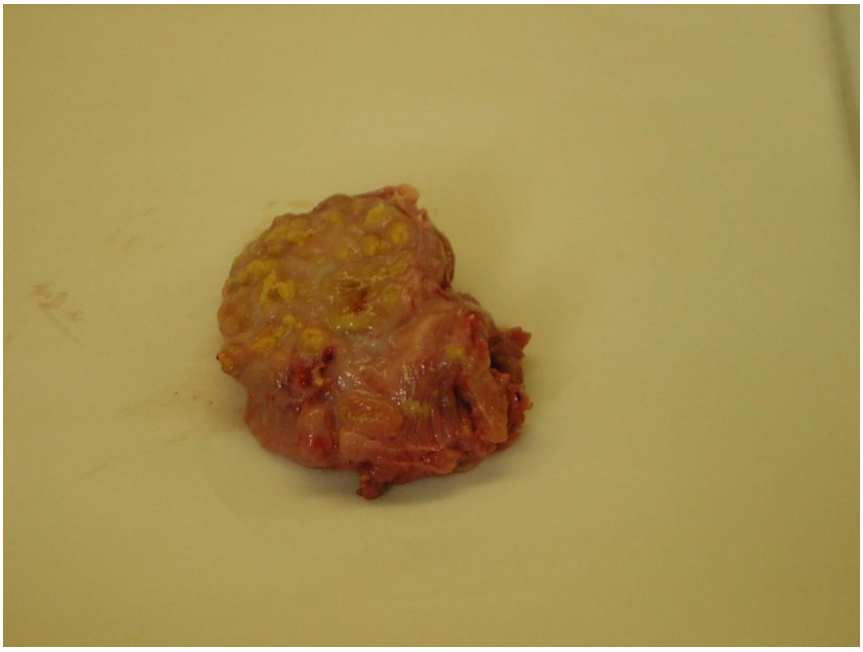


Figure 1.4 Tuberculosis Lesion in a Retropharyngeal Lymph node (www.agriculture.gov.ie/Costello presentation)

1.5.2 Culture of *M. bovis*

Most mycobacteria grow slowly, taking 2-6 weeks or longer at 37 ºC±1 to produce visible growth. The 'gold standard' method of culturing *M. bovis*, involves a combination of solid and liquid culture media. This is the method used in the laboratory, CVRL. A portion of lymph node is firstly decontaminated with 5% oxalic acid. Decontamination is performed to prevent overgrowth by contaminating bacteria.

Solid culture media is prepared in tubes or bottles as *M. bovis* is generally present in small numbers, therefore large inoculums are required to be spread over the surface of the medium. Löwenstein-Jensen and Stonebrinks (Media for Mycobacteria, Glamorgan,

United Kingdom) are the egg based media of choice in the CRVL. They contain eggs, phosphate buffer, magnesium salts, pyruvate or glycerol and Malachite green. Löwenstein-Jensen with glycerol (LJG) promotes the growth of the human strain *M. tuberculosis* whereas Löwenstein-Jensen with pyruvate (LJP) promotes the growth of *M. bovis* as *M. bovis* cannot use glycerol as a carbon source (Collins and Grange, 1983; Corner, 1994).

The BBL MGIT Mycobacteria growth indicator is an automated liquid medium system commonly used when isolating mycobacteria including *M. bovis*. The system utilises a 7ml tube containing 7H9 broth base, together with OADC enrichment (212240, Becton Dickinson, care of Unitech, City West, Dublin) and PANTA (245124, Becton Dickinson, care of Unitech, City West, Dublin) antibiotic mixture that provides the necessary ingredients for the rapid growth of mycobacteria (see section 2.1). The PANTA antibiotic mixture contains a lyophilized mixture of the antimicrobial agents, polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin. The tubes contain a chemical that will fluoresce when oxygen levels are reduced by the aerobically metabolisingmycobacteria . The BACTEC MGIT 960 tubes are incubated at 37ºC ±1 and automatically scanned by the BACTEC MGIT 960 unit every 60 mins for increasing fluorescence. A positive tube can contain 10^5 to 10^6 colony forming units (CFU) per ml (Tortoli *et al.*, 1999; Somoskövi *et al.*, 2000; Hines *et al.*, 2006).

1.5.3 Identification of *M. bovis*

Preliminary identification of *M. bovis* is based on colonial morphology and staining characteristics. *M. bovis* colonies on solid media are typically small, rounded, and off white to pale yellow in colour with irregular edges and a granular surface. When stained with Ziehl-Neelsen (ZN) and microscopically examined mycobacteria, including *M. bovis,*are described as being 'acid fast'. They appear as red coccobacilli or short rods 3 µm or 4µm long and are arranged in serpentine cords, **Figure 1.5** (Corner, 1994; Collins *et al*., 1997).

Previously, definitive diagnosis was based on a series of biochemical tests. These required good culture yields and sometimes gave ambiguous results (Richter *et al*., 2003). These tests have now largely been replaced by DNA based tests. There are commercial molecular identification test kits available such as the Genotype MTBC (Hain Lifescience, Nehren, Germany). This is a PCR and hybridisation assay that differentiates between the members of the *Mycobacterium tuberculosis* complex. Specific oligonucleotides targeting the gyr B DNA sequence and the RD 1 deletion of *M. bovis* are immobilised onto membrane strips. Amplicons derived from a multiplex PCR react with these probes during hybridisation resulting in a banding pattern as shown in **Figure 1.6.** The assay is easy to perform and results are easily interpreted (Richter *et al*., 2003).

A PCR and hybridisation method of strain tying known as 'spoligotyping' (refer to section 1.7.8.) can also be used for definitive identification of *M. bovis* isolates (Kamerbeek *et al*., 1997). Like the Genotype MTBC test this is an easy method to use, and has the advantage of differentiating strains as well.

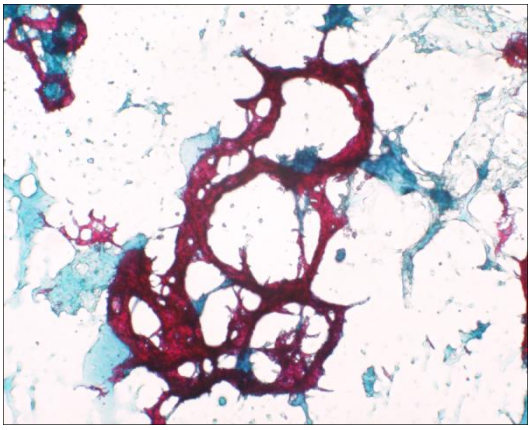
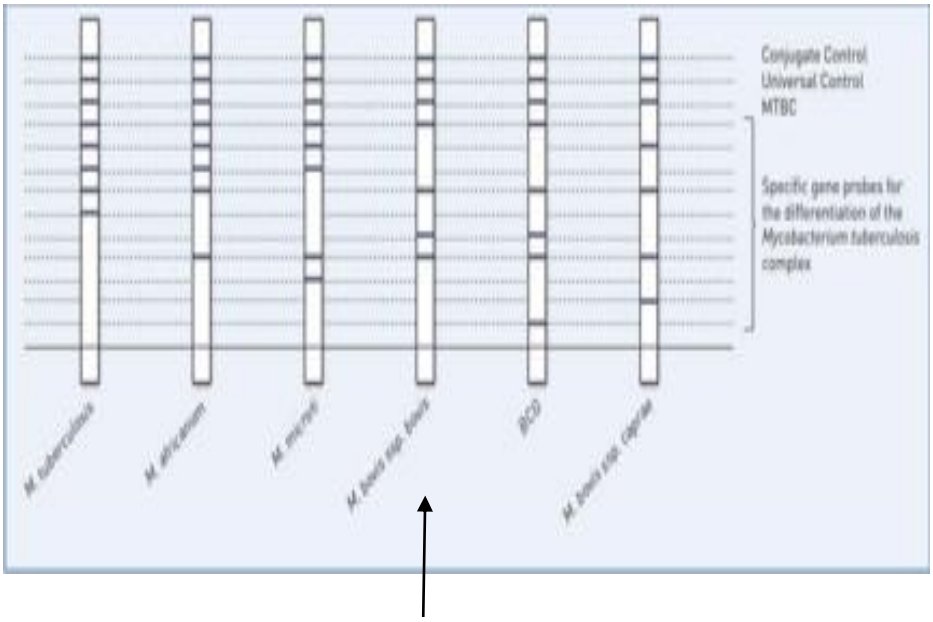


Figure 1.5 AFB Serpentine Cords stained with Zn (www.agricuture.gov.ie/Costello presentation).



Strip Number 4

Figure 1.6 The banding patterns obtained with the Genotype MTBC assay. Strip number 4 represents *M. bovis* subspecies *bovis*. This image is reproduced from www.hain-lifescience.de

1.6 Genotyping

Genotyping is the characterisation of an organism in terms of its DNA. Genomic differences can be used to identify different strains. Prior to the development of genotyping techniques there was no satisfactory method of differentiating strains of *M. bovis* as phenotypic methods such as serotyping were not informative. All bacteria are capable of undergoing mutations of DNA sequences. When essential enzymes or structural proteins are not affected by these mutations, the daughter generation should survive. If or when the typing technique detects the genetic change then a new clone is identified (Durr *et al.*, 2000b).

Genotyping has proved very valuable for investigating the epidemiology of tuberculosis both in humans and animals (Durr *et al.*, 2000a). When an infectious bacterium passes from one host to another the bacterium retains the same genetic make-up. If bacterial strains isolated from two individual hosts are found to have two different genotypes, it can be inferred that the infection has not spread from one host to the other although the two hosts may have been in close proximity to one another (Durr *et al.*, 2000b). When two different genotypes are found in the same host, it suggests that two independent infections have occurred. Recently individual badgers infected with more than one *M. bovis* strain have been reported (Furphy *et al*., 2012).

In Ireland, genotyping has shown that the same strains of *M. bovis* are common to isolates from cattle, badgers and deer within the same locality, providing strong evidence for inter-species transmission of infection. However, genotyping cannot indicate the direction of transmission (Collins *et al*., 1994; Costello *et al*., 1999; Olea-Popelka *et al*., 2005). It has also proved useful in the investigation of specific disease outbreaks, for example, VNTR typing showed that the same strains of *M. bovis* were

shared by cattle and alpacas in a TB outbreak in county Clare, (Connolly *et al*., 2008).

1.7 Genotyping Methods

These can be divided into two categories; whole genome methods and PCR based methods. Whole genome typing involves the digestion of whole genomic DNA using enzymes produced by bacteria that are known as restriction endonucleases. These restriction endonucleases cleave the DNA strands at highly specific nucleotide sequences, resulting in many small fragments of DNA, which can be separated by agarose gel electrophoresis. The fragments migrate within the gel due to an electrical current, the smallest fragment moving the furthest. There are three whole genome typing methods that have been used for the differentiation of *M. bovis* strains, namely; restriction endonuclease analysis (REA), restriction fragment length polymorphism analysis (RFLP) and pulsed field gel electrophoresis (PFGE). All of the whole genome methods have the disadvantage that relatively large quantities of DNA are required and the end result is a pattern of bands that makes analysis difficult and somewhat subjective, making inter laboratory comparison difficult. The PCR-based methods do not have these disadvantages as relatively small quantities of DNA are required and results can be expressed in a digital format (Durr *et al.*, 2000a). The most commonly used PCR-based typing methods are spoligotyping and variable number tandem repeat (VNTR) analysis.

1.7.1 Restriction Endonuclease Analysis (REA)

This was one of the first *M. bovis* typing techniques to be developed (Durr *et al.*, 2000a). It is the 'gold standard' method used in New Zealand and is a valuable part of their bovine tuberculosis control scheme (Collins, 2011). It is the easiest of the whole genomic methods to perform. The extracted DNA is digested with three restriction endonucleases *BstE*II, *Puv*II and *Bc*II as shown in **Figure 1.7**. These enzymes cleave the DNA strands at highly specific nucleotide sequences, resulting in numerous small DNA fragments, which are separated by gel electrophoresis. The gel is stained with ethidium bromide to show the patterns of fragments, which are then recorded photographically by transillumation with ultraviolet light (Collins *et al*., 1985; Durr *et al*., 2000a). REA gives the greatest discrimination between strains when the whole genome is used. However, the interpretation of the REA patterns isdifficult due to the complex fragment patterns (Cousins *et al*., 1993; Durr *et al*., 2000a). REA also does not allow for a numeration catalogue to be established for the different strain types. Therefore, comparison between laboratories is problematic. As a result, REA has not been widely used outside of New Zealand (Durr *et al*., 2000a).

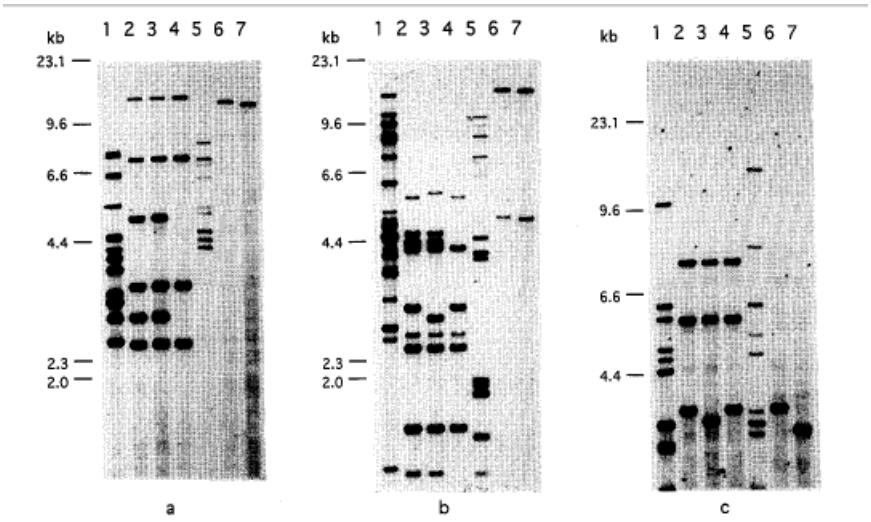


Figure 1.7 REA of reference strains of the *Mycobacterium tuberculosis* complex using a) *BstE*II, b) *Bc*II and c) *Pvul*I. This image is reproduced from Cousins *et al*., 1993.

1.7.2 Pulsed Field Gel Electrophoresis (PFGE)

The steps of PFGE are fundamentally the same as REA. However, the restriction endonuclease enzymes used cut the genome less frequently resulting in a smaller number of large DNA fragments. These larger fragments are too big to be separated by standard agarose gel electrophoresis, but can be separated by applying a constant pulsing electrical current. The banding pattern is easier to interpret compared to REA. However, the method is labour intensive, requiring meticulous technique to avoid breaking the large DNA fragments, and therefore, it is rarely used for typing *M. bovis (*Durr *et al.*, 2000a). Nevertheless, Feizabadi *et al.*, (1996) have demonstrated good discrimination between *M. bovis* strains as shown in **Figure 1.8.**

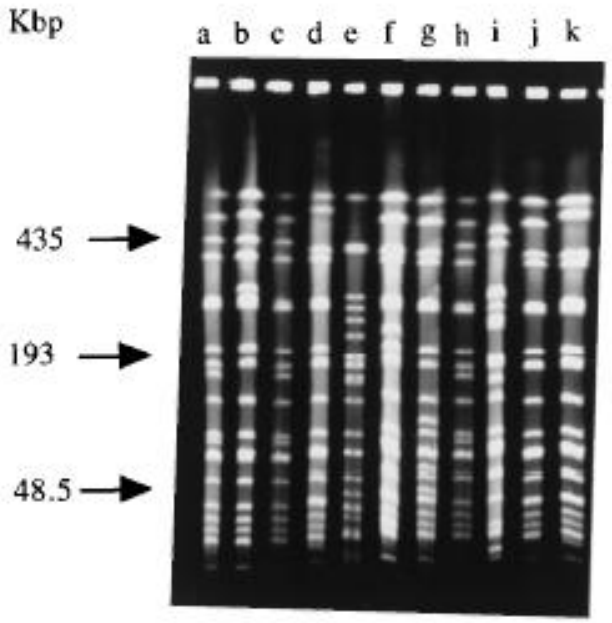


Figure 1.8 PFGE of *M. bovis* strains digested with *Dra*I. This image is reproduced from Feizabadi *et al*., 1996.

1.7.3 Restriction Fragment Length Polymorphism Analysis (RFLP)

RFLP involves the digestion of whole genomic DNA using either *Pvu*II or *Alu*I endonucleases. These two endonucleases cleave the DNA strands at highly specific nucleotide sequences, resulting in many small fragments of DNA, which are separated by agarose gel electrophoresis. This initial part of the process is essentially similar to REA. In order to produce a less complex banding pattern, a labelled DNA probe that will hybridise to specific repeat sequences or insertion sequences within the genome is used. Only those restriction fragments that contain the specific repeat sequence or insertion sequence will be visualised. After digestion by a restriction endonuclease the DNA is transferred onto a nitro-cellulose membrane, a process known as southern blotting. The labelled probe is then hybridised to the DNA fragments on the

nitro-cellulose membrane (Durr *et al.,* 2000a). A detection system is then applied to the membrane that interacts with the probe label, usually producing a luminescent reaction that can be photographed (**Figure 1.9)** (Durr *et al.,* 2000a). Preferably an RFLP method should detect between eight and 15 fragments of varying molecular weight so sufficient discrimination between bovine strains can be achieved and results can be compared between laboratories and databases can be compiled (Cousins *et al.,* 1993; Aranaz *et al.,* 1998; Roring *et al.,* 1998).

The most commonly used probes are those that are specific to the insertion sequence IS*6110,* the polymorphic guanine and cytosine rich repeat sequences (PGRS) and the direct repeat (DR) region. Frequently a combination of all three of these probes was used (Skuce *et al*., 1996; Costello *et al*., 1999). Another useful probe was pUCD which hybridised to three different tandemly repeated sequences (O'Brien *et al*, 2000a,b).

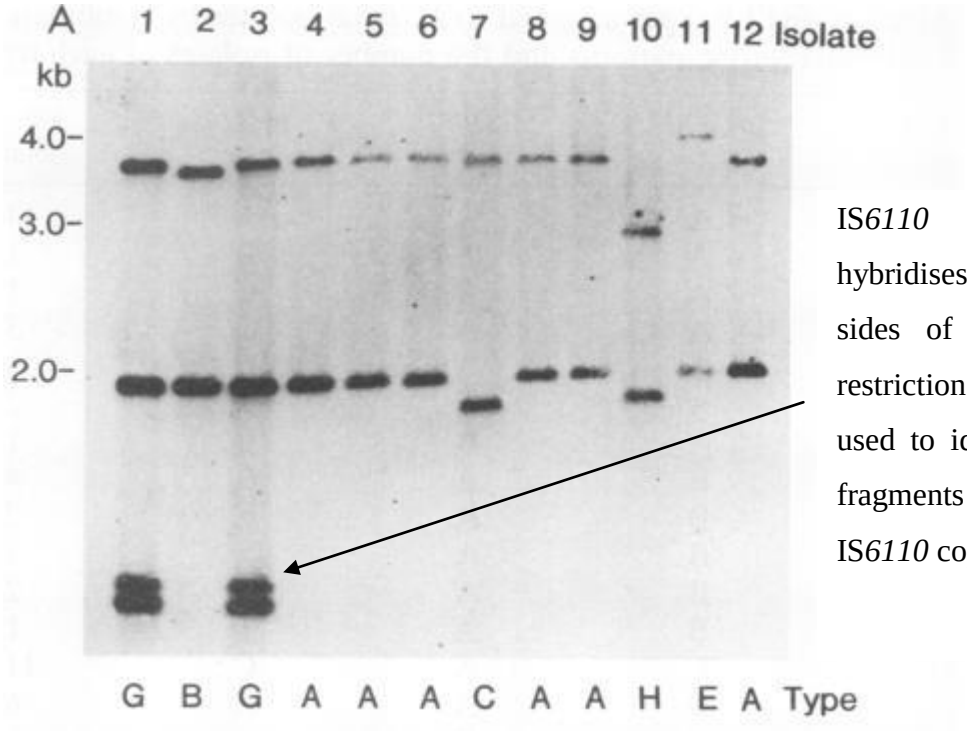


Figure 1.9 Photograph of *M. bovis* strains genotyped by RFLP-IS*6110* restricted with *Pvu*II and probed with ^{32}P-labelled IS*6110* (Skuce *et al*., 1994)

1.7.4 RFLP based on the Insertion Sequence IS*6110*

Insertion sequences (IS) are small mobile genetic elements that are widely distributed in most bacterial genomes (Mathema *et al*., 2006). IS*6110* is a member of the IS3 family of enterobacterial insertion sequence elements and is considered to be specific to the members of the *Mycobacterium tuberculosis* complex. (van Soolingen *et al*., 1992; Haddad *et al*., 2004). It is a 1,361 bp fragment and was previously isolated and known as IS*986* and IS*987* before these elements were recognised as being the same as IS*6110*. The difference in location and number of the IS*6110* copies is the source of polymorphism between Mycobacterial strains. There can be up to 20 copies of IS*6110* in *M. tuberculosis*, giving high levels of polymorphism within the tuberculosis genome.

Hence, RFLP-IS*6110* is an ideal tool for typing *Mycobacterium tuberculosis* complex and it is described as the "gold standard" technique for molecular epidemiology studies of human tuberculosis (van Soolingen *et al.,* 1994; Skuce *et al.,* 1996; Roring *et al.,* 1998;, Durr *et al.,* 2000a; Haddad *et al.,* 2004; Flores *et al.,* 2010),

Fewer copies of IS*6110* have been found in *M. bovis,* with the majority of bovine isolates only containing one copy (O'Brien *et al.,* 2000a; Skuce *et al.,* 1996; van Soolingen *et al*., 1992). However, the number of copies can depend to some extent on the type of species infected as multiple copies have been found in *M. bovis* isolates from zoo and wild animals (Aranaz *et al.,* 1998). The restriction endonuclease used for RFLP-IS*6110* is *Pvu*II and there is a *Pvu*II restriction site present in IS*6110*. Therefore, if a probe that hybridises to both sides of this restriction site is used it will identify two fragments for each IS*6110* copy as shown in **Figure 1.9**. This increases the discriminatory power of IS*6110* RFLP and it has been widely used for typing *M. bovis* isolates (Collins *et al*., 1993; Skuce, *et al*., 1996; Costello *et al*., 1999). It has been recommended that if strains contain three or more copies of the sequence then RFLP-IS*6110* should be the method of choice when typing isolates due to the good discrimination that is offered by this technique. However, when bovine isolates contain only one copy of IS*6110* then other supplementary typing methods should be used along with RFLP-IS*6110* so that good discrimination can be achieved (Cousins *et al.,* 1998; Durr *et al.,* 2000a; O'Brien *et al.,* 2000a; Flores *et al.,* 2010).

1.7.5 RFLP using Polymorphic Guanine and Cytosine Rich Repeat Sequences (PGRS-RFLP)

Polymorphic G-C rich sequences (PGRS) are short repeat sequences found throughout the *M. bovis* genome that have a G-C content of 80% or greater (Durr *et al.,* 2000a; Haddad *et al.,* 2004). These 24 bp repeats are separated by spacer sequences of variable lengths **(Figure 1.10)** and are scattered in at least 26 clusters around the genome (Durr *et al.,* 2000a; O'Brien *et al.,* 2000a; Skuce *et al.,* 1996). The fingerprint contains a large number of bands therefore to facilitate interpretation only the larger PGRS fragments (2 to 5 kb) are included in the analysis (O'Brien *et al.,* 2000a; Durr *et al.,* 2000a). Studies have shown that RFLP using PGRS probe has generally given greater differentiation of strains than RFLP with either IS*6110* or Direct Repeat (DR) probes (Costello *et al.,* 1999; O'Brien *et al.,* 2000a; Durr *et al.,* 2000a).

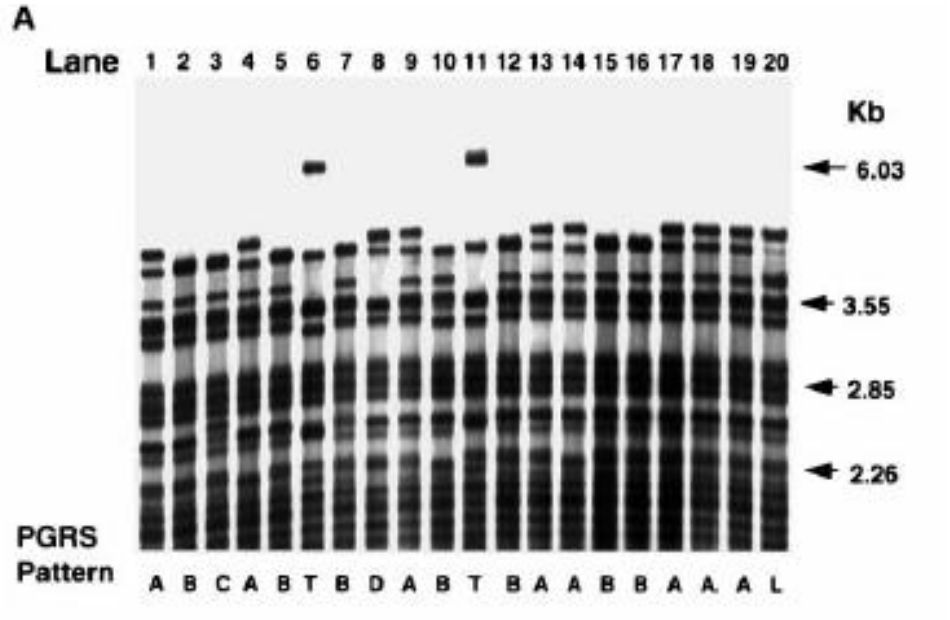


Figure 1.10 Photograph of *M. bovis* strains genotyped by PGRS-RFLP. This image is reproduced from Skuce *et al*., 1996.

1.7.6 Direct Repeat (DR)-RFLP

DR-RFLP is based on DNA polymorphism found at one specific chromosomal locus the "Direct Repeat" region. This region is unique to *M. tuberculosis* complex bacteria. (Kamerbeek *et al*., 1997; Durr *et al*., 2000a; Haddad *et al*., 2004; Spoligo Manual) The DR cluster consists of multiple 36 bp repetitive sequences that are interspersed with non-repetitive spacer sequences (spacers). The spacers range in size between from 35 bp to 41 bp in length. (Kamerbeek *et al*., 1997; Durr *et al*., 2000a; van Embden *et al*., 2000; Spoligotyping manual available online). Each DR sequence and the adjacent variable spacer sequence are termed a direct variable repeat (DVR) (van Embden *et al*., 2000; Haddad *et al*., 2004). The DR-RFLP probe targets the 36 bp DR sequence (O'Brien *et al*., 2000a; van Embden *et al*., 2000). The number of DVRs varies between

strains, thus generating the polymorphism detected by DR-RFLP (**Figure 1.11**)

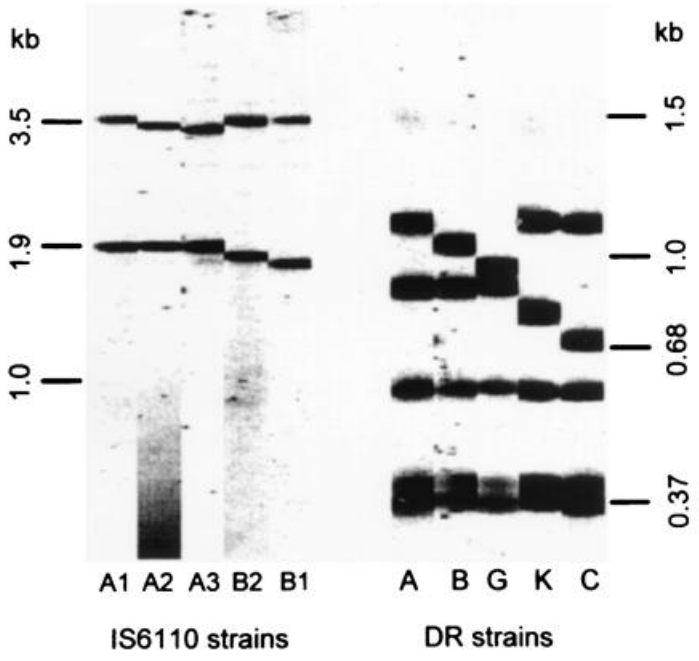


Figure 1.11 Photograph of *M. bovis* strains genotyped by RFLP-IS*6110* & RFLP-DR. This image is reproduced from Costello *et al*., 1999.

1.7.7 pUCD-RFLP

This method was developed after RFLP based on IS*6110*, PGRS and DR had been used routinely in laboratories (O'Brien *et al*., 2000a). It detected polymorphism in three different tandem repeat sequences located closely together on the *M. bovis* genome (O'Brien *et al*., 2000b). Polymorphism was generated by variation in the number of repeats in each of the tandem repeat sequences, this resulted in a step-like variation in the size of the *Alu*I cleaved fragments containing those repeat sequences. This produced simple patterns that were easier to interpret compared to other RFLP probes (**Figure 1.12**), nevertheless a high level of strain differentiation was achieved (O'Brien *et al*., 2000a,b).

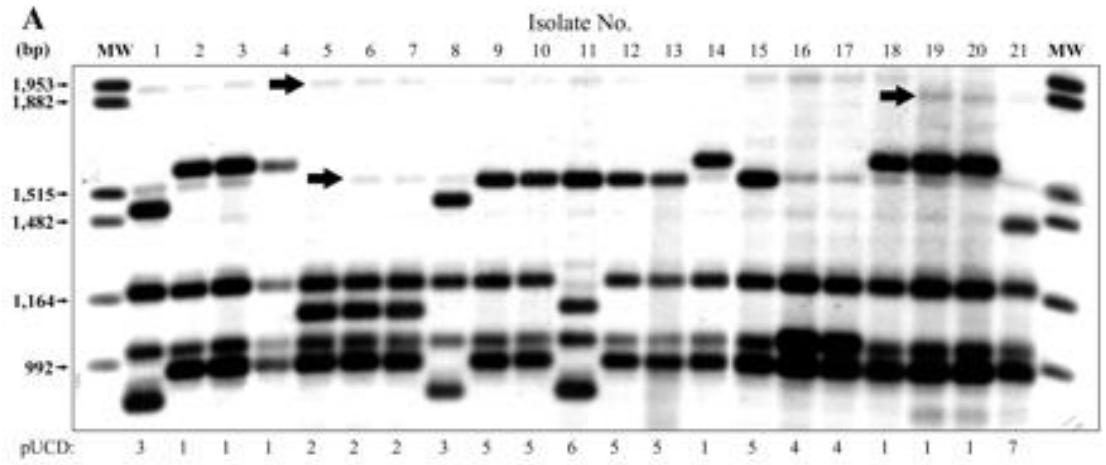


Figure 1.12 Photograph of *M. bovis* strain type A1 A1 A, subdivided by pUCD to a further seven strains. This image is reproduced from O'Brien *et al*., 2000a.

1.7.8 Spoligotyping

Similar to DR-RFLP, spoligotyping is based on DNA polymorphisms found at one specific chromosomal locus the "Direct Repeat" region. However spoligotyping is a PCR and hybridisation technique that has the advantage of being a significantly faster and less labour intensive method to execute than DR-RFLP. It is the most commonly used method for typing *M. tuberculosis* complex strains (Smith and Upton, 2012). An international database of the recorded spoligotypes of *M. bovis* and other RD9-deleted lineages has been compiled and is now freely available online (Smith and Upton, 2012).

The DR region may contain over 60 DVR units (direct repeat plus unique spacer sequence) which are arranged sequentially. The sequential order does not vary; polymorphism is due to the loss of individual DVRs or blocks of adjoining DVRs. A total of 43 of the spacer units were selected for the standard spoligotyping method which was developed at the Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands (Kamerbeek *et al*., 1997). The 43 spacer units are immobilised on a membrane in their naturally occurring sequential order. The presence or absence of spacer units in an isolate can be detected by hybridisation of PCR amplified spacer DNA to this membrane. The end result is a digital type pattern based on the presence or absence of specific spacer sequences, that is simple to interpret, as shown in the spoligoblot in **Figure 1.13** (SpoligotypingManual; Kamerbeek *et al.*, 1997; Durr *et al.*, 2000a; Haddad *et al.*, 2004). For *M. bovis* isolates the spacers 3,9,16 and 39-43 are always absent. (Kamerbeek *et al.*, 1997; Haddad *et al.*, 2004). The level of strain differentiation by spoligotyping is less compared to that of the RFLP-IS*6110* for strains that have five or more IS6110 copies present but for strains with five or less copies of IS*6110* the level of differentiation is higher than that of RFLP- IS*6110*

(Spoligotyping Manual; Kamerbeek *et al.*, 1997). In general, spoligotyping alone does not provide sufficient discrimination between *M. bovis* strains, therefore it cannot be used solely as a typing method, but can be combined with other techniques such as RFLP or VNTR. (Costello *et al*., 1999; Durr *et al*., 2000).

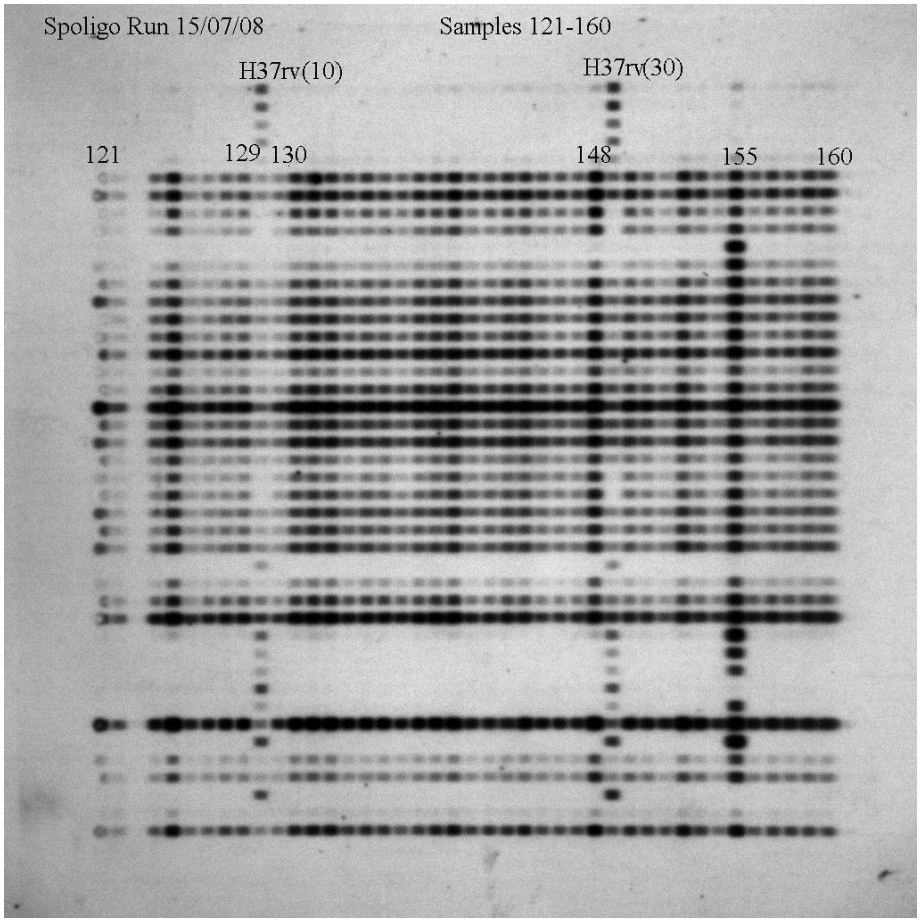


Figure 1.13 Spoligoblot of *M. bovis* strains genotyped by Spoligotyping.

1.7.9 Variable Number Tandem Repeat (VNTR) & Mycobacterial Interspersed Repetitive Units (MIRUs).

Tandem repeat sequences were first discovered in large quantities in eukaryotic genomes and have been gradually identified in prokaryotes. Several to thousands of copies of short tandem repeated sequences are distributed throughout the genome of many if not all higher eukaryotes (van Belkum *et al.*, 1998). It is these tandem repeat sequences that are used for human forensic and paternity testing (Frothingham & Meeker-O'Connell, 1998).

When the *M. bovis* genome was sequenced by the Sanger Institute in 2003, it enabled polymorphic regions within the genome to be identified. The majority of these polymorphisms occur in the regions of the tandem repeated DNA. Tandem repeat polymorphisms can consist of nucleotide sequence changes between individual repeat units or the variation in the number of repeat units. It is the variation in the number of repeat units that VNTR typing is based on (Roring *et al.*, 2002). Polymorphic regions with short sequence repeats of 1-3 bp are referred to as microsatellites, whereas polymorphic regions with 10-100 bp are referred to as minisatellites (Supply *et al.*, 2000; Haddad *et al.*, 2004).

In mycobacteria the majority of the VNTR correspond to the mycobacterial interspersed repetitive units (MIRUs). MIRUs are 40 -100 bp repetitive sequences dispersed in the intergenic regions of the *M. tuberculosis* complex genomes. MIRUs can be found in 41 locations within the *M. tuberculosis* H37Rv genome (Supply *et al.*, 2000; Haddad *et al.*, 2004). It is MIRU-VNTRhypervariablity that allows for the exploitation for typing *M. bovis* strains (Roring *et al.*, 2002; Supply *et al.*, 2000; Haddad *et al.*, 2004). Depending on the MIRU-VNTR, between two and eight alleles can be detected at each of the 12

loci analysed for the majority of *M. bovis* strains. This then produces high levels of polymorphisms. The level of polymorphism is supported by the fact that MIRU-VNTR typing is a multilocus system (Haddad *et al.*, 2004). MIRU-VNTR typing relies on PCR amplification of multiple loci. Primers that are specific for the flanking regions of each repeat locus are used, the sizes of the amplicon are determined and the numbers of target tandem repeat sequences are established. The sizing of the amplicon can be done by either gel electrophoresis or by using capillary arrays. The data is then expressed in a numerical format and can be easily compared between laboratories.

1.8 Aims

1.8.1 Overarching Aim

To evaluate MIRU-VNTR and spoligotyping for the genotyping *M. bovis* strains.

1.8.2 Individual Aims

- To identify MIRU-VNTR loci that gave good discrimination between *M. bovis* strains.
- To compare agarose gel electrophoresis and MegaBACE^{tm} 1000 detection methods.
- To compare the genotyping methods; RFLP, MIRU-VNTR and Spoligotyping for their ability to discriminate between *M. bovis* strains.

Chapter two

Materials & Methods

2.1 Media

2.1.1 7H9 Broth

The 7H9 broth (2.35 g) (Sigma, M0178) was weighed and mixed in distilled H_2O (450 ml). Tween 80 (0.5 ml) (Sigma, P1754), Pyruvate (2 g) (Sigma, P8574) and Middlebrook OADC enrichment (50 ml) (Becton, Dickinson 212240) was added. The broth was mixed thoroughly and autoclaved at 121°C for 15 minutes (min). After sterilisation the broth was dispensed into 3ml volumes in sterile bottles and stored at 4°C.

2.2 Mycobacterial Strains

2.2.1 Mycobacterial strains and Culture Procedure

Stored *M. bovis* strains (n=386) that had previously been typed by RFLP analysis (Costello *et al*., 1999, Costello *et al*., 2006) were used in this study. *M. bovis* strainsthat had been stored at –20^{o}C were thawed and cultured in 3 ml of Middlebrook 7H9 broth at 37°C for 7 days. Aliquots (0.5 ml) of the Middlebrook 7H9 broth were inoculated onto Stonebrinks and Löwenstein-Jensen medium (Media for Mycobacteria, Glamorgan, United Kingdom) containing pyruvate (prepared as solid slants in screw-cap tubes), incubated at 37°C and monitored on a weekly basis. Cultures suitable for DNA extraction were obtained for 386 *M. bovis* strains. The strains had been obtained from 243 badgers, 119 cattle and 24 deer during the years 1996 to 2002. The *M. bovis* strains were obtained from all areas of the Republic of Ireland. The majority (206) of the badger strains came from the four study areas described by Griffin *et al*., 2005. In

the four area study *M. bovis* was isolated from 450 of the culled 2,360 badgers and strain typed by RFLP. Three controls were used during this study, *M. bovis* reference strain, *M. tuberculosis* H37Rv reference strain (Public Health Agency, Porton Down, Salisbury) and a blank reaction without DNA template.

2.2.2 DNA Extraction of Mycobacterial Strains

Colonies were transferred from the slopes into microtubes containing 500 µl of phosphate buffered saline with Tween 20 (PBS-Tw) (P1379, Sigma Aldrich, Wicklow, Ireland). The microtubes were placed in a heating block at 100 °C for 15 min to heat lyse the cells.The microtubes were vortexed periodically to assist in the heat lysis of the cells. Microtubes were centrifuged at 6000 × *g* for 2 min. The supernatant was transferred into a clean, labelled 1.5 ml Eppendorf tube. DNA template was stored at -20°C.

2.3 Primer Selection

An initial panel of 20 primers , **Table 2.1** (Sigma Aldrich, Wicklow, Ireland) were selected to amplify the MIRU-VNTR loci of a range of strains (*n* = 60) (Frothingham & Meeker-O'Connell 1998, Roring *et al*., 2002, Skuce *et al*., 2002, Supply *et al*., 2006). The forward primer of the primer pair was labelled with a Fluorescent dye, **Table 2.1**, to facilitate with the detection of the amplified product by the MegaBACE^{TM} Instrument as described in section **2.9.**

The first 60 amplified strains were detected using both the MegaBACE^{TM} Instrument and agarose gel electrophoresis to compare the two detection methods and to reduce the

genomic loci panel, to use only those that showed the largest degree of polymorphism, **Table 2.2**. The first 60 strains were selected as they were the first 60 strains to grow and DNA to be extracted.

Table 2.1 Primer Sequences for MIRU-VNTR Typing

MIRU-VNTR Locus	MIRU-VNTR Length (bp)	Primer Pair with Label (5'-3')	Reference
QUB 11a	69	Hex CCC ATC CCG CTT AGC ACA TTC GTA TTC AGG GGG GAT CCG GGA	Skuce *e. al.* 2002
QUB 11b	69	Hex CGT AAG GGG GAT GCG GGA AAT AGG CGA AGT GAA TGG TGG CAT	Skuce *et al.* 2002
QUB 1895	57	Fam GGT GCA CGG CCT CGG CTC C AAG CCC CGC CGC CAA TCA A	Roring *et al.* 2002
QUB 3232	56	Fam CAG ACC CGG CGT CAT CAA C CCA AGG GCG GCA TTG TGT T	Roring *et al.* 2002
QUB 3336	59	Fam ATC CCC GCG GTA CCC ATC GCC AGC GGT GTC GAC TAT CC	Roring *et al.* 2002
QUB 26	111	Hex AAC GCT CAG CTG TCG GAT GGC CAG GTC CTT CCC GAT	Skuce *et al.* 2002
ETR A	75	Fam AAA TCG GTC CCA TCA CCT TCT TAT CGA AGC CTG GGG TGC CCG CGA TTT	Frothinham & Meeker 1998
ETR B	57	Hex GCG AAC ACC AGG ACA GCA TCA TG GGC ATG CCG GTG ATC GAG TGG	Frothinham & Meeker 1998
ETR C	58	Hex GTG AGT CGC TGC AGA ACC TGC AG GGC GTC TTG ACC TCC ACG AGT G	Frothinham & Meeker 1998
ETR D	77	Fam CAG GTC ACA ACG AGA GGA AGA GC GCG GAT CGG CCA GCG ACT CCT C	Skuce *et al.* 2002
ETR E	53	Fam CTT CGG CGT CGA AGA GAG CCT C CGG AAC GCT GGT CAC CAC CTA AG	Frothinham & Meeker 1998
ETR F	79	Hex CTC GGT GAT GGT CCG GCC GGT CAC GGA AGT GCT CGA CAA CGC CAT GCC	Frothinham & Meeker 1998
MIRU 2	53	Fam TGG ACT TGC AGC AAT GGA CCA ACT TAC TCG GAC GCC GGC TCA AAA T	Supply *et al.* 2006
MIRU 10	53	Hex GTT CTT GAC CAA CTG CAG TCG TCC GCC ACC TTG GTG ATC AGC TAC CT	Supply *et al.* 2006
MIRU 16	53	Hex TCG GTG ATC GGG TCC AGT CCA AGT A CCC GTC GTG CAG CCC TGG TAC	Supply *et al.* 2006
MIRU 20	77	Fam TCG GAG AGA TGC CCT TCG AGT TAG GGA GAC CGC GAC CAG GTA CTT GTA	Supply *et al.* 2006
MIRU 24	54	Tamra CGA CCA AGA TGT GCA GGA ATA CAT GGG CGA GTT GAG CTC ACA GAA	Supply *et al.* 2006
MIRU 26	51	Hex TAG GTC TAC CGT CGA AAT CTG TGA AC CAT AGG CGA CCA GGC GAA TAG	Supply *et al.* 2006
MIRU 39	53	Fam CGC ATC GAC AAA CTG GAG CCA AAC CGG AAA CGT CTA CGC CCC ACA CAT	Supply *et al.* 2006
MIRU 40	54	Fam GGG TTG CTG GAT GAC AAC GTG T GGG TGA TCT CGG CGA AAT CAG ATA	Supply *et al.* 2006

[a] The forward primer of the primer pair was labelled with a Fluorescent dye to facilitate with the detection of the amplified product .

Table 2.2 Reduced panel of Primer Sequences for MIRU-VNTR Typing

MIRU-VNTR Locus	Alias	Primer Pair with Label (5'-3')[a]	Reference
2163a	QUB 11a	Hex CCC ATC CCG CTT AGC ACA TTC GTA TTC AGG GGG GAT CCG GGA	Skuce *et. al.* 2002
2163b	QUB 11b	Hex CGT AAG GGG GAT GCG GGA AAT AGG CGA AGT GAA TGG TGG CAT	Skuce *et. al.* 2002
1895	QUB 1895	Fam GGT GCA CGG CCT CGG CTC C AAG CCC CGC CGC CAA TCA A	Roring *et. al.* 2002
4052	QUB 26	Hex AAC GCT CAG CTG TCG GAT GGC CAG GTC CTT CCC GAT	Skuce *et. al.* 2002
2165	ETR A	Fam AAA TCG GTC CCA TCA CCT TCT TAT CGA AGC CTG GGG TGC CCG CGA TTT	Frothinham & Meeker 1998
2996	MIRU 26	Hex TAG GTC TAC CGT CGA AAT CTG TGA AC CAT AGG CGA CCA GGC GAA TAG	Skuce *et. al.* 2002

[a] The forward primer of the primer pair was labelled with a Fluorescent dye to facilitate with the detection of the amplified product .

2.4 PCR Amplification using Hotstar *Taq* Polymerase

2.4.1 PCR Amplification using 50 µl reaction volumes

All 20 genomic loci were amplified in separate PCR reactions with primers described in **Table 2.1**. Reaction volumes were 50 µl containing 5 µl of 10X PCR buffer (Qiagen West Sussex, UK, 203203), 0.4 µl of 50 pmol primer set, 4 µl (100 µM) of the four deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP (Promega U1240, Medical Supply Company, Dublin, Ireland), 10 µl of Q solution (Qiagen), 0.25 µl of Hotstar *Taq* (1 unit) (Qiagen) and 20.35 µl of pure H_20. Template DNA (10 µl) was added to each PCR reaction mix including *M. bovis* control, *M. tuberculosis* H37Rv control and a negative non-template control. Amplification was performed in a

Flexigene thermal cycler with an initial activation step of 95°C for 15 min followed by 40 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 2 min. The final extension was 72°C for 10 min.

2.4.2 PCR Amplification using 25 μl reaction volumes

Reaction volumes were reduced to 25 μl to save on reagents as 3 μl of amplified PCR product is only required for the 1:50 dilution for the MegaBACE^{tm} 1000 detection The 25 μl reaction volumes contained 2.5 μl of 10X PCR buffer (Qiagen), 0.2 μl of 50 pmol primer set, 2 μl (100 μM) of the four deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP, 5 μl of Q solution, 0.125 μl of Hotstar *Taq* (1 unit) and 10.175 μl of pure H_20. Template DNA (5 μl) was added to each PCR reaction mix including *M. bovis* control, *M. tuberculosis* H37Rv control and a negative non-template control. Amplification was performed as described in **2.4.1**

The PCR product was stored light protected at 4°C until ready to analyse.

2.5 Gen Elute PCR Clean-up

Gen Elute PCR clean-up kit (NA1020, Sigma) purifies single-stranded or double-stranded PCR amplification products from other components in the reaction mix. Gen Elute was performed on the first 60 amplified *M. bovis* strains to remove the excess salt from the PCR product as this interferes with the capillary electrophoresis on the MegaBACE^{TM} 1000. The Gen Elute was performed as per manufacturer's instructions. Briefly, the PCR product was combined with an ethanol based binding solution. PCR product/binding solution was washed through the Gen Elute spin column. An elution

solution was added to extract the DNA from the spin column. DNA was transferred into a clean-labelled eppendorf tube. DNA template was stored light protected at -18°C until ready to run on the MegaBACE^{TM} 1000.

2.6 1:50 Dilution of the Amplified PCR Product

Amplified PCR product (3 µl) were mixed with distilled, DNase/RNase free H_2O (147 µl). The 1:50 dilution was stored at -18°C until ready for MegaBACE^{TM} detection.

2.7 Gen Elute vs. 1:50 Dilution

Gen Elute PCR clean up and 1:50 dilution was performed on the first 60 amplified *M. bovis* strains. The cleaned & diluted strains were then detected using the MegaBACE^{TM} 1000. Results were compared to determine if any differences in repeat sizes were detected between the Gen Elute PCR clean up and 1:50 dilution.

2.8 Agarose Gel Electrophoresis Detection

The first 60 amplified *M. bovis* strains were electrophoresed on 1.5% (w/v) 400 mls agarose gels (Sigma, A9414) with 20 µl ethidium bromide (10mg/ml) with a final concentration of 0.5µg/ml (Sigma, E1510) in 1X Tris Borate-EDTA buffer (Sigma, T9650-4L) at 80 volts (v) for 2 hours. A 100 bp DNA ladder (Biolabs, N3236L, Ipswich, UK) was included on each gel. Product sizes were visualised and photographed by using an ultraviolet (UV) transilluminator. Product sizes were

established by comparing the bands with the DNA ladder bands. The number of repeats was determined by comparing the product sizes to an allele sizing table, **Table 2.3**.

Table 2.3 Allele Sizing Table

LOCUS	REPEAT	PARTIAL REPEAT	bp FLANKING REGION	0'	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ETR-A(VNTR 2165)	75	23	195	172	195	270	345	420	495	570	645	720	795	870	945	1020	1095	1170	1245	1320
VNTR 1895	57	0	86	86	86	143	200	257	314	371	428	485	542	599	656	713	770	827	884	841
MIRU26(VNTR 2996)	51	8	285	277	285	336	387	438	489	540	591	642	693	744	795	846	897	948	999	1050
QUB11a(VNTR 2163a)	69	15	167	152	167	236	305	374	443	512	581	650	719	788	857	926	995	1064	1133	1202
QUB11b(VNTR 2163b)	69	9	67	58	67	136	205	274	343	412	481	550	619	688	757	826	895	964	1033	1102
QUB26(VNTR 4052)	111	24	153	129	153	264	375	486	597	708	819	930	1041	1152	1263	1374	1485	1596	1707	1818

0'=flanking region without partial repeat

0=flanking region with partial repeat

2.9 MegaBACE™ 1000

2.9.1 MegaBACE™ 1000 Detection

The MegaBACE™ 1000 (GE Healthcare Life Sciences, UK) DNA analysis system is a high-throughput, fluorescence based DNA system utilizing capillary electrophoresis. The forward primer of the primer pair was labelled with a Fluorescent dye (**Table 2.1)**, to facilitate the detection of the amplified product. The 1:50 dilution of the PCR products (*n* = 386) were separated on the 96-capillary MegaBACE ™ 1000 Sequencer using Rox-labelled MegaBACE ET900-R as a size standard. The electrophoresis was run for 120 min using MegaBACE matrix with an injection voltage of 3 kV for 45 s and a running voltage of 10kV. Each peak was identified according to colour and size and assigned to a distinct allele number.

2.9.2 Analysis of Data

Raw data from notepad was converted into an excel format for easier analysis of the data. The product size displaying the highest peak for each labelled PCR product was the size selected for that particular MIRU-VNTR locus. The number of repeats for that MIRU-VNTR locus was determined by comparing the product sizes to an allele sizing table, **Table 2.3**.

2.10 Spoligotyping

Spoligotyping was performed according to the method described by Kamerbeek *et al*. except that a digoxigenin labelling and detection system (Roche Diagnostics, West Sussex, UK) was used. Spoligotype patterns were given the names assigned in the *M. bovis* spoligotyping database on http://www.mbovis.org **Figure 3.4 & Table 3.3.**

2.11 Statistical analysis

Calculation of the discriminatory power of each typing method was based on Simpsons index of diversity as described by Hunter and Gaston (Hunter and Gaston, 1988). This value is commonly referred to as the Hunter Gaston discriminatory index (HGDI). The Equation for HDGI is shown in **Figure 2.1.** Wallace's coefficient was used to quantify the level of concordance between typing methods (Carriço *et al*., 2006). This calculates the degree to which one typing method can predict the result of another typing method. A high value of Wallace's coefficient suggests the use of both methods is redundant. Wallace's coefficient was calculated using the web tool http://www.comparingpartitions.info. The allelic diversity at the different VNTR loci was calculated using the formula $h = 1\sum x_i^2[n/(n-1)]$ where x_i is the frequency of the *i*th allele at the locus, *n* is the number of isolates or ETs in the sample and $n/(n-1)$ is a correction for bias in small samples (Selander *et al*., 1986).

Figure 2.1 The formula used to calculate the Hunter Gaston discriminatory index (HGDI)

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

N is the total number of strains in the sample population, S the total number of types described, n_j is the number of strains belonging to the *j*th type.

2.12 Quality Control and Safety

As *M. bovis* is a Hazard Group (HG) three organism, all *M. bovis* strains were manipulated in a class I biosafety cabinet in a level 3 containment laboratory under negative air pressure.

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Chapter three

Results

3.1 Evaluation of VNTR methods and the selection of suitable loci

3.1.1 Assessment of the level of strain resolution achieved by the VNTR loci

An initial analysis was performed on a panel of 60 *M. bovis* strains using 20 VNTR loci in order to identify the loci that demonstrated the largest degree of discrimination between *M. bovis* strains. The chosen loci were then used to test the remaining 326 isolates. Analysis of the results showed that MIRU's 2, 10, 20, 24, 39 & 40 as well as ETR C and ETR E displayed no discrimination between strains as they had only one fragment size for all 60 *M. bovis* strains. ETR's B, D & F, MIRU 16, QUB 3232 and QUB 3336 displayed some degree of discrimination. However, six loci (QUB 11a, QUB 11b, 1895, 4052, ETR A and MIRU 26) displayed the highest degree of discrimination as shown in **Table 3.1** and were selected to strain type the remaining 326 *M. bovis* strains.

3.1.2 Comparison of agarose gel electrophoresis and MegaBACE^{TM} 1000.

VNTR analyse was performed on the first 60 amplified *M. bovis* strains and were detected using both the MegaBACE^{TM} Instrument and agarose gel electrophoresis, for each of the 20 VNTR loci examined. The concordance between both detection methods was assessed for each locus and the results are displayed in **Table 3.2**. The results show that there was a high degree of concordance (>93%) between both methods for the selected six VNTR loci. There was a low level of concordance for locus QUB 3232 (0%) and locus QUB 3336 having a higher concordance of 68%. However, these two loci did not display high levels of discrimination between *M. bovis* strains and were not

included in the loci used to amplifiy the remaining 326 *M. bovis* strains. Examples of agarose gels are shown in **Figures 3.1**, **3.2.** and **3.3.**

Table 3.1 The MIRU-VNTR profiles of the first 60 strains examined with 10 VNTR loci that displayed varying levels of strain discrimination.

	Spoligotype	RFLP	QUB 11a*	QUB11b*	ETR A*	MIRU 26*	4052*	1895*	3336	ETR B	MIRU 40	MIRU 20
1	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
2	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
3	SB0140	A1A5A	10	4	6	5	4	4	4	5	8	7
4	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
5	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
6	SB0140	A1A1A	11	4	7	5	2	4	4	5	8	7
7	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
8	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
9	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
10	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
11	SB0143	B1C1C	11	4	7	6	4	3	4	5	8	7
12	SB0144	B1C1C	11	4	7	6	4	3	4	5	8	7
13	SB0145	B1C1C	11	4	7	6	4	3	4	5	8	7
14	SB0146	B1C1C	11	4	7	6	4	3	4	5	8	7
15	SB0147	B1C1C	11	4	7	6	4	3	4	5	8	7
16	SB0993	B2A3Y	3	3	5	5	4	3	4	5	8	7
17	SB0994	B2A3J	10	3	5	5	4	3	4	5	8	7
18	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
19	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
20	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
21	SB0130	G1H5P	9	3	6	5	4	4	9	5	8	7
22	SB0130	G1H5P	9	3	6	5	4	4	9	5	8	7
23	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
24	SB0130	C1H1J	11	3	7	3	3	2	7	5	8	7
25	SB0130	C1H1J	11	3	7	4	3	2	7	5	8	7
26	SB0269	A1L2D	11	2	6	5	4	4	7	5	8	7
27	SB0130	C1H7J	11	3	7	3	3	2	7	5	8	7
28	SB0140	A2A5T	11	4	7	5	2	4	4	5	8	7
29	SB0140	A1A8A	11	4	7	5	2	4	4	7	8	7
30	SB0140	A2A2B	11	4	7	5	4	4	4	5	8	7
31	SB0140	A1E2A	10	3	6	5	4	4	4	5	8	7

Table 3.1 continued;

	Spoligotype	RFLP	QUB 11a*	QUB 11b*	ETR A*	MIRU 26*	4052*	1895*	3336	ETR B	MIRU 40	MIRU 20
32	SB0995	D2C1L	11	4	7	6	4	3	4	5	8	7
33	SB0140	A1T2A	11	4	7	5	2	4	4	5	8	7
34	SB0142	B1C2C	11	4	7	6	4	3	4	5	8	7
35	SB0142	B1C1R	11	4	7	6	4	3	4	5	8	7
36	SB0140	A1T3A	10	4	6	5	4	4	4	5	8	7
37	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
38	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
39	SB0130	G1H5P	9	3	6	5	4	4	9	5	8	7
40	SB0146	D1H3I	11	3	6	3	3	2	7	5	8	7
41	SB0140	A1E2A	10	4	6	5	4	4	4	5	8	7
42	SB0273	A1A1F	9	3	6	5	4	4	9	5	8	7
43	SB0130	N3H11J2	11	3	7	3	3	2	7	5	8	7
44	SB0140	A1A5A	10	4	6	5	4	4	4	5	8	7
45	SB0140	F1A1C4	10	4	6	5	4	4	4	5	8	7
46	SB0140	A1B5A	11	4	9	2	4	4	9	5	8	7
47	SB0140	A1A1A	11	4	7	5	2	4	4	5	8	7
48	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
49	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
50	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
51	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
52	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
53	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
54	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
55	SB0142	B1C1C	11	4	7	6	4	3	4	5	8	7
56	SB0273	A1A1F	10	4	6	5	4	4	4	5	8	7
57	SB0140	A1E2A	10	3	6	5	4	4	4	5	8	7
58	SB0146	D1H3I	11	3	6	3	3	2	7	5	8	7
59	SB0140	T1A5A	10	4	6	5	4	4	4	5	8	7
60	SB0141	A1A8A	11	4	7	5	2	4	4	7	8	7

* These six VNTR Loci displayed the highest degree of discrimination.
3336, ETR B and E displayed some degree of discrimination.
MIRU 20 and 40 displayed no discrimination.

Table 3.2 The degree of concordance between the 20 different MIRU-VNTR loci using agarose gel electrophoresis and MegaBACE^{TM} detection

MIRU-VNTR Locus	Degree of Concordance
QUB 11a*	95%
QUB 11b*	100%
QUB 1895*	100%
QUB 3232	0%
QUB 3336	68%
4052*	93%
ETR A*	98%
ETR B	95%
ETR C	100%
ETR D	95%
ETR E	100%
ETR F	98%
MIRU 2	100%
MIRU 10	95%
MIRU 16	91%
MIRU 20	86%
MIRU 24	96%
MIRU 26*	96%
MIRU 39	98%
MIRU 40	96%

* These six MIRU-VNTR Loci displayed the highest degree of discrimination.

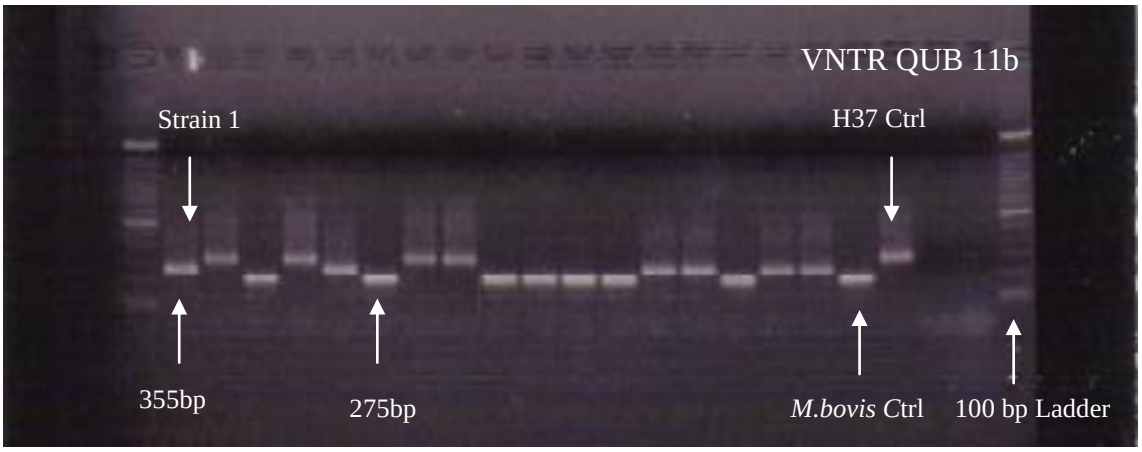


Figure 3.1 Agarose gel electrophoresis for strains 1-17, *M. bovis* Ctrl and *M. tuberculosis* H37Rv amplified with VNTR locus QUB11b.

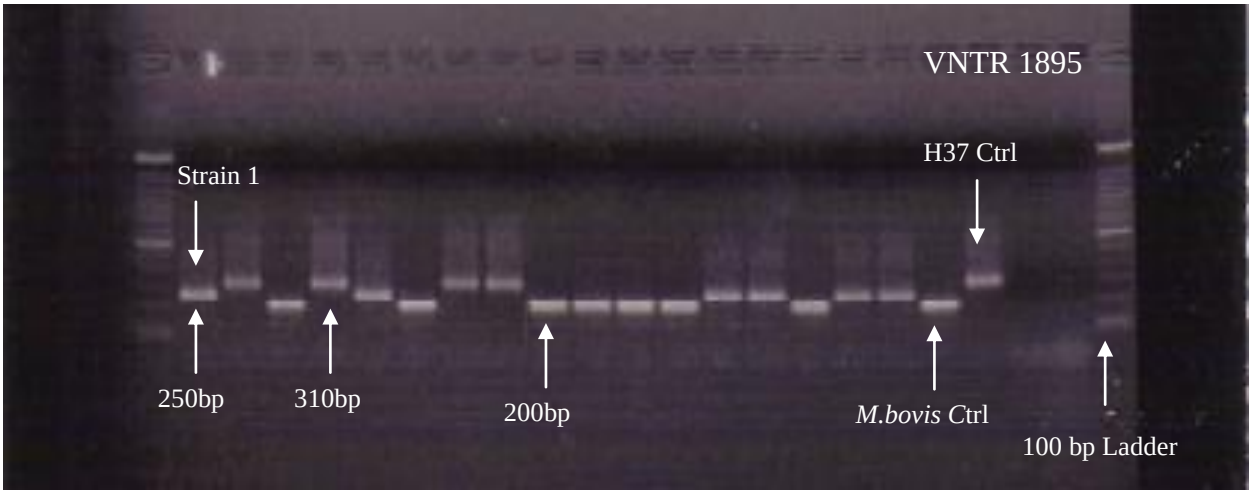


Figure 3.2 Agarose gel electrophoresis for strains 1-17, *M. bovis* Ctrl and *M. tuberculosis* H37Rv amplified with VNTR locus 1895

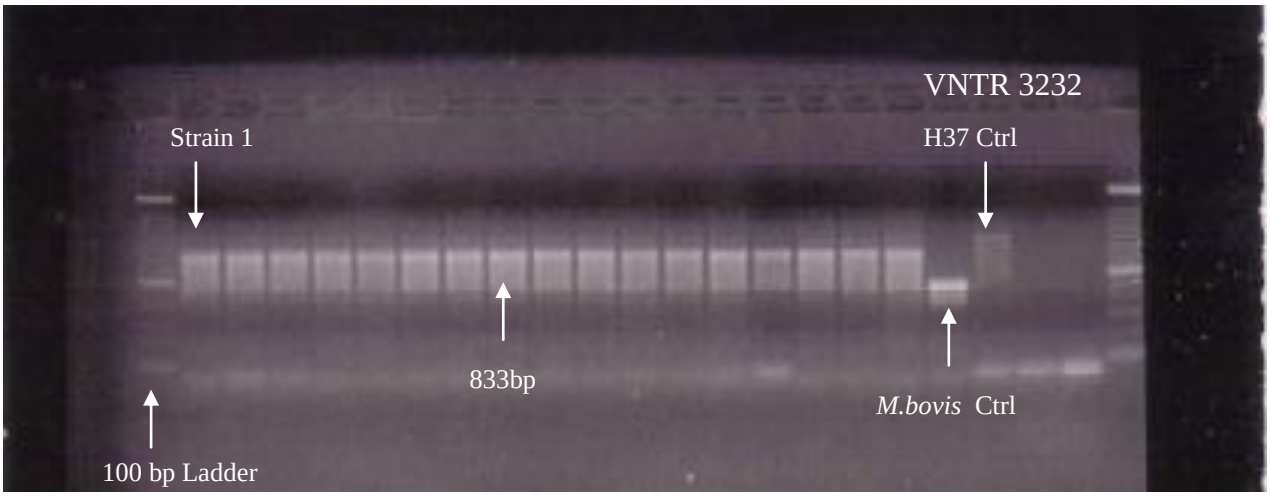


Figure 3.3 Agarose gel electrophoresis for strains 1-17, *M. bovis* Ctrl and *M. tuberculosis* H37Rv amplified with VNTR locus 3232

3.1.3 Comparison of Gen Elute PCR clean-up and 1:50 dilution of PCR product.

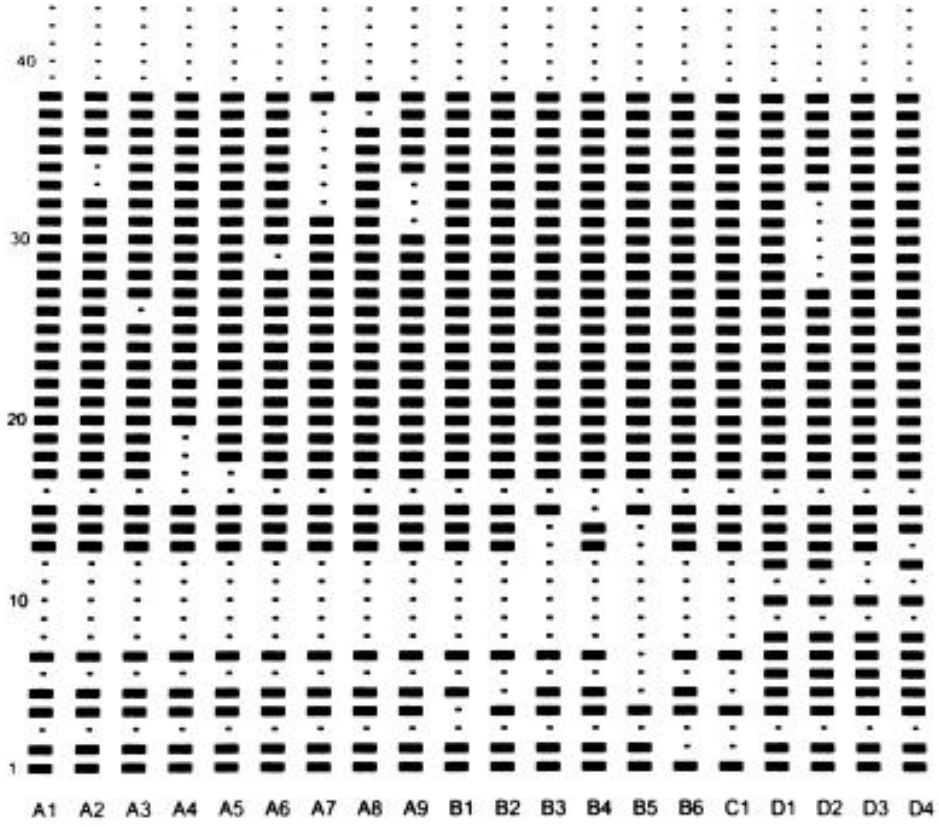
The Sigma Gen Elute PCR clean-up and the 1:50 dilution was performed on the 60 amplified isolates. The two PCR clean-up methods were compared to determine which method gave the most accurate sizing of PCR product result on the MegaBACE^{TM} Instrument for each VNTR loci. The two methods produced the same sizing results for the VNTR loci tested. Therefore the 1:50 dilution of the PCR product was used for the remaining 326 isolates for MegaBACE^{TM} Instrument detection as this method is less expensive and less time consuming than the Sigma Gen Elute PCR clean up kit.

3.1.4 Spoligotyping Results

Spoligotyping of the 386 isolates was performed using the method described by Kamerbeek *et al.*, 1997. However, the detection element used a digoxigenin labelling and detection system. Spoligotype is determined by the presence or absence of the

spacer units in the DR region of the isolate as shown in **Figure 3.4**. The most common Spoligotype was found to be SB0140. SB0140 represented 50% (n = 194) of the 386 isolates examined. The second most common spoligotype is SB0130 with this spoligotype representing 17% (n=64) of the isolates tested (**Table 3.3**).

Figure 3.4 Schematic representation of 20 spoligotype patterns identified among 386 *M. bovis* strains. The 43 spacer oligonucleotides are numbered as described by Kamerbeek *et al*., 1997.



Spoligotype pattern names are specific to the CVRL

Table 3.3 The International Spoligotyping Name for the 20 CVRL spoligotype patterns and the most common spoligotpyes present in the 386 *M. bovis* strains analysed.

CVRL Spoligotype Name	International Spoligotype Name	Number of Strains	%	Spoligotype Pattern
A1	SB0140	194	50.26	11011010000011101111111111111111111111100000
A2	SB0142	49	12.69	11011010000011101111111111111111100111100000
A3	SB0993	5	1.30	11011010000011101111111111111111100111100000
A4	SB0144	10	2.59	11011010000011100001111111111111100111100000
A5	SB0298	0	0	11011010000011100111111111111111111111100000
A6	SB0978	0	0	11011010000011101111111111111011111111100000
A7	SB0995	4	1.04	11011010000011101111111111111111000000100000
A8	SB0273	20	5.18	11011010000011101111111111111111111110100000
A9	SB0668	0	0	11011010000011101111111111111110001111100000
B1	SB0054	7	1.81	11001010000011101111111111111111111111100000
B2	SB0141	5	1.30	11010010000011101111111111111111111111100000
B3	SB0486	7	1.81	11011010000000101111111111111111111111100000
B4	SB0269	3	0.78	11011010000011001111111111111111111111100000
B5	SB0145	6	1.55	11010000000000101111111111111111111111100000
B6	SB0996	0	0	10011010000011101111111111111111111111100000
C1	SB0997	0	0	10010010000011101111111111111111111111100000
D1	SB0130	64	17	11011111010111101111111111111111111111100000
D2	SB0146	5	1.30	11011111010111101111111111000001111111100000
D3	SB0129	4	1.04	11011111010011101111111111111111111111100000
D4	SB0998	3	0.78	11011111010101101111111111111111111111100000

3.2 Comparison of VNTR analysis, RFLP analysis and Spoligotyping for strain differentiation of 386 *M. bovis* isolates

3.2.1 Resolution of strains

All strains used in this study had previously been typed by RFLP analysis (Costello *et al*., 1999, Costello *et al*., 2006). MIRU-VNTR and spoligotyping was performed on all 386 *M. bovis* strains as described in Chapter 2. Calculation of the discriminatory power of each typing method was based on Simpsons index of diversity as described by Hunter and Gaston (Hunter and Gaston, 1988). This value is commonly referred to as the Hunter Gaston discriminatory index (HGDI). RFLP, with a HGDI value of 0.927 was more discriminating than MIRU-VNTR, which had a HGDI value of 0.918, while spoligotyping was the least discriminatory of the three methods (**Table 3.4**). There were 65 RFLP profiles that were divided into 33 clusters and 32 unique isolates compared to 41 VNTR profiles comprising 26 clusters and 15 unique isolates. The largest RFLP cluster contained 58 isolates, while the largest MIRU-VNTR cluster contained 65 isolates. Spoligotyping identified 14 clusters and 1 unique isolate, the largest cluster, represented by spoligotype pattern SB0140, contained approximately 50% (194/386) of the isolates. Spoligotyping with RFLP and VNTR showed the greatest degree of discrimination with a HGDI value of 0.958 Spoligotyping with 4 MIRU-VNTR (0.93) was slightly less discriminating than spoligotyping with RFLP and VNTR with a HGDI value of 0.958. Spoligotyping was then performed to further resolve eight of the 26 MIRU-VNTR clusters, with the largest MIRU-VNTR 11 3 7 3 3 2 cluster (n=65) being subdivided into 3 different spoligotypes (**Table 3.5**). RFLP clusters were not resolved to the same extent by spoligotyping with only three being further subdivided. Spoligtyping can subdivide some MIRU-VNTR clusters and, therefore, is a useful addition to VNTR

typing. The allelic diversity of the VNTR loci ranged from 0.44 for VNTR QUB 11a to 0.57 for VNTR 1895 (**Table 3.6**).

Table 3.4 Comparison of the discriminatory power of various genotyping protocols

Procedure	HGDI[a]	Profiles (n=)	Clusters (n=)	Unique strains (n=)	Largest group (n=)
RFLP	0.927	65	33	32	58
VNTR	0.918	41	26	15	65
Spoligotyping	0.7	15	14	1	194
Spoligotyping + RFLP	0.929	68	35	33	58
Spoligotyping + VNTR (6 loci)	0.933	54	36	18	61
Spoligotyping + VNTR (5 loci)[b]	0.93	51	34	17	63
Spololigotyping + VNTR (4 loci)[c]	0.93	49	34	15	63
Spololigotyping + RFLP + VNTR	0.958	104	51	53	55

[a]Hunter Gaston Discriminatory Index.

[b]VNTR loci QUB 11a, QUB 11b, ETR A, 4052 and 1895

[c]VNTR loci QUB 11a, QUB 11b, ETR A and 4052

(n=) The number of profiles, clusters and the number of strains

Table 3.5 Resolution of the eight VNTR clusters by Spoligotyping

VNTR profile[a]	Spoligotype	Number of strains	Total Number of strains
10 3 5 5 4 3	SB0140	20	23
	SB0993	3	
10 4 6 5 4 4	SB0140	19	29
	SB0273	10	
11 2 6 5 4 4	SB0140	2	3
	SB0269	1	
11 3 7 5 4 4	SB0140	6	20
	SB0144	12	
	SB0486	2	
11 4 5 5 4 4	SB0140	2	8
	SB0054	6	
11 4 7 5 3 4	SB0140	14	31
	SB0141	7	
	SB0486	5	
	SB0145	5	
11 4 7 6 4 3	SB0140	1	49
	SB0142	46	
	SB0995	2	
11 3 7 3 3 2	SB0120	61	65
	SB0146	3	
	SB0998	1	

[a] The VNTR loci are listed in the order QUB 11a, QUB 11b, ETR A, MIRU 26, 4052 and 1895.

Table 3.6 Allelic diversity of six MIRU-VNTR loci

Locus	Allelic diversity	Number of strains with VNTR allele										
		1	2	3	4	5	6	7	8	9	10	11
QUB 11a	0.44			7			8			13	82	276
QUB 11b	0.48	5	5	130	245	1						
ETR A	0.45		1	4	1	48	54	277		1		
MIRU 26	0.49		1	68	2	260	55					
4052	0.55		45	110	231							
1895	0.57		70	89	227							

3.2.2 Typing system concordance

The level of concordance between the typing systems varied according to spoligotype. Wallace's coefficient (Carriço *et al.*, 2006) which is a measure of the degree to which one typing method can predict the result of another typing method, was used to quantify the level of concordance between typing methods (**Table 3.7**). Strains bearing spoligotype SB0140 showed highly variable RFLP and VNTR profiles and a low level of concordance (0.25) between these two typing methods. The highest level of concordance (0.89) was found in the strains that did not have the SB0140 spoligotype pattern. When all isolates were examined and RFLP was the Reference typing system the Wallace coefficient had a value of 0.56 to predict the VNTR result, the Secondary typing system. However, when the MIRU-VNTR method was the Reference typing system the Wallace coefficient had a value of 0.49 to predict the RFLP result, the Secondary typing system. A high Wallace coefficient value suggests that the use of the two typing methods is redundant. Therefore, the MIRU-VNTR typing method is the superior method to predict the outcome of the RFLP typing method (Carriço *et al.*, 2006).

There was a close correlation between the number of alleles identified at MIRU 26 and spoligotype. A five repeat allele at the MIRU 26 loci was characteristic of 187 of the 194 isolates represented by spoligotype SB0140 a three repeat allele was present in 62 of the 64 isolates represented by spoligotype SB0130 and a six repeat allele was present in all of the 49 isolates represented by spoligotype SB0142. At the VNTR 1895 locus a two repeat allele was present in all of the spoligotype SB0130 isolates. Consequently, when a combined spoligotyping and MIRU-VNTR typing protocol was used the omission of MIRU 26 and 1895 resulted in only a slight reduction in strain resolution

(Table 3.4).

3.2.3 Geographic and species distribution

Spoligotype SB0140 was widely distributed throughout Ireland. Nine VNTR types represented 71% of the SB0140 isolates and were also widely distributed geographically. Another 23% of isolates represented by spoligotype SB0140 were subdivided by MIRU-VNTR typing into geographically localised clusters. Sixty four isolates were represented by spoligotype SB0130 and were widely distributed throughout the south of the country. In contrast to spoligotype SB0140, there was little diversity of VNTR types within the spoligotype SB0130 cluster, with 95% of the isolates represented by a single VNTR profile. The third most frequent spoligotype was SB0142. This was found predominantly in three counties in the North East. Like spoligotype SB0130 there was little diversity of VNTR types with 94% of the isolates represented by a single VNTR type. Isolates represented by spoligotype SB0273 were found in two widely separated counties (Donegal and Kilkenny). However, differences in RFLP and VNTR profiles suggested that these were two phylogenetically unrelated groups. All of the prevalent VNTR profiles were shared by strains from cattle, badgers and deer. This is consistent with previous findings that spoligotypes and RFLP types were shared by strains from all three species.

Table 3.7 Potential of one typing system (reference typing system) to predict the outcome of an alternative typing system (secondary typing system) as measured by the Wallace coefficient

Reference typing system	Secondary typing system	Wallace coefficient		
		Group 1 (n = 386)	Group 2 (n = 194)	Group 3 (n = 192)
RFLP	VNTR	0.56	0.25	0.89
VNTR	RFLP	0.49	0.35	0.66
Spoligotyping	RFLP	0.24	0.14	0.74
Spoligotyping	VNTR	0.22	0.1	0.87

Group 1 = all isolates,
Group 2 = isolates with spoligotype pattern SB0140
Group 3 = isolates that did not have spoligotype pattern SB0140

Chapter four

Discussion

4.1 DISCUSSION

Bovine TB still poses a major economic problem for agriculture both in Ireland and the UK (Pollock, O'Neill, 2002). It leads to economic losses, disruption to trade and farming practices, and has the potential for zoonotic spread (O'Reilly and Daborn, 1995). It is usually a slow progressive disease that can take a number of years for clinical signs to become apparent. The absence of clinical signs in the majority of affected cattle is just one of the factors that cause difficulties in disease investigations. A high frequency of movement of cattle between herds and the presence of infection in wild deer and badgers are other factors that pose a problem for epidemiological investigations.

The most common means of detecting new outbreaks of tuberculosis in cattle herds are either routine tuberculin testing or detection of tuberculosis lesions in animals at abattoir post mortem examination. In Ireland, whenever a new outbreak is detected in a herd, an assessment is carried out by the local Department of Agriculture, District Veterinary Office to try to determine the source of the outbreak and to investigate the possibility of spread of infection to other herds. It is possible for investigators to trace the movement of cattle between herds as it is a legal requirement that each animal has an ear tag with a unique identity number. The Department of Agriculture maintains a database with the identity numbers of the cattle in each herd in the country and movements of cattle between herds are recorded in the database. This database is linked to a Geographical Information System (GIS) in order to facilitate epidemiological investigations. Data on the location of badger setts and the post mortem examination of culled badgers or deer is also included in the GIS (Sheridan, 2011; More and Good, 2006). The effectiveness of GIS for tracing spread of infection can be further increased

if information is available on the strains of *M. bovis* present in the cattle and wildlife populations (Skuce *et al*., 2010) and to observe if the *M. bovis* strains were common among cattle and wildlife. In this study the species examined, cattle, badger and deer, shared the *M. bovis* strains. For example 23 *M. bovis* strains had the MIRU-VNTR profile 10 3 5 5 4 3, and, of these, 20 were found in cattle and 3 in badgers.

Effective methods of strain typing *M. bovis* isolates were not available until the development of DNA based methods. Initially REA, RFLP and spoligotyping were used to differentiate *M. bovis* strains. More recently MIRU-VNTR has become the method of choice, often used in combination with spoligotyping (Skuce *et al*., 2010). The aims of this study were firstly to identify MIRU-VNTR loci that gave good resolution of *M. bovis* strains and secondly to compare MIRU-VNTR typing and spoligotyping with RFLP.

Between two to eight alleles can be found in 12 of the 20 MIRU-VNTR loci used in the study when typing the *M. bovis* strains providing high levels of discrimination between strains (Haddad *et al*., 2004). Results of MIRU-VNTR screening are numerical and therefore inter-laboratory comparisons can be made. When used alone as a typing tool spoligotyping does not provide sufficient discrimination between strains, however when combined with another typing method such as MIRU-VNTR high levels of discrimination can be achieved (Costello *et al*., 1999; Durr *et al*., 2000). To date RFLP has been the ‘gold standard’ technique when strain typing, however RFLP is labour intensive, requires large quantities of DNA and interpretation of the banding patterns are difficult. Inter-laboratory comparisons using RFLP can also be difficult.

The initial aim of this study was firstly to identify MIRU-VNTR loci that gave good resolution of *M. bovis* strains. In order to identify MIRU-VNTR loci that gave good resolution 60 *M. bovis* strains were amplified with 20 MIRU-VNTR loci (**Table 2.1**). Results of this study indicated that six MIRU-VNTR loci (QUB 11a, QUB11b, 1895, ETR A, MIRU 26 and 4052) gave the highest degree of discrimination between the 60 isolates of varying *M. bovis* strain types (**Table 3.1**).

Following a comparison of the two detection methods, agarose gel electrophoresis and MegaBACE^{TM} 1000, and for high throughput of isolates the MegaBACE^{TM} was the detection method of preference (Results section 3.1.2).

The allelic diversities recorded for loci QUB 11b, ETR A, MIRU 26 and 4052 was very similar to findings in other studies in Northern Ireland (Roring *et al.*, 2004), Italy (Boniotti *et al.*, 2009), Spain (Romero *et al.*, 2008) and the USA (Martinez *et al.*, 2008). The allelic diversity of VNTR locus QUB 11a was found to be low in studies in Spain (Romero *et al.*, 2008) and the USA (Roring *et al.*, 2004), but was satisfactory in the present study as was the case in Northern Ireland (Roring *et al.*, 2004). In this study VNTR 1895 had the highest allelic diversity of the six loci in contrast to previous studies (Roring *et al.*, 2004 Boniotti *et al.*, 2009, Martinez *et al.*, 2008). ETR B (VNTR 2461) produced good resolution of *M. bovis* strains in a number of studies (Hilty *et al.*, 2005, Skuce *et al.*, 2005, Martinez *et al.*, 2008, Boniotti *et al.*, 2009). However, ETR B did not display high levels of discrimination between the first 60 *M. bovis* strains. In a study of seven VNTR loci in Northern Ireland VNTR 3232 produced the greatest resolution of *M. bovis* strains (Skuce *et al.*, 2005). In the present study VNTR 3232 did not display high levels of discrimination between the first 60 *M. bovis* strains. VNTR 3232 showed 0% concordance between the two typing methods, agarose gel

electrophoresis and MegaBACE^{TM} 1000. These difficulties have also been reported in other studies (Martinez *et al.*, 2008, Boniotti *et al.*, 2009).

There is little information available on the discriminatory power of MIRU-VNTR typing compared to RFLP. Allix *et al.*, 2006 found that in a panel of 68 *M. bovis* strains a combination of three VNTR loci (3232, ETR A and ETR B) had a genotypic diversity of 0.86 compared to 0.73 for IS*6110* RFLP (Allix *et al.*, 2006). In the present study RFLP analysis using three probes (IS*6110*, PGRS and DR) produced 65 different profiles while MIRU-VNTR typing of this panel of strains gave 41 different profiles. However, almost 50% of the RFLP profiles were unique to one strain and the discriminatory power of both methods as measured by the Hunter-Gaston discriminatory index was comparable (**Table 3.4**).

A combination of spoligotyping and MIRU-VNTR typing offers some advantages over MIRU-VNTR typing alone. A number of MIRU-VNTR profiles were common to more than one spoligotype (**Table 3.5**) and were identified in *M. bovis* strains from diverse geographic regions. Strains bearing these MIRU-VNTR types could usually be subdivided into geographically localised clusters by spoligotyping. In addition, spoligotyping may provide useful phylogenetic information (Smith *et al.*, 2006a). Some alleles of MIRU 26 loci and 1895 had a linkage disequilibrium with spoligotyping. Consequently these two loci were to a large extent redundant in a combined spoligotyping and MIRU-VNTR protocol and only the four VNTR loci QUB 11a, QUB 11b, ETR A and 4052 were required.

SB0140 is the spoligotype most frequently identified in *M. bovis* strains in Ireland and Great Britain. This spoligotype has previously been referred to as type A1 (Costello *et al.*, 1999) and VLA type 9 (Smith *et al.*, 2006a). There was a high level of diversity of

RFLP and MIRU-VNTR profiles within the group of 194 *M. bovis* strains represented by SB0140. There was also a low degree of concordance between MIRU-VNTR and RFLP profiles compared to that found within groups of strains represented by other spoligotypes. The majority of strains with the spoligotype pattern SB0130 had the matching RFLP and MIRU-VNTR profiles. In agreement with our findings a high level of both genetic and phenotypic diversity was found amongst strains bearing SB0140 in Great Britain (Winder *et al.*, 2006). The most common MIRU-VNTR types within the SB0140 group were widely distributed geographically which limited their usefulness for tracing geographic spread of infection. In contrast, most of the less common MIRU-VNTR types within the SB0140 group were largely concentrated in defined geographic areas.

With few exceptions the other spoligotypes were also concentrated in defined geographic areas. The most geographically dispersed was SB0130 which was distributed over several counties in the South. This was the second most common spoligotype identified and interestingly it does not belong to the SB0140 clonal complex described by Smith *et al*., 2006. This clonal complex is identified by the deletion of spacers 6 and 8 to 12 and it includes the majority of *M. bovis* strains in Ireland and Great Britain. However, in contrast to SB0140, there was very little diversity of MIRU-VNTR profiles within the SB0130 group, which suggests that it has undergone a more recent clonal expansion in Ireland compared to SB0140.

The optimal procedure to use for strain typing of *M. bovis* will depend on the strains present in a region, the number of strains to be typed, the resources available and the degree of resolution required. A combination of spoligotyping and typing of four MIRU-VNTR loci offers a relatively uncomplicated procedure suitable for high throughput typing. This study has shown using MIRU-VNTR loci QUB 11a, QUB 11b,

ETR A and 4052 gave a level of strain discrimination that was comparable to that produced by the combined typing methods IS*6110*, PGRS and DR RFLP when typing Irish *M. bovis* strain types.

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Publication

Journal of Clinical Microbiology

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J. Clin. Microbiol. **2010, 48(12):4541. DOI: 10.1128/JCM.01175-10.**
Published Ahead of Print 29 September 2010.

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2010, p. 4541–4545 Vol. 48, No. 12
0095-1137/10/$12.00 doi:10.1128/JCM.01175-10

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Received 10 June 2010/Returned for modification 13 August 2010/Accepted 23 September 2010

Common strain typing methods for differentiation of *Mycobacterium bovis* isolates include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, spoligotyping, and, more recently, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing. MIRU-VNTR typing and spoligotyping were evaluated in this study, and these typing methods were compared with RFLP typing. A total of 386 *M. bovis* isolates from cattle, badgers, and deer in the Republic of Ireland that had previously been typed by IS*6110*, polymorphic GC-rich sequence (PGRS), and direct-repeat (DR) RFLP were included in the study. Spoligotyping and analysis of six VNTR loci (QUB 11a, QUB 11b, ETR A, 4052, MIRU 26, and 1895) were performed on the samples. RFLP analysis was the method that gave the greatest differentiation of strains, with a Hunter-Gaston discriminatory index (HGDI) of 0.927; the HGDI recorded for MIRU-VNTR typing was marginally lower at 0.918, and spoligotyping was the least discriminatory method, with an HGDI of 0.7. Spoligotype SB0140 represented approximately 50% of the isolates. Within the group of isolates represented by SB0140, there was a much lower level of concordance between RFLP and MIRU-VNTR typing than for groups represented by other spoligotypes. A combination of spoligotyping and MIRU-VNTR typing offered advantages over MIRU-VNTR typing alone. In a combined spoligotyping and MIRU-VNTR typing protocol, the number of VNTR loci could be reduced to four (QUB 11a, QUB 11b, ETR A, and 4052) while maintaining a high level of strain differentiation.

The development of molecular techniques for differentiation of *Mycobacterium bovis* isolates has been of considerable benefit in epidemiological studies. Typing methods that have been commonly used include restriction endonuclease analysis (REA), restriction fragment length polymorphism (RFLP) analysis, spoligotyping, and, more recently, mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing (7, 19).

RFLP analysis of *M. bovis* isolates has commonly utilized polymorphism of the insertion sequence IS*6110* and repetitive DNA elements such as the polymorphic GC-rich sequence (PGRS) and the direct-repeat (DR) region. Analysis of polymorphism of IS*6110*, the PGRS, and the DR region in combination has provided a high level of discrimination between strains (7, 19). REA has been widely used in New Zealand and has also given excellent resolution of strains (4). However, both RFLP analysis and REA require relatively large quantities of DNA and are laborious and time-consuming procedures. Complex banding patterns make analysis and interlaboratory comparisons difficult. Spoligotyping is a PCR-based typing method that reveals the presence or absence of unique spacer sequences located between the direct-repeat sequences of the DR region (12). It is a relatively easy procedure to perform, and the results can be expressed in a digital format. However, spoligotyping does not differentiate *M. bovis* strains to the same extent as RFLP analysis or REA (7, 19). Minisatellite-like loci in the *Mycobacterium tuberculosis* complex genome, described as mycobacterial interspersed repetitive units, may show polymorphism of the number of tandem repeats. A wide range of *M. tuberculosis* complex MIRU-VNTR loci have been evaluated, and loci which are informative for *M. bovis* isolates have been identified (8, 16, 17, 20, 23). Similar to spoligotyping, MIRU-VNTR typing has the advantages of ease of procedure and the generation of results in a digital format.

In recent years, genotyping by IS*6110*, PGRS, and DR RFLP has been used in epidemiological studies of *M. bovis* infection in the Republic of Ireland (5, 6, 14). While RFLP analysis has given a high level of strain differentiation, its replacement by MIRU-VNTR typing or by a combination of MIRU-VNTR typing and spoligotyping offers potential advantages. The objective of this study was to evaluate MIRU-VNTR typing or a combination of MIRU-VNTR typing and spoligotyping for discrimination of *M. bovis* strains, to compare the discriminatory powers of the two methods against RFLP analysis, and to investigate the level of concordance between the three typing systems.

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▽ Published ahead of print on 29 September 2010.

TABLE 1. Primer sequences for MIRU-VNTR typing

VNTR locus	Alternate name	Primer pair with label (5′ → 3′)[a]	Reference
QUB 11a	2163a	Hex-CCCATCCCGCTTAGCACATTCGTA, TTCAGGGGGGATCCGGGA	20
QUB 11b	2163b	Hex-CGTAAGGGGGATGCGGGAAATAGG, CGAAGTGAATGGTGGCAT	20
1895		Fam-GGTGCACGGCCTCGGCTCC, AAGCCCCGCCGCCAATCAA	16
2165	ETR A	Fam-AAATCGGTCCCATCACCTTCTTAT, CGAAGCCTGGGGTGCCCGCGATTT	8
2996	MIRU 26	Hex-TAGGTCTACCGTCGAAATCTGTGAC, CATAGGCGACCAGGCGAATAG	23
4052	QUB 26	Hex-AACGCTCAGCTGTCGGAT, GGCCAGGTCCTTCCCGAT	20

[a] The forward primer of the primer pair was labeled with a fluorescent dye to facilitate with the detection of the amplified product. Hex, hexchlorofluorescein; FAM, 6-carboxyfluorescein.

MATERIALS AND METHODS

Mycobacterial strains and culture procedure. Stored *M. bovis* isolates that had previously been typed by RFLP analysis (5, 6) were used in this study. Isolates that had been stored at −20°C were thawed and cultured in 3 ml of Middlebrook 7H9 broth at 37°C for 7 days. Aliquots (0.5 ml) of the Middlebrook 7H9 broth were streaked onto Stonebrinks medium and Lowenstein-Jensen medium containing pyruvate (prepared as solid slants in screw-cap tubes), incubated at 37°C, and monitored on a weekly basis. Cultures suitable for DNA extraction were obtained for 386 isolates. The isolates had been obtained from 243 badgers, 119 cattle, and 24 deer during the years 1996 to 2002. The isolates were obtained from all areas of the Republic of Ireland; however, a total of 206 originated in four study areas described by Griffin et al. (9).

DNA extraction. Colonies were transferred from the slopes into microtubes containing 500 μl of phosphate-buffered saline with Tween 20 (PBS-Tw) (Sigma Aldrich, Wicklow, Ireland). The microtubes were placed in a heating block at 100°C for 15 min to heat lyse the cells and vortexed periodically. Microtubes were centrifuged at 6,000 × *g* for 2 min. The supernatant was transferred into a clean, labeled 1.5-ml Eppendorf tube. DNA template was stored at −20°C.

VNTR typing. VNTR typing was performed using the six loci QUB 11a, QUB 11b, ETR A, MIRU 26, 4052, and 1895. The six genomic loci were amplified in separate PCRs with the primers described in Table 1. Reaction volumes of 25 μl containing 2.5 μl of 10× PCR buffer (Qiagen, West Sussex, United Kingdom), 0.2 μl of 50 pmol primer set, 2 μl (100 μM) of each of four deoxynucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), 5 μl of Q solution, 0.125 μl of Hotstar *Taq* polymerase (1 unit) (Qiagen), and 9.175 μl of pure H_2O. Template DNA (5 μl) was added to each PCR mix. A DNA extract from *M. bovis* and *M. tuberculosis* H37 was included in each set of reactions as a positive control and sterile distilled water as a negative nontemplate control. Amplification was performed in a Flexigene thermocycler with an initial activation step of 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min. The final extension was 72°C for 10 min. When the PCR was complete, the amplified products were stored light protected at −18°C until ready to be run on the MegaBACE 1000 (GE Healthcare Life Sciences, United Kingdom). The forward primer of the primer pair was labeled with a fluorescent dye (Table 1) to facilitate the detection of the amplified product. PCR products were diluted 1:50 in molecular-grade water and separated on a 96-capillary MegaBACE 1000 sequencer using Rox-labeled MegaBACE ET900-R as a size standard. The electrophoresis was run for 120 min using MegaBACE matrix, with an injection voltage of 3 kV for 45 s and a running voltage of 10 kV. Each peak was identified according to color and size and assigned to a distinct allele number.

Spoligotyping. Spoligotyping was performed according to the method described by Kamerbeek et al. (12) except that a digoxigenin labeling and detection system (Roche Diagnostics, West Sussex, United Kingdom) was used. Spoligotype patterns were given the names assigned in the *M. bovis* spoligotyping database at http://www.mbovis.org.

Statistical analysis. Calculation of the discriminatory power of each typing method was based on Simpson's index of diversity as described by Hunter and Gaston (11). This value is commonly referred to as the Hunter-Gaston discriminatory index (HGDI). Wallace's coefficient was used to quantify the level of concordance between typing methods (3). This calculates the degree to which one typing method can predict the result of another typing method. A high value of Wallace's coefficient suggests that the use of both methods is redundant. Wallace's coefficient was calculated using the Web tool http://www.comparingpartitions.info. The allelic diversity at the different VNTR loci was calculated using the method described by Selander et al. (18).

RESULTS

Resolution of strains. RFLP analysis, with an HGDI of 0.927 was more discriminating than MIRU-VNTR typing, which had a HGDI of 0.918, while spoligotyping was the least discriminatory of the three methods (Table 2). There were 65 RFLP profiles, which were divided into 33 clusters and 32 unique isolates, compared to 41 VNTR profiles comprising 26 clusters and 15 unique isolates. The largest RFLP cluster contained 58 isolates, while the largest MIRU-VNTR cluster contained 65

TABLE 2. Comparison of discriminatory powers of various genotyping protocols

Procedure	HGDI[a]	No. of:			
		Profiles	Clusters	Unique isolates	Isolates in largest group
RFLP analysis	0.927	65	33	32	58
VNTR typing	0.918	41	26	15	65
Spoligotyping	0.700	15	14	1	194
Spoligotyping + RFLP analysis	0.929	68	35	33	58
Spoligotyping + VNTR typing with:					
6 loci	0.933	54	36	18	61
5 loci[b]	0.930	51	34	17	63
4 loci[c]	0.930	49	34	15	63
Spoligotyping + RFLP analysis + VNTR typing	0.958	104	51	53	55

[a] HGDI, Hunter-Gaston discriminatory index.
[b] VNTR loci QUB 11a, QUB 11b, ETR A, 4052, and 1895.
[c] VNTR loci QUB 11a, QUB 11b, ETR A, and 4052.

TABLE 3. Resolution of eight VNTR clusters by spoligotyping

VNTR profile[a]	Spoligotype	No. of isolates
10 3 5 5 4 3	SB0140	20
	SB0993	3
10 4 6 5 4 4	SB0140	19
	SB0273	10
11 2 6 5 4 4	SB0140	2
	SB0269	1
11 3 7 5 4 4	SB0140	6
	SB0144	12
	SB0486	2
11 4 5 5 4 4	SB0140	2
	SB0054	6
11 4 7 5 3 4	SB0140	14
	SB0141	7
	SB0486	5
	SB0145	5
11 4 7 6 4 3	SB0140	1
	SB0142	46
	SB0995	2
11 3 7 3 3 2	SB0120	61
	SB0146	3
	SB0998	1

[a] The VNTR loci are listed in the order QUB 11a, QUB 11b, ETR A, MIRU 26, 4052, and 1895.

isolates. Spoligotyping identified 14 clusters and one unique isolate; the largest cluster, represented by spoligotype pattern SB0140, contained approximately 50% of the isolates. Spoligotyping produced further resolution of eight MIRU-VNTR clusters (Table 3). RFLP clusters were not resolved to the same extent by spoligotyping, with only three being further subdivided. The allelic diversity of the VNTR loci ranged from 0.44 for VNTR QUB 11a to 0.57 for VNTR 1895. (Table 4).

Typing system concordance. The level of concordance between the typing systems varied according to spoligotype. Wallace's coefficient (3), which is a measure of the degree to which one typing method can predict the result of another typing method, was used to quantify the level of concordance between typing methods (Table 5). Strains bearing spoligotype SB0140 showed highly variable RFLP and VNTR profiles and a low level of concordance between these two typing methods. The highest level of concordance was found in the strains that did not have the SB0140 spoligotype pattern.

TABLE 4. Allelic diversity of VNTR loci

Locus	Allelic diversity	No. of isolates with VNTR allele:										
		1	2	3	4	5	6	7	8	9	10	11
QUB 11a	0.44			7			8			13	82	276
QUB 11b	0.48	5	5	130	245	1						
ETR A	0.45		1	4	1	48	54	277		1		
MIRU 26	0.49		1	68	2	260	55					
4052	0.55		45	110	231							
1895	0.57		70	89	227							

TABLE 5. Potential of one typing system (reference typing system) to predict the outcome of an alternative typing system (secondary typing system) as measured by the Wallace coefficient

Reference typing system	Secondary typing system	Wallace coefficient for group[a]:		
		1 (n = 386)	2 (n = 194)	3 (n = 192)
RFLP analysis	VNTR typing	0.56	0.25	0.89
VNTR typing	RFLP analysis	0.49	0.35	0.66
Spoligotyping	RFLP analysis	0.24	0.14	0.74
	VNTR typing	0.22	0.10	0.87

[a] Group 1, all isolates; group 2, isolates with spoligotype pattern SB0140; group 3, isolates that did not have spoligotype pattern SB0140.

There was a close correlation between MIRU 26 alleles and spoligotype. A five-repeat allele at the MIRU 26 locus was characteristic of 187 of the 194 isolates represented by spoligotype SB0140, a three-repeat allele was present in 62 of the 64 isolates represented by spoligotype SB0130, and a six-repeat allele was present in all of the 49 isolates represented by spoligotype SB0142. At the VNTR 1895 locus, a two-repeat allele was present in all of the spoligotype SB0130 isolates. Consequently, when a combined spoligotyping and MIRU-VNTR typing protocol was used, the omission of MIRU 26 and 1895 resulted in only a slight reduction of strain resolution (Table 2).

Geographic and species distribution. Spoligotype SB0140 was widely distributed throughout Ireland. Nine VNTR types represented 71% of the SB0140 isolates and were also widely distributed geographically. Another 23% of isolates represented by spoligotype SB0140 were subdivided by MIRU-VNTR typing into geographically localized clusters. Sixty-four isolates were represented by spoligotype SB0130 and were widely distributed throughout the south of the country. In contrast to the case for spoligotype SB0140, there was little diversity of VNTR types within the spoligotype SB0130 cluster, with 95% of the isolates represented by a single VNTR profile. The third most frequent spoligotype was SB0142. This was found predominantly in three counties in the northeast. Like for spoligotype SB0130, there was little diversity of VNTR types, with 94% of the isolates represented by a single VNTR type. Isolates represented by spoligotype SB0273 were found in two widely separated counties (Donegal and Kilkenny). However, differences in RFLP and VNTR profiles suggested that these were two phylogenetically unrelated groups. All of the prevalent VNTR profiles were shared by strains from cattle, badgers, and deer. This is consistent with previous findings that spoligotypes and RFLP types were shared by strains from all three species (5).

DISCUSSION

In this study, MIRU-VNTR typing using a panel of six loci was an easy-to-apply and reliable technique that provided good differentiation of strains. The six VNTR loci were selected based on an initial evaluation of a panel of 24 loci in 60 *M. bovis* isolates (unpublished data). The allelic diversities recorded for loci QUB 11b, 2165, MIRU 26, and 4052 were very similar to findings in other studies in Northern Ireland (17), Italy (2), Spain (15), and the United States (13). The allelic

diversity of VNTR locus QUB 11a was found to be low in studies in Spain (15) and the United States (13) but was satisfactory in the present study, as was the case in Northern Ireland (17). In this study VNTR 1895 had the highest allelic diversity of the six loci, in contrast to previous studies (2, 13, 17). There are other VNTR loci that have proved useful for discrimination of *M. bovis* strains that were not evaluated in this study. ETR B (VNTR 2461) produced good resolution of *M. bovis* strains in a number of studies (2, 10, 13, 21). In a study of seven VNTR loci in Northern Ireland, VNTR 3232 produced the greatest resolution of *M. bovis* stains (21). However, difficulties with the reproducibility of typing VNTR 3232 have been reported (2, 13).

There is little information available on the discriminatory power of MIRU-VNTR typing compared to RFLP analysis. Allix et al. (1) found that in a panel of 68 *M. bovis* isolates, a combination of three VNTR loci (3232, ETR A, and ETR B) had a genotypic diversity of 0.86, compared to 0.73 for IS*6110* RFLP. In the present study, RFLP analysis using three probes (IS*6110*, the PGRS, and the DR region) produced 65 different profiles, while MIRU-VNTR typing of this panel of isolates gave 41 different profiles. However, almost 50% of the RFLP profiles were unique to one isolate, and the discriminatory powers of the two methods as measured by the Hunter-Gaston discriminatory index were comparable (Table 2).

A combination of spoligotyping and MIRU-VNTR typing offers some advantages over MIRU-VNTR typing alone. A few VNTR profiles were common to more than one spoligotype (Table 3) and were identified in isolates from diverse geographic regions. Isolates bearing these VNTR types could usually be subdivided into geographically localized clusters by spoligotyping. In addition, spoligotyping may provide useful phylogenetic information (22). Some alleles of the MIRU 26 and 1895 loci had a linkage disequilibrium with spoligotyping. Consequently, these two loci were to a large extent redundant in a combined spoligotyping and VNTR protocol, and only the four VNTR loci QUB 11a, QUB 11b, ETR A, and 4052 were required.

SB0140 is the spoligotype most frequently identified in *M. bovis* isolates in Ireland and Great Britain. This spoligotype has previously been referred to as type A1 (5) and VLA type 9 (22). There was a high level of diversity of RFLP and VNTR profiles within the group of 194 isolates represented by SB0140 and a lower degree of concordance between VNTR and RFLP types than found within groups of isolates represented by other spoligotypes. In agreement with our findings, high levels of both genetic and phenotypic diversity were found among strains bearing SB0140 in Great Britain (24). The most common VNTR types within the SB0140 group were widely distributed geographically, which limited their usefulness for tracing the geographic spread of infection. In contrast, most of the less common VNTR types within the SB0140 group were largely concentrated in defined geographic areas.

With few exceptions, the other spoligotypes were also concentrated in defined geographic areas. The most geographically dispersed was SB0130, which was distributed over several counties in the south. This was the second most common spoligotype identified, and interestingly, it does not belong to the SB0140 clonal complex described by Smith et al. (22). This clonal complex is identified by the deletion of spacers 6 and 8 to 12, and it includes the majority of *M. bovis* strains in Ireland and Great Britain. However, in contrast to the case for SB0140, there was very little diversity of VNTR profiles within the SB0130 group, which suggests that it has undergone a more recent clonal expansion in Ireland than SB0140.

The optimal procedure to use for strain typing of *M. bovis* will depend on the strains present in a region, the number of isolates to be typed, the resources available, and the degree of resolution required. A combination of spoligotyping and typing of four VNTR loci offers a relatively uncomplicated procedure suitable for high-throughput typing. This study has shown that a protocol using VNTR loci QUB 11a, QUB 11b, ETR A, and 4052 combined with spoligotyping gave a level of discrimination of *M. bovis* strains that was comparable to that produced by IS*6110*, PGRS, and DR RFLP.

ACKNOWLEDGMENTS

We thank our colleagues within the laboratory for all their help and support throughout the duration of this study.

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