Mycobacterium microti and vole tuberculosis: improved diagnostics and molecular epidemiology

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Mohamed Ali Ibrahim

November 2009

Declaration

I declare that this thesis has not been previously submitted for a degree at this or any other university, and I further declare that the work incorporated in it is my own.

....

Mohamed Ali Ibrahim

Contents

Declaration i

Table of contents ii

Acknowledgements vi

Abstract vii

Chapter 1: General introduction 1

1.1 Historical perspective 2

1.2 Epidemiology of *M. microti* infections in small mammals 7

1.3 Pathology of vTB in field voles 8

1.4 *M. microti* pathogenicity 10

1.5 *M. microti* as a pathogen of medical and veterinary importance 12

1.6 General microbiology 16

1.7 Genetic homology of MtC members 17

1.8 Molecular delineation of MtC strains 20

1.9 Laboratory diagnosis of tuberculosis 24

1.10 Aims of this study 28

Chapter 2: *M. microti* infections in field voles at Kielder Forest 30

2.1 Introduction 31

2.2 Material and Methods 34

2.2.1 Animals 34

2.2.2 Post-mortem examination 35

2.2.3 Ziehl-Neelsen (ZN) stain on lesion impression smears 36

2.2.4 Culture 37

2.2.5	Histological examination	38
2.3	**Results**	38
2.3.1	Prevalence and distribution of macroscopic lesions	39
2.3.2	Observation of acid-fast bacilli in ZN-stained impression smears	42
2.3.3	Isolation of *M. microti*	43
2.3.4	Histopathology	44
2.3.5	Ecological data	54
2.4	**Discussion**	60
Chapter 3:	PCR-based diagnosis of *M. microti* infections	66
3.1	**Introduction**	67
3.2	**Materials and Methods**	74
3.2.1	Lung and lymph node tissues	74
3.2.2	Extraction of DNA from clinical samples	74
3.2.3	DNA amplification assays	76
3.2.4	Detection of field vole DNA	76
3.2.5	Detection of *Mycobacterium tuberculosis* complex DNA	78
3.3	**Results**	79
3.3.1	Overall findings	79
3.3.2	Performance of PCR in field voles yielding a *M. microti* isolate	85
3.3.3	Performance of PCR in field voles with visible lesions and acid fast bacilli	86
3.4	**Discussion**	87

Chapter 4: Molecular epidemiology of tuberculosis among Kielder Forest field voles 93

4.1 **Introduction** 94

4.2 **Materials and methods** 102

4.2.1 Exploring regions of difference (RD) 102

4.2.1.1 *M. microti* isolates 102

4.2.1.2 PCR amplification of RDs 102

4.2.2 Spoligotyping 103

4.2.2.1 *M. microti* isolates 103

4.2.2.2 PCR amplification of the direct repeat (DR) region 104

4.2.2.3 Attachment of DR spacer oligonucleotides to the spoligotype membrane 105

4.2.2.4 Hybridisation of PCR products to membrane-bound oligonucleotides 106

4.2.3 Variable number of tandem repeat (VNTR) analysis 107

4.2.3.1 *M. microti* isolates 107

4.2.3.2 PCR-based amplification of VNTR loci 110

4.2.3.4 Fragment analysis of VNTR amplification products 111

4.2.3.5 Sequencing of VNTR amplification products 111

4.3 **Results** 112

4.3.1 Exploring regions of difference 112

4.3.2 Spoligotyping 113

4.3.3 VNTR analysis 114

4.3.4 Sequencing of VNTR amplification products 120

4.3.5 Molecular epidemiology of *M. microti* in Kielder Forest 121

4.4 **Discussion** 123

Chapter 5: Immunological response to *M. microti* infection in field voles 129

5.1 **Introduction** 130

5.2 **Materials and methods** 133

5.2.1 Experiment 1 133

5.2.2 Experiment 2 134

5.2.3 Detection of IFN-γ by ELISA 136

5.3 **Results** 137

5.3.1 Experiment 1 137

5.3.2 Experiment 2 138

5.4 **Discussion** 140

Chapter 6: General Discussion 144

6.1 The nature of vTB skin lesions 145

6.2 *M. microti* as an emerging zoonotic pathogen 148

6.3 *M. microti* as a confounder of bovine tuberculosis epidemiology 149

References 152

Acknowledgements

I am grateful for the support of the Libyan people, who funded my study. I wish to express my sincere appreciation to my supervisor Dr Richard Birtles, for his patience, encouragement and guidance in this project. Also, I wish to extend my gratitude to my second supervisor Professor Anja Kipar for help and advice in the pathology chapter. My thanks must go to the people in the TB Research Group in Veterinary Laboratories Agency, New Haw, Surrey, especially Dr Steven Gordon, Dr Noel Smith, Dr James Dale and Dr Phillip Hogarth for help and advice with spoligotyping, VNTR, deletion analysis and the interferon gamma detection assay.

I would like to thank all who work in the Department of Veterinary Pathology on the Leahurst Campus for their friendship, invaluable help and advice on countless occasions. Particular thanks to Dr Lutfi Altunesi for being my best friend and for his continued support and interest in what I do, and further I would like to mention my officemates, especially Kevin Bown, Claire Johnston and Gemma Chaloner. Lastly, and most importantly, my thanks most go to my family, to whom I would like to dedicate this work: my wife Hanan, my lovely son Jehad and my family in Libya especially my father. I want them to know that I am very grateful for their unreserved love and encouragement throughout my studies. You have all been a source of inspiration and moral support. Without their love and patience, I would not have become who I am now.

Abstract

This study aimed to extend work carried out at the University of Liverpool over the past decade that has been part of a revival of interest in the microbiology of the bacterium *Mycobacterium microti* and the epidemiology of the disease it causes, vole tuberculosis (vTB), in woodland rodents in the United Kingdom. The study utilised field voles (*Microtus agrestis*) inhabiting Kielder Forest, Northumberland, UK, which were surveyed in a manner that permitted the determination of both field vole population dynamics and *M. microti* transmission dynamics between 2001 and 2006. These surveys took place every six months, with a total of 11 being completed, however, data relating to *M. microti* infection status had only been generated for the first four. Hence, the first aim of this study was to extend this data are set to include another five surveys. The diagnosis of vTB both *post mortem* and, in particular, *ante mortem* remains imperfect. Hence, the study also explored new approaches to diagnosis. Finally, the study began exploration of the molecular epidemiology of vTB through the application of contemporary molecular typing methodologies developed for use on the closely related species *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

The voles examined in this study were captured during the five surveys between March 2005 and March 2007. In total 1270 animals were subjected to detailed post-mortem examination and dissection, and the presence of *M. microti* in their tissues was determined, initially, on the basis of two criteria, namely (i) the observation of macroscopic tuberculous lesions shown to contain acid-fast bacilli microscopically and/or (ii) isolation of *M. microti*. Subsequently, the value of histology and a PCR-based assay were explored. The general findings were that isolation of *M. microti* by

culture remained the most sensitive single approach to detection, but many cases of vTB in animals that did not yield a culture were also diagnosed. Histology showed much promise as an additional diagnostic tool, but the sensitivity of the PCR assay was only moderate compared to *M. microti* isolation. Macroscopic lesions were almost always present in the lymph nodes of infected voles, suggesting this to be the primary niche of infection. The skin was the second most frequent site of lesions, then lungs, and these three sites were the only sites were macroscopic lesions were observed. However, detailed histopathological examination of voles revealed granulomas not only in these tissues but also in the spleen and, most frequently, the liver.

Molecular typing was applied to a large panel of representative isolates of *M. microti*. The isolates were monomorphic when tested using deletion analysis and spoligotyping. However, variable number tandem repeat analysis based on the six previously described exact number tandem repeat (ETR) loci delineated six genotypes, albeit with a low allelic diversity index of 0.39. As expected the discriminatory power of each ETR locus varied, although the relative performance of each in discriminating among *M. microti* strains was different to that observed when the scheme has been applied to *M. bovis*. No obvious epidemiological relevance to the *M. microti* genotypes was discernible, and the epidemiology of vTB in the Kielder Forest field vole population appears to be characterised by a stable population of strains in circulation, with one genotype dominating both temporally and spatially.

Attempts to apply an established murine interferon gamma (IFN-γ) detection assay to the diagnosis of vTB were thwarted as preliminary experiments indicated that antibodies raised against murine IFN-γ did not recognise vole IFN-γ.

Chapter 1.

General introduction

1.1. Historical perspective

Vole tuberculosis (vTB) was first described over 80 years ago, when Wells (1937) encountered the disease during the survey of field voles (*Microtus agrestis*) in the Scottish borders. Wells' article described the presence of caseous lesions filled with acid-fast bacilli that resembled *Mycobacterium tuberculosis*. These bacteria were isolated using media developed for *M. tuberculosis*. This discovery was considered remarkable as hitherto it had been generally believed that tuberculosis was almost entirely a disease of man and of domesticated or captive animals and birds (Elton, 1942). Wells (1937) speculated on the medical or veterinary importance of these organisms, suggesting they represented strains of either *M. tuberculosis* or *Mycobacterium bovis* or a new member of the genus. Subsequent characterisation led to differences being recognised between the agent of vTB and *M. tuberculosis* and *M. bovis*, thus the agent vTB was classified as *Mycobacterium tuberculosis* subspecies *muris* (Wells, 1953). More recently, following re-classification of all *M. tuberculosis* subspecies, the taxon was renamed as *Mycobacterium microti* and incorporated into a complex that includes the primary agent of human tuberculosis, *M. tuberculosis*, *Mycobacterium bovis*, the agent of bovine tuberculosis, also a disease of major veterinary and public health concern, *Mycobacterium africanum*, a prevalent cause of human TB in Africa, *Mycobacterium canetti*, a far rarer cause of human TB in Africa, and *Mycobacterium pinnipedii*, which has been recovered from seals in the southern hemisphere (Wayne & Kubica, 1986). This complex is commonly referred to as the *M. tuberculosis* complex (MtC).

The commentary that accompanied Wells' original article highlighted how his discovery resulted from an innovative collaboration between pathologists and ecologists and how such interdisciplinary studies represented an exciting new approach to scientific investigation (Anon, 1937). This collaboration was established to explore the potential role of parasites in regulating vole population dynamics (Elton, 1942) but ultimately failed to identify any links between parasite burden and vole declines. This failure led to the influence of parasites on host population dynamics being discounted for many subsequent years (Chitty, 1954). However, interest in the epidemiology of *M. microti* infections in UK wildlife has been revived in the last decade. In 2002, Cavanagh and colleagues reported studies of small mammal populations in Northumberland in which characteristic skin lesions had been observed on a small proportion of individuals. Burthe and colleagues (2008) extended these studies with more structured horizontal and longitudinal survey of vTB epidemiology among Northumbrian field voles, and were able to collate the effect of infection on the condition and survival of voles.

Despite the failure of Wells' collaboration with Elton to demonstrate a role of vTB in vole population dynamics, their discovery of *M. microti* led to a great deal of further work. Wells (1946) reported a series of studies aimed at determining the geographical range of *M. microti*, the pathology of infection and the diversity of small mammal species naturally infected with the bacterium. Infected field vole populations were identified in many parts of the UK including Scotland, Wales and the Thames valley and infected bank voles (*Myodes glareolus*), wood

mice (*Apodemus sylvaticus*) and common shrews (*Sorex araneus*) were also recorded (Wells, 1946). Post-mortem examination led to a detailed description of lesions in the organs and tissues of infected animals, and the use of Zeihl-Neelsen staining and isolation methods to confirm the presence of *M. microti* were described (Wells, 1946). Wells (1946) also initiated studies into the antigenicity of *M. microti* that ultimately led to the use of the species as a vaccine for *M. tuberculosis* (Wells, 1946). *M. microti* was introduced as a vaccine in 1946 and over 100,000 people in the UK were vaccinated with it before its withdrawal in 1961 (Hart et al., 1977). During the same period, a *M. microti* vaccine was also used in the Czech Republic, with an estimated half million newborns being vaccinated (Sula & Radkovsky, 1976). The vaccine was safe and was found to be as effective as the *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine, with a 77% protection efficacy (Hart & Sutherland, 1977). Although the *M. microti* vaccine offered some advantages over BCG, for example the species was naturally attenuated and could therefore be passaged in voles to maintain its antigenic integrity (Wells & Wylie, 1954), it was withdrawn because 3 to 17 % of the vaccinated patients developed a lipoid skin reaction (Wells & Wylie, 1954; Tuberculosis Vaccine Trials Committee, 1956). Interest in *M. microti* as a vaccine has recently been revived by Manabe and colleagues (2002), who, in an attempt to avoid the adverse cutaneous reactions observed earlier, tested the efficacy of aerosol and oral vaccination routes in a murine model. They demonstrated that high-dose orogastric vaccination resulted in a statistically significant improvement in protection against aerosol challenge with virulent *M. tuberculosis* compared with subcutaneous BCG vaccination.

Following the flurry of activity surrounding the development, evaluation and administration of *M. microti* as a vaccine for human tuberculosis, scientific interest in the bacterium waned. However, during the 1970s and 1980s, the emergence of cellular microbiology was reflected in a series of studies into the interactions between *M. microti* and macrophages. Such studies were a natural progression of early experimental work carried out 40 years earlier. These first attempts to model vTB in laboratory animals were reported by Wells (1937), Griffith (1941) and Wells (1946). Wells (1937) briefly reported the provocation of symptoms and tuberculin test reactivity in guinea pigs and rabbits inoculated with *M. microti*. Griffith (1941) produced a far more detailed report of experiments in which golden hamsters were inoculated with *M. microti* and developed a generalized disease resembling tuberculosis. However, although characteristic tuberculous lesions were formed, with bacilli abundant in the lesions, there was no necrosis or caseation. Furthermore, *M. microti* was only virulent when administrated in large quantities (Griffith 1941). Interestingly, Griffith (1939) had earlier tested the susceptibility of voles to both *M. tuberculosis* and *M. bovis*, finding the latter to be far more infectious than the former, and they cited earlier work by Robert Koch who, reportedly, infected continental voles (*Microtus arvalis*) with some of his original isolates of *M. tuberculosis*. Wells (1946) explored the route by which *M. microti* infected voles, showing that voles could acquire infection by consuming tissues of infected animals or by drinking contaminated water. Wells (1946) also tested the ability of *M. microti* to infect other laboratory animals, inducing infections of varying severity, longevity and pathology in guinea pigs, white mice, hamsters, Orkney

voles (*Microtus orcadensis*), continental voles, wood mice and bank voles. In addition to laboratory animals, Griffith and Darling (1940) inoculated calves with *M. microti*, demonstrating the generation of tuberculous lesions lacking caseation or necrosis, and Griffith (1942) reported *M. microti* was not virulent for fowl, having observed viable bacilli in the apparently unaltered spleen of high-dose inoculated birds for over four months. Somewhat later, Hejlicek (1959) reported experimental infection of pigs, observing that lesions were generally more severe than in the other animals.

Cellular microbiological studies began in 1970, when Sula and Konradov presented ultrastructural studies of *M. microti*-infected rabbit macrophages. A similar study using mouse macrophages followed (Hart et al., 1972), as part of the development of a model system for the study of *M. tuberculosis* complex members with their target host cell. This model system was used to explore the basis of mycobacterial resistance to macrophage killing (e.g. Lowrie et al., 1975; Lowrie et al., 1979; Walker & Lowrie, 1981), and other similar models were developed and utilised (Coates et al., 1986; Kaye et al., 1986; Kaye & Feldmann, 1986). *M. microti* exploitation of macrophages was found to be mediated by inhibition of phagosome-lysosome fusion, a process that appeared to result from the inhibition of lysosomal movements (Hart et al., 1987).

1.2. Epidemiology of *M. microti* infections in small mammals.

Following on from his first description of vTB (Wells, 1937), Wells reported study of the epidemiology of infections in UK vole populations. He demonstrated a prevalence in field voles of between nine and 31%, depending on place and season of capture, and lower values of 11% in bank voles, 2% in wood mice and 1.5% in shrews (Wells 1946). Wells (1946) also reported a clear seasonality to infections; for example at Greskine Forest in Dumfrieshire, surveys of field voles in spring revealed an infection prevalence of between 37 and 66%, whereas only between 11 and 20% of animals were symptomatic in summer and autumn surveys. Chitty (1954) reported more detailed survey of vTB in vole populations at Lake Vyrnwy in North Wales, confirming the seasonality observed by Wells (1946). Chitty concluded that the spring peak in vTB prevalence was because during this time of the year the vole population was dominated by old, overwintered animals in which infection had had time to progress enough to provoke overt symptoms. Chitty also observed that vTB was unevenly distributed in space, being absent from voles in some areas around the Lake, but very prevalent in others.

No further studies of vTB epidemiology in voles appeared for almost 50 years, but then, in 2002, Cavanagh and colleagues revived interest with the description of vTB lesions in Northumbrian field voles and wood mice and bank voles from Cheshire. External lesions were observed on up to 8% of individuals within the various populations. The study also applied various molecular typing techniques to the *M. microti* isolates obtained to confirm their identity and compare them

with isolates made by Wells and colleagues during the 1930s, concluding that all isolates were very similar to one another (Cavanagh et al., 2002). Two further reports from the same research group have followed, focusing on ecological aspects of vTB in vole populations that exhibit multi-annual cycles of population density. Cavanagh and colleagues (2004) studied vole population dynamics and vTB symptoms at 12 sites in Kielder Forest District (Northumberland) reporting that *M. microti* infection showed delayed density dependence in relation to vole population size, with a lag of three to six months. Subsequently, Burthe and colleagues (2008) carried out longitudinal and cross-sectional studies to confirm that vTB prevalence increased with age and that seasonality was due to a higher proportion of older animals in spring. This study was also able to confirm that external lesions only appeared during the advanced stages of infection, and that the body condition of individuals declined as these lesions began to appear. However, once lesions were present, the survival time of individuals was not significantly shorter than in asymptomatic animals (Burthe et al., 2008).

1.3. Pathology of vTB in field voles

The course of *M. microti* infections in naturally-infected field voles has been studied in some detail. Wells (1946) performed post-mortem examination for macroscopic lesions (using a x10 dissecting microscope) of almost 900 field voles and reported encountering two courses of disease evolution. In the majority of cases he concluded that infection began in the lymphatic system then spread to other sites, most frequently the lungs. However, he also noted that infection in some animals was characterised by infiltration of the subcutaneous tissue,

usually in the axilla, groin and between the scapulae, often with further spread to the lungs and other organs. This form of interscapular infection was fairly common, and was considered noteworthy by Wells, who described the subcutaneous site of infection as being made up of white necrotic material swarming with acid-fast bacilli, with little evidence of lymphoid tissue. He also noted that spreading infection from this site through the outer layers of the skin led to the development of the characteristic external ulcerations by which vTB had first been recognised (Wells, 1937). Robb-Smith (1946) produced a more detailed pathological report that accompanied Wells' paper (1946), describing caseating necrotic lesions with calcification in the subcutaneous tissue and the muscle layers. He observed that the lesions were surrounded by macrophages, plasma cells and lymphocytes, with few multinucleated giant cells.

More recently, Cavanagh and colleagues (2002) briefly reported the pathology of the dermal lesions of *M. microti*-infected voles, describing "exudative" tuberculosis with necrotic macrophages and large numbers of intracellular bacteria and "proliferative" tuberculosis with fewer intracellular mycobacteria. Examination of internal tuberculous lesions also confirmed a granulomatous pathology typical of MtC infections.

The course of vTB has also been studied in experimentally infected animals. Wells (1946) reported that voles inoculated artificially via intraperitoneal injection were all progressively affected. Microscopically, caseation within the lymph nodes contained necrotic cellular material with vast number of acid fast

bacilli. Epithelioid cells and occasional giant cells encompassed the necrotic tissue.

More recently, Manabe and colleagues (2002) reported markedly different infection course in BALB/c mice and *Microtus ochrogaster* voles. Whereas the mice remained relatively healthy during the study, the condition of the voles deteriorated dramatically, developing extensive caseous lesions containing acid-fast bacilli and macrophage infiltrations.

1.4. *M. microti* pathogenicity

The mechanisms by which *M. microti* is able to infect and persist within small mammals (and other, accidental, mammalian hosts) have yet to be explored. However, as the gene content of *M. microti* is so similar to that of other MtC members, it is likely that they all share common modes of virulence. Recent analyses of the *M. microti* genome have revealed the absence of some genes that have been implicated in *M. tuberculosis* virulence. Brodin and colleagues (2002) identified 10 regions of difference between the genomes of the type strains of the two species. One region missing from the *M. microti* genome overlapped with the RD1 deletion in the *M. tuberculosis* BCG vaccine strain, a deletion that is assumed to be involved in its attenuation. Also missing from the *M. microti* genome were the some of the genes encoding the early secretory antigenic target (ESAT-6) family proteins, which have been shown to be potent T-cell antigens, and genes encoding proteins with Pro-Glu (PE) and Pro-Pro-Glu (PPE) N-terminal motifs. These proteins are thought to be of immunological

importance as a source of antigenic variation (Cole et al, 1998) and knockout of PE-PPE encoding genes leads to attenuation of *M. tuberculosis* in a murine model (Camacho et al., 1999). Thus, the absence of ESAT-6 and PE-PPE proteins may play a part in the attenuation of *M. microti* for some species (Garcia-Pelayo et al, 2004).

Interaction with macrophages is thought to play a key role in host exploitation by all MtC species, with the development of granulomas as much a characteristic of vTB pathology as that observed for human tuberculosis. Work in the 1970s and 1980s, outlined above, explored the interaction between *M. microti* and macrophages suggesting that inhibition of phagosome-lysosome fusion was a key stage in this process, as observed for other MtC species (Hart et al., 1987). *M. tuberculosis* bacteria interact with recognised macrophage surface receptor molecules, including complement receptor 3, and are then internalised into phagosomes (Ernst, 1998). However, normal maturation of this vacuole is disrupted. Studies have shown that molecules such as major histocompatibility complex (MHC) class I and II complexes and coronin 1, that are normally lost with maturation, continue to be expressed by *M. tuberculosis* containing phagosomes (Ferrari et al, 1999) and have been implicated in the prevention of phagosome-lysosome fusion. Although some lysosomal glycoproteins appear to enter the phagosome, they are evidently ineffective against the mycobacteria. The unusual lipid-rich mycobacterial cell wall may play an important role in this resistance (Glickman et al, 2000). Several studies have attempted to determine the molecular mechanisms possessed by *M. tuberculosis* and other

Mycobacterium species that underlie this parasitism. In addition to cell wall lipids, a number of proteins are involved in the arrest of phagosomal maturation (Stanley et al., 2003). Recent micro-array based studies indicating that *M. tuberculosis* expresses a significant "differential intraphagosome transcriptome" affecting about 600 genes (Schnappinger et al., 2003). Amongst these were the 50 or so members of the *dosR* regulon, a regulon influenced by signals such as low oxygen and nitrous oxide (Park et al., 2003).

1.5. *M. microti* as a pathogen of medical and veterinary importance

Although several early studies demonstrated the susceptibility of various mammal species to experimental infections due to *M. microti*, natural infections were only reported in small woodland mammals (see above). However, in 1958, Wagner and colleagues isolated a mycobacterium they referred to as the "dassie bacillus" from Cape hyraxes (*Procavia capensis*) in South Africa and, on the basis of its low virulence in various laboratory animals, considered it to be an attenuated strain of *M. microti*. This identification was supported by Smith (1960) on the basis of morphology and growth requirements, although Wagner and Bokkenheuser (1961) did find some differences in growth requirements between the "dassie bacillus" and *M. microti*. In 1994, Cousins and colleagues reported the isolation of a MtC strain from the lungs of a tuberculous hyrax (*Procavia capensis*) imported into Perth Zoo, Australia from South Africa, and used a variety of molecular methods to compare it to *M. microti* and the "dassie bacillus". Restriction fragment length polymorphism analysis indicated that their isolate was most similar to the dassie bacillus, but quite distinct from *M. microti*.

A similar organism was isolated from hyraxes imported into Canada (Lutze-Wallance et al., 2006) and spoligotyping (see below) indicated it to be quite distinct from *M. microti*. Parsons and colleagues (2008) reported tuberculosis in free-living Cape hyraxes and again used spoligotyping to show that the *Mycobacterium* strains involved in this infection were distinct from *M. microti* and other recognised MtC members. However, others (Guden et al., 2008) have suggested Cape hyrax-associated mycobacteria are strains of *M. africanum* on the basis of the performance of isolates in the commercial GenoType MTBC test.

Away from this confusion, other case reports of non-rodent infections by (apparently) "true" *M. microti* strains have been published. Huitema and Vloten (1960) described the presence of *M. microti*-like organisms in tuberculous lesions in the lungs and lymph nodes of a cat, and Huitema and Jaartsveld (1967) described a similar presentation in pigs surveyed immediately after slaughter in laryngeal, submandiblar and mesenteric lymph nodes. Much more recently, *M. microti* (confirmed by spoligotyping) was also isolated from pigs possessing calcified granulomas in submandibular lymph nodes (observed on slaughter) in the UK (Taylor et al., 2006). In 1970, Pattyn and colleagues reported a presumptive *M. microti* infection in a llama *(Lama vicugnain)* in a Belgian zoo, based on the observation of curved, hooked and S-shaped acid-fast bacilli in lesions of the lung, kidney, liver and intestine. Subsequently, *M. microti* infections have been encountered in two members of a different llama species (*Lama lama*) living in a Swiss zoo with generalised tuberculosis (Oevermann et al., 2006). The identity of the infectious agent was confirmed by spoligotyping.

In 1998, Kremer and colleagues reported spoligotyping data to support the involvement of *M. microti* in disease in seven cats, a badger and a cow from the UK, and a pig, a llama and a ferret from the Netherlands. Later Jahans and colleagues (2004) described the isolation of *M. microti* from another cow. In 2004, Deforges and colleagues reported a mycobacterial isolate from a dog with lesions of acute peritonitis and, on the basis of spoligotyping, identified it as *M. microti*. Following on from the study of Kremer and colleagues (1998), Emmanuel and colleagues (2007) diagnosed *M. microti* infections in two Scottish cats, suggesting that cats, as active hunters of rodents, may be at particular risk of accidental infection. The same group (Emmanuel et al., 2007) also detected *M. microti* in a badger, a llama and a ferret from Scotland. Most recently, Henrich and colleagues (2007) reported multiple granulomas in three squirrel monkeys (*Saimiri sciureus*) living in a German zoo and identified *M. microti* as the causative agent by isolation and spoligotyping. Thus, as proposed by Kremer and colleagues (1998), "*M. microti* is (demonstratively) more widespread than previously thought".

Although *M. microti* has long been perceived as being, at worst, of limited virulence for humans, and indeed has been successfully employed as a vaccine against *M. tuberculosis*, its ability to cause disease in humans has recently become apparent. In 1998, van Soolingen and colleagues retrospectively obtained a diagnosis of *M. microti* infection in four Dutch patients by using newly developed molecular identification tools. Three of the four patients were immunocompromised and suffered from generalized forms of tuberculosis. The

fourth patient was a 34-year-old immunocompetent male with a persistent cough and undefined radiographical abnormalities. The source of these infections was not clear. In one case, the authors found circumstantial evidence for transmission from rodents to a human, because this patient stored his dialysis fluid bags under non-hygienic conditions, where contamination with faecal material from mice or voles was likely to occur. For one of the other patients zoonotic transmission is also conceivable, as an infestation of mice occurred in his house a few months prior to the manifestation of tuberculous symptoms. For the two remaining patients no direct indications for zoonotic transmission were found, although one of the patients, a 41-year-old immunocompetent male, was living in a caravan camp, where the chances of contact with the habitat of mice may be greater than in an ordinary household. In 2000, Neimann and colleagues described a further two cases of *M. microti* infection causing pulmonary tuberculosis (TB) in HIV-seronegative immunocompetent patients in Germany. The isolates were identified as *M. microti* by spoligotyping. No source of infection could be identified for either patient. Another case of *M. microti* induced tuberculosis was reported in a German HIV-infected patient in 2001 (Horstkotte et al., 2001). *M. microti* has also been implicated in immune restoration disease, a condition associated with highly active antiretroviral treatment (HAART) for HIV infection. Diagnosis was made in a UK patient undergoing HAART presenting with breathlessness, nausea, diarrhoea and fever, and enlarged lymph nodes were evident in the right axilla and over the left parotid gland. A chest radiograph showed increased shadowing and a computed tomographic scan of the chest showed a large solid mass in the

upper left chest, reported as "almost certainly a solid neoplasm". Fortunately his symptoms resolved with appropriate treatment (Jenkins et al., 2006). Recently, retrospective diagnosis of *M. microti* infection was made for four Scottish patients with pulmonary tuberculosis, three of whom were immunocompetent. The authors indicated that inherited defects of interleukin receptor function are known to specifically predispose to intracellular infections, particularly mycobacterial infection, therefore, some persons with apparently normal immunity infected with *M. microti* may in fact have undetected specific immune defects (Emmanuel et al., 2007).

Several of the reports described above suggested that the underestimation of the prevalence of the *M. microti* in human cases may have been due to the unavailability (until recently) of suitable tools for species identification and the slow growth rate of *M. microti* in vitro. Many of the standard operating procedures used in tuberculosis diagnostic laboratories discard cultures after four weeks incubation, by which time they are considered negative for *M. tuberculosis*. However, *M. microti* takes at least eight weeks to grow. (van Soolingen et al., 1998).

1.6 General microbiology

Like other members of the MtC, *M. microti* is a curved or rod–shaped (0.2-0.6 x 1.0-10.0 μm) acid-fast bacterium. It has long been suggested that, when observed in infected tissue, *M. microti* has a very characteristic morphology, appearing sickle-shaped, or spiral, or S-shaped, or even in a complete circle.

However, this pleomorphism is generally lost on axenic culture (Wells, 1946). The isolation of *M. microti* requires great patience, with colonies taking at least eight weeks and often as long as fifteen weeks to become visible on Lowenstein-Jensen (LJ) medium. Work by Pattyn and colleagues (1970) characterised the phenotype of the species and demonstrated phenotypic differences among MtC members. Subsequently, Tsukamura and colleagues (1985) applied a numerical taxonomic approach, incorporating 88 phenotypic characters, in an attempt to delineate the MtC species. Four *M. microti* strains were included in this analysis, but these, as with strains of the other MtC species, did not group together in a discrete cluster.

Although LJ medium is perhaps the most widely used medium for cultivation of *M. microti*, others have been described and used. Middlebrook 7H9 Medium (Manabe et al., 2002), MB/BacT media (Cavanagh et al., 2002), modified Dubos medium (Wagner &Kubica 1980), Accuprobe culture (van Soolingen et al., 1998), Dubos broth and Dubos medium with albumin (Frota et al., 2004) have all been used and reportedly work adequately.

1.7 Genetic homology of MtC members

Genetic studies have demonstrated that the species within the MtC are extremely similar to one another. Imaeda (1985) compared 25 strains of *M. tuberculosis, M. microti, M. bovis* and *M. africanum* by measuring their genome size and the G+C% content of their genomes, and carrying out whole genome restriction digests and DNA:DNA hybridisation. For the first three of these

measurements, the 25 strains were virtually indistinguishable, and for DNA:DNA hybridisation the strains shared over 85% relatedness, a level far greater than the 70% threshold usually used in bacteriology to delineate species, and thus, using this criterion alone, all MtC members would be considered members of the same species. Furthermore, in their survey of 16S rRNA sequence diversity among *Mycobacteria* species, Böddinghaus and colleagues (1990) reported that the 16S rRNA sequenced of the MtC members were almost identical. In 1997, Sreevatsan and colleagues sequenced two megabases in 26 structural genes or loci in 842 strains belonging to the MtC, including nine strains of *M. microti*, revealing remarkably restricted variation. What diversity there was between species was largely attributed to the presence, absence and/or copy number of insertion sequences rather than mutations in structural genes. Further application of comparative genomics has supported the evolutionary homology of MtC members but more thoroughly differences between them. In 2002, Brodin and colleagues used pulsed field gel electrophoresis to examine differences in a set of bacterial artificial chromosome *M. microti* clones, revealing 10 regions of difference (RD) of between two kilobases (kb) and 14.6 kb in size between *M. microti* and *M. tuberculosis*. One of these RDs, $RD1^{mic}$ contained genes that, in *M. tuberculosis*, encode the potent antigens ESAT-6 and culture filtrate protein (CFP-10), and thus its absence from *M. microti* was thought to account for the apparent attenuation of the species. Brodin and colleagues (2002) also compared RDs among nine *M. microti* strains, six of which were isolated from human beings and three of which were isolated from voles. Interestingly, the vole isolates all lacked three RDs that were present in all six

human isolates. In 2004, two microarray-based analyses of *M. microti* gene content were published. Frota and colleagues (2004) compared 12 *M. microti* isolates from a variety of hosts including voles and human beings and identified 13 deletions that included the 10 RDs described earlier. Again, not all *M. microti* strains lacked all the RDs, and only one, RD3, was absent in all strains examined. The authors speculated that that there may be an association between RD presence/absence and host range, but that it was difficult to ascribe virulence to any particular *M. microti* RD pattern. Garcia-Pelayo and colleagues (2004) carried out a very similar study that compared the gene content of *M. tuberculosis* H37Rv strain with three *M. microti* strains. They too were able to confirm the earlier findings of Brodin and colleagues (2002) and identify a novel RD, termed MiD4, that contained genes encoding proteins belonging to the ESAT-6 family and the similarly antigenic PE-PPE family. Although all these studies were focused on identifying genetic differences among *M. microti* isolates or differences between *M. microti* and *M. tuberculosis*, all emphasised the extreme limitation of these differences; the underlying observation is that genomes of members of the MtC share greater than 99.9% sequence identity.

Analysis of the distribution of RDs among MtC members has provided the basis for the current evolutionary model for the complex in which progenitor strains diverged into three distinct lineages, the oldest leading to *M. canetti*, the second carrying *M. tuberculosis*, and the most recent carrying *M. africanum*, *M. microti* and *M. bovis* (Brosch et al., 2002). A similar study, carried out at the same time, reached similar conclusions, but extended the analysis to demonstrate the

divergence of *M. bovis* from *M. africanum* and *M. microti* (Mostowy et al., 2002). This work has now been extended even more by examining the patterns of loss of spoligotype spacer units and the distribution of single nucleotide mutations to produce a robust model in which all MtC species are distinguished from one another (Smith et al., 2006).

1.8 Molecular delineation of MtC strains

Several approaches to delineating genotypes among MtC species have now been described, and among these, three methods have been widely adopted, namely IS6110 fingerprinting, spoligotyping and, most recently, mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing (Gillespie et al., 2003). The relative usefulness of each of these methods in differing epidemiological scenarios has been explored, particularly for *M. tuberculosis* and *M. bovis* infections (Gillespie et al., 2003). Also the use of these protocols for members of the MtC has provided powerful tools to identify organisms that resemble the vole bacillus, and thus those strains have been classified as *M. microti* (Niemann et al., 2000; van Soolingen et al., 1998).

IS6110 fingerprinting remains the "gold-standard" against which other approaches are compared and is widely used in reference laboratories around the world. This approach uses the IS6110 insertion element as a probe in a Southern blot against electrophoretically resolved restriction enzyme -digested genomic DNA (Eisenach et al., 1990; Hermans et al., 1990; Thierry et al., 1990). The difference in the location and number of copies of this element is a source of

polymorphism among MtC strains. Most strains of the MtC species carry between five and 15 copies of the insertion element in different positions of the genome. For *M. tuberculosis, M. africanum* and *M. bovis* the approach usually offers a high degree of discrimination. IS6110 fingerprinting has been standardised so results from different laboratories can be meaningfully compared (van Embden et al., 1993). Nonetheless, this approach is not without drawbacks as it is complex, time consuming, and requires reference facilities where isolates can be safely grown and where high-quality DNA extractions can be prepared. Furthermore, in some communities, low IS6110 copy number strains make up a significant proportion of the *M. tuberculosis* population, and, occasionally strains will lack the insertion element altogether (Haddad et al., 2004)

IS6110 typing has been applied to *M. microti*. Van Soolingen and colleagues (1998) examined 13 isolates, including five isolated from UK field voles in the 1930s, finding that they possessed distinct yet highly similar profiles. The five UK vole isolates had slightly different profiles from one another. Cavanagh and colleagues (2002) added to this by obtaining data for four recent vole isolates. The profiles of these isolates were most similar to, but nonetheless different from those obtained from those isolates recovered from UK voles in the 1930s. Emmanuel and colleagues (2007) obtained IS6110 profiles from Scottish *M. microti* isolates made from various animal species, including human beings, but not voles. These profiles appeared to be similar to, but distinct from, those reported earlier (although precise comparison of profiles was difficult).

The technical difficulties associated with IS6110 typing provoked the development of other, less demanding approaches to MtC strain delineation. Among these, spoligotyping has emerged as the technique of choice for exploring global and national diversity and molecular epidemiology of MtC members. Spoligotyping is a define PCR-based method that exploits polymorphism at a hypervariable locus known as the chromosomal direct repeat (DR) locus, which is present in the genomes of all MtC species and which contains a variable number of short direct repeats interspersed with non-repetitive spacers. Strains are distinguished by their spoligotype patterns, characterised by the number and identity of non-repetitive spacers (van Soolingen et al., 1995). The presence of spacer sequences varies among strains and are visualised by a spot on a fixed site of a hybridisation membrane. Unlike IS6110 typing, the results of spoligotyping are totally transferable, meaning that comparison of results obtained at different times in different places is straightforward. This characteristic has permitted the establishment of an international MtC spoligotyping database (http://www.pasteur-guadeloupe.fr/tb/bd_myco.html). The most recent iteration of this database, SpolDB4 (Brudey et al., 2006) contains data for almost 40,000 isolates, which have been delineated into over 5,000 different spoligotypes. Spoligotype results are available for about 40 *M. microti* isolates, most of which, including all those from UK voles, possess the "classical" ST539 spoligotype. This spoligotype is characterised by the presence of only spacers 37 and 38 so lacks almost all the 43 non-repetitive spacers that are present in the most complete spoligotypes

(Kremer et al., 1998; Cavanagh et al., 2002; Emmanuel et al., 2007). However, a minority of *M. microti* isolates from other hosts possess other spoligotypes that include other spacers in addition to 37 and 38. One of these spoligotypes is referred to as "llama type" possessing spacers 4-7, 23, 24, 37 and 38 (Kremer et al., 1998) and has been isolated from llamas, cats and humans (Kremer et al., 1998; Emmanuel et al., 2007).

In most situations, the sensitivity of spoligotyping is lower than that of IS6110 typing. However, when MtC strains have a low copy number of IS6110 insertion sequences in their genomes, spoligotyping has better resolving power (Kamerbeek et al., 1997). For *M. microti*, about 25 isolates have been typed by both methods and these have been delineated into about 20 IS6110 profiles and only five spoligotypes (Kremer et al., 1998; Cavanagh et al., 2002; Emmanuel et al., 2007). However, criteria for confidently distinguishing between very similar IS6110 profiles have not been evaluated for *M. microti* as they have for other Mtc species. Many of the slightly different *M. microti* profiles may well be considered "not confidently distinguishable" if these criteria were rigorously applied (van Soolingen et al., 1998).

The most recently developed approach to MtC strain delineation is variable number tandem repeat (VNTR) analysis. Examination of MtC genomes revealed the existence of much polymorphism within regions of tandemly repeated DNA. Polymorphism at a tandem repeat locus can occur either as a result of nucleotide sequence changes between individual repeat units or, more often, as a result of

variation in the number of repeat units, thereby creating genetic variants. VNTR analysis exploits variation in the number of repeat units and, as with spoligotyping it is PCR based and produces results that are easily transferable. Many different tandem repeat loci (sometimes referred to as *Mycobacterium*-interspersed repetitive units, or MIRUs) have been discovered in the genomes of MtC members, and about 30 have been used in MtC diversity studies (e.g. Smittipat & Pallittapongarnpim, 2000; Supply et al., 2000). A panel of six loci has been adopted for international studies of *M. tuberculosis* diversity (Frothingham et al., 1998) and the performance of this panel relative to spoligotyping and IS6110 profiling has been well evaluated (Kremer et al., 1999). These and other VNTR panels have also been applied to the delineation of strains of other MtC species, in particular *M. bovis* (e.g. Roring et al., 2002). In general, the combined discriminatory power of spoligotyping and VNTR analysis is about the same as IS6110 profiling, although different approaches perform with different degrees of power in different epidemiological circumstances (e.g. Kremer et al., 1999; Allix et al., 2006).

1.9 Laboratory diagnosis of tuberculosis

The isolation of *M. microti* remains the most reliably sensitive method available for the diagnosis of vTB. Processing of clinical material for culture usually involves firstly alkaline decontamination of samples, then inoculation of LJ medium, usually poured as slopes in screw-top glass tubes. *M. microti* is particularly slow-growing, hence inoculated agars need to be incubated for at least 12 weeks. Concern about the detrimental effect of the decontamination

step on recovery rates have been raised (Pfyffer et al, 2003), but given the fastidious nature of *M. microti*, ensuring the removal of faster growing bacteria from the inoculum is essential.

Although Cavanagh and colleagues (2002) used PCR to demonstrate the presence of *M. microti* in DNA extracts prepared from skin lesions, lymph nodes and internal caseous abscess tissues of infected voles, the use of PCR for vTB diagnosis in living animals that do not possess external signs of disease has not yet been described. PCR-based methods are becoming increasingly used in certain settings for the diagnosis of infections caused by other MtC species. *M. tuberculosis* DNA has been detected in a wide range of clinical samples collected from infected individuals. For example, Burggraf and colleagues (2005) reported the usefulness of numerous respiratory and non-respiratory clinical samples for the detection of *M. tuberculosis*. Non-invasive samples have also been used for diagnosis of tuberculosis in cattle. Vitale and colleagues (1998) tested milk samples for the presence of *M. bovis* DNA. PCR has also been employed to detect *M. bovis* DNA in environmental samples, for example in soil taken from farm with a history of bovine tuberculosis (Young et al., 2005).

Various studies have evaluated different protocols for DNA extraction from different tissues. Buck and colleagues (1992) and Kirschner and colleagues (1993) reported the value of mechanical disruption of clinical samples whereas others have tested a variety of biochemical methods (e.g. de Lamballerie et al., 1992; Wilson et al., 1993; Amicosante et al., 1995; Bodmer et al., 1997).

Numerous different PCRs have been described and evaluated for MtC infection diagnosis, targeting a variety of loci around the genome (e.g. Plilkaytis et al., 1992; Comincini et al., 1998; Lee et al., 2000). More recently, real-time PCR technology has been applied to MtC DNA detection in clinical material (e.g. Torres et al., 2000; Taylor et al., 2001; Broccolo et al., 2003; Parra et al., 2007; Taylor et al., 2007), although the performance of both forms of PCR in this role remains imperfect (e.g. Bruijinesteijn et al., 2004; Parra et al., 2007; Taylor et al., 2007). As expected, the sensitivity of PCR appears to be dependent on the sort of clinical sample collected. For example, Wards and colleagues (1995) suggested that in comparison to the large number of bacilli present in the sputum of *M. tuberculosis* infected patients, the intensity of *M. bovis* infection in various bovine tissues was low.

Serological assays that detect circulating antibodies have shown inadequate sensitivity and specificity for early diagnosis of MtC infections (Rhodes et al., 2000). Their shortcomings are due to the nature of the immunological response generated in MtC infections, which is dominated by a Th1 cellular response, although this defence is exploited by MtC bacteria to generate chronic inflammation and persistence (Plackett et al., 1989). Antibodies are produced in abundance with the progression of disease (Dlugovitzky et al., 2000). The dominance of cellular immunity during the early stages of MtC infection has necessitated the development of appropriate assays such as the tuberculin skin test (TST) and interferon gamma (IFN-γ) (Vordermeier et al., 2004). The TST was developed over 100 years ago and determines cell- mediated immune response

by measuring the delayed-type hypersensitivity (DTH) response to intradermal inoculation of tuberculin purified protein derivative (PPD) (Lalvani et al., 2007), which is a crude mixture of antigens many of which are shared between members of the *M. tuberculosis* complex and other *Mycobacteria* species (Andersen et al., 2000). In the UK, the TST is now used to determine *M. tuberculosis* and *M. bovis* infection status in humans and cattle respectively. TST testing is at the centre of the UK Government's bovine TB eradication programme and is the international standard ante -mortem diagnosis of bovine TB in cattle herds and individuals. The Office International des Epizooties has prescribed the skin tests for the international trade of cattle (Anon, 2004).

IFN-γ is the cytokine predominantly released by Th1 lymphocytes, hence is an ideal target for determining Th1 activity. IFN-γ assays exploit the principle that T cells of infected individuals produce IFN-γ when they re-encounter mycobacterial antigens (Andersen et al., 2000). IFN-γ detection and quantification assays for the early diagnosis of MtC infections were first developed during the early 1990s by scientists working with *M. bovis* (Rothel et al., 1990; Wood et al., 1990). A variety of antigens have been incorporated into IFN-γ assays ranging from crude whole cell preparations to specific recombinant proteins. The specificity of IFN-γ assays has been enhanced through the incorporation of antigens such as ESAT-6 and CFP10, which are recognised by T cells but which are not present on environmental *Mycobacterium* species or on the attenuated *M. bovis* BCG strain widely used for vaccination (Mahairas et al, 1996; Lalvani et al., 1997; Vordermeier et al., 1999; Pollock et al., 2000; Buddle et al., 2001; Chapman et al.,

2002; Waters et al., 2004). Several commercial IFN-γ detection assays are now available. Comparison of the performance of the TST and IFN-γ detection assays for MtC infection diagnosis has been reported. IFN γ-based diagnostics appear to identify infected individuals at an earlier stage of infection than TST assays (Pollock et al., 2005). IFN γ assays also appear to detect infections in some individuals that do not respond to the tuberculin test (Nell et al., 1994).

1.10 Aims of this study

The intention of this study was to build on previous work carried out at the University of Liverpool exploring the biology of *M. microti* infections in UK field voles. The majority of this work has focused on populations of field voles inhabiting Kielder Forest on the England/Scotland border. Since 2001, these populations and their *M. microti* infection status has been monitored in surveys at six-monthly intervals in which rodents are trapped at numerous sites across the forest and their infection status determined. For some spatial surveys (1, 5, 6 and 7) infection status was determined solely by the observation of external skin lesions, but for others (2-4, 8-12), animals were collected and stored to allow diagnosis to be made *post-mortem* by a combination of pathological and microbiological diagnostics. Prior to this study, these diagnostics had been performed only on animals captured in surveys 2 to 4 (March 2002 to September 2003). The principal aims of the study were as follows:

i. to determine the infection status in voles trapped during spatial surveys 8 to 12 (March 2005 to March 2007) using established post-mortem diagnostics.

ii. to describe the pathology of vole tuberculosis.

iii. to select, adapt and evaluate a PCR-based approach for the diagnosis of *M. microti* infections.

iv. to apply molecular typing methods to a representative sample of *M. microti* isolates in order to explore the molecular epidemiology of vole tuberculosis in Kielder Forest field voles.

v. to determine the suitability of an established murine IFN γ- detection assay for use in field voles.

It was hoped that the result of these efforts would facilitate future studies, in particular by providing a means of ante-mortem diagnosis of vTB. Furthermore, more sensitive diagnostics, and/or the ability to determine infection status sooner after acquisition of infection, would be extremely useful for efforts to better understand the epidemiology of vTB.

Chapter 2.

M. microti infections in field voles at Kielder Forest

2.1 Introduction

M. microti infections have been recognised in field voles inhabiting Kielder Forest, which lies at the England-Scotland border in the county of Northumberland, since before the Second World War (Wells, 1937). Kielder Forest is England's largest forest, covering about 620 square kilometres. However, the majority of the forest is managed by the Forestry Commission for commercial spruce production, hence, at any one time, a quarter of the area of the Forest is open space. The felling of trees generates clear-fell patches of 5-12 hectares, which are subsequently replanted and progress through grassland and thicket stage after 12-15 years. During this period, clear-cut patches provide excellent habitat for field voles. Within each clear-cut, the field vole population density can reach in excess of 700 voles per hectare (Burthe et al., 2006). The bulk of Kielder Forest lies within three valleys, Kershope to the south west, Kielder in the middle and Redesdale to the north east (Figure 2.1). In was in the first of these valleys that Elton (1924; 1942) carried out fieldwork that resulted in his description of small rodent population cycles during the 1920s, and where, in the following decade, vole tuberculosis (vTB) was first recognised by members of the Bureau of Animal Population in the University of Oxford (Wells, 1937).

Wells (1937) reported that in January 1937 a wild vole that had been held in captivity for one month developed, then succumbed, with symptoms of a tubercular disease. Diagnosis was based on post-mortem examination which showed "*caseous areas throughout the subcutaneous tissues of the body, involving the glands of the neck, axillae, inguinal region, and back, with*

ulceration of the skin round the right pinna; both lungs contained caseous areas with sharply defined edges; the mediastinal and mesenteric glands were much enlarged and caseous; the spleen was enlarged. The caseous material in the subcutaneous tissues contained a very large number of acid-fast bacilli, which have the morphology of Mycobacterium tuberculosis. *The caseous areas in the lungs similarly contained a great number of acid-fast bacilli, but there was in section very little cellular reaction"*. The caseous material from this animal was inoculated onto suitable media and after six weeks incubation colonies with a morphology similar to that of *M. tuberculosis* appeared (Wells, 1937). Wells goes on to report the subsequent detection of macroscopic tuberculous lesions in 134 other field voles submitted to him from various parts of Great Britain. Furthermore, inoculation of an emulsion of caseous material into laboratory-reared voles, guinea pigs and rabbits resulted in systemic disease in the former two species, localised disease in the latter, and reactivity to the tuberculin skin test in all guinea pigs and rabbits inoculated (Wells, 1937). Subsequently, Wells performed post-mortem examinations on a far larger number of field voles and produced a far more comprehensive gross pathological study of vTB. This study was published in 1946 (Wells, 1946) and was based on detection of macroscopic lesions in the bodies of 882 infected animals. Lymph nodes were the most frequently affected tissues (92% of animals), and infections were observed in lymph nodes throughout the body. The sub-maxillary lymph nodes were those most often affected (66%) although often more than one site was involved (74% of animals). Pathology was also observed in other organs including, most commonly the lungs (54% of animals) (Wells, 1946). Wells (1946) highlighted the

unusual presentation of lesions in the interscapular region (seen in 31% of animals) which consisted of white necrotic material containing numbers of acid-fast bacilli, with little or no evidence of granulation tissue. He reported that interscapular infections spread in the subcutaneous tissue provoking ulceration through the skin. On the basis of his observations, Wells (1946) speculated that *M. microti* caused two types of disease in field voles: the first type of disease was characterized by primary infection of the lymph nodes with early spread to other organs, especially the lungs. The second type was characterised by lesions in the subcutaneous tissue, usually in the axilla, groin and between the scapulae, often with further spread to the lungs, liver and spleen. Robb-Smith (1946) produced a more detailed pathological report that accompanied Wells' paper (1946), describing caseating necrotic lesions with calcification in the subcutaneous tissue and the muscle layers. He observed that the lesions were surrounded by macrophages, plasma cells and lymphocytes, with few multinucleated giant cells.

A more recent study of wild rodent populations, including wild voles, field voles and wood mice, was carried out in Northumberland and Cheshire (Cavanagh et al., 2002) and detected external tuberculosis lesions in 8% of the animals. These were represented by an ulcerative dermatitis similar to that described by Wells (1937, 1946). In a post-mortem study of snap-trapped field voles (Cavanagh et al., 2002), a 21% prevalence of tuberculosis based on external and internal tuberculous lesions was identified, compared to just 7% based on external signs only (ulcerative dermatitis). Microscopically, Cavanagh and colleagues (2002) identified the dermal lesions as exudative tuberculosis, with necrotic

macrophages and large numbers of intracellular mycobacteria, with fewer bacteria in macrophages.

This study aimed to determine the dynamics of tuberculosis (vTB) and *M. microti* infection among Kielder field voles between March 2005 and March 2007, by diagnosing infections in animals surveyed in a structured manner at six-monthly intervals. The study also aimed to determine the distribution of infection in infected individuals on the basis of observation of macroscopic lesions and, on a subset of sample, on histopathological analysis of dissected tissues.

2.2 Materials and Methods

2.2.1 Animals

The field voles used in this study were collected from transient grassland habitats ("clearcuts") within Kielder Forest (Figure 2.1), as part of an ongoing, systematic and structured survey, described in detail elsewhere (Cavanagh et al.,2002). Briefly, field voles were surveyed biannually, in March and in September, at 27 sites across Kielder Forest District (Figure 2.1). The times were chosen to reflect differences in the vole population structure, with the March session occurring pre-breeding and samples therefore consisting of over-wintered, adult animals, and the September session occurring towards the end of the breeding season and samples dominated by juvenile animals. At each site, three rodent traps were set within 1 metre of each corner of a 15 m x 15 m square grid. Traps were pre-baited with wheat and carrots for three days, then set overnight and then checked over three consecutive days. The animals included in this study were

drawn from spatial survey (SS) 8 (March 2005), SS9 (September 2005), SS10 (March 2006), SS11 (September 2006) and SS12 (March 2007).

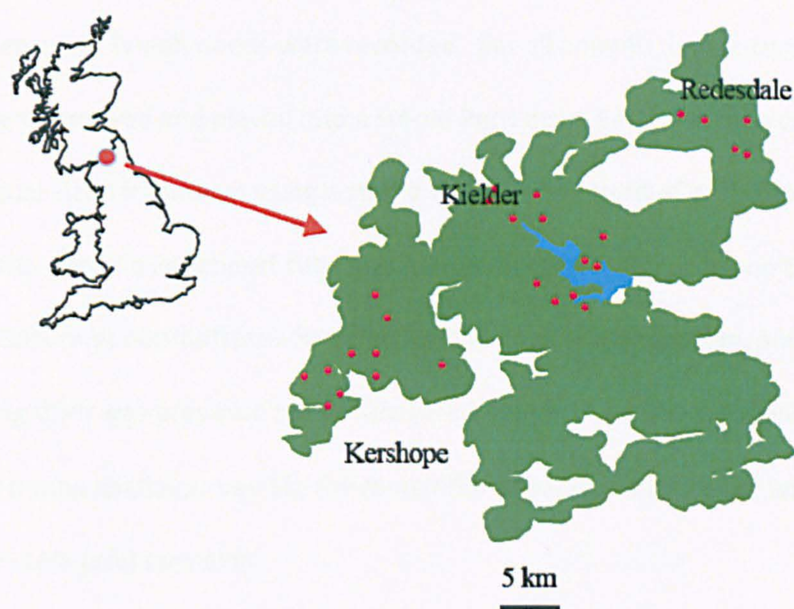


Figure 2.1 Location of Kielder Forest and relative positions of the three catchments and the 27 survey grids within each catchment.

Captured animals were removed from traps and immediately euthanized by exposure to isofluorane (Merial Animal Health, Harlow, UK) and subsequent dislocation of the cervical vertebrae. Animals were frozen as soon after killing as possible, and kept frozen (-20°C) until dissected.

2.2.2 Post-mortem examination

The body of each vole was palpated to check for lumps under the skin and internal lesions indicative of vTB (Wells, 1937). Each vole was placed on its back

and, using sterile scissors, scalpel and forceps, the body was opened in the ventral midline by a longitudinal incision from below the chin to the anus. Animals were carefully dissected and the location and appearance of any gross lesions typical of vTB (Wells, 1937; Cavanagh et al., 2002) or any abnormalities such as enlarged lymph nodes were recorded. For all animals, lymph nodes and lungs were removed and placed into a sterile Petri dish. Each was then cut into three equal-sized fragments using a sterile scalpel. One third of each tissue was placed into a sterile Eppendorf tube and frozen for DNA extraction, one third was fixed in 10% (v/v) non-buffered formalin for histological examination, and the remaining third was prepared for culture (see below). In addition, for animals trapped during spatial survey 10, the remainder of each animal's body was placed in 10% (v/v) formalin.

2.2.3 Ziehl-Neelsen (ZN) stain on lesion impression smears

Tissues from all field voles in spatial surveys 8 - 11 that exhibited gross skin or internal lesions suggestive of vTB were examined for the presence of acid-fast bacilli in impression smears. Smears were prepared from the cut surface of dissected lesions, lungs and/or lymph nodes then stained using the Kinyoun Cold method (Crookham et al., 1991). The slides were flooded with filtered Kinyoun carbol fuchsin (1 g basic fuchsin in 10 ml absolute ethyl alcohol) and incubated for 5 min, then rinsed with tap water. Slides were then flooded with 3% (v/v) acid-alcohol (3% (v/v) hydrochloric acid in 95% (v/v) ethyl alcohol) and incubated for 5 min and subsequently rinsed with tap water. Slides were counterstained with Loeffler's methylene blue (0.25% (w/v) methylene blue in 1% (v/v) acetic

acid) for 30 sec, rinsed with tap water, blotted with paper and left to dry. The slides were examined by light microscopically under an oil immersion lens (1000x magnification). Mycobacteria exhibited a red/pink staining on a blue background (Crookham et al., 1991).

2.2.4 Culture

Isolation of *M. microti* was attempted on lungs and lymph nodes removed from all field voles and was performed in a Category 3 laboratory. Tissue samples, as described above, were sliced finely with a sterile scalpel blade, placed into a sterile 1.5 ml Eppendorf tube with an equal volume of sterile phosphate buffered saline (PBS), then further disrupted and mixed thoroughly using the blunt end of a sterile inoculation loop. An equal volume of CPC decontaminant (5 g cetyl-pyridinium chloride, 10 g sodium chloride in 1000ml water) (Sigma-Aldrich Co, Gillingham, UK) was added to the sample, vortexed to mix and agitated in an incubator at room temperature overnight. Decontaminated samples were centrifuged at 3000 x g for 15 min and the supernatant discarded. Sediment was resuspended in 250 µl of distilled water and vortexed for 30 sec. Finally, 200 µl of suspension was inoculated onto Lowenstein–Jensen pyruvate-based (LJP) culture slopes (SGS Laboratory Supplies Ltd, Ellesmere Port, UK). Inoculated slopes were incubated at 37°C for two weeks, then examined and discarded if contaminated. Uncontaminated slopes were incubated for a further 26 weeks before being re-examined and incubations continued for up to 6 months after which sterile slopes were discarded and the sample scored as not infected. The

identity of isolates obtained was confirmed using a variety of molecular methods (see below and chapters 3 and 4).

2.2.5 Histological examination

Histological examination was carried out almost exclusively on tissues dissected from field voles captured during spatial survey 10, however examinations were also performed during the early stages of the study on a handful of symptomatic animals captured during spatial survey 8. Fixed tissue samples were embedded into paraffin wax by Veterinary Laboratory Services technical staff in the Faculty of Veterinary Science at the University of Liverpool, using standard procedures. Sections (3-5 μm thick) were stained with haematoxylin-eosin (HE) for histopathological assessment. From any suspected vTB lesions, subsequent sections were stained with the ZN stain to identify mycobacteria.

2.3 Results

Diagnosis of the vTB and *M. microti* infection was made based on the presence of gross and/or histological lesions consistent with tuberculosis together with the visualisation of acid-fast bacilli on ZN-stained lesion impression smears, and/or the isolation of *M. microti* by culture. A total of 1270 field voles were trapped during the five spatial surveys (Figure 2.2) and all were examined externally and internally for macroscopic lesions, and dissected tissues put up for *M. microti* culture. In addition, all 228 field voles trapped during SS10 were examined by histology.

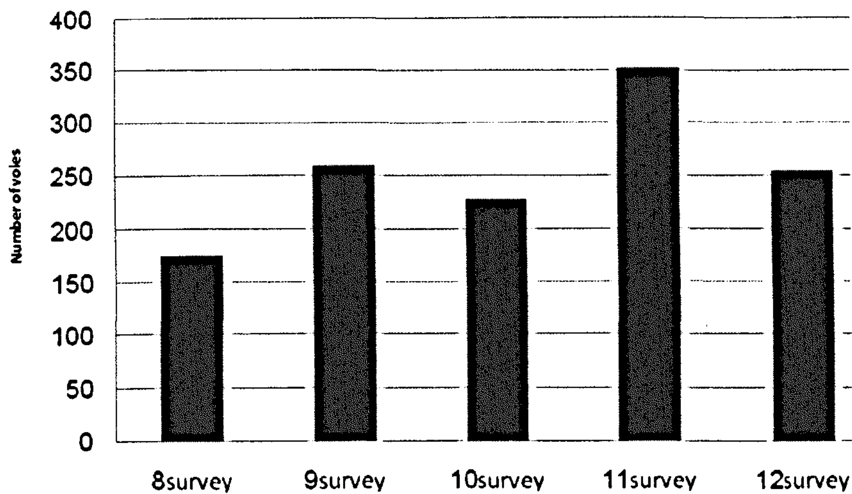


Figure 2.2 Number of field voles examined grossly and by culture in each spatial survey included in the study.

2.3.1 Prevalence and distribution of macroscopic lesions

Gross lesions consistent to vTB were detected in the skin, the subcutaneous tissue, in lymph nodes (sub mandibular, axillary and cervical lymph nodes) and lungs. Whenever gross vTB lesions were detected, these included lesions in lymph nodes, except for one case in SS9 where a gross lesion was detected in the lung only. Lesions in the skin were usually represented by hard, raised, circular scabs with a crusty surface (Figure 2.3).

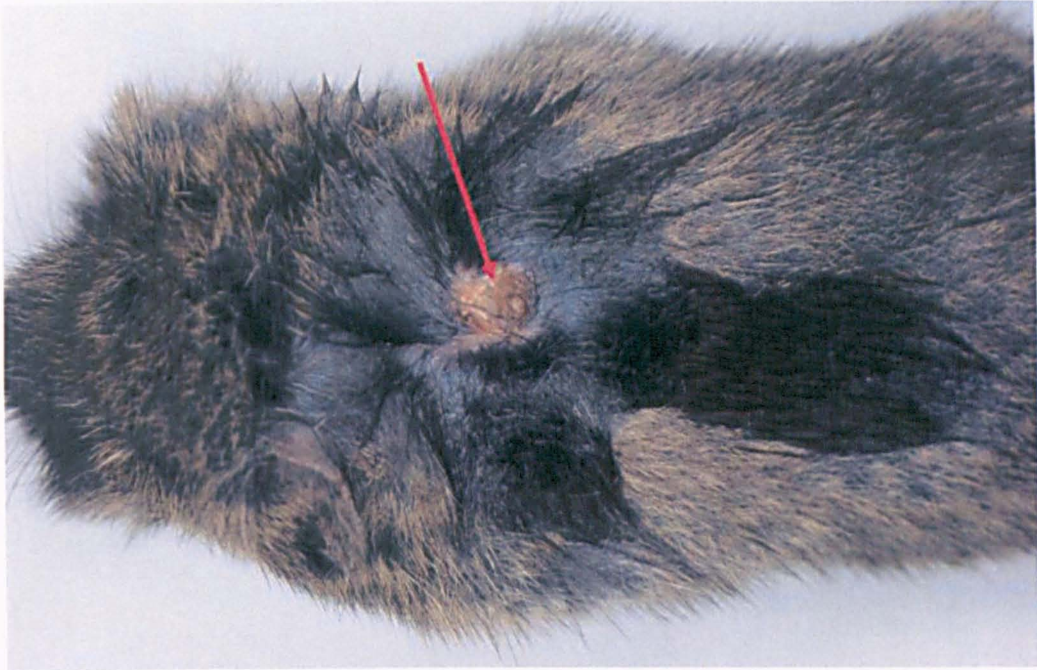


Figure 2.3 Vole with interscapular vTB lesion in the skin, represented by a raised, circular scab with a crusty surface (arrow).

These were often red-brown or dark grey/blackish in colour, and skin lumps were often yellow coloured. Skin lesions were often located on the leg, the chest and neck, dorsally in the interscapular region (Figure 2.3) and on the shoulder. Subcutaneous lesions often appeared as foci of whitish, caseous material. Gross lesions in the lungs were often represented by small white nodules that appeared calcified upon dissection (granulomas) (Figure 2.4).

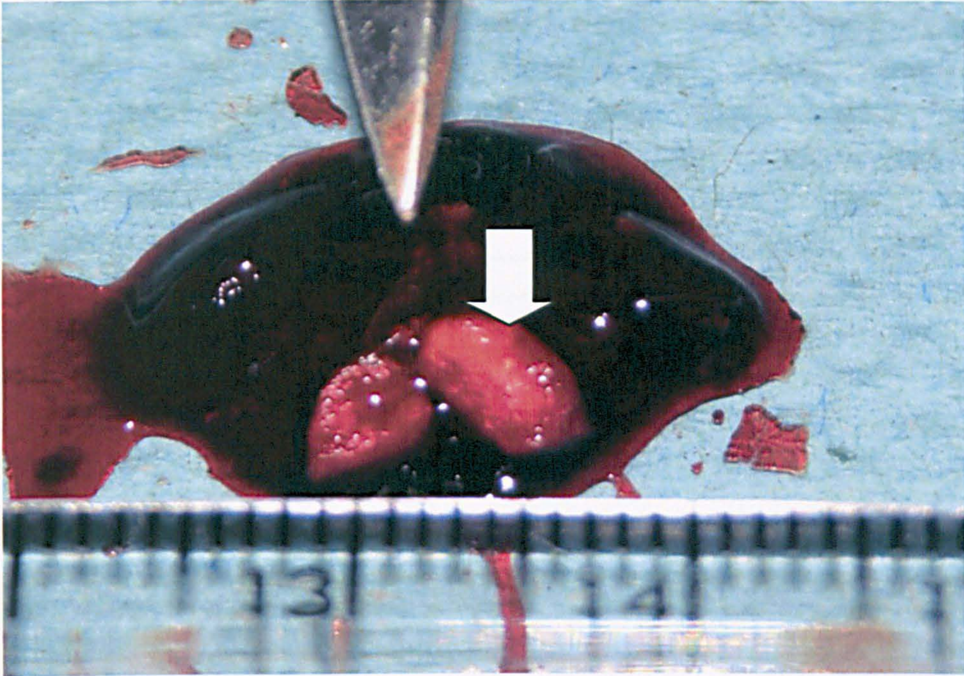


Figure 2.4 Vole lung lobe containing granulomas consistent with vTB. The cut surface (arrow) is whitish and gritty due to the mineralisation.

Affected lymph nodes were enlarged and exhibited whitish caseous material on the cut surface. A total of 68 (5.3%) of the 1270 field voles examined exhibited macroscopic lesions consistent with vTB. The prevalence of macroscopic lesions varied between surveys, ranging from <1% in SS11 to >20% in SS8.

The distribution of lesions in symptomatic field voles was explored (Table 2.1). Lymph nodes were the most common site for macroscopic lesions, with all bar one animal (99%) with lesions having lymph node involvement. Skin lesions were more common than lung lesions (21% v 13%) and no other sites were affected by macroscopic lesions.

spatial survey	animals possessing macroscopic lesions	location of macroscopic lesions (%)			
		lymph nodes	lungs	skin	other
8	37	37	3	8	0
9	8	7	4	2	0
10	11	11	2	4	0
11	2	2	0	0	0
12	10	10	0	0	0
TOTAL	68	67 (99)	9 (13)	14 (21)	0 (0)

Table 2.1 Distribution of macroscopic lesions in different tissues.

2.3.2 Observation of acid-fast bacilli in ZN-stained impression smears

Acid-fast bacilli were observed microscopically in 37 (54%) of the 68 animals that had macroscopic lesions (Figure 2.5). The percentage of positive smears from organs with gross vTB lesions varied; acid fast bacilli were detected in 54% of lymph node tissues with gross lesions, and in 98 % of lung and skin tissues that exhibited gross lesions.

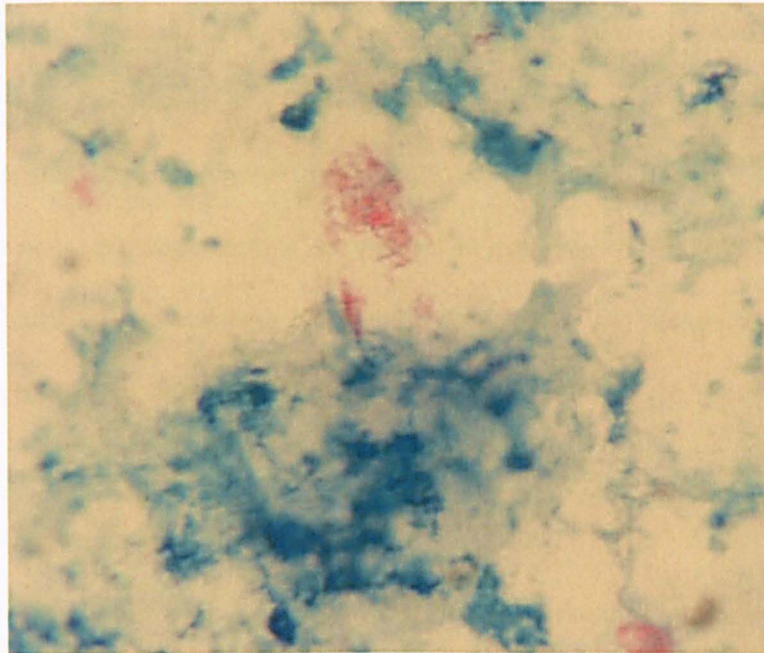


Figure 2.5 ZN-strained impression smear of lymph node containing a macroscopic vTB lesion. Acid fast bacilli appear as red bundles against dark blue tissue debris.

2.3.3 Isolation of M. microti

Colonies characteristic of *Mycobacteria* spp. were obtained from 167 of 1270 (13%) field voles. The prevalence of the *M. microti* isolation varied between surveys (Table 2.2)

spatial survey	number of isolations attempted	number of *M. microti* isolates obtained (%)
8	175	48(27)
9	260	20(7.6)
10	228	11(4.8)
11	353	11(3.1)
12	254	77(30)
TOTAL	1270	167 (13)

Table 2.2 Prevalence of *M microti* isolation in each of the five surveys

Among the 167 voles that were culture positive, an isolate was obtained from only lung tissue of 34 voles and from only lymph node tissue of 107 voles, and from both lung and lymph node tissues of 26 animals. The number of voles that exhibited no macroscopic lesions but yielded an isolate was 133 (80%). Of those, 87(66%) isolates were obtained from lymph nodes, 26 (19%) isolates were obtained from lungs and 20 (15%) were obtained from both.

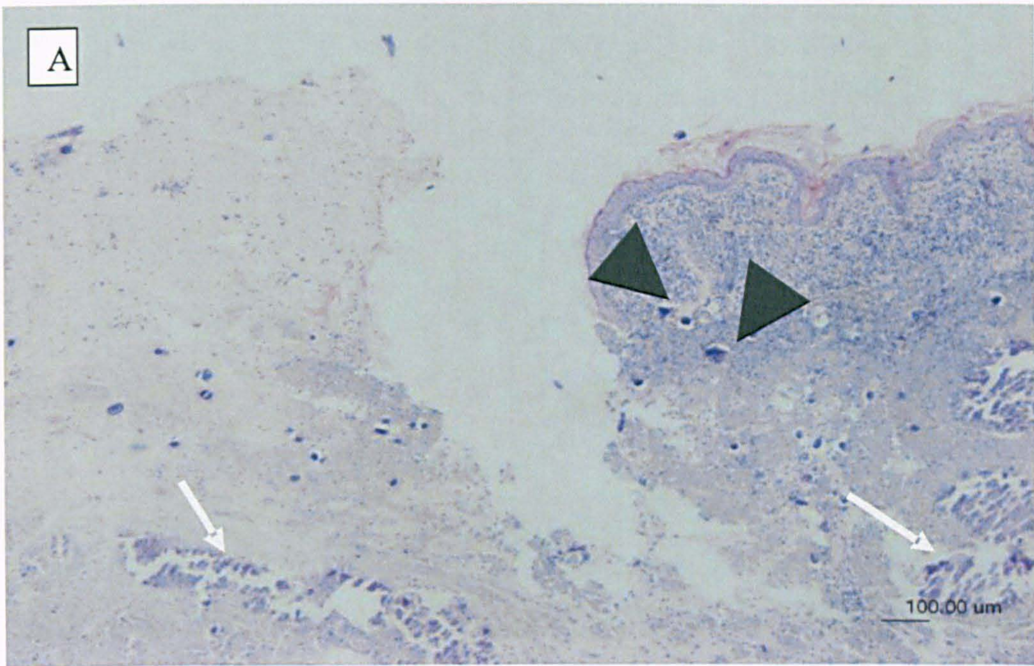
2.3.4 Histopathology

The histological examination of the suspected vTB skin lesions in three field voles captured during SS8 identified a common pathology, presenting as focal, extensive, deep ulcerative and granulomatous dermatitis, extending into the deep dermis. Extensive necrosis and multifocal mineralisation was also observed and there were often very numerous intra- and extracellular acid fast bacilli (Figure 2.7). The lesions extended into the subcutaneous adipose tissue and underlying skeletal muscle layers. The inflammatory infiltrate was dominated by macrophages, epithelioid cells and fewer multinucleated giant cells, intermingled with lymphocytes and some neutrophils.

Suspected vTB gross lesions in the lymph nodes were confirmed as a granulomatous lymphadenitis with morphological features (cellular composition, necrosis, mineralisation, presence of acid-fast bacilli) very similar to those described for the skin lesions. Suspected gross vTB lesions in the lungs were in all cases identified as a multifocal granulomatous pneumonia with variable degrees of focal necrosis and mineralisation. The number of acid-fast bacteria

within the lesions and in cells varied considerably (Figure 2.8). In addition, nine animals without gross vTB lung lesions were found to exhibit a mild to moderate multifocal granulomatous pneumonia with intra-lesional acid-fast bacilli, thereby confirming that the animals had developed vTB. Isolates of *M. microti* were obtained from only three of these animals.

The histological examination of kidneys and adrenal glands with perirenal/adrenal adipose tissue in the one case (from SS8) that exhibited gross lesions consistent with vTB in these tissues, confirmed the presence of a focal extensive granulomatous steatitis and focal granulomatous nephritis and adrenalitis with intralesional acid-fast bacteria (Figures 2.9 and 2.10).



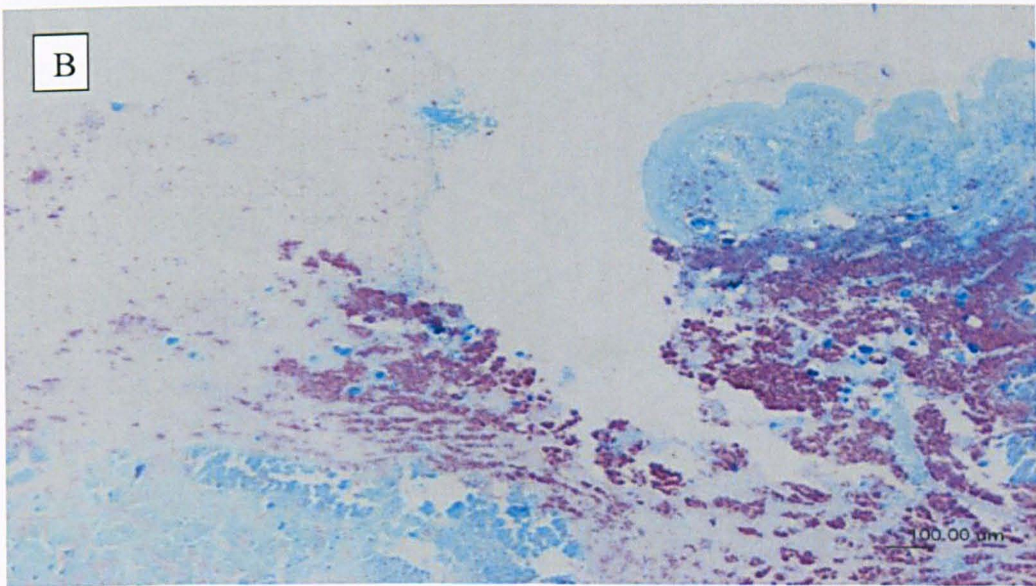
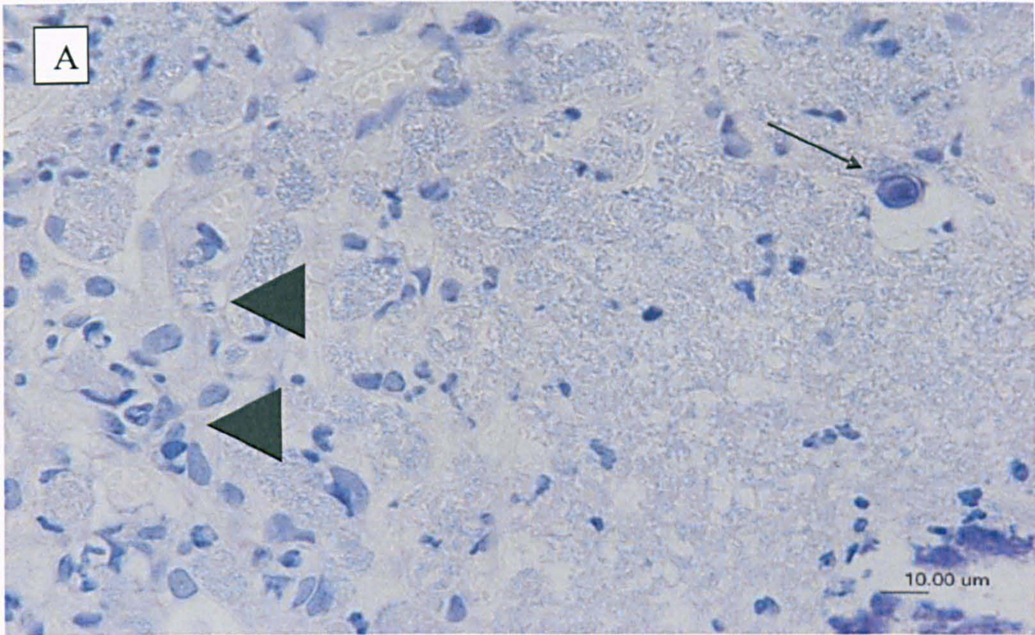


Figure 2.7 Skin, interscapular region; [A] HE-stained section demonstrating extensive focal ulcerative and granulomatous dermatitis with extensive necrosis and multifocal large areas of mineralization (white arrows) and mineralisation pearls (black arrows), and [B] ZN-stained section in which numerous acid-fast bacilli are seen intracellularly and cell-free within the inflammatory process.



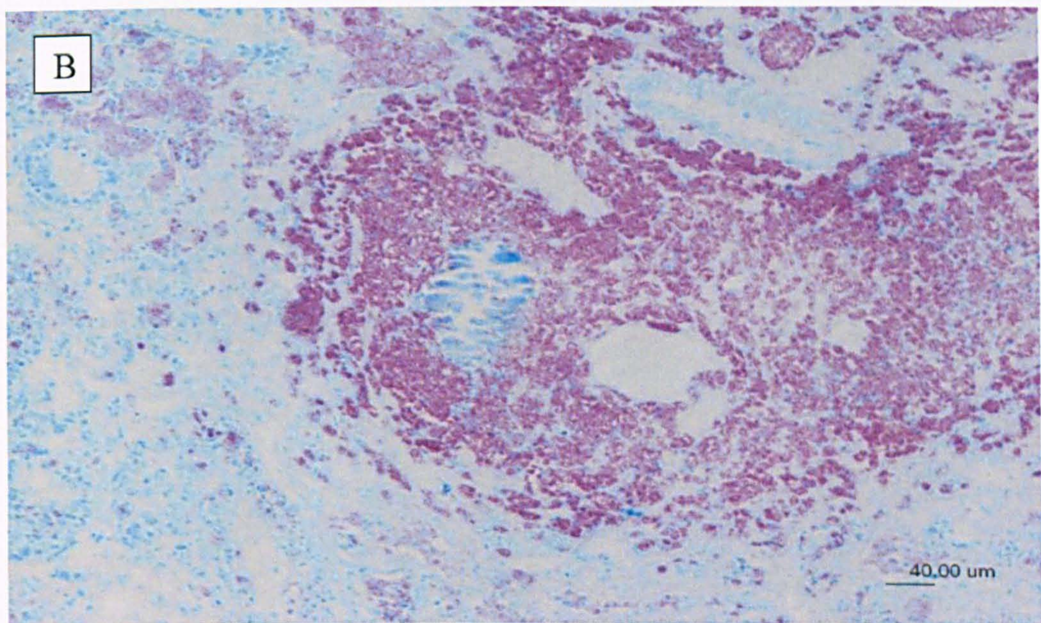
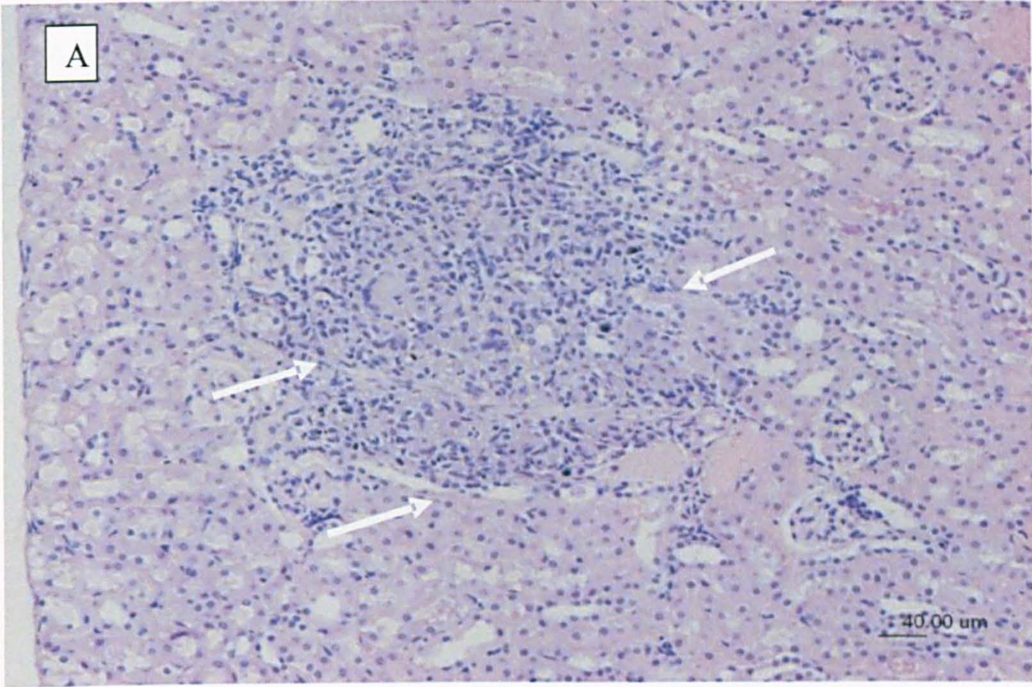


Figure 2.8 Lung, [A] HE-stained section showing focal granulomatous pneumonia with central necrosis and mineralisation pearls (arrow). Numerous rod-shaped bacteria are seen as unstained shapes both within macrophages and cell-free in the necrotic area (arrowheads), and [B] ZN-staining confirms the presence of numerous acid-fast bacilli in the lesion.

In liver and spleen, macroscopic lesions were not detected. The histological examination, however, detected a mild to moderate multifocal granulomatous hepatitis, often located adjacent to the portal areas, with acid fast bacilli (Figure 2.11). In 16 cases, the liver lesions were either seen together with vTB lesions in other organs (n=10) or represented the only tuberculous lesions in an animal (n=6). Five of the latter animals yielded a *M. microti* isolate. A multifocal granulomatous splenitis with acid-fast intra-lesional bacilli was detected in nine animals (Figure 2.12). The granulomatous infiltrates were often located adjacent to the follicles.



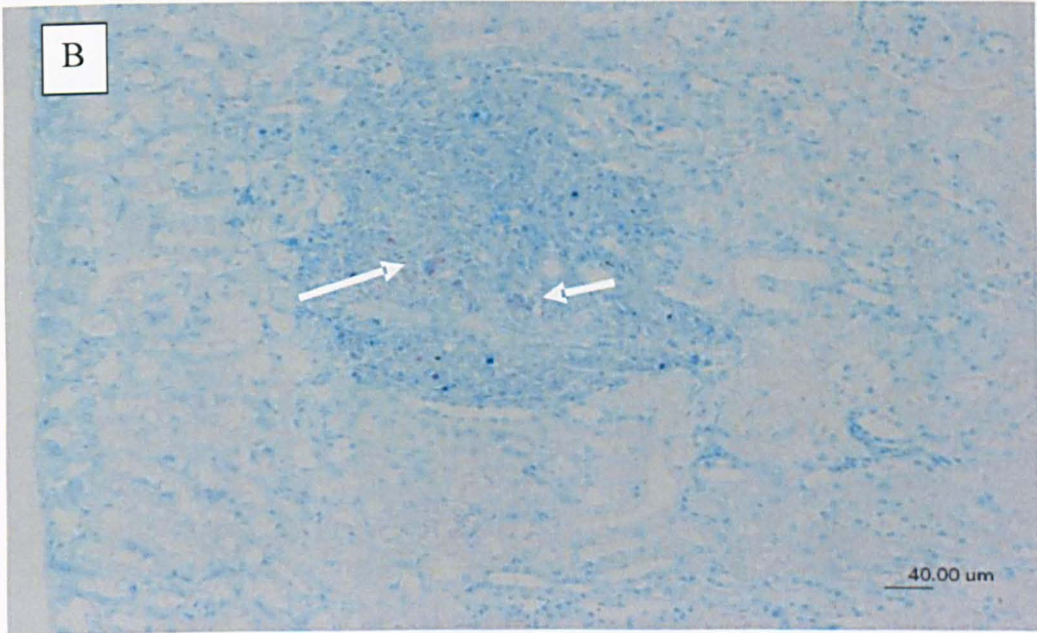
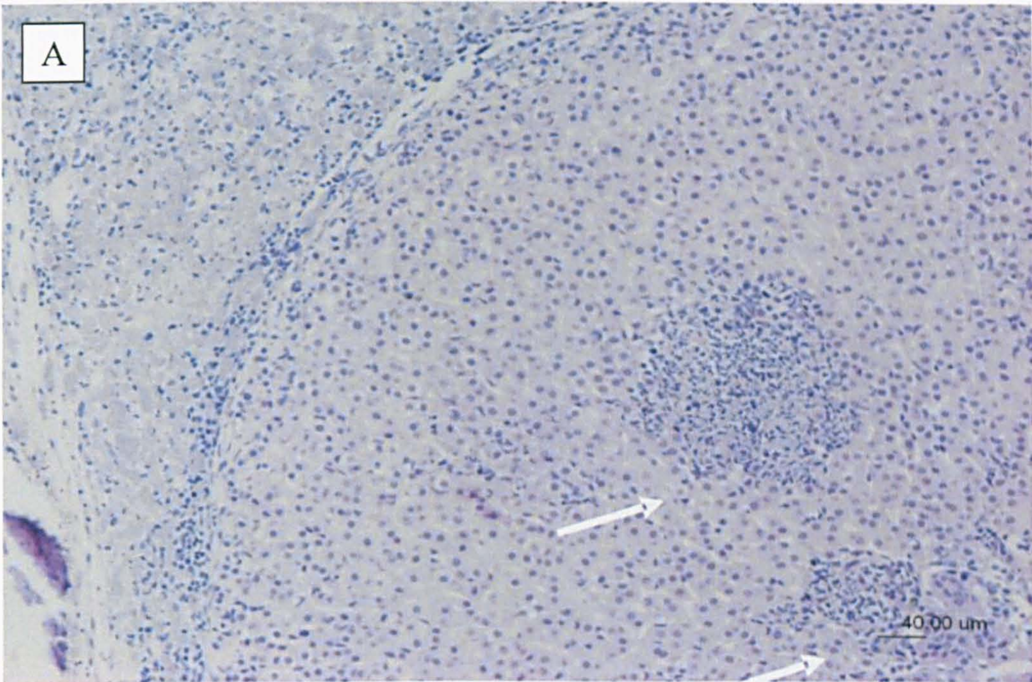


Figure 2.9 Kidney, [A] HE-stained section revealing focal granulomatous nephritis (white arrows), and [B], ZN-staining confirms the presence of intra-lesional acid-fast bacteria (white arrows).



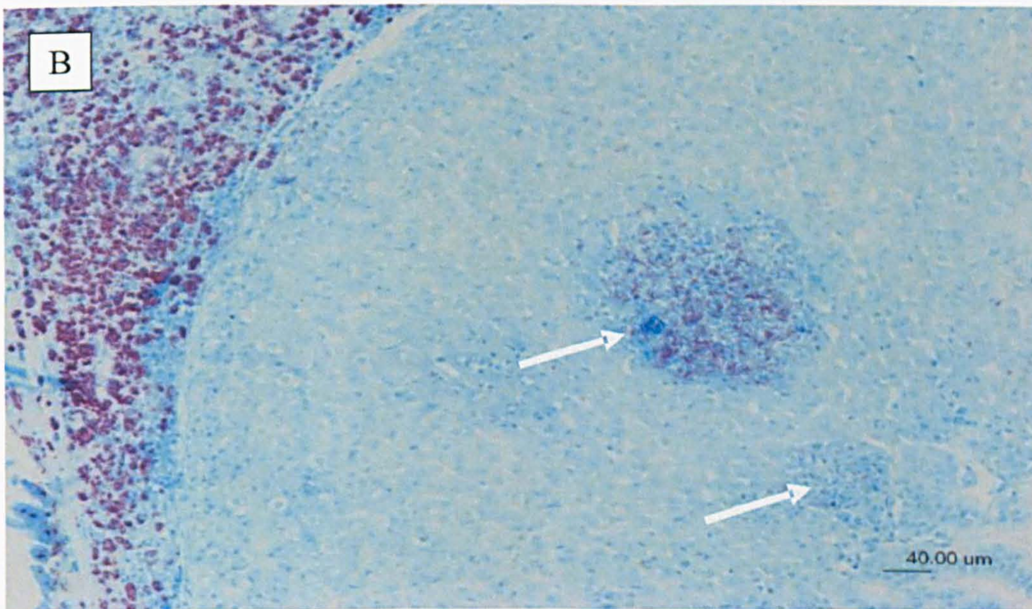
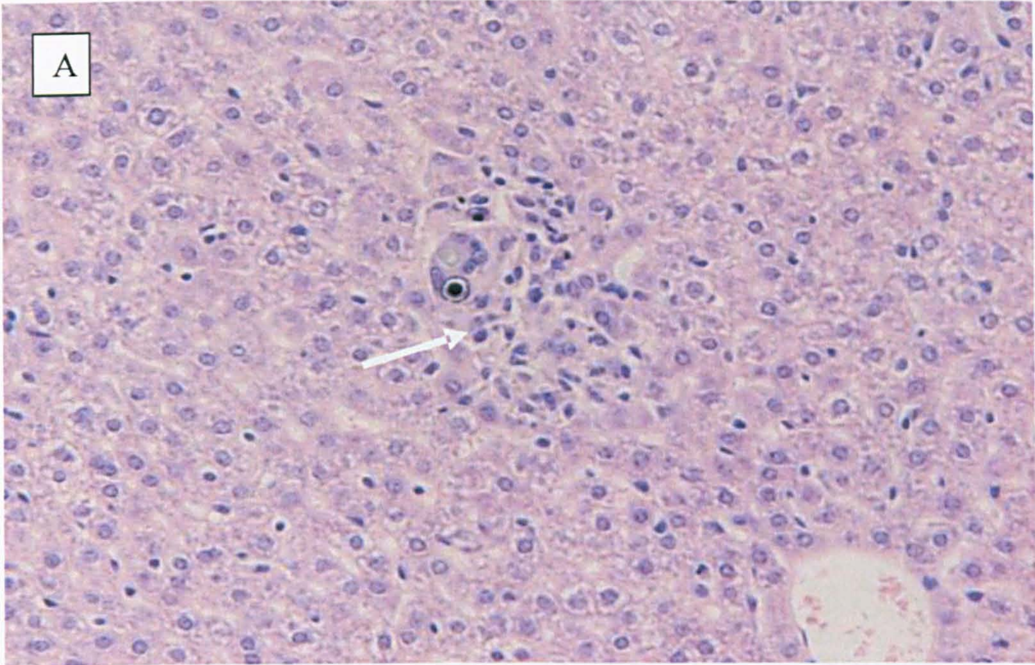


Figure 2.10 Adrenal gland with perirenal/adrenal adipose tissue, [A] HE-stained section showing focal extensive granulomatous adrenalitis (white arrows) and steatitis (black arrow), and [B] ZN-staining confirms the presence of intra-lesional acid-fast bacteria (white arrows).



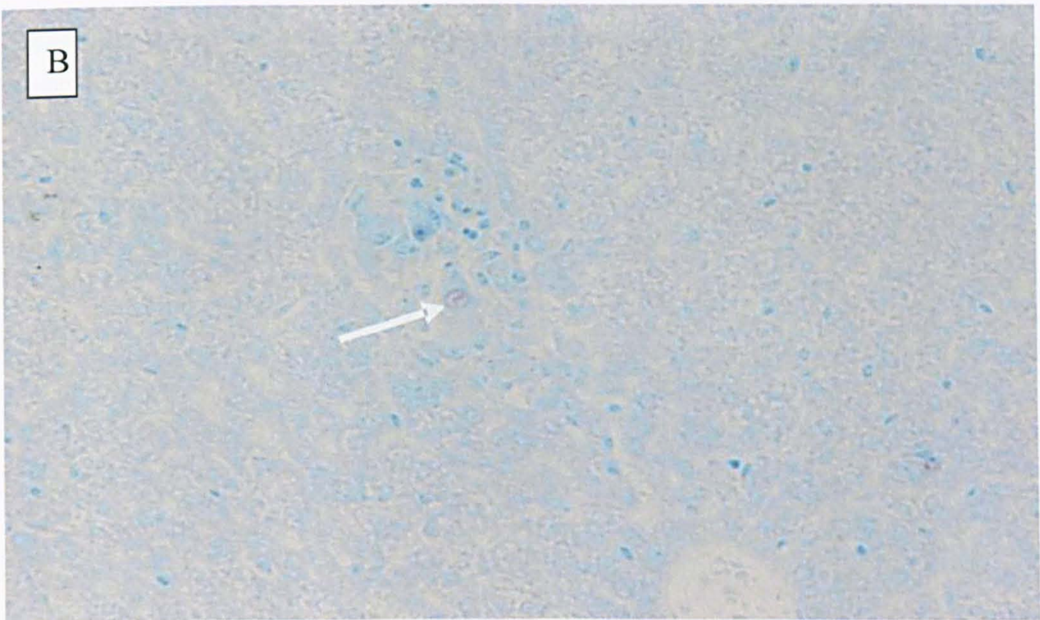
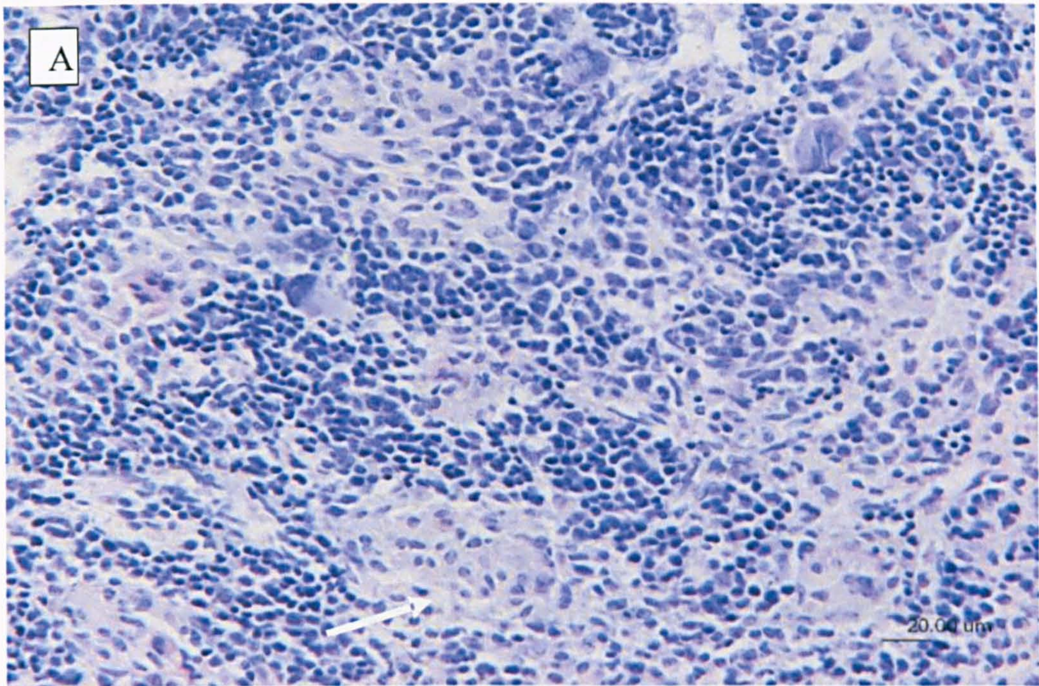


Figure 2.11 Liver, [A] HE-stained section showing focal granulomatous hepatitis and [B] ZN-staining confirms the presence of intra-lesional acid-fast bacteria.



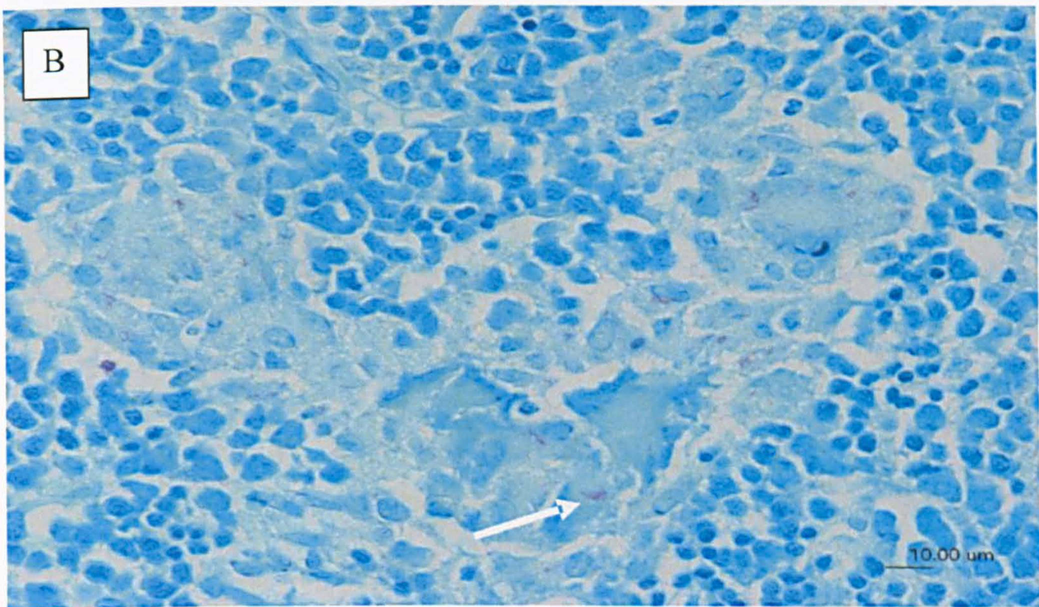


Figure 2.12 Spleen, [A] HE stained section shows multifocal granulomatous splenitis (arrow), and [B] ZN staining confirms the presence of numerous acid-fast bacilli in the lesion.

The relative performance of each of three different approaches, namely (i) the presence of macroscopic lesions containing acid-fast bacilli, (ii) the isolation of *M. microti* by culture, and (iii) the presence of histological TB lesions, to diagnosing vTB among the field voles collected during SS10 was assessed (Figure 2.13). Of the 228 field voles included in this study, 11 exhibited macroscopic lesions consistent with vTB; *M. microti* was isolated from 11, and 19 animals exhibited histopathological lesions consistent with vTB. All animals with macroscopic lesions also had histological evidence of tuberculosis, although a further nine animals had histological evidence of infection in the absence of macroscopic lesions. Culture only confirmed two of these nine infections, but a further eight animals yielded an isolate despite being asymptomatic and having no histological evidence of infection.

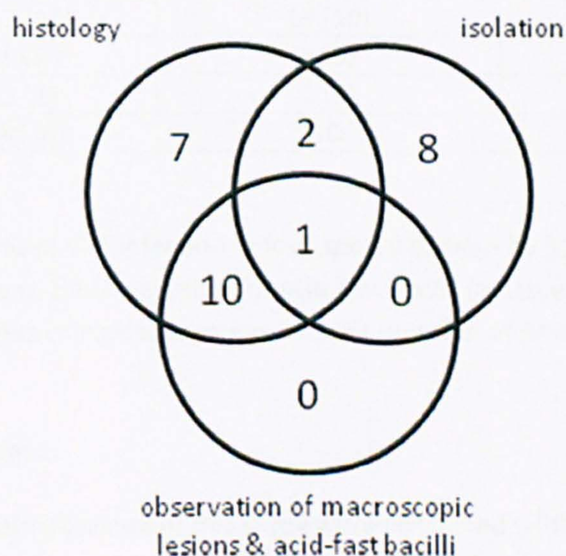


Figure 2.13 Venn diagram demonstrating the relative performances of the three approaches to determining *M. microti*-infection status in the 28 field voles captured during SS10 with evidence of infection.

Diagnosis of vTB among the field voles collected during SS8, 9, 11, and 12 was assessed by detection of macroscopic lesions, observation of acid-fast bacilli in tissue with macroscopic lesions using a ZN smear and isolation of *M. microti* by culture (Table 2.3). As seen for SS10, the prevalence of vTB varied according to diagnostic approach; in all spatial surveys the prevalence of vTB detected by isolation of *M microti* was markedly higher than that detected by observation of the macroscopic lesions containing acid-fast bacilli (Table 2.3). However, not all voles with these latter symptoms yielded an isolate; in SS8, 9 and 11, for which data on ZN staining were available, of the 29 animals with macroscopic lesions containing acid-fast bacilli, only 16 yielded an isolate.

spatial survey	number of voles with macroscopic lesions (%)	number of macroscopic lesions positive with acid fast bacilli(%)	number of *M. microti* isolates obtained (%)
8	37 (21)	19 (10)	48 (27)
9	8 (3)	8 (3)	20 (8)
11	2 (1)	2 (1)	11 (3)
12	10 (4)	ND[1]	77 (30)

[1]not done

Table 2.3 Diagnosis of vTB infection in four spatial surveys by (i) detection of macroscopic lesions, (ii) Observation of acid fast bacilli in tissue exhibited macroscopic lesions using tissue smear and (iii) isolation of *M microti* by culture.

2.3.5 Ecological data.

The laboratory data obtained in this study were combined with field data available for all 12 spatial surveys carried out to date (beginning September 2001) and with previously obtained laboratory data relating to SS2, 3 and 4 (March 2002, September 2002 and March 2003) (Burthe, 2005). In total, 3264 field voles have been trapped at the 27 sites during these surveys (Table 2.4).

grid	grid ref	spatial survey number												total
		1	2	3	4	5	6	7	8	9	10	11	12	
BBH	694E914N	5	10	3	16	12	16	2	6	9	12	13	9	113
BCR	663E869N	3	0	3	8	5	16	5	8	10	7	7	16	88
BGB	659E924N	3	7	9	7	18	5	7	15	16	11	10	15	123
BHP	753E997N	3	19	16	17	19	16	6	13	14	12	10	14	159
BLB	608E948N	2	3	16	11	18	2	7	6	5	7	18	9	104
CRF	502E781N	14	6	14	19	16	4	3	18	10	9	8	3	124
CWS	525E824N	5	18	13	18	10	15	5	6	3	3	6	7	109
FRD	643E949N	1	2	12	14	19	10	2	10	10	8	7	6	101
KCS	625E929N	3	1	6	4	18	2	6	6	16	11	8	8	89
KRB	532E859N	14	9	20	22	2	0	10	9	3	6	27	22	144
LSB	626E878N	10	9	14	22	14	5	5	14	6	11	11	4	125
LWH	678E854N	0	3	17	7	10	3	3	3	14	7	11	7	85
PLJ	674E896N	18	9	13	19	18	6	2	6	16	13	16	1	137
QRY	786E970N	12	2	17	15	18	3	9	2	8	12	25	3	126
RLB	517E826N	10	14	24	19	16	14	23	7	9	11	22	9	178
ROB	792E995N	8	14	9	14	16	6	4	4	19	11	14	9	128
RPC	523E814N	12	13	15	8	1	13	8	4	2	5	13	9	103
SGS	483E805N	7	16	17	15	8	7	6	4	7	1	2	4	94
SLB	496E775N	7	2	1	8	14	11	12	14	7	7	5	1	89
SQC	613E957N	3	6	15	11	10	18	14	19	9	12	12	14	143
SWB	650E864N	0	1	7	13	20	4	7	11	15	11	20	17	81
SWC	745E100	5	13	15	20	13	9	6	8	9	11	9	14	132
TBB	554E823N	13	5	9	22	10	24	5	10	5	3	17	16	139
TDH	524E873N	9	13	16	14	6	19	17	9	4	4	10	17	138
TRI	498E804	8	8	12	10	10	9	12	13	13	4	15	7	121
WBN	783E973N	1	4	10	12	26	9	0	4	9	13	13	8	109
WNH	687E892N	10	8	13	16	12	6	1	1	17	10	24	10	128
TOTAL		186	215	336	381	359	252	187	230	265	232	362	259	3264

Table 2.4 Number of field voles trapped on each grid during SS1-12.

The number of captured voles is used as an indication of the relative size of the field vole population at each grid, in each catchment and overall across Kielder Forest. The size of the field vole population on all three levels was not constant, but rather varied seasonally and annually (Figure 2.14). Although the number of animals caught in the late summer surveys (1695) was very similar to that caught in spring surveys (1569), the number trapped each year varied markedly, for

example, 740 animals being trapped in 2003 but only 439 being trapped in 2004 (Figure 2.14). The number of animals trapped on different grids ranged from a total of 81 at SWB to almost double, 159, at BHP (Table 2.4).

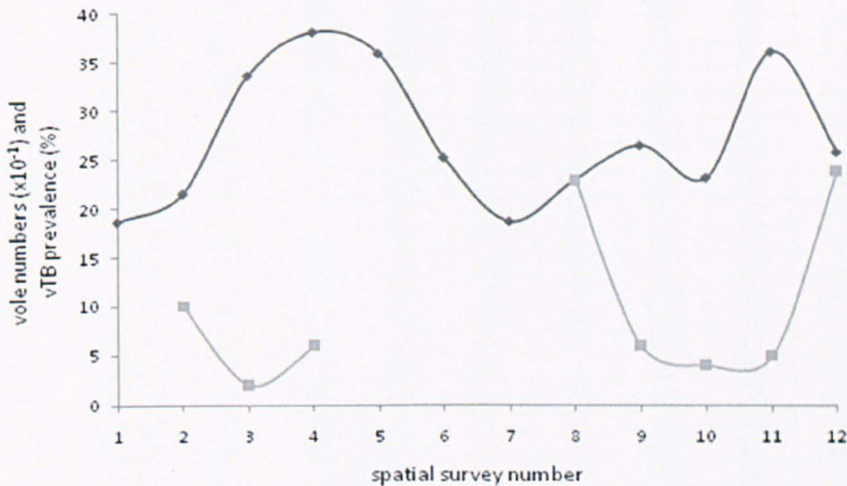


Figure 2.14 Total number of field voles caught during each spatial survey (dark grey line) and overall prevalence of *M. microti* infection in the vole population (light grey line).

The prevalence of the vTB infection in field voles has been assessed for only eight spatial surveys (2-4 and 8-12) (Table 2.5). Assessment of vTB infection status was determined for a total of 1752 animals. The diagnosis of vTB has been based on the following criteria: (i) the observation of macroscopic caseous lesions in which acid fast bacilli are microscopically observed, and/or (ii) the isolation of *M microti*. The overall prevalence of the vTB infection was 14.3%, however the range in prevalence was from 4.2% in SS3 to over 30% in SS12 (Table 2.5 and Figure 2.14). The prevalence of the vTB infection recorded during the spring surveys was 18% compared to 7% during the late summer surveys (Table 2.5).

site	catch-ment	spatial survey number								TOTAL
		2	3	4	8	9	10	11	12	
BBH	Kielder	3/10	1/2	1/10	0/4	0/9	2/11	1/4	2/8	10/58
BCR	Kielder	0/ 0	0/1	1/7	2/6	1/10	0/7	0/3	6/16	10/50
BGB	Kielder	1/6	1/6	2/7	2/5	3/16	0/11	0/8	6/13	15/72
BHP	Redesdale	1/13	1/8	1/17	5/9	1/13	3/12	0/8	2/14	14/94
BLB	Kielder	1/3	1/8	0/8	1/4	1/6	2/7	1/1	4/8	11/45
CRF	Kershope	0/5	0/10	2/19	3/14	0/10	0/5	0/4	0/3	5/70
CWS	Kershope	2/16	0/7	0/18	2/4	0/3	0/4	0/3	1/1	5/56
FRD	Kielder	0/2	0/8	0/9	2/17	2/10	1/9	0/3	2/6	7/64
KCS	Kielder	0/0	0/5	0/4	1/5	2/15	0/11	0/5	0/5	3/50
KRB	Kershope	1/9	0/14	3/21	1/9	0/3	0/7	1/7	4/21	10/91
LSB	Kielder	0/5	0/10	0/10	2/9	0/6	0/12	0/0	0/4	2/56
LWH	Kielder	0/2	0/11	0/7	0/2	2/14	0/8	0/4	0/7	2/55
PLJ	Kielder	1/7	0/6	2/19	2/2	0/8	0/9	0/2	1/1	6/54
QRY	Redesdale	0/1	0/9	2/10	0/1	2/7	1/12	2/7	2/3	9/50
RLB	Kershope	0/8	0/16	2/18	1/7	0/9	0/11	1/12	1/9	5/90
ROB	Redesdale	2/9	0/7	3/10	2/3	3/17	0/11	2/9	2/8	14/74
RPC	Kershope	2/11	1/11	1/8	1/4	0/2	0/5	1/9	3/9	9/59
SGS	Kershope	0/15	0/11	0/9	0/4	0/7	0/1	0/1	1/4	1/52
SLB	Kershope	0/1	0/0	1/8	3/14	0/6	0/7	0/0	1/1	5/37
SQC	Kielder	1/5	2/12	0/10	5/8	0/9	2/12	1/2	3/15	14/73
SWB	Kielder	0/1	1/6	0/10	6/8	2/16	3/10	1/8	7/17	20/76
SWC	Redesdale	4/9	0/10	1/20	3/6	1/9	0/12	0/7	6/14	15/87
TBB	Kershope	1/5	0/6	6/20	4/11	1/5	1/3	0/11	5/16	18/77
TDH	Kershope	2/11	0/8	0/10	3/9	0/4	1/4	1/4	4/17	11/67
TRI	Kershope	2/8	0/9	0/9	1/13	0/12	1/4	0/2	0/6	4/63
WBN	Redesdale	0/0	0/7	8/12	1/3	0/9	0/12	0/4	4/8	13/55
WHH	Kielder	1/7	1/7	2/16	0/1	1/17	1/10	0/8	7/11	13/77
TOTAL		25/169	9/215	38/326	53/182	22/252	18/227	12/136	74/245	251/1752

Table 2.5 Number of *M. microti* infected voles per spatial per trapping grid. x/y, where x is the number of animals in which vTB was diagnosed and y is the number of animals for which clinical material was successfully processed.

Comparison of the vTB prevalence at each grid also revealed marked differences. The overall prevalence of infection ranged from less than 2% (1 of 52 animals) at SGS to over 26% (20 of 76 animals) at SWB. However, even at high vTB prevalence grids, in some surveys no infected animals were detected, although it is fair to say that, in general, some sites had consistently higher vTB prevalence than others (Table 2.5 and Figure 2.15). Only a preliminary analysis of the spatial pattern to vTB occurrence or prevalence was attempted; the prevalence in each catchment varied, with 11% in Kershope, 15% in Kielder and 18% in Redesdale, and the range of vTB prevalence at the grids within each catchment varied - from 2% to 23% in Kershope, 3.1% to 24% in Kielder, and 15% to 24% in Redesdale. A

further general observation is that temporal variation in the overall vTB prevalence was reflected across the Forest, such that each catchment and, to some extent, each grid, was "behaving" in a similar manner. Thus, regardless of the overall vTB prevalence at a grid, it was still susceptible to the temporal dynamics experienced by the Forest as a whole (Figure 2.15).

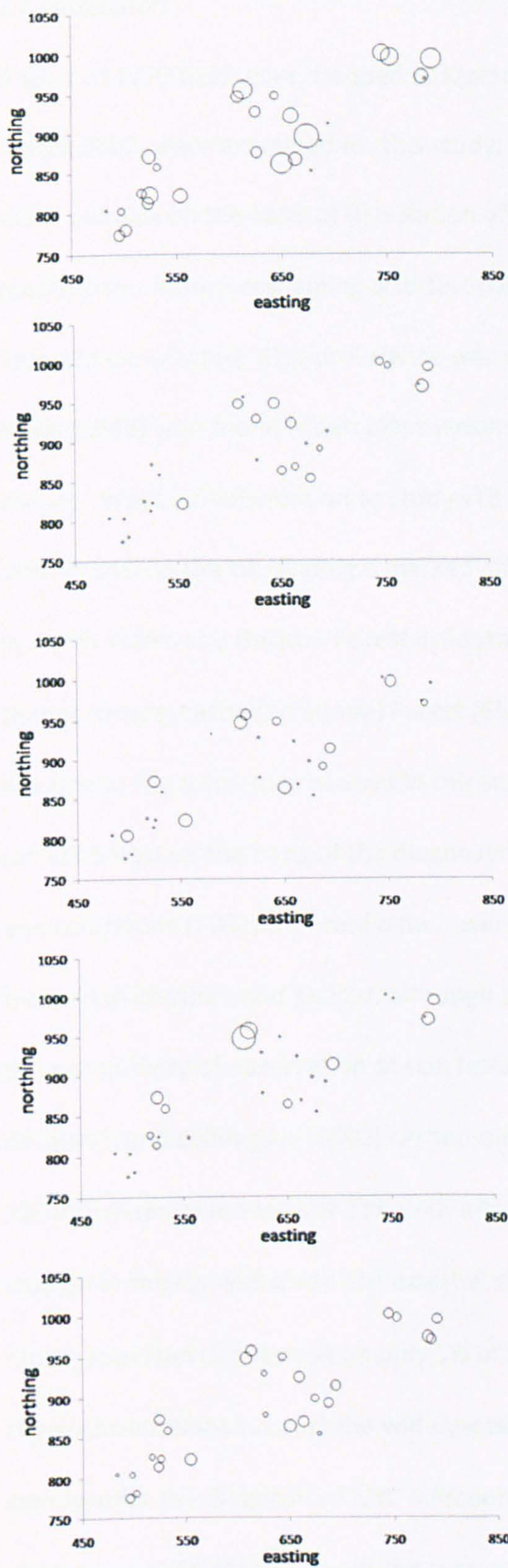


Figure 2.15 The prevalence of the vTB infection among 27 sites in Kielder Forest during SS8 (top), 9, 10, 11 and 12 (bottom).

2.4 Discussion

A total of 1270 field voles, trapped at Kielder Forest between March 2005 and March 2007, were examined for this study. Vole tuberculosis was diagnosed in these animals on the basis of (i) isolation of *M. microti* and/or (ii) observation of macroscopic lesions containing acid-fast bacilli. In total, 200 animals (15%) were found to be infected. This prevalence was slightly lower than that reported by Wells (1946) who found tuberculous lesions in 20% of 4309 voles from around the UK. Wells (1946) went on to study vTB prevalence in three geographically distinct sites in the UK, finding a marked difference between that at Lake Vyrnwy in North Wales and Geskine Forest in Southern Scotland (27-31%) compared to that at Newcastleton (Kershope) Forest (6%). Burthe and colleagues (2008) working at the same sites as used in this study, reported a vTB prevalence 11% (of 733 voles) on the basis of the diagnostic criteria used in this study. Cavanagh and colleagues (2002) reported a far lower prevalence of 2% among voles trapped in Cheshire and Kielder, although this low estimate is most likely due to the insensitivity of observation of skin lesions as a means of diagnosis. Indeed, Cavanagh and colleagues (2002) carried out a smaller study of 180 voles, finding 7% with external lesions but 21% with internal lesions. Only 14 (1%) of the voles studied in the current study had external signs of vTB, and Burthe and colleagues (2008) reported skin lesions on only 5% of the voles they examined. Although these observations support the widely-held belief that culture is the gold standard for the diagnosis of MtC infections, it is important to note that for almost one third of the voles in the current study, a diagnosis of vTB was made on the basis of observation of macroscopic lesions containing acid-fast bacilli.

The reliability of observation of macroscopic lesions alone for the accurate diagnosis of vTB is debatable; in the current study, and previously, lesions suggestive of vTB, particularly on the skin surface, have been confused with wound scabs or other pathologies (Dr Sarah Burthe, University of Liverpool, personal communication). Hence throughout this study it was used only in conjunction with observation of acid-fast bacilli in ZN-stained impression smears. However, this conservative decision has probably compromised the sensitivity of this diagnostic criterion. For example, in spatial survey 8, nine *M. microti* isolates were obtained from the 18 voles found to have macroscopic lesions in which acid-fast bacilli could not be observed. Furthermore, in spatial survey 10 (for which histopathology data were available), 11 voles had macroscopic lesions, of which nine were found to contain acid-fast bacilli. However, the two lesions in which acid-fast bacilli were not observed by impression smear (and were culture negative) were granulomatous associated with acid-fast bacilli when examined histologically. Nonetheless, as demonstrated in the spatial 8 data above, for a significant proportion of animals with suspected vTB lesions, no other evidence of *M. microti* infection was forthcoming. A valid general conclusion is probably that although culture would be the method of choice if only one diagnostic approach was to be used, the most sensitive way of determining *M. microti* infection status is to employ a combination of approaches. The data obtained from spatial survey 10 suggests that a combination of culture and histology is as sensitive as a combination as culture, histology and observation of macroscopic lesions infected with acid-fast bacilli, although a larger study is required to test the reliability of this result.

In the current study, external skin lesions were most commonly found on or between the scapulas of the infected individuals, and subcutaneous lesions were often associated with the external ulcerated lesion in the skin. This apparent preferred location of external lesions has previously been reported by Wells (1946). Lymph nodes were by far the most common internal site of vTB lesions; only one animal that possessed internal lesions did not have evidence of lymph node involvement. Wells (1946) also found lymph nodes to be the most common infection sites. The location of affected lymph nodes were not recorded in the current study, however my findings do support Wells' (1946) hypothesis that *M. microti* first affects lymph nodes, then spreads to other organs. Wells (1946) classed lungs as the most important of these "other" organs but in the current study, skin lesions were more frequently observed than lung lesions.

Histological examination of a set of tissues (lung, lymph node, liver and spleen) showed that vTB infection status was well assessed by this method. Of 228 animals subjected for histology, 19 (8.3%) exhibited histology that was consistent with vTB lesions (Robb-Smith (1946). Eight (42%) animals had been misclassified as uninfected based on gross lesions alone. The distribution of histological lesions consistent with vTB among infected organs was varied, in which the liver was mostly affected. Liver lesions were detected in 16 cases. In six of these animals there were no other microscopical lesions, but in the remainder, additional lesions were detected in the lymph nodes (10), spleen (9) and lungs (7). This pathology study reveals some interesting information regarding possible routes of infection of tuberculosis in the field voles. All the individuals with

lesions had macroscopic lesions in the lymph nodes of the neck, which may suggest that infection had arisen from the ingestion of infected material. Wells (1946) investigated tuberculosis transmission experimentally by introducing a field vole with an external tuberculous lesion into a cage of fourteen uninfected voles. The vole with tuberculosis was killed and consumed by the uninfected voles. Four voles died within the first five days of the experiment, and large number of acid-fast bacteria was found in the stomach contents of these animals. The remaining voles were killed 98 days after the introduction of the infected vole. Two individuals showed macroscopic tuberculous lesions in the lymph nodes draining the intestinal tract and the lungs. In a repeat of this experiment, this time with 18 uninfected voles, one vole died four days after consuming the infected vole and there was evidence of mycobacteria in the stomach. Seven of the remaining seventeen voles showed macroscopic tuberculous lesions in the lymph nodes draining the intestine and in the lungs when killed within 100 days. These experiments reveal that ingestion of infectious material could lead to the type of the infection observed in wild populations of field voles at Kielder. The involvement of the liver and spleen beside the lymph nodes as the most frequent organs that exhibited vTB lesions is indicative of the presence of systemic bacterial infection spread that may occur after the enteric stage of the infection.

Lung tissue was found to be infected, which suggests that at least in some individuals, infection is likely to have been through the respiratory route. Field voles inhabit enclosed nests and tunnels in vegetation and respiratory

transmission could occur directly between voles during aggressive or reproductive interactions. Kidneys showed evidence of infection with *M. microti* indicating possible excretion via urine, although Cavanagh and colleagues (2002) were unable to detect *M. microti* DNA in urine collected from field voles.

A small percentage of animals (0.4%) were infected with *M. microti* but did not show any evidence of tuberculosis, based on the absence of histological lesions in lungs, lymph nodes, liver and spleen. It is possible that these animals were in the early stages of infection, when number of bacteria was still too small to have induced vTB lesions. Alternatively, the animals showed lesions but so few and/or so small that they were not detected by histology. Finally, the effect of the freezing process on the histological sections may have affected the detection of early pathological lesions.

As previously observed, the overall prevalence of vTB was greater in spring spatial surveys compared to late summer spatial surveys (Wells, 1946; Chitty, 1954; Cavanagh et al., 2004; Burthe et al., 2008). This phenomenon appears to be the result of vTB being a chronic, insidious disease that manifests far more often in older animals, which dominate the vole population in spring, but not in late summer (Wells, 1946; Chitty, 1954; Cavanagh et al., 2004; Burthe et al., 2008). However, the results of spatial survey 10 in the current study are contrary to these general observations. The reason for this may well be that vTB transmission dynamics has also been shown to be correlated to vole population density. Cavanagh and colleagues (2004) and Burthe and colleagues (2008) both

calculated that vTB showed delayed density dependence with a lag time of three to six months. Although statistical demonstration of this relationship was beyond the limits of the current study, the data presented in Figure 2.14 give a strong indication that this relationship is reflected in the number of voles caught and vTB cases diagnosed during spatial surveys 8 to 12. The spatial pattern of vTB during these surveys also supports previous work (Burthe et al., 2008) that Kielder Forest as a whole is affected by multi-annual cycles of field vole population density and thus the transmission dynamics of vTB are generally the same across the Forest. However, some sites seem to be consistently more vTB "rich" than others, although this observation has yet to be rigorously tested and the reason for it is unclear.

In summary, this study has extended the monitoring of *M. microti* infection status of field voles inhabiting Kielder Forest to include a further five spatial surveys. Infection status has been determined using a variety of diagnostic approaches, among which culture remains the most sensitive, but histology shows much promise. Histological examination of symptomatic and asymptomatic animals has confirmed the presence of classical tuberculous granulomas in a variety of tissues, most frequently the lymph nodes, which appear to be the primary niche of infection in the body. The dynamics of vTB in the Kielder field vole population surveyed during this study appear to be in keeping with previously established correlations.

Chapter 3.

PCR-based diagnosis of *M. microti* infections

3.1 Introduction

At present, the diagnosis of *M. microti* infections in field voles ante-mortem can only be achieved by observation of external lesions. Laboratory diagnosis, using a range of established methods such as microscopic examination of stained tissue smears, isolation of bacteria, or even PCR, has only been performed on animal s' *post-mortem*. However, for other *M. tuberculosis* complex (MtC) members, numerous approaches for ante-mortem diagnosis have been described. These include the demonstration of a cellular and/or humoral immune response and, for acute-phase infections, the demonstration or isolation of bacteria in/from clinical samples. The identification of acid-fast bacilli by microscopic observation of Ziehl-Neelsen (ZN) stained smears is rapid, but does not permit identification of the *Mycobacteria* species involved and lacks sensitivity because a relatively large number of organisms (at least 10^4) must be present to be reliably detected (Bates, 1979). Culture is far more sensitive and permits subsequent species identification, and is therefore considered the "gold standard" for tuberculosis diagnosis, but its usefulness as a diagnostic tool is hindered by the slow growth of mycobacteria. *M. tuberculosis* and *M. bovis* typically take between 3 and 6 weeks to grow, whereas *M. microti* takes at least 12 weeks. Furthermore, many clinical samples used for culture require alkaline decontamination which causes a substantial decrease in the number of colony-forming units recovered (Pfyffer et al., 2003).

PCR-based detection methods have been the subject of much attention from scientists interested in mycobacterial diagnostics as they have the potential to be species specific, as sensitive as culture and almost as rapid as ZN staining. The first assays for the amplification of mycobacterial DNA appeared soon after the arrival of PCR technology (Böddinghaus et al., 1990) and since then numerous different PCR assays have been described in the literature for the diagnosis of active pulmonary tuberculosis in humans. Several commercial kits are available (Drobniewski et al., 2003). Similarly, many assays for the detection of *M. bovis* infection in cattle have also been described and evaluated (Sreedevi & Krishnappa, 2004). PCR can equally be used to confirm and identify mycobacterial infections *post mortem*. Due to the highly infectious nature of MtC species, rapid confirmation of infection is necessary if effective control measures are to be implemented.

Although for *M. tuberculosis* diagnosis, sputum is by far the most commonly used clinical sample, the suitability of a wide range of clinical samples for PCR testing has been assessed. Cheng and colleagues (2005) reviewed the sensitivity and specificity of PCR using either in-house or commercial methods for the detection of *M. tuberculosis* from direct clinical samples. High sensitivity (>90% relative to culture) was obtained for sputum, bronchoalveolar lavage fluid, endotracheal aspirates and pleural biopsies, but assays performed less well when testing samples collected from other anatomical sites. Assays performed on non-invasive samples such as urine had an overall sensitivity of between 55 and 95%. In general, the specificities reported for all the assays were high, ranging

between 94 and 100% (Cheng et al., 2005). As many different assays were included in this comparison, the results must be interpreted with care as inherent limitations of specific assays, rather than the material tested, may have compromised their performance. Wards and colleagues (1995) described the first PCR for the specific detection of *M. bovis* in clinical tissues. The assay was evaluated on a variety of clinical samples from a variety of species, as they were submitted for routine *M. bovis* isolation. Further description of the tissues used was not provided. Subsequently, *M. bovis* PCRs have been applied almost always to samples collected from lymph nodes, with or without visible tuberculous lesions. Liebana and colleagues (1995) compared a PCR with culture for the detection of *M. bovis* in lymph nodes and reported that only 71% of culture positive samples were PCR positive. Taylor and colleagues (2007) reported a similar sensitivity in a similar study but using a real-time PCR. Parra and colleagues (2008) described a real-time PCR for use in abattoir surveillance of bovine tuberculosis and reported a sensitivity (compared to culture) of 61% for samples with non-visible lesions and 81% for chronic lesions, with an average sensitivity of 74%. In their review of ante-mortem diagnosis of tuberculosis in cattle, De la Rua-Domenech and colleagues (2006) considered that PCR diagnosis of infections using non-invasive or minimally-invasive clinical samples such as blood, urine, faeces, nasal swabs and lymph node biopsies, were unlikely to be particularly sensitive due to the low numbers of bacilli in these samples. Thus, "it is unrealistic to consider them a viable alternative to immunological tools for routine diagnosis of tuberculosis in live cattle" (De la Rua-Domenech et al., 2006).

The performance of MtC PCRs is not only influenced by the clinical material from which DNA extracts are prepared. It is also influenced by the method by which DNA is extracted. A number of different extraction methods have been used, ranging from simple boiling (Afghani & Stutman, 1996) or trapping of DNA on chelex resin (Heginbotham et al., 2003) to bead disruption (Tell et al., 2003), sonication (David et al., 1984) or enzymatic digestion (Zhang et al., 1997). Today, commercial kits are widely used (Aldous et al., 2005). The relative performance of these various extraction methods has been evaluated. Wards and colleagues (1995) compared five in-house and commercial approaches. In general they found that a proteinase K digestion step was common to all of the most successful methods although the best was time consuming, involving multiple proteinase K digestions, disruption with detergents, the addition of tRNA "carrier" molecules and nucleic acid precipitations. Taylor and colleagues (2007) reported an in-house adaptation of a commercial extraction kit to enhance killing and lysis of mycobacteria in infected clinical material. Their adaptation allowed processing of samples outside the Category 3 facilities required for laboratory work with living mycobacteria. In another study, Parra and colleagues (2008) demonstrated that the sensitivity of their PCR was 74% when using manual extraction but dropped to only 48% when using an automated robotic system. Recently, Barcelos and colleagues (2008) have presented a study of the influence of different extraction methods on the relative performance of PCRs detecting *M. tuberculosis* in formalin-fixed and paraffin wax-embedded tissues.

Numerous loci in the genomes of MtC members have been targeted by different PCR assays. These include housekeeping genes such as the 16S rDNA (Böddinghaus et al., 1990), *hsp65* (Pai et al., 1997), *rpoB* and *rpoV* (Whelen et al., 1995; Comincini et al., 1998), non-coding regions such as the 16S rRNA-23S rRNA intergenic spacer (Gürtler et al., 2006) and loci either specific to MtC members such as the MPB70 antigen gene (Young et al., 2005) or loci specific to particular species within the complex, for example Ghadersohi and colleagues (1997) identified regions of *M. bovis* that were absent from other *Mycobacterium* species, then designed PCRs to specifically target these regions. PCRs targeting some of the widely recognised "regions of difference" (RDs) between MtC species have been described (e.g. Parsons et al., 2002; Huard et al., 2003) and have been used not only to confirm the identity of species yielding a PCR product but also to confirm the identity of species not yielding a PCR product (and therefore missing a specific RD) (Courtenay et al., 2006). Similarly, Huard and colleagues (2003) reported that a PCR targeting a transposase pseudogene, IS1561', yielded amplification products for all MtC members except *M. microti*.

For the PCR assays that were not species specific, several post-amplification steps have described to identify which *Mycobacterium* species has been detected. For example, Lee and colleagues (2000) reported that the use of the restriction fragment length polymorphism (RFLP) analysis of a 360 base pair region of *rpo*B to differentiate over 50 *Mycobacterium* species. RFLP analysis has also been used in conjunction with other PCRs, for example Plikaytis and colleagues (1992) used RFLP of *hsp65* amplification products to identify all

Mycobacterium species, and Sansila and colleagues (1998) differentiated between isolates of *M. tuberculosis* and *Mycobacterium ovium* by using RFLP of 16S-23S rRNA gene spacer sequence amplification products. Sequencing of PCR products has also been widely reported (e.g. Senna et al., 2008).

In an attempt to enhance the sensitivity of MtC PCRs, genetic loci present in multiple copies in the genome have been targeted. Insertion sequences make suitable candidates for such assays as they are often present in many copies in bacterial genomes (Siguier et al., 2006). The insertion sequence IS6110 is found in the genomes of MtC members at a copy number ranging from one to over 20 (McEvoy et al., 2007). Although single copy-containing strains are common among *M. bovis* isolates, other species contain more (McEvoy et al., 2007). The development of a PCR to detect *M. tuberculosis* IS6110 was concurrently described by Hermans and colleagues (1990) and Thierry and colleagues (1990) and numerous variations on this have been described subsequently. The presence of IS6110 in the genome of a *M. microti* representative was demonstrated in one of these early studies (Thierry et al., 1990) at a medium copy number (about 10 copies), and more recently (Cavanagh et al., 2002; Emmanuel et al., 2007).

For *M. bovis*, which possesses single or low copies of IS6110, other insertion sequences have been targeted. Taylor and colleagues (2007) described PCR for the amplification of IS1081, which is present in six copies in the MtC. This assay proved remarkably sensitive, detecting *M. bovis* DNA in 91% of culture-positive

tuberculous lesions. The sensitivity of a PCR targeting a single copy locus run at the same time was only 51%.

Real-time PCR assays have been introduced over recent years, providing a means of quantifying mycobacterial load as well as a more convenient and rapid tool (Soini & Musser, 2001). Several different assays have been described (e.g. Desjardin et al., 1998; Torres et al., 2000; Taylor et al., 2001; Broccolo et al., 2003; Parra et al., 2007; Taylor et al., 2007), although, as with conventional PCRs, the sensitivity of these assays was variable. Some, however, appeared to offer a diagnostic advantage over other approaches. For example, Bruijnesteijn Van Coppenraet and colleagues (2004) tested samples collected from 67 children with suspected mycobacterial lymphadenitis based on a positive mycobacterial skin test using conventional culture and a real time PCR assay. Whereas isolates were obtained from only 41% of samples, the real time PCR detected mycobacterial DNA in 72%.

The ultimate aim of this study was the development of a PCR-based diagnostic assay that could be used on living field voles to determine their *M. microti* infection status. Review of the literature suggested that a recently described real-time assay (Taylor et al., 2007), targeting the multicopy IS1801, was potentially the best assay to use. Description of this assay included a novel adaptation of a commercial DNA extraction kit that was reported to enhance the sensitivity of the PCR assay. This approach was evaluated by using DNA extracts prepared from tissues taken from the voles described in the previous chapter for

which infection status had been determined by post-mortem examination, acid-fast staining and culture.

3.2 Materials and Methods

3.2.1 Lung and lymph node tissue

The lung and lymph node tissues used for this study were those removed from field voles trapped during spatial survey carried out during the spring of 2005. Tissues from a total of 175 animals were analysed. At the time of their dissection, lungs were divided into three equal sized portions, then one portion was prepared for culture, the second was submerged in formalin for histological studies and the third was immediately frozen at -20°C and stored for use in PCR. The jugular, axillary and inguinal lymph nodes were also collected from all animals and pooled together. This pool of tissue was minced using a sterile scalpel then divided into three equal sized portions. Each portion was processed as described above for lung tissue.

3.2.2 Extraction of DNA from clinical samples

Nucleic acid extracts were prepared from all tissues using a NucliSens Kit (Biomerieux UK Ltd, Basingstoke, UK) according to a previously described amended protocol (Taylor et al., 2007). This extraction protocol is based on the initial cell lysis in guanidinium buffer containing detergents followed by trapping of released DNA onto silica beads and partial purification with the DNA isolated reagents. An Eppendorf tube containing tissue was thawed, heat-killed by

boiling for 5 min, and then snap frozen three times by submersion in liquid nitrogen. A 60 μl aliquot of lysis buffer was added to the tube together with a sterile tungsten bead. The tube was then placed in a Tissue Lyser (Qiagen Ltd Crawley, UK) and the tissue within the tube was homogenised at 20 Hz for 3 min. Next, the tube containing homogenised tissue was placed in a boiling water bath for 5 min then centrifuged for 5 minutes at 1100 x g. The tube was gently removed from the centrifuge and the lid carefully opened so not to disturb the pellet at its base. Using a sterile, DNA-free filtered pipette tip, the supernatant was removed from the tube and transferred into a clean 1.7 ml Eppendorf tube. An aliquot of 50 μl of the silica suspension was added to the supernatant and the tube was agitated on a rotating wheel for 30 min at room temperature. After further centrifugation at 1100 x g for 5 min, the supernatant was removed from the tube and pellet was washed twice, firstly with absolute ethanol and then with acetone. Once the acetone was removed, the pellet was dried by leaving the lid of the tube open at room temperature for about 30 min. A 25 μl aliquot of elution buffer was then added to the dried pellet and the tube with its lid closed was incubated at 60°C for 10 min. The tube was vortexed for 10 sec, centrifuged at 1100 x g for 5 min, and then the supernatant was transferred into clean Eppendorf tube and stored frozen at -20°C until required.

Several samples were usually processed concurrently. To control for cross-contamination between these tubes, one dummy tube, that did not contain any tissue, was processed as described above at the same time as every 5 tubes containing samples.

3.2.3 DNA amplification assays

All PCRs were prepared and performed in laboratories designed for purpose. Reaction mixes minus DNA were stored and prepared in a laboratory into which DNA extracts had never been introduced. This laboratory was physically removed from other laboratories and was under positive air pressure reducing the passage of unfiltered air into the room. Once reaction mixes were prepared they were transferred to a laboratory designated for the addition of DNA templates. This laboratory was physically removed from other laboratories and was also under positive air pressure. Once DNA templates had been added to reaction mixes, tubes were transferred on ice to a third laboratory housing thermal cyclers. A fourth laboratory was used for downstream processing of PCR products. Each laboratory had dedicated lab coats and single-use disposable latex gloves were worn in each.

3.2.4 Detection of field vole DNA

The first PCR assay used in this study was used to confirm the success of the DNA extraction procedure by targeting a 450 base pair fragment of the field vole 18S rRNA-encoding gene. The assay incorporated the primer pair MA1F (5'-CTA GAG CTA ATA CGT GCA AC-3') and MA2R (5'-GAT AAG GTT CAG TGG ACT TC-3'). These primers were manufactured commercially (Eurofins MWG Operon, Ebersberg, Germany) and supplied freeze-dried. Each primer was resuspended to a concentration of 100 pmol μl^{-1} using sterile distilled DNA-free water. Working strength aliquots (100 µl) of each primer at a concentration of 25 pmol μl^{-1} were prepared from this stock solution by adding 25 µl of stock to 75 µl of sterile

distilled DNA-free water. Both working strength aliquots and stock solutions were stored at -20°C until required. Remnants of working strength aliquots were never refrozen but rather discarded after single use.

The reaction mixture consisted of 1µl each primer at 25 pmol μl^{-1}, 12.5 µl of 2x PCR Mastermix (Thermo Scientific Ltd, Epsom, UK) (which contains 75mM Tris-HCl (pH 8.8 at 25°C), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01 %(v/v) Tween 20, 0.2mM each of dATP, dCTP, dGTP and dTTP) and 7.5 µl of sterile distilled DNA-free water. To each reaction mixture, 3 µl of template DNA was added. The thermal programme used for DNA amplification consisted of 95°C for 5 minutes, followed by 35 cycles of 95°C for 10 sec, 55°C for 10 sec and 72°C for 50 sec. The programme was completed with a final elongation step of 72°C for 5 min. This programme was carried out on a PTC-200 DNA Engine (Bio-Rad, Hemel Hempstead, UK). The post-reaction mixes were then removed from the machine and a 5 µl aliquot of each was added to one well of a 96 well round-bottom plate containing 5 µl of sterile distilled water and 2 µl of 0.5% (w/v) xylene cyanol solution containing 50% (v/v) glycerol.

The success of this PCR was determined by visualisation of amplification products following their resolution in1.5% (w/v) agarose gels. Agarose gels were made by dissolving 1.5 g of agarose (Biogene Ltd, Huntingdon, UK) in Tris-Acetate-EDTA (TAE) buffer using a microwave oven to heat the buffer. The agarose solution was allowed to cool then ethidium bromide was added to it to a final concentration of 1.5%. The solution was then poured into a perspex gel tray, a

plastic well comb was placed into the solution, and it was left for 30 min to cool and solidify. The solid agarose gel was then transferred to an electrophoresis tank and submerged in TAE and the well comb was removed. A 10 µl aliquot of each post-reaction mix/xylene cyanol/glycerol mixture was then added to each well in the agarose gel. A DNA size ladder (*Hae*III digest of ϕX174 phage, (New England Biolabs, Hitchin, UK) diluted to 50 µg $µl^{-1}$, was also loaded into at least one empty well on every gel. Once all wells in the gel were loaded, an electric current with a potential difference of 100 volts was applied to the gel for 30 min. After this time, the gel was removed from the gel tank and transferred to a transilluminater to be visualised under UV illumination and images were stored digitally.

3.2.5 Detection of MtC DNA

The PCR assay designed to detect MtC DNA targeted a 236 base pair fragment of IS1081, as previously described (Taylor et al., 2007). For each sample, the reaction mix consisted of 1 µl of primer F2 (5'-CTG CTC TCG ACG TTC ATC GCC G - 3) and 1 µl of primer R3 (5' –TGG CGG TAG CCG TTG CGC -3), at a concentration of 25 pmol $µl^{-1}$, 12.5 µl of 2 x Absolute qPCR SYBR Green Master Mix (Thermo Scientific), 7.5 µl of distilled water and 3 µl of template DNA. The thermal programme consisted of an initial denaturation step of 95°C for 3 min, then 45 cycles of amplification were performed as follows 95°C for 10 sec, 58°C for 30 sec, 72°C for 20 sec and acquisition of fluorescent signal at 85°C. A final extension at 72°C for 2 min completed the programme.

3.3 Results

3.3.1 Overall findings

DNA extracts were prepared from tissues removed from 175 field voles trapped during spatial survey 8 (March 2005) at Kielder Forest. All 175 extracts yielded a 450 base pair product when tested with the PCR that targeted the field vole 18S rDNA fragment, confirming the success of the extraction procedure used on all samples.

An overview of performance of direct PCR in comparison with observation of macroscopic lesions and acid fast bacilli and culture, is shown in Table 3.1.

Table 3.1 vTB diagnostic results for 175 field voles.

vole number	visible lesions	acid-fast bacilli observed	lung culture	lung PCR	lymph node culture	lymph node PCR	culture PCR
T010	no	not tested	negative	negative	negative	negative	not tested
T011	no	not tested	negative	negative	negative	negative	not tested
T015	no	not tested	negative	negative	negative	negative	not tested
T016	no	not tested	negative	negative	positive	negative	positive
T017	no	not tested	negative	negative	negative	negative	not tested
T023	LN[1]	no	negative	negative	negative	negative	not tested
T024	no	not tested	positive	negative	positive	positive	positive
T025	no	not tested	negative	negative	negative	negative	not tested
T026	no	not tested	negative	negative	negative	negative	not tested
T027	no	not tested	negative	negative	positive	negative	positive
T028	no	not tested	negative	negative	negative	negative	not tested
T029	no	not tested	negative	negative	negative	negative	not tested
T037	LN	no	negative	negative	positive	negative	negative
T039	LN	yes	negative	negative	positive	positive	positive
T040	no	not tested	negative	negative	negative	negative	not tested
T041	no	not tested	negative	negative	negative	negative	not tested
T042	LN	no	negative	negative	negative	negative	not tested
T058	no	not tested	negative	negative	negative	negative	not tested
T059	no	not tested	positive	negative	positive	negative	positive
T060	no	not tested	negative	negative	negative	negative	not tested
T061	no	not tested	negative	negative	negative	negative	not tested
T067	no	not tested	negative	negative	negative	negative	not tested
T068	no	not tested	negative	negative	negative	negative	not tested
T069	no	not tested	negative	negative	negative	negative	not tested
T071	no	not tested	negative	negative	negative	negative	not tested
T072	no	not tested	negative	negative	negative	negative	not tested
T074	no	not tested	negative	negative	negative	negative	not tested
T077	LN	no	negative	negative	negative	negative	not tested
T078	no	not tested	negative	negative	negative	negative	not tested
T079	no	not tested	negative	negative	negative	negative	not tested
T081	no	not tested	negative	negative	negative	negative	not tested
T082	no	not tested	negative	negative	negative	negative	not tested
T084	no	not tested	negative	negative	negative	negative	not tested
T085	no	not tested	negative	negative	negative	negative	not tested
T089	no	not tested	negative	negative	negative	negative	not tested
T090	LN	no	negative	negative	negative	negative	not tested
T092	no	not tested	negative	negative	negative	negative	not tested
T093	no	not tested	negative	negative	negative	negative	not tested
T107	no	not tested	negative	negative	negative	negative	not tested

Table 3.1 continued

vole number	visible lesions	acid-fast bacilli observed	lung culture	lung PCR	lymph node culture	lymph node PCR	culture PCR
T108	no	not tested	negative	negative	negative	negative	not tested
T111	no	not tested	negative	negative	negative	negative	not tested
T115	no	not tested	positive	negative	positive	positive	positive
T117	no	not tested	negative	negative	negative	negative	not tested
T118	no	not tested	negative	negative	negative	negative	not tested
T122	no	not tested	negative	negative	negative	negative	not tested
T128	no	not tested	negative	negative	negative	negative	not tested
T129	no	not tested	negative	negative	negative	negative	not tested
T134	no	not tested	negative	negative	negative	negative	not tested
T141	no	not tested	negative	negative	negative	negative	not tested
T143	no	not tested	negative	negative	negative	negative	not tested
T145	no	not tested	negative	negative	negative	negative	not tested
T146	no	not tested	negative	negative	negative	negative	not tested
T149	no	not tested	negative	negative	negative	negative	not tested
T151	no	not tested	negative	negative	positive	negative	positive
T153	no	not tested	negative	negative	negative	negative	not tested
T155	no	not tested	negative	negative	negative	negative	not tested
T156	no	not tested	negative	negative	negative	negative	not tested
T157	no	not tested	negative	negative	negative	negative	not tested
T163	no	not tested	negative	negative	negative	negative	not tested
T164	no	not tested	negative	negative	negative	negative	not tested
T169	no	not tested	negative	negative	negative	negative	not tested
T171	no	not tested	negative	negative	negative	negative	not tested
T173	no	not tested	negative	negative	negative	negative	not tested
T178	no	not tested	negative	negative	positive	negative	positive
T183	no	not tested	negative	negative	positive	negative	positive
T184	no	not tested	negative	negative	negative	negative	not tested
T185	no	not tested	negative	negative	negative	negative	not tested
T188	no	not tested	negative	negative	negative	negative	not tested
T189	no	not tested	negative	negative	positive	negative	positive
T190	no	not tested	negative	negative	negative	negative	not tested
T191	no	not tested	negative	negative	negative	negative	not tested
T202	no	not tested	negative	negative	negative	negative	not tested
T204	LN & LUNG	yes	negative	positive	negative	positive	not tested
T208	LN	no	positive	negative	negative	positive	positive
T209	no	not tested	negative	negative	negative	negative	not tested
T210	LN	yes	negative	negative	positive	positive	positive
V002	LN	no	negative	negative	positive	negative	positive
V003	no	not tested	negative	negative	negative	negative	not tested

Table 3.1 continued

vole number	visible lesions	acid-fast bacilli observed	lung culture	lung PCR	lymph node culture	lymph node PCR	culture PCR
V011	LN	yes	negative	negative	positive	positive	positive
V012	no	not tested	negative	negative	negative	negative	not tested
V018	no	not tested	negative	negative	negative	negative	not tested
V019	no	not tested	negative	negative	positive	negative	positive
V020	no	not tested	negative	negative	negative	negative	not tested
V029	no	not tested	negative	negative	negative	negative	not tested
V030	no	not tested	negative	negative	positive	negative	positive
V031	LN	no	negative	negative	negative	negative	not tested
V032	no	not tested	negative	negative	negative	negative	not tested
V033	no	not tested	negative	negative	negative	negative	not tested
V034	no	not tested	negative	negative	negative	negative	not tested
V039	LN	yes	negative	negative	negative	positive	not tested
V040	no	not tested	negative	negative	negative	negative	not tested
V041	LN	no	negative	negative	negative	negative	not tested
V042	no	not tested	negative	negative	negative	negative	not tested
V043	no	not tested	positive	negative	positive	negative	positive
V046	LN	yes	positive	positive	positive	positive	positive
V047	no	not tested	positive	negative	negative	negative	positive
V048	no	not tested	negative	negative	negative	negative	not tested
V049	no	not tested	negative	negative	negative	positive	not tested
V050	LN	yes	negative	negative	positive	positive	positive
V051	no	not tested	positive	negative	negative	negative	negative
V052	LN	yes	negative	negative	negative	negative	not tested
V053	no	not tested	negative	negative	negative	negative	not tested
V057	no	not tested	positive	negative	negative	negative	negative
V058	no	not tested	negative	negative	negative	negative	not tested
V059	no	not tested	negative	negative	negative	negative	not tested
V062	no	not tested	positive	negative	positive	negative	positive
V063	no	not tested	negative	positive	positive	negative	positive
V064	no	not tested	positive	negative	negative	positive	positive
V065	no	not tested	negative	negative	negative	negative	not tested
V075	no	not tested	negative	negative	negative	negative	not tested
V076	LN	no	positive	positive	negative	positive	positive
V077	no	not tested	negative	negative	negative	negative	not tested
V078	no	not tested	negative	negative	negative	negative	not tested
V079	no	not tested	negative	negative	negative	negative	not tested
V080	no	not tested	negative	negative	negative	negative	not tested
V083	LN	no	negative	negative	negative	negative	not tested
V084	LN	no	negative	negative	negative	negative	not tested

Table 3.1 continued

vole number	visible lesions	acid-fast bacilli observed	lung culture	lung PCR	lymph node culture	lymph node PCR	culture PCR
V090	LN	yes	negative	negative	positive	positive	positive
V091	no	not tested	negative	negative	negative	positive	not tested
V092	no	not tested	negative	negative	negative	negative	not tested
V093	no	not tested	negative	negative	negative	negative	not tested
V094	no	not tested	negative	negative	negative	negative	not tested
V106	no	not tested	negative	negative	negative	negative	not tested
W009	no	not tested	negative	negative	negative	negative	not tested
W010	no	not tested	negative	negative	negative	negative	not tested
W011	no	not tested	negative	negative	negative	negative	not tested
W014	no	not tested	negative	negative	negative	negative	not tested
W016	no	not tested	negative	negative	positive	negative	positive
W019	no	not tested	negative	negative	negative	negative	not tested
W020	no	not tested	negative	negative	negative	negative	not tested
W022	no	not tested	negative	negative	negative	negative	not tested
W023	no	not tested	negative	negative	negative	negative	not tested
W025	LN	no	negative	negative	positive	negative	positive
W027	no	not tested	negative	negative	positive	negative	positive
W028	no	not tested	negative	negative	negative	negative	not tested
W029	no	not tested	positive	negative	positive	negative	positive
W033	no	not tested	negative	negative	negative	negative	not tested
W044	no	not tested	negative	negative	negative	negative	not tested
W048	no	not tested	negative	negative	negative	negative	not tested
W049	no	not tested	positive	negative	negative	negative	negative
W051	no	not tested	negative	negative	negative	negative	not tested
W053	no	not tested	negative	negative	negative	negative	not tested
W054	no	not tested	positive	negative	positive	negative	positive
W057	no	not tested	negative	negative	negative	negative	not tested
W059	LN	yes	negative	negative	positive	positive	positive
W060	LN	no	negative	negative	positive	negative	positive
W064	no	not tested	negative	negative	negative	negative	not tested
W065	LN	yes	negative	negative	positive	negative	positive
W067	LN	no	positive	negative	positive	positive	positive
W073	no	not tested	negative	negative	positive	negative	positive
W077	no	not tested	negative	negative	negative	negative	not tested
W081	LN	no	positive	positive	positive	negative	positive
W082	LN	yes	negative	negative	positive	negative	positive
W085	LN	no	negative	negative	negative	negative	not tested
W094	no	not tested	negative	negative	negative	negative	not tested
W096	no	not tested	positive	negative	negative	negative	negative

Table 3.1 continued

vole number	visible lesions	acid-fast bacilli observed	lung culture	lung PCR	lymph node culture	lymph node PCR	culture PCR
W098	no	not tested	negative	negative	negative	negative	not tested
W099	LN	yes	negative	negative	negative	positive	not tested
W100	no	not tested	negative	negative	negative	negative	not tested
W107	no	not tested	negative	negative	negative	negative	not tested
W108	no	not tested	negative	negative	negative	negative	not tested
W109	no	not tested	negative	negative	negative	negative	not tested
W114	LN	no	positive	negative	positive	negative	positive
W116	LN & LUNG	yes	negative	negative	negative	positive	not tested
W121	LN	yes	negative	negative	negative	positive	not tested
W123	no	not tested	negative	negative	positive	negative	positive
W125	LN	yes	negative	negative	positive	positive	positive
W135	LN & LUNG	yes	negative	positive	positive	positive	positive
W137	LN	yes	negative	negative	negative	positive	not tested
W142	no	not tested	negative	negative	negative	negative	not tested
W144	no	not tested	negative	negative	negative	negative	not tested
W146	no	not tested	negative	negative	negative	negative	not tested
W147	no	not tested	negative	negative	negative	negative	not tested

[1]LN = lymph node

A total of 26 (15%) of extracts yielded an amplification product when tested with the PCR (Table 3.1). Evidence of *M. microti* infection, based on isolation of *M. microti* confirmed by PCR on the isolate, or observation of macroscopic lesions AND microscopic observation of acid-fast bacilli in ZN-stained impression smears, was recorded in a total of 55 (31%) of the 175 voles (Table 3.1 and Figure 3.1).

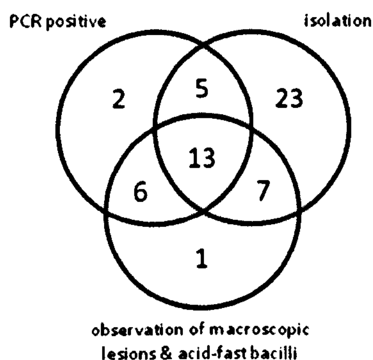


Figure 3.1 Venn diagram demonstrating the relative performances of the three approaches to determining *M. microti*-infection status in the 57 field voles with evidence of infection.

	PCR positive	PCR negative	TOTAL
"true positive"	24	31	55
"true negative"	2	118	120
TOTAL	26	149	175

Table 3.2 Summary of the performance of PCR relative to established ("true") diagnostics.

Only 24 of 55 animals considered as "truly" infected yielded a PCR product (Table 3.2), hence the sensitivity ((true positives / true positives + false negatives) x 100) of PCR relative to the established diagnostics was only 47%. Two animals that lacked macroscopic lesions and that did not yield an *M. microti* isolate tested PCR positive. Thus, by comparison to the two established approaches to infection status determination, the specificity of the PCR assay ((true negatives / true negatives + false positives) x 100) was estimated to be 98%.

Estimation of the positive predictive value (i.e. the proportion of animals with positive PCR results that are correctly diagnosed) of the PCR assay was:

24 (true positives) / 26 (true positives + false positives) x 100 = 92%

Estimation of the negative predictive value (i.e. the proportion of animals with negative PCR results who are correctly diagnosed) of the PCR assay was:

118 (true negatives) / 149 (true negatives + false negatives) x 100 = 79%

3.3.2 Performance of PCR in field voles yielding a M. microti isolate.

An isolate of *M. microti* (confirmed by PCR of cultured organism) was obtained from 48 of the 55 animals with evidence of infections (87%). Direct PCR-based

evidence of infection was found in only 18 of these animals (38%). Twelve animals yielded an isolate from both lung and lymph node cultures. Of these, 6 (50%) tested positive using the IS1081 PCR. *M. microti* was isolated only from the lymph nodes of 28 animals. Of these, nine animals (32%) also tested IS1081 PCR positive. *M. microti* was isolated only from the lungs of eight field voles. Of these, three (38%) tested PCR positive. Of the 20 animals yielding an isolate from lung tissue, nine (45%) tested PCR positive. Of the 40 animals yielding an isolate from lymph node tissue, 15 (38%) tested PCR positive. Eight animals that did not yield an isolate tested PCR positive, however, six of these animals had macroscopic lesions containing acid-fast bacilli.

3.3.3 Performance of PCR in field voles with visible lesions and acid fast bacilli.

A total of 36 of the 175 field voles studied possessed visible lesions consistent with vTB and for 27 of these, either acid-fast bacilli could be observed in ZN stained tissue smears or a *M. microti* isolate was obtained. A PCR product was obtained from 19 (70%) of these animals. Three animals had visible lesions in both lungs and lymph nodes and all tested IS1081 PCR positive. Sixteen (67%) of the 24 animals with lesions only in the lymph nodes tested IS1081 PCR positive. Seven animals without visible lesions yielded a IS1081 PCR product; *M. microti* isolates were obtained from 5 of these animals.

3.4 Discussion

This study was the first to apply real-time PCR to determining the *M. microti* infection status in field voles. The assay chosen was a generic MtC assay, targeting IS1081 insertion sequences, as described by Taylor and colleagues (2007). This assay was chosen over other assays not only as a result of its published performance, but also following consultation with scientists routinely using the assay (Dr Michael Taylor, Imperial College, personal communication). Evaluation of the IS1081 assay, although described for use in rapid detection of *M. bovis* DNA, included three *M. microti* strains, all of which were successfully detected by the assay (along with strains of other MtC member species) (Taylor et al., 2007). Other assays targeting IS1081 have been frequently used for detection of MtC species, either directly in infected clinical material or to confirm the identity of isolates. For example, Shin and colleagues (1995) used an IS1081-targetting assay to detect *M. tuberculosis* in a captive orang-utan in Korea. Also, Ahmed and colleagues (1998) used a similar assay to detect *M. tuberculosis* in blood from immunocompetent patients with pulmonary tuberculosis. Finally, Fatolahzadeh and colleagues (2007) used IS1081 PCRs to diagnose *M. tuberculosis* infections in pleural fluid samples from Iranian patients with tuberculous pleuritis. In this final report, the performance of the IS1081 assay was compared to an assay targeting an *rpoB* fragment. The sensitivity, specificity, positive predictive value and negative predictive value for the IS1081 PCR were 94%, 56%, 37% and 97%, respectively, which were generally better than values of 94%, 26%, 26% and 94% obtained for the *rpoB* assay (results of both assays were compared to traditional diagnostic approaches) (Fatolahzadeh

et al., 2007). Interestingly, the presence of IS1081 in multiple copies in the genome of MtC species, which is one reason why it is a popular PCR target, did not enhance the sensitivity of the assay used by Fatolahzadeh and colleagues when compared to the (single copy) *rpoB* assay. Taylor and colleagues (2007) compared the performance of their IS1081 assay (i.e. that used in this study) relative to an assay targeting a fragment of the single copy RD4 region. The former assay was able to detect at least five times less MtC DNA (genome copies) than the latter, and when applied to *M. bovis*-infected lymph nodes, the detection rate (compared to culture) was found to be 91% using the IS1081 assay and only 59% using the RD4 assay (Taylor et al., 2007).

Only one previous attempt to detect *M. microti* in infected field voles by PCR has been reported. Cavanagh and colleagues (2002) prepared DNA extracts from various clinical samples and used these as templates for spoligotyping. Twenty four of the 60 samples tested yielded a PCR product. The make-up of these samples is not entirely clear, but it is reported that amplification products were obtained from skin and internal macroscopic lesions and lymph nodes, but not from faeces, urine or lungs. No attempt to evaluate the performance of the PCR compared to isolation of other approaches to diagnosis was made (Cavanagh et al., 2002).

The performance of the IS1081 assay in this study was far from perfect, with a sensitivity of only 47% compared to a combination of the two established approaches of culture and presence of visible lesions containing acid-fast bacilli.

When individual approaches were compared, culture was the best, identifying 48 of the 55 infected animals encountered in the survey. Twenty seven of these animals possessed macroscopic lesions associated with acid-fast bacilli and only 24 yielded a PCR product. However, clearly, no one method was capable of identifying all the infected animals. Although diagnosis by PCR and observation of macroscopic lesions associated with acid-fast bacilli identified a similar proportion of infected animals, there was not congruence between the two approaches, with only 19 animals being diagnosed by both. This observation is at odds with other studies that have reported good agreement between the two approaches. In their study using a LightCycler assay for detection of *M. tuberculosis* in clinical samples, Burggraf and colleague (2005) reported that all the samples yielding a PCR product were found to contain acid-fast bacilli. Similarly, Dalovisio and colleagues (1996), in their comprehensive comparison of the various commercial and in-house approaches to nucleic-acid amplification of *M. tuberculosis* DNA in respiratory specimens, reported high levels of agreement between PCR results and those obtained from examination of ZN-stained bronchial smears. Neither of these reports, or others, quantified the number of acid-fast bacilli observed, and this quantification was not done during the current study. Thus, I can only speculate that better agreement between PCR and smear results were obtained in these studies because patients had overt disease and thus more intense infections. However, as the animals in the current study were included because they were caught rather than because they were ill, this explanation is plausible.

The inadequacy of the sensitivity of PCR diagnostics when compared to culture for MtC diagnosis is well recognised, and no reference could be found to a molecular assay that out-performed culture in this respect. For example, even in the study by Taylor and colleagues (2007), which involved only lymph nodes with macroscopic lesions, the IS1081 assay only detected DNA in 91% of 109 samples from which a *M. bovis* isolate was obtained. It appears that the reason for this shortfall is the difficulty associated with extracting mycobacterial DNA from infected tissues rather than the presence of PCR inhibitors (Taylor et al., 2007). My finding that vole DNA could be amplified from all extracts goes some way to supporting this hypothesis. The lysis of mycobacterial cells to release DNA is well-recognised as being problematic and results from the robust cell wall they possess (Brennan & Nikaido, 1995). As highlighted in the Introduction, a wide variety of approaches have been developed and employed, and none has been proven to be the best. The methodology chosen in this study was shown by Taylor and colleagues (2007) to have good efficiency.

Despite its position as the diagnostic "gold standard", isolation of MtC is not 100% sensitive (as already discussed in Chapter 2). In this study, seven animals had evidence of *M. microti* infection but did not yield an isolate. DNA extracts derived from six of these animals produced a product when tested in the IS1081 PCR. Taylor and colleagues (2007), working with macroscopic lesions, found nine that yielded an IS1081 PCR product but no isolate. They concluded that this finding probably reflects amplification by mycobacterial DNA from dead organisms, although, in our study at least, the presence of intact acid-fast bacilli

suggests this argument may not always be correct. Other studies have also reported this situation. Liebana and colleagues (1995) found that tissue samples from two animals were negative when they were cultured but were positive when tested by PCR. These samples were from skin-test positive cattle, which, on post-mortem examination, were found to possess caseous lesions containing acid-fast bacilli. The authors considered that their finding resulted from the effects of the harsh decontaminates which are routinely used to remove contaminating bacteria from suspected MtC-infected tissue samples. A similar explanation is possible for this study. Among the MtC, *M. microti* is the most difficult to isolate, requiring prolonged incubation times. Furthermore, anecdotal evidence (Dr James Dale, TB Laboratories, VLA Weybridge, personal communication) suggests that different strains of *M. microti* grow better on different media, so my use of only Lowenstein-Jensen agar may have compromised the efficiency of the isolation approach.

Interestingly, in this study, a PCR product was obtained from the lymph nodes of two animals for which no other indication of *M. microti* infection was obtained. Cautiously, these results have been treated as false positives. However, no reports of a lack of specificity from IS1081 PCRs have been forthcoming. Furthermore with these samples, as with all others reported in this study, negative control dummy samples processed concurrently did not yield a PCR product, thus there was no apparent cross-contamination between samples. One feasible explanation is that these samples did indeed contain *M. microti* DNA, but this DNA was not associated with viable and/or intact bacteria.

Alternatively, knowing the shortfalls in the sensitivity of culture and, in particular, ZN staining, for *M. microti* diagnosis, these samples may have contained infectious bacteria that were missed by the established diagnostic approaches.

In summary, the PCR used in this study showed good specificity but only moderate sensitivity. This result is not entirely surprising given the low numbers of viable bacteria recovered from infected tissues by isolation (the majority of samples yielded less than 10 bacteria). However, despite this shortfall, the application of PCR does permit the rapid detection of *M. microti* in some samples. This is particularly appealing for a bacterium that typically takes up to three months to grow. The sensitivity of PCR is similar to that obtained when observing macroscopic lesions and associated acid-fast bacilli – this approach is widely considered of value for MtC diagnosis. For all MtC infections, it is clear that although culture remains the most sensitive means of assessing infection status, the inclusion of other, often more convenient, approaches enhances overall diagnostic sensitivity.

Chapter 4.

Molecular epidemiology of tuberculosis among Kielder Forest field voles

4.1 Introduction

The earlier chapters in this thesis have focused on pathology associated with vole tuberculosis (vTB) and diagnosis of infection. This chapter addresses a different area by applying contemporary molecular tools to study the genetic diversity among *M. microti* isolates recovered from field voles, and using these results to explore the epidemiology of vTB in the Kielder Forest.

A wide variety of molecular approaches has been used to explore the genetic diversity of *M. tuberculosis* complex (MtC) species (Haddad et al., 2004). However, as discussed in Chapter 1, the MtC is characterised by extreme genetic homology and clonality (Brosch et al., 2002). A consequence of this second characteristic is that once DNA is lost from a genome, it cannot return, hence relatedness between strains can be deduced by evaluating the presence or absence of specific genomic regions. Brosch and colleagues (2002) exploited deletions together with single nucleotide polymorphisms (SNPs) to infer the phylogenetic relatedness between MtC species. This study indicated that *M. microti* was closely allied to *M. pinnipedii* within the lineage leading from *M. tuberculosis* to *M. bovis*. Both *M. microti, M. pinnipedii* and *M. bovis* were all found to lack several genetic regions (RDs) present in *M. tuberculosis*, termed RD7, RD8, RD9 and RD10, however the former two species had RD4, RD12 and RD13, all of which were missing from *M. bovis* (Brosch et al., 2002). The deletion of RD4 appears to distinguish *M. bovis* from the goat species *M. caprae* (Smith et al., 2006). Three further RDs, RD1, RD2 and RD14, were found to be present in some *M. bovis* strains, but not others, for example, RD14 was missing from the

M. bovis BCG Pasteur vaccine strain (Brosch et al., 2002). Differences between *M. microti* and *M. pinnipedii* were identified by Huard and colleagues (2003) who identified a number of *M. microti*-specific SNPs within RD13 and the 16S rRNA-encoding gene. Brodin and colleagues (2002) also explored MtC RDs and found some, including RD^{mic} that were missing from all nine *M. microti* isolates they included in their study. RD^{mic} is a 14 kilobase region that, in *M. tuberculosis*, includes the genes encoding the potent T-cell antigens ESAT-6 region (Tekaia et al., 1999) and CFP-10 (Harboe et al., 1998). Brodin and colleagues (2002) also identified another RD, termed MiD1 that was missing from all *M. microti* strains studied. This RD lay within the direct repeat-containing locus targeted by spoligotyping (see below), and this RD was responsible for the reduced spoligotype pattern that is characteristic of *M. microti* (Brodin et al., 2002). The determination of the presence or absence of RDs is now considered a key genetic means of differentiating between MtC members and for assigning strains to species within the complex (Smith et al., 2006). RD analysis is also widely used for intra-species delineation, particularly among *M. tuberculosis* strains on a global scale (e.g. Lazzarini et al., 2007; Thwaites et al., 2008), although for MtC species in which many of the RDs present in *M. tuberculosis* are missing, this approach has less sensitivity hence other methods are required.

Spoligotyping is probably the most widely used approach to genetic intra-species differentiating between MtC members and has been applied to all species within the complex (Kamerbeek et al., 1997). Unlike other contemporary MtC typing schemes, spoligotyping quantifies genetic variation in a single genomic region

called the direct repeat (DR) locus (Kamerbeek et al., 1997). This locus comprises of multiple, virtually identical, 36 base pair regions interspersed with unique DNA spacer sequences of a similar size, termed direct variant repeats (DVRs). The sequences of different DVRs are dissimilar and each DVR exists only in the DR locus (van Embden et al., 2000). The number of DVRs within the DR locus may be as many as 60, however, 43 have been selected and are now used in the international standard application of spoligotyping. This application exploits PCR to amplify the DR locus of a particular MtC strain, then Southern blotting using oligonucleotides specific to each of the 43 DVRs to probe for their presence or absence in the amplified DR locus (Kamerbeek et al., 1997). Genetic variation at the DR locus results from the deletion of single or multiple DVRs, giving rise to a huge number of variants. These variants are catalogued on international databases. SpolDB4 is the latest iteration of the *M. tuberculosis* spoligotype database (Brudey et al., 2006), which contains information about almost 40,000 isolates obtained from 122 different countries. For MtC strains other than *M. tuberculosis*, the www.Mbovis.org database is used. This resource now contains almost 2,000 distinct spoligotype patterns.

M. microti was not included in the original description of a standardised spoligotyping protocol (Kamerbeek et al., 1997), but the following year, van Soolingen and colleagues (1998) published the first application of spoligotyping for the species. Their study included 13 isolates including five obtained from voles in the UK during the 1930s and six more recent isolates made from non-rodent hosts, including human beings, a pig and a ferret in The Netherlands.

These 11 isolates all possessed the same spoligotype, referred to as SB0118 in the www.Mbovis.org database, which is characterised by the possession of only two DVRs, 37 and 38 and is now considered as the "classic" *M. microti* spoligotype (Emmanuel et al., 2007). The study also included two other isolates, the "dassie bacillus", which is no longer considered as *M. microti* (Mostowy et al., 2004) and an isolate obtained from a llama in a Belgian zoo. The spoligotype of this isolate contained DVRs 37 and 38, but also seven additional DVRs. In a follow-up study, Kremer and colleagues (1998) reported spoligotype data for 11 more *M. microti* isolates. Four of these isolates possessed SB0118 or a very similar spoligotype comprised of DVR 4, 37 and 38. Six of these isolates possessed a spoligotype that differed only by the absence of DVR 26 from the spoligotype of the isolate obtained from the Belgian llama. The final isolate also possessed a spoligotype that differed by the absence of a single DVR from the llama isolate, but the missing DVR was 38, one of those also present in SB0118 (Kremer et al., 1998). Brodin and colleagues (2002) also reported spoligotyping of nine *M.microti* strains and encountered SB0118 and the profile obtained from the Belgian llama. However several, if not all, of these isolates were the same as those previously typed. These data confirmed the existence of rather more spoligotype diversity among *M. microti* strains than was previously thought, and suggested the existence of two genetically different groups of strains within the species, the "vole type" and the "llama type". All of the five vole isolates (all probably from Kielder) studied up to this point possessed the same "vole type" spoligotype, but otherwise the epidemiological significance of these two types remained unclear. In 2002, Cavanagh and colleagues reported detecting the

SB0118 spoligotype in infected tissues collected from 24 vTB symptomatic field voles at Kielder Forest in 1998 and 1999. Similarly, Burthe (2005) reported the same spoligotype in 35 *M. microti* isolates obtained from 29 voles trapped at Kielder Forest during 2001 and 2002.

Variable number tandem repeat (VNTR) typing is another PCR-based approach to delineation of bacterial strains. It is very much akin to mini-satellite typing widely used in eukaryotic population genetics (Haddad et al., 2004) as it measures variation in the number of repeats of specific DNA motifs at a series of loci dispersed throughout the genome (Jeffreys et al., 1985). These loci tend to be non-coding and contain what is often referred to as "junk" DNA. Each DNA motif is, typically, 10 to 20 base pairs long and may exist as a single copy or in multiple repeats, ranging from two to several dozen (Riley, 2004). A PCR is designed to bridge the entire VNTR region and variation in the size of the amplification product obtained is used as the basis of strain differentiation. Usually several different VNTR loci are included in a typing scheme to increase its sensitivity. VNTR analysis has been applied to numerous bacterial pathogens, particularly those which are considered of limited genetic diversity such as *Yersinia pestis* (Adair et al., 2000), *Bacillus anthracis* (Jackson et al., 1998) and *M. tuberculosis* (Frothingham et al., 1998). VNTR analysis is widely considered a highly sensitive means of distinguishing between highly related strains of a pathogen species, and is therefore often used to unravel epidemiological issues on a local scale (e.g. Jackson et al., 1998; Sola et al., 2001).

VNTR analysis was first described from *M. tuberculosis* in 1998 (Frothingham & Meeker-O'Connell, 1998) and this approach has subsequently been applied to other members of the MtC (Frothingham et al., 1999; Roring et al., 2002; 2005; Moser et al., 2008). VNTR analysis is now widely used in conjunction with spoligotyping as a means of obtaining a sensitive genetic identifier for a particular MtC member. The use of VNTR analysis has, to some extent, replaced another form of genotyping, which is based in Southern blotting electrophoretically-resolved genomic digests with a probe for the IS6110 insertion sequence. IS6110-based genotyping of MtC members was developed in the early 1990s (Cave et al., 1991) and rapidly became the gold-standard. However, this approach is problematic as it is technically demanding, requires a large biomass of bacteria from which to isolate sufficient DNA, and requires extensive use of standards to obtain profiles that are meaningfully comparable with one another (Kanduma et al., 2003). Both VNTR and IS6110-based genotyping are widely used, particularly for *M. tuberculosis* molecular epidemiological studies. However, for other MtC members, and a subset of *M. tuberculosis* strains, the low copy number of IS6110 elements present in the genome restricts the usefulness of this approach (Haddad et al., 2004). For *M. bovis* in particular, the copy number of IS6110 is low, with some strains possessing only a single copy of the insertion sequence (Haddad et al., 2004). Comparison of the relative performances of IS6110 and VNTR typing have yielded conflicting results (Allix et al., 2006; Hanekom et al., 2008) but, in the majority of epidemiological settings, the two approaches appear to be of similar sensitivity and yield congruent results (Allix et al., 2006; Oelemann et al., 2007;

Allix-Beguec et al., 2008). These observations support the usefulness of VNTR typing as a convenient and discriminatory technique for the analysis of the population structure of MtC members, particularly non-*M. tuberculosis* species, and when fine-scale discrimination is required.

Several different types of VNTRs have been described and exploited for the development of VNTR typing of MtC members. Hermans and colleagues (1992) detected and characterised a "major polymorphic tandem repeat" (MPTR) in the *M. tuberculosis* genome, made up of a 10-bp sequence that was tandemly repeated and invariably separated by 5 bp unique spacer sequences. The authors demonstrated the presence of this feature in the genome of other *Mycobacterium* species and discussed its potential usefulness in the epidemiology of mycobacterioses, although no further exploration of MPTR in this role has been published. The first description of the VNTR scheme most widely used today for *M. tuberculosis* and *M. bovis* was published by Frothingham and Meeker-O'Connell in 1998. In this study, 11 tandem repeat loci in the *M. tuberculosis* genome were analysed. These comprised of five major polymorphic tandem repeat (MPTR) loci, characterised by 15 bp repeats with substantial sequence variation in adjacent copies, and six exact tandem repeat (ETR) loci, characterised by large (40-100 bp) DNA repeats with identical sequences in adjacent repeats. Exploration of these loci in 48 MtC strains revealed one of the five MPTR loci and all six ETR loci had length polymorphisms corresponding to insertions or deletions of tandem repeats, and each ETR locus had multiple alleles in the panel. Combined analysis identified 22 distinct allele

profiles in 25 wild-type MtC strains and five allele profiles in 23 *M. bovis* BCG strains. The study also found that allelic profiles were reproducible and stable, as demonstrated by analyses of multiple isolates of particular reference strains obtained from different laboratories. Additional VNTR loci, termed *Mycobacterium* interspersed repetitive units (MIRUs) were first described by Supply and colleagues (2000). These authors found 41 MIRU loci in the *M. tuberculosis* H37Rv chromosome, some of which corresponded to the ETR loci previously described. After PCR and sequence analyses of these loci in 31 MtC strains, 12 of them were found to display variations in tandem repeat copy numbers. Even more VNTR loci have been described subsequently (e.g. Roring et al., 2002). Numerous studies have evaluated the relative discriminatory power of various sets of VNTR loci in relation to one another and other mycobacterial typing schemes such as spoligotyping and IS6110 typing. In the UK, the six VNTR loci described by Frothingham and Meeker-O'Connell (1998) are routinely used (by the VLA) to type *M. bovis*, and the discriminatory power and epidemiological relevance of VNTR typing of *M. bovis* on this country has been well documented (e.g. Smith et al., 2003; Smith et al., 2006; Evans et al., 2007).

This study aimed to explore the local diversity of *M. microti* and the epidemiology of vTB among Kielder field voles by applying contemporary molecular fingerprinting (spoligotyping and VNTR) to isolates derived from structural spatial surveys of the area. The study aimed to explore the evolutionary relatedness among *M microti* isolates and other members of the *M tuberculosis* complex using deletion analysis.

4.2 Materials and methods

4.2.1 Exploring regions of difference (RD)

4.2.1.1 M. microti isolates

Eighteen representatives were selected from the archive of *M. microti* isolates obtained from field voles at Kielder Forest since 2001. For each isolate, a sweep of colonies was harvested from LJP agar slopes into a sterile Eppendorf tube containing 50 µl of sterile water. This cell suspension was then held at 100°C on a heating block for 10 min to kill and lyse bacteria. *M. tuberculosis* and *M. bovis* control strains (*M. tuberculosis* H37RVb & *M. bovis* BCG) were prepared in the same way.

4.2.1.2 PCR amplification of RDs

Three previously-described PCR assays were used to determine the presence or absence of three RDs, namely RD^{mic}, RD4 and RD14 (Brosch et al., 2002). Each reaction mix consisted of 0.5 µl of a 25 pmol μl^{-1} solution of each primer (see Table 4.1), 10 µl of HotStart Taq Mastermix (Qiagen), 7 µl of sterile distilled water and 2 µl of template DNA. Reaction mixes were subjected to the following thermal programme: an initial denaturation at 95°C for 15 min, then 35 cycles of 94°C for 60 sec, 61°C for 60 sec and 72°C for 150 sec, then a final elongation step at 72°C for 10 min. Each PCR run included, in addition to test strains, the two control strains and a reagent-only (no DNA added) negative control. Amplification products were electrophoretically resolved on 1.5% agarose gels containing 7 µg ml^{-1} ethidium bromide in Tris-acetate-EDTA (TAE) buffer, then visualised under UV illumination.

region of difference	primer sequences	expected fragment size (bp)
RD4	forward 5'-ctg gtc gaa ggc cac taa ag-3' reverse 5'-aag gcg aac aga ttc agc at-3'	447
$RD1^{mic}$	forward 5'-gca gtg caa agg tgc aga ta-3' reverse 5'-gca gtg caa agg tgc aga ta-3'	642
RD14	forward 5'-cga tgg tgt ttc ttg gtg ag-3' reverse 5'-gga tcg gct cag tga ata cc-3'	481

Table 4.1 Sequences of primers used in PCR amplification of RDs as described by Brosch et al., 2002.

4.2.2 Spoligotyping

4.2.2.1 M. microti isolates

Thirty nine representatives were selected from the archive of *M. microti* isolates obtained from field voles inhabiting Kielder Forest District since 2001. Isolates of diverse provenance were chosen, by selecting strains infecting voles in different areas of the District and from different years (April 2002 to September 2005), as described in Table 4.2. For each isolate, a sweep of colonies was harvested from LJP agar slopes into a sterile Eppendorf tube containing 50µl of sterile water. This cell suspension was then held at 100°C on a heating block for 10 min to kill and lyse bacteria. *M. tuberculosis* and *M. bovis* control strains were prepared in the same way.

survey date	isolate	grid	catchment
April 2002	A016	SWC	Redesdale
	A031	BGB	Kielder
	A061	PLJ	Kielder
	A085	WNB	Kielder
	A097	SWB	Redesdale
	A102	BLB	Kielder
	B009	TBB	Kershope
	B022	TBB	Kershope
	B042	TDH	Kershope
	B063	RPC	Kershope
	B091	RPC	Kershope
	B103	KRB	Kershope
	B198	KRB	Kershope
September 2002	C040	BLB	Kielder
	C167	LSB	Kielder
	C183	BLB	Kielder
	D036	WNH	Kielder
	D037	WNH	Kielder
April 2003	E004	LSB	Kielder
	E069	BBH	Kielder
	E088	PLJ	Kielder
	F003	QRY	Redesdale
	F010	WBN	Kielder
	F030	CWS	Kershope
	F148	KRB	Kershope
	F182	TBB	Kershope
	F186	TBB	Kershope
April 2005	T100	CWS	Kershope
	T115	TBB	Kershope
	T189	KRB	Kershope
	V007	SQC	Kielder
	V057	BBH	Kielder
	W060	BHP	Redesdale
	W099	WBN	Kielder
	W123	PLJ	Kielder
	W135	CWS	Kershope
September 2005	AA101	KCS	Kielder
	AA368	LSB	Kielder
	AC068	TBB	Kershope

Table 4.2 Provenance of *M. microti* isolates on which spoligotyping was performed.

4.2.2.2 PCR amplification of the DR region

PCR-based amplification of the DR region was achieved using a previously-described assay (Kamerbeek et al., 1997). Each reaction mixture comprised 2 µl

of a 25 pmol μl^{-1} solution of biotinylated primer DRa (5'-ggt ttt ggg tct gac gac-3'), 2μl of a 25 pmol μl^{-1} solution of primer DRb (5'-ccg aga ggg gac gga aac-3'), 12.5μl of HotStart Taq Mastermix (Qiagen), 5.5μl of sterile distilled water and 5μl of boiled cell suspension. Reaction mixes were subjected to the following thermal programme: an initial denaturation at 95°C for 15 min, then 35 cycles of 96°C for 60 sec, 55°C for 60 sec and 72°C for 50 sec, then a final elongation step at 72°C for 10 min. Each PCR run included, in addition to test strains, two positive control strains (*M. tuberculosis H37RVb* and *M. bovis BCG*) and a reagent-only (no DNA added) as a negative control.

4.2.2.3 Attachment of DR spacer oligonucleotides to the spoligotype membrane. Oligonucleotides corresponding to 43 unique DR spacer regions, were synthesized commercially (Eurofins MWG Operon) with a 5'-terminal amino group modification, permitting their covalent linkage to a negatively charged nylon membrane. Each oligonucleotide was diluted to a pre-determined optimal concentrations (typically 125 to 5000 pmol) in 150μl of 500mM $NaHCO_3$, pH8.4.

A negatively charged nylon membrane (Biodyne C, Pall Europe, Portsmouth, UK) was cut to the appropriate size and activated by a 10 min incubation in 16% (w/v) 1-ethyl-3(3-dimethyaminpropyl) carbodiimide (EDAC) in distilled water in a rolling hybridisation bottle at room temperature. The activated membrane was then placed on a support foam cushion (PC200, Immunetics, Cambridge, UK) and the two layers were sandwiched in a clean miniblotter system (MN45, Immunetics) with the membrane on the slatted side of the apparatus. After

removal of residual liquid, 150µl of each of the 43 oligonucleotides was added to the different slats spanning the membrane. After 1 min incubation, excess oligonucleotide was removed from each slat by aspiration, the membrane was removed from the miniblotter using forceps and inactivated by soaking in 100 mM NaOH in a rolling bottle at room temperature for 8 min. The membrane was washed in 250ml of primary buffer (2xSSPE [0.18 M Na Cl, 10 mM Na H_2 PO_4, 1 mM EDTA (pH7.7)], 0.1% SDS) for 5 min at 60°C then stored at 4°C until required.

4.2.2.4 Hybridisation of PCR products to membrane-bound oligonucleotides

A 150 µl aliquot of the primary buffer was dispensed into each well of a 96 well microtitre plate, then 20µl of PCR product was added to each well. The plate was sealed and heated to 96°C for 10 min to denature the PCR product.

The membrane with DR spacer oligonucleotides attached was removed from the fridge and equilibrated in 250ml of pre-warmed primary buffer for 10 min in the 60°C oven on the shaking platform. The membrane was placed on a foam cushion and the two layers were sandwiched in the miniblotter as before, ensuring that the lines of oligonucleotides ran perpendicular to the slats. An aspirator was used to remove any residual fluid from the slats. The denatured PCR products were removed from the heating block onto ice, then each was aliquoted into one of the slats on the miniblotter, which was then hybridized for 60 min at 60°C on a horizontal surface without shaking. Next, excess PCR products were removed from each slat by aspiration and the membrane was transferred into 250ml of secondary buffer (2XSSPE-0.5% SDS) using forceps. The

membrane was washed in this buffer twice at 60°C for 10 min on a shaking platform before being transferred to a rolling bottle containing distilled water and allowed to cool. The distilled water was discarded and replaced with 2.5µl of streptavidin-peroxidase conjugate (500U ml^{-1}) (Boehringer Ltd, Bracknell, UK) diluted in 10ml of secondary buffer. The membrane was incubated at 42°C for 60 min then was removed from the bottle and placed in a plastic container containing 250ml of secondary buffer. The membrane was washed twice in this buffer for 10 min at 42°C on a shaking platform, then twice with 2X SSPE for 5 min at room temperature. The membrane was transferred to a plastic pouch to which 15 ml of chemiluminescent peroxidase substrate (ECL Solution, Amersham Biosciences, Little Chalfont, UK) was added. After 2 to 10 min, the membrane was removed from the pouch and wrapped in clingfilm. In a dark room the membrane was placed in contact with X-ray film in an autorad cassette case and exposed at room temperature for between 20 min to overnight, depending on signal strength. Following exposure, the X-ray film was developed using an automatic X-ray developer and results were read by placing the developed film on a light box.

4.2.3 Variable number of tandem repeat (VNTR) analysis

4.2.3.1 M. microti isolates

One hundred and twenty one representatives were selected from the archive of *M. microti* isolates obtained from field voles inhabiting Kielder Forest District since 2001. Isolates of diverse provenance were chosen, by selecting strains infecting voles in different areas of the District and from different years (April

2002- September 2005), as described in Table 4.3. For each isolate, a sweep of colonies was harvested from agar slopes into a sterile Eppendorf tube containing 50µl of sterile water. This cell suspension was then held at 100°C on a heating block for 10 min to kill and lyse bacteria. *M. tuberculosis* and *M. bovis* control strains were prepared in the same way.

survey date	isolate	grid	catchment
Apr-02	A016	SWC	Redesdale
Apr-02	A031	BGB	Kielder
Apr-02	A061	PLJ	Kielder
Apr-02	A063	RPC	Kershope
Apr-02	A085	WNH	Redesdale
Apr-02	A097	SWC	Redesdale
Apr-02	A100	RPC	Kershope
Apr-02	A101	RPC	Kershope
Apr-02	A102	BLB	Kielder
Apr-02	B022	TBB	Kershope
Apr-02	B042	TDH	Kershope
Apr-02	B063	RPC	Kershope
Apr-02	B091	RPC	Kershope
Apr-02	B103	KRB	Kershope
Apr-02	B148	KRB	Kershope
Apr-02	B198	KRB	Kershope
Sep-02	C167	LSB	Kielder
Sep-02	C183	BLB	Kielder
Sep-02	D037	WNH	Redesdale
Apr-03	E004	BGB	Kielder
Apr-03	E036	BCR	Kielder
Apr-03	E069	BBH	Kielder
Apr-03	E088	PLJ	Kielder
Apr-03	F003	QRY	Redesdale
Apr-03	F010	WBN	Kielder
Apr-03	F016	ROB	Redesdale
Apr-03	F018	ROB	Redesdale
Apr-03	F022	ROB	Redesdale
Apr-03	F117	TBB	Kershope
Apr-03	F120	TBB	Kershope
Apr-03	F121	TBB	Kershope
Apr-03	F148	KRB	Kershope
Apr-03	F182	TBB	Kershope
Apr-03	F185	TBB	Kershope
Apr-03	F186	TBB	Kershope
Apr-05	T016	TRI	Kershope
Apr-05	T024	CGF	Kershope
Apr-05	T027	CGF	Kershope
Apr-05	T037	SLB	Kershope
Apr-05	T039	SL B	Kershope
Apr-05	T059	RPC	Kershope
Apr-05	T100	CWS	Kershope
Apr-05	T115	TBB	Kershope
Apr-05	T151	CGF	Kershope

survey date	isolate	grid	catchment
Apr-05	T178	CGF	Kershope
Apr-05	T183	TBB	Kershope
Apr-05	T208	TDH	Kershope
Apr-05	T210	TDH	Kershope
Apr-05	V002	SQC	Kielder
Apr-05	V007	SQC	Kielder
Apr-05	V011	SQC	Kielder
Apr-05	V019	BLB	Kielder
Apr-05	V022	BLB	Kielder
Apr-05	V023	BLB	Kielder
Apr-05	V027	KCS	Kielder
Apr-05	V029	KCS	Kielder
Apr-05	V030	KCS	Kielder
Apr-05	V040	SLB	Kershope
Apr-05	V043	BCR	Kielder
Apr-05	V046	SWB	Kielder
Apr-05	V047	SWB	Kielder
Apr-05	V048	SWB	Kielder
Apr-05	V050	SWB	Kielder
Apr-05	V051	SWB	Kielder
Apr-05	V057	BBH	Kielder
Apr-05	V062	SQC	Kielder
Apr-05	V063	SQC	Kielder
Apr-05	V064	SQC	Kielder
Apr-05	V057	BBH	Kielder
Apr-05	V073	LSB	Kielder
Apr-05	V076	LSB	Kielder
Apr-05	V085	BCR	Kielder
Apr-05	V090	SWB	Kielder
Apr-05	V097	SQC	Kielder
Apr-05	W016	RLB	Kershope
Apr-05	W025	CWS	Kershope
Apr-05	W027	CWS	Kershope
Apr-05	W029	TBB	Kershope
Apr-05	W037	WBN	Redesdale
Apr-05	W042	WBN	Redesdale
Apr-05	W054	CWS	Kershope
Apr-05	W055	CWS	Kershope
Apr-05	W059	BHP	Redesdale
Apr-05	W060	BHP	Redesdale
Apr-05	W062	BHP	Redesdale
Apr-05	W065	BHP	Redesdale
Apr-05	W067	CWS	Kershope
Apr-05	W073	FRD	Kielder
Apr-05	W081	CHS	Kielder
Apr-05	W082	CHS	Redesdale
Apr-05	W085	CHS	Redesdale
Apr-05	W096	ROB	Redesdale
Apr-05	W097	ROB	Redesdale
Apr-05	W098	SWC	Redesdale
Apr-05	W099	WBN	Kielder
Apr-05	W114	FRD	Kielder
Apr-05	W125	PLJ	Kielder
Apr-05	W126	PLJ	Kielder
Apr-05	W135	CWS	Kershope
Sep-05	AA012	QRY	Redesdale
Sep-05	AA062	SWC	Redesdale

survey date	isolate	grid	catchment
Sep-05	AA101	KCS	Kielder
Sep-05	AA102	KCS	Kielder
Sep-05	AA103	KCS	Kielder
Sep-05	AA146	ROB	Redesdale
Sep-05	AA152	ROB	Redesdale
Sep-05	AA156	ROB	Redesdale
Sep-05	AA183	BLB	Kielder
Sep-05	AA261	CHS	Redesdale
Sep-05	AA267	PLJ	Kielder
Sep-05	AA366	SWB	Kielder
Sep-05	AA368	LSB	Kielder
Sep-05	AA369	BCR	Kielder
Sep-05	AC068	TBB	Kershope
Sep-05	AC090	RLB	Kershope
Apr-06	BA042	TBB	Kershope
Apr-06	BA081	SWB	Kielder
Apr-06	BA123	WBN	Redesdale
Apr-06	BB095	SWB	Kielder
Sep-06	CB052	ROB	Redesdale

Table 4.3 Provenance of *M. microti* isolates on which VNTR typing was performed.

4.2.3.2 PCR-based amplification of VNTR loci

PCR-based amplification of six VNTR loci was performed according to a previously-described protocol (Frothingham et al., 1998). Each 25µl reaction mixture comprised of 1µl of a 25 pmol μl^{-1} solution of the forward primer, 1µl of a 25 pmol μl^{-1} solution of the reverse primer (Table 4.4), 2x PCR Mastermix (Thermo Scientific), 9µl of sterile distilled water and 3µl of boiled cell suspension. Reaction mixes were subjected to the following thermal programme: an initial denaturation at 95°C for 3 min, then 45 cycles of 96°C for 10 sec, 58°C for 10 sec and 72°C for 60 sec, then a final elongation step at 72°C for 10 min. Each PCR run included, in addition to test strains, two positive control strains (*M. tuberculosis* H37RVb and *M. bovis* BCG and a reagent-only (no DNA added) as a negative control. PCR products were analysed either by fragment analysis, sequencing (see below) or by UV illumination following electrophoretic

resolution through a 1.5% (w/v) agarose gel containing 7μg ml^{-1} ethidium bromide in TAE buffer.

VNTR locus	primers
ETR-A	forward 5'-aaa tcg gtc cca tca cct tct ta-3' reverse 5'-cga agc ctg ggg tgc ccg cga ttt-3'
ETR-B	forward 5'-gcg aac acc agg aca gca tca tgg gtt reverse 5'-ggc atg ccg gtg atc gag tgg cta ta-3'
ETR-C	forward 5'-gtg agt cgc tgc aga acc tgc ag-3' reverse 5'-ggc gtc ttg acc tcc acg agt g-3'
ETR-D	forward 5'-cag gtc aca acg aga gga aga gc-3' reverse 5'-gcg gat cgg cca gcg act cct c-3'
ETR-E	forward 5'-ctt cgg cgt cga aga gag cctc-3' reverse 5'-cgg aac gct ggt cac cac cta ag-3'
ETR-F	forward 5'-ggt gat ggt ccg gcc ggt cac-3' reverse 5'-gtg ctc gac aac gcc atg cc-3'

Table 4.4 Oligonucleotide primers used for VNTR analysis

4.2.3.4 Fragment analysis of VNTR amplification products

Fragment analysis was performed commercially on PCR products amplified as described above except that the forward primer used in each reaction was fluorescein-labelled. Products of these reactions were submitted directly to a commercial service.

4.2.3.5 Sequencing of VNTR amplification products

Amplification products were purified for sequencing using the QIAquick PCR clean-up kit (Qiagen). Briefly, 50 μl of PCR product was added to 250 μl of PBI buffer, mixed thoroughly then added to a spun column. The column was then centrifuged at 13,000 x g for 60 sec and the flow-through discarded. Next, 750μl of the PE buffer was added to the column, which was then spun centrifuged

again at 13,000 x g for 60 sec. The flow-through was discarded and the column re-centrifuged as before. The column was then placed in a sterile 1.7ml Eppendorf tube and 15µl of sterile distilled water was added to it. The column was centrifuged for a final time and the purified DNA solution that was eluted from the column was collected. The success of this protocol was assessed by UV illumination of a 1.5% (w/v) agarose gel containing 5 µg ml^{-1} ethidium bromide in TAE buffer on which 1µl of purified PCR product had been electrophoretically resolved. Provided a strong band was visible on this gel, the PCR product was submitted to a commercial sequencing service. Sequencing reactions were performed with the same primers as used for amplification of the VNTR locus.

4.3 Results

4.3.1 Exploring regions of difference

Eighteen *M. microti* representatives were tested for the presence/absence of three regions of difference, RD^{mic}, RD 4 and RD14. The same result was obtained for all 18 isolates (Table 4.5), with RD^{mic} being absent but RD4 and RD14 being intact. The two control strains used in this assay also gave anticipated results.

Strain	**RD^{mic}**	**RD4**	**RD14**
A100	deleted	intact	intact
B042	deleted	intact	intact
F016	deleted	intact	intact
F120	deleted	intact	intact
T210	deleted	intact	intact
V047	deleted	intact	intact
V050	deleted	intact	intact
V062	deleted	intact	intact
V076	deleted	intact	intact
W054	deleted	intact	intact
W060	deleted	intact	intact
W067	deleted	intact	intact
W096	deleted	intact	intact
W099	deleted	intact	intact
AA010	deleted	intact	intact
BA012	deleted	intact	intact
BB095	deleted	intact	intact
CB052	deleted	intact	intact
M.tuberculosis H37RVb	intact	intact	intact
M. bovis BCG	intact	deleted	deleted

Table 4.5 RD analysis in 18 *M. microti* strains and *M. tuberculosis* and *M. bovis* controls.

4.3.2 Spoligotyping

Spoligotyping was attempted on 39 *M. microti* isolates. A clear signal was obtained for 32 of these isolates, with all possessing the spoligotype, SB0118, which comprised of DVRs 37 and 38 only (Figure 4.1).

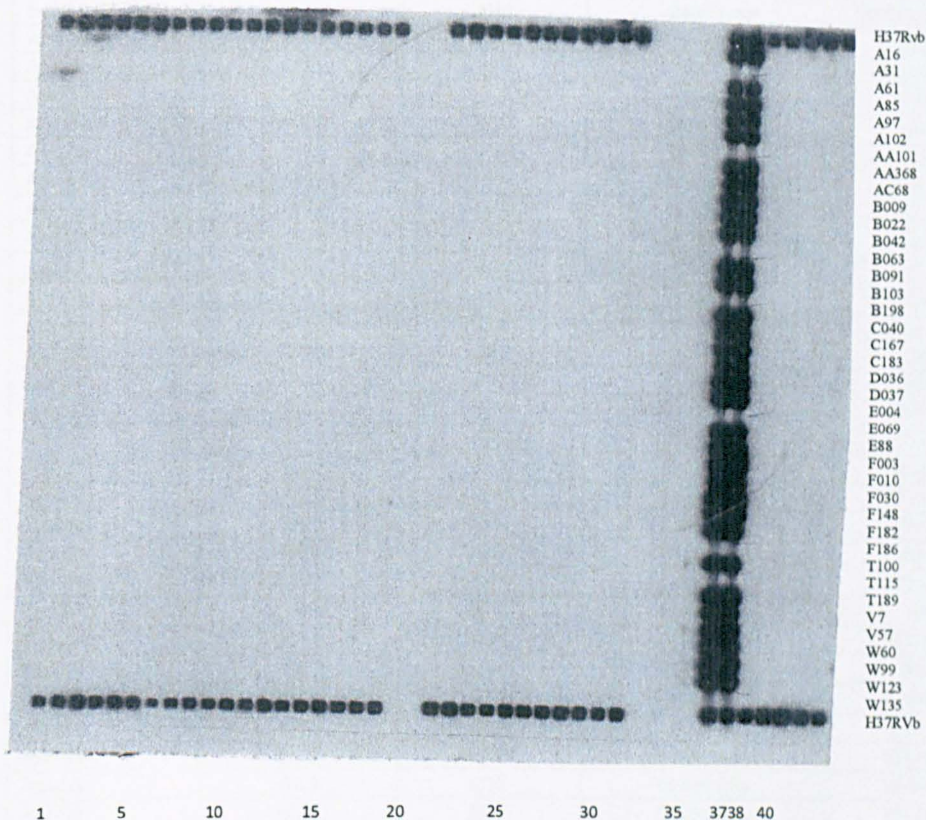


Figure 4.1 Spoligotyping patterns for *M microti* isolates from field voles. All isolates possess the same pattern in which only two spacers (spacers 37 and 38) are present. The pattern from a reference strain (*M. tuberculosis* H37RVb) is shown at the top and bottom of the blot.

4.3.3 VNTR analysis

VNTR analysis was attempted on 121 *M. microti* isolates, and a complete profile was obtained for 79 of these isolates (Table 4.6).

survey date	isolate	grid	catchment	VNTR genotype
Apr-02	A016	SWC	Redesdale	KFMM1
Apr-02	A031	BGB	Kielder	KFMM1
Apr-02	A061	PLJ	Kielder	KFMM1
Apr-02	A063	RPC	Kershope	NR[1]
Apr-02	A085	WNH	Redesdale	NR[1]
Apr-02	A097	SWC	Redesdale	KFMM1
Apr-02	A100	RPC	Kershope	KFMM1
Apr-02	A101	RPC	Kershope	KFMM2
Apr-02	A102	BLB	Kielder	KFMM1
Apr-02	B022	TBB	Kershope	KFMM1
Apr-02	B042	TDH	Kershope	KFMM3
Apr-02	B063	RPC	Kershope	NR[1]
Apr-02	B091	RPC	Kershope	KFMM1
Apr-02	B103	KRB	Kershope	KFMM3
Apr-02	B148	KRB	Kershope	NR[1]
Apr-02	B198	KRB	Kershope	KFMM1
Sep-02	C167	LSB	Kielder	KFMM1
Sep-02	C183	BLB	Kielder	KFMM1
Sep-02	D037	WNH	Redesdale	NR[1]
Apr-03	E004	BGB	Kielder	KFMM1
Apr-03	E036	BCR	Kielder	NR
Apr-03	E069	BBH	Kielder	KFMM1
Apr-03	E088	PLJ	Kielder	NR[1]
Apr-03	F003	QRY	Redesdale	KFMM1
Apr-03	F010	WBN	Kielder	KFMM1
Apr-03	F016	ROB	Redesdale	KFMM1
Apr-03	F018	ROB	Redesdale	KFMM2
Apr-03	F022	ROB	Redesdale	KFMM1
Apr-03	F117	TBB	Kershope	KFMM9
Apr-03	F120	TBB	Kershope	KFMM1
Apr-03	F121	TBB	Kershope	KFMM2
Apr-03	F148	KRB	Kershope	KFMM1
Apr-03	F182	TBB	Kershope	KFMM1
Apr-03	F185	TBB	Kershope	KFMM1
Apr-03	F186	TBB	Kershope	KFMM1
Apr-05	T016	TRI	Kershope	NR[1]
Apr-05	T024	CGF	Kershope	KFMM1
Apr-05	T027	CGF	Kershope	NR[1]
Apr-05	T037	SLB	Kershope	KFMM1
Apr-05	T039	SL B	Kershope	KFMM1
Apr-05	T059	RPC	Kershope	KFMM1
Apr-05	T100	CWS	Kershope	KFMM1
Apr-05	T115	TBB	Kershope	KFMM1
Apr-05	T151	CGF	Kershope	NR[1]
Apr-05	T178	CGF	Kershope	KFMM1
Apr-05	T183	TBB	Kershope	NR[1]
Apr-05	T208	TDH	Kershope	KFMM1
Apr-05	T210	TDH	Kershope	KFMM7
Apr-05	V002	SQC	Kielder	KFMM1
Apr-05	V007	SQC	Kielder	KFMM1
Apr-05	V011	SQC	Kielder	KFMM1
Apr-05	V019	BLB	Kielder	NR[1]
Apr-05	V022	BLB	Kielder	NR[1]
Apr-05	V023	BLB	Kielder	NR[1]
Apr-05	V027	KCS	Kielder	NR[1]
Apr-05	V029	KCS	Kielder	NR[1]
Apr-05	V030	KCS	Kielder	NR[1]
Apr-05	V040	SLB	Kershope	NR[1]
Apr-05	V043	BCR	Kielder	KFMM1

survey date	isolate	grid	catchment	VNTR genotype
Apr-05	V046	SWB	Kielder	KFMM1
Apr-05	V047	SWB	Kielder	KFMM4
Apr-05	V048	SWB	Kielder	KFMM1
Apr-05	V050	SWB	Kielder	KFMM5
Apr-05	V051	SWB	Kielder	NR[1]
Apr-05	V057	BBH	Kielder	KFMM6
Apr-05	V062	SQC	Kielder	KFMM4
Apr-05	V063	SQC	Kielder	KFMM1
Apr-05	V064	SQC	Kielder	KFMM1
Apr-05	V073	LSB	Kielder	NR
Apr-05	V076	LSB	Kielder	KFMM1
Apr-05	V085	BCR	Kielder	NR
Apr-05	V090	SWB	Kielder	KFMM4
Apr-05	V097	SQC	Kielder	NR
Apr-05	W016	RLB	Kershope	KFMM1
Apr-05	W025	CWS	Kershope	KFMM1
Apr-05	W027	CWS	Kershope	KFMM1
Apr-05	W029	TBB	Kershope	KFMM1
Apr-05	W037	WBN	Redesdale	NR
Apr-05	W042	WBN	Redesdale	NR
Apr-05	W054	CWS	Kershope	KFMM1
Apr-05	W055	CWS	Kershope	KFMM2
Apr-05	W059	BHP	Redesdale	KFMM1
Apr-05	W060	BHP	Redesdale	KFMM1
Apr-05	W062	BHP	Redesdale	NR
Apr-05	W065	BHP	Redesdale	KFMM1
Apr-05	W067	CWS	Kershope	KFMM1
Apr-05	W073	FRD	Kielder	NR
Apr-05	W081	CHS	Kielder	KFMM1
Apr-05	W082	CHS	Redesdale	NR
Apr-05	W085	CHS	Redesdale	NR
Apr-05	W096	ROB	Redesdale	KFMM3
Apr-05	W097	ROB	Redesdale	KFMM8
Apr-05	W098	SWC	Redesdale	KFMM5
Apr-05	W099	WBN	Kielder	KFMM1
Apr-05	W114	FRD	Kielder	KFMM1
Apr-05	W125	PLJ	Kielder	NR
Apr-05	W126	PLJ	Kielder	NR
Apr-05	W135	CWS	Kershope	KFMM1
Sep-05	AA012	QRY	Redesdale	NR
Sep-05	AA062	SWC	Redesdale	KFMM1
Sep-05	AA101	KCS	Kielder	KFMM1
Sep-05	AA102	KCS	Kielder	KFMM2
Sep-05	AA103	KCS	Kielder	NR
Sep-05	AA146	ROB	Redesdale	KFMM1
Sep-05	AA152	ROB	Redesdale	NR
Sep-05	AA156	ROB	Redesdale	NR
Sep-05	AA183	BLB	Kielder	NR
Sep-05	AA261	CHS	Redesdale	NR
Sep-05	AA267	PLJ	Kielder	NR
Sep-05	AA366	SWB	Kielder	NR
Sep-05	AA368	LSB	Kielder	KFMM1
Sep-05	AA369	BCR	Kielder	NR
Sep-05	AB084	LWH	Kielder	NR
Sep-05	AC068	TBB	Kershope	KFMM1
Sep-05	AC090	RLB	Kershope	NR
Apr-06	BA042	TBB	Kershope	KFMM1
Apr-06	BA081	SWB	Kielder	KFMM1
Apr-06	BA123	WBN	Redesdale	KFMM1

survey date	isolate	grid	catchment	VNTR genotype
Apr-06	BB095	SWB	Kielder	KFMM5
Sep-06	CB052	ROB	Redesdale	KFMM1
Sep-06	CB031	QRY	Redesdale	NR[1]

NR[1]= No results

Table 4.6 Results of VNTR typing on 121 *M. microti* isolates.

A total of nine VNTR profiles were distinguished among these 79 isolates. The most common profile was encountered in 61 isolates, 5 strains possessing the second most common profile, three profiles encountered 3 isolates each, and four profiles being encountered only once (Table 4.7). The discriminatory power of the VNTR typing scheme was quantified by measuring allelic diversity (the probability of identifying different types in two isolates chosen at random) using the equation $h = 1- \sum x_i^2 (n / n-1)$ where n is the number of isolates and x_1 the frequency of the *i*th allele (Selander et al., 1986). Using this equation, the allelic diversity among the 79 isolates determined using VNTR was 0.385.

N° of isolates	VNTR locus						allelic profile
	ETR-A	ETR-B	ETR-C	ETR-D	ETR-E	ETR-F	
61	5	5	5	4	2	3.3	KFMM1
5	5	5	5	6	2	2.2	KFMM2
3	5	4	5	4	2	3.3	KFMM3
3	4	5	5	4	2	3.3	KFMM4
3	3	5	5	4	2	3.3	KFMM5
1	5	5	5	4	2	3.2	KFMM6
1	5	5	5	4	2	3.4	KFMM7
1	5	5	5	3	2	3.3	KFMM8
1	3	4	5	4	2	3.3	KFMM9

Table 4.7 Details of the Nine VNTR profiles distinguished in this study

Amplification of the ETR-A locus yielded three different sized fragments of 575 bp, 500 bp and 420 bp (Figure 4.2), representing 3, 4 and 5 copies of the 75 bp repeat respectively. Seventy two of the 79 isolates possessed 5 repeats, whereas three possessed 4 repeats and four possessed 3 repeats. The allelic diversity among the isolates using this locus only was 0.16.

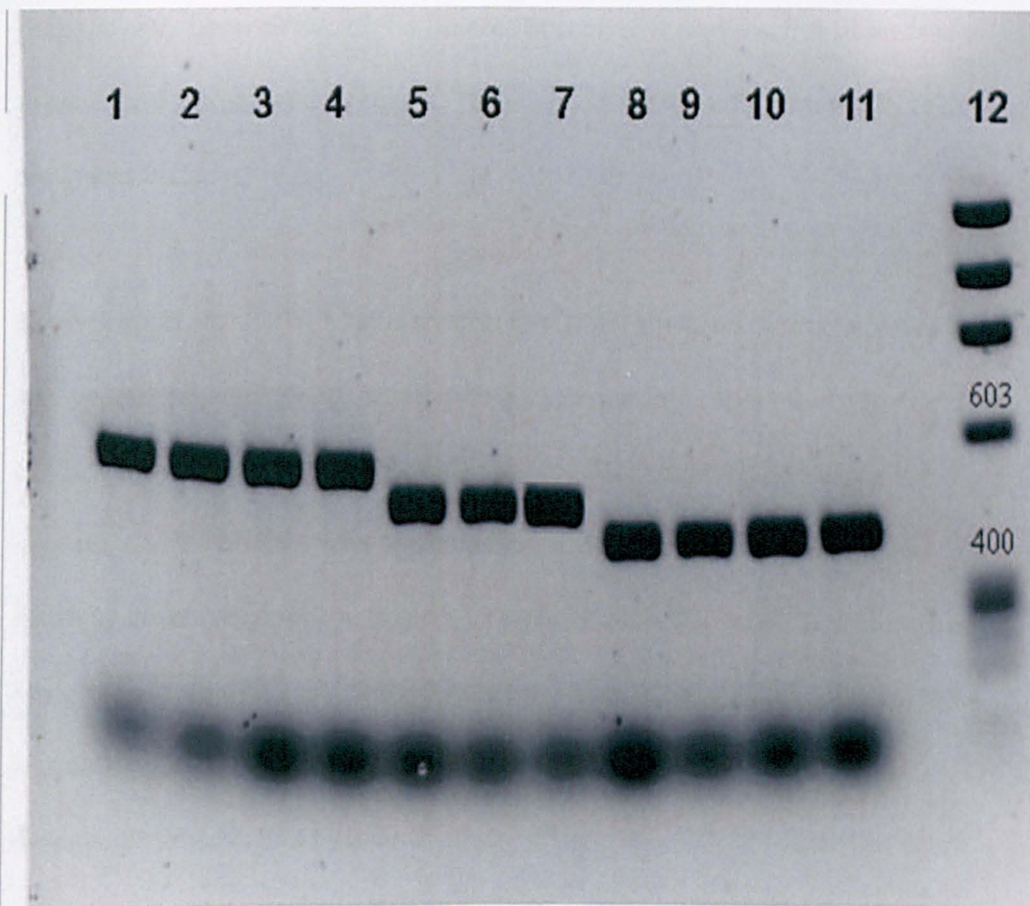


Figure 4.2 ETR-A PCR products obtained from a representative panel of *M. microti* isolates. Lane 1-4: isolates have ETR-A allele A3,lanes 5-7: ETR-A allele A4, and lanes 8-11: ETR-A allele A5. Lane 12 contains *Hind*III digest of ϕx174 size marker (400bp and 603 bp bands indicated).

Amplification of the ETR-B locus yielded two different sized fragments of 330 bp and 360 bp size, representing 4 and 5 copies of the 57 bp repeat respectively.

Seventy five of the isolates possessed 5 repeats and for isolates possessed 4 repeats. The allelic diversity among the isolates as determined by solely this locus was 0.11.

Amplification of the ETR-D locus yielded three different size products of 305bp and 381bp and 538bp, representing 3, 4 and 6 copies of the 77 bp repeat respectively. Seventy two of the isolates possessed 4 repeats, five possessed 6 repeats and 1 possessed 3 repeats. The allelic diversity as determined by this locus was 0.15.

Amplification of the ETR-F locus yielded four different sized products of 375 bp, 445 bp, 502 bp and 559 bp, representing 55 bp tandem repeat and 79bp tandem repeat. Seventy two of the isolates possessed 3.3 repeats, 5 possessed 2.2 repeats, and 3.2 and 3.4 were each encountered in one isolate. The allelic diversity determined for the isolates using only this locus was 0.16). The three alleles consisted of 3.2, 3.3 and 3.4 tandem copies of the 79 &55 bp repeat unit. As this locus can contain various permutations of 55bp and 79bp tandem repeats, distinguishable by only small size differences, PCR products can prove difficult to size, hence, to confirm the results above, some sequencing was done (see below).

Amplification of the ETR-C and ETR-E loci yielded products of 305 bp and 170 bp respectively for all 79 isolates, hence neither locus was discriminatory (allelic diversity = 0).

4.3.4 Sequencing of VNTR amplification products

On occasion, the results of fragment analysis and/or agarose gel-based estimates of VNTR PCR product size were unexpected. To help clarify their identity, nucleotide base sequencing was performed. ETR-F was investigated in this manner, with amplification products from the previously-unseen F2.2 allele being sequences from four isolates (T110, V057, V062 and CB052). An indistinguishable sequence was obtained from all four isolates and comparative analysis of this sequence confirmed it contained two pairs of tandem repeats, one of 55 bp and one of approximately 80 bp (Figure 4.3).

```
V062  1   GTGAGCCTGCAAGCCGCGCCGCACCACCTCGACTTCGGGCAGCTCGGGCAT‖CCAGTGATG
CB052 1   GTGAGCCTGCAAGCCGCGCCGCACCACCTCGACTTCGGGCAGCTCGGGCAT‖CCAGTGATG
T210  1   GTGAGCCTGCAAGCCGCGCCGCACCACCTCGACTTCGGGCAGCTCGGGCAT‖CCAGTGATG
V057  1   GTGAGCCTGCAAGCCGCGCCGCACCACCTCGACTTCGGGCAGCTCGGGCAT‖CCAGTGATG

V062  61  ATCGCAAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGC
CB052 61  ATCGCAAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGC
T210  61  ATCGCAAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGC
V057  61  ATCGCAAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGC

V062  121 AAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGCAAGCG
CB052 121 AAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGCAAGCG
T210  121 AAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGCAAGCG
V057  121 AAGCGCGGCGAAGCCGGGCGCAGCGGGTCATCACCATCGAA‖CCAGTGATGATCGCAAGCG

V062  181 CGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGGGAGGTGCCCCCAGGTCATCACCATCGAA‖
CB052 181 CGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGGGAGGTGCCCCCAGGTCATCACCATCGAA‖
T210  181 CGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGGGAGGTGCCCCCAGGTCATCACCATCGAA‖
V057  181 CGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGGGAGGTGCCCCCAGGTCATCACCATCGAA‖

V062  241 CCAGTGATGATCGCAAGCGCGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGCGGCAAAGCC
CB052 241 CCAGTGATGATCGCAAGCGCGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGCGGCAAAGCC
T210  241 CCAGTGATGATCGCAAGCGCGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGCGGCAAAGCC
V057  241 CCAGTGATGATCGCAAGCGCGGCGAAGCCGGGCGCAGTCCCCCGCAAGCGCGGCAAAGCC

V062  301 GGCGCCCCCAGGTCATCACCATCAA‖TCCAGTTAGGCGGAGGTTTTGCCC
CB052 301 GGCGCCCCCAGGTCATCACCATCAA‖TCCAGTTAGGCGGAGGTTTTGCCC
T210  301 GGCGCCCCCAGGTCATCACCATCAA‖TCCAGTTAGGCGGAGGTTTTGCCC
V057  301 GGCGCCCCCAGGTCATCACCATCAA‖TCCAGTTAGGCGGAGGTTTTGCCC
```

Figure 4.3 Alignment of ETR-F sequences for the four isolates studied. Tandem repeats are in bold and junctions between them are marked with ‖. The ETR contains two identical copies of a 55 bp repeat, then two near-identical copies of an 80/86bp repeat. Regions of difference between the copies of the second tandem repeat are underlined.

4.3.5 Molecular epidemiology of *M. microti* in Kielder Forest

The VNTR data obtained for the 79 *M. microti* isolates was compared to epidemiological data were relating to the provenance of these isolates. For each isolate, details were available regarding the date and location where the animal was trapped. Population level data were relating to the size of the field vole population on a local and forest-wide scale was also available.

One *M. microti* VNTR type, KFMM1 dominated the population. At least 75% of the samples tested from each spatial survey possessed KFMM1 and this genotype was present (Table 4.8), at some point, in each of the three Kielder catchments and even on all of the 24 grids where *M. microti*-infected animals were trapped.

spatial survey	voles caught	TB %	voles sampled	KFMM genotype									allelic diversity
				1	2	3	4	5	6	7	8	9	
2	215	13	12	9	1	2	0	0	0	0	0	0	0.355
3	336	4	2	2	0	0	0	0	0	0	0	0	NT
4	381	12	14	11	2	0	0	0	0	0	0	1	0.313
8	230	29	40	30	1	1	3	2	1	1	1	0	0.410
9	265	9	6	5	1	0	0	0	0	0	0	0	NT
10	232	8	4	3	0	0	0	1	0	0	0	0	NT
11	362	9	1	1	0	0	0	0	0	0	0	0	NT
total	2021	11	79	61	5	3	3	3	1	1	1	1	0.385

Table 4.8 Details of VNTR typing during each spatial survey

Four genotypes were encountered infrequently, but more than once. Among these KFMM2 was encountered five times in four different spatial surveys, and at five different sites spread across all three catchments. KFMM3 was encountered three times in 2 spatial surveys at different sites in two catchments. KFMM4 was

also encountered three times, but all isolates were made during the same spatial survey and all of them were from Kielder catchment, two of them were from the same grid. KFMM5 was encountered three times in two different spatial surveys and at two grids in two different catchments (Table 4.9).

genotype	strain	spatial survey	catchment	grid
KFMM2	A101	2 (April 2002)	Kershope	RPC
	F018	4 (April 2003)	Redesdale	ROB
	F121	4	Kershope	TBB
	W055	8 (April 2005)	Kershope	CWS
	AA102	9 (Sept 2005)	Kielder	KCS
KFMM3	B024	2	Kershope	TDH
	B103	2	Kershope	KRB
	W096	8	Redesdale	ROB
KFMM4	V047	8	Kielder	SWB
	V062	8	Kielder	SQC
	V090	8	Kielder	SWB
KFMM5	V050	8	Kielder	SWB
	W098	8	Redesdale	SWC
	BB095	10 (April 2006)	Kielder	SWB

Table 4.9 Provenance of the 14 isolates possessing uncommon VNTR genotypes.

The diversity of genotypes present at different times during the study period and at different places across the study sites was also explored. Estimates of allelic diversity were made for spatial surveys in which more than 10 isolates yielded a complete VNTR profile. In April 2002 the allelic diversity was 0.355, in April 2003 it was 0.313 and in April 2005 it was higher, at 0.410. These values compare to an allelic diversity of 0.385 for all 79 *M. microti* isolates studied (Table 4.8). For a spatial perspective, the allelic diversity in each catchment was calculated (Table 4.10). Kielder had the highest allelic diversity, 0.439, and Kershope and Redesdale had similar values of 0.356 and 0.347 respectively.

catchment	total	KFMM genotype									allelic diversity
		1	2	3	4	5	6	7	8	9	
Kershope	33	26	3	2	0	0	0	1	0	1	0.356
Kielder	28	21	1	0	3	2	1	0	0	0	0.439
Redesdale	18	14	1	1	0	1	0	0	1	0	0.347
total	79	61	5	3	3	3	1	1	1	1	0.385

Table 4.10 Allelic diversity in three of catchments in Kielder Forest.

4.4 Discussion

This is the first study to quantify the genetic diversity of *M. microti* strains circulating in field vole populations inhabiting Kielder Forest, and to use these typing data to attempt to explore the molecular epidemiology of vTB infections in endemically infected animals. Two of the typing methods used in this study, deletion analysis and spoligotyping, have been previously applied to a handful of *M. microti* isolates, but VNTR analysis has not previously been used in conjunction with this species other than during the development and evaluation of the methodology (Frothingham & Meeker-O'Connell, 1998).

As expected, deletion analysis revealed that all *M. microti* strains studied lacked RD^{mic} but were intact for the RD4 and RD14. Although the usefulness of deletion analysis for delineating MtC species and, in some instances, important lineages within species, is well-established (Brosch et al., 2002; Smith et al., 2006), to date, only a few *M. microti* strains have been included in these studies (Brosch et al., 2002; Brodin et al., 2002). The 18 isolates analysed in this study more than doubles the number of *M. microti* strains for which published deletion analysis data are available. Although the provenance of previously studied *M. microti*

strains is not absolutely clear, several were isolated from field voles in the UK during the 1930s, undoubtedly as part of the surveys reported by Wells (1946). Thus, our results provide evidence for a genomic stability in terms of retention of RD4 and RD14, among the *M. microti* population circulating in UK field voles over a 70 year period.

All 32 *M. microti* isolates for which spoligotyping data were obtained possessed the same spoligotype, which comprised of DVRs 37 and 38 only. This spoligotype is considered the "classic" *M. microti* spoligotype and has previously been obtained from 24 field voles captured in Kielder and Cheshire in the late 1990s (Cavanagh et al., 2002) and from UK field voles captured in the 1930s (van Soolingen et al., 1998). It is tempting to assume that all *M. microti* strains circulating in field voles are likely to possess this spoligotype. However, as most strains examined were derived from animals that may well have been trapped in the same geographic location (i.e. Kielder Forest), this assumption may be premature. In the UK, *M. microti* strains with this spoligotype have been recovered from mammals other than field voles, including cats, cows and badgers (Kremer et al., 1998; Emmanuel et al., 2007). It is therefore reasonable to speculate that these animals acquired their infection following interaction with field voles. Cats and even badgers can actively hunt field voles so are among the species that more likely to acquire accidental infections. Field voles also inhabit pasture margins and thus may come into contact with cattle. The prevalence of *M. microti* infections in populations of "professional" field vole

hunters such as weasels or mink has not yet been assessed, but such studies may be worthwhile.

Interestingly, a proportion of the *M. microti* strains for which spoligotypes data have been published possessed markedly different spoligotypes to that found in field voles (Kremer et al., 1998; van Soolingen et al., 1998; Emmanuel et al., 2007). The natural reservoir for these spoligotypes is not yet known, but, as suggested above, to dismiss field voles would be foolhardy. *M.bovis* spoligotypes in the UK are not randomly distributed geographically but rather show strong geographical localisation (Smith et al., 2003) and the same may be true for *M. microti*.

The application of VNTR, using the six loci described by Frothingham and Meeker-O'Connell (1998) was able to delineate genotypes among the 79 *M. microti* isolates from which a complete VNTR profile was obtained. The "sensitivity" of VNTR typing, as assessed by calculating allelic diversity, was 0.39. This value is low compared to previous studies in which VNTR typing has been applied to other MtC species. For example, Roring and colleagues (2002) calculated an allelic diversity of 0.67 when the ETR VNTRs were examined among 42 *M. bovis* strains from Ireland. However, these isolates were selected on the basis of being epidemiologically unrelated to one another, unlike the *M. microti* isolates included in this study, many of which came from animals sampled in the same location at the same time. In a different epidemiological setting, Hilty and colleagues (2005) explored the discriminatory power of VNTR typing based on 12 MIRUs, 3 ETRs and VNTR 3232 among 67 *M. bovis* strains collected sequentially a

slaughterhouse in Chad. They concluded that VNTR typing was highly discriminative with an overall allelic diversity of 0.922. Perhaps more akin to the epidemiological situation found in the current study, Jeon and colleagues (2008) applied VNTR typing to distinguish *M. bovis* isolates obtained from cattle inhabiting a region of South Korea. A total of 59 strains were included in the study, collected over a two year period from 20 farms. The ETR VNTR panel discriminated these isolates into five allelic profiles with an allelic diversity of 0.57. The most common profile was obtained from 35 isolates, and on most of the farms included only one or two allelic profiles. As with spoligotyping, VNTR genotypes of *M. bovis* are geographically localised in the UK (Smith et al., 2006) such that a single allelic profile can dominate an area of 250 km^2. The pattern of allelic diversity observed among *M. microti* isolates from field voles across Kielder Forest therefore appears similar to that observed on a local scale among the UK *M. bovis* population.

The discriminatory power of individual ETR loci among the *M. microti* isolates were, as expected, varied. Several studies focused on VNTR discrimination of *M. bovis* have observed the same (Sola et al., 2003; Roring et al., 2004). For example, in the study described above, Hilty and colleagues (2005) reported that ETR-A and ETR-B were "highly discriminatory" with an allelic diversity of >0.25, whereas other loci showed no polymorphism at all. In the current study, ETR-A and ETR-F were the most discriminatory loci, whereas ETR-C and ETR-E had no discriminatory power. This finding is somewhat at odds with what is observed

from *M. bovis* in the UK where ETR-E and ETR-F are virtually monomorphic (Smith et al., 2003).

The application of *M. microti* genotyping to the study of vTB molecular epidemiology at Kielder did not reveal any dramatic findings. The dominant VNTR type (KFMM1) dominated the population in all three catchments and during all seven surveys. Its distribution was ubiquitous, being present on all grids where infected field voles were trapped. Generally, there were no obvious epidemiological links between the few animals that were infected with uncommon VNTR genotypes. For example, the five strains with a KFMM2 genotype were encountered during four different spatial surveys at sites spread across all three catchments. The only exception was KFMM4 that was encountered three times in total and twice in two animals caught on the same grid at the same time. The diversity of *M. microti* genotypes was not obviously proportional to the size of the field vole population. A slightly elevated diversity was observed in one survey (SS8, April 2005) compared to others, but this survey was also the one from which the largest sample was available. There was also no obvious difference in strain diversity among isolates obtained from animals living in different catchments. Strains from Kielder were slightly more diverse than those from either of the two other catchments but the significance of this observation is unclear.

In summary, molecular typing using methods of proven value among other MtC members, were applied to a large panel of representative isolates of *M. microti*

from Kielder field voles. As expected these isolates were monomorphic when tested using deletion analysis and spoligotyping. VNTR analysis, however, was discriminatory, delineating the isolates into six genotypes with an allelic diversity index of 0.39. This pattern is akin to that observed on a parish-scale for *M. bovis* in the UK. Comparison of the relative discriminatory power of individual ETR loci indicates expected variation, but the relative performance of each locus for *M. microti* discrimination is different to that observed for *M. bovis* discrimination. The molecular epidemiologic picture for vTB at Kielder suggests a stable population of strains in circulation, with one genotype dominating both temporally and spatially. No evidence of epidemic or geographically localised genotypes could be found.

Chapter 5.

Immunological response to *M. microti* infection in field voles

5.1 Introduction

M. microti infections have, to date, been diagnosed solely by clinical signs or post-mortem examination. Work reported in this thesis and elsewhere (Burthe et al., 2008) has demonstrated the insensitivity of a clinical approach to diagnosis compared to post-mortem pathology and microbiology. The absence of a sensitive means of detecting infections in living animals represents a significant hindrance to efforts aimed at better understanding the epidemiology of vole tuberculosis. In an earlier Chapter (3), attempts to develop and evaluate a PCR-based approach to vTB diagnosis were described. However, for other *M. tuberculosis* complex (MtC) members at least, tests for cellular immunity have long represented an important means of ante-mortem diagnosis (de la Rua-Domenech et al., 2006). This chapter therefore reports on attempts to adapt a commercial assay used for the detection of interferon gamma in response to MtC stimulation in mice, for use in voles.

M. microti, like other MtC species is an intracellular pathogen of monocytic cells. The predominant immunological response in MtC-infected hosts is effected by T lymphocytes, and the Th1 response provoked is both the mechanism of host defence (Walker & Lowrie, 1981) and the cause of the chronic granulomas that characterise the pathology of MtC infections (Pritchard, 1988). Interferon gamma (IFN-γ) is a cytokine predominantly secreted by activated Th1 cells, which represent a subset of CD4+ T cells. T cell differentiation towards the Th1 subset is itself driven by interleukin 12 and IFN-γ produced by natural killer cells in response to antigenic (in this case mycobacterial) stimulation (Benjamini et al.,

1996). The production of IFN-γ and other cytokines including interleukin 2, by Th1 cells activates a cellular immune response dominated by CD8+ T cells, natural killer cells and macrophages. The absence of Th2 cells and associated cytokines such as interleukin 4 and interleukin 5, particularly during the early stages of mycobacterial infection, results in no B- cell activation, hence no humoral response. Antibodies are produced only during the advanced stages of disease or when a host is exposed to a high infective dose (Welsh et al., 2005).

Two forms of test for cellular immunity to MtC are widely used, the intradermal tuberculin test and the IFN-γ detection assay. The former of these tests detects a delayed-type hypersensitivity reaction to intradermal inoculation of tuberculin, which is a generic name for a crude protein derivative prepared from one or other MtC members (Snider, 1982). The latter assay detects the release of IFN-γ in whole blood cultures stimulated, most commonly, with tuberculin using an enzyme-linked immunosorbent assay (ELISA) (Rothel et al., 1990). Around the world, the tuberculin skin test (or Heaf/Mantoux test) is used as the principle means of determining infection status in human beings (for *M. tuberculosis*) and livestock (for *M. bovis*). For the latter species, disease control programmes, based on the systematic testing of cattle herds accompanied by slaughter of reactors, have eradicated bTB from many countries such as Australia and most of the EU member states (Caffrey, 1994; Cousins et al., 1998).

Although the tuberculin skin test is cheap and simple to do, increasing concerns about its performance (de la Rua-Domenech et al., 2006) resulted in the

development of ancillary diagnostic tests such as the IFN-γ test. This assay was first developed in Australia in the late 1980s for the diagnosis of bovine TB in cattle (Woods et al., 1991). In this assay, aliquots of whole blood collected from a test animal are incubated with bovine tuberculin, avian tuberculin and a no antigen control for 16 to 24 hours, after which the amount of IFN-γ in plasma supernatants harvested from each mixture are quantified using an ELISA. Several commercial ELISA kits are now available for the detection of IFN-γ from humans, cattle and, increasingly, other species. Numerous evaluations and trials have demonstrated the value of IFN-γ assays for the diagnosis of MtC infections. For example, for *M. bovis* diagnosis in cattle, the test is as sensitive as and perhaps even more sensitive than the skin test and it can detect infections earlier in their course. Furthermore, it is amenable to the inclusion of more defined antigens than tuberculin, thereby allowing the differentiation of IFN-γ responses to infecting "pathogenic" strains from those resulting from exposure to sensitising environmental mycobacteria or attenuated vaccines (e.g. Pollock & Andersen, 1997; Waters et al., 2004). Antigens such as ESAT-6 and CFP10 have been proposed as alternatives to tuberculin (Lalvani et al., 1997).

Animal models are widely used to study molecular, cellular, immunological and pathological aspects of MtC infections (e.g. Dharmadhikari & Nardell, 2008; Nuermberger, 2008). Rodents are most commonly used, particularly mice, rabbits and guinea pigs, the latter species being extremely vulnerable to *M. tuberculosis* infection. Mice, although generally resistant to TB infection compared to guinea pigs and "natural" hosts such as humans or cattle, are often

used for experiments on immune responses to tuberculosis (Dharmadhikari & Nardell, 2008). Although mice develop an atypical pathology following experimental inoculation (Rhoades et al., 1997), the chronic nature of this infection has helped scientists explore T-cell function in response to prolonged exposure to mycobacteria (Lazarevic et al., 2005). The murine immune response to tuberculosis has been carefully explored (reviewed by Orme, 2003) thanks to the wide selection of antibodies and assays for cytokines and immune cells that are available for mice, which include IFN-γ tests. Antibodies for detecting IFN-γ are also available for rats, rabbits and guinea pigs and numerous non-rodent species, but not for voles.

The initial aim of this study was to determine the suitability of currently available rodent MtC IFN-γ assays for use in field voles. If these assays proved suitable, then a further aim was to determine the extent of IFN-γ production by field vole splenocytes in response to first non-specific, then MtC specific stimulants.

5.2 Materials and methods

5.2.1 Experiment 1

Two Balb/c mice were obtained from Biomedical Services Department, University of Liverpool. Two field voles were obtained from captive colonies maintained by the Faculty of Veterinary Science, University of Liverpool. Each animal was euthanized using an overdose of isoflurane (Merial Animal Health) and exsanguination by cardiac puncture. Whole blood samples were collected

into heparin-coated Vacutainers (Becton-Dickinson Biosciences Ltd, Oxford, UK) and these tubes were transferred to a laminar flow cabinet to reduce the risk of environmental contamination. Next, an equal volume of Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen Ltd, Paisley, UK) supplemented with 10% (v/v) foetal calf serum (Invitrogen), 2% (v/v) penicillin (10 units) and streptomycin (100ug/ml) was added to each blood sample. Six 25µl aliquots of each blood/supplemented DMEM mixture was added to six wells of a 96 well round-bottomed tissue culture plate (Corning Inc, NY, USA) and to three of these wells an equal volume of supplemented DMEM containing 0.2 mg ml^{-1} of staphylococcal enterotoxin B (Sigma-Aldrich Ltd) was added. To the three remaining wells, 25µl of supplemented DMEM alone was added. The plate was incubated at 37°C in a 5% CO_2 atmosphere overnight. After this incubation, the plate was centrifuged at 4°C at 300xg for 5 min. The supernatant was then removed from each well and placed into a sterile Eppendorf tube. These tubes were frozen at -80°C until tested.

5.2.2 Experiment 2

Two Balb/C mice and two field voles were obtained and euthanized as described above. The body of each animal was placed on its back and, by using sterile scissors, scalpel and forceps; the ventral side of the body was opened by a longitudinal incision. The spleen was located and removed intact and transferred into a 50 ml polypropylene tube (Corning Inc) containing 10 ml of ice-cold supplemented DMEM held on ice. Tubes were moved into a laminar flow cabinet to reduce the risk of environmental contamination. Next, the tissue and

media were transferred to 45µm pore-size sterile cell strainer (Becton-Dickinson Biosciences Ltd) placed within a sterile Petri dish. Using the sterile end of a plunger removed from a 2ml syringe, the tissue was worked through the strainer into the Petri dish below. The Petri dish was tipped to an angle and, by using a sterile pipette, the media and cells were carefully aspirated and added to a fresh 50ml tube containing 30ml of supplemented DMEM. This tube was centrifuged at 4°C at 1100xg for 10 min. The supernatant was carefully discarded and the pellet of cells was resuspended in 1ml of supplemented DMEM. A 5µl aliquot of this cell suspension was were added to 45 µl of white cell counting fluid (Becton-Dickinson Biosciences Ltd) and this diluted suspension was added to a cytometric microscope slide and observed at 40x magnification to enumerate the splenocytes. On the basis of this count, the remaining cell suspension was adjusted to $5x10^6$ cells ml^{-1} with supplemented DMEM.

A 1ml aliquot of the splenocyte suspension was added to each of the wells of a 48 well cell culture plate (Corning Inc). Concanavalin A (Sigma-Aldrich Ltd) was added to three wells to a final concentration of 5µg ml^{-1}, phytohaemagglutinin (Sigma-Aldrich Ltd) was added to three more wells to a final concentration of 10µg ml^{-1}, and pokeweed mitogen (Sigma-Aldrich Ltd) was added to three further wells to a final concentration of 10µg ml^{-1}. Nothing was added to the final three wells. The plate was incubated at 37°C in an atmosphere containing 5 % CO_2 for 24 hours. After this time, the plate was centrifuged at 4°C at 300xg for 5 min and the supernatant was removed from each well into a sterile Eppendorf tube and immediately frozen to -80°C and stored until tested in the IFN-γ ELISA.

5.23 Detection of IFN-γ by ELISA

The ELISA used for this study was based on the mouse DuoS ELISA development system (DY485, R&D Systems Ltd, Abingdon, UK) and incorporated monoclonal antibodies specific to murine IFNγ both as capture and detection antibodies. ELISAs were performed by Dr Phillip Hogarth, TB Research Group, Veterinary Laboratories Agency, Weybridge.

Capture antibody, AN-18, a monoclonal antibody raised against mouse IFN-γ (Prat et al., 1984) was diluted in 1ml of sterile phosphate buffer saline (PBS) to a concentration of 720 μg ml^{-1} then aliquoted into 100 μl volumes and stored at -20°C. Detection antibody, a biotin conjugate of R4-6A2, a monoclonal antibody raised against mouse IFN-γ (Spitalny & Havell, 1984) was diluted in 1ml of reagent diluent (0.1% (w/v) bovine serum albumin, 0.05% (v/v) Tween20 in Tris buffered saline (2.42 g Trizma base, 8.7 g NaCl in 1000 ml distilled H_2O) to a concentration of 72 μg ml^{-1} then aliquoted into 100 μl volumes and stored at -20°C. IFN-γ standards were diluted in 500 μl of reagent diluent to a concentration of 45 ng ml^{-1}, then aliquoted into 50 μl volumes and stored at -20°C.

A volume of 100 μl of diluted capture antibody diluted to 4 μg ml^{-1} in sterile coating buffer (0.05M $NaCO_3$- Na HCO_3 in deionised H_2O) was added to each well of a Maxisorp 96 well ELISA plate (Corning Inc). The plate was wrapped in tinfoil to protect it from light, and incubated overnight at 4°C. The capture antibody

was removed and replaced with 200 µl of blocking buffer (1% (w/v) Bovine serum albumin (BSA) , 5% (w/v) sucrose, 0.05% (w/v) NaN_3 in PBS), and the plate was incubated at 37°C for 1 hour wrapped in tinfoil. The wells were washed using washing buffer (PBS with 0.05% (v/v) Tween 20) three times then plates were blotted dry. A volume of 100 µl of each test sample was added to each well. To each plate, a series of IFNγ standards (100 µl volumes) was added to 8 wells at 2 fold dilutions beginning with 2000 pg ml^{-1} (diluted in reagent diluent). An aliquot of 100 µl of reagent diluent only served as a blank. The plate was incubated at 37°C for 1 hour wrapped in tinfoil. The plate was washed three times as described above. Next, 100 µl of detection antibody was added to each well and the plate was incubated at 37°C for 1 hour wrapped in tinfoil. The plate was washed again, as described above, then 100 µl of streptavidin-horseradish peroxidise conjugate, diluted in reagent diluent (typically 1; 200), was added to each well and the plate was incubated at 37° C for 20 min wrapped in tinfoil. The plate was washed again, then 100 µl of substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added to each well. The plate was incubated at 37°C for 20 min wrapped in tinfoil then 50 µl of stop solution (2N H_2SO_4) was added to each well. The plate was taped gently to ensure the content of each well was fully mixed, then the colour intensity in each well was assessed spectrophotometrically at 450 nm wavelength.

5.3 Results

5.3.1 Experiment 1

No detectible signal was produced by any of the samples tested. Hence, blood samples from both mice and voles, either stimulated with the addition of staphylococcal enterotoxin B or not, contained insufficient IFN-γ to be detected by the assay.

5.3.2 Experiment 2

IFN-γ was detected in murine splenocytes stimulated with both phytohaemagglutinin and pokeweed mitogen but not concanavalin A (Figure 5.1). Splenocytes from one mouse produced high levels of IFN-γ, with >500pg ml^{-1} being detected. Splenocytes from the other mouse produced lower levels of IFNγ, but nonetheless at a concentration markedly higher than that produced by unstimulated murine splenocytes (Figure 5.1). However, splenocytes derived from both voles used in this study did not produce IFN-γ that was detectible in this assay. For both voles, the signals obtained for stimulated splenocytes were not greater than those obtained from unstimulated cells (Figure 5.1).

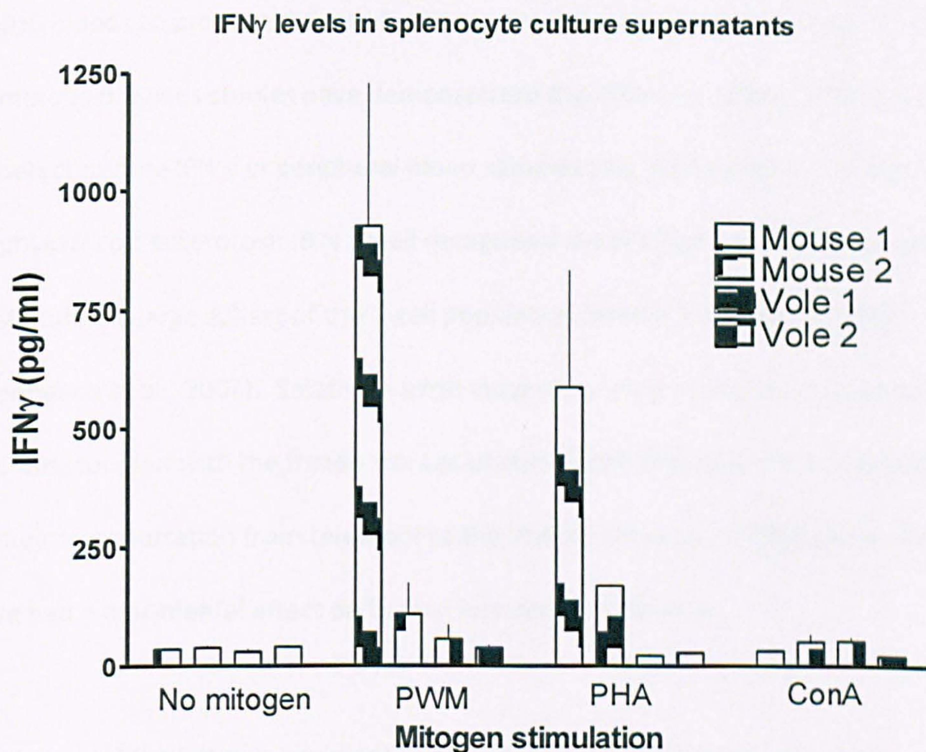


Figure 5.1 IFN-γ detected for mouse and vole splenocytes either unstimulated (no mitogen) or stimulated with pokeweed mitogen (PWM), phytohaemagglutinin (PHA) or concanavalin A (ConA). Each test was performed in triplicate and the mean value obtained is presented.

5.4 Discussion

The first experiment attempted involved the stimulation of T cells in whole blood using a generic stimulant staphylococcal enterotoxin B. No evidence of IFN-γ production was obtained from any blood samples, including blood samples collected from the "positive control" Balb/c mice. The failure of the stimulated Balb/C bloods to produce detectible IFN-γ to be detectible was surprising. Numerous previous studies have demonstrated the efficiency of the ELISA used to detect murine IFN-γ in peripheral blood samples (e.g. Hempel et al., 2002) and staphylococcal enterotoxin B is a well recognised superantigen with the capacity to stimulate a large subset of the T-cell population (Marrack & Kappler, 1990; Buenopane et al., 2007). Relatively small volumes of blood samples were used, and this, coupled with the frozen storage of stimulated bloods and the necessity of their transportation from Liverpool to the VLA laboratories in Weybridge, may have had a detrimental effect on the performance of the assay.

The failure of the initial experiment necessitated the identification of a more reliable, richer source of IFN-γ producing cells, hence I attempted to culture field vole and Balb/C mouse splenocytes. The culture of field vole splenocytes has not been reported previously, but proved relatively straight-forward using the generic protocol chosen. Microscopic observation of splenocyte cultures derived from both mice and field voles revealed healthy cells of differing morphologies, probably representing lymphocytes and cells of the myeloid lineage. Good growth was obtained within 24 hours of isolation and cultures remained intact

for at least one week. Thus, stimulation assays were performed on cell cultures that were between 48 and 72 hours old.

Stimulation of mouse splenocytes using either pokeweed mitogen (PWM), phytohaemagglutinin (PHA) produced expected results, with significant amounts of IFN-γ being detected from both animals tested. The reliability of these two mitogens to stimulate cells of the immune system is well documented (e.g. Lieberman et al., 2007). However, concanavalin A (ConA) failed to provoke IFN-γ production by murine splenocytes, an unexpected finding since, like PWM and PHA, this mitogen is widely recognised as a reliable stimulator of murine splenocytes (e.g. Prat et al., 1984; Spitalny & Havell, 1984; Fenaux et al., 2005). The use of an inactive reagent or experimental error is therefore the most likely explanation; no attempt to repeat the experiment was made, hence this was not investigated further.

Despite the detection of IFN-γ production by stimulated murine splenocytes, the ELISA failed to detect IFN-γ in supernatants collected from stimulated field vole splenocytes. Two alternative explanations are most feasible, firstly, the mitogens used failed to stimulate field vole splenocytes or secondly, the ELISA used failed to detect field vole IFN-γ. As PWM and PHA are recognised as generic stimulants of immune cells (Lieberman et al., 2007) from many different hosts, including human beings (Winek et al., 2008) the first of these explanations seems unlikely. Far more plausible is the second explanation. The ELISA used incorporated two monoclonal antibodies against murine IFN-γ (Prat et al., 1984;

Spitalny & Havell, 1984) that are likely to be specific to this host species. In the original description of these antibodies, their inactivity with human and rat IFN-γ was recorded (Prat et al., 1984; Spitalny & Havell, 1984). Cross-reactivity between these monoclonal antibodies and arvicolar IFN-γ has not been studied.

Although there are no data yet available regarding the sequence of the field vole IFN-γ encoding gene, or indeed field vole IFN-γ structure itself, the extent of variation between IFN-γ in different hosts is recognised. Gray & Goeddel (1983) reported that the nucleotide homology between the murine and human IFN-γ genes is only 60-65%, whereas the encoded proteins are only 40% homologous. This suggests that inter-species IFN-γ variation is marked, and thereby provides evidence to support the likely murine-specificity of the antibodies used in this study. No details on the epitopes for the two antibodies could be found.

The unavailability of field vole specific reagents prevented further work characterising IFN-γ production in field voles. However, given more time, a number of alternative approaches could have been attempted. Other anti-IFN-γ antibodies have been described either for mice (e.g. XMG12, Pharmigen) or for other murine rodents such as *Rattus* and *Sigmoden* species (R&D Systems Ltd). However, perhaps a more successful approach would be to develop assays to determine IFN-γ transcription. Similar assays have been used in studies involving other wild rodent species (Schountz et al., 2007), and ongoing work in Liverpool has resulted in the development of field vole specific reverse-transcriptase-PCR (RT-PCR) assays for various cytokines (Dr Richard Birtles, personal

communication). The detection of IFN-γ transcription has been described for the diagnosis of MtC infections in multiple animal species, including wildlife (Harrington et al., 2007).

Chapter 6.

General discussion

6.1 The nature of vTB skin lesions

In this study and previous work by Cavanagh and colleagues (2002) and Wells (1946), skin lesions were detected in voles infected with vTB. However, the origin of these lesions is not clear. An insight of their possible provenance can perhaps be gained from studying the presentation of disease caused by *M. tuberculosis* complex members, and other *Mycobacterium* species, in other hosts. Tuberculosis affecting the skin in humans can divided into two broad categories: cutaneous TB (mycobacterium infection of the skin) and tuberculids (cutaneous reactions to extracutaneous TB) (Burgin et al., 2004). Cutaneous TB can also be subdivided according to the organism i.e. exogenous or endogenous (Burgin et al., 2004). Exogenous sources of mycobacteria can cause warty TB and tuberculous chancres. Endogenous infections may arise secondary to lymphatic or haematogenous dissemination. Infection of the skin may also occur following autoinoculation from the underlying infected tissue or from bodily excretions. These are known by names such as lupus vulgaris, acute miliary TB of the skin, tuberculous gumma, orificial TB or scrofuloderma (Burgin et al., 2004). Inoculation into skin injuries is also a well known route of infection in humans. The lesions that develop at the inoculation site can progress to form secondary lesions along draining lymphatics and regional lymphadenopathy but the infection is usually self-limiting (Grange & Zumla, 2002). In humans, a major form of cutaneous mycobacterium is leprosy, caused by *M. leprae*. Cutaneous leprosy occurs due to a lack in T cell mediated immune response whereas tuberculoid leprosy is a paucibacillary disease with an intense T cell-mediated response (Samuelson, 1999). *M.leprae* is also capable of infecting nine-banded

armadillos, which could act as a reservoir for human infection (Paige et al., 2002). The mode of transmission of *M. leprae* is unclear; the main portal of entry and exit seems to be the respiratory system, particularly the nose. However, dissemination from skin lesions may also have some importance, although there is currently little evidence to suggest that direct entry of the organism through the skin plays a significant role (Visschedijk et al., 2000).

Skin lesions are also a common presentation of *M. microti* and *M. bovis* infections in cats (Gunn-Moore et al.,1996). The cutaneous form of the classical tuberculosis in cats causes non-healing lesions on the face , neck and shoulders with lymphadenopathy and draining sinus tracts. Lesions are thought to arise from infected bites or scratches, or by local or even haematogenous spread (Snider et al., 1971). Feline leprosy is primarily a cutaneous disease involving multiple ulcerated nodules on the head and limb (Schieffer & Middleton 1983). It can be caused by *M.lepraemurium* or other *Mycobacterium* species, including *M.avium* (Barrs et al., 1999), and , as with its human equivalent, delineation of feline tuberculoid or lepromatous leprosy has been proposed (Barrs et al., 1999), although this partition was not be supported in the later studies (Kipar et al., 2003). Systemic disease only develops occasionally, but regional lymphadenopathy is common (Gunn-Moore et al.,1996). Atypical mycobacterial infection , thought mainly to the enter via skin wounds, generally only causes cutaneous lesions with variable regional lymphadenopathy (Kipar et al., 2003). Feline (muco)cutaneous mycobacteriosis cases have been attributed to mycobacterium of the MtC (Kipar et al.,2003); these pyogranulomatous lesions,

with variably sized areas of necrosis, exhibit multinucleated giant cell and lymphocyte infiltration with many acid fast bacilli (Kipar et al.,2003).

The lesions observed in cats with cutaneous mycobacterium infections appear similar to the lesions observed in naturally infected *M microti*-infected voles. As it is strongly suspected that cutaneous mycobacteriosis infections can be acquired via the skin in cats and humans, that the same mechanism also provokes these symptoms in voles is feasible. Furthermore, subsequent systematic spread of infection, as observed in cats, may also provoke the widespread foci of infections observed in voles. However, the observations presented in this thesis and elsewhere indicate that lymphatic and respiratory foci for vTB are far more common in voles than cutaneous lesions, suggesting that the latter may well result from dissemination of infection established at the former sites (Wells, 1946; Cavanagh et al., 2002). However, acquisition of *M. microti* by various routes is also a possibility, including via the skin and via the respiratory and/or digestive tracts. Furthermore, the skin may be an important route by which *M. microti* is shed by voles. Kipar and colleagues (2003) noted that although the axillary lymph nodes draining the ulcerated interscapular tissue were frequently affected, the crust material overlying these areas also contained mycobacteria. However, salivary glands and pulmonary exudates of voles with pneumonia associated with *M microti* infection have also been shown to contain mycobacteria (Kipar et al., 2003). Consequently, it seems conceivable to hypothesise that excretion of the organisms in saliva , pulmonary exudates and skin lesions may lead to the spread of infection within the family group.

Wells' descriptions of lesions in the interscapular region of voles with *M microti* infection (1946) has recently lead to further investigation into the inoculation process required to produce such lesions (Kipar et al 2003). In this study, interscapular lesions, exhibiting large necrotic areas containing mycobacteria, were demonstrated in 53% of infected animals (compared to 31% described by Wells in 1946 and 21% in this study). Given the lack of lymphoid tissue in this area it is not understood why these lesions form in the wild voles. There is the possibility that infection could occur directly into the areas through damage or abrasion to the skin surface. Field voles, particularly males, are aggressive, yet fighting alone would produce generalized skin damage rather than the localised lesions observed. An alternative hypothesis could be the introduction of *M microti* into the scruff of the neck of off-spring via the saliva of infected mothers, who lift and carry their off-spring by clasping this region of the body in their mouths. However, the extent to which the skin of off-spring is damaged during these manoeuvres is uncertain, although only superficial damage may be necessary to introduce bacteria into a naïve host.

6.2 *M. microti* as an emerging zoonotic pathogen

The feasibility of transmission of *M. microti* from voles to other wild or domesticated animals, including humans, has been demonstrated by a handful of case reports in the literature (e.g. Huitema & Jaartsveld, 1967; Taylor et al., 2006; Oevermann et al., 2006; Emmanuel et al., 2007). However, contrary to these observations, we have failed to detect tuberculous lesions on the skin on

numerous weasels trapped at sites in Kielder where vTB is present in the vole populations that represent the diet of these predators (unpublished observations). This observation suggests that some species, perhaps specifically those evolved to prey on field voles, are not susceptible to *M. microti* infection. Conversely, *M. microti* infections in cats are being increasingly reported (Smith et al., 2009); perhaps this is the result of feline tuberculosis now being a reportable disease (as a result of cat's susceptibility to *M. bovis*), however it may also reflect a variety of epidemiological changes such as (i) the spread of vTB to rodent populations where it was previously absent, (ii) the increasing size of infected rodent populations nationally, (iii) an increased prevalence of infection in affected populations, (iv) increased hunting of/contact between cats and infected rodent populations, or (v) a combination of all of the above. Perhaps of relevance is that the amount of grassland, the preferred habitat of field voles, is rapidly increasing in the UK. The reversion of pasture to grassland, as favoured by the Government's "green" agenda, is likely to lead to a significant increase in the size and distribution of the UK's field vole population, and with this may come the increased risk of the opportunistic transmission of *M. microti* infection to non-reservoir species.

6.3 *M. microti* as a confounder of bovine tuberculosis epidemiology

A recent study has demonstrated an apparent mutual geographic exclusivity between *M.bovis* and *M. microti* infections in the UK (Smith et al., 2009). Although this observation can only be considered preliminary, since the *M. microti* infections were documented in domestic cats, which are an accidental

host, rather than in field voles and other woodland rodents, its remarkable nature warrants discussion.

The reliability of the evidence presented by Smith and colleagues (2009) was supported by a relatively large dataset of 141 feline *M. microti* isolates that were submitted to the Veterinary Laboratories Agency over a 14 year period to 2008. Of these 140 isolates, details of geographic provenance were available for 67, and although this provenance was widely distributed across Eastern and Northern England, North Wales and Scotland, only one originated in a part of the UK affected by bTB. Counter to this, 34 of 35 cats infected with *M. bovis* for which geographic data were available, lived in parts of the UK where bTB was present in cattle.

It is interesting to speculate what biological processes might underlie this geographic exclusion, and one possible explanation centres on the importance of badgers (*Meles meles*) in bTB epidemiology. Badgers are recognised as reservoir hosts for *M. bovis* in the UK (Donnelly et al., 2007), however, as omnivores, their diet consists partly of rodents. Thus, in places where *M. microti* infections circulate in rodent communities, badgers may well consume infected rodents as prey thereby risking being exposed to, or even infected by *M. microti*. However, although such infections may induce morbidity in badgers, the relatively benign nature of *M. microti* means that infections are unlikely to be as severe as *M. bovis*. Furthermore, exposure to *M. microti* is likely to lead to cross-protection against *M. bovis*. Indeed, as described in Chapter 1, *M. microti* has, historically

been used as a vaccine against other MtC members including *M. tuberculosis* and *M. bovis*. (Manabe et al., 2002). Thus, badgers living in regions of the UK where *M. microti* infections are common in rodents may have the opportunity to acquire natural resistance to *M. bovis*, thereby preventing *M. bovis* infections becoming established in these areas. An extension of this hypothesis is that the presence of infected field voles on the fringes of pasture, may lead to exposure of cattle to *M. microti*, thereby offering them some protection from *M. bovis*. There is some evidence that MtC members can persist in the environment (Sweeney et al., 2007) having been shed from infected hosts, so direct contact between rodents and cattle or badgers may not be necessary. Clearly, the hypotheses presented above require thorough testing, but should these tests yield support for them, the use of *M. microti*, or *M. microti*-infected rodent populations may serve as a natural vaccination against *M. bovis*. Indeed, the presence of *M. microti* in rodents inhabiting farmland may contribute to control of bTB in livestock. Intriguingly, bTB appears to be less prevalent on wildlife-friendly farms (Mathews et al., 2006).

References

Adair, D. M., Worsham, P. L., Hill, K. K., Klevytska, A. M., Jackson, P. J., Friedlander, A. M., Keim, P. (2000). Diversity in a variable-number tandem repeat from *Yersinia pestis*. J Clin Microbiol **38**:1516-9.

Afghani, B., Stutman, H. R. (1995). Diagnosis of tuberculosis: can the polymerase chain reaction replace acid-fast bacilli smear and culture? J Infect Dis **172**:903-5.

Afghani, B., Stutman, H. R. (1996). Polymerase chain reaction for diagnosis of M. tuberculosis: comparison of simple boiling and a conventional method for DNA extraction. Biochem Mol Med **57**:14-8.

Ahmed, N. A., Mohanty, K., Mukhopadhyay, U., Batish, V. K. Grover, S. (1998). PCR-based rapid detection of *Mycobacterium tuberculosis* in blood from immunocompetent patients with pulmonary tuberculosis. J Clin Microbiol **36**:3094-5.

Ahmed, S. A. (2005). Altered splenocyte function in aged C57BL/6 Mice prenatally exposed to diethylstilbestrol. J Immunotoxicol **2**:221-9.

Kipar, A ., Schiller, I., Baumgartne , W. (2003). Immunopathological studies on feline cutaneouse and (muco)cutaneous mycobacteriosis . J Veterinary Immunol Immunopathol **91**:169-82

Aldous, W. K., Pounder, J. I., Cloud, J. L., Woods, G. L. (2005). Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative real-time PCR. J Clin Microbiol **43**:2471-3.

Allix, C., Walravens, K., Saegerman, C., Godfroid, J., Supply, P., Fauville-Dufaux, M. (2006). Evaluation of the epidemiological relevance of variable-number tandem-repeat genotyping of *Mycobacterium bovis* and comparison of the method with IS6110 restriction fragment length polymorphism analysis and spoligotyping. J Clin Microbiol **44:** 1951-62.

Allix-Beguec, C., Fauville-Dufaux, M., Supply, P. (2008). Three-year population-based evaluation of standardized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol **46**:1398-1406.

Amicosante, M., Richeldi, L., Trenti, G., Paone, G., Campa, M., Bisetti, A., Saltini, C. (1995). Inactivation of polymerase inhibitors for *Mycobacterium tuberculosis* DNA amplification in sputum by using capture resin. J Clin Microbiol **33**:629-30.

Andersen, H., Dempsey, D., Chervenak, R., Jennings, S. R. (2000). Expression of intracellular IFN-gamma in HSV-1-specific CD8+ T cells identifies distinct responding subpopulations during the primary response to infection. J Immunol, **165**:2101-7.

Anon, (2004). Bovine Tuberculosis. In: Manual of Diagnostic T tests and Vaccines for Terrestrial Animals, fifth ed. Office International des Epizooties, Paris, Chapter 2.3.3.

Anon, A, S. (1937). Tuberculosis in wild voles. Lancet 1221

Barcelos, D., M. Franco,M. F., Leao, S. C. (2008). Effects of tissue handling and processing steps on PCR for detection of *Mycobacterium tuberculosis* in formalin-fixed paraffin-embedded samples. Rev Inst Med Trop Sao Paulo **50**:321-6.

Bates, J. H. (1979). Diagnosis of tuberculosis. Chest **76**:757-63.

Benjamini, E., Sunshine, G., Leskowitz. (1996). Immunology A short course. Third Ed.Wiley –Liss. New York

Boddinghaus, B., Rogall, T., Flohr, T., Blocker, H., Bottger, E. C. (1990). Detection and identification of mycobacteria by amplification of rRNA. J Clin Microbiol **28**:1751-9.

Boddinghaus, B., Wolters, J., Heikens, W., Bottger, E. C. (1990). Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol Lett **58:**197-203.

Bodmer, T., Gurtner, A., Scholkmann, M., Matter, L. (1997). Evaluation of the COBAS AMPLICOR mtb system. J Clin Microbiol **35:**1604-5.

Brennan, P. J., Nikaido, H. (1995). The envelope of mycobacteria. Ann Rev Biochem **64**:29-63.

Broccolo, F., Scarpellini, P., Locatelli, P., Zingale, G. A., Brambilla, A. M., Cichero, P., Sechi, L. A., Lazzarin, A., Lusso, P., Malnati, M. S. (2003). Rapid diagnosis of mycobacterial infections and quantitation of *Mycobacterium tuberculosis* load by two real-time calibrated PCR assays. J Clin Microbiol **41**:4565-72.

Brodin, P., Eiglmeier, K., Marmiesse, M., Billault, A., Garnier, T., Niemann, S., Cole, S. T., Brosch, R. (2002). Bacterial artificial chromosome-based comparative genomic analysis identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant. Infect Immun, **70:**5568-78.

Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L. M., Pym, A. S., Samper, S., van Soolingen, D., Cole, S. T. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci USA **99:**3684-89.

Brudey, K., Driscoll, J. R., Rigouts, L., Prodinger, W. M., Gori, A., Al-Hajoj, S. A., Allix, C., Aristimuno, L., Arora, J., Baumanis, V., Binder, L., Cafrune, P., Cataldi, A., Cheong, S., Diel, R., Ellermeier, C., Evans, J. T., Fauville-Dufaux, M., Ferdinand, S., Garcia de Viedma, D., Garzelli, C., Gazzola, L., Gomes, H. M., Guttierez, M. C., Hawkey, P. M., van Helden, P. D., Kadival, G. V., Kreiswirth, B. N., Kremer, K., Kubin, M., Kulkarni, S. P., Liens, B., Lillebaek, T., Ho, M. L., Martin, C., Mokrousov, I., Narvskaia, O., Ngeow, Y. F., Naumann, L., Niemann, S., Parwati, I., Rahim, Z., Rasolofo-Razanamparany, V., Rasolonavalona, T., Rossetti, M. L., Rusch-Gerdes, S., Sajduda, A., Samper, S., Shemyakin, I.G., Singh, U. B., Somoskovi, A., Skuce, R. A., van Soolingen, D., Streicher, E. M., Suffys, P. N., Tortoli, E., Tracevska, T., Vincent, V., Victor, T. C., Warren, R. M., Yap, S. F., Zaman, K., Portaels, F., Rastogi, N., Sola, C. (2006). *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol **6**:23.9

Bruijnesteijn Van Coppenraet, E. S., Lindeboom, J. A., Prins, J. M, Peeters, M. F., Claas, E. C, Kuijper, E. J. (2004) Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children. J Clin Microbiol **42**:2644–50.

Buck, G. E., O'Hara, L. C., Summersgill, J. T. (1992). Rapid, sensitive detection of *Mycoplasma pneumoniae* in simulated clinical specimens by DNA amplification. J Clin Microbiol **30:** 3280-83.

Buddle, B. M., Ryan, T. J., Pollock, J. M., Andersen, P., de Lisle, G. W. (2001). Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol **80:**37-46.

Burggraf, S., Reischl, U., Malik, N., Bollwein, M., Naumann, L., Olgemoller, B. (2005). Comparison of an internally controlled, large-volume LightCycler assay for detection of *Mycobacterium tuberculosis* in clinical samples with the COBAS AMPLICOR assay. J Clin Microbiol **43:**1564-69.

Burgin, S., Keltz-Pomeranz, M., Orbuch., P., Shupack, J. (2004). *Mycobacteria* and the skin. In :Tuberculosis. Ed: Rom, W.N and Garay ,S.M.London, Lippincott Williams and Wikins, pp.593-606.

Burthe, S. (2005). The dynamics of cowpox infection and vole tuberculosis in cyclic wild field vole populations. PhD thesis, University of Liverpool.

Burthe, S., Bennett, M., Kipar, A., Lambin, X., Smith, A., Telfer, S., Begon, M. (2008). Tuberculosis (*Mycobacterium microti*) in wild field vole populations. Parasitol **135**:309-17.

Burthe, S., Telfer, S., Lambin, X., Bennett, M., Carslake, D., Smith, A., Begon, M. (2006). Cowpox virus infection in natural field vole *Microtus agrestis* populations: delayed density dependence and individual risk. J Anim Ecol **75**:1416-25.

Caffrey, J. P. (1994). Status of bovine tuberculosis eradication programmes in Europe. Vet Microbiol **40**:1-4.

Camacho, L. R., Ensergueix, D., Perez, E., Gicquel, B., Guilhot, C. (1999). Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. Mol Microbiol **34**:257-67.

Cavanagh, R. D., Lambin, X., Ergon, T., Bennett, M., Graham, I. M., van Soolingen, D., Begon, M. (2004). Disease dynamics in cyclic populations of field voles (*Microtus agrestis*): cowpox virus and vole tuberculosis (*Mycobacterium microti*). Proc Biol Sci **271**:59-67.

Cavanagh, R., Begon, M., Bennett, M., Ergon, T., Graham, I. M., De Haas, P. E., Hart, C. A., Koedam, M., Kremer, K., Lambin, X., Roholl, P., van Soolingen, D. (2002). *Mycobacterium microti* infection (vole tuberculosis) in wild rodent populations. J Clin Microbiol **40**:3281-85.

Cave, M. D., Eisenach, K. D., McDermott, P. F. Bates, J. H. Crawford, J. T. (1991). IS6110: conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. Mol Cell Probes **5**:73-80.

Cheng, V. C, Yam, W. C, Hung, I. F, Woo, P. C, Lau, S. K, Tang, B. S, Yuen, K. Y. (2004). Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. J Clin Pathol **57**:281–85.

Cheng, V. C., Yew, W. W., Yuen, K. Y. (2005). Molecular diagnostics in tuberculosis. Eur J Clin Microbiol Infect Dis **24**:711-20.

Chitty, D. (1954). Tuberculosis among wild voles : With a discussion of other pathological conditions among certain mammals and birds. Ecology **35**:227-37.

Chitty, D. (1960). Population processes in the vole and their relevance to general theory. Can J Zool **38**:99-113.

Coates, S. R., Hansen, D., Schecter, G., Slutkin, G., Hopewell, P., Affronti, L., Echenberg, D. F. (1986). Identification of *Mycobacterium tuberculosis* antigens in Seibert fractions by immunoblotting. J Clin Microbiol **24**:126-30.

Cole, R. N., Skeiky, Y. A., Vedvick, T., Bement, T., Ovendale, P., Campos-Neto, A., Alderson, M. R., Reed, S. G. (1998). Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of *Mycobacterium tuberculosis*. J Immunol **161**:2356-64.

Comincini, S., Barbarini, D., Telecco, S., Bono, L., Marone, P. (1998). Rapid identification of *Mycobacterium tuberculosis* and *Mycobacterium avium* by polymerase chain reaction and restriction enzyme analysis within sigma factor regions. New Microbiol **21**:391-95.

Courtenay, O., Reilly, L. A., Sweeney, F. P., Hibberd, V., Bryan, S., Ul-Hassan, A., Newman, C., Macdonald, D. W., Delahay, R. J., Wilson, G. J., Wellington, E. M. (2006). Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis? Biol Lett **2**:460-2.

Cousins, D. V., Peet, R. L., Gaynor, W. T., Williams, S. N., Gow, B. L. (1994). Tuberculosis in imported hyrax (*Procavia capensis*) caused by an unusual variant belonging to the *Mycobacterium tuberculosis* complex. Vet Microbiol **42**:135-45.

Cousins, D. V., Skuce, R. A., Kazwala, R. R., van Embden, J. D. (1998). Towards a standardized approach to DNA fingerprinting of *Mycobacterium bovis*. Int J Tuberc Lung Dis **2**:471-8.

Crookham, J., Dapson, R. (1991). Hazardous chemicals in the histopathology laboratory, 2nd Edition, Anatech, USA.

Dalovisio, J. R., Montenegro-James, S., Kemmerly, S. A., Genre, C. F., Chambers, R., Greer, D., Pankey, G. A., Failla, D. M., Haydel, K. G., Hutchinson, L., Lindley, M. F., Nunez, B. M., Praba, A. Eisenach, K. D., Cooper, E. S. (1996). Comparison of the amplified *Mycobacterium tuberculosis* (MTB) direct test, Amplicor MTB PCR, and IS6110-PCR for detection of MTB in respiratory specimens. Clin Infect Dis **23**:1099-106.

David, H. L., Levy-Frebault V., Dauguet, C., Grimont, F. (1984). DNA from *Mycobacterium leprae*. Acta Leprol **2**:129-36

de la Rua-Domenech, R., Goodchild, A. T., Vordermeier, H. M., Hewinson, R. G., Christiansen, K. H., Clifton-Hadley, R. S. (2006). Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay and other ancillary diagnostic techniques. Res Vet Sci **81**:190-10.

De Lamballerie, X., Zandotti, C., Vignoli, C., Bollet, C., de Micco, P. A. (1992). One-step microbial DNA extraction method using Chelex 100 suitable for gene amplification. Res Microbiol **143**:785-90.

Deforges, L., Boulouis, H. J., Thibaud, J. L., Boulouha, L., Sougakoff, W., Blot, S., Hewinson, G., Truffot-Pernot, C., Haddad, N. (2004). First isolation of *Mycobacterium microti* (Llama-type) from a dog. Vet Microbiol **103**:249-53.

Desjardin, L. E., Chen, Y., Perkins, M. D., Teixeira, L., Cave, M. D., Eisenach, K. D. (1998). Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis. J Clin Microbiol **36**:1964-8.

Dharmadhikari, A. S., Nardell, E. A. (2008). What animal models teach humans about tuberculosis. Am J Respir Cell Mol Biol **39**:503-8.

Dlugovitzky, D., Bay, M. L., Rateni, L., Fiorenza, G., Vietti, L., Farroni, M. A., Bottasso, O. A. (2000). Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis (TB). Clin Exp Immunol **122**:343-49.

Drobniewski, F. A., Caws, M. M., Gibson, A., Young, D. (2003). Modern laboratory diagnosis of tuberculosis. Lancet Infect Dis **3**:41-7.

Elton, C. (1924). Periodic fluctuations in the numbers of animals: their causes and effects. J Exp Biol **2**:110-63.

Elton, C. (1942). Voles, mice and lemming, Oxford University Press, Oxford, UK.

Emmanuel, F., Seagar, A. L., Doig, C., Rayner, A., Claxton, P., Laurenson, I. (2007). Human and animal infections with *Mycobacterium microti*, Scotland. Emerg Infect Dis **13**:1924-7.

Ernst, J. D. (1998). Macrophage receptors for *Mycobacterium tuberculosis*. Infect Immun **66**:1277-81.

Evans, J. T., Smith,E. G., Banerjee, A., Smith, R. M., Dale, J., Innes, J. A., Hunt, D. Tweddell, A., Wood, A., Anderson, C., Hewinson, R. G., Smith, N. H., Hawkey, P. M., Sonnenberg, P. (2007). Cluster of human tuberculosis caused by *Mycobacterium bovis*: evidence for person-to-person transmission in the UK. Lancet **369** :1270-6.

Fatolahzadeh, B., Maleknejad, P., Bahador, A., Peeri-Dogaheh, H., Alikhani, M. Y., Radmanesh-Ahsani, R. (2007). Evaluation of different primer sets for the rapid diagnosis of tuberculosis. Pak J Biol Sci **10**:107-11.

Fenaux, J. B., Gogal, R. M., Jr., Lindsay, D., Hardy, C., Ward, D. L., Saunders, G., Ahmed, S. A. (2005). Altered splenocyte function in aged C57BL/6 Mice prenatally exposed to diethylstilbestrol. J Immunotoxicol **2**:221-29.

Ferrari, G., Langen, H., Naito, M., Pieters, J. (1999). A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell **97**:435-47.

Frota, C. C., Hunt, D. M., Buxton, R. S., Rickman, L., Hinds, J., Kremer, K., van Soolingen, D., Colston, M. J. (2004). Genome structure in the vole bacillus, *Mycobacterium microti*, a member of the *Mycobacterium tuberculosis* complex with a low virulence for humans. Microbiology **150**:1519-27.

Frothingham, R., Meeker-O'Connell, W. A. (1998). Genetic diversity in the Mycobacterium tuberculosis complex based on variable numbers of tandem DNA repeats. Microbiology **144**:1189-96.

Frothingham, R., P., Strickland, Bretzel, G. Ramaswamy, S. Musser, J. M. Williams, D. L. (1999). Phenotypic and genotypic characterization of *Mycobacterium africanum* isolates from West Africa. J Clin Microbiol **37**:1921-6.

Garcia-Pelayo, M. C., Caimi, K. C., Inwald, J. K., Hinds, J., Bigi, F., Romano, M. I., van Soolingen, D., Hewinson, R. G., Cataldi, A., Gordon, S. V. (2004). Microarray analysis of *Mycobacterium microti* reveals deletion of genes encoding PE-PPE proteins and ESAT-6 family antigens. Tuberculosis **84**:159-66.

Ghadersohi, A., Coelen, R. J., Hirst, R. G. (1997). Development of a specific DNA probe and PCR for the detection of *Mycoplasma bovis*. Vet Microbiol **56**:87-98.

Gillespie, S. H., Charalambous, B. M. (2003). A novel method for evaluating the antimicrobial activity of tuberculosis treatment regimens. Int J Tuberc Lung Dis **7**:684-9.

Glickman, M. S., Cox, J. S., Jacobs, W. R., (2000). A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. Mol Cell **5**:717-27.

Grange, J.M., Zumla, A. (2002). Tuberculosis. In: Manson's tropical disease. Eds. Cook, G., Zumla, A. London, Saundes. 1-59

Gray, P. W., Goeddel, D. V. (1983). Human immune interferon (IFN-gamma) gene sequence and structure. Basic Life Sci **25**:35-61.

Greene ,C.E and Gunn-Moore, D.A.1998. Mycobacterial infections. In: Infectious Diseases of the dog and cat: Ed: Greene, C.E. London Saunders, 313-25.

Griffith, A. S. (1939). The relative susceptibility of the field voles to bovine, human and avian types of tubercle bacilli and to the vole strain of acid-fast bacillus. J Hyg **39**:244-59.

Griffith, A. S. (1941). Further experiments on the golden hamster (*Cricetus auratus*) with tubercle bacilli and the vole strains of acid – fast bacillus. J Hyg **41**:260-65.

Griffith, A. S. (1942). The cultural characters and pathogenicity for some laboratory animals of the vole strains of acid-fast bacillus. J Hyg **42**:527-31.

Griffith, A. S., Dalling, T. (1940). Inoculation and immunity experiments on calves with the vole strain of acid fast bacillus. J Hyg **40**:673-80.

Gudan, A., Artukovic, B., Cvetnic, Z., Spicic, S., Beck, A., Hohsteter, M., Naglic, T., Bata, I., Grabarevic, Z. (2008). Disseminated tuberculosis in hyrax (*Procavia capensis*) caused by *Mycobacterium africanum*. J Zoo Wildl Med **39**:386-91.

Gurtler, V., Harford, G., Bywater, J., Mayall, B. C. (2006). Direct identification of slowly growing *Mycobacterium* species by analysis of the intergenic 16S-23S rDNA spacer region (ISR) using a GelCompar II database containing sequence based optimization for restriction fragment site polymorphisms (RFLPs) for 12 enzymes. J Microbiol Method **64**:185-99.

Haddad, N., Masselot, M. Durand, B. (2004). Molecular differentiation of *Mycobacterium bovis* isolates. Review of main techniques and applications. Vet Sci **76**:1-18.

Hanekom, M., van der Spuy, G. D., Gey van Pittius, N. C., McEvoy, C. R., Hoek, K. G., Ndabambi, S. L., Jordaan, A. M., Victor, T. C., van Helden, P. D., Warren, R. M. (2008). Discordance between mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing and IS6110 restriction fragment length polymorphism genotyping for analysis of *Mycobacterium tuberculosis* Beijing strains in a setting of high incidence of tuberculosis. J Clin Microbiol **46**:3338-45.

Harboe, M., Wiker, H.G. (1998). Secreted proteins of *Mycobacterium leprae*. Scand J Immunol **48**:577-84.

Harrington, N. P., Surujballi, O. P., Waters, W. R., Prescott, J. F. (2007). Development and evaluation of a real-time reverse transcription-PCR assay for quantification of gamma interferon mRNA to diagnose tuberculosis in multiple animal species. Clin Vac Immunol **14**:1563-71.

Hart, D. A., Wang, A. L., Pawlak, L. L., Nisonoff, A. (1972). Suppression of idiotypic specificities in adult mice by administration of antiidiotypic antibody. J Exp Med **135**:1293-300.

Hart, P. D., Armstrong, J. A., brown, C. A., Draper, P. (1972). Ultrastructural study of the behaviour of macrophages toward parasitic mycobacteria. Infect Immun **5**:803-7.

Hart, P. D., Sutherland, I. (1977). BCG and vole bacillus vaccines in prevention of tuberculosis in adolescence and early adult life - final report to medical-research-council. BMJ **2**:293-95.

Hart, P. D., Young, M. R., Gordon, A. H. Sullivan, K. H. (1987). Inhibition of phagosome-lysosome fusion in macrophages by certain mycobacteria can be explained by inhibition of lysosomal movements observed after phagocytosis. J Exp Med **166**:933-46.

Heginbothom, M. L., Magee, J. T., Flanagan, P. G. (2003). Evaluation of the Idaho Technology LightCycler PCR for the direct detection of *Mycobacterium tuberculosis* in respiratory specimens. Int J Tuberc Lung Dis **7**:78-83.

Hejlicek, K. (1959). Study of the properties of a murine strain of tubercle bacillus in pigs. Vet Bull **30**:3473-93

Hempel, D. M., Smith, K. A., Claussen, K. A., Perricone, M. A. (2002). Analysis of cellular immune responses in the peripheral blood of mice using real-time RT-PCR. J Immunol Methods **259**:129-38.

Henrich, M., Moser, I., Weiss, A., Reinacher, M. (2007). Multiple granulomas in three squirrel monkeys (*Saimiri sciureus*) caused by *Mycobacterium microti*. J Comp Pathol **137**:245-48.

Hermans, P. W., van Soolingen, D., Dale, J. W., Schuitema, A. R., McAdam, R. A., Catty, D., van Embden, J. D. (1990). Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. J Clin Microbiol **28**:2051-58.

Hermans, P. W., van Soolingen, D., van Embden, J. D. (1992). Characterization of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. J Bacteriol **174**:4157-65.

Hilty, M., Diguimbaye, C., Schelling, E., Baggi, F., Tanner, M., Zinsstag, J. (2005). Evaluation of the discriminatory power of variable number tandem repeat (VNTR) typing of *Mycobacterium bovis* strains. Vet Microbiol **109**:217-22.

Horstkotte, M. A., Sobottka, I., Schewe, C. K., Schafer, P., Laufs, R., Rusch-Gerdes, S., Niemann, S. (2001). *Mycobacterium microti* llama-type infection presenting as pulmonary tuberculosis in a human immunodeficiency virus-positive patient. J Clin Microbiol **39**:406-7.

Huard, R. C., Lazzarini, L. C., Butler, W. R., van Soolingen, D., Ho, J. L. (2003). PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J Clin Microbiol **41**:1637-50.

Huitema, H., Jaartsve, F. H. (1967). *Mycobacterium microti* infection in a cat and some pigs. Antonie Van Leeuwenhoek J Microbiol **33**:209-15.

Huitema, H., van, V. (1960). Murine tuberculosis in a cat. Antonie Van Leeuwenhoek J Microbiol **26**:235-40.

Imaeda, T. (1985). Deoxyribonucleotide acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. Int J Syst Bacteriol **35**:147-50.

Jackson, P. J., Hugh-Jones, M. E., Adair, D. M. Green, G., Hill, K. K., Kuske, C. R., Grinberg, L. M., Abramova, F. A., Keim, P. (1998). PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: the presence of multiple *Bacillus anthracis* strains in different victims. Proc Natl Acad Sci U S A **95**:1224-9.

Jahans, K., Palmer, S., Inwald, J., Brown, J., Abayakoon, S. (2004). Isolation of *Mycobacterium microti* from a male Charolais-Hereford cross. Vet Rec **155**:373-74.

Jeffreys, A. J., Wilson, V. Thein, S. L. (1985). Individual specific fingerprints of human DNA. Nature **316**:76-9.

Jenkins, N. E., Beadsworth, M. B., Anson, J. J., Nye, F. J., Martlew, V. J., Beeching, N. J., (2006). Immune restoration disease in HIV patient. Emerg Infect Dis **12**:689-91.

Jeon, B., Je, S., Park, J., Kim, Y., Lee, E. G., Lee, H., Seo, S., Cho, S. N. (2008). Variable number tandem repeat analysis of *Mycobacterium bovis* isolates from Gyeonggi-do, Korea. J Vet Sci **9**:145-53.

Kamerbeek, J., Schouls, L., Kolk, A., van Agterveld, M., van Soolingen, D., Kuijper, S., Bunschoten, A., Molhuizen, H., Shaw, R., Goyal, M., van Embden, J. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol **35**:907-14.

Kanduma, E., T. McHugh, T. D., Gillespie, S. H. (2003). Molecular methods for *Mycobacterium tuberculosis* strain typing: a user's guide. J Appl Microbiol **94**:781-91.

Kaye, P. M., Sims, M., Feldmann, M.. (1986). Regulation of macrophage accessory cell activity by mycobacteria. II. In vitro inhibition of Ia expression by *Mycobacterium microti*. Clin Exp Immunol **64**:28-34.

Kaye, P., Feldmann, M. (1986). Regulation of macrophage accessory cell activity by mycobacteria. I. Ia expression in normal and irradiated mice infected with *Mycobacterium microti*. Clin Exp Immunol **64**:20-7.

Kirschner, P., Springer, B., Vogel, U., Meier, A., Wrede, A., Kiekenbeck, M., Bange, F. C., Bottger, E. C. (1993). Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. J Clin Microbiol **31**:2882-89.

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W. H., Hermans, P. W., Martin, C., Palittapongarnpim, P. P., likaytis, B. B., Riley, L. W., Yakrus, M., Musser, J., van Embden, J. D. (1999). Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol **37**:2607-18.

Kremer, K., van Soolingen, D., van Embden, J., Hughes, S., Inwald, J., Hewinson, G., (1998). *Mycobacterium microti*: more widespread than previously thought. J Clin Microbiol **36**:2793-94.

Lalvani, A. (2007). Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest **131**:1898-1906.

Lalvani, A., Brookes, R., Hambleton, S. (1997). Rapid effector function in CD8+ memory T cells. J Exp Med **186**:859-65

Lazarevic, V., Yankura, D. J., DiVito, S. J., Flynn, J. L. (2005). Induction of *Mycobacterium tuberculosis*-specific primary and secondary T-cell responses in interleukin-15-deficient mice. Infect Immun **73**:2910-22.

Lazzarini, L. C., Huard, R. C., Boechat, N. L., Gomes, H. M., Oelemann, M. C., Kurepina, N., Shashkina, E., Mello, F. C., Gibson, A. L., Virginio, M. J., Marsico, A. G., Butler, W. R., Kreiswirth, B. N., Suffys, P. N., Lapa, E. S. J. R., Ho, J. L. (2007). Discovery of a novel *Mycobacterium tuberculosis* lineage that is a major cause of tuberculosis in Rio de Janeiro, Brazil. J Clin Microbiol **45**:3891-3902.

Lee, H. S. N., Cho, S. N., Bang, H. E., Lee, J. H., Bai, G. H., Kim, S. J., Kim, J. D.(2000). Exclusive mutations related to isoniazid and ethionamide resistance among *Mycobacterium tuberculosis* isolates from Korea. Int J Tuberc Lung Dis **4**:441-7.

Liebana, E., Aranaz, A., Mateos, A., Vilafranca, M., Gomez-Mampaso, E. Tercero, J. C., Alemany, J., Suarez, G., Domingo, M., Dominguez, L. (1995). Simple and rapid detection of *Mycobacterium tuberculosis* complex organisms in bovine tissue samples by PCR. J Clin Microbiol **33**:33-6.

Lieberman, M. M., Clements, D. E., Ogata, S., Wang, G., Corpuz, G., Wong, T., Martyak. T., Gilson, L., Coller, B. A., Leung, J., Watts, D. M., Tesh, R. B., Sirin, M., Travassos da Rosa, A., Humphreys, T., Weeks-Levy, C. (2007). Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. Vaccine **25**:414-23.

Lowrie, D. B., Andrew, P. W., Peters, T. J. (1979). Analytical subcellular fractionation of alveolar macrophages from normal and BCG-vaccinated rabbits with particular reference to heterogeneity of hydrolase-containing granules. Biochem J **178**:761-7.

Lowrie, D. B., Jackett, P. S., Ratcliffe, N. A. (1975). *Mycobacterium microti* may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes. Nature **254**:600-2.

Lutze-Wallace, C., Turcotte, C., Glover, G., Cousins, D., Bell, J., Berlie-Surujballi, G., Barbeau, Y., Randall, G. (2006). Isolation of a *Mycobacterium microti*-like organism from a rock hyrax (*Procavia capensis*) in a Canadian zoo. Can Vet J **47**:1011-13.

Mahairas, G. G., Sabo, P. J., Hickey, M. J., Singh, D. C., Stover, C. K. (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol **178**:1274-82.

Manabe, Y. C., Scott, C. P., Bishai, W. R. (2002). Naturally attenuated, orally administered *Mycobacterium microti* as a tuberculosis vaccine is better than subcutaneous *Mycobacterium bovis* BCG. Infect Immun **70**:1566-70.

Mathews, F., Lovett, L., Rushton, S., Macdonald, D. W. (2006). Bovine tuberculosis in cattle: reduced risk on wildlife-friendly farms. Biol Lett **2**:271-4.

McEvoy, C. R., Falmer, A. A., Gey van Pittius, N. C., Victor, T. C., van Helden, P. D., Warren, R. M. (2007). The role of IS6110 in the evolution of *Mycobacterium tuberculosis*. Tuberculosis **87**:393-404.

Moser, I., Prodinger, W. M., Hotzel, H., Greenwald, R., Lyashchenko, K. P., Bakker, D., Gomis, D., Seidler, T., Ellenberger, C., Hetzel, U., Wuennemann, K., Moisson, P. (2008). *Mycobacterium pinnipedii*: transmission from South American sea lion (*Otaria byronia*) to Bactrian camel (*Camelus bactrianus bactrianus*) and Malayan tapirs (*Tapirus indicus*). Vet Microbiol **127**:399-406.

Mostowy, S., Cousins, D., Brinkman, J., Aranaz, A., Behr, M. A. (2002). Genomic deletions suggest a phylogeny for the *Mycobacterium tuberculosis* complex. J Infect Dis **186**:74-80.

Mostowy, S., D. Cousins, Behr., M. A. (2004). Genomic interrogation of the dassie bacillus reveals it as a unique RD1 mutant within the *Mycobacterium tuberculosis* complex. J Bacteriol **186**:104-9.

Neill, S. D., Cassidy, J., Hanna, J., Mackie, D. P., Pollock, J. M., Clements, A., Walton, E., Bryson, D. G. (1994). Detection of *Mycobacterium bovis* infection in skin test-negative cattle with an assay for bovine interferon-gamma. Vet Rec **135**:134-5.

Niemann, S., Richter, E., Dalugge-Tamm, H., Schlesinger, H., Graupner, D., Konigstein, B., Gurath, G., Greinert, U., Rusch-Gerdes, S. (2000). Two cases of *Mycobacterium microti* derived tuberculosis in HIV-negative immunocompetent patients. Emerg Infect Dis **6**: 539-42.

Oelemann, M. C., Diel, R., Vatin, V., Haas, W., Rusch-Gerdes, S., Locht, C., Niemann, S., Supply, P. (2007). Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. J Clin Microbiol **45**:691-97.

Oevermann, A., Pfyffer, G. E., Zanolari, P., Meylan, M., Robert, N. (2004). Generalized tuberculosis in llamas (*Lama glama*) due to *Mycobacterium microti*. J Clin Microbiol **42**:1818-21.

Orme, I. M. (2003). The mouse as a useful model of tuberculosis. Tuberculosis **83**:112-15.

Pai, S., Esen, N., Pan, X., Musser, J. M. (1997). Routine rapid *Mycobacterium* species assignment based on species-specific allelic variation in the 65-kilodalton heat shock protein gene (hsp65). Arch Pathol Lab Med **121**:859-64.

Paige, C., Scholl, D.T., Truman, R.W. (2002). Prevalance and incidence density of *Mycobacterium leprae* and *Typanosma cruzi* infections within a population of wild nine-banded armadillos . Am J Trop Med Hyg **67**:528-32.

Park, H. D., Guinn, K. M., Harrell, M. I., Liao, R., Voskuil, M. I., Tompa, M., Schoolnik, G. K., Sherman, D. R. (2003). Rv3133c/dosR is a transcription factor that mediates the hypoxic response of *Mycobacterium tuberculosis*. Mol Microbiol **48:**833-43.

Parra, A., Garcia, N., Garcia, A., Lacombe, A., Moreno, F., Freire, F., Moran, J., Hermoso de Mendoza, J. (2008). Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis. Vet Microbiol **127:**315-24.

Parsons, L. M., Brosch, R., Cole, S. T., Somoskovi, A., Loder, A., Bretzel, G., Van Soolingen, D., Hale, Y. M., Salfinger, M. (2002). Rapid and simple approach for identification of *Mycobacterium tuberculosis* complex isolates by PCR-based genomic deletion analysis. J Clin Microbiol **40:**2339-45.

Parsons, S., Smith, S. G., Martins, Q., Horsnell, W. G., Gous, T. A., Streicher, E. M., Warren, R. M., van Helden, P. D., Gey van Pittius, N. C. (2008). Pulmonary infection due to the dassie bacillus (*Mycobacterium tuberculosis* complex sp.) in a free-living dassie (rock hyrax-*Procavia capensis*) from South Africa. Tuberculosis **88:**80-3.

Pattyn, S. R., Antoine-Portaels, F., Kageruka, P. Gigase, P. (1970). *Mycobacterium microti* infection in a zoo-llama: *Lama vicugna* (Molina) . Acta Zool Pathol Antwerp **51:**17-24.

Pfyffer, G. E., Brown-Elliott, B. A, Wallace, R. Jr. (2003). *Mycobacterium*: General characteristics, isolation, and staining procedures. In: Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH (eds) Manual of Clinical Microbiology, 8th edn. Washington, District of Columbia, USA. 532–59

Plackett, P., Ripper, J., Corner, L., Small, A., K.de Witte, K., Melville, L., Hides, S., Wood, P. R. (1989). An ELISA for the detection of anergic tuberculous cattle. Aust Vet J **66**:15-9.

Plikaytis, B. B., Plikaytis, B. D., Yakrus, M. A., Butler, W. R., Woodley, C. L., Silcox, V. A., Shinnick, T. M. (1992). Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis*, by gene amplification and restriction fragment length polymorphism analysis. J Clin Microbiol **30**:1815-22.

Pollock, J. M., Andersen, P. (1997). The potential of the ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J Infect Dis **175**:1251-4.

Pollock, J. M., Girvin, R. M., Lightbody, K. A., Clements, R. A., Neill, S. D., Buddle, B. M., Andersen, P. (2000). Assessment of defined antigens for the diagnosis of bovine tuberculosis in skin test-reactor cattle. Vet Rec **146**:659-65.

Pollock, J. M., Welsh, M. D., McNair, J. (2005). Immune responses in bovine tuberculosis: towards new strategies for the diagnosis and control of disease. Vet Immunopathol **108**:37-43.

Prat, M., Gribaudo, G., Comoglio, P. M., Cavallo, G., Landolfo, S. (1984). Monoclonal antibodies against murine gamma interferon. Proc Natl Acad Sci USA **81**:4515-9.

Pritchard, D. G. (1988). A century of bovine tuberculosis 1888-1988: conquest and controversy. J Comp Pathol **99**:357-99.

Rhoades, E. R., Frank, A. A., Orme, I. M. (1997). Progression of chronic pulmonary tuberculosis in mice aerogenically infected with virulent *Mycobacterium tuberculosis*. Tuber Lung Dis **78**:57-66.

Rhodes, S. G., Palmer, N., Graham, S. P., Bianco, A. E., Hewinson, R. G., Vordermeier, H. M. (2000). Distinct response kinetics of gamma interferon and interleukin-4 in bovine tuberculosis. Infect Immun **68**:5393-400.

Riley, L. W. (2004). Molecular Epidemiology of Infectious Diseases. Principles and Practices. AMS Press, American Society for Microbiology: Washington DC, USA.

Robb-Smith, A. H. T. (1946). Notes on the morphology of infection by the vole acid-fast bacillus. MRC Rep **259:**43-48.

Roring, S., Scott, A. N., Glyn Hewinson, R., Neill, S. D., Skuce, R. A. (2004). Evaluation of variable number tandem repeat (VNTR) loci in molecular typing of *Mycobacterium bovis* isolates from Ireland. Vet Microbiol **101:**65-73.

Roring, S., Scott, A., Brittain, D., Walker, I., Hewinson, G., Neill, S., Skuce, R. (2002). Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J Clin Microbiol **40:**2126-33.

Rothel, J. S., JonesCorner, L. A., Cox, J. C., Wood, P. R. (1990). A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. Aust Vet J **67**:134-7.

Samuelson, J. (1999). Leprosy. In: Pathological basis of disease. Eds: Cotran, R. S., Kumar, V., Collins, T. Philadelphia, Saunders, USA. 385-87.

Sansila, A., Hongmanee, P., Chuchottaworn, C., Rienthong, S., Rienthong, D., Palittapongarnpim, P. (1998). Differentiation between *Mycobacterium tuberculosis* and *Mycobacterium avium* by amplification of the 16S-23S ribosomal DNA spacer. J Clin Microbiol **36:**2399-03.

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P.D., Nathan, C., Schoolnik, G. K. (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: Insights into the phagosomal environment. J Exp Med **198:**693-704.

Schountz, T., Calisher, C. H., Richens, C, H., T. R. Rich, A. A., Doty, J. B., Hughes, M. T., Beaty, B. J. (2007). Rapid field immunoassay for detecting antibody to Sin Nombre virus in deer mice. Emerg Infect Dis **13**:1604-7.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N., Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol **51**:873-84.

Senna, S. G., Battilana J., Costa, J. C., Silva, M. G., Duarte, R. S., Fonseca, L. S., Suffys, P. N., Bogo, M. R. (2008). Sequencing of hsp65 gene for identification of *Mycobacterium* species isolated from environmental and clinical sources in Rio de Janeiro, Brazil. J Clin Microbiol **46**:3822-5.

Shin, N. S., Kwon, S. W., Han, D. H., Bai, G. H., Yoon, J., Cheon, D. S., Son, Y. S., Ahn, K., Chae, C., Lee, Y. S. (1995). *Mycobacterium tuberculosis* infection in an orangutan (*Pongo pygmaeus*). J Vet Med Sci **57**:951-53.

Siguier, P., Filee, J., Chandler, M. (2006). Insertion sequences in prokaryotic genomes. Curr Opin Microbiol **9**:526-31.

Smith, N. (1960). The 'Dassie' bacillus. Tubercle, **41**:203-12.

Smith, N.H., Crawshaw, T., Parry, J., Birtles, R.J. (2009). *Mycobacterium microti*: More diverse than previously thought. J Clin Microbiol **47**:2551-9.

Smith, N. H., Dale, J., Inwald, J., Palmer, S., Gordon, S. V., Hewinson, R. G., Smith, M. J. (2003). The population structure of *Mycobacterium bovis* in Great Britain. Proc Natl Acad Sci USA. **100**:15271-5.

Smith, N. H., Gordon, S. V., de la Rua-Domenech, R., Clifton-Hadley, R.S., Hewinson, R. G. (2006). Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis*. Nat Rev Microbiol **4**:670-81.

Smittipat, N., Palittapongarnpim, P. (2000). Identification of possible loci of variable number of tandem repeats in *Mycobacterium tuberculosis*. Tub Lung Dis **80**:69-74.

Snider, D. E., Jr. (1982). The tuberculin skin test. Am Rev Respir Dis **125**:108-18.

Soini, H., J. M. Musser (2001). Molecular diagnosis of mycobacteria. Clin Chem **47**:809-14.

Sola, C., Filliol, I., Legrand, E., Lesjean, S., Locht, C., Supply, P., Rastogi, N. (2003). Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol **3**:125-33.

Sola, C., Filliol, I., Legrand, E., Mokrousov, I., Rastogi, N. (2001). *Mycobacterium tuberculosis* phylogeny reconstruction based on combined numerical analysis with IS1081, IS6110, VNTR, and DR-based spoligotyping suggests the existence of two new phylogeographical clades. J Mol Evol **53**:680-9.

Spitalny, G. L., Havell, E. A. (1984). Monoclonal antibody to murine gamma interferon inhibits lymphokine-induced antiviral and macrophage tumoricidal activities. J Exp Med **159**:1560-5.

Sreedevi, B., Krishnappa. (2004). Standardization of poly merase chain reaction for the detection of mycobacterium tuberculosis complex organisms from bovines . Ind J Anim Sci **74:**1120-23.

Sreevatsan, S., Pan, X., Stockbauer, K. E., Connell, N. D., Kreiswirth, B. N., Whittam, T. S., Musser, J. M. (1997). Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci USA **94**:9869-74.

Stanley. S. A., Raghavan, S., Hwang, W. W., Cox, J. S. (2003). Acute infection and macrophages subversion by *Mycobacterium tuberculosis* require a specialized secretion system. Proc Nat Acad Sci USA **100**:13001-6

Sula, L., Radkovsky, I. (1976). Protective effects of *M. microti* vaccine against tuberculosis. J Hyg Epidemiol Microbiol Immunol **20**:1-6.

Supply, P., Mazars, E., Lesjean, S., Vincent, V., Gicquel, B., Locht, C. (2000). Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol, **36**:762-71.

Sweeney, F.P., Courtenay, O., Hibberd, V., Hewinson, R.G., Reilly, L.A., Gaze, W.H., Wellington, E.M. (2007). Environmental monitoring of *Mycobacterium bovis* in badger feces and badger sett soil by real-time PCR, as confirmed by immunofluorescence, immunocapture, and cultivation. Appl Environ Microbiol **73**:7471-3.

Taylor, C., Jahans, K., Palmer, S., Okker, M., Brown, J., Steer, K. (2006). *Mycobacterium microti* isolated from two pigs. Vet Rec **159**:59-60.

Taylor, G. M., Worth, D. R., Palmer, S., Jahans, K., Hewinson, R. G. (2007). Rapid detection of *Mycobacterium bovis* DNA in cattle lymph nodes with visible lesions using PCR. BMC Vet Res **3**:12.

Taylor, M. J., Hughes, M. S., Skuce, R. A., Neill, S. D. (2001). Detection of *Mycobacterium bovis* in bovine clinical specimens using real-time fluorescence and fluorescence resonance energy transfer probe rapid-cycle PCR. J Clin Microbiol **39**:1272-8

Tekaia, F., Gordon, S.V., Garnier, T., Brosch, R., Barrell, B.G., Cole, S.T. (1999). Analysis of the proteome of *Mycobacterium tuberculosis* in silico. Tuber Lung Dis **79**:329-42.

Tell, L. A., Foley, J., Needham, M. L., Walker, R. L. (2003). Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three *Mycobacterium* spp. that affect birds. Avian Dis **47**:1486-90.

Thierry, D., Cave, M. D., Eisenach, K. D., Crawford, J. T., Bates, J. H., Gicquel, B., Guesdon, J. L. (1990). IS6110, an IS-like element of *Mycobacterium tuberculosis* complex. Nucleic Acids Res **18**:188.

Thwaites, G., Caws, M., Chau, T. T., D'Sa, A., Lan, N. T., Huyen, M. N., Gagneux, S., Anh, P. T., Tho, D. Q., Torok, E., Nhu, N.T., Duyen, N.T., Duy, P.M., Richenberg, J., Simmons, C., Hien, T. T., Farrar, J. (2008). Relationship between *Mycobacterium tuberculosis* genotype and the clinical phenotype of pulmonary and meningeal tuberculosis. J Clin Microbiol **46**:1363-68.

Torres, L., Arazo, P., Blas Perez, J., del Pilar Amador, M., Antonia Lezcano, M., Jose Revillo, M., Bautista Garcia-Moya, J. (2000). Resistance of *Mycobacterium tuberculosis* in Zaragoza, Spain (1993-1997) and related factors. Med Clin (Barc) **115**:605-9.

Tsukamura, M. (1985). Numerical classification of *Mycobacterium nonchromogenicum, Mycobacterium terrae* and *Mycobacterium triviale*. Kekkaku **60**:265-5.

Tuberculosis vaccine trails committee. (1956). BCG and vole bacillus vaccine in the preventions of tuberculosis in adelosence. Br. Med J. 413-27.

van Embden, J. D., Cave, M. D., Crawford. J. T., Dale, J. W., Eisenach, K. D., Gicquel, B., Hermans, P., Martin, C., McAdam, R., Shinnick, T. M. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting recommended for a standarized methodology. J Clin Microbiol **33**:3234-8.

van Embden, J. D., van Gorkom, T., Kremer, K., Jansen, R., van Der Zeijst, B.A., Schouls, L. M. (2000). Genetic variation and evolutionary origin of the direct repeat locus of *Mycobacterium tuberculosis* complex bacteria. J Bacterial **182**:2393-401.

van Soolingen, D., Qian,de Hass, P. E, Douglas, J. T., Traore, H., Portaels, F., Qian, H. Z, Enkhsaikan, D., Nymadawa, P., van Embden, J. D. (1995). Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J Clin Microbiol **33**:3234-8.

van Soolingen, D., van der Zanden, A. G., de Haas, P. E., Noordhoek, G. T., Kiers, A., Foudraine, N. A., Portaels, F., Kolk, A. H., Kremer, K., van Embden, J. D. (1998). Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. J Clin Microbiol **36:**1840-45.

Visschedijk,J., van de Broek ,J,.Eggens .H,. Lever,P,. van Beers,S., Klatser,P. (2000). *Mycobacterium leprae* – millennium resistance . Leprosy control on the threshold of a new era. Trop Med Int Health **5**:388-99.

Vitale, F., Capra, G., Maxia, L., Reale, S., Vesco, G., Caracappa, S. (1998). Detection of *Mycobacterium tuberculosis* complex in cattle by PCR using milk, lymph node aspirates, and nasal swabs. J Clin Microbiol **36**:1050-5.

Vordermeier, H. M., Cockle, P. C., Whelan, A., Rhodes, S., Palmer, N., Bakker, D., Hewinson, R.G. (1999). Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. Clin Diagn Lab Immunol **6:**675-82.

Vordermeier, M., Goodchild, A., Clifton-Hadley, R., de la Rua, R. (2004). The interferon-gamma field trial: background, principles and progress. Vet Rec **155**:37-8.

Wagner, J. C., Bokkenheuser, V. (1961). The mycobacterium isolated from the dassie *Procavia capensis* (Pallas). Tubercle **42:**47-56.

Wagner, J. C., Buchanan, G., Bokkenheuser, V., Leviseur, S. (1958). An acid-fast bacillus isolated from the lungs of the Cape hyrax, *Procavia capensis* (Pallas). Nature **181:**284-85.

Walker, L., Lowrie, D. B. (1981). Killing of *Mycobacterium microti* by immunologically activated macrophages. Nature **293**:69-71.

Wards, B. J., Collins, D. M., de Lisle, G. W. (1995). Detection of *Mycobacterium bovis* in tissues by polymerase chain reaction. Vet Microbiol **43:**227-40.

Waters, W. R., Nonnecke, B. J., Palmer, M. V., Robbe-Austermann, S., Bannantine, J. P., Stabel, J. R., Whipple, D. L., Payeur, J. B., Estes, D. M., Pitzer, J. E., Minion, F. C. (2004). Use of recombinant ESAT-6:CFP-10 fusion protein for differentiation of infections of cattle by *Mycobacterium bovis* and by *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. Clin Diagn Lab Immunol **11**:729-35.

Wayne, L. G., Kubica, G. P. (1986). The Mycobacteria. The Williams and Wilkins Co., Baltimore, MD, USA.

Wells, A. Q. (1937). Lancet **1**:1221.

Wells, A. Q. (1953). *Mycobacterium-tuberculosis* var *muris*. J Gen Microbiol **9**:149-159.

Wells, A. Q., Wylie, J. A. H. (1954). Vaccination against tuberculosis with the vole bacillus. Brit Med Bull **10**:96-100.

Wells, A.Q. (1946). The murine type of tubercle (the vole acid fast bacillus). Special Report Series in Medicine, Council of London **259**:1-48

Welsh, M. D., Cunningham, R. T., Corbett, D. M., Girvin, R. M., McNair, J., Skuce, R. A., Bryson, D. G., Pollock, J. M. (2005). Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunol **114**:101-11.

Whelen, A. C., Felmlee, T. A., Hunt, J. M., Williams, D. L., Roberts, G. D., Stockman, L., Persing, D. H. (1995). Direct genotypic detection of *Mycobacterium tuberculosis* rifampin resistance in clinical specimens by using single-tube heminested PCR. J Clin Microbiol **33**:556-61.

Wilson, S. M., McNerney, R., Nye, P. M., Godfrey-Faussett, P. D., Stoker, N. G., Voller, A. (1993). Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. J Clin Microbiol, **31**:776-82.

Winek, J., Rowinska-Zakrzewska, E., Demkow, U., Szopinski, J., Szolkowska, M., Filewska, M., Jagodzinski, J., Roszkowski-Sliz, K. (2008). Interferon gamma production in the course of *Mycobacterium tuberculosis* infection. J Physiol Pharmacol **59**:751-9.

Wood, P. R., Corner, L. A., Plackett, P. (1990). Development of a simple, rapid in vitro cellular assay for bovine tuberculosis based on the production of gamma interferon. Res Vet Sci **49**:46-9

Wood, P. R., Corner, L. A., Rothel. J. S., Baldock., C., Jones, S. L., Cousins, D. B., McCormick, B. S., Francis. B. R., Creeper, J., Tweedle, N. F. (1991). Field comparison of the interferon-gamma assay and intradermal tuberculin test for the diagnosis of bovine tuberculosis. Austral Vet J **68:**286-90.

Young, J. S., Gormley, E., Wellington, E. M. (2005). Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. Appl Environ Microbiol **71**:1946-52.

Zhang, Z. Q., Ishaque, M. (1997). Evaluation of methods for isolation of DNA from slowly and rapidly growing mycobacteria. Int J Lepr Other Mycobact Dis **65:**469-76.