Systemic *Mycobacterium avium* subspecies *paratuberculosis* infection in sheep.

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Abstract

The systemic infection of organs and skeletal muscle outside the alimentary tract with *Mycobacterium avium* subspecies *paratuberculosis* (Map) has sparingly been mentioned in the many scientific studies undertaken in sheep, yet within the past decade a zoonotic association has been proposed. The occurrence of systemic Map infection at the time of slaughter might enable this organism to be present in food products, such as meat, destined for human consumption, creating a potential link to public health and may therefore attract some attention by the meat industry. There have been very few studies investigating whether meat has potential to expose humans to Map. With this lack of information, it is difficult for the meat industry to make informed decisions in the event that public perception establishes a link with Crohn's disease. Chapter one provides a brief history of Map infection in ruminants and suggests there may be a need to identify steps that could be implemented to mitigate human exposure to Map. The aims for this thesis therefore were to i) determine whether skeletal muscle from naturally infected animals provides a source of Map for humans, ii) provide information on systemic Map infection in sheep, identifying classes of stock that may pose a risk for exposure iii) develop a histological diagnostic test for quantifying the cost of systemic Map infection in sheep with potential use in therapeutic efficacy studies, and iv) provide a potential means to mass screen sheep at time of slaughter using real time spectroscopy to identify systemically infected animals.

Chapter two reviews the source of Map, transmission pathways and subsequent availability of modern diagnostic tests for identifying sheep infected with this organism. There is a lack of published information on systemic Map infection, with little known about how this event develops, how the immune system reacts when Map bacteraemia occurs, whether systemic

Map infection has a cost to production and whether quantification of this cost can be assessed with currently available diagnostic tests.

The aim of Chapter three was to determine whether skeletal muscle from ewes with clinical Johne's disease contained Map and therefore provided a potential source of Map for humans. Fifty one mixed-age, low body condition score ewes (1.5/5), from a farm where clinical Johne's disease had been diagnosed, were necropsied. This included 48 ewes with Map infection confirmed by ileal BACTEC radiometric culture and 21 with clinical Johne's disease confirmed by ileal histopathology. In 18 ewes with clinical Johne's disease, Map was found in the culture of blood (n=13), blood and muscle (n=10) and muscle (n=5). In ewes without clinical Johne's disease, Map was found in 5/30 animals including muscle (n=4) and blood (n=1). It was concluded that meat from ewes with clinical Johne's disease is likely to contain Map and suggested that systemic Map infection may also occur in sheep without clinical disease when managed in direct contact with clinically affected ewes shortly before slaughter.

The presence of Map within skeletal muscle was further investigated in Chapter four with 24 healthy mixed age ewes selected from one farm, which were not in contact with clinically affected ewes. Ileal and mesenteric lymph node cultures identified Map infection in 12/24 ewes. All other tissues and faeces were culture negative, and only 1/24 animals sero-converted. In flocks where Map is present, it appears that up to 50% of animals may be latently infected. Lack of positive culture from blood and muscle samples in latently infected sheep suggests that meat from healthy sheep may not be a source of human exposure to Map.

In New Zealand, the current measure to mitigate human exposure to Map from meat products is the identification of clinically affected sheep prior to slaughter through ante-mortem inspection with emaciated animals rejected at time of slaughter and processed as pet food.

However, this screening process is non-specific with many different causes of emaciation. Currently there are no legal requirements or recommendations from the meat industry for the downgrading of meat from carcases with macroscopic signs of clinical Johne's disease and, as such, meat from these sheep enters the human food chain. Identifying sheep with systemic Map infection is problematic, with diagnosis requiring solid or liquid media culture of Map or polymerase chain reaction (PCR) to identify Map specific DNA. These diagnostic tests are expensive, time consuming and require a high level of expertise. They are therefore unlikely to be adopted by the meat industry as a screening tool for systemic Map infection in sheep. With the aim to develop a diagnostic tool that is relatively quick, simple and cheap, 126 mixed age ewes in poor body condition were euthanised as described in Chapter five and their Johne's disease status determined through histopathology and Ziehl Neelsen stain of the ileum and mesenteric lymph nodes. Sixty ewes were differentiated histopathologically with 51 clinically affected including Type 3b (n=40) and 3c (n=11) and nine not clinically affected with Type 1 (n=5), Type 2 (n=3) and Type 3a (n=1) ileal lesions. Hepatic epithelioid macrophage micro-granulomas (HEM) were observed only in ewes with Type 3b or 3c ileal lesions, all of which were ELISA positive. When present, HEM were in equal densities in liver section and biopsy samples. The sensitivity and specificity for liver histopathology (section or biopsy) for predicting clinical OJD was 96% (95% CI, 87-99%) and 100% (95% CI, 95-100%), respectively, and Cohen's Kappa had an almost perfect level of agreement between HEM formation, ileal pathology and ELISA sero-positivity. This study determined that the presence of HEM provided a surrogate measure of ileal pathology, identified ewes with clinical Johne's disease, and that biopsy samples and post mortem sections were equally suitable for the diagnosis of HEM.

Encouraged by the predictive quality of HEM in Chapter five, it was hypothesised that the identification of HEM from biopsies may provide a method to follow the progression of Map

infection through serial sampling and to quantify the production cost of systemic Map infection. The longitudinal challenge study in Chapter six utilised the identification of HEM as an indicator of systemic Map infection in naïve lambs orally challenged with 1 x 10^9 organisms on ten occasions over 30 days. The presence of HEM was related to live weight gain, body condition score, development of clinical disease or occurrence of self-cure (recovery), and ELISA serology All challenged lambs developed HEM, a higher density of HEM was associated with increased ELISA S/P ratios with a Cohen's kappa substantial level of agreement, and mean weight loss (-2.03kg) from 51 to 154 days post challenge with an almost perfect level of agreement. Thereafter, lower weight gain led to a mean body weight difference of -8kg at 195days compared to non-challenged lambs. Four challenged lambs had to be euthanised due to clinical OJD. After this period, the HEM density and ELISA S/P ratios declined, growth rates increased in the challenged lambs up to 482 days after which no HEM were detected and growth rates were equal between challenged and unchallenged groups. The challenged lambs failed to regain equivalent weights over the 820 days being 11kg lighter at the end of the study despite having equal body condition scores. The challenged lambs were smaller than the unchallenged lambs both in body height and length with multivariate ANOVA analysis determining the post mortem mean skeletal measurements of the poll to rump length and metacarpal/meta-tarsal bones being 4% and 5% shorter, respectively. There were no positive ELISA blood samples or histopathological lesions in any tissues sampled at necropsy from both groups of lambs at the end of the study, suggesting complete cure of the surviving challenged lambs. The findings demonstrated i) that artificial challenge can cause systemic Map infection, ii) systemic infection results in negative growth rates and a loss of body condition, iii) and in addition to the period of retarded growth losses occur from death of some lambs (4/18), iv) that the temporary poor weight gain impacted on the final weight, and v) that recovery to systemic Map infection

appears to occur in survivors of acute disease. Moreover, it was postulated that the identification of HEM from serial liver biopsies may have the potential to determine the therapeutic efficacy of new anti-mycobacterial drugs (such as thalidomide, Appendix one) or vaccines for preventing systemic Map infection.

Chapter seven revisits the histopathological findings described in Chapter five, expanding from the microscopic visual identification of HEM to utilising spectroscopy and hyperspectral image analysis. The aims of this final study included identifying whether a spectral signature for skeletal muscle or liver exists in sheep with Johne's disease and developing an algorithm that can identify the presence of systemic Map infection in sheep. Ninety five mixed aged ewes, of low body condition score from nine farms were euthanised and OJD was confirmed by histopathology in 10 animals. The liver and transected longissimus dorsi muscle were scanned using a visible light to near infrared (Vis-NIR) detector as well as 200 lamb livers from a slaughter house. The histological identification of HEM was used as a surrogate measure of systemic Map infection with HEM recorded in the 10 ewes with Johne's disease and none of the 85 ewes without or the 200 lamb livers. There was no histopathological or hyperspectral differences identified for the transected longissimus dorsi muscle in the 95 ewes. However a a computer generated algorithm identified a hyperspectral signature for liver tissue that when applied, blind to the Johne's disease status of the ewes was able to differentiate all 10 animals with Johne's disease from the 85 ewes and 200 lambs without. This pilot study suggests that spectroscopy may have potential to be a useful real time tool for the identification of sheep with systemic Map infection at the time of slaughter.

In conclusion, disseminated Map infection does occur in sheep with clinical OJD, and meat from these animals can be a source of Map for humans. Meat from healthy sheep or sheep without clinical OJD does not appear to expose consumers to Map. The identification of

HEM in liver biopsies has diagnostic value for identifying sheep with clinical OJD. In naturally infected sheep, HEM only appear when Map infection has progressed to clinical OJD. As opposed to high–dose artificial challenge, systemic Map infection under natural challenge conditions appears to require ileal pathology, suggesting different mechanisms for the occurrence of systemic infection in these two challenge types. Examination of serial liver biopsies and the identification of HEM has enabled the investigation of the production loss due to temporary progression and subsequent resolution of Map infection. The final study in this thesis has tested proof of concept for a new real time diagnostic test that has potential to mass screen sheep within abattoirs at point of slaughter using spectroscopy and hyperspectral analysis. However further research is required to validate this spectroscopic test.

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I would like to thank the BBC for being the catalyst for initiating this entire thesis, which, in 2002, while I was living in England, aired a programme on the use of thalidomide for the successful treatment of the Mycobacterial disease erythema nodosum leprosum or leprosy in humans.

Last but not least, thanks to my family who have been patient, have had no use of the home computer for the past nine months and have not been out in the weekends as I have put together the finishing touches to this project.

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Chapter One

General Introduction

Delia

1.1 Introduction – A brief history of *Mycobacterium avium* subsp. *paratuberculosis*

In the 1820's an emaciating disease of unknown origin was recorded that affected cattle across Europe. Some seventy years later in 1894, Frothingham and Johne described and published their findings regarding an enteric wasting disease in cattle that involved thickened and inflamed intestines, although they considered it to be a form of bovine tuberculosis (Johne and Frothingham 1895). Further confirmation that this was not tuberculosis and a separate enteric disease was determined by Bang in 1906 who satisfied Kochs' postulates and replicated the disease in calves fed intestinal scrapings from an affected cow (Bang 1906). Bang also noted that infected animals responded to avium but not bovine purified protein derivative (PPD) tuberculin coining the term 'para' or resembling tuberculosis and established the use of the first diagnostic tests for identifying animals with this disease. In 1910, Twort and Holth, two independent researchers developed methods to isolate the organism naming it *Mycobacterium enteritidis chronicaea pseudotuberculosis* (Nielsen 2001). In 1923 the organism was officially recognised as *Mycobacterium paratuberculosis*. At the same time control measures were being established in attempts to control the spread of *Mycobacterium paratuberculosis* throughout the world and the first reference to *Mycobacterium paratuberculosis* posing a potential zoonotic threat to humans was proposed by a Scottish doctor, Dalziel, who stated that the granulomatous lesions found in cattle were similar to that in humans and that the two diseases may have the same aetiology (Dalziel 1923). In 1932, Crohn, Ginzberg and Oppenheimer reclassified the pathological lesions found in humans that were broadly known as regional ileitis to the current nomenclature of Crohn's disease, although, at the time a Mycobacterial aetiology was not considered (Crohn *et al*, 1932). The disease in domesticated ruminants throughout the world is now recognised

as Johne's disease with *Mycobacterium avium* subspecies *paratuberculosis* (Map) the aetiological agent (Chiodini 1993; Clarke 1997; Begg and Whittington 2008).

Where Map is present in a flock, categories of infection include sheep that are either uninfected, or infected without any ileal pathology (latently infected), or infected with ileal pathology (Perez *et al,* 1996). Sheep with ileal pathology are classified into different Types. Animals with Type 1 and 2 ileal lesions are referred to as being sub-clinically affected, with no overt signs of clinical disease and have normal production parameters recorded. These lesions may not progress to cause clinical disease and can be found in healthy sheep. Type 3a ileal lesions are also considered sub-clinical, although once these develop, progression to clinical disease always occurs (Perez *et al,* 1999). Type 3a lesions progress to either multibacillary (many AFO) Type 3b or paucibacillary (low numbers of AFO) Type 3c ileal lesions. Johne's disease is defined in this thesis as the period when Type 3b or 3c ileal lesions have developed.

Scientific interest in Map currently arises from both the pastoral sector, where production losses appear to only occur from sheep with clinical disease (Thompson *et al,* 2002; Morris *et al,* 2006), and more recently, the public health sector, with a systematic meta-analysis and epidemiological review demonstrating a significant and specific zoonotic association (Feller *et al,* 2007; Uzoigwe *et al,* 2007; Behr *et al,* 2008). Human exposure to Map comes from many different sources, with Map intrinsically linked with agriculture and subsequent contamination of the environment (Whittington *et al,* 2004), waterways (Pickup 2005,2006) and animal products destined for the food chain such as dairy (Elthoth *et al,* 2009; Millar *et al,* 1996; Stephan *et al,* 2007) and meat (Manning *et al,* 2003; Mutharia *et al,* 2010; Reddacliff *et al,* 2010; National Advisory Committee on Microbial Criteria for Foods Review: 2010; Gill *et al,* 2011).

Traditionally, Map is considered to have an affinity for the alimentary tract, associated with ileal and local mesenteric lymphatic pathology. However, active shedding of viable Map from the mammary gland into milk has been well recorded in sheep, goats and cattle (Sweeney *et al,* 1992; Grant *et al,* 2001; Manning *et al,* 2003; Singh and Vihan 2004; Grant 2006; Slana *et al,* 2008) and provides evidence of the occurrence of systemic Map infection in healthy animals, providing a route of exposure for humans from the consumption of these products. Systemic Map infection may also culminate in the infection of other organs and tissues distant to the alimentary tract such as the liver and skeletal muscle, providing another potential source of Map infection for human exposure (Buergelt *et al,* 1978; Juste *et al,* 1994, Burrells *et al,* 1998; Gwozdz *et al,* 2000; Nelli *et al,* 2008; Reddacliff *et al,* 2010). There is little data available on the prevalence of Map infection within meat products, with Map recorded in the gastrocnemius muscle of sheep and cattle with Johnes disease (Nelli *et al,* 2008), cattle diaphragm (Alonso-Hearn *et al,* 2010), and in an Australian study where Map was cultured from skeletal muscle in cull ewes (Reddacliff *et al,* 2010). The ability to identify sheep with active systemic Map infection may become important should the public become aware of viable Map organisms within food products and the recognised epidemiological zoonotic association with Map. The maintenance of the national food safety record and that of the public perception on how safe meat is to consume is paramount (Eltholth *et al,* 2009; National Advisory Committee on Microbial Criteria for Foods 2010; Gill *et al,* 2011). Consumers, already wary of food safety issues arising through food scares such as Bovine Spongiform Encephalopathy in the United Kingdom throughout the 1980's, and more recently from Shiga toxin-producing *Escherichia coli* O157:H7 (Doyle *et al,* 2006) are readily influenced and have easy access to public media and potential (mis)-information. Worldwide awareness of this issue already exists, with a systematic review concluding there is likelihood of human exposure to Map from contaminated / infected dairy or meat products

at point of retail sale (Eltholth *et al*, 2009; Gill *et al*, 2011). This is further supported by another independent American review that concluded the fundamental risk of human exposure to Map comes not only from the environment but also from contaminated food, including milk and meat, and that the cattle farming sector is a major contributor (National Advisory Committee on Microbial Criteria for Foods Review: 2010). This review suggested that the current diagnostic tests were limited and inadequate to prevent this exposure and that further research is required to develop new diagnostic tests that may reduce human exposure to Map from food and that mitigating this risk could be achieved by controlling the shedding of Map from cattle and thereby reducing the prevalence of Map within the environment and in other domesticated livestock. One conclusion from this committee included the encouragement of further research be undertaken to identify further steps that could be taken to further reduce the risk of human exposure to Map from food and that the meat and dairy industries needed to take a pro-active stance on mitigating any potential negative effects should systemic Map infection become public knowledge (National Advisory Committee on Microbial Criteria for Foods Review: 2010;).

There is currently little information available for the identification of sheep where systemic Map infection is actively occurring and as such the mechanism for the establishment of systemic Map infection and the prevalence of Map infection in naturally infected sheep is unknown.

The aims of this study were to; 1) Determine whether skeletal muscle from sheep is infected with Map; 2) Identify which class of livestock poses a risk for human exposure; 3) Develop a diagnostic test, through hepatic histopathology, that can identify these animals providing a potential means to quantify the therapeutic efficacy of drugs such as thalidomide for controlling or preventing systemic Map infection; 4) Apply this diagnostic test to a longitudinal artificial challenge study to establish a method to quantify the effects of systemic

Map infection on production parameters such as live weight and body condition in sheep and 5) Develop a new real time diagnostic test that can identify sheep with Johne's disease at time of slaughter using hyperspectral analysis.

1.2 Thesis Organisation

This thesis consists of a general introduction, six papers, four already published in peer reviewed journals, a general discussion and conclusion. Chapter one includes the introduction, thesis organisation and author published papers from this thesis. Chapter two includes the literature review and the rational for the research undertaken. Chapters three and four investigate the occurrence of systemic Map infection within the hind limb skeletal muscle of thin cull ewes, both with and without clinical Johne's disease, and healthy mixed aged ewes from the same farm, recording the likelihood of human exposure to this organism from consumption of meat from these two different classes of stock. These two chapters identify a need to mitigate this risk and a need to develop a new, rapid and affordable real time diagnostic test to identify ewes with systemic Map at time of slaughter. Chapter five explores the development of such a diagnostic test encompassing liver biopsy histopathology and the identification of Map specific hepatic epithelioid macrophage micro-granulomas (HEM). Chapter six explores histopathological uses of HEM, as a surrogate measure for the occurrence of systemic Map infection, in a longitudinal intervention study determining the occurrence of systemic Map infection in artificially challenged lambs, the time line for HEM formation and resolution, the relationship between the humoral immune response and systemic Map infection, resolution and recovery from systemic Map infection and the cost of this on production parameters such as live weight and body condition in sheep. This chapter further questions whether artificial challenge studies truly represent natural infection with

Map and suggests this diagnostic test has potential for use in therapeutic efficacy studies with the identification of HEM enabling comparison to be made between animals with and without systemic Map infection and the effectiveness of an anti-mycobacterial drug or vaccine at preventing this in sheep. Appendix one continues this theme with information from human clinical trials identifying thalidomide as a likely anti-pro-inflammatory cytokine therapeutic candidate for use in sheep. Currently there is no data available on the pharmacokinetic properties of thalidomide in this species and this is addressed here with the elimination half-life ($T_{1/2}$), maximum plasma concentration (Cmax) and the time to reach Cmax (Tmax) determined. This is the first data set and chromatogram ever published for use of this drug in ruminants and now enables a protocol to be developed for the evaluation of the therapeutic effectiveness of thalidomide on systemic Map infection in sheep. Chapter seven re-visits the food safety issue of systemic Map infection of skeletal muscle in sheep and examines the use of hyperspectroscopy to evaluate whether a hyperspectral signature exists for either the liver or skeletal muscle. It is proposed that if an algorithm can be developed to identify sheep with Johne's disease then there may be potential for this to be applied at time of slaughter using simple spectral imaging cameras. This would enable sheep with positive signatures to be removed from the slaughter line for further inspection. Chapter eight provides a general discussion, discusses limitations on these studies and provides some suggestions for future research.

1.3 Publications

Smith SL, West DM, Wilson PR, Heuer C, de Lisle GW, Collett MG, Chambers JP. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in skeletal muscle and blood of ewes from a New Zealand sheep farm. *New Zealand Veterinary Journal* 59(5), 240–243, 2011, (Chapter Three).

Smith SL, West DM, Wilson PR, de Lisle GW, Collett MG, Heuer C, Chambers JP. The prevalence of disseminated *Mycobacterium avium* subsp. *paratuberculosis* infection in tissues of healthy ewes from a New Zealand farm with Johne's disease present. *New Zealand Veterinary Journal,* 61 (1), 41-44, 2012, (Chapter Four).

Smith SL, Wilson PR, Collett MG, Heuer C, West DM, Stevenson M, Chambers JP. Liver biopsy histopathology for diagnosis of Johne's disease in sheep. *Veterinary Pathology*, 51 (5), 915-918, 2014, (Chapter Five).

Smith SL, Singh P, Harding D, Lun D, Chambers JP. Thalidomide pharmacokinetics in sheep. *New Zealand Veterinary Journal*, 64(4), 238-242, 2016, (Appendix One).

Chapter Two

Literature Review

2.1 Nomenclature

Mycobacteria belong to the bacterial phylum Actinobacteria, suborder Corynebacterineae, family Mycobacteriaceae and genus *Mycobacterium*, forming part of the *Mycobacterium avium* complex (MAC) and the *Mycobacterium avium* species with Map being a subspecies of this group which includes *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis* and *M. avium* subsp. *silvaticum*. The *Mycobacterium* genus consists of over 120 different species and these are classified into three main groups including the *M. tuberculosis* complex, the non-tuberculosis group and the *M. leprae* group. *Mycobacterium avium* subsp. *paratuberculosis* belongs to the non-tuberculosis group and genetically shares 99% of its DNA homology with *M. avium* subsp. *avium* with identical ribosomal RNA sequences (Van der Giessen *et al,* 1992). The insertion sequences IS900 and IS1311 are Map specific and occur as multiple copies (polymorphism) within the genome (Collins *et al,* 1990; Vary *et al,* 1990). Initially Map was classified as either the ovine (S) or bovine (C) strain type with each differentiated by genetic DNA typing using polymerase chain reaction (PCR) or restriction fragment length polymorphism (RFLP) with the insertion sequences IS1311 or IS900 respectively (Collins *et al,* 1990 and 2002; Whittington *et al,* 1998a; Marsh *et al,* 1999 and 2006). However, this initial classification of Map failed to adequately identify the host animal with misleading results. In New Zealand and Australia, S strain types predominantly affect sheep (Whittington *et al,* 2000; Cousens *et al,* 2000). However in Europe the C strain type commonly affects sheep, cattle and non-ruminant hosts including wildlife (Pavlik *et al,* 2000) and the pigmented S strain causes clinical disease in cattle (Taylor 1953; Watt 1954).

In 2002, a study by Stevenson *et al,* using a pulsed field gel electrophoresis (PFGE) genetic DNA probe, identified sub-strain isolates within the S and C Strains of Map and that these sub-strain isolates had two genetically distinct dendrographic clusters. This finding contributed to a change in the classification of Map to a more robust Type 1 and 2 nomenclature. Type I strain type has a strong host preference for sheep and appears to be more virulent for sheep. Included within the Type 1 strain are the slow growing pigmented isolates only found in England, Scotland and Spain and the non-pigmented strains found in Iceland, Morocco, South Africa, New Zealand and Australia (Stevenson *et al,* 2002). Type II cattle strains are faster growing, have a broader host range and have been isolated from cattle in New Zealand and Australia, humans (Scanu *et al,* 2007) and wildlife (Beard *et al,* 2001).

2.2 Map – the organism

Mycobacterium avium subsp. *paratuberculosis* bacteria are small (0.5 x 1.5 micron), waxy coated, gram positive, spore forming (Lammont *et al,* 2010), Ziehl Nielsen acid fast, immobile, obligate aerobe bacillus organisms that are mycobactin dependant (Collins 1996) and slow growing (Lambrecht *et al,* 1988), with DNA specific polymorphism IS900 and IS 1311 insertion sequences (Hines *et al,* 1987; Green *et al,* 1989; Collins *et al,* 1990 and 2002; Whittington *et al,* 1998a; Marsh *et al,* 1999 and 2006) that can be identified using the Polymerase Chain Reaction (PCR) test (Whittington *et al,* 1998; Garrido *et al,* 2000*;* Juste *et al,* 2005). Spheroplastic forms of Map also exist with non-cell walled spheroid in shape structures that do not readily stain with traditional acid fast stains although their genetic integrity is maintained, allowing PCR and immuno-histochemistry (IHC) diagnosis (Chiodini *et al,* 1986; Naser *et al,* 2004, Delgado *et al,* 2011).

2.2.1 Host Species

Map infection appears not to be species specific, with intra-species and interspecies infection occurring (Greig 2005; Stevensen *et al,* 2009) and has been cultured from the faeces, terminal ileum and mesenteric lymph nodes from many different domesticated species clinically affected by Map throughout the world including cattle (Buergelt *et al,* 1978; Collins *et al,* 1990; Antognoli *et al,* 2008; Martinson *et al,* 2008), goats (Greig 2000; Manning *et al,* 2003; Singh and Vaughn 2004; Tripathi *et al,* 2006), sheep (West 1997; Perez *et al,* 1996; Michel and Bastianello 2000; Whittington *et al,* 2000; Stewart *et al,* 2004;) and deer (Mackintosh *et al,* 2004; Stringer *et al,* 2009). Wildlife also act as a reservoir of infection, including the stoat, weasel, hare, rat, hedgehog, badger, crow, fox, and mouse in Scotland (Beard *et al,* 2001, Motiwala *et al,* 2004), rodents in Greece (Florou *et al,* 2008), wild rabbits in England and Scotland (Greig *et al,* 1997; Judge *et al,*. 2006), Australian kangaroos (Cleland *et al,*. 2010), Tule elk in California (Jessup *et al,* 1981), American deer (Chiodini and Van Kruiningen 1983, Raizman 2005), big-horn sheep in Colorado (Williams *et al,* 1979), Equine (Mobius *et al,* 2012), and within zoos including stump-tail Macaque primates (McClure *et al,* 1987) and rhinocerous (Bryant 2012). In humans, diagnostic investigations have determined the presence of antibodies to Map (Elsaghier *et al,* 1992) and systemic Map infection in blood cultures and from PCR detection of IS900 and IS1311 polymorphed sequences in humans with Crohn's disease (CD) (Chiodini *et al,* 1984; McFadden *et al,* 1987; Bull *et al,* 2003; Scanu *et al,* 2007; Feller *et al,* 2007; Behr *et al,* 2008; Naser *et al,* 2000, 2004; Rocca *et al,* 2010). Interestingly, there appears to be a background level of faecal infection in humans with up to 23% of healthy people PCR and histopathologically positive to Map (Sanderson *et al,* 1992; Bull *et al,* 2003; Scanu *et al,* 2007; Juste *et al,* 2007) and may be a reflection of Mycobacterial diseases where typically many individuals are infected yet few develop clinical disease as seen in *M. tuberculosis* (Cardona and Ruiz-Manzano 2004).

Comparatively, similar figures may occur in the New Zealand sheep industry whereby the prevalence of clinical disease within Map infected flocks was estimated at less than 1% (West 1997, Brett *et al,* 1998) and more recently 0.13% (Heuer *et al,* 2011; Verdugo *et al,* 2014). The prevalence of Map infection within these flocks is currently unknown and has been investigated in a New Zealand flock in Chapter four.

2.3 Sources of Map.

2.3.1 Oral - Environmental Sources

2.3.1.1 Soil and Supplements

Mycobacteria, like other living cells, require free iron for metabolic processes and multiplication to occur (Merkal and Curan 1974). Free iron is rare in the environment with transferrin, lactoferrin, ferritin and other iron chelating complexes competing for free ferric Fe^{+3} ions. Most Mycobacteria produce two types of iron chelating siderophores for transportation of iron into the bacterial cell, including exochelins which are secreted outside the bacterial cell wall and mycobactin which is associated within the cell wall (McCullough and Merkal 1982; Barclay and Ratledge 1983; Roosenberg *et al,* 2000). Siderophores allow bacteria to solubilize and store insoluble Fe^{+3} from extracellular sources making the iron available to the cell by reducing Fe^{+3} to Fe^{+2} through breaking down the siderophore intracellularly (Roosenberg *et al,* 2000). However, in Map the cell walls produce low amounts of mycobactin with a low affinity for Fe^{+3} requiring Map to live intracellularly within macrophages where the pH is low, freeing up Fe^{+3} ions (Thorel *et al,* 1990, 2000). Mycobactin has no function at an intra-cellular level where free Fe^{+3} ions are available and is only required in-vitro for Map colonies to form (Francis *et al,* 1953; Merkal and Curran 1974). Therefore, being unable to replicate outside living cells and as an obligate

intracellular bacterium, the prevalence of Map within any environment is determined solely by the rate of contamination from both farmed and wild animals and human effluent (Thorel *et al,* 1990; Lambrecht *et al,* 1992; Sanderson *et al,* 1992; Collins *et al,* 1996). Map has efficient mechanisms to survive within the soil, fresh water reservoirs, soil microbia (protozoal) and nematode environments, in a dormant state, for prolonged periods of time (Whittington *et al,*. 2001, 2004, 2005; Pickup *et al,* 2005, 2006, Nura *et al,* 2006; Salgado *et al,* 2011; Whan *et al,* 2006). Environmental survival may be enhanced by the structure of the organism. A small cell encased in a thick hydro-phobic, waxy, desiccation resistant cell wall and the ability to sporulate and form spores (Lammont *et al,* 2010).

Environmental contamination from grazing ruminants is a continuous process via the faecal shedding of Map with up to 1 x 10^8 Map / gram faeces recorded in sheep (8 x 10^{10} per day) (Whittington *et al,* 2000). The practice of slurry spreading onto soil during crop or pasture establishment also contributes to this with prolonged Map survival (Jorgansen 1977). In studies by Lovell (1944), Doyle (1956) and Johnson–Ifearulundu and Kaneene (1997) viable Map was recovered from soil 250 days after artificial inoculation. Similar results were recorded by Whittington (2004) where viable Map was recovered from dry, shaded plots 55 weeks after inoculation and 25 weeks from pasture grown through Map infected soil. This study referred to these results as evidence of a 'dormancy state' for Map to survive in the environment and suggested that it was the most significant factor for survival, above and beyond the effects of moisture, UV radiation, pH and heat fluctuations. Dormancy may be explained by the 'dormancy associated genes' carried within Map's genome, although more recent research suggests that this may also be explained by the discovery that Map is a spore forming bacterium (Whittington *et al,* 2004; Lammont *et al,* 2010). It is accepted that the transmission of Map to animals occurs mainly via the faecal oral route with intestinal colonisation occurring in sheep from ingesting organisms from contaminated pasture and that

mitigating this exposure can be undertaken via influencing farm management practices whereby feed contamination or exposure to contaminated feed is reduced (Sweeney *et al,* 1996). The survival on pasture leaf was significant at 25 weeks post germination, although appears to be limited by physical removal due to rainfall, providing a route of exposure to livestock from either direct grazing or from supplementary feeds contaminated with Map (Whittington *et al,* 2004). However, infections from supplementary feed sources appear to be minimal despite Map surviving the hay and ensilaging processes (Arrigoni *et al,* 2009; Kohl 2010; Cook *et al,* 2013).

Despite the 'dormancy effect' and subsequent prolonged survival times within the environment, without the continual deposition of Map into the environment from animal grazing, Map numbers eventually do decline to a point where there are none detectable within soil substrates via PCR or culture. The time frame for this occurrence appears to be between five and twelve months after de-stocking (Whittington *et al,* 2003). This apparent loss of Map presence from the environment may be due to the obligate parasitic nature of this organism and suggests this management practice could be used as a tool in eradication protocols. However, maintaining a 'clean' environment would be completely dependent on the prevention of the re-introduction of Map infected animals and with the current inability of any such diagnostic tests to identify all sub-clinically affected or any latently infected animals, this is unlikely to occur. Additionally, wildlife may also have a role to play in the maintenance of Map infection within farming systems and contribute to environment contamination with Map organisms found in the faeces of wildlife throughout the world including small carnivores and rabbits in Scotland (Daniel *et al,* 2001; Beard *et al,* 2001), wild deer and rabbits in America (Raizman *et al,* 2005), birds (Corn *et al,* 2005), rodents in Greece (Florou *et al,* 2008), environmental nematodes (Whittington *et al,* 2001) and invertebrates (Kopecna *et al,* 2005).

The distribution of Map within the environment appears to be evenly distributed with greater soil prevalence and pasture contamination associated with farming practices, compared to non-farming areas, with cattle farming systems appearing to have greater influence than sheep based systems throughout the UK, Europe and America (Caldow *et al*, 2007; Nielsen and Toft 2009**)**. It also appears, from previous studies, that soil pH, carbon concentration, rainfall, temperature, altitude all influence Map survival within the soil (Emmet *et al,* 2010). However this may be misleading as these parameters also support cattle grazing systems more favourably than sheep systems and it is suggested that the demographic of where cattle can graze has greater influence on the presence of Map in soils (Rhodes *et al,* 2013).

2.3.1.2 Water

Another environmental source of Map for livestock comes from the survival of Map in waterways and appears to be proportionally influenced by agriculture with increasing numbers of Map organisms recorded downstream of farming areas, likely as a result of natural water "run off" from rainfall (Whittington *et al,* 2004; Pickup *et al,* 2005,2006). Access of stock to water courses may also act as a faecal source of Map contamination through defecation directly into the waterway. The survival of Map in wet environments has been well documented with Map identified in water and sediments of ponds, stock reservoirs and rivers (Whittington *et al,* 2004, 2005; Pickup *et al,* 2005, 2006, Salgado *et al,* 2011). Survival times in water concluded that Map viability remained for 632 days with PCR still identifying cell densities at 841 days (Pickup *et al,* 2005). Therefore the use of natural watering holes, dams or reservoirs and access to streams and rivers as a source of drinking water for stock has potential to provide a source of Map exposure and may play a role in the maintenance and development of clinical Johne's disease on farms infected by Map.

2.3.1.3 Aerosol

The survival of Map in a dry environment, spore formation and apparent resistance to desiccation provides opportunity for Map to contaminate environments distant to where it was faecally deposited in the form of moisture vapour or dust particulate aerosols. In a study by Eisenberg *et al,* (2010, 2011) dust particles containing 1 x 10^8 colony forming units (cfu) Map / gram dust were recovered within buildings housing cattle and that environmental contamination surrounding those buildings increased over time through the spread of these dust particles. The ability for bacteria, including mycobacteria, suspended in liquid water, to transition to water vapour and aerosol droplets with the subsequent concentration of bacteria within these droplets is well documented (Blanchard and Syzdek 1970; Parker *et al,* 1983; Falkinham 2003). Evidence of this with Map contaminating water vapour aerosols was determined by the identification of Map, both by PCR and culture, in water vapour collected immediately above the surface of the river Taff in Wales and from shower vapours in counties across the UK (Rhodes *et al,* 2014). This suggests there is potential for a route of inhalational infection for livestock and humans from faecal shedding into the environment (soil and water). This is supported in a recent study where Map intranasal and tracheal inoculation lead to the establishment of intestinal and mesenteric lymph node infection in calves (Eisenberg 2011). With Map also isolated from the saliva of cattle (Kubica *et al,* 1963; Sorge *et al,* 2013) aerosols may have a role to play in the development of Johne's disease in ruminants as postulated by Corner *et al,* (2004), especially in farming systems where housing and close contact is inevitable.

2.3.2 Maternal Sources:

Maternal sources include pre and postnatal infection, with trans-placental transfer of Map to fetuses occurring in sheep (Lambeth *et al,* 2004), cattle (Doyle 1958; Sweeney *et al,* 1992; Buergelt and Williams 2003; Whittington and Windsor 2009) and deer (Van Kooten *et al,* 2006). Post natal shedding of occurs, with Map isolated from the colostrum and milk of ruminants (Sweeney *et al,* 1992; Giese and Aherns 2000; IG Grant 2001; Manning *et al,* 2003; Singh and Vihan 2004; Slana *et al,* 2008; Bradner *et al,* 2013) and humans (Nasser *et al,* 2000), providing exposure to this organisms early in life. In the dairy industry, the practice of 'pooling colostrum' occurs, with all neonates on these properties being manually dosed with the requisite volume of colostrum from this pooled source within the initial 12 hours of life to ensure passive transfer of immunity. It has been suggested that this practice may increase the risk of neonates to the exposure of Map (Orpin *et al,* 2012; Bradner *et al,* 2013), although this risk may be minimal (Nielsen *et al,* 2008), with suggested steps to mitigate neonate exposure to Map including the feeding of milk replacer (milk powder) or dam milk only to each calf (Nielsen *et al,* 2008).

2.3.3 Iatrogenic and Parenteral Sources

There have been many intervention studies in rodents and ruminants where Map has been artificially administered in order to replicate that of natural infection. Administration routes studied include intra-nasal and tracheal, aerosol (Kluge *et al,* 1968; Merkal *et al,* 1968; Robbe-Austerman 2007; Eisenberg 2011), intra-tonsil (Gwodz *et al,* 2000; Begg *et al,* 2005), intravenous (Kluge *et al,* 1968; Merkal *et al,* 1968; Larsen 1977; Tripathi and Kolhe 2007), sub-cutaneous (Larsen *et al,* 1977; Golan *et al,* 2009) intra-peritonal (Tripathi *et al,* 2007; Stabel *et al,* 2009) and oral (Gilmour *et al,* 1965, 1978; Kluge *et al,* 1968; Merkal *et al,* 1968;

Larsen *et al,* 1977; Begara – McGorum *et al,* 1998; Gwozdz *et al,* 2000; Storset *et al,* 2001; Kurade *et al,* 2004; Reddacliff *et al,* 2003; Waters *et al,* 2003; Stewart *et al,* 2004; Begg *et al,* 2005; Sweeney *et al,* 2006; Robbe-Austerman *et al,* 2007; Stabel *et al,* 2009). With many studies utilising different methods of artificial inoculation it is difficult to comparatively analyse and interpret the immune responses recorded as these parenteral and iatrogenic routes of infection are less likely to occur in nature with the oral route considered the primary source of Map infection. With the exception of the Robbe-Austerman (2007) study above, where killed Map organisms were inoculated, live Map organisms were used and although there were marked differences between the pathologies that developed and subsequent expression of resilience, all recorded tropism of Map for the alimentary tract irrespective of the route of infection. The Robbe-Austerman study, recorded no immune response, supporting other studies where killed Map had no virulence and could be considered of little risk for the development of clinical Johne's disease in sheep (Brotherston *et al,* 1961b). This finding may have important implications re the mitigation of potential Map related food scares as consumers can be informed that the adequate cooking of tissues or the purchase of pasteurised milk provides food considered 'safe' for human consumption (Chiodini and Herman-Taylor 1993; Gao *et al,* 2002; Mutharia *et al,* 2010; Gill *et al,* 2011).

2.4 Pathogenesis of Johne's disease

Immune Responses to Map infection

Infection is thought to start from an early age, with neonates more susceptible (Juste *et al,* 1994; Windsor and Whittington 2010), although adult animals can also become naturally infected by Map when continuously exposed from Map shedding cohorts (Perez *et al,* 1996; Reddacliff *et al,* 2004; Gonzalez *et al,* 2005; Mackintosh *et al,* 2010; Dennis *et al,* 2011,

McGregor *et al*, 2015). In naturally infected animals the main route of infection is considered to be the faecal oral route (Sweeney *et al,* 1997; Greig 2000) with Map having an apparent tropism for the terminal jejunum, ileum and mesenteric lymph nodes of the alimentary tract where clinical pathology occurs (Buergelt *et al,* 1978; Perez 1996, 1997, Whitlock and Buergelt 1996; Greig 2000; Sweeney *et al,* 2006). This tropism also appears to occur from artificial parenteral challenge with Map infection of the alimentary tract and subsequent pathology occurring irrespective of initial infection site suggesting a systemic route of infection may also play a role in the pathogenesis of disease (Gwozdz *et al*, 2000; Begg *et al,* 2005; Tripathi and Kohle 2007; Eisenberg 2011).

2.4.1 Cell Mediated Response

Post ingestion, Map enters the lumen of the ileum where it either passes through, with passive excretion in the faeces recorded (Sweeney *et al,* 1992; Pradham *et al,* 2011), or is taken up, predominantly through M dome cells, domes of lymphoid tissue within the ileal epithelium situated between the villi of the Peyer's patch (Momotani *et al, 1988;* Garcia-Marin *et al,* 1992; Bermudez *et al,* 2010), where rapid colonisation of the local mesenteric lymph nodes occurs (Wu *et al*, 2007). Local histiocytic and endothelial macrophages phagocytose the invading Map, providing an initial cell mediated immune response (CMIR), sequestering Map and preventing further systemic spread (Steadham *et al,* 2003; Weis and Souza 2008). The CMIR can be identified by histopathological changes within Map infected tissues and although the number of animals infected within New Zealand flocks is currently unknown (Chapter four), it appears that this initial CMIR is effective at the prevention of further establishment of infection and progression to clinical Johne's disease, with only 0.13% animals developing clinical disease (Verdugo *et al,* 2014). However, in animals where

clinical disease develops, Map survives phagocytosis and becomes an intra-cellular parasite, proliferating within ileal mucosal and sub-mucosal macrophages (Woo *et al,* 2008). This survival appears to be accomplished by Map down-regulating the macrophage mRNA expression for the production of phagosomal hydrogen peroxide and up-regulating interleukin 10 (IL10), preventing bacteriolysis and allowing Map to multiply within the macrophage (Rao *et al,* 2001; Weis and Souza 2008). Intracellular colonisation of the macrophage also stimulates the expression and up regulation of mRNA protein synthesis for the pro-inflammatory cytokines Tumour Necrosis Factor alpha (TNFα), the interleukins 1,4 and 6 (IL1,4 and 6) and gamma interferon (IFN-γ) (Alzuherri *et al,* 1996; Begara-McGorum *et al,* 1998; Aho *et al,* 2003; Reddacliff *et al,* 2003; Robbe-Austerman *et al,* 2006; Coussens *et al,* 2004; Stewart *et al,* 2004; Nielsen and Toft 2008; Weis and Sousa 2008; Delgado *et al,* 2012). It is proposed that this unregulated and over expression of these pro-inflammatory cytokines produces an inappropriate immune response, with subsequent further macrophage recruitment leading to the progressive granulomatous development of this disease (Alzuherri *et al,* 1996; Aho *et al,* 2003; Steadham *et al,* 2003; Weis and Souse 2008).

In all cases of clinical Johne's disease in ruminants, there is progressive loss of body condition, with emaciation the fundamental clinical sign. The currently accepted aetiology for this terminal weight loss is considered to be from a progressive granulomatous enteritis and resultant protein losing enteropathy, with or without diarrhoea, due to pathological changes of the ileum and associated lymphatics (Patterson and Berret 1969; Beurgelt *et al,* 1978, Carrigan and Seaman 1990). These include histopathological segmental epithelioid macrophage micro-granuloma formation packed with intracellular acid fast organisms (AFO) within the ileal, ileo-caecal valve and terminal jejunum mucosa and lamina propria, progressing to sheets of coalesced epithelioid macrophage dominant micro-granulomas producing a grossly visible and thickened, corrugated ileal mucosa (Figure 2.1), blunting of

mucosal villi (Figure 2.2), and the micro and macroscopic enlargement (lymphadenitis) and inflammation (lymphangitis) of the of regional lymphatics (Buergelt *et al,* 1978; Carrigan and Seaman 1990; Perez *et al,* 1996; Whitlock and Buergelt 1996, Stringer *et al,* 2009) (Figures 2.3, 2.4)

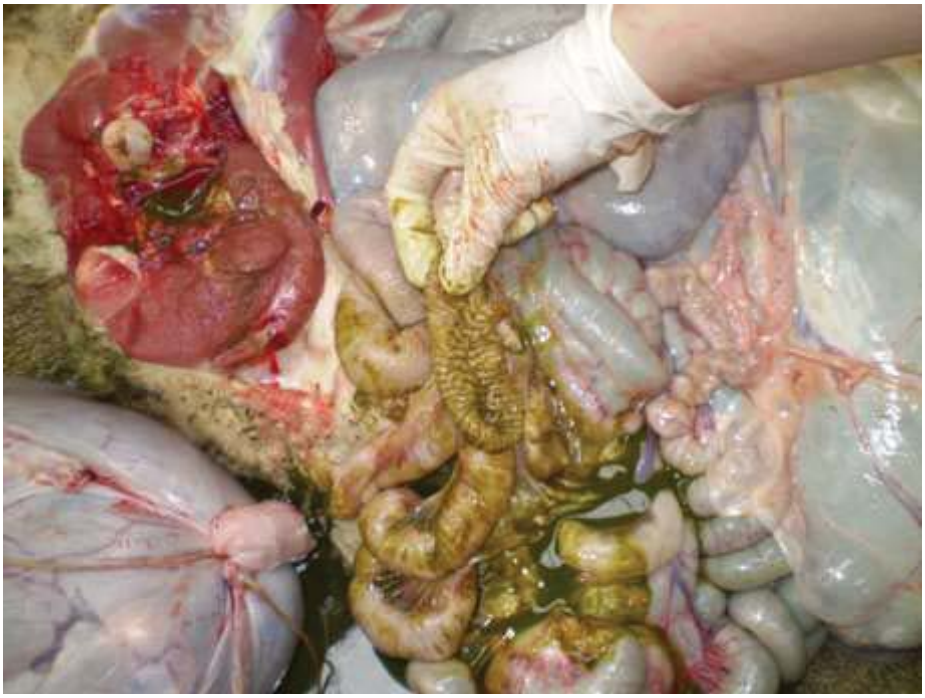


Figure 2.1. Gross thickening of terminal ileal mucosa with granulomatous enteritis in sheep with clinical Johne's disease.

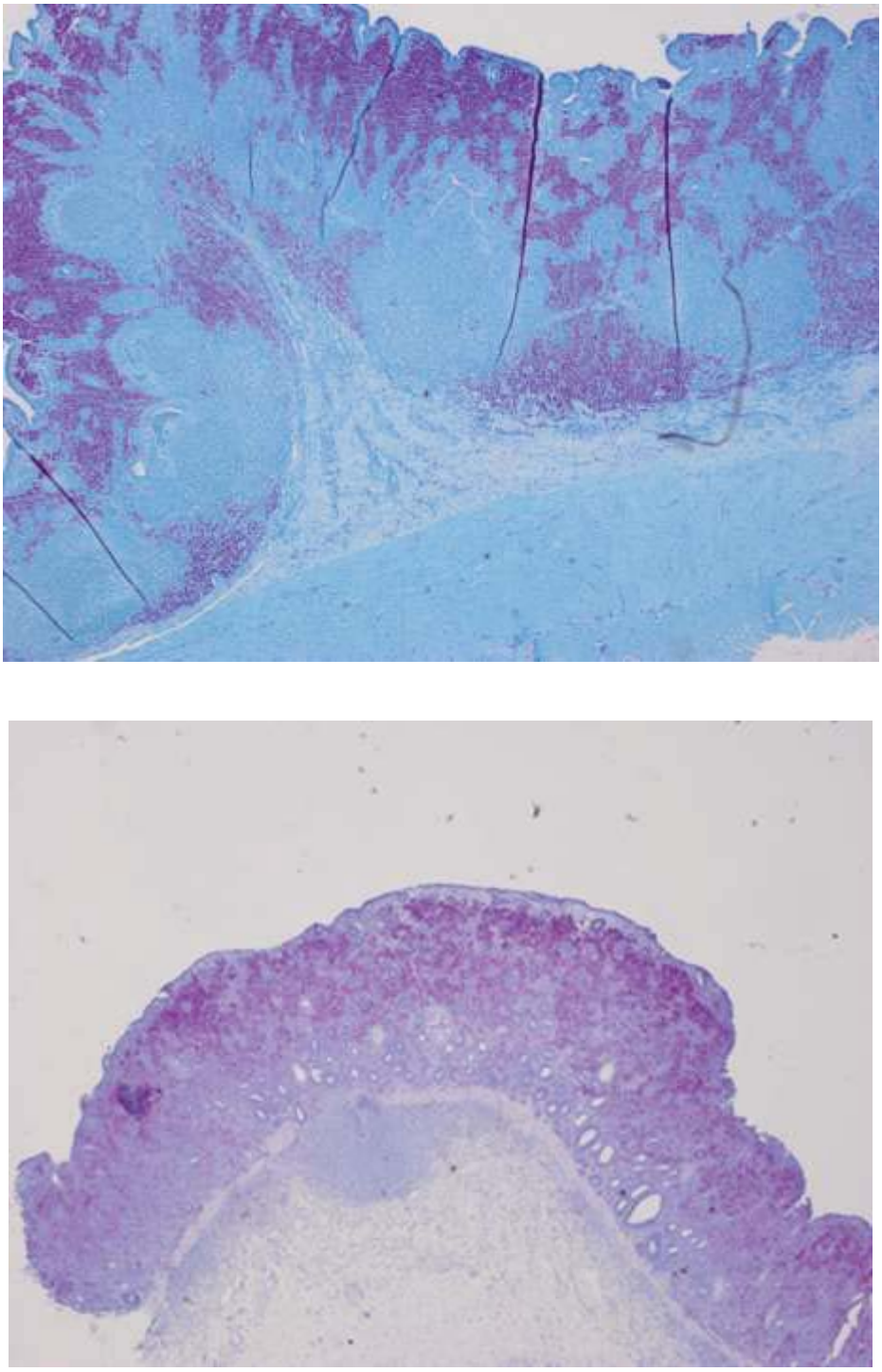


Figure 2.2. Histopathological sections of terminal ileal mucosa showing blunting of mucosal villi and infiltration by sheets of coalesced epithelioid macrophage micro-granulomas packed with *Mycobacterium avium* subsp *paratuberculosis* AFO, x100, ZN

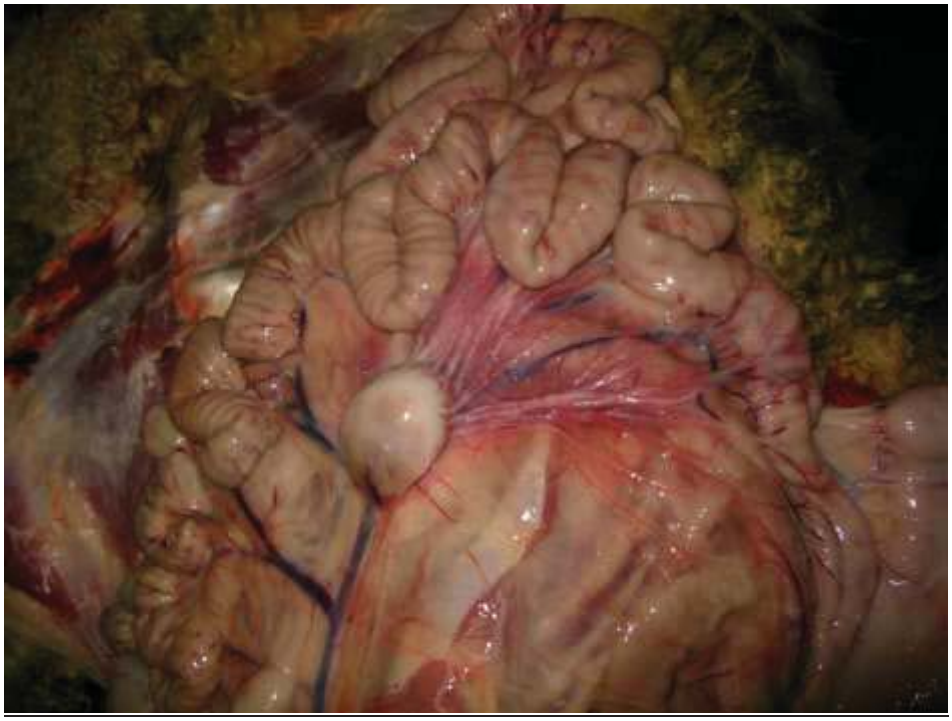


Figure 2.3 Ovine lymphadenitis of 1st mesenteric lymph node.

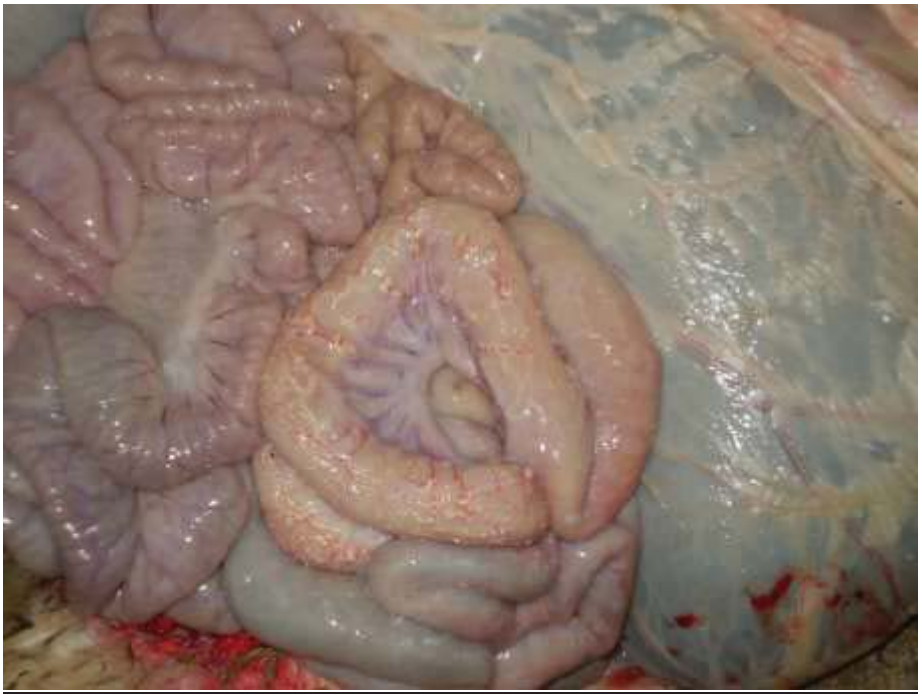


Figure 2.4 Lymphangitis or cording of lymphatics over serosal surface of terminal jejunum and ileum.

However, in sheep and goats diarrhoea is not always featured in animals with clinical Johne's disease (Buergelt 1996; Greig 2000; Manning *et al,* 2003; Begg *et al,* 2005; Begg and Whittington 2008) suggesting that terminal cachexia may not be solely due to a protein losing enteropathy but may also involve protein losses due to alternate mechanisms such as cytokine up-regulation and / or over expression or systemic cell mediated immune (CMI) stimulation (Dennis *et al,* 2011). This requires further consideration and is addressed in the intervention study in Chapter six where another pathogenesis for this cachexia is proposed.

2.4.2 Humoral Response

In naturally infected sheep, sero-conversion often occurs in the later stages where Map infection has progressed to clinical disease. At this point it appears that the overwhelming of the local CMI enables systemic Map infection to occur whereby a subsequent humoral response produces Map specific antibodies that can be measured using AGID and ELISA serological diagnostic tests (Hilbink *et al,* 1994; Burrells *et al,* 1998; Whittington and Sergeant 2001; Sergeant *et al,* 2003; Steadham *et al,* 2003; Robbe-Austerman *et al,* 2006). Unfortunately, there is a wide spectrum of sensitivity and specificity for the ELISA blood test (16-85% and 95-100% respectively), (Nielsen and Toft 2008) with not all animals that develop clinical Johne's disease sero-converting. The aetiology of the production of Map antibodies and their contribution to resilience from Map infection is currently unknown although, in naturally infected animals, it appears that a relationship may exist between the onset of clinical Johne's disease and sero-conversion, with sero-conversion appearing to have little effect on the terminal nature of clinical disease. However, this appears not to be the case in experimental artificial infection where sero-conversion has been recorded in otherwise healthy lambs (Bower *et al,* 2011; Kurade *et al,* 2004; Begg *et al,* 2005), placing

significant limitations on the ability to interpret serological results when trying to identify Map infection, suggesting additional diagnostic assessment should be considered (Michel and Bastianello 2000; Sergeant *et al, 2003;* Nielsen and Toft 2008).

In naturally infected ruminants, both sero-conversion and systemic Map infection appear to occur at the end stages of Johne's disease suggesting there may be a relationship between these two events. Systemic Map infection occurs in animals with Johne's disease with Map identified in organs peripheral to the alimentary tract, including the diaphragmatic muscle of cattle (Alonso-Hearn *et al,* 2010), liver of goats (Manning *et al,* 2003) and gastrocnemius and thigh skeletal muscle of adult sheep (Nelli *et al,* 2008; Reddacliff *et al,* 2010). Currently there is no information on the prevalence of systemic Map infection in New Zealand sheep. Further research is required to determine this and the role this may have on the development of Map anti-body titres (Nielson and Toft 2008). Chapter four provides data on the prevalence of systemic Map infection in naturally infected sheep from a single flock with the longitudinal study in Chapter six addressing this for artificial experimental infection.

2.5 Diagnosis and Quantification of Map

There are four categories of animal on any farm infected by Map. Animals that are uninfected, or those where Map is found within the ileal or mesenteric tissue without the formation of any pathological lesions (latently infected and not affected) (Whitlock and Buergelt1996), or those with pathology of the mesenteric lymph nodes and ileal tissues without any outward clinical signs (infected and sub-clinically affected) and those with Map found within the ileal or mesenteric lymph node tissues with pathology and where clinical disease is grossly evident (infected and clinically affected). There are currently no diagnostic tests available to identify latently or non-shedding sub-clinically affected sheep, with most

diagnostic tests identifying sheep with clinical Johne's disease with varying levels of sensitivity and specificity (Nielsen and Toft 2008). Current diagnostic tests available include;

- Microscopy and identification of Acid Fast Organisms (AFO)
- Microscopy and Immunohistochemistry (IHC)
- Culture – solid and liquid media
- Humoral response - sero-conversion
- Genetic identification of Map – Polymerase chain reaction (PCR), pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP)
- Cell Mediated Immune Response – delayed hypersensitivity reactions and cytokine assays
- Cell Mediated Immune Response – microscopy and histopathology alimentary tract and systemic infection
- Cell Mediated Immune Response - Spectroscopy

2.5.1 Microscopy and Acid Fast Organism(AFO) identification.

The Ziehl–Neelsen stain, was first described by two German doctors; a bacteriologist, Franz Ziehl (1859 to 1926), and a pathologist Friedrich Neelsen (1854 to 1898). The development of the Ziehl Neelsen (ZN) staining method, using a combination of carbol fuschin, acid alcohol (ethanol) and methylene blue, initailly enabled the identification of Mycobacteria organisms (tuberculosis) in human medicine. Mycobacteria have a high concentration of mycolic acid / mycolates within the cell wall, providing a waxy hydrophobic layer which contributes to their acid fast nature, with ZN positive organisms staining red and being identified as acid fast organisms (AFO) (Rowe and Grant 2006) (Figure 2.5)

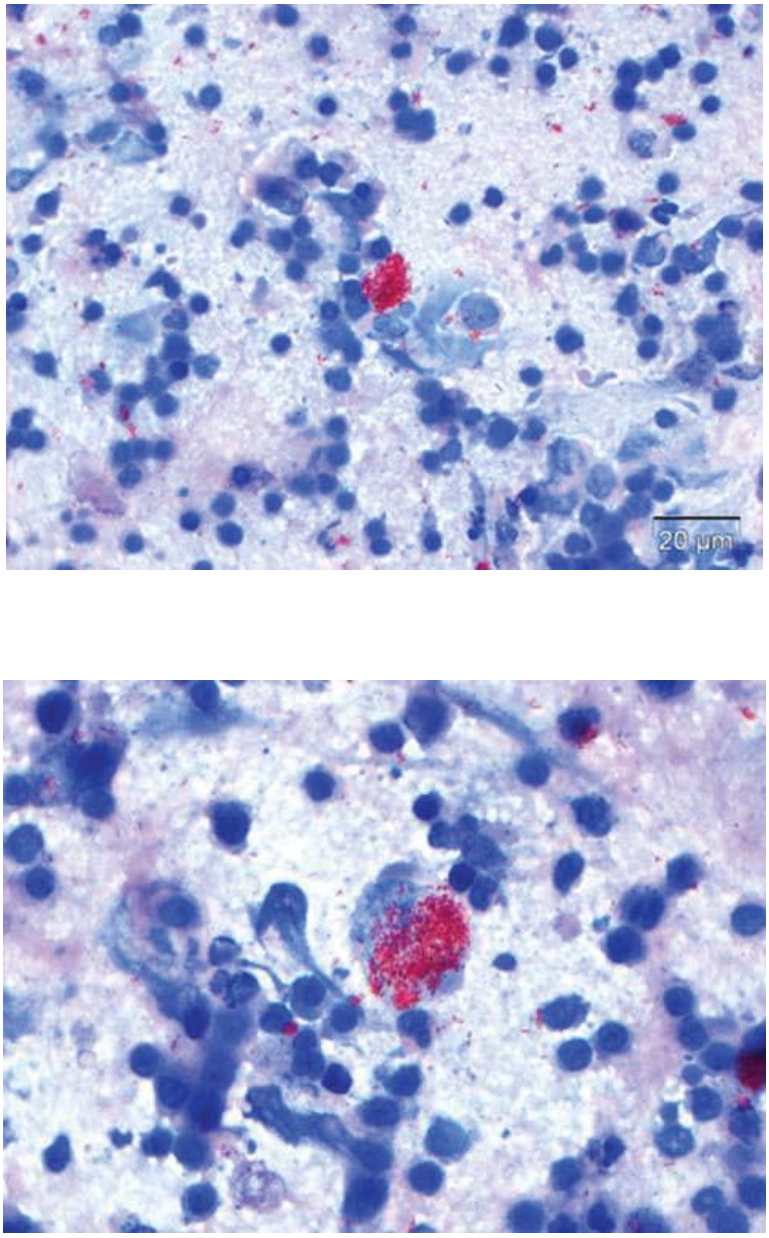


Figure 2.5. Ovine ileal impression smear showing clumped Ziehl Neelsen positively stained acid fast organisms, identified from PCR as Type 1, sheep strain, *Mycobacterium avium* subsp. *paratuberculosis*.

Identification of AFO from faecal shedding or at necropsy with tissue impression smears using Ziehl Neelsen (ZN) or auramine and rhodamine fluorochrome (AR) stains are both

quick and effective techniques with the AR fluorescence providing a more sensitive assessment for the presence of AFO and enabling larger sample areas to be examined in a shorter time frame (Huebner *et al,* 1993). The sensitivity and specificity for ZN and AR stains to identify AFO in faeces and tissue samples appears to be influenced by the initial number of organisms within the specimen, the preparation of samples, and the temperature of the stain at time of application (McCarter and Robinson 1994; Jayanathan *et al,* 2006) and even though the presence of AFO enables direct measurement of the organism with dimensions compared to other known AFO it does not distinguish Map from other AFO species. This can be achieved via further analysis using the genetic Polymerase Chain Reaction (PCR) test and identifying Map specific insertion sequences IS900 and IS1311 (Whittington *et al,* 1998; Garrido *et al,2000;* Juste *et al,* 2005).

2.5.1.1 Microscopy and Immuno-histochemsitry (IHC)

Map not only exists as a cellular structure where acid fast stains attach to the cell wall producing visible identification (ZN and AR stains) but also as a cell wall deficient or spheroplastic structure. These have been recorded in humans with Crohn's disease (Chiodini *et al,* 1986; Sechi *et al,* 2001, 2004; Delgado *et al,* 2009, 2011; Rocca *et al,* 2010) and in cattle with Johne's disease (Buergelt *et al,* 1996). Spheroplasts can be identified using Map specific genetic probes via PCR diagnostic tests and through immune-histochemistry (IHC). Techniques using IHC have application in fresh and formalin fixed samples (Brees *et al,* 2000; Delgado *et al,* 2009) and involve the use of fluorescing dyes, such as fluorescin iso-thiocyanate, in combination with polyclonal antibodies for *M. bovis*, which has been shown to have affinity for Mtb and Map, with positive samples identified through visual microscopic examination. The sensitivity and specificity of IHC versus acid fast organism

identification appears to be higher (Thorensen *et al,* 1994; Brees *et al,* 2000), with AFO stains limited by the wall deficient or spheroplastic form of Map. In contrast, there appears to be mixed results when comparing IHC and PCR, although both outperform AFO identification as a method to identify animals infected with Map. (Chiodini *et al,* 1989; Gey *et al,* 1999; Hulton *et al,* 2000; Sechi *et al,* 2001; Nasser *et al,* 2004; Delgado *et al,* 2009; Rocca *et al,* 2010).

2.5.2 Culture –

2.5.2.1 Solid Media Culture -

Identification of Map can be achieved via bacteriology and culture using solid and liquid media techniques. Culture of Map can take up to 6 months for colonies to appear, initially forming clear small smooth colonies that may require visualisation under a dissecting microscope for early confirmation (Gwozdz *et al,* 1997), increasing in size and becoming rough over time. The choice of media for culture also impacts on visualisation with dyes such as malachite green providing contrast and transparent Modified Middlebrook 7H9 and 7H10 media (Damato and Collins 1990), providing clear backgrounds for viewing.

Decontamination

Overcoming culture contamination and overgrowth from commensal bacteria and fungi is a fundamental step for successful Map culture on solid media. There are four main chemicals commonly used in mycobacterial decontamination assays, namely sodium hydroxide, oxalic acid, benzalkonium chloride and hexadecylpyridinium chloride (Yajko *et al,* 1993) which utilise Map's thick, waxy cell wall structure and accompanying natural resistance to acid and

alkaline environments (Rowe and Grant 2006). Pre-culture decontamination steps include sample digestion with 5% oxalic acid and 2% NaOH, neutralised with phosphate buffer and inoculated onto Lowenstein-Jensen or modified Middlebrook 7H9 / 7H10 growth media or digestion with hexadecylpyridinium chloride (HPC), neutralised with egg yolk and inoculated onto Herrold's egg yolk media, with the yolk providing essential fatty acids for Map growth (Merkal 1974; Whittington *et al,* 1999; Johansen *et al,* 2004; Payeur *et al,* 2005; Wiszniewska *et al,* 2005). Antibiotic and anti-fungal combinations are also added to prevent commensal overgrowth including ANV (amphotericin B, nalidixic acid and vancomycin) or PANTA (polymixin B, amphotericin B, Nalidixic acid, trimethoprim, and azlocilin) (Merkal and Richards 1972; Jorgansen 1982; Whitlock and Rosenberger 1991; Stabel 1997; Johnasen *et al,* 2004).

Mycobactin

In the Mycobacterial family, there appears to be an inverse relationship between the presence of iron and mycobactin formation, with an increase in mycobactin production when iron availability is low (Morrison 1965). Unlike other Mycobacterial species that secrete mycobactin from their cell walls, some members of the *M. avium* complex, including *M. sylvaticum* and Map are mycobactin dependant in that they do not produce this siderophore used for the transportation of iron into the bacterial cell rendering Map an obligate intracellular parasite, requiring a host cell (macrophages) to provide the iron for growth (Francis *et al,* 1953; Morrison 1965; Merkal and Curren 1974; Lambrecht *et al,* 1992; Collins *et al,*1996). Thus, mycobactin is not required in-vivo. However, in-vitro growth of Map requires exogenous supplementation of mycobactin (Thorel *et al,* 1990). This was initially produced and purified from *M. phlei* (mycobactin P), (Francis *et al,* 1953). New strains of mycobactin independent *M. paratuberculosis* were identified and cultured on artificial media

with an improved new mycobactin J harvested from *M. johnei*. This strain of mycobactin has provided a reduced time for colony formation and an increased range of samples from different species where colonies were successfully grown and is currently used throughout the world for in-vitro Map culture (Merkal and McCullough 1982).

The identification of Map from solid media requires the visualisation of colonies and the sub-culture, on the same media, with and without mycobactin present. The failure of colonies to form in media without mycobactin strongly suggests the presence of Map, although again this does not definitively identify Map, as there are other mycobactin dependent bacterial species (Francis *et al,* 1953).

For solid culture media, specimens are considered positive for Map when colonies are identified, colonies grow on media with mycobactin J present and don't grow on the same media without mycobactin J, they are strongly acid fast and they have Map specific IS900 insertion sequences as determined by polymerase chain reaction (PCR). In circumstances where media contamination has occurred, PCR identification of IS900 insertion sequences may be the only identification method available (Begg and Whittington 2008). However, this insertion sequence is also shared by other bacteria so further PCR diagnostic tests identifying Map specific genetic sequences such as IS1311 may also be required for definitive identification (Englund *et al,* 2002; Cousins *et al,* 1999).

Quantification on Solid Media

On solid media, Map has a tendency to clump within these colonies making it difficult to access individual bacteria, with separation techniques such as passing suspended colonies through fine hypodermic needles followed by titration onto culture media used to quantify the number of organisms present (Begg *et al,* 2005; Begg and Whittington 2008). Despite

these techniques, colony counts on solid media only provide an estimate of actual numbers present. To address this problem, liquid media cultures have been developed and have substantially improved the ability to quantify the number of organisms present with both radiometric and non-radiometric techniques used (Whittington *et al,* 2000; Reddacliff *et al,* 2003).

2.5.2.2 Liquid Media Map Culture

The visualisation and identification of Map from colony formation on solid culture media is labourious and prone to complications, such as overgrowth from commensal contaminants and has limitations re determining the number of Map initially inoculated (Whittington *et al,* 1998; 2000). Liquid media culture methods exist that provide a quicker and more quantitative assessment of the presence of Mycobacteria and include the BACTEC radiometric culture and the non-radiometric BACTEC MGIT 960 systems.

Radiometric liquid media culture

The BACTEC radiometric system utilises a liquid media (BACTEC 12B) containing C_{14} radio-labelled palmitic acid which, when metabolised by Mycobacteria, releases radiolabeled CO_2 indicating Mycobacterial growth which is subsequently detected by the BACTEC 460 reader (Cousins *et al,* 1995; Whittington *et al,* 1999; Payeur *et al,* 2005) with weekly measurements of CO_2 taken over a six week period providing a growth index (GI). Quantifying the number of Mycobacteria present can be achieved indirectly using growth index standard curves based on the time taken to record a cumulative growth index >1000 (CGI1000) (Reddacliff *et al,* 2003). As with solid media cultures, the number of organisms

within the initial inoculant determines the rate of identification with an inverse relationship, with samples containing > 1 x 10^8 Map organisms per gram recording positive results within the first week of culture (Reddacliff *et al,* 2003: Whittington *et al,* 2003). Media with growth indices > 10 are re-selected for Map differentiation with further sub-cultures performed, AFO identification and PCR analysis (Whittington *et al,* 1999; Payeur *et al,* 2005). The BACTEC 12B radiometric system was considered to be the most reliable and 'gold standard' diagnostic test for identifying animals infected with Map, with detection limits as low as 3 Map organisms per gram of tissue compared to 10^2 organisms for solid media (Lambrecht *et al,* 1988; Whittington *et al,* 2000; Stewart *et al,* 2004). However, the use of radioactive isotopes has limitations and as such this radiometric method has recently been replaced with the BACTEC MGIT 960 non-radiometric liquid media system.

Non-radiometric liquid media culture systems

BACTEC MGIT 960 system utilises liquid media (BD MGIT ParaTB) with a fluorescent compound (Tris 4, 7-diphenyl-1,10-phenanthroline ruthenium chloride pentahydrate) incorporated within the media tube. In the presence of oxygen dissolved within the media this compound does not fluoresce. However as bacterial growth metabolises oxygen the fluorescence increases with Mycobacteria producing a unique signature, identified by hourly measurements and computer analysis. Suspect positive samples are selected for further sub-culture, acid fast staining and PCR (Fyock *et al,* 2005; Payeur *et al,* 2005; Thomas *et al,* 2005).

ESP Liquid Culture System II (TREK Diagnostics) measures the consumption of gases by Mycobacteria within the test tube. Bacterial growth is measured as an increase in negative pressure within the sealed media tube with Mycobacterial growth again having a computer recognised specific signature (Begg and Whittington 2008; Nielsen and Toft 2008). Suspect positive results can appear quickly, within 4 days post media inoculation, with further steps taken for Map differentiation including AFO staining and PCR identification of IS 900 insertion sequences (Payeur *et al,* 2005; van Maanen *et al,* 2005)

2.5.3 Humoral Response - Antibody Identification

Serum antibody levels appear to increase as the CMIR declines and occur as Map infection progresses into the more advanced stages of clinical disease (Burrels *et al,* 1995; Stewart *et al,* 2004). Antibodies specific to Map can be measured using AGID and ELISA serological diagnostic tests (Hilbink *et al,* 1994; Burrells *et al,* 1998; Sergeant *et al,* 2003; Robbe-Austerman *et al,* 2006). In New Zealand, the commercially available ELISA serological blood test records the optical density (OD) of Map specific immunoglobulins and converts the data into a sample/positive (s/p) ratio using the following formula with animals over 50 units considered positive for Map (Institut Pourquier, Montepellier, France/Idexx Laboratories Inc).

(Sample OD) - (-ve Control OD) / (+ve Control OD) – (-ve Control OD) x 100/1 = s/p ratio for sero-conversion to Map.

As with other serological diagnostic tests for Map, ELISA has a wide sensitivity and specificity range (Nielsen and Toft 2008) yet despite this, it does provide a relatively cheap and rapid opportunity to screen large numbers of animals with additional diagnostic options

available to confirm cases of clinical disease. Currently there is very little information on whether a relationship exists between sero-conversion and systemic Map infection despite both appearing to occur at the later, more progressive stages of infection and when clinical Johne's disease has developed (Chapter six).

2.5.4 Genetic Identification – Polymerase Chain Reaction (PCR), Pulsed Field Gel Electrophoresis (PFGE) and Restriction Fragment Length Polymorphism (RFLP).

The identification of Map specific immune responses via detection of sero-conversion, recording the expression or suppression of pro-inflammatory cytokines, visualising the associated pathologies with CMI including the delayed hypersensitivity in the Johnin Skin test (Robbe-Austermann 2006) can be problematic due to the variability in immune response between animals, the apparent sharing of immune responses and the cross reactivity for M. tuberculosis and M. avium complex, producing a wide range of sensitivities and specificities (Nielsen and Toft 2008). However, there appears to be very little variation within the genetic code for Map (Stevenson *et al,* 2002; Sevilla *et al,* 2005), with a Map specific genetic sequence IS900 identified (Hines *et al,* 1987; McFadden *et al,* 1987; Green *et al,* 1989, Garrido *et al,* 2000). Unfortunately IS900 also appears not to be completely unique to Map, with IS900-like sequences found in non-pathogenic bacteria from animal and human samples (Englund *et al,* 2002; Cousins *et al,* 1999). However, when combined with other Map specific repeating (polymorphism) genetic sequences such as IS1311, F57 and ISMav2 (Mobius *et al,* 2008) the diagnostic efficacy of PCR, PFGE and RFLP is enhanced, allowing the identification and quantification of the number of organisms present for both cellular and spheroplastic forms of Map (Bull *et al,* 2003).

2.5.5 Cell Mediated Immune Response

2.5.5.1 Cell Mediated Cytokine Expression and delayed Hypersensitivity testing

Current diagnostic tests available to determine a CMIR to Map infection include the intradermal Johnin test, with a sensitivity and specificity of 74% and 84% respectively (Robbe-Austerman 2006) and measurement of increased genetic expression of pro-inflammatory cytokines using assays for Interferon gamma (IFN-γ) (Burrells *et al,* 1998; Perez *et al,* 1999; Manning *et al,* 2003;Stewart *et al,* 2004; Storset 2006), the interleukins 1, 4, 6, 8 and 10 (IL-1, IL-4, IL-6, IL-8, IL10) (Coussens *et al,* 2004) and Tumour Necrosis Factor alpha (TNFα)(Aho *et al,* 2003; Alzuherri *et al,*1996; Begara-McGorum *et al,* 1998*;* Robbe-Austerman *et al,* 2006). Cytokine assays provide opportunity to assess the genetic up- or down-regulation occurring from Map infection, with in-vitro cell culture being used to determine this expression at a cellular level. However, cytokine assays are not reliable in younger animals (Huda *et al,* 2003) nor are they Map specific, with other disease processes also increasing expression (Davies *et al,* 2004; Rogas-Caraballo *et al,* 2014), suggesting cytokine assays have a limited place in the diagnosis of Map infection in ruminants and humans and need to be interpreted carefully and in conjunction with other supporting diagnostic tests.

2.5.5.2 Histopathology - Local Alimentary Tract Infection.

The CMIR to Map infection occurs early in the disease and is considered to be the most efficient form of immune response for controlling mycobacterial infections, with the initial prevention of Map penetrating the ileal mucosa and progression to deeper tissues and establishing infection within the local lymphatics (Clarke 1997; Perez *et al,* 1999; Stabel

2006; Kurade and Tripathi 2008; Dennis *et al,* 2011, Kruger et al 2015). Infection with Map stimulates a local CMIR within the alimentary tract lymphatics, terminal jejunal and ileal mucosa (Peyer's Patch), where circulating monocytes become immobilised at sites of inflammation and morphologically change into phagocytes or epithelioid macrophages. These recruit other epithelioid macrophages, producing an epithelioid macrophage micro-granuloma, which is considered to be a Map specific pathological lesion that can be determined histopathologically (Perez *et al,* 1996, Buergelt *et al,* 1996; Kurade *et al,* 2004; Reddacliffe *et al,* 2004). In some animals, Map phagocytosis produces an unregulated CMI expression of pro-inflammatory cytokines, encouraging an over recruitment of additional macrophages with subsequent formation of the characteristic histopathological lesions of Johne's disease with coalesced sheets of epithelioid macrophage dominant granulomas, packed with IS900 and IS1311 PCR positive intracellular AFO bacilli and blunted ileal mucosal villi (Papadimitrou *et al,* 1971; Adams 1976).

At necropsy, gross CMIR lesions, considered to be collectively pathognomonic for clinical Johne's disease in ruminants, can be identified, and include terminal jejunal / ileal granulomatous enteritis, lymphadenitis (enlarged mesenteric lymph nodes) and lymphangitis (cording) accompanied with thickening and formation of permanent corrugations of the ileal mucosa (Carrigan and Seaman 1990; Whitlock and Buergelt 1996; Greig 2000; Kurade *et al,* 2004).

Currently, histopathology is considered the gold standard diagnostic test for identifying animals clinically affected by Map (Kurade *et al,* 2004). In human patients with Crohn's disease a standardised intestinal histopathological scoring system exists, the Crohn's disease activity index, >150 or <150 (active or inactive scores) which enables measurement of the progressive nature of this disease and enables objective assessment of therapeutic efficacy studies in these patients utilising serial endoscopic biopsy examination (Seghal and Koltun

2010). In sheep a similar standardised method to classify the progression of these pathological lesions from clinically normal sheep through to those with Johne's disease has been developed, albeit from necropsy, enabling animals to be classified objectively into six main groups (Perez *et al,* 1996). Classification is determined by the histopathological assessment of tissues infected by Map, recording the number of epithelioid macrophage micro-granulomas, their positions relative to organised lymphoid tissue such as the ileal Peyer's patch and the number of AFO present. Animals in the first category have no histopathological evidence of Map infection. These animals are either un-infected by Map or have latent Map infection (Whitlock and Buergelt 1996). The next category includes animals where the ileal epithelioid macrophage micro-granulomas are non-invasive, discrete and walled off. This Type 1 lesion is the most common lesion found in sheep and often occurs in young animals recently infected by Map (Juste *et al,* 1994; Perez *et al,* 1996; Kurade *et al,* 2004), although they have also been recorded in adult sheep (Perez *et al,* 1996). In older sheep, these lesions are unlikely to be an active infection and may either represent ewes recently exposed to Map or ewes that have recovered from previous Map infection (Gilmour *et al,* 1978; Reddacliff *et al,* 2004; Dennis *et al,* 2011). Type 1 lesions are considered to be the precursors for Type 2 lesions, with the change in classification representing a progression of the CMIR to a more diffuse, less encapsulated lesion which then further evolves to a more invasive Type 3a ileal lesion. Sheep within these three histopathological categories are often referred to as being sub-clinically affected with no overt signs of clinical disease and have normal production parameters recorded (Thompson *et al,* 2002; Morris *et al,* 2006; McGregor *et al*, 2015). In sheep, all Type 3 lesions irreversibly progress through to clinical Johne's disease (Clarke 1997; Perez *et al,* 1999) with Type 3a progressing to Type 3b or Type 3c ileal lesions (Carrigan and Seaman 1990; Juste *et al,* 1994; Whitlock and Buergelt 1996; Perez *et al,* 1996; Dennis *et al,* 2011).

Within the Perez classification, Type 3c ileum lesions differ from the epithelioid macrophage dominant Type 3a and 3b lesions with epithelioid macrophages and AFO being less common, replaced by a lymphocytic dominant granulomatous CMIR and subsequent enteritis. As such a parallel classification for Types 3b and 3c exists with the terms multibacillary or lepromatous (many AFO/organisms, macrophage dominant expression of the CMIR) or paucibacillary or tuberculoid (few AFO / organisms, lymphocytic expression of the CMIR) used respectively (Buergelt 1978; Perez *et al,* 1996).

Factors that appear to influence the CMIR to Map infection include the initial oral dose, with artificial challenge studies having mixed results regarding the effect of high or low dose exposure (Begg and Whittington 2008). High doses (>5 x 10^8 organisms) were considered necessary (Reddacliff and Whittington 2003), although a more recent study has found that a majority of animals become infected irrespective of the initial oral dose, with histopathology occurring proportionately to dose rate (Delgado *et al,* 2012). The strength of this local ileal CMIR from this initial infection appears to be affected by the age of the animal with older animals mounting a larger and more effective CMIR than young animals. In older sheep, the Map infection is limited to discrete small granulomas within the ileal Peyer's Patch (Type 1 lesions). However, in young animals Map appear to penetrate further into the intestinal mucosal tissues with epithelioid micro-granulomas appearing as more diffuse, Type 2, ileal lesions (Juste *et al,* 1994; Perez *et al,* 1996; Gwozdz *et al,* 2000; Delgado *et al,* 2012). In these studies it is hypothesised that this difference in apparent CMI maturity comes from previous sensitisation of the ileal mucosa to Map infection with older animals having greater opportunity to develop this efficiency and therefore possess a greater capacity to resist Map infection (Shield 1983). Further support of this hypothesis comes from artificial vaccination studies where resilience to Map infection and the ability to wall off infection and prevent progression into deeper tissues occurs in lambs vaccinated against Map with discrete focal

ileal micro-granulomatous lesions forming compared to the more diffuse and penetrating lesions recorded in unvaccinated lambs (Brotherston *et al,* 1961b; Juste *et al,* 1994; Gwozdz *et al,* 2000).

2.5.5.3 - Histopathology - Systemic Map Infection

Infection with Map and the development of Johne's disease is primarily considered to be an enteric disease with histopathology found within the alimentary tract. Historically, there have been few recordings of histopathology outside the alimentary tract, and until fairly recently, systemic Map infection had not been investigated or identified. The exception to this statement would be the large number of studies where Map has been found within mammary tissue, with passive and active shedding into milk recorded in many different ruminant species both with and without clinical Johne's disease and also in humans with Crohn's disease (Doyle 1954; Sweeney *et al,* 1992; Nasser *et al,* 2000, 2004; Grant 2001; Gao *et al,* 2002; Manning *et al,* 2003; Singh and Vihan 2004; Slana *et al,* 2008).

Systemic infection has been recorded in many different species and identified in organs distant to the alimentary tract. In naturally infected goats with clinical Johne's disease these include the spleen, mediastinal lymph nodes, liver, uterus, testes, lung, skeletal muscle, kidney, mammary gland and milk (Manning *et al,* 2003; Singh and Vihan 2004). In cattle disseminated Map infection has been recorded in the spleen, popliteal lymph nodes, mammary glands (Hasonova *et al,*2009), diaphragmatic skeletal muscle (Alonso-Hearn *et al,* 2010), cheek skeletal muscle (Meadus *et al,* 2008; Okura *et al,* 2011), uterus and foetuses (Kopecky *et al,* 1967; Sweeney *et al,* 1992; Buergelt and Williams 2003; Whittington and Windsor 2007), kidney, liver, blood (Buergelt *et al,* 1978; Juste *et al,* 2005; Mutharia *et al,* 2010), semen (Larsen and Kopecky 1970), supra-mammary lymph nodes and milk (Sweeney *et al,* 1992; Miller *et al,* 1996; Corti and Stephan 2002; Gao *et al,* 2002; Ayele *et al,* 2005;

Grant 2006; Pinedo *et al,* 2008; Eltoth *et al,*2009; Gill *et al,*. 2011; Khol *et al,* 2012). Systemic Map infection has also been documented in wildlife involving the testes, uterus, placenta, and milk of rabbits (Judge *et al,* 2006). In primates acid fast bacilli in clinically affected Macaque monkeys were identified in tissues such as the spleen, kidney, liver, and bone marrow (McClure *et al,* 1987). Disseminated Map infection has also been identified in humans with Crohn's disease, with Map detected in breast milk (Nasser *et al,* 2000), and blood (Naser *et al,* 2004; Scanu *et al,* 2007; Bentley *et al,* 2008).

In sheep, the progression of Map infection into deeper tissue structures including the lymphatics occurs, with Map isolated more frequently from mesenteric lymph nodes than terminal ileum in Map infected individuals (Dennis *et al,* 2011). Systemic infection occurs in both naturally and artificially infected sheep, and has been identified in the lungs, liver, hepatic lymph nodes (Juste *et al,* 1994; Greig 2000; Gwozdz *et al,* 2000; Kurade et a 2004; Bower *et al,*. 2011), blood (Gwozdz *et al,* 1997, 2000; Begg *et al,* 2005; Juste *et al,* 2005; Coehlo *et al,* 2008; Reddacliff *et al,* 2010, Bower *et al,* 2010, 2011; Stewart *et al,* 2004), uterus (Lambeth *et al,* 2004), mammary gland, milk (Lambeth *et al,* 2004), pre-scapular and pre-femoral lymph nodes, and peripheral skeletal muscle of the fore and hind limbs (Nelli *et al,* 2008; Reddacliff *et al,* 2010).

The aetiology for the systemic spread of Map from the alimentary tract to distant organs and the prevalence of this event in naturally infected is currently unknown. Likewise, in artificial challenge studies, Map specific liver lesions have been recorded in sheep although it appears that this is very different to natural systemic infection in that systemic infection can occur in young animals without clinical disease (Juste *et al,* 1994; Begg *et al,* 2005; Buergelt and Whitlock 2003; Kurade *et al,* 2004; Bower *et al,* 2010; Stewart *et al,* 2004).

In naturally infected sheep, systemic Map infection appears to occur in adult animals in the later stages of the disease and once animals are clinically affected by Map. In these animals Map disseminates from the alimentary tract and invades distant organs such as the liver. In artificially infected sheep a similar occurrence has been recorded with liver lesions identified in animals where clinical Johne's disease was induced from artificial oral dosing (Begg *et al,* 2005; Kurade *et al,* 2004). However there also appears to be another well recorded systemic event where the reverse process also appears to occur in artificial challenge studies, with Map having an innate tropism for the alimentary tract despite inoculation sites being from outside the digestive tract. In these challenge studies, it appears that Map has the ability to travel from the point of inoculation to the alimentary tract with the development of pathology within the ileum and associated lymphatics recorded. These studies include the administration of Map via intra-nasal and tracheal, aerosol (Kluge *et al,* 1968; Merkal *et al,* 1968; Robbe-Austerman 2007;Eisenberg 2013), intra-tonsil (Gwodz *et al,* 2000; Begg *et al,* 2005), intravenous (Kluge *et al,* 1968; Merkal *et al,* 1968; Larsen *et al,*, 1977; Tripathi and Kolhe 2007), sub-cutaneous (Larsen *et al,* 1977) and intra-peritoneal (Tripathi *et al,* 2007; Stabel *et al,* 2009). Currently there is no information available on how this event occurs nor is there information on the aetiology and prevalence of systemic Map infection after artificial oral challenge.

2.5.5.4 - Hepatopathology – Map Specific Lesions

The liver is the first organ to receive blood from the alimentary tract via the portal vein and as such is the first organ to receive absorbed toxins or bacteria originating from this tract with subsequent development of histopathological lesions. There are many diseases where hepato-pathology occurs from systemic exposure to toxins or bacteria or parasites, including

biliary hyperplasia from ingestion of the sporidesmin toxin produced by the fungi *Pithomyces chartarum* (Facial Eczema), (Di Menna *et al,* 2009), bacterial hepatitis (*Campylobacter fetus fetus* ; *Fusobacterium necrophorum*; *Pasteurella multocida*; *Corynebacterium pseudotuberculosis; Salmonella typhimurium*) and eosinophilic hepatitis from parasitic migration (*Meuleris capillaris; Dictyocaulis filaria; Fasciola hepatica*). In human patients with leprosy, the liver is the second most affected organ with histopathological micro-granulomas present (Chen *et al,* 1976). Similar findings were recorded in cattle both with clinical and sub-clinical Johne's disease, with liver biopsy first being proposed as a potential diagnostic test for Johne's disease in this species (Buergelt *et al,* 1978). Murine experimental challenge models have also found histopathology and culture from ileal and liver samples to be a useful diagnostic tool for evaluating the therapeutic efficacy of vaccines against Map infection and have potential to screen large numbers of artificially infected animals for relatively little cost (Shin *et al,* 2006). In a review of experimental challenge models for Johne's disease, it was implied that mice were the only species where liver histology had a diagnostic role to play in identifying Map infection (Hines *et al,* 2007). However, this may not be the case, and the use of liver histopathology as a diagnostic tool to identify Map infection in sheep is addressed in Chapters five and six.

The dissemination of Map from the alimentary tract to the liver has been recorded (Buergelt *et al,* 1978; Juste *et al,* 1994; Gwozdz *et al,* 2000; Burrells *et al,* 1998; Kurade *et al,* 2004; Hines *et al,* 2007; Bower *et al,* 2010, 2011;) with two potential bacteraemic routes, including either passing from the infected ileal tissues directly into the portal vein or from the mesenteric lymphatics into the venous circulation to the heart via the cisterna chyli located within the abdomen (Felinski *et al,* 1964; Hines *et al,* 1987). The CMIR to systemic Map infection in the liver has not been investigated although micro-granuloma formation has been recorded (Beurgelt *et al,* 1978; Manning *et al,* 2003; Kurade *et al,* 2004; Begg *et al,* 2005)

and as such an opportunity exists to measure this response via liver biopsy histopathology. Identification of Map specific lesions within the liver would enable the occurrence of systemic Map infection in naturally and artificially infected sheep to be readily determined and would provide an ante-mortem diagnostic test to identify sheep where not only Map bacteraemia is occurring but may also provide information on whether systemic infection has occurred previously. Currently, there is no information recorded on whether systemic Map infection produces a Map specific hepatic CMI lesion (Chapter five and six).

2.5.6 – Spectral Imaging – Spectral Signatures.

Hyper-spectral imaging is currently used successfully across many different disciplines including pastoral science where the remote assessment of crop and pasture cover analysis and plant speciation can be achieved from satellite imagery using visible light to near infrared (Vis-NIR) cameras (Asner *et al,* 1998; Raun *et al,* 2001; Irisami *et al,* 2009; Pullanagari *et al,* 2011, 2011a, 2012a). The meat industry currently utilises spectroscopic techniques on animal tissue samples to record protein and fat composition by Vis-NIR or from white light fluorescence reflectivity measuring the excitation-emission matrix (EEM) of a sample (Prieto *et al,* 2006; Wold *et al,* 2011; Karoui and Blecker 2011). Animal tissue quality can also be objectively measured, with unique spectral signatures identified for meat character such as steak tenderness, with different cuts of meat having different spectral reflectivities (Naganathan *et al,* 2008). Advantages of spectral imagery include low input costs, non-invasive collection of data and real time recording making it an ideal candidate for use in the diagnostic testing of diseases where changes occur within tissues. Visible changes include CMI, where the normal structure of the tissue is altered through the recruitment of macrophages and other inflammatory cells. This is traditionally identified visually by

microscopic examination and the recording of histopathological changes which in many diseases have a unique histopathological signature or pathognomonic characteristic. Hyperspectral analysis identifies changes in the Vis-NIR emission with computer generated algorithms developed to identify the hyperspectral signature of tissues. This is the fundamental principle of spectral analysis of tissue (Lu and Fei 2014) and has been used successfully in developing spectral indices for identifying different plant diseases (Mahleim *et al,* 2013) and in human studies identifying pre-cancerous stages of epithelial carcinoma using a light emitting probe and recording changes in epithelial reflectivity (Backman *et al,* 2000; Muller *et al,* 2002) There have been very few studies where liver tissue has been examined using spectral analysis for the identification of disease processes. In one intervention study in rats, hyperspectral imaging of the liver was undertaken via ante-mortem fibre optic probe. Successful assessment of liver fibrosis and cirrhosis was achieved with diagnostic sensitivity and specificity of 86% and 98% respectively (Nazeer *et al,* 2015).

Systemic infection with Map produces hepatic pathology including micro-granulomatous lesions in cattle and sheep (Buergelt *et al,* 1978; Juste *et al,* 1994; Burrells *et al,* 1998; Kurade *et al,* 2004; Bower *et al,* 2011) and muscle wasting. These hepatic or skeletal muscle changes may have a distinct frequency of reflectivity that may be identified by hyperspectral imaging cameras. The identification of hyperspectral signature for Johne's disease in sheep, using hyperspectral imaging, has never been recorded and as such there may be an opportunity to establish a new real time diagnostic test that could be used at time of slaughter to identify animals with systemic Map infection. Currently the prevalence of hepatic or skeletal muscle lesions from systemic Map infection is unknown (Chapter five). Likewise, no data exists on whether Map infection of these tissues has a unique spectral signature and if so, there is no information for the possible aetiology of this signature. This is addressed in Chapter seven.

2.6 Longitudinal Intervention Studies

Methodology and Standardisation

In a review undertaken by Begg and Whittington (2008), there were 97 publications available on experimental artificial Map infection in animals. In this review Begg and his co-authors concluded that the interpretation of results from these intervention studies has historically been difficult with many different time points' recorded, different experimental design and methodologies used, often without replication, producing data sets that in many cases are difficult to interpret or compare. One criticism of some of these studies was that the duration of the study was dictated by a pre-set outcome and as such the result may have been misleading and may have been very different had the study duration been extended and animals been allowed to recover from Map exposure. There have been few studies that have recorded this recovery phase or resilience to Map infection. Additionally, many of the outcomes of these studies had been determined from a single sampling time point, such as histopathology at necropsy, and as such may not be truly representative with serial sampling suggested as having greater potential for providing more informed measurement of the immune responses occurring post Map exposure and providing a more accurate assessment of the cost of recovery. There have been few longitudinal studies where serial histopathology samples have been collected (Dennis *et al,* 2011; Mackintosh *et al,* 2011*;* Kruger *et al*, 2015). Similar critiques have been recorded with study design and the non-standardised methodology used for the recording of results being blamed for the inability of many Map studies to be compared with any significant outcome achieved (Whittington and Sergeant 2001, Nielsen and Toft 2007).

The overriding conclusion from these reviews was a need to develop a model of artificial Map infection in order to mitigate these confounding variables, enabling the standardisation of data recorded (Hines *et al,* 2007; Begg and Whittington 2008). Several key areas were identified for consideration in such a model including; ensuring the duration of the study is long enough to measure the full range of possible immune responses post exposure to Map; Identifying the strain type of Map prior to inoculation using PCR IS900 and IS1311 RFLP sequences to confirm the strain being used has known virulence in the study target species; Recognising that strain Types 1 and 2 produce different immunological responses in sheep or cattle (Stewart *et al,* 2006) and that these responses are influenced by the geographical location and an appropriate strain type should be used in the target species in that location for that study (Cousins *et al,* 2000, Whittington *et al,* 2001; Stevenson *et al*, 2009). Standardising the source of Map, using quantified in-vitro harvested colonies from a bank of registered paratuberculosis strain types versus Map harvested from homogenated diseased tissue; recognising in-vitro strains are often less virulent than Map sourced from naturally diseased tissue (Stewart *et al,* 2004); Ensuring the artificial infection route is representative of natural infection with oral dosing the only route used; appreciating the oral Map dose rate and dosing interval appears to be proportionally related with higher doses of Map organisms at shorter interval producing greater immune responses and development of clinical disease (Reddacliff *et al,* 2003; 2004; Delgado *et al,* 2012) and that recovery and resilience to Map infection may be influenced by both (Mackintosh *et al,* 2011).

Recently, guidelines have been proposed for improving the robustness of data sets collected for longitudinal challenge studies with specific reference to methodology and study design (Nielsen *et al,* 2011). Recommendations include the structured recording and reporting of twenty five key information points with the purpose of not only increasing the accuracy and

robustness of the data collected but also increase the compatibility of this data with other independent studies (Gardner *et al,* 2011).

2.6.1 Serial Histopathological Sampling

Resilience, or the ability to continue to produce despite being infected by Map, occurs with infection in a majority of animals failing to progress to clinical disease. Quantification of resilience is difficult with current diagnostic tests unable to identify all infected animals or those with latent infections (Buergelt *et al,* 1996; Nielsen and Toft 2008). Therefore the cost of resilience and the ability of a Map infected animal to recover from infection has yet to be quantified, with losses only recorded as being attributable to deaths from clinical disease (Thompson *et al,* 2002; Morris *et al,* 2006; Bush *et al,* 2006, 2008; McGregor *et al*, 2015).

The recovery from Map infection is most likely due to the CMIR (Perez *et al,* 1997, 1999; Stabel 2006) and is a dynamic process with ileal lesions reported to resolve over time (Dennis *et al,* 2011). Histopathological classification is recognised as the most accurate diagnostic test to identify animals affected by Map (Kurade *et al,* 2004) and is currently the only diagnostic test available that can identify Type 1, 2, or 3a ileal lesions and identify sub-clinically affected animals. Therefore, quantification of the sub-clinical effects of Map infection on production parameters such as blood albumin, live weight or body condition will require histopathological assessment of the ileal tissues. The accurate interpretation of data from single point sampling can be difficult and subject to error from confounding variables (Begg and Whittington 2008). Determining the production cost of sub-clinical Map infection using histopathology would therefore require a longitudinal study where serial sampling of ileal and mesenteric lymphatic tissues is undertaken throughout. There are few longitudinal studies where serial histopathological assessment of alimentary tract tissues have been

undertaken and none utilising hepatic tissues. In one study, serial ileal and mesenteric lymph node samples were collected from lambs, naturally exposed to Map, over a 36 month period with a proportional relationship recorded between ileal lesions and the progression towards clinical disease (Dennis *et al,* 2011). Evidence of recovery to Map infection was recorded with ileal lesions resolving despite initially having more advanced, diffuse and mucosally invasive Type 2 micro-granulomas. This study enabled the assessment of the transition of ileal lesions post Map exposure allowing resilience and recovery to Map infection to be identified and objectively measured in sub-clinically affected sheep.

In human medicine, a similar application of this diagnostic test is undertaken on patients clinically affected with Crohn's disease where serial intestinal biopsy samples are collected and compared with previous samples to asses response to treatment with reductions in the intestinal disease activity index used as a measure of therapeutic efficacy (Seghal and Koltun 2010). As such, there is also potential for analogous therapeutic efficacy studies to be undertaken in sheep for anti-mycobacterial drugs or vaccines. However, serial biopsies from the alimentary tract is cumbersome, time consuming, requires competent veterinary skill and adequate surgical and aftercare facilities (McConnel *et al,* 2004). There is a need to develop new diagnostic tests that are less invasive, using more accessible organs that may allow identification of animals infected with Map and identify animals that are either developing clinical Johne's disease or recovering from Map infection. This is the subject for chapters five and six in the thesis.

2.7 Research Aims

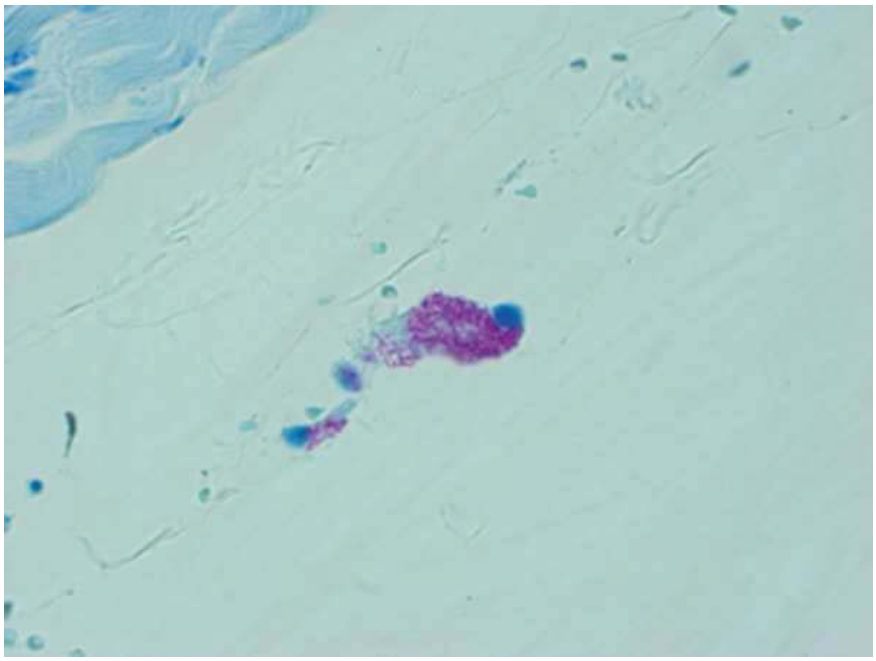
Systemic Map infection has been recorded in many different species, with tissues outside the alimentary tract harbouring Map organisms. There is potential risk of human exposure to these organisms from the consumption of infected meat, although the level of risk is

unknown as no information exists on the prevalence of systemic infection in New Zealand sheep. This is further exacerbated by the inability of currently available diagnostic tests to identify animals where systemic Map infection has occurred and as such quantification of this risk is unlikely without the development of novel diagnostic tests.

The aims of this thesis were to; a/ Identify whether systemic Map infection occurs in New Zealand sheep and whether meat, destined for the human food chain, from these animals contains Map organisms; b/ Determine which class of stock pose the greatest risk for human exposure; c/ Develop a novel diagnostic test, hepatic histopathology, for the identification of animals where systemic Map infection has occurred; d/ Determine whether hepatic biopsy samples provide an adequate amount of tissue to also identify animals where systemic Map infection has occurred; e/ Identify the time frame for the formation and resolution of hepatic CMI post systemic Map infection using serial liver biopsy histopathology in a longitudinal intervention study; f/ Compare natural and artificial Map infection models and the potential use of serial hepatic biopsies in assessing therapeutic efficacy of anti-mycobacterial drugs such as thalidomide; g/ Record whether recovery from systemic Map infection occurs in sheep and if so identify the cost of this on commonly measured production parameters such as live weight and body condition score; h/ Determine whether the liver or skeletal muscle from sheep with Johne's disease has a hyperspectral signature that can be identified using real time hyperspectral analysis.

Chapter Three

Detection of *Mycobacterium avium* subspecies *paratuberculosis* in skeletal muscle and blood of ewes from a New Zealand sheep farm.



Acid fast organisms inside a macrophage in ovine skeletal muscle

3.1 Abstract

AIM: To determine whether viable *Mycobacterium avium* subsp. *paratuberculosis* (Map) is present in skeletal muscle and blood in ewes with and without clinical Johne's disease.

METHODS: A total of 51 mixed-aged ewes, in poor body condition, from a farm with a history of clinical Johne's disease, were culled and examined at necropsy. BACTEC radiometric culture was performed on samples of skeletal muscle from the biceps femoris, mononuclear cells in peripheral blood, and ileum. Histological sections and Ziehl-Neelsen (ZN) stained impression smears of terminal ileum and mesenteric lymph nodes were examined.

RESULTS: Eighteen of 21 ewes with confirmed clinical Johne's disease were culture positive for *Mycobacterium avium* subsp. *paratuberculosis* (Map) from sites peripheral to the alimentary tract, comprising 15 from skeletal muscle and 13 from blood. Five of 30 ewes that did not have clinical Johne's disease were also culture positive from sites peripheral to the alimentary tract, with four from skeletal muscle and one from blood.

CONCLUSION: The prevalence of Map infection of skeletal muscle and blood in ewes with clinical Johne's disease was 71% and 62%, respectively, and in unaffected ewes was 13% for muscle and 3% for blood.

CLINICAL RELEVANCE: Skeletal muscle and blood are potential sources of exposure of humans to Map, and the risk appears higher from sheep with clinical signs of Johne's disease.

KEY WORDS: *Mycobacterium avium* subsp. *paratuberculosis, Johne's disease, Skeletal Muscle, blood, BACTEC Radiometric Culture, Crohn's Disease.*

Abbreviations

AFO	Acid Fast Organisms
Blood	Mononuclear cells in peripheral blood hereafter referred to as blood
Map	*Mycobacterium avium* subsp. *paratuberculosis*
ZN	Ziehl-Neelsen

3.2 Introduction

Many sheep flocks in New Zealand are infected with Map, although the annual incidence of clinical cases is estimated at less than 1% (Unpublished West 1997; Unpublished Brett 1998), with clinical cases of Johne's disease within a flock thought to represent a small proportion of animals actually infected (Verdugo *et al,* 2014). *Mycobacterium avium* subsp. *paratuberculosis* is the bacterial aetiological agent for Johne's disease in domestic ruminants. In sheep, infection is believed to occur early in life, with clinical disease usually seen in older animals (Whitlock and Buergelt 1996). In sheep, infection with Map can result in granulomatous inflammation of the ileum, with associated lymphangitis and mesenteric lymphadenitis resulting in a loss of body condition and ultimately death (Perez *et al,* 1996). With a wide range of known host species, including primates, all with similar disease pathogenesis, and its constant environmental presence, Map may also have public health implications for humans (McClure *et al, 19*87; Beard *et al,* 2001). For many years it has been known that Mycobacteria are present in a proportion of human patients with Crohn's disease with Map identified as the most likely species (Chiodini *et al,* 1984; McFadden *et al,* 1987; Sanderson *et al,* 1992). Whilst an association between this bacterium and some cases of Crohn's disease in humans is now generally accepted (Feller *et al,* 2007), a causal link has

yet to be established. Potential sources of infection for humans include contaminated water supplies, contact with animals and their environments (Whittington *et al*, 2004, 2005; Pickup *et al,* 2005, 2006) and consumption of animal products such as unpasteurised fresh milk from cows and goats (Singh and Vihan 2004; Ayele 2005; Pinedo *et al*, 2008).

Several studies in ruminants with clinical Johne's disease have shown Map to be disseminated throughout tissues that are peripheral to the alimentary tract, such as the uterus and fetus of sheep (Lambeth *et al,* 2004), cattle (Whittington and Windsor 2009) and deer (Van Kooten *et al,* 2006).

Disseminated infection can occur in cattle with or without clinical signs of Johne's disease, with Map having been detected in diaphragm (Alonso-Hearn et al, 2010), liver, kidney, heart, lung, popliteal and supra-mammary lymph nodes (Sweeney *et al* 1992; Antognoli *et al*, 2008), and in skeletal muscle (Mutharia *et al,* 2010). Manning *et al,* (2003) reported Map from the liver, kidney, lung, uterus, testes and skeletal muscle from the hind limb of goats. Recently, Reddacliff *et al,* (2010) demonstrated disseminated infection in sheep with Map cultured from peripheral lymph nodes and skeletal muscle of the hind limb in both animals with clinical Johne's disease (59%) and those considered not to have the disease (4.5%).

This study describes the first detection of Map in skeletal muscle and blood from confirmed clinical and sub-clinical cases of Johne's disease in mixed-age ewes from a farm in New Zealand.

3.3 Materials and Methods

In July 2008, and 2009, 21 and 30 mixed-age ewes, respectively, were selected from a single farm with a history of clinical Johne's disease in the ewe flock. Johne's vaccination was not practiced on this farm. Ewes, consisting of East Friesian, Romney and Highland cross breeds, were culled due to their poor body condition score, with an average score 1.5 / 5 (Jefferies 1961).

Whole blood samples were taken from the jugular vein from each ewe into sterile lithium heparinised 10ml vacutainers (Franklin Lakes NJ USA) for bacterial culture. Ewes were then euthanised via captive bolt and exsanguinated for necropsy. Gross pathology suggestive of Johne's disease included the presence of lymphatic cording (lymphangitis) over the serosal surface of either, the ileum, caecum, distal jejunum or mesenteric lymphatics, enlarged mesenteric lymph nodes (lymphadenitis) including the ileo-caecal lymph node and mucosal thickening with corrugations of the distal jejunum and ileum. Samples of ileo-caecal valve, terminal ileum, terminal jejunum, ileo-caecal and 1st mesenteric lymph nodes were stained with haematoxylin and eosin (HE) and examined histologically with epithelioide macrophage granulomata formation, blunting of the mucosal villi recorded with ileal tissues classified into Type 1, 2, 3a, 3b or 3c lesions (Perez *et al*, 1996). Histological specimens and mucosal impression smears were also stained with Ziehl Neelsen (ZN) with the identification of acid fast organisms (AFO) indicative of Johne's disease. Further confirmation was undertaken using BACTEC Radiometric culture and Polymerase Chain Reaction (PCR) analysis, identifying the presence of Type 1 strain Map infection. Histopathology is considered the 'gold standard' diagnostic test for identifying sheep with or without clinical Johne's disease (Kurade *et al*, 2004) and as such ewes were classified as those with clinical Johne's disease if they were in poor body condition and had Type 3b or 3c histopathological lesions (Perez *et al,* 1996). Ewes in poor body condition without these histopathological lesions were classed as not having clinical Johne's disease.

Tissues were collected, using sterile instruments, with the biceps femoris muscle sample collected prior to opening the abdomen and submitted for BACTEC radiometric culture. The mesenteric lymph node(s) and samples of ileum were then collected and divided, with half fixed in 10% neutral buffered formol saline and submitted for histology, and the remaining tissue submitted for fresh impression smear, ZN staining and the fresh ileum for

Radiometric BACTEC culture and PCR. Ewes were classified as systemically infected when Map had disseminated away from the alimentary tract and was identified in blood or skeletal muscle.

The association between ewes with and without Johne's disease and the likelihood of systemic MAP infection occurring was determined using ODD's Ratio and Fisher's two tail test.

3.4 Culture Procedure

A total of 8 ml of heparinised blood was lysed with 40 ml of sterile tris ammonium chloride (pH 7.2, 0.2% tris and 0.75% ammonium chloride). The lysed cells were centrifuged at 3500g for 20min at 5^0C and the supernatants discarded. The pellets were re-suspended in 1ml of sterile H_2O. BACTEC 12B (Becton Dickinson, Sparks, Maryland, USA) vials supplemented with the antibiotic cocktail PANTA (Becton Dickinson, Sparks, Maryland, USA), 1 ml of sterile egg yolk and mycobactin (Allied Monitor, Fayette, Missouri, USA) were inoculated with 1ml of the re-suspended pellet. For culturing tissues, approximately 1gm of sample was homogenised in 20ml of sterile water in a Stomacher (Coleworth). The tissue suspension was filtered through sterile cheese cloth and 10ml of the homogenate was decontaminated with an equal volume of 0.75% cetyl pyridinium chloride for 40 min, centrifuged at 3,500 g for 20 min. The supernatant was discarded and the pellet re-suspended in 1ml of sterile water and 0.75 ml inoculated into a supplemented BACTEC 12B vial. The inoculated vials were incubated at 37^0C and read at weekly intervals with positive Map cultures and growth indices determined from the time taken (days) to measure the presence of $^{14}CO_2$. The time required for detection of growth is inversely proportional to the number of Map organisms present in the inoculum sample (Lambrecht *et al*, 1988). Vials registering a positive growth index were examined for the presence of ZN staining organisms and also inoculated onto blood agar for the presence

of microbial contamination. *Mycobacterium avium* subsp. *paratuberculosis* was identified on the basis of the presence of slow growing clumps of ZN staining organisms that were mycobactin dependent. Randomly selected isolates from 2008 and 2009 were examined using a PCR that can differentiate "ovine" and "bovine" strains of Map (Collins *et al,* 2002).

3.5 Results

Skeletal muscle, blood and ileum culture results, with both years combined, are presented in Table 3.1. All positive Map cultures examined by PCR were confirmed to be the "ovine" type. Twenty one ewes were confirmed, by histopathology, as having clinical Johne's disease. Of those, 15 (71%) had positive cultures from the biceps femoris muscle, and 13 (62%) from blood. Ten of these ewes had positive cultures from both tissues. In total, 18 of 21 ewes with clinical Johne's disease had systemic Map infection.

Table 3.1 The number of cultures positive for *Mycobacterium avium* subsp. *paratuberculosis* from samples of biceps femoris muscle, blood and ileal tissue in ewes confirmed histologically with (n=21) and without (n=30) clinical Johne's disease (JD), collected from one farm in 2008 and 2009

Tissue	**Clinical Johne's Disease Status**	
	With JD (n=21)	**Without JD (n=30)**
Blood and muscle	10	0
Muscle only	5	4
Blood only	3	1
Neither muscle or blood	3	25
Ileum	21	27

Five of 30 ewes without clinical Johne's disease had Map cultured from the ileum and had systemic infection of Map with four positive cultures from the biceps femoris muscle and one from blood. All 21 ewes with and 27 of 30 ewes without clinical Johne's disease had positive cultures from the ileum. The likelihood that ewes with clinical Johne's disease have systemic Map infection compared to ewes without was determined as OR=30 (95%CI 6.3-142, $p<0.001$).

Data for time to positive growth index (positive culture) is presented in Table 3.2. The median time to positive culture for skeletal muscle for ewes both with and without clinical Johne's disease was 28 days and for blood 27 days. For the ileum samples, culture was positive for ewes with and without Johne's disease at medians of ≤ 7 days and ≥ 14 days, respectively.

Table 3.2 Median Time for Map Radiometric BACTEC Positive Culture (days)

Tissue	**Clinical Johne's disease status**	
	Yes	**No**
Muscle	28	28
Blood	27	27
Ileum	≤ 7	≥14

3.6 Discussion

In this study, Map infection was found to be common for ewes both with and without clinical Johne's disease. Positive ileum cultures were found in 48 of 51 ewes despite 30 ewes not having clinical Johne's disease. Disseminated Map infection of the skeletal muscle and blood was also found in ewes both with and without clinical Johne's disease.

Despite the high prevalence of Map in the ileum, not all infected ewes had Map in their peripheral tissues. Positive cultures were from 71% and 13% from the biceps femoris muscle from ewes with and without clinical Johne's disease respectively and 62% and 3% of blood samples from ewes with and without Johne's disease respectively. Overall, 18 of 21 clinically affected ewes (86%) and 5 of 30 ewes without signs of clinical JD (17%), respectively, had Map infection disseminated in the skeletal muscle and/or blood.

The procedures for culturing blood used in this study are similar to those recently described by Bower *et al,* (2010), although there were differences that may have enhanced the sensitivity, isolation and growth rate of Map in liquid culture. In this study, blood cultures were performed on mononuclear cells in peripheral blood after red blood cells had been removed by osmotic shock and centrifugation, the blood samples were not frozen prior to culture, there was no decontamination with cetyl pyridinium chloride, and the antibiotic supplement PANTA was added to the BACTEC 12B vials.

Estimates of the number of Map present in samples can be obtained from BACTEC 12B cultures, with a linear and negative relationship between the numbers of Map present and the time taken to record a positive growth index . Positive growth indices of ≤ 7 days indicate very high levels of Map, with levels above 1 x 10^6 Map / gram tissue previously recorded (Lambrecht *et al,*1988; Reddacliff *et al*, 2003).

In this study, 48 of 51 ewes were detected as infected with Map. All ewes were grazed closely together for up to a week prior to necropsy and were exposed to high levels of faecal-contaminated pasture. This may have resulted in a potentially false inference of infection per se in clinically unaffected ewes, since positive ileal culture alone may represent a 'passing through effect' that has been previously reported to occur from faecal cultures in cattle (Sweeney *et al*, 1992, Wu *et al,* 2007, Pradhan *et al*, 2011). Ewes with and without Johne's disease had different ileum tissue growth indices, being ≤ 7 days and ≥ 14 days, respectively.

This indicates that ewes with clinical Johne's disease had higher numbers of Map present per gram of ileum tissue than those without. Ewes with high numbers of ileal Map present were statistically more likely to be systemically infected and have Map cultured from peripheral tissues including skeletal muscle and blood than ewes without clinical Johne's disease (OR= 30, $p<0.001$).

Map bacteraemia was present in 13 of 21 clinically affected ewes, and one of the non-affected ewes, with positive growth indices of 27 days for both groups, suggesting the presence of very low numbers of Map in the blood. Map infection of peripheral tissues has been observed in other livestock species with clinical Johne's disease, though the prevalence of Map infection in limb skeletal muscle has rarely been documented. This study cultured Map and visualized phagocytised AFO within macrophages, circulating through the muscle tissue of ewes with clinical Johne's disease, supporting the recent findings of Reddacliff *et al,* (2010) demonstrating skeletal muscle destined for human consumption may be a source of exposure of humans to Map (Figure 3.1).

The significance of Map as a human pathogen is unknown, but limiting human exposure to Map is currently considered a reasonable precautionary measure. However, the findings here should not be overlooked as the potential risk to human health from the consumption of Map from retail animal products exists. (Eltholth *et al,* 2009) To reduce this risk, the pasteurisation of raw milk, and the cooking of meat prior to consumption, will significantly reduce the numbers of Map and produce a product that may be regarded as being microbiologically safe (Gao *et al,* 2002; Mutharia *et al,* 2010; Whittington *et al,* 2010). However, these practices are subject to limitations based on the initial concentration of Map in the milk or meat sample and the temperature and duration of cooking (Grant *et al,* 2002; Whittington *et al,* 2010). Further mitigating steps include the banning of the submission of clinically diseased animals for slaughter in New Zealand, although clinically normal culled

ewes destined for human consumption may contain Map in their tissues albeit in low numbers. This study has shown viable Map to be present systemically in adult ewes from an infected flock, with and without clinical Johne's disease. In previous studies, the prevalence of Map infection in New Zealand flocks was recorded to be at least 68% (Heuer *et al,* 2011; Verdugo *et al,* 2014). These findings suggest that peripheral skeletal muscle from clinically normal adult sheep, from a majority of New Zealand flocks, may be a potential source of Map for humans. The overall risk of human infection with Map from animal products is yet to be established and further studies are warranted to determine the prevalence of Map infection in tissue, especially skeletal muscle, from sheep flocks across New Zealand.

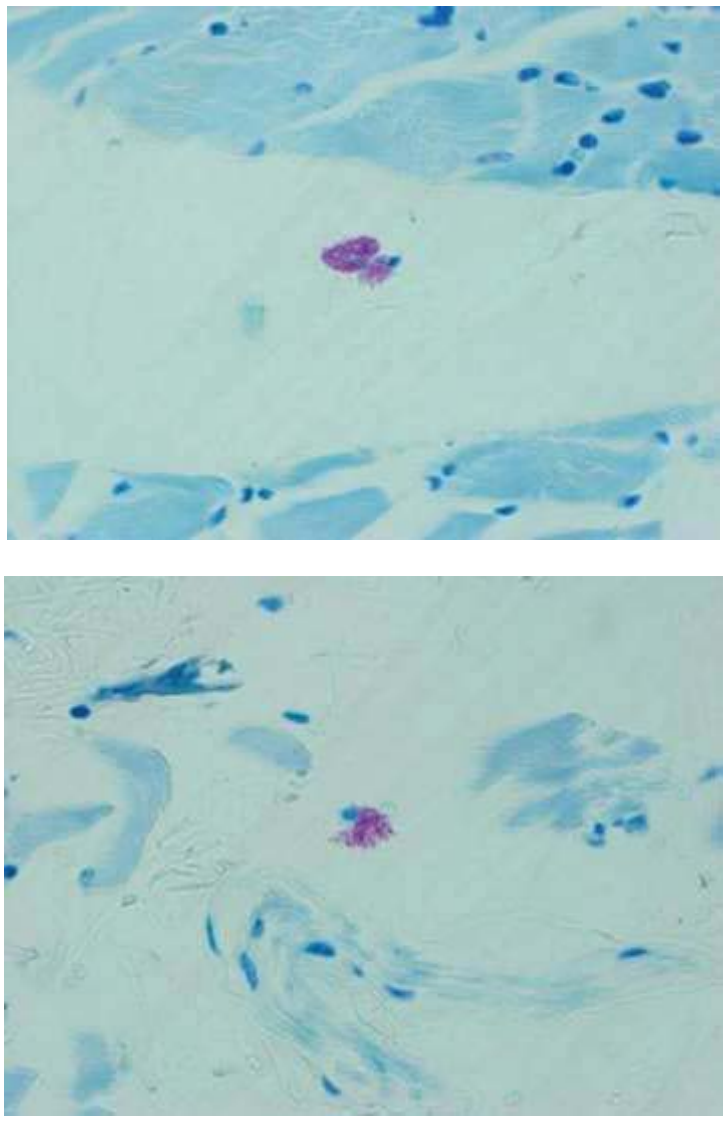


Figure 3.1 Macrophages with AFO within ovine biceps femoris muscle (x100, ZN).

3.7 Acknowledgements.

We would like to thank Geoff de Lisle, Gary Yates and the team at Ag Research laboratory in Wallaceville, New Zealand, for performing the cultures and PCR diagnostics and the C Alma Baker Trust for financial support.

Chapter Four

The prevalence of disseminated *Mycobacterium avium* subsp. *paratuberculosis* infection in tissues of healthy ewes from a New Zealand farm with Johne's disease present.

Mixed age ewes

4.1 Abstract

AIM: To determine the prevalence of disseminated *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection in healthy ewes in a flock with a history of clinical Johne's disease.

METHODS: Twenty four healthy ewes, from a large sheep and cattle farm with a history of clinical Johne's disease in the ewe flock, were selected from a mob of 400 ewes, with body condition scores greater than 3/5, by drafting out the first 24 animals entering the yards, euthanised, blood sampled, and examined at necropsy. These animals were from the same farm as those in Chapter three. BACTEC radiometric culture for Map was performed on samples of faeces, ileum, mesenteric lymph node, biceps femoris muscle and mononuclear cells in peripheral blood. Serum antibody ELISA tests were performed. Histological sections and Ziehl Neelsen (ZN) stains of impression smears of ileum and mesenteric lymph node were examined for pathological lesions characteristic of Johne's disease and acid fast organisms (AFO). Indirect quantification of Map was estimated, using BACTEC radiometric growth indices measuring the time taken for the production of $^{14}CO_2$.

RESULTS:

No histological evidence of Johne's disease or AFO were found in the ileum and mesenteric lymph nodes. Twelve of the 24 ewes (50%) had Map cultured from the ileum (n=6) and/or mesenteric lymph nodes (n=8) while none had Map cultured from the faeces, biceps femoris muscle or blood mononuclear cells. One of the 12 Map culture positive ewes was serum ELISA positive. The culture growth rates in liquid medium suggest low numbers of Map were present in the tissues of the culture positive ewes.

CONCLUSION: Fifty per cent of clinically healthy ewes exposed to Map within a Johne's infected flock were Map culture positive in the ileum and / or mesenteric lymph node(s), while the ELISA was positive in (n=1) 8% of those animals. There was no faecal shedding of

Map and no Map was cultured from skeletal muscle or from blood mononuclear cells suggesting that systemic Map infection in healthy mixed age ewes without clinical Johne's disease may be uncommon.

CLINICAL RELEVANCE:

ELISA serology detected 1 of 12 ewes infected with Map whilst none were detected from faecal BACTEC radiometric culture, suggesting biosecurity measures used to control the spread of Map may be of limited use. Map was not cultured from blood or skeletal muscle, indicating that meat from healthy ewes, from farms where Johne's disease is present, is an unlikely source of Map exposure for humans. Further research is warranted to establish the prevalence and dissemination of Map in tissues outside the alimentary tract of healthy ewes from farms throughout New Zealand where Map is present.

Key Words

Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis* (Map), prevalence, sheep, New Zealand, skeletal muscle, blood, ileum, mesenteric lymph node, BACTEC radiometric culture

Abbreviations and definitions

AFO **acid fast organism**

Blood **Mononuclear cells in blood hereafter referred to as blood**

dCGI1000 **days taken for Map Cumulative Growth Index to reach 1000**

Map ***Mycobacterium avium* subsp. *paratuberculosis***

Systemic Infection **Positive culture of Map from skeletal muscle and/or blood**

ZN **Ziehl Neelsen**

4.2 Introduction

Clinical Johne's was first diagnosed in New Zealand sheep in 1952 (Williamson and Salisbury 1952) and despite efforts to control this disease, the aetiological agent, *Mycobacterium avium* subsp. *paratuberculosis* (Map) is now widespread, with a recent survey showing at least 68% (CI = 95%,60.3-75.4%) of flocks to be infected (Heuer *et al,* 2011). Map infection without clinical disease occurs in ruminants throughout the world, with infection occurring in farmed deer (estimated 45% prevalence) (Stringer *et al,* 2009), cattle (Whitlock and Buergelt 1996) and sheep (Bower *et al,* 2011). However, only a small proportion of sheep infected with Map experience clinical disease (West *et al,* 1997(unpublished); Brett *et al,* 1998(unpublished)) with the mean annual prevalence of clinical cases of Johne's disease estimated to be 0.13% in New Zealand infected flocks (Verdugo *et al,* 2014). The infection of domesticated ruminants with Map occurs by the ingestion of contaminated colostrum and milk from infected dams (Sweeney *et al,* 1992; Singh and Vihan 2004), the ingestion of milk, pasture and/or water contaminated with faeces (Gill *et al,* 2011**),** as well as transplacentally in sheep (Lambeth *et al,* 2004), cattle (Whittington and Windsor 2009) and deer (Van Kooten *et al,* 2006). Map has a prolonged environmental survival in soil and water (Whittington *et al,* 2004, 2005; Pickup *et al,* 2005) and is shed continuously in the faeces, from both clinically and sub-clinically affected ewes, with daily excretion rates of up to 1×10^{10} organisms per ewe as recorded by Whittington *et al,* 2000a. In sheep with clinical Johne's disease, disseminated Map infection of tissues outside the alimentary tract such as blood and hind limb skeletal muscle has been demonstrated to occur in 18 of 21 (86%) of cases with and 17% of cases without Johne's disease (Smith *et al, NZVJ,* 59, 240-3, 2011). In contrast to that study which used low body condition score sheep and clinically affected sheep, this pilot study aimed to investigate the

prevalence of disseminated Map infection in clinically healthy mixed-age ewes from the same New Zealand flock.

4.3 Materials and Methods

In July 2010, 24 mixed-age East Friesian, Romney and Highland cross ewes were selected from a flock of 9,000 mixed age ewes on a 4,000 hectare farm that had a history of clinical Johne's disease affecting <1% of the flock (West *et al,* 2001(unpublished), (Figure 4.1). Johne's vaccination was not practiced on this farm because of the low clinical incidence. These ewes, were from the same farm as those in Chapter Three and were selected from a mob of 400 ewes, with body condition scores greater than 3/5, (Jefferies 1961), by drafting out the first 24 animals entering the yards, euthanized, blood sampled, and examined at necropsy. Prior to euthanasia they were clinically examined, showed no signs of clinical illness and were considered to be healthy. They were euthanised on farm using a captive bolt and exsanguinated immediately prior to necropsy. Faecal samples were collected for Map BACTEC radiometric culture. Whole blood for culture and and blood samples for serum collection were taken from the jugular vein from each ewe into sterile 10ml lithium heparinised and 10ml plain vacutainers (Franklin Lakes NJ USA) for Map BACTEC radiometric bacterial culture and serum ELISA (Institut Pourquier, Montpeliier, France/Idexx Laboratories Inc, OD+ >0.35). Samples of ileum, ileo-caecal valve, mesenteric lymph nodes and biceps femoris muscle, were collected using sterile surgical instruments. A subsample of each was fixed in 10% phosphate buffered formol saline for histology with fresh impression smears taken for ZN staining while another subsample was collected for Map BACTEC radiometric culture.

For histopathology, fixed tissues were processed routinely and sections were cut at 3 μm and stained with H&E and ZN. Ewes were assessed as not having clinical Johne's disease as

described by Smith *et al,* (2011), based on gross pathology at necropsy, the failure to find AFOs in impression smears of fresh ileum or mesenteric lymph node, and from the absence of characteristic histopathological lesions (Perez *et al,* 1996).

The prevalence of Map culture positive ewes was determined using Collett's (1999) model on binary data.

Figure 4.1 Healthy mixed age ewes, body condition score > 3/5, drafted onto concrete pens immediately prior to slaughter.

4.4 Culture procedure

The procedure used for culturing Map from blood in this study was that described previously (Smith *et al,* 2011). In summary, the red cells were lysed using tris ammonium chloride, and then centrifuged. The pellets were re-suspended in sterile water and inoculated into a BACTEC 12B vial (Becton Dickinson, Sparks, Maryland, USA) supplemented with

antibiotics and mycobactin. The tissue samples were homogenised in sterile water and decontaminated with cetyl pyridinium chloride and then centrifuged. The re-suspended pellet was inoculated into BACTEC 12B vials as above. The vials were incubated at 37^0C and examined weekly for the production of $^{14}CO_2$. The number of Map per gram of tissue was estimated indirectly from time taken to record a positive cumulative growth index (CGI) of $^{14}CO_2$ production from the BACTEC 12B cultures with a negative linear relationship between the number of Map present in the culture, and the time taken (days) for the CGI to reach 999 (dCGI1000) (Lambrecht *et al,* 1988; Reddacliff *et al,* 2003).

Vials registering a positive growth index were examined for the presence of AFO and also inoculated onto blood agar for the presence of microbial contamination. Map was identified on the basis of the presence of slow growing clumps of AFO that were mycobactin dependent. Randomly selected isolates were examined using a PCR that can differentiate "ovine" and "bovine" strains of Map (Collins *et al*, 2002).

4.5 Results

All 24 ewes were confirmed as not having clinical or pathological evidence of Johne's disease. The median body condition score was 3 (range 2.5-3.5/5). Twelve (50%) were culture positive either in the ileum (n=6), mesenteric lymph node(s) (n=8) or in both tissues (n=2). PCR identified the Type 1 or ovine strain of Map. The prevalence of positive Map culture was 50% (CI95%, 29-71%), (Collett 1999). No Map was cultured from the faeces, biceps femoris muscle or from mononuclear cells in blood. One of the 12, Map culture positive ewes was ELISA positive, with Map cultured from the ileum.

The mean time to reach dCGI1000 for the culture positive samples for the ileum and mesenteric lymph nodes was 26(SD2.42) and 43(SD7.56) days respectively.

4.6 Discussion

In New Zealand the screening of sheep for Map infection to establish prevalence within flocks has not been undertaken. This pilot study was undertaken to investigate the prevalence of Map infection within a New Zealand sheep flock with a history of clinical Johne's disease. Healthy mixed age ewes from this farm had a 50% prevalence of Map infection in the ileum and/or mesenteric lymph nodes but no Map was detected in faeces, muscle or blood mononuclear cells. Data is consistent with that from Australia (Whittington and Sergeant 2001) showing clinically healthy ewes can be infected with Map and carry this bacterium within their alimentary tract and mesenteric lymph nodes.

In live sheep, current diagnostic tests, as shown with the ELISA and faecal culture in this study, are unable to identify all animals that are sub-clinically infected with Map. Currently, BACTEC radiometric culture of faeces is considered the best surveillance test for identifying sheep infected with Map (Whittington *et al,* 2000b). However, with Map sequestered in the mesenteric lymph node(s) and with no faecal shedding in these ewes after infection, the effectiveness of this surveillance tool has limitations, especially in many sub-clinically infected sheep.

In a recent epidemiological survey, the prevalence of Map infection in New Zealand sheep flocks was estimated to be at least 68% (Heuer *et al,* 2011). Combined with a prolonged environment survival time, a wide host range, and the general lack of diagnostic efficacy at detecting Map infection in the live animal, the effectiveness of biosecurity control protocols for Map infection in New Zealand may be questionable.

The dissemination of Map infection to tissues from ruminants destined for human consumption has interest, since there is an association between Map and at least some cases

of Crohn's disease in humans (reviewed by Feller *et al*, 2007; Uzoigwe *et al,* 2007; Behr and Kapur 2008). This will be of much greater importance if causation is established. Thus the potential risk to public health from exposure to Map in animal products should not be overlooked (Eltholth *et al,* 2009) since it is now accepted that food may be a source of human exposure to Map (Ransom 2010)). Map has been cultured from the blood and skeletal muscle of sheep with and without clinical Johne's disease in Australia (Reddacliff *et al,* 2010) and recently in New Zealand (Smith *et al,* 2011). In the latter study, the mixed age ewes both with and without clinical Johne's disease were from the same farm as the healthy ewes in this study, were in poor body condition (<1.5 / 5) and were grazed as a single group for five days prior to slaughter. Map was cultured from the ileum in all ewes with Johne's disease and 27 / 30 ewes without at time of slaughter with the first observation of a positive growth index recorded at 14 days. Map was also cultured from the hind limb skeletal muscle in 18 of 21 ewes with clinical Johne's disease and 5 of 30 ewes without suggesting that skeletal muscle from cull ewes without Johne's disease may also be a source of Map for humans. By contrast, in the current study, healthy ewes were selected and penned on concrete, with no mixing or co-grazing immediately prior to slaughter. Map was cultured from the ileum and mesenteric lymph node samples from 12 of 24 ewes with none cultured from skeletal muscle. This suggests that there may be little risk of exposure to Map from the consumption of skeletal muscle from ewes considered to be healthy with body condition scores >3 / 5 and those that are kept isolated from clinically affected ewes prior to slaughter.

The factors that may contribute to the presence of Map in the skeletal muscle of sheep without Johne's disease are currently unknown. We hypothesise that this may be attributable, at least in part, to ewe grazing and management practices immediately prior to slaughter. Hence, further research is justified to validate the prevalence of Map infection in muscle tissues of ewes without Johne's disease and whether there is potential for

disseminated distribution of Map by the pre-slaughter co-grazing of healthy sheep with shedding cohorts, posing a potential public health risk.

In this flock, it appears that 50 % of healthy mixed age ewes may have been infected with Map, yet fewer than 1% go on to develop clinical disease in their lifetime. The circumstances that trigger clinical disease in some sheep are still unknown. This pilot study has indicated that further research is warranted to more robustly establish the prevalence of Map infection in healthy ewes in flocks throughout New Zealand. The ewes reported here were from one farm only, and while there was no evidence of Map in meat, bacterial challenge will be different between farms, and sheep from other farms may show a different manifestation and distribution of infection.

If the skeletal muscle finding from this study is consistent across a larger sample of the sheep population in New Zealand, it would be a positive finding for the sheep meat industry since it would confirm that the risk of the organism transferring to humans from meat from healthy sheep managed appropriately prior to slaughter was minimal. Thus, further Map prevalence studies will be critical in underpinning strategies aimed at reducing or eliminating the risk of Map entering the human food chain via sheep meat. This should permit the sheep meat industry to respond in an informed manner to concerns among consumers and other stakeholders in the marketplace.

4.7 Acknowledgements

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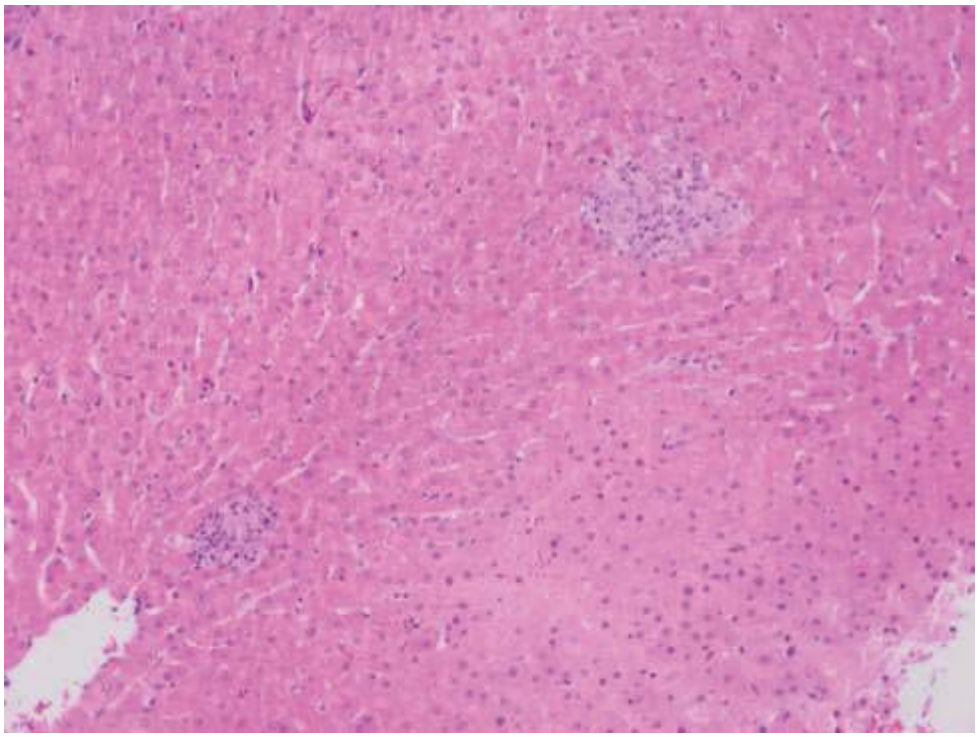
Chapter Five

Liver Biopsy Histopathology for Diagnosis of Johne's Disease in Sheep



Hepatic Epithelioid Macrophage Micro-granulomas (HEM)

5.1 Abstract

Sheep with Johne's disease develop epithelioid macrophage micro-granulomas, specific to *Mycobacterium avium* subsp. *paratuberculosis* (Map) infection, in the terminal ileum, mesenteric lymph nodes, and organs distant to the alimentary track such as the liver. The aims of this study were to determine whether liver pathology was present in ewes affected by Map and whether liver cores provide adequate tissue for this potential diagnostic marker. One hundred and twenty-six adult, low body condition ewes were euthanized, necropsied, and underwent simulated liver biopsy. Ileal lesions typical of Map were found in 60 ewes. Hepatic epithelioid micro-granulomas were observed in all ewes with Type 3b (n = 40) and 82% (n = 11) with Type 3c ileal lesions. None were found in ewes unaffected by Map or with Type 1, 2, or 3a lesions. Liver biopsy core samples provided adequate tissue for histopathology with a sensitivity and specificity of 96% (95% confidence interval [CI], 0.87-0.99) and 100% (95%CI, 0.95-1), respectively.

Keywords

diagnostic test, ELISA, hepatic granuloma, hepatic epithelioid macrophage, histopathology, Johne's disease, liver biopsy, *Mycobacterium avium* subsp. *paratuberculosis* (Map), New Zealand, serology, sheep, sensitivity, specificity

5.2 Introduction

The diagnosis of clinical Johne's disease (JD) in sheep is often made post-mortem with examination of gross lesions including cording or lymphangitis of the ileal serosal lymphatics, lymphadenitis, and enlargement of the mesenteric lymph nodes and corrugated

granulomatous thickening of the ileal mucosa with confirmation made histopathologically. In live sheep, available diagnostic tests include faecal culture, Ziehl Neelsen stained faecal smears identifying acid fast organisms (AFO), ELISA, Map-specific DNA using polymerase chain reaction (PCR), identification of Map specific peptides, and measuring the mRNA expression of pro-inflammatory cytokines such as gamma interferon. Sheep with clinical JD have advanced ileal lesions with Map cultured from organs distant to the alimentary tract such as the peripheral lymph nodes, skeletal muscle, and liver as a result of Map bacteraemia and systemic infection (Bower *et al,* 2011; Gwozdz *et al,* 2002; Reddacliff *et al,* 2010; Smith *et al,* 2011). The portal blood system facilitates Map infection of the liver, which is an organ readily accessed using routine biopsy in the live animal. Liver biopsy histopathology may provide an opportunity to identify JD in live sheep without radical surgery. The aims of this study were to determine whether sheep with ileal lesions typical of Map have concurrent hepatic epithelioid macrophage micro-granulomas (HEM), compare the sensitivity and specificity of liver histopathology with ELISA serology for diagnosis of ewes affected by Map, defined as those with Type 3b and 3c ileal lesions (Perez *et al*, 1996) and determine whether liver biopsy samples provide enough tissue for use as a diagnostic test.

5.3 Materials and Methods

Adult, Romney composite mixed breed and mixed age ewes (>3 years old) from seven North Island New Zealand farms, with a history of clinical JD and flock size >3000 ewes, were body condition scored (BCS) over a two year period. Ewes with a body condition score $\leq$1.5 (scale 1–5), (Jefferies 1961), were selected. Vaccination against ovine JD was not practiced on these farms. A subsample of 80 ewes were blood tested for serology using ELISA (Institut Pourquier, Montpelier, France/Idexx Laboratories Inc; New Zealand Veterinary Pathology Ltd commercial diagnostic laboratory, Palmerston North). Ewes were euthanized and a

simulated liver biopsy was performed using a styletted, 3-mm inner core diameter trocar (Shoof

International) with the liver core fixed in 10% buffered formalin (West and Vermunt 1995). Necropsies were then performed, with samples of terminal ileum, including ileo-caecal valve, mesenteric lymph node, and a 200 mm^2 section of liver (1 cm x 2 cm) collected from the dorso-cranial right lobe from all ewes and fixed in 10% buffered formalin.

Histopathology

For histopathology, fixed tissue sections of ileum, mesenteric lymph node, liver, and liver biopsy cores were processed routinely and examined for the presence of granulomatous inflammation and AFO. One section per tissue was cut at 3 μm and stained with haematoxylin and eosin (HE) and Ziehl Neelsen (ZN). For liver biopsy cores, 24 serial longitudinal sections at 3 μm were taken. Alternate sections were discarded and the remainder serially mounted onto 4 glass slides (3 sections per slide) with 6 sections stained using either HE or ZN. Each biopsy section measured approximately 3 mm x 12 mm, with 6 sections providing approximately 200 mm^2 of liver for examination, equivalent to the size of the single section examined from the necropsy liver sample (Figure 5.1). Ewes were classified retrospectively as either not histologically affected (not infected or latently infected) or histologically affected with Type 1, 2,3a, 3b, or 3c ileal lesions (Table 5.1), (Perez *et al,* 1996).

Figure 5.1 Alternate serial liver sections stained with HE from single liver biopsy sample.

Table 5.1 Criteria for classification of lesion types associated with natural paratuberculosis infection in sheep (Perez et al 1996).

Classification	Definition and Histopathological Description
Type 1	Microscopic lesions only with few granulomas in ileum Peyer's patch, none extending into the mucosa of intestine or jejunum at Peyer's patch or between lymphoid tissue of the ileum Peyer's patches. Few granulomas found in regional mesenteric lymph nodes. No acid-fast organism (AFO) found in either ileum Peyer's patch or mesenteric lymph node.
Type 2	Microscopic lesions only. Multiple granulomas extending into lamina propria mucosae and villus of ileum at the Peyer's patch. Non-diffuse with ileum villus integrity maintained. No lesions in mucosa of ileum outside Peyer's patch lymphoid tissue areas. Similar mesenteric lymph node lesions as Type 1. AFO detected in very few granulomas of ileum Peyer's patch.
Type 3	Granuloma lesions present in large numbers within the lymphoid tissue of the Peyer's patch, also extending into the mucosa between Peyer's patches including villi. Macroscopic lesions with 3 different subtypes:
Type 3a	Multiple granulomas extending into mucosa between Peyer's patches and causing the villi to enlarge. Always found in ileum and less frequently in jejunum. Lymphocytic and macrophage infiltration around lymphatic and blood vessels of submucosa and serosa. Granulomas always present in mesenteric lymph nodes. Lymphangitis visible in ileum serosal lymphatics. AFO present in ileum mucosal sections and Peyer's patches.
Type 3b	Diffuse granulomatous enteritis with gross mucosal thickening and corrugation with lymphangitis of serosal lymphatics. Oedematous and swollen mesenteric lymph nodes with lymphatic cording over ileum and jejunum. Peyer's patches lose structure due to macrophage dominant granulomas. The lamina propria and mucosa between Peyer's patches has diffuse enteritis with swelling, blunting, and coalescing of villi in ileum through to jejunum. Submucosa lymphocyte and plasma cell infiltrates are common, lymphadenitis with macrophage thrombi present with infiltration of blood vessels and smooth muscle layers of the ileum and jejunum. Mesenteric lymph node and ileo-caecal lymph node lost structure with multifocal granuloma formation. Numerous AFO throughout mucosal tissues and mesenteric lymph nodes.
Type 3c	Diffuse granulomatous enteritis. Similar lesions to Type 3b although the predominant inflammatory cell is lymphocytic infiltration of the lamina propria of Peyer's patches and areas between the Peyer's patches including the villi with less macrophage infiltration. Formation of mesenteric lymph node Langhans multinucleate giant cells. AFO absent in most tissues or present in small numbers. Macroscopically similar to Type 3b.

Livers were examined for the presence of spherical HEM consisting of epithelioid macrophages with clear and large nuclei, prominent nucleoli, with lightly staining HE foamy vacuolated cytoplasm, with or without acid-fast organisms with some lymphocytes present. Histopathology was performed blinded to the identity of the ewe.

Statistical Analysis

The sensitivity and specificity of liver biopsy histopathology and ELISA as diagnostic tests for ewes with ileal lesions and JD were determined as described by Altman and Bland (1994). The level of agreement between the presence of HEM in liver tissue and either the ileal lesion score or ELISA positivity were determined using Cohen's kappa coefficient (Cohen 1960).

5.4 Results

Data on ileal and liver lesions, AFOs, and ELISA results are presented in Tables 5.2 and 5.3. Sixty ewes had ileum histopathology typical of that caused by Map including 51 classified with Type 3b (n = 40) or Type 3c (n = 11) lesions and 9 classified with Type 1 (n = 5), Type 2 (n = 3), or Type 3a (n = 1) lesions. Hepatic epithelioid macrophage micro-granulomas were observed only in ewes with Type 3b (100%, n = 40) and with Type 3c ileal lesions (82%, 9/11). When present, they were in equal numbers per 200 mm^2 for both the liver and liver biopsy sections, suggesting a uniform lesion pattern throughout the right cranio-dorsal lobe of the liver. No HEM were found in either liver sections or biopsies of any ewe without ileal lesions (n = 66) or ewes with Type 1, Type 2, or Type 3a ileal lesions.
Of the 80 blood tested subsample of ewes, 45 had ileum histopathology comprising 3 Type 1 and Type 2, 1 Type 3a, 29 Type 3b, and 9 Type 3c lesions.

ELISA was positive in 28 of 29 Type 3b and 7 of 9 with Type 3c lesions, 2 of 35 ewes without Map ileal lesions, and the ewe with the Type 3a ileal lesions. Hepatic epithelioid macrophage micro-granulomas were present in all ewes with Type 3b, 8 of 9 with Type 3c lesions, none with Type 1, 2, or 3a ileal lesions or ewes without Map ileal lesions.

Table 5.2 Number of ewes sampled with ileum and liver (biopsy and section) histopathology and identification of HEM and acid fast organisms (AFO).

All Ewes						
Ileum Lesion	No. Ewes	Ileal	Liver	AFO	AFO	AFO
Category		Histology +	Histology +	Ileum +	MLN +	Liver +
No Lesion	66	0	0	0	0	0
Type 1	5	5	0	0	0	0
Type 2	3	3	0	0	0	0
Type 3a	1	1	0	1	0	0
Type 3b	40	40	40	40	37	27
Type 3c	11	11	9	7	3	1
Total	126	60	49	48	40	28

Table 5.3 Comparison between ELISA and HEM detection for identifying ewes with clinical Johne's disease.

Ewes Blood Sampled for ELISA					
Ileum Lesion	No. Ewes	Ileal		Liver	Both ELISA
Category		Histology +	ELISA +	Histology +	& Liver +
No Lesion	35	0	2	0	0
Type 1	3	0	0	0	0
Type 2	3	0	0	0	0
Type 3a	1	1	1	0	0
Type 3b	29	29	28	29	28
Type 3c	9	9	7	8	7
Total	80	39	38	37	35

Neither liver histology nor ELISA were positive in ewes with Type 1 or Type 2 ileal lesions, and HEM were found in all ELISA positive ewes with Type 3b and 3c ileal lesions (Figure 5.2).

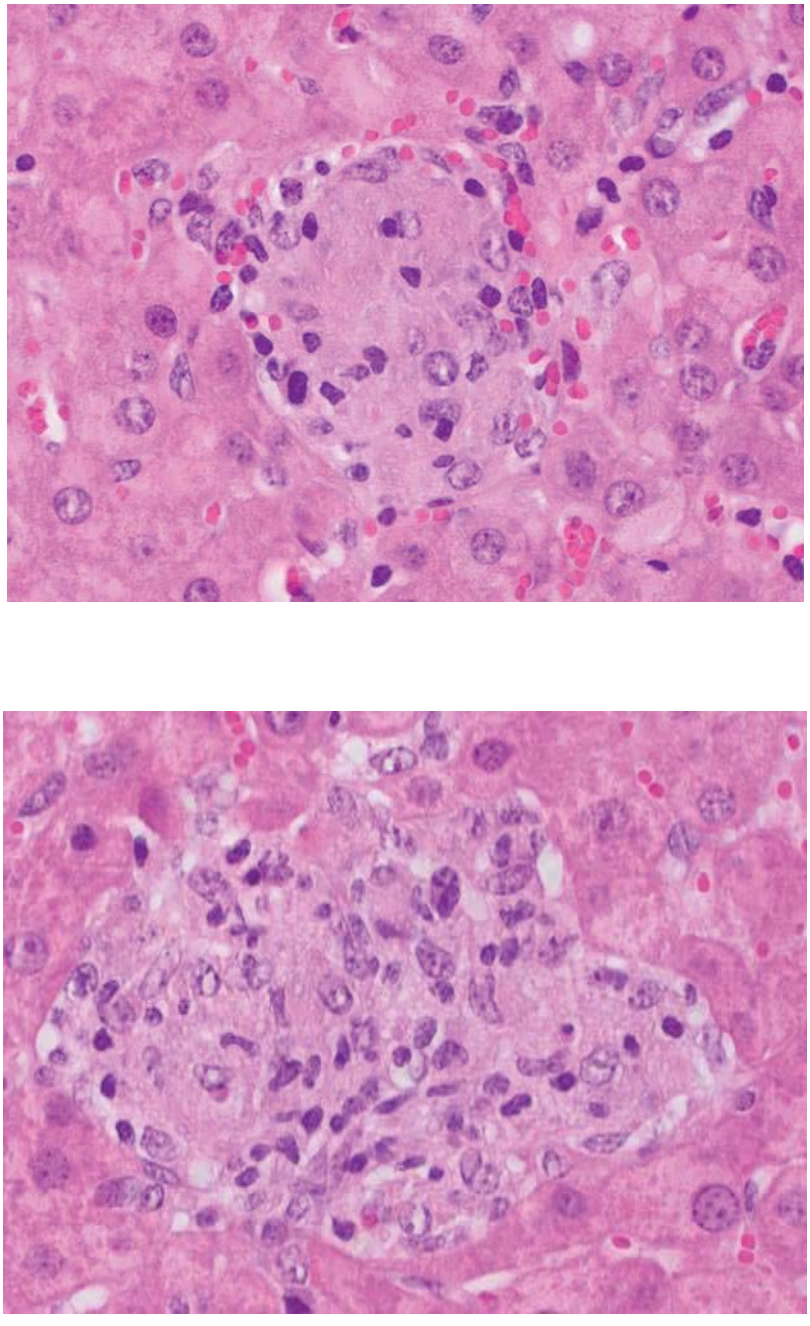


Figure 5.2 Hepatic Epithelioid Macrophage micro-granulomas (HEM), (x 100, HE)

The sensitivity and specificity for liver histopathology (section or biopsy) for predicting the presence of Type 3b and 3c ileal lesions was 96% (95% confidence interval [CI], 0.87-0.99) and 100% (95% CI, 0.95-1), respectively, and for ELISA, 92% (95% CI, 0.79-0.97) and 93% (95% CI, 0.81-0.98), respectively. Almost perfect level of agreement was shown between ileum and liver histopathology (Cohen's kappa = 0.82, P < .001), and a substantial level of agreement was shown between ileum histopathology and ELISA positivity (Cohen's kappa = 0.73, P < .001). Agreement between liver histopathology and ELISA sero-positivity was almost perfect (Cohen's kappa = 0.87, P < .001).

5.5 Discussion

No HEM or hepatic AFO were found in Type 1, 2, or 3a ewes or in ewes without ileal Map lesions, suggesting that portal vein Map bacteraemia may not have occurred. In contrast, bacteraemia and disseminated Map infection appears to have occurred in ewes with Type 3b and 3c ileal lesions, with HEM found in 96% of cases. In these ewes, AFO did occur in HEM (<10/granuloma) although not every granuloma revealed AFO in stained slides (Figure 5.3). The use of immune-histochemistry may have increased the identification of Map within HEM (Delgado *et al,* 2009; Thorensen *et al,* 1994*;* Brees *et al,* 2000) and should be considered in future validation studies. Infrequently, other forms of micro-granulomas without AFO were observed in the liver sections from Type 1, 2, 3a, 3b, and 3c ewes including ones dominated by polymorph neutrophils or lymphocytes, with or without eosinophil infiltration, the aetiology of which is unknown although they may be as a result of possible migrating parasite larvae.

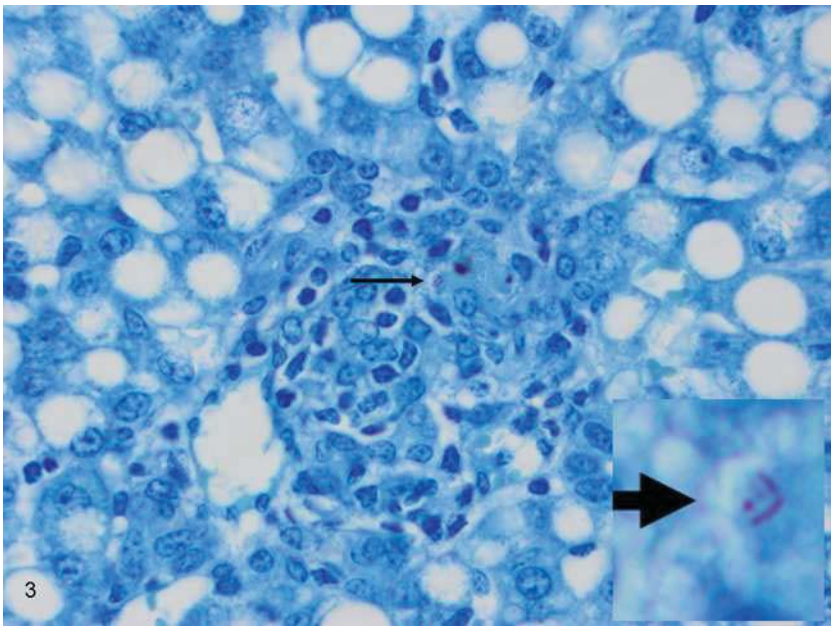


Figure 5.3 Hepatic Epithelioid Macrophage micro-granuloma with AFO (x100, ZN)

Despite the availability of diagnostic tests to identify clinically affected sheep, there are currently none that reliably identify sheep with subclinical JD or where early Map bacteraemia has occurred. Blood culture has little application for diagnosis in early Map infections (Bower *et al,* 2011). ELISA has a wide range for sensitivity and specificity, being 16% to 85% and 95% to 100%, respectively (Nielsen and Toft 2008). It also appears that liver histopathology was unable to identify sheep sub-clinically affected by Map (Type 1, 2, or 3a ileal lesions), although the numbers of sheep in this study, with these lesions, was limited and further research is now required for validation. However, previous longitudinal studies where lambs have been orally dosed with high numbers of Map have found young animals with Map infection disseminated to tissues outside the alimentary tract, including haematogenous spread, with potential Map bacteraemia, prior to the onset of clinical disease (Bower *et al,* 2011; Gwozdz *et al,* 2002; Juste *et al,* 1994). In those studies, where hepatic

lesions were described, no conclusions were made as to their significance Gwozdz *et al*, 2002). Nevertheless, they did demonstrate the occurrence of systemic and disseminated Map infection in young animals and are evidence that Map bacteraemia and histopathological involvement of the liver is not just limited to adult sheep with Type 3b and Type 3c ileal lesions.

Hepatic epithelioid macrophage micro-granuloma lesions do occur in young sheep sub-clinically affected by Map. We therefore hypothesize that in sheep destined to develop clinical JD, an early Map bacteraemia occurs and liver biopsy histopathology may provide a surrogate measure of the progression of ileal lesions in lambs and indirectly describe the relationship between the development of HEM and clinical JD.

The principal conclusions from this study are that there is a high level of agreement between HEM and Type 3b and 3c ileal lesions in ewes, that liver biopsy histopathology in sheep has high sensitivity and specificity for identifying ewes with Type 3b and Type 3c ileal lesions and that liver biopsy cores provide adequate tissue for this diagnostic test in live animals. Liver biopsy histopathology is a potential research diagnostic tool, and while this study has provided a test of concept, further research is now needed to fully validate the diagnostic sensitivity and specificity for sheep with Types 1, 2, and 3a ileal lesions. Further longitudinal study is also needed to establish a timeline for the formation of liver granulomas after oral Map exposure and whether lambs destined to eventually develop clinical JD can be identified in the early subclinical stages using this technique.

5.6 Acknowledgements

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Chapter Six

Hepatic epithelioid micro-granuloma formation, sero-conversion and production cost following artificial oral infection with *Mycobacterium avium* subsp. *paratuberculosis* in lambs.

6.1 Abstract

Aim

The aims of this longitudinal study were to: i) identify the formation of hepatic epithelioid macrophage micro-granulomas (HEM) by serial liver biopsy histopathology and use this as a surrogate measure to record the occurrence of systemic infection; ii) determine the time frame for the formation of HEM; iii) record the humoral response; and iv) quantify the production cost of systemic infection, following artificial infection of lambs with *Mycobacterium avium* subspecies *paratuberculosis* (Map).

Materials & Method

Thirty six, four month old, Map-free Texel/Romney/Finnish Landrace crossed ewe lambs (Highlanders) were selected from a low prevalence Johne's disease farm and randomly allocated to two groups of 18 by selecting odds and evens as the lambs were run up a drafting race. In the challenged group, each lamb was orally dosed, on 10 separate occasions, three days apart, with >1 x 10^9 Type-1 Map organisms, using a homogenate of terminal ileum, jejunum and mesenteric lymph nodes sourced from a ewe from another farm, diagnosed with clinical Johne's disease. The unchallenged lambs were orally dosed on the same occasions with 10mL sterile phosphate buffered saline. All lambs were grazed outdoors, each group on isolated paddocks, on the same farm with pasture covers > 1250kg Dry Matter per hectare (kgDM/Ha), maintaining no opportunity for cross contamination for 820 days. Blood samples were collected before and at 4, 7, 22, 29, 43, 51, 69, 93, 114, 154, 195, 233, 279, 364, 482, 596, 820 days for ELISA testing (positive S/P ≥50). Body condition score (BCS), (Jefferies 1961), and live weights were recorded before and at 51, 114, 154, 195, 233, 338, 364, 394, 450, 482, 519, 596, 729 and 820 days, and liver biopsy samples were collected before and at 51, 114, 154, 195, 233, 364, 482, 596 and 820 days after the date of first oral

Map challenge. Serology was determined by ELISA. Fixed liver biopsy cores were serially sectioned at 3µm with every 3rd section collected for haematoxylin and eosin stain. Nine sections in total were examined with the number of HEM counted over approximately 324mm^2 liver. At 820 days, all ewes were euthanised using captive bolt and necropsied with samples of ileum, ileo-caecal valve, mesenteric lymph node and liver collected for histopathology. Skeletal structures were measured at necropsy using engineering vernier callipers (± 0.1mm) and a tape measure (± 1mm).

Results

Hepatic epithelioid macrophage micro-granulomas were first observed in some animals at day 51, in 17/18 lambs at day 114 and in all lambs at 195 days with a Cohen kappa perfect level of agreement between oral dosing with Map and the formation of HEM (K =1). The number of HEM per biopsy sample increased up to 195 days in 15/18 lambs, and continued to increase in the four lambs that developed clinical Johne's disease, but decreased in others. There were no HEM recorded in 9/14 challenged sheep at 364 days and none in any sheep at 482-820 days. The four lambs that developed clinical Johne's disease were euthanised between 270 and 450 days. In challenged lambs, sero-conversion and ELISA S/P ratios ≥ 50 occurred in 13/18 with positives appearing at 93 days. Peak S/P ratios occurred at 233 days, then subsequently decreased, with a Cohen kappa substantial level of agreement between artificial oral dosing and sero-positivity (K= 0.72) and between the number of animals where HEM formation occurred and sero-positivity (K=0.72). In the unchallenged sheep, no ELISA positives, HEM or clinical disease were recorded.

Live weight loss occurred in 15/18 challenged animals between 51 and 195 days with a mean loss of ¯3.53kg (¯0.5 - ¯8.3kg). Three challenged lambs gained weight over this period averaging 2.77kg (range 0.7 – 3.8kg). The mean weight gain and BCS for the unchallenged

lambs over the same period was 6.37kg and one BCS, respectively, higher than the challenged lambs. Weight loss or reduced weight gain occurred in the challenged lambs as HEM appeared with a Cohen's kappa almost perfect level of agreement (K= 0.89). There was a poor level of agreement between sero-positivity and weight loss (Cohen's kappa = 0.1).

At 364 days the unchallenged lambs were 9.8kg heavier than the challenged lambs., after which both groups had similar growth rates and BCS, with the unchallenged animals being 11kg heavier at 820 days ($p < 0.001$). Post mortem skeletal measurements determined the challenged animals to be smaller than the unchallenged group, with the mean poll to rump length 4% shorter and the length of the 3rd metacarpus, tibia, calcaneous, 3rd metatarsus, and ulna all 5% shorter (ANOVA Microsoft Excel, $p<0.001$). There was no significant difference between the diameters of the 3rd metatarsal or 3rd metacarpal bones.

Conclusion

This study has determined that systemic Map infection may be initiated from artificial oral dosing, and liver histopathology can be used, as a surrogate measure, to identify this event. Hepatic epithelioid macrophage micro-granulomas were identified in all of the challenged and none of the unchallenged lambs. Serial liver biopsy histopathology provided a timeline for the formation of HEM and a surrogate measure of systemic Map infection post artificial oral challenge, with HEM first identified at 51 days, peaking at 195 days, with none present by 482 days. This timeline enables comparisons to now be made between similar timelines for ELISA sero-positivity, live weight and body condition score changes between challenged and unchallenged sheep, allowing an objective method to quantify the effects of systemic Map infection on these production parameters. Positive ELISA S/P ratios followed HEM formation, peaking at 233days, then decreasing along with HEM numbers until none were present at the end of the study suggesting stimulation of the humoral immune response may

require systemic infection and Map bacteraemia. ELISA S/P ratios were poor predictors of weight loss from systemic Map infection.

In contrast, there were two periods of systemic Map infection in this study with animals forming increasing numbers of HEM and losing weight and body condition in both periods. The first period occurred immediately after oral challenge in all lambs and the second period occurred after 195 days in the four lambs that developed Johne's disease suggesting another mechanism may be occurring that also produces systemic Map infection in sheep. After 195 days HEM numbers decreased, suggesting a decrease in systemic infection with weight gain, improved body condition scores and 'self cure' occurring in 14 animals. The number of HEM identified within liver samples, at 195 days may be predictive for the outcome of systemic infection with increasing HEM numbers predictive for the development of Johne's disease (n=4) and decreasing numbers predictive of resolution or recovery (n=14).

Production losses in sheep occur with systemic Map infection and this has a permanent effect on skeletal size and live weight. Serial liver biopsy histopathology enables these losses to be quantified and as such has potential to objectively assess the therapeutic efficacy of drugs, including vaccines, on systemic Map infection. Differences exist between artificial and natural infection and this study suggests artificial infection in lambs may provide a poor model of representation for that recorded in sheep naturally infected by Map and that extrapolation of these results should be interpreted carefully.

Key Words:

Mycobacterium avium subsp. *paratuberculosis*, Map, Johne's Disease, hepatic epithelioid macrophage granuloma, sheep, liver, granuloma, systemic, artificial infection, natural infection, weight, skeletal size.

6.2 Introduction

Systemic infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map) occurs in naturally infected sheep with clinical Johne's disease, with the organism being identified in tissues distant to the alimentary tract including blood (Gwozdz *et al,*1997*;* Smith *et al,* 2011; Bower *et al,* 2011), skeletal muscle (Reddacliff *et al,* 2010; Smith *et al,* 2011), and liver, in hepatic epithelioid macrophage micro-granulomas (HEM), (Smith *et al*, 2014). The formation of HEM indicates systemic spread of Map from the alimentary tract, with the portal vein being the most likely route. In naturally occurring Johne's disease, systemic infection appears to occur once Type 3b or 3c ileal lesions have developed (Smith *et al,* 2014). In these cases the presence of HEM and ELISA sero-positivity were reliable predictors of clinical disease.

Systemic Map infection has also been recorded following artificial challenge and can be induced within a shorter time frame (<9 months), compared with natural infection, when lambs were artificially orally dosed with > 5 x 10^8 Map organisms (Juste *et al,* 1994; Gwozdz *et al,* 2000; Begg *et al,* 2005; Hines *et al*, 2007; Begg and Whittington 2008; Bower *et al,* 2011). There have been no longitudinal artificial challenge studies where the occurrence of systemic Map infection and its consequences have been assessed in sheep using histopathology and the identification of HEM. Serial liver biopsy histopathology and the identification of HEM may provide a surrogate measure for systemic infection and as such may provide a method that may enable quantification of the effects of this infection on production parameters such as live weight and body condition. This may also provide insight into the effects on the humoral immune response and whether HEM identification is predictive of Johne's disease in sheep.

The aims of this study were to use serial liver biopsy histopathology and the identification of HEM to determine the prevalence of systemic Map infection in lambs, establish a time frame for the development of this infection, identify any humoral responses and determine whether this method can be used to quantify the cost of artificial systemic Map infection on production in lambs using body condition score, live weight and skeletal size as production parameters.

6.3 Materials and Methods

This was a longitudinal study over 820 days in which sheep were orally challenged with Map. Serial liver biopsies were taken for histopathology to record HEM, blood was collected for serology, weights and body condition were recorded, and at the termination of the study, post-mortem measurement of skeletal size was undertaken. All procedures using animals were approved by the Massey University Animal Ethics Committee, approval number 99/2011).

Lambs for the study were sourced from a Manawatu sheep farm considered in a previous study to have a low risk of Map infection based on parallel pooled faecal culture and ELISA negative screening (Verdugo *et al*, 2014). Prior to selection of lambs for this study, faecal samples from 200 mixed age ewes were collected from a mob of 3000 and submitted for BACTEC radiometric culture (AgResearch, Wallaceville Infectious Disease Unit, Wellington, New Zealand) as 10 pooled samples. At 60 days no Map colonies were recorded. In February, 36, four month old, Texel/Romney/Finnish Landrace crossed ewe lambs (Highlanders) from the tested mob were selected by the farm owner based on equivalent live weights (36kg) and transported to clean concrete pens at the large animal teaching unit, Massey University. Here they were randomly allocated to two groups of 18 by running up a race and drafting into odds and evens in the order of appearance at the drafting gate. They were all orally dosed with 'Matrix' (Merial Ancare, New Zealand), a triple

combination anthelmintic (Levamisole HCL(40g/L) / Abamectin (1g/L) / Oxfendazole (22.7g/L) and injected sub-cutaneously with 2mL 'Lamb Vaccine' containing tetanus antitoxin and pulpy kidney toxoid Coopers Schering Plough, New Zealand). The average live weight was 36kg (range 35.5-36.5kg) with all lambs having the same body condition score (3/5), (Jefferies 1961). All lambs were blood sampled (jugular vein, 10mL heparinised vacutainer, Franklin Lakes NJ USA), with fresh samples centrifuged at 3000rpm for 10 minutes. Plasma was submitted for ELISA (Paratuberculosis test kit, Pourquier, Idexx Test Laboratories Inc) with S/P ratios ≥50 considered positive. All lambs were liver biopsied through the right 11^{th} intercostal space following local anaesthetic (5mL Lignocaine 2%, Phoenix New Zealand Ltd) , and using a 3mm inner core diameter liver biopsy probe (Shoof, New Zealand) as described by West and Vermunt (1995). Biopsy samples were immediately fixed in 10% formol phosphate buffered saline and prepared as described by Smith *et al,* (2014).

The unchallenged lambs were then orally dosed with 10ml phosphate buffered saline and the challenged lambs with 10ml ileal, jejunal and mesenteric lymph node homogenate containing approximately 1 x 10^8 Map / mL, sourced from a euthanised adult ewe from a different Manawatu farm, that was confirmed, by histopathology, to have clinical Johne's disease as described by Perez *et al,* (1996) and Smith *et al,* (2011), and infected by Type 1 strain Map, as confirmed by BACTEC radiometric culture and PCR. The ewe with Johne's disease was euthanised and the homogenate prepared one week prior to beginning the challenge doses. Homogenate preparation involved the collection of samples of ileum, terminal ileum, terminal jejunum and the mesenteric lymph node chain where gross pathology for clinical Johne's disease was evident. These intestines were opened longitudinally with the luminal contents removed through washing in water. Homogenates were made by adding 1kg of this fresh ileal, jejunal and mesenteric lymph node tissue, to 1.4L sterile phosphate buffered

saline, homogenated (IKA Ultra-Turrax T25, Janke and Kunkel Labortechnik, Staufen) producing 10 sub-samples of 240mL each. All homogenated samples were subsequently frozen at -20'C. For each treatment session, 240mL homogenate was thawed in warm water with a 2mL sample collected for quantitative analysis using BACTEC Radiometric culture and titration plots of the time taken (days) to cumulatively reach 1000 colony units, (cumulative growth index, CGI), or dCGI1000, (Wallaceville, Infectious Disease Unit, Wellington, New Zealand) as described by Reddacliff *et al,* (2003). The dCGI1000 for the Wallaceville homogenate and that used to dose the lambs was 13 days and 12 days respectively equating to approximately 1 x 10^8 organisms per ml of homogenate for both samples (Figure 6.1). For each 2mL thawed sample, approximately 1 x 10^8 organisms per mL were recorded using the dCGI1000, (Table 6.1 and Figure 6.1), with PCR identifying IS900 sequences for Type 1, sheep strain of Map

Table 6.1 Estimated number of Map organisms/mL in the homogenates used to dose lambs in this study using the Wallaceville laboratory standardised growth index curve.

Accession Number	Dilution	Date cultured	Days to cumulatively grow 1000 colony units (dCGI1000)	Final Predicted No. of Map / mL Homogenate
W12/0142	1/1000	15/02/2012	13	~10^8
W12/0142	1/1000	15/02/2012	12	~10^8
W12/0233	1/500	6/03/2012	11	~10^8
W12/0289	1/1000	14/03/2012	14	~10^8
W12/0289	*1/1000*	14/03/2012	14	~10^8
W12/0296	1/1000	16/03/2012	11	~10^8
W12/0340	1/1000	27/03/2012	11	~10^8
W12/0357	1/1000	5/04/2012	12	~10^8

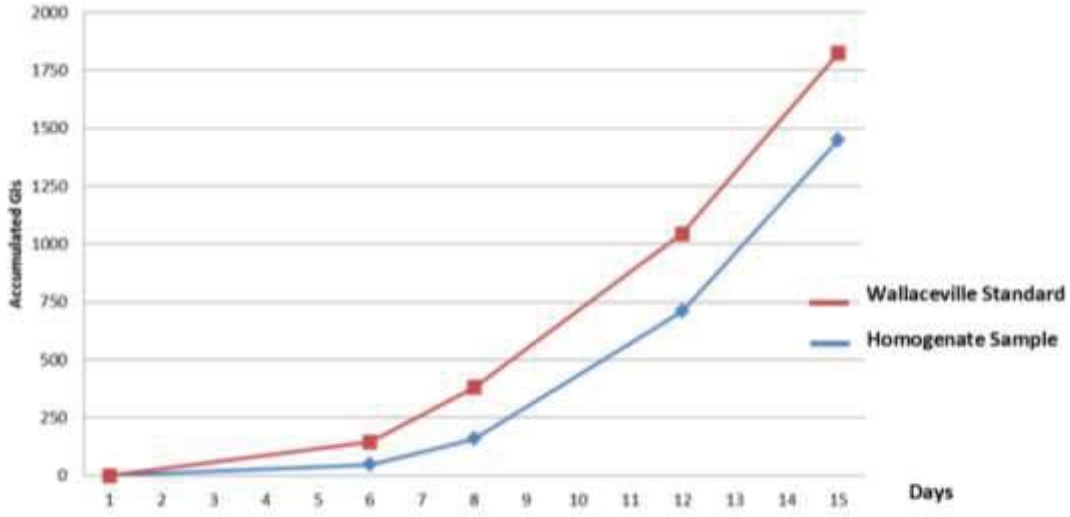

Figure 6.1 Cumulative Growth Index curves for *Mycobacterium avium* subsp *paratuberculosis for* the 1st sample of homogenate orally dosed to lambs at (T = 0),(blue line - 12 days to reach dCGI1000, with estimated 1 x 10^8 organisms/ml), compared to a validated Wallaceville laboratory sample,(red line - 13 days to reach dCGI1000 with estimated 1 x 10^8 organisms/ml).

After the initial blood and liver biopsies were collected and the initial oral dosing completed, all lambs were moved from the concrete penned sheep handling facility at Massey University to a 24 hectare farmlet where sheep had not been grazed for three years. Here they were orally dosed with either 10mL homogenate (challenged) or 10mL phosphate buffered saline (unchallenged), on days 4, 7, 10, 13, 16, 19, 22, 25, and 29. Heparinised blood samples were collected from all sheep on days 4, 7, 22, 29, 43, 51, 69, 93, 114, 154, 195, 233, 279, 364, 482, 596, and 820. Blood samples were centrifuged at 3000rpm for 10 minutes with fresh plasma used for ELISA serology (Paratuberculosis test kit, Pourquier, Idexx Test Laboratories Inc,) using a cut-point S/P ratio ≥50 as positive.

Body weight, body condition score data and liver biopsies were collected on days 0, 51, 114, 154, 195, 233, 290, 364, 482, 596 and 820. For each liver biopsy core, 27 serial longitudinal

sections at 3 µm were taken, with sections collected in order with one for HE, one for ZN and one discarded, providing a distance of 9µm between each HE or ZN stained section. Six glass slides were prepared with three sections per slide with nine sections stained using either HE or ZN. Each biopsy section measured approximately three mm x 12 mm, resulting in approximately 324 mm^2 of liver surface area for examination. The total surface area for all nine sections was examined, at x10 magnification, for the presence of spherical HEM, consisting of epithelioid macrophages with clear and large nuclei, prominent nucleoli, with lightly staining HE foamy vacuolated cytoplasm, and with some lymphocytes present. For ZN prepared sections, HEM were examined at x100 magnification for the presence of acid-fast organisms. Histopathology was performed blinded to the identity of the sheep.
All animals were administered 'Matrix' oral drench (1mL / 5kg live weight) every four weeks for the duration of the study to reduce the effects of gastro-intestinal parasitism. Subcutaneous injection of '5 in 1 vaccine' (Ultravac, Pfizer, New Zealand) was also administered to all lambs in March and April of the first year and annually to prevent Clostridial diseases. Lambs were grazed on isolated paddocks on the same farm with >1250 kgDM/Ha feed allocations maintaining no opportunity for cross contamination. Feeding levels and pasture allocation were determined using a pasture stick (Beef & Lamb, New Zealand).

The criteria for euthanasia of challenged sheep where clinical Johne's disease had developed included body condition below BCS 1.5 (Jefferies 1961), weight loss despite anthelmintic treatment and provision of >1250kgDM/Ha food, and the development of faecal shedding with AFO identified in faecal ZN smears. Euthanasia was performed by captive bolt to the cranium.

The study ended at day 820 with all surviving animals euthanised as above. Samples of ileum, liver, and mesenteric lymph node were fixed in 10% phosphate buffered saline and

submitted for histopathology. Blood samples were collected for ELISA. Post mortem skeletal structures including poll to rump, right 3rd metacarpal length and diameter (medio-lateral), tibial crest to medial calcaneus, calcaneus length, right meta-tarsal length and diameter (cranio-caudal) and right ulna length (olecranon to 1st metacarpal joint) were measured as described by Davis (1996) using engineers vernier callipers (±0.1mm) and a tape measure (± 1mm).

Statistical Analysis

Cohen's kappa was used to determine the level of agreement between artificial oral challenge with Map and the formation of HEM, sero-positivity and live weight changes and to determine the level of agreement between animals where HEM formation occurred, sero-positivity, and live weight differences and the level of agreement between increasing HEM numbers per liver biopsy sample with increasing S/P ratios.

Analysis for the liveweight differences between the challenged and unchallenged animals was undertaken using a repeat measure ANOVA (Microsoft Excel) with live weight as the outcome variable, Map dosing as a between subject effect, and time, as categorised in order of measurement, as a within subject effect. All data are estimated marginal means from the ANOVA model.

The results for Mauchly's test of sphericity was significant with Huynh-Feldt's correction used to estimate the within subject effects.

The skeletal measurements were analysed using multivariate ANOVA (Microsoft Excel) with the skeletal measurement being the outcome variable and Map dosing being the fixed effect.

6.4 Results

HEM Formation

In the unchallenged animals no sero-conversion or HEM development was recorded throughout the study. In contrast, the formation of HEM occurred in the entire challenged group, with the first observation at 51 days, while 17/18 had them at 114 days and all had them at 195 days. Cohen's kappa recorded a perfect level of agreement between the formation of HEM and artificial oral challenge with Map (K=1). In three sheep, HEM were first observed at 114 days then the number decreased, with none present at 233 days. In the remaining 15 animals the number of HEM increased up to 195 days. From 195 days, four sheep progressed to clinical Johne's disease with weight loss and faecal shedding of AFO between 290 and 450 days. At necropsy all four had developed Type 3b histopathological ileal lesions (Perez *et al,* 1996). In these animals HEM numbers continued to increase and remained high (≥25 per 324mm^2 sample) until euthanasia (Table 6.2). In the surviving 14 sheep, HEM numbers began to decrease in three animals from 114 days, in eleven animals from 195 days, two animals from 233 days, one animal from 364 days, with no HEM identified in any animal from 482-820 days, (Figure 6.2), (Appendix 6.1a, 6.1b).

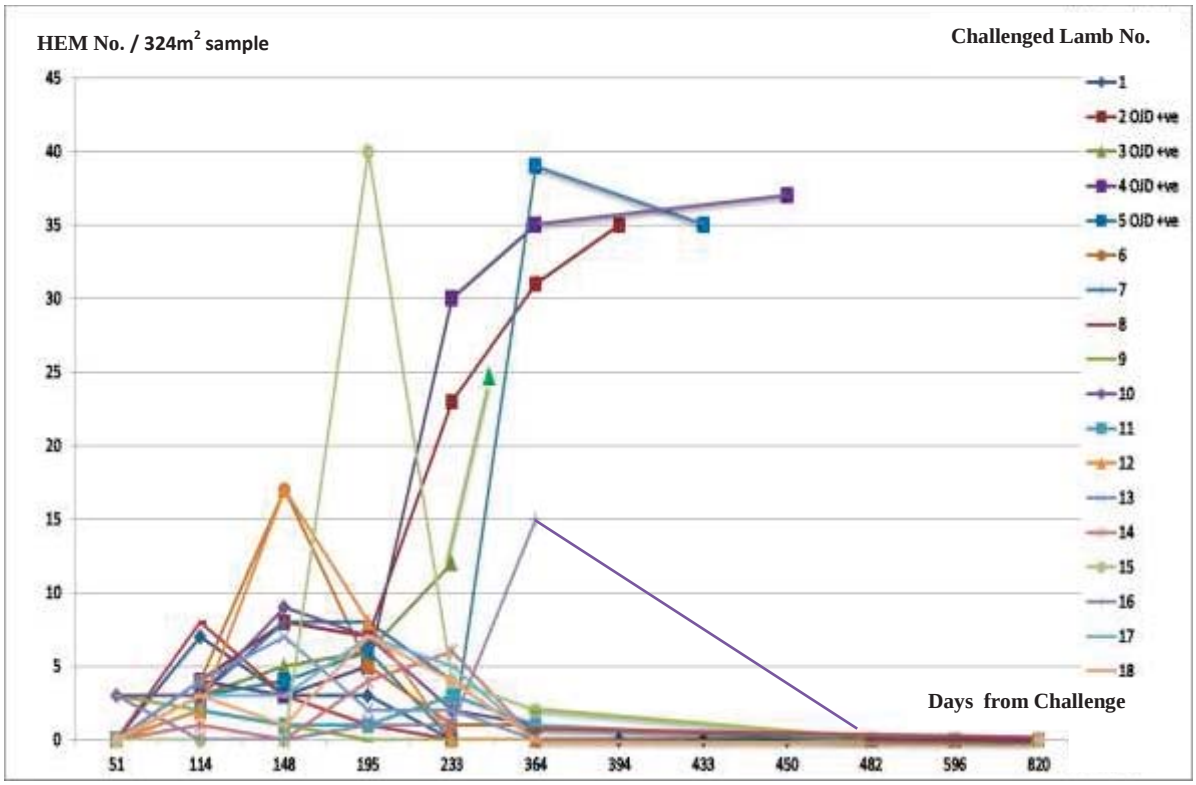

Figure 6.2 Number of hepatic epithelioid macrophage micro-granulomas (HEM) per 324mm^2 liver biopsy histological section, at each sampling, in each animal artificially challenged with Map (Note: OJD +ve indicates those sheep euthanised for clinical disease; no HEM were observed in survivors at days 482 - 820).

Table 6.2 Number of HEM in challenged sheep recorded from nine histological sections per liver biopsy sample. Liver samples collected at time points t = 0 – 820 days.

Date	DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
01.03.12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20.04.12	51	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	3	0	0
22.06.12	114	7	4	3	4	3	4	3	8	2	3	2	2	4	1	0	0	3	3
25.07.12	154	3	8	5	3	4	17	8	3	1	9	1	17	7	0	0	0	3	1
06.09.12	195	3	7	6	5	6	5	8	1	0	7	1	8	2	4	40+	1	7	7
09.10.12	233	0	23	12	30	0	1	4	0	0	2	3	0	2	6	4	1	5	4
05.12.12	290	--	--	25 XX	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
28.02.13	364	0	31		35	39	1	0	0	0	1	1	0	0	0	2	15	0	0
29.03.13	394	--	35 XX	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
07.05.13	433	--	--	--	--	35 XX	--	--	--	--	--	--	--	--	--	--	--	--	--
24.05.13	450	--	--	--	37 XX	--	--	--	--	--	--	--	--	--	--	--	--	--	--
26.06.13	482	0	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0
17.10.13	596	0	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0
29.05.14	820	0	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0

Key: DPI = Days Post Infection; XX = time when ewe was euthanised; 1-18 = ewe ear tag

Serology

ELISA S/P ratios ≥ 50 were recorded in the challenged sheep, with 4/18 positive at 93 days, and 13/18 at 233 days. None of the unchallenged sheep sero-converted with no ELISA S/P ratios recorded. There was a Cohen's Kappa substantial level of agreement between artificial oral infection and becoming ELISA S/P positive (K=0.72). In 2/13 animals the S/P ratios began to decline at 114 days, with another animal declining at 154 days. Four sheep developed clinical Johne's disease with ELISA sero-positivity occurring in two at 233 days. In these two animals, ELISA S/P ratios continued to increase until euthanasia. In the remaining 11 ELISA positive sheep, the S/P ratios in 10 declined at 233 days and all at 279 days (Figure 6.3). Most were negative at day 364, and all but one were negative at day 596 and all were negative at day 820.

All sheep with positive ELISA S/P ratios developed HEM with a Cohen's kappa substantial level of agreement between the presence of HEM and sero-positivity recorded (K=0.72). As the number of HEM per 324mm^2 liver sample increased so did the S/P ratio and *vice versa* with a Cohen's kappa substantial level of agreement (K=0.72). However, S/P ratio's were not predictive of animals losing weight, with a Cohen's kappa poor level of agreement (K=0.1)

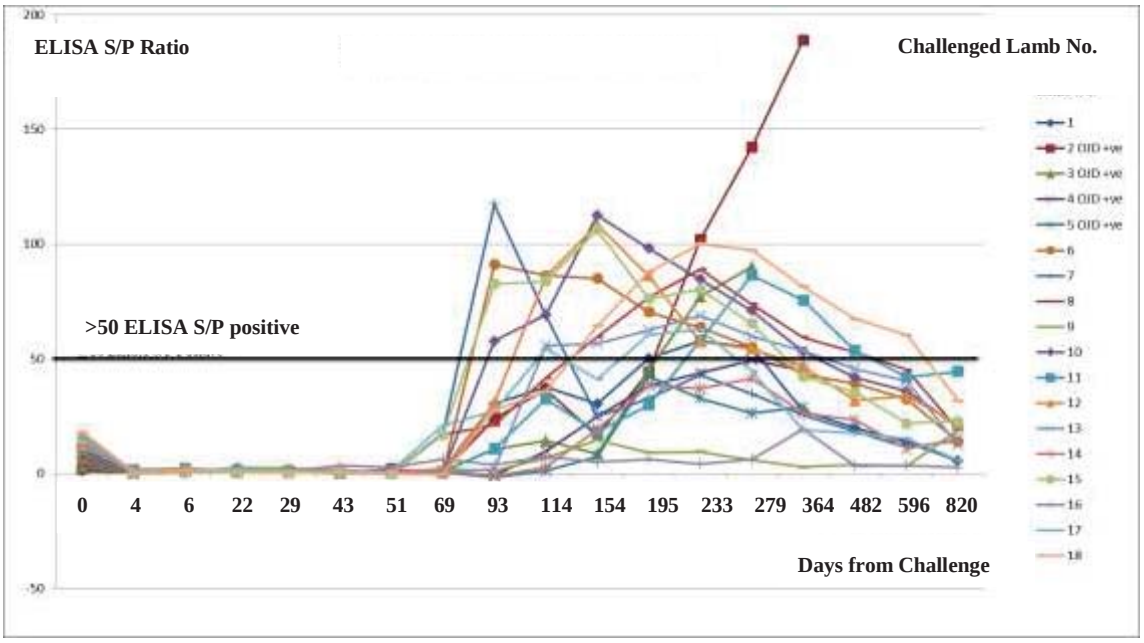

Figure 6.3 ELISA S/P ratios for each sheep orally challenged from day 0 through to Day 30. (S/P ratio ≥50 is considered +ve). (OJD +ve indicates those sheep euthanised for development of clinical disease).

Pasture Allowance

The mean pasture cover for the entire study for the unchallenged and challenged sheep was recorded as 1550kgDM/Ha (800-2270kgDM/Ha) and 1591kgDM/Ha (800-2270kgDM/Ha) respectively, with levels only falling below 1250kgDM/Ha in the winter months (July/August, 990-1140kgDM/Ha) and for the last 100 days of the study due to drought (800kgDM/Ha), (Figure 6.4).

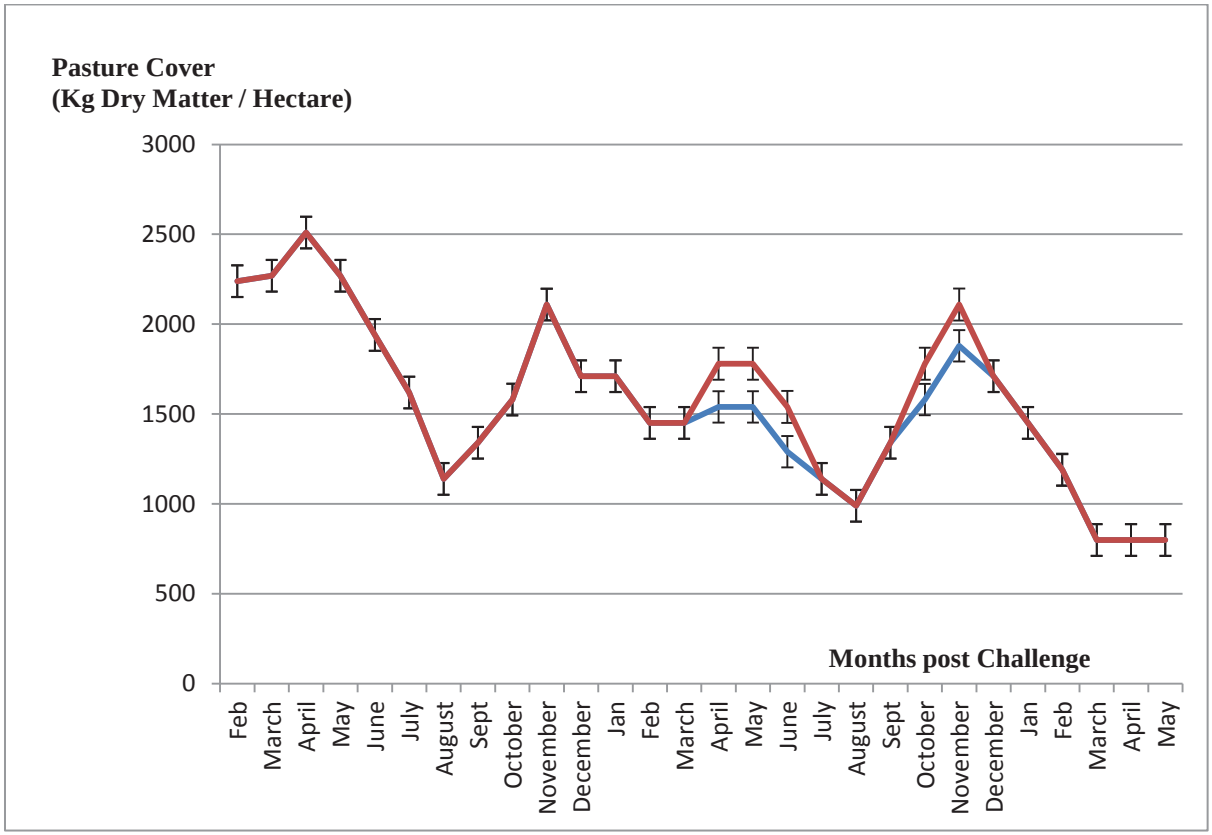

Figure 6.4 Mean feed allowance with standard errors for the two separate areas grazed by challenged (Brown Line) and unchallenged sheep (Blue Line). Measured monthly (Kg dry matter/hectare) using a pasture stick (Beef & Lamb, NZ).

Live weight and Body Condition Score

Mean live weights for both groups were recorded as 36kg (35.5-36.5kg) at the beginning of the study. At 51 days the mean live weights for both the unchallenged and challenged groups had increased to 45.8kg (range 41.4-49.2kg) and 45kg (range 40.2-50.4kg) respectively. Between 51 and 195 days, animals in the unchallenged group gained an average weight of 6.37kg (range 0.9-10.8kg). Over the same period, 15/18 of the challenged animals lost an average of 3.53kg (range ¯0.4 – ¯8.3kg) and three challenged sheep gained an average of 2.77kg (range 0.7-3.8kg). At 364 days there was a mean live weight difference of 9.8kg

between the two groups with Cohen's kappa determining an almost perfect level of agreement between artificial oral Map challenge and live weight loss (K=0.89) and an almost perfect level of agreement between animals forming HEM and live weight loss (K=0.89). Weight gain occurred from 195 days in all lambs with equivalent growth rates and BCS recorded from 482–820 days (Appendix 6.3). At the end of the study, the average live weights for the unchallenged (n=18) and challenged sheep that survived (n=14) were 88.2kg (77.5-104kg) and 77.7kg (71-83kg) respectively, a 10.5kg weight difference (Figures 6.5, 6.6), with both groups having equivalent BCS. There was a significant interaction between the effect of time (CI95%, $p < 0.001$) and time and oral dosing (CI95%, $p<0.001$) and oral dosing (CI95%, $p < 0.001$), where the challenged sheep grew more slowly (Repeat Measure ANOVA, Excel).

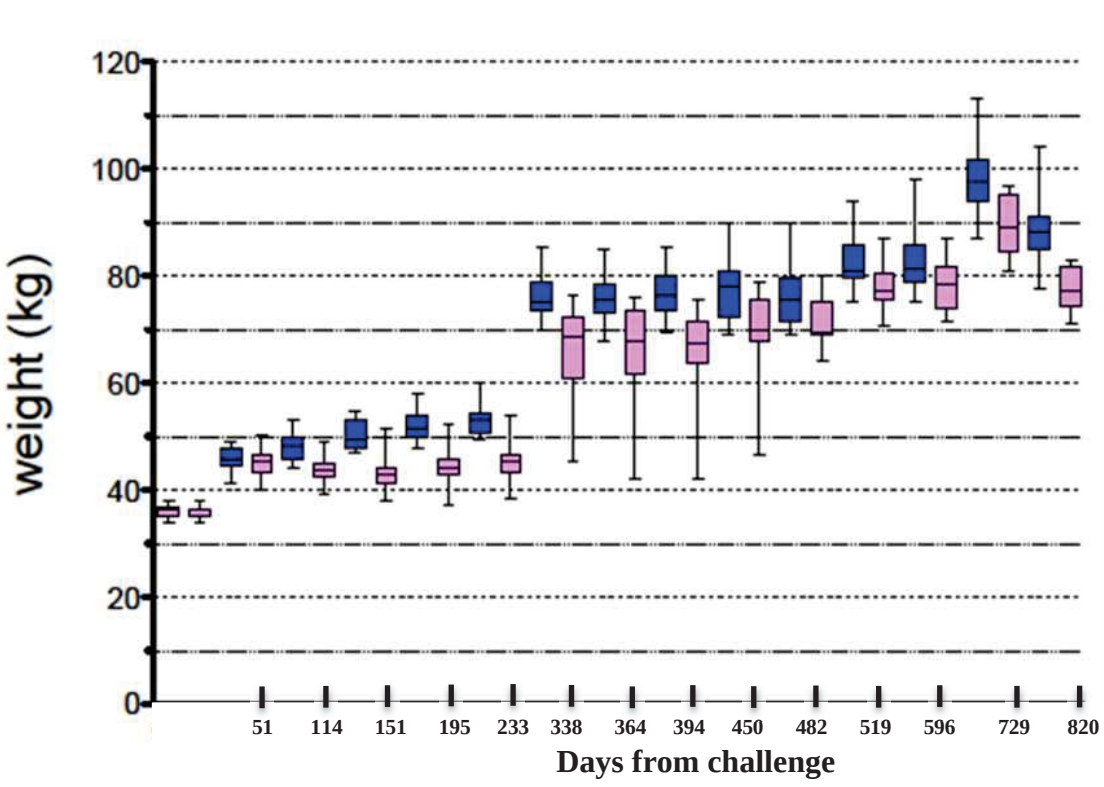

Figure 6.5 Boxplot of live weight data (kg) collected at each time point (days post challenge) for challenged (■) and unchallenged sheep (■).

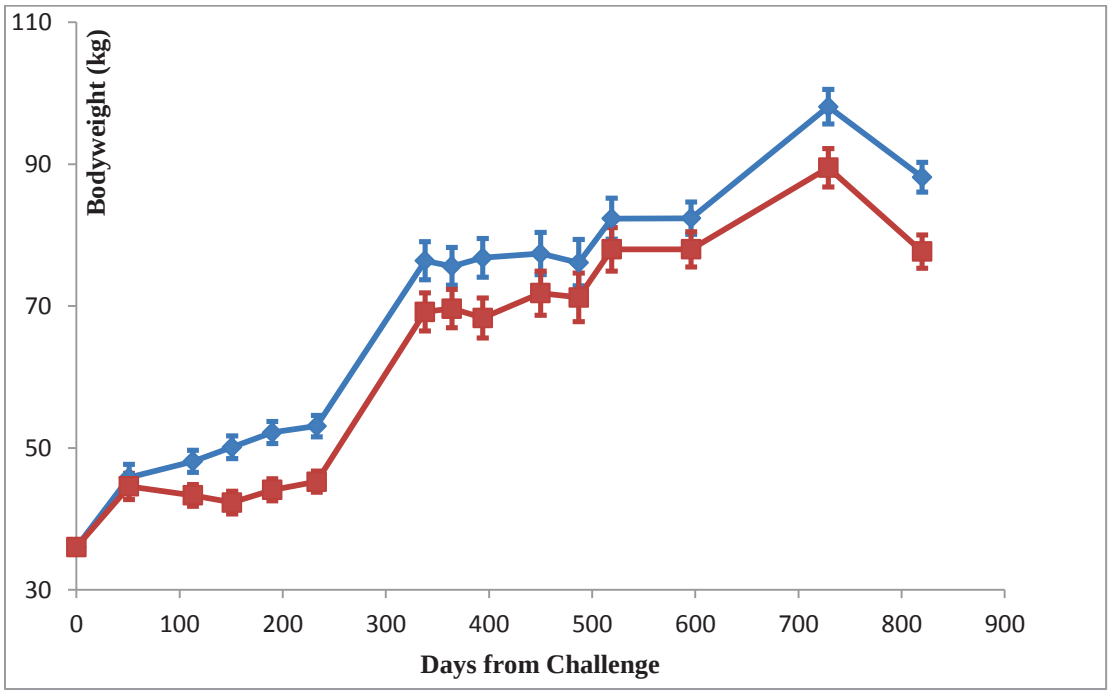

Figure 6.6 Mean weights with standard errors for challenged ( ) and unchallenged ( ) sheep collected at each time point post oral challenge.

Skeletal Structures

At the end of the study the challenged sheep were shorter in both body length and in stature. Post mortem skeletal measurement determined the poll to rump length to be 4% shorter (50mm) and the 3rd metacarpal length, calcaneous length, 3rd metatarsal length and diameter to be 5% shorter in the challenged lambs compared to the unchallenged lambs (multivariate ANOVA, CI95%, $p<0.001$), (Table 6.3, Appendix 6.5a, 6.5b).

Table 6.3 Post mortem skeletal measurements (cm) of unchallenged (0) and challenged (1) lambs at 820 days from challenge.

Dependent Variable		Mean Distance (cm)	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
Poll/Rump	0	116.150	.789	114.540	117.760
	1	110.993	.894	109.167	112.819
Right Metacarpal length	0	15.522	.213	15.086	15.958
	1	14.286	.242	13.791	14.780
Right Metacarpal diameter	0	2.728	.033	2.659	2.796
	1	2.671	.038	2.594	2.749
Tibial Crest to calcaneus	0	25.894	.393	25.092	26.697
	1	25.079	.446	24.168	25.989
Calcaneus length	0	7.367	.059	7.247	7.486
	1	6.893	.066	6.757	7.028
Right Metatarsal length	0	21.083	.230	20.613	21.554
	1	20.121	.261	19.588	20.655
Right Metatarsal Diameter	0	2.306	.033	2.239	2.372
	1	2.129	.037	2.053	2.204
Right ulna length	0	22.428	.246	21.925	22.931
	1	22.086	.279	21.516	22.656

6.5 Discussion

Serial liver biopsy histopathology and the identification of HEM have provided a surrogate method to measure the occurrence of systemic Map infection in sheep. This diagnostic test enables objective comparison to be made between animals with and without systemic infection and is a fundamental step in being able to quantify production losses or immune responses that may occur as a result of Map bacteraemia and systemic infection. This study compared live weight, body condition score and humoral immune response changes between artificially challenged and unchallenged sheep and determined that systemic Map infection can be identified in artificially infected lambs, that serial biopsies enable the progressive nature of this infection to be followed, supporting previous longitudinal challenge studies (Juste *et al,* 1994; Kurade *et al,*2004; Dennis *et al,* 2011), and that costs to production such as

live weight and body condition losses along with sero-conversion coincide with periods when systemic Map infection is occurring.

The humoral immune response in naturally infected sheep is often recorded in adult sheep in the later stages of the disease (Hilbink *et al,* 1994; Burrells *et al,* 1998; Whittington and Sergeant 2001; Sergeant *et al,* 2003) whereas in this study the humoral response was recorded in 7-12 month old lambs with 13/18 becoming sero-positive. In the surviving 14 sheep, HEM lesions began to resolve from 195 days with none present in any animal at 482 days. A similar trend of decreasing ELISA S/P ratios was recorded with no sero-positive animals present at the end of the study. However, in this study, as with previous studies (Nielsen and Toft 2008), ELISA serology was not predictive for live weight or body condition losses for artificially challenged sheep or for their outcomes regarding whether they would go on to develop clinical disease or recover. In a previous study, there was an almost perfect level of agreement between the presence of HEM and ELISA sero-positivity (Cohen's kappa = 0.87, $P < 0.001$), (Smith *et al*. 2014). In this longitudinal study, there was a Cohen's kappa substantial level of agreement between the formation of HEM and ELISA sero-positivity and a similar level of agreement between increasing HEM numbers per liver biopsy sample and increasing ELISA S/P ratios. It is hypothesised here that Map infection is initially sequestered by the cell mediated immune response within macrophages and that the humoral response is only initiated once free Map enters the circulation and Map bacteraemia occurs with subsequent systemic infection and the formation of HEM.

Hepatic epithelioid macrophage micro-granulomas have only been identified in animals where ileal pathology has progressed to Type 3b or 3c lesions (Smith *et al,* 2014). However, in this study, the oral dosing of naïve lambs with $> 1 \times 10^9$ Map organisms on 10 occasions over a thirty day period, resulted in all 18 lambs developing HEM by 195 days, with two animals at 51 days and 17/18 occurring by 114 days. It is unlikely that Type 3b or 3c ileal

pathology was present by 195 days, suggesting, in artificial challenge circumstances, at least at the dose rate used here, ileal pathology may not be required for HEM to form. This is supported in a previous study where ileal lesions were not found in lambs artificially challenged with Map within this time frame (Juste *et al,* 1994) and suggests there may be a different aetiology or mechanism occurring for the development of systemic Map infection and the formation of HEM for natural and artificial infection.

Quantifying the cost of Map infection on production is difficult and it is proposed that there are two periods of loss; the sub-clinical or non-systemic period where ileal and mesenteric lymphatic invasion occurs; and a period of systemic infection where Map escapes the alimentary tract, bacteraemia occurs and HEM form. The study by Dennis *et al*, (2011) identified progressive ileal pathological changes from Map infection using serial ileal biopsy. Whether systemic infection had occurred at the same time was not explored, as hepatic histopathology was not undertaken with no HEM detected. Production losses were also not addressed, although recovery from major surgery after each biopsy session may have been a confounding factor. Quantifying sub-clinical costs on production has yet to be achieved and it is proposed this may be unattainable, as identifying animals where sub-clinical ileal pathology is occurring is currently difficult and as such comparison on the effects on production such as live weight and body condition cannot be made between animals with and without these sub-clinical lesions.

In contrast, serial liver biopsy and the identification of HEM has potential to provide a new diagnostic tool to identify animals where systemic Map infection is occurring, enabling these comparisons to be made, making quantification of the cost of systemic infection on these production traits possible. In naturally infected sheep, HEM formation has only been identified in animals that develop Type 3b or 3c ileal lesions, with no recovery recorded in animals where clinical Johne's disease has developed (Perez *et al.* 1996, 1997). In these

animals, losses are attributable to animals dying from clinical disease (Thompson *et al*, 2002; Morris *et al,* 2006; Bush et al, 2006, 2008). In a recent Australian study, losses were recorded up to 10 months prior to the development of clinical Johne's disease with naturally infected sheep losing serum albumin and liveweight (McGregor *et al*, 2015). This result suggested losses attributable to sub-clinical or non-systemic infection had been quantified. However, it is proposed, that these losses may not have been sub-clinical as the sheep had developed Type 3b or 3c ileal lesions and although liver histopathology was not reported, sheep with these ileal lesions become systemically infected and develop HEM (Smith *et al,* 2014). Therefore, the production losses recorded in this Australian study may also have been attributable to clinical disease.

A similar result was attained in this longitudinal study, where losses were only recorded when systemic Map infection was occurring. No losses were recorded in periods where systemic Map infection or HEM numbers were decreasing or absent. There were two periods of production loss and systemic Map infection with subsequent HEM formation. The first period was initially recorded at 51 days after artificial oral challenge with all the lambs developing HEM. It is proposed that this represents the beginning of a systemic period of infection that lasted until 195 days when the mean number of HEM per liver biopsy sample peaked. Over this period, animals were clinically affected by systemic Map infection, with losses in liveweight and body condition measured. After this period HEM numbers began to decline and live weight gains occurred, suggesting a decline in systemic infection. For 14/18 sheep this period continued until there were no HEM present at 482-820 days, with these animals recovering. At the end of the study (t = 820 days), there were no histopathological lesions found within the alimentary tract or liver of any of the unchallenged group or the 14 surviving challenged lambs suggesting a full recovery post Map infection had occurred. However, this recovery came at a cost that was attributable to a period of systemic infection,

which was quantified as smaller skeletal structures and an average 11kg lighter live weight difference, when compared to the unchallenged sheep, at the end of the study. In four of the challenged lambs, a second period of systemic Map infection occurred after 195 days with a corresponding second period of HEM formation and production losses, with the cost on production attributable to not only live weight and body condition losses but also from animals dying with Johne's disease. For these four animals, the number of HEM per liver biopsy sample increased continuously after 195 days, whereas for the 14 survivors HEM numbers in this period declined. This suggests that in artificial challenge studies, serial liver biopsies and the identification of HEM after 195 days may be predictive of Johne's disease if HEM numbers are increasing and predictive of resolution and recovery with declining HEM numbers. This study has determined the timeline for the formation and resolution of HEM and with further validation may have potential use in future therapeutic efficacy studies.

Whilst this study was not replicated, every attempt was made to ensure both the challenged and unchallenged lambs were grazed on the same pasture species with the same allocation of food throughout the entire study. Regular pasture cover assessments were made with animals maintained on >1250kgDM/Ha feed for the entire study with the exception of the winter months and the period of drought at the end of the study. However, no nutritional analyses were performed, hence differences in liveweight between the two groups as a result of potential different nutritional levels cannot be fully excluded. Feed supplements were not provided. Thus, confounding variables may exist regarding differences in weight. Nevertheless, the growth rates experienced in the unchallenged sheep and the challenged group after 195 days would suggest that feeding levels and management practices that were put in place were not limiting factors for these animals. Additionally, the fact that both groups had similar BCS yet the challenged sheep had lower live weight and skeletal size adds

further evidence that these differences were a likely result of a disease process rather than being nutritional.

This study has determined serial liver biopsy histopathology and the identification of HEM can be used to identify systemic infection in lambs, has potential predictive values for Johne's disease and recovery with potential commercial application and can be used to quantify the cost on production parameters such as live weight and body condition in artificially infected sheep. However, with the outcome of systemic Map infection being very different for natural and artificial infection, this study suggests there may be different aetiologies and / or mechanisms for the development of this event and as such extrapolation of results from this study or other infection models may need to be done with caution. Using artificial infection to study ovine Johne's disease may have benefits in that the re-creation of clinical Johne's disease, using the current infection model, can be achieved in a shorter time frame than that found in natural infection (Begg *et al,* 2005). This longitudinal study suggests artificial infection in lambs provides a poor model of representation for that recorded in sheep naturally infected by Map and that artificial results may be unlikely to occur under normal grazing practices.

6.6 Acknowledgements

Dean and Debbie Sparkes for their immense patience and stock handling throughout the longitudinal study, Gary Yates, Wallaceville Infectious Disease Laboratory for culturing Map, Mike Reilly for providing the large animal teaching unit (LATU) facility at Massey University, Palmerston North, for the liver biopsy sampling, Phil O'Reilly for processing the sheep at the end of the study, Mark Collett, IVABS, for the histopathology and Richard Laven, IVABS, for providing the ANOVA analysis.

Chapter Seven

Hepatic spectroscopy as a diagnostic test for the identification of sheep with clinical Johne's disease.

7.1 Abstract

Aim: A pilot study to determine whether there is a different hyperspectral signature for liver or skeletal muscle from sheep with or without Johne's disease and if so, to develop an algorithm that enables those with this disease to be identified at the time of slaughter.

Methods: Ninety five mixed age ewes in poor body condition were euthanised and necropsied for gross and histological evidence of Johne's disease. The longissimus-dorsi muscle was transected at the 3rd lumbar vertebra and the cut surface of this muscle and the cranial serosal surface of the left liver lobe were scanned at three sites using a hyperspectral spectroscope sensor (ASD Fieldspec, USA) recording a signature (Matlab®) for each tissue. Ileal sections were histologically prepared and stained with both Haematoxylin/Eosin (HE) and Ziehl Neelsen (ZN) stains. Longissimus dorsi muscle and liver sections were histologically prepared and stained with HE. An additional 200 four-month-old, mixed sex lambs from 12 farms throughout the Manawatu, New Zealand, were slaughtered at an abattoir and livers scanned as above. Liver samples were collected from the scanned sites using a 3mm inner core biopsy probe, fixed in 10% phosphate buffered formalin and submitted for HE staining and histopathology.

Results: Ten ewes with gross pathology resembling Johne's disease were confirmed by histopathology to have Type 3b multibacillary ileal lesions and hepatic epithelioid macrophage micro-granulomas (HEM). Eighty five ewes did not have HEM or alimentary

tract lesions and no histopathological lesions were recorded in the longissimus dorsi muscle. No HEM were identified in any lamb liver samples.

There were no hyperspectral differences identified in the longissimus dorsi muscle from all ewes with differences only found in hepatic tissue. A learning model algorithm was developed from a single liver, from a ewe confirmed to have Type 3b Johne's disease, that identified a hepatic hyperspectral signature that, when applied blinded to the Johne's status of the ewes and lambs, successfully differentiated all 10 animals with Johne's disease from the 95 ewes and 200 lambs without.

Conclusion: This study has developed a learning model algorithm that can differentiate the hyperspectral signature of liver from sheep with or without Johne's disease. Hyperspectral imaging of the liver may have a role to play in identifying sheep with Johne's disease and may provide a real time diagnostic test at time of slaughter.

Key Words

spectroscopy, spectral, hyperspectral, *Mycobacterium avium* subsp. *paratuberculosis*, Map, hepatic epithelioid macrophage, liver, muscle, granuloma, Johne's disease, sheep, systemic Map infection

7.2 Introduction

The first compound optical microscope was invented by Hans and Zacharias Janssen in 1650, opening the door to the study of objects smaller than that detected by the human eye (Helden *et al*, 2010). The electromagnetic spectrum (EMS), including ultraviolet light through to infra-red, has a relatively narrow range of wavelength from 100nm through to 1mm respectively, with optical microscopy utilising the reflection and absorption of visible light

within this range encompassing violet (380nm) through to red (750nm) light wavelengths (Brand 1995). Spectroscopy and hyperspectroscopy utilise similar electromagnetic radiation wavelengths to 'visualise' an object. However, the data collected and its analysis is different. Spectroscopy records an image, comprising of all the continuous data available for visible light from 380nm to 750nm, as a single data point. By contrast, with hyperspectral imaging, this continuous visible spectrum is sub-divided into 10nm band widths with the reflectivity, absorption and electromagnetic emission recorded across the entire visible range, providing a set of images for each band width that are combined to form a three dimensional hyperspectral 'data cube' for every pixel within an image (Lu and Fei 2014). This data is then analysed using mathematical algorithms producing a unique hyperspectral signature for that object.

Identifying the electromagnetic signature of objects using hyperspectroscopy has been reported across many disciplines including the assessment of pasture quality and quantity (Pullanagari *et al,* 2011a), the identification of different grass species within a pasture sward (Irisami *et al,* 2009), the prediction of crop yields by satellite hyperspectral imaging (Singh *et al*, 1992), and the identification of canopy species in farming systems (Asner 1998). The diagnostic application of hyperspectroscopy appears to be vast and has been utilised in plant and animal biology with the identification and development of unique spectral indices for specific plant diseases enabling a non-invasive, rapid and relatively cost-efficient means for diagnosis (Mahlein *et al,* 2013). In animals, hyperspectral analysis of carcases in abattoirs has been used as a real time diagnostic test to determine carcase characteristics including protein and fat composition (Pullanagari *et al,* 2015), and prediction of meat tenderness (Naganathan *et al,* 2008), using the visible to near infra-red light spectrum (Vis-NIRS).

There have been many similar diagnostic applications in human medicine with hyperspectral analysis of tissues or products of metabolism used to identify clinical disease in patients.

Some of these studies include analysis of serum for identification of gynaecological cancers (Leiner *et al,* 1986); serum and urine for patients with hepatic cirrhosis and hepatic cancer (Li *et al,* 2004; Alsalhi *et al,* 2012) and identification of cancer cells from colonic biopsy (Massood and Rajpoot 2008). Direct, in situ spectroscopy has also been used to identify pre-cancerous epithelial cells in humans (Backman *et al,* 2000; Muller *et al,* 2002) and in a recent murine study, structural liver damage induced by exposure to carbon tetrachloride (CCl_4) was recorded using trans-abdominal spectroscopy (Nazeer *et al,* 2015). That ability to identify changes in organs such as the liver using hyperspectral analysis suggests that this may also have efficacy for diagnosing other hepatic diseases where structural changes are observed histopathologically, such as may be related to hepatic epithelioid macrophage micro-granulomas (HEM) that form in sheep with Johne's disease after systemic infection with *Mycobacterium avium* subsp. *paratuberculosis* (Map), (Smith *et al*, 2014). Currently there has been no research undertaken to identify whether a hyperspectral signature exists for liver from sheep with Johne's disease or livers containing HEM.

The aims of this pilot study were to determine whether liver or skeletal muscle from sheep with clinical Johne's disease have different hyperspectral signatures than those without, and whether an algorithm can be developed from Vis-NIRS to differentiate affected animals at time of slaughter.

7.3 Materials and Methods

Over a period of one year, ninety five mixed age ewes of body condition score 1.5 / 5 (Jefferies 1961) were necropsied on nine North Island farms in New Zealand. Each farm had a history of animals diagnosed with clinical Johne's disease. No Johne's vaccination had been administered to any of the ewes examined. All animals were humanely euthanised using a

captive bolt to the cranium and subsequently exsanguinated (Massey University Animal Ethic approval 100/2015). Samples of ileum, ileo-caecal valve and mesenteric lymph node were collected from each ewe, placed in 10% phosphate buffered formalin and submitted for HE and ZN staining and histopathology. The vertebral column was transected at the 3rd lumbar vertebra exposing the right and left longissimus dorsi muscles. The liver was removed from the carcase, and positioned with the gall bladder ventral, exposing the cranial serosal surface of the right and left lobes. Three different areas of the transected surface of the left longissimus dorsi muscle and cranial surface of the left lobe of the liver were scanned within three hours of collection using a high intensity probe (ASD, Boulder, CO) connected to a hyperspectral spectroscope sensor, Vis-NIR spectro-radiometer (ASD, Fieldspec,USA). Illumination was achieved by halogen bulb with scanned areas measuring 10mm diameter. The visible to near infrared range (Vis-NIR) was 350nm-2500nm with a spectral resolution of 3nm and 10nm at 350nm-1000nm and 1000nm-2500nm respectively. The longissimus dorsi muscle fibres were scanned with the probe directed in the same direction as the muscle fibres, end on, across the transected surface. The liver was scanned perpendicular to the surface. Three spectral measurements were collected for each of the above tissues, with the average for each calculated for further analysis. After scanning had been completed, transverse sections (30x20x10mm) of the longissimus dorsi muscle and liver were collected from the same areas as scanned, fixed in 10% phosphate buffered formalin and submitted for HE staining and histopathology. Ewes were classified according to ileal lesion scores (Perez *et al,* 1996) as either those with or without Johne's disease.

In addition, livers from 200 four-month-old, mixed-sex lambs from 12 different Manawatu farms were sampled at an abattoir with hepatic hyperspectral imaging performed as described above. Samples of liver from these scanned sites were then collected using a 3mm inner core

liver biopsy probe, (Shoof, New Zealand), fixed in 10% phosphate buffered formalin and submitted for histopathology using HE stain as described by Smith *et al,* (2014).

Analysis

This study used the support vector machine (SVM) to develop a learning model and algorithm to classify the hyperspectral data of the longissimus dorsi muscle and liver with the Johne's disease status of each ewe.

Hyperspectral data requires pre-processing steps to remove background noise that may be attributable to operator or environmental conditions at time of sampling. Currently there is no standardisation technique to achieve this for longissimus dorsi or liver tissue in sheep so the method used was the same as that described in a previous lamb fatty acid study (Pullanagari *et al,* 2015). These steps included 1) data trimming and scaling of the Vis-NIR from 350nm-2500nm to 400nm-2450nm; 2) log transformation ; 3) first order derivative transformation and statistical analysis was performed using Matlab® programme with this method of analysis used for both linear and non-linear SVM classification as described by Vapnik (2000).

Briefly, in this study, hyperspectral data from the liver and longissimus dorsi muscle was collected from the initial 10 ewes that were necropsied. Of these ewes, histopathology confirmed one to have Type 3b ileal lesions, the presence of HEM and Johne's disease. Each data point for the ewe with and the nine ewes without Johne's disease was viewed as a support vector, analysed by the SVM and divided into two different classes. This initial training data enabled a learning algorithm to be developed, identifying a hyperplane that provided the greatest separation between the two classes. As new hyperspectral data from additional ewes was added to the study, the SVM learning algorithm classified this data and

predicted which side of the hyperplane separation it belonged to, being either Johne's positive, predicted class one, or Johne' s negative predicted class zero (Figure 7.1).

7.4 Results

Ten ewes had gross lesions typical of Johne's disease including lymphangitis (cording of lymphatics on serosal surfaces of ileum and caecum), lymphadenitis (enlargement of mesenteric lymph nodes (MLN), including the 1st lymph node in the mesenteric chain and the ileo-caecal lymph node), and granulomatous thickening of the ileal mucosa. Histopathology confirmed all of these animals to have Type 3b, multi-bacillary ileal lesions with abundant intracellular acid fast organisms (AFO) within coalesced sheets of epithelioid macrophage dominant micro-granulomas extending away from the Peyer's patch into the surrounding ileal mucosal and sub-mucosal tissues, with lymphadenitis within the MLN, accompanied by intracellular AFO, blunting of ileal mucosal villi and thickened granulomatous mucosa. Hepatic epithelioid macrophage micro-granulomas (HEM) were identified in all ten ewes with confirmed Johne's disease. There were no ileal or mesenteric lymph node histopathological lesions in the remaining 85 ewes with no HEM recorded in these ewes or the 200 lambs.

There were no histological or hyperspectral differences identified for the longissimus dorsi muscle between ewes with and without Johne's disease. A hepatic hyperspectral signature was identified between ewes with and without Johne's disease. The SVM algorithm , when tested blind to the histopathology results, classified all the livers from the 10 ewes with Johne's disease as being one class and informative for Johne's disease and classed all livers from the 85 other ewes and the 200 lambs as a separate group and coming from sheep without Johne's disease (Figure 7.1).

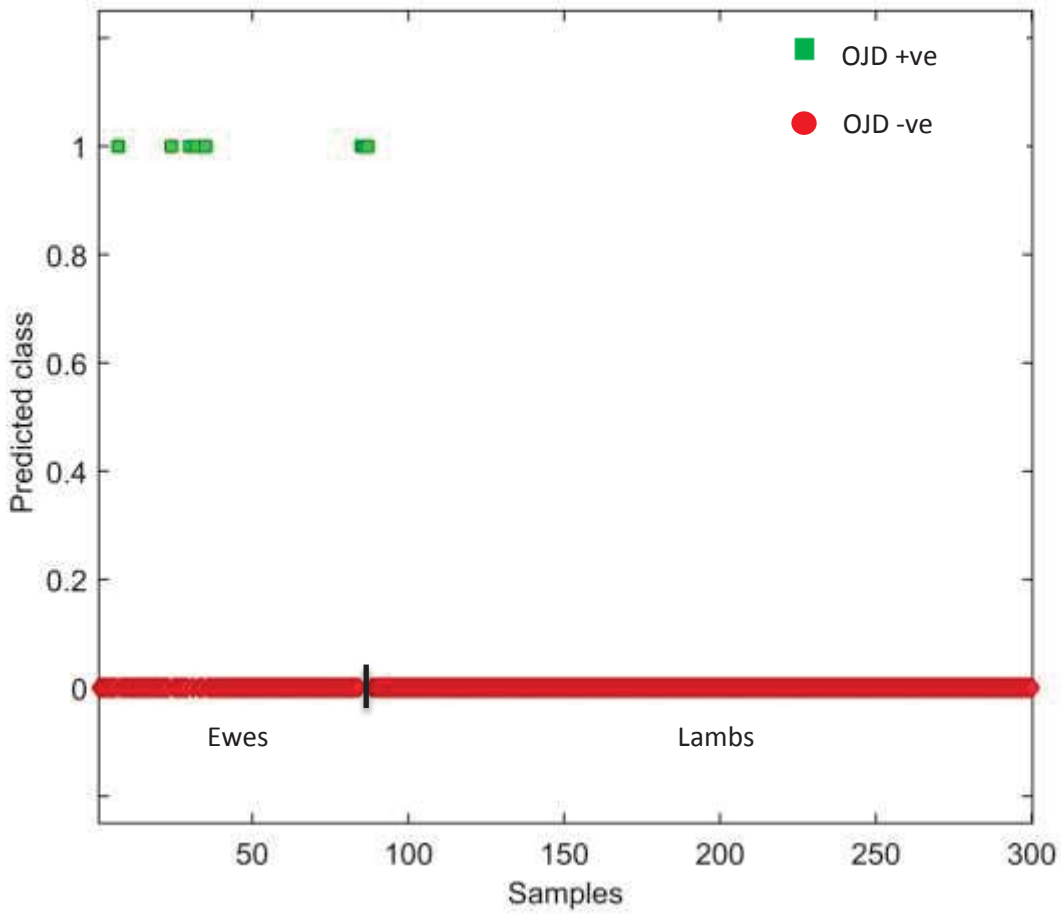

Figure 7.1 Hepatic hyperspectral differences and Support Vector Machine (SVM) classification of ewes with Johne's disease (n = 10) and ewes (n=85) and lambs without (n=200); Predicted class one is informative of ewes with Johne's disease and class zero predictive of those without; x-axis defines number of samples and y-axis represents cross-validated SVM classification index.

7.5 Discussion

This pilot study has developed an algorithm based on hyperspectral analysis of liver tissue, that accurately distinguished sheep clinically affected with Type 3b ileal lesions and Johne's

disease from those without. No difference in hyperspectral analysis was detected in muscle from either group of sheep. However, muscle samples in this study were not cultured and despite systemic Map infection, including peripheral skeletal muscle infection, being present in a majority of sheep with clinical Johne's disease (Smith *et al,* 2011), it may be possible that Map was not present within the longissimus muscle fibres for any of these ewes in this study. If this were the case then the hyperspectral signature for this muscle would be the same for both ewes with and without Johne's disease. Further research is now required to determine the Map infection status of the longissimus dorsi muscle and whether there are hyperspectral differences between ewes with and without Johne's disease.

Clinical Johne's disease occurs in a minority of sheep, with the annual incidence within infected flocks in New Zealand reported between 0.13-1% (West 2002; Verdugo *et al,* 2014). Systemic infection has been recorded in sheep with Map cultured from thigh skeletal muscle (Reddacliff *et al*, 2010; Smith *et al*, 2011). Currently there are no legal requirements or recommendations from the meat industry for discrimination of meat from carcases with macroscopic signs of clinical Johne's disease and as such meat from these sheep enters the human food chain. With recent food safety scares around the world, the current scientific association between Map infection and Crohn's disease (Fellar *et al,* 2007; Uzoigwe *et al,* 2007; Behr *et al,* 2008), and the presence of Map within skeletal muscle, the meat industry could be at risk from consumer rejection if the association between Map infection and Crohn's disease is found to be causal. Therefore it may be prudent for industry to pro-actively develop new diagnostic tests that can be applied to limit the exposure of humans to potentially zoonotic bacteria such as Map in meat products. This would require the development of a real-time or animal-side diagnostic test that has a high sensitivity and specificity, is able to be readily incorporated into abattoirs and is cost effective to install and operate.

Currently available diagnostic tests include culture (Cousins *et al,* 1995; Reddacliff *et al,* 2003: Whittington *et al,* 2000), the identification of DNA sequences using polymerase chain reaction (PCR) (Begg and Whittington 2008) and the histopathological identification of HEM (Smith *et al*. 2014). All of these are unlikely to be adopted by industry as they are not cost effective and do not provide real time results. This study has tested the concept of identifying a hyperspectral signature for ovine Johne's disease from tissues such as skeletal muscle and liver.

Initial choices of skeletal muscle for hyperspectral analysis included the diaphragm, lateral thigh, lateral shoulder and external abdominal oblique as these were considered to be readily accessible at time of slaughter. However, fat or connective tissue coverage across these muscles was different for each carcase so standardisation of the muscle tissue surface for imaging was achieved by transection of the longissimus dorsi muscle at the 3rd lumbar vertebrae. Hyperspectral imaging of this muscle surface failed to identify a hyperspectral signature that could be used to differentiate between ewes with or without clinical Johne's disease whereas imaging of the liver did identify a difference in the initial phase of this investigation enabling an algorithm to be developed that was then tested on the Johne's positive sheep as they were identified throughout the study.

In naturally infected sheep, HEM are only found in animals with Type 3b or 3c ileal lesions and where clinical Johne's disease is occurring (Smith *et al,* 2014). In these animals, disseminated Map infection is most likely to be occurring (Reddacliff *et al,* 2010; Smith *et al,* 2011). While HEM were identified in all 10 ewes with Johne's disease, whether they are responsible for the hepatic hyperspectral signature identified has yet to be determined as other factors such as biochemical changes may also be involved. It is proposed that there may be potential for hepatic hyperspectral imaging to identify carcases sourced from sheep

with Johne' s disease, where systemic Map infection has occurred. Liver tissue is readily available with carcases and offal trays accompanying each other throughout processing. Hyperspectral imaging cameras and the associated software algorithm are relatively inexpensive and could be readily installed into an abattoir killing chain, providing potential for the meat industry to mass screen animals destined for human consumption, cost effectively at point of slaughter.

The extent of this cost would depend on how hyperspectral screening was implemented with respect to which animals to assess. It is proposed that the mass hyperspectral screening of livers from all sheep may not be justified as systemic Map infection in naturally infected sheep has not been recorded in healthy sheep with body condition score > 2.5 / 5 (Jefferies 1961), (Smith *et al,* 2012) and is most likely to occur in animals with Type 3b or 3c ileal lesions (Reddacliff *et al,* 2010; Smith *et al*, 2011, 2014). Therefore if hyperspectral screening was to be adopted, it would be best targeted at the class of stock where Type 3b or 3c ileal lesions and systemic Map infection is most likely to occur, namely poor condition cull mixed-age ewes or those in direct contact with poor condition ewes immediately prior to slaughter (Smith *et al,* 2011, 2012).

This was a pilot study to test the concept that a hyperspectral signature could be predictive of clinical Johne's disease. It has shown that there appears to be a spectral signature and an algorithm suitable for this purpose. Further research is now justified to determine the diagnostic efficacy of this technique for predicting the risk that a given carcass is infected by Map. Should the technique be proven to be both sensitive and specific in sheep, its adoption in other species such as cattle, deer and goats would be justified.

7.6 Acknowledgements

To all the farm clients who donated their sheep for this project, Reddy Pullanagari who gave up his time to scan the tissues samples and run the analysis and Russell Wilson for providing the initial contact for the spectral team at Massey University and Ovation Abattoir, Feilding, New Zealand for access to the lamb livers.

Chapter Eight

General Discussion

8.1 Introduction

The scientific community agrees that there is an association between Map and Crohn's disease (Feller *et al,* 2007; Uzoigwe *et al,* 2007; Behr and Kapur 2008), and as such, minimising human exposure to this organism may be prudent should this association be found to be causal. The presence of Map, within skeletal muscle from ewes with Johne's disease, should be of worldwide concern for the meat industry as, despite current food hygiene standards, some of this product is processed for human consumption.

In sheep, *Mycobacterium avium* subsp *paratuberculosis* infection can be categorised into those sheep that are uninfected, or those that are infected without any ileal pathology (latently infected), or those infected with ileal pathology (Perez *et al,* 1996). Sheep with ileal pathology are further classified into different Types. Animals with Type 1 and 2 ileal lesions are referred to as being sub-clinically affected with no overt signs of clinical disease with normal production parameters recorded. These lesions may not progress onto clinical disease and can be found in healthy sheep. Type 3a ileal lesions are also considered sub-clinical although once these develop, progression to clinical disease always occurs (Perez *et al,* 1999). Type 3a lesions progress to either multibacillary, (many AFO), Type 3b or paucibacillary, (Low numbers of AFO), Type 3c ileal lesions. The reason for this differentiation is not understood. Johne's disease is defined in this thesis as the period when Type 3b or 3c ileal lesions have developed.

Despite the annual incidence of Johne's disease in New Zealand flocks being 0.13% (Verdugo *et al,* 2014), meat products from some of these clinically affected sheep continues to enter the human food chain, providing a source of Map for human exposure.

The aims of this study were to; 1) Determine whether skeletal muscle from sheep is infected with Map; 2) Identify which class of livestock poses a risk for human exposure; 3) Develop a diagnostic test, through hepatic histopathology, that can identify these animals, providing a potential means to quantify the therapeutic efficacy of drugs such as thalidomide for controlling or preventing systemic Map infection; 4) Apply this diagnostic test to a longitudinal artificial challenge study to establish a method to quantify the effects of systemic Map infection on production parameters such as live weight and body condition in sheep and 5) Develop a new real time diagnostic test that can identify sheep with Johne's disease at time of slaughter using hepatic hyperspectral analysis.

8.2 Map infection of skeletal muscle

There have been few studies undertaken identifying systemic Map infection in sheep and even fewer looking at the infection of skeletal muscle. Only recently has this been recorded (Reddacliff *et al,* 2010; Smith *et al,* 2011), with Map identified in skeletal muscle using PCR, BACTEC radiometric culture and also through visualisation in histological sections, (Figure 3.1). Systemic Map infection occurs when Map has escaped the ileal tissues and infected organs distant to the alimentary tract. In Chapter three this event was detected in 86% of ewes clinically affected with Johne's disease, with 71% having positive cultures from muscle and 62% in blood. This supports a similar Australian study where 94% of ewes with clinical Johne's disease had systemic Map infection including 59% with skeletal muscle and 85% with pre-scapula/pre-femoral lymph nodes infected (Reddacliff *et al,* 2010). The number of Map recorded in the skeletal muscle from both studies was determined to be low, with days

to reach a cumulative growth index of 1000 (dCGI1000) of 27 and 24 days, respectively, suggesting 1 x 10^{2-3}organisms / gram tissue (Reddacliff *et al,* 2010). However, the suggested numbers from the dCGI1000 above may be an under-estimation of the actual numbers of Map present per gram of tissue (Figure A2.7) as photomicrographs in Chapter three demonstrate > 1 x $10^{2}$of AFO inside single macrophages within the connective tissue of skeletal muscle, and several macrophages with AFO present per histological section suggesting >1 x 10^{7} organisms / gram tissue.

The presence of Map within skeletal muscle appears to be limited to the lumen of blood vessels and vascular system throughout the muscle bundles and fascial sheets of the muscle tissue, with Map only identified as phagocytosed intracellular AFO within macrophages (Figure 3.1). Free Map organisms within the muscle fibres or the development of muscle pathology from systemic Map infection was not histologically recorded. It is proposed that skeletal muscle may not be a tissue where Map multiplication occurs but may be a tissue where Map is 'passing through'. The number of Map per gram of skeletal muscle may be influenced by the number of organisms circulating as a bacteraemia at the time of slaughter. It is believed that Map bacteraemia most commonly occurs as clinical Johne's disease progresses from Type 3a ileal lesions through to the multibacillary Type 3b or paucibacillary Type 3c lesions and terminal disease. Currently, there is no information available for quantifying systemic Map infection and the associated occurrence of clinical disease. Further research is required to establish whether the quantity of Map in tissues increases as clinical disease progresses.

In addition, both this study and the Australian study above identified systemic Map infection in sheep without clinical Johne's disease, with a prevalence of 5/30, (17%), (Smith *et al,* 2011) and 7/22 (32%), (Reddacliff *et al,* 2010) respectively. This suggests that meat from these sheep may also pose a potential source of Map for humans. However, in both studies,

all studied ewes were either co-grazed in small holding paddocks with clinically affected, likely heavily shedding cohorts (Smith *et al,* 2011), or came from other sources where they had been exposed to unusually heavy burdens of oral Map immediately prior to slaughter (Reddacliff *et al,* 2010). It is proposed that in these circumstances, animals destined for entry into the human food chain could be readily exposed to large numbers of oral Map where shedding rates of up to 1 x 10^8 organisms / gram faeces have been recorded (Whittington *et al,* 2000). The oral uptake of Map in these clinically unaffected animals could therefore rapidly increase over a short period of time, with Map entering the ileal lumen and rapidly passing through the Peyer's patch into deeper tissues within hours of exposure (Momotani *et al,* 1988; Wu *et al,* 2007).

The ability to withstand a sudden influx of Map and prevent bacteraemia and systemic infection would be dependent on the robustness or capacity of the primary line of defence, the ileal cell mediated immunity (CMI), (Buergelt 1978; Whitlock and Buergelt 1996; Perez *et al,* 1996; Kurade *et al,* 2004). The ileal CMI capacity takes time to develop and is enhanced in adult animals, yet remains undeveloped in young, naïve animals. Adults have the capacity to sequester and prevent the diffuse spread of invading Map, with this capacity increasing gradually through macrophage recruitment into the ileal tissues, as animals are exposed to Map from the consumption of contaminated pasture, or when vaccination against Map is practised (Juste *et al,* 1994; Kurade *et al,* 2004; Begg and Whittington 2008; Bush *et al,* 2008; Dennis *et al,* 2011). Skeletal muscle sourced from sheep with clinical Johne's disease is likely to be infected with Map (Smith *et al,* 2011, Chapter three) whereas Map was not cultured in meat from healthy animals (Smith *et al,* 2012, Chapter four). However, it was cultured from skeletal muscle in adult ewes without Johne's disease when they were suddenly mixed and co-grazed with heavily shedding cohorts (Smith *et al,* 2011, Chapter three). It is proposed that in these adult ewes there is little time for the ileal CMI to up-regulate and

adapt, as Map invasion is rapid (Wu *et al,* 2007). Therefore opportunity exists in these adult sheep for the ileal CMI to be completely overwhelmed, producing a transient Map bacteraemia that may be recorded in skeletal muscle culture (Reddacliff *et al,* 2010; Smith *et al,* 2011). It is suggested here that the management practice of mixing clinically affected and unaffected sheep may allow systemic Map infection of skeletal muscle of the latter and may constitute a risk for exposing humans to Map. This could readily be mitigated by preventing the co-grazing of healthy sheep with poor body condition score sheep or avoiding the use of contaminated holding paddocks immediately prior to slaughter, with the use of concrete or slatted pens as holding facilities prior to transportation to slaughter premises.

8.3 Map infection in New Zealand flocks

The prevalence of Map infection and systemic Map infection within healthy sheep being grazed in conditions that represent normal farming practices is currently unknown in New Zealand, as addressed in Chapter four. In New Zealand, Map infection has been reported on 75% of sheep farms, with the annual incidence of Johne's disease estimated to be 0.13 on these farms (Verdugo *et al,* 2014), and in a previous study the prevalence of animals developing clinical Johne's disease over a year was estimated to be <1% (West 1997). Within these infected flocks, the proportion of animals infected by Map is currently unknown, although clinical cases of Johne's disease are considered to be the 'tip of the iceberg' when considering the number of animals likely to be infected.

In Chapter four, twenty four mixed age healthy ewes, from a single infected flock, were removed from their grazing cohorts and immediately housed onto cleaned concrete pens and euthanised, thus preventing any cross contamination that may occur by grazing overused holding paddocks close to the yards. Samples of ileum, mesenteric lymph node, skeletal muscle, faeces and blood were collected for BACTEC radiometric culture and the tissue

samples for histology. Histopathology demonstrated no lesions in any of the tissue samples and culture results for faecal, blood and muscle samples were also negative. Sero-conversion occurred in one ewe and latent Map infection was identified in 12 ewes with Map cultured from the ileal and mesenteric lymph nodes. These results support other studies where latent Map infection has been identified (Whitlock and Buergelt 1996, Nielsen and Toft 2008) and suggest that up to 50% of sheep in New Zealand's infected flocks may be infected. Similar results were reported in a New Zealand nationwide cross-sectional study in deer slaughtered in abattoirs. In this study, mesenteric lymph nodes from healthy deer were cultured with a national carcase level prevalence of 45% (Stringer *et al,* 2009).

Although the sheep study in Chapter four suggests a similar level of prevalence, it was not a national survey, with mixed age ewes selected from a single farm where Map infection was present. There is currently little information available for the national prevalence of Map infection in individual sheep within the New Zealand flock and it is proposed here that a similar nationwide survey, as undertaken in the deer, be initiated to establish the level of infection across New Zealand.

An industry pro-actively prepared is more likely to maintain its local and international meat trading agreements than an industry looking for answers after the event has occurred. It may be in New Zealand's meat industry best interests to take a pro-active stance and undertake steps that go some way to mitigate any food safety issues that may be perceived negatively by the public.

A nationwide survey will enable more informed decisions to be made regarding mitigating human risk of exposure to Map by providing information such as which class of stock may pose the greatest risk, provide information on possible regional differences and allow more

specific targeting of new diagnostic tests, should animals with Johne's disease need to be identified and removed at time of slaughter.

Currently there are no commercially available diagnostic tests available to identify these latently infected animals ante-mortem. This was achieved in this study only after necropsy and BACTEC radiometric culture of the ileum (n=6) and mesenteric lymph nodes (n=8). This suggests the currently accepted protocols for controlling the spread of Map such as quarantine and pre-purchase testing animals prior to introduction to a property, region or country may be ineffective.

In healthy mixed age ewes (Chapter four), systemic Map infection was not identified, with infection limited to alimentary track tissue and, when present, was only in a latent, non-invasive and non-pathological state. Although this was a small observational study that requires further validation, this result suggests that despite being infected by Map, systemic infection may not occur in naturally infected sheep without ileal pathology. By inference, therefore, meat sourced from these animals is unlikely to pose a risk for humans, although this assertion needs to be validated by further research, (see below). It also suggests that if carcase screening was to be undertaken within abattoirs at the time of slaughter, targeting the classes of stock most likely to pose the greatest risk for human exposure would be more cost effective for the meat industry than blanket screening. This would likely be cull ewes, particularly those in low body condition, as shown in Chapters three and five and in Chapter seven, where a proposed hyperspectral screening methodology is discussed.

Data in Chapters three and four have provided some insights into the occurrence of systemic Map infection, including that this may be limited to sheep with clinical Johne's disease. These data justify further work to increase the robustness of these findings, using a larger sample size and number of flocks. Additionally, a new diagnostic test will need to be

developed to more readily identify the occurrence of systemic infection in sheep as currently, liquid or solid media culture of Map from blood and tissues distant to the alimentary tract and the identification of DNA sequences, via PCR analysis of tissue samples, are the only diagnostic methods available for identifying systemic Map infection. These are time consuming, expensive and are unlikely to be adopted if a screening tool is deemed necessary for the meat industry. Chapter five proposes a new histological diagnostic test that may enable the identification of animals where systemic Map infection is occurring (see 8.4.1).

8.4 Identifying Map contaminated meat

In New Zealand, meat hygiene standards are in place within abattoirs that include the ante and post mortem inspection of animals and carcasses that enable the early identification and subsequent prevention of grossly diseased animals entering into the human food chain. The removal of poor body condition score (ill-thrift) ewes at ante-mortem inspection provides an important step towards mitigating the potential exposure of humans to Map from infected meat, as the number of Map in meat may be proportional to the severity of clinical Johne's disease, with emaciation a characteristic trait in more advanced cases (Reddacliff *et al,* 2003). However, there are many causes of ill-thrift for sheep throughout the world, including lameness, chronic pneumonia, gastro-intestinal parasitism, chronic facial eczema, liver fluke, dental issues and intestinal adenocarcinoma to name a few. Currently, there is no real time diagnostic test available to identify carcases where systemic Map infection is occurring. Pathogenic bacterial contamination of any kind, in any food product, is likely to be unaccepted by the public. When the bacterium involved has zoonotic potential, and the public becomes informed that the meat industry knew about product contamination prior to sale yet failed to put in place steps to mitigate this human exposure, then it could be expected

that the response may be quite negative towards the industry as a whole. Well-publicised food scares and perceived risks of food products can be very detrimental and expensive to any industry as was demonstrated in the United Kingdom with Bovine Spongiform Encephalopathy, and eggs contaminated with Salmonella.

The current inability to identify animals with Johne's disease amongst the many ill-thrift ewes at the time of slaughter poses the greatest risk to the meat industry as this has potential to be portrayed to the public as a risk to public health as inevitably some of these animals are still routinely processed for human consumption (Link 2012). Reports by Elthoth *et al,* (2009) and the National Advisory Committee in Microbial Criteria for Foods (2010), presented data warning the industries that Map contamination of food products was a national and international problem and that steps needed to be undertaken to address this issue including management of perceived risk to public health. In previous studies the oral administration of non-viable or killed Map antigens was reported to play no role in the pathogenesis of Johne's disease in sheep (Brotherston *et al,* 1961b; Juste *et al,* 1994). Further research is required to determine the pathogenic role, if any, that Map may play in Crohn's disease and whether non-viable organisms pose a risk to human health. Public education programmes on the preparation and cooking of meat >60'C prior to consumption may be one way to help manage this perceived risk (Whittington *et al,* 2010). However, relying on preparation by the end consumer to ultimately provide a product for consumption that is 'safe' still puts consumers at risk. It is arguably irresponsible for an industry that is aware of the accepted association between Map and Crohn's disease, as proposed by Feller *et al,* (2007), Uzwoizge *et al,* (2007), Elthoth *et al,* (2009); Behr *et al,*(2008), NACMCF (2010) and Gill *et al,* (2011), to permit potentially contaminated product, as shown by Reddacliff *et al,* (2010) and Smith *et al,* (2011), to be offered to consumers in the first instance.

It is proposed that there may be ways to manage this perceived risk at the level of the processing industry. This would firstly involve the validation of the findings in Chapter four on ascribing poor condition sheep as high risk, followed by validation of tests proposed in this thesis, namely identification of hepatic epithelioid micro-granulomas (HEM) (Chapter 5 and 6) and a specific hyperspectral analysis technique (Chapter 7) .

8.4.1 Hepatic epithelioid macrophage micro-granulomas (HEM) – a new diagnostic test.

Map bacteraemia does occur and has been identified in sheep with clinical Johne's disease (Gwozdz *et al,* 1997; Bower *et al,* 2011; Begg *et al,* 2005; Begg and Whittington 2008; Readdacliff *et al,* 2010; Smith *et al,* 2011). Liver lesions including the appearance of micro-granulomas have been identified in cattle (Beurgelt *et al,* 1978), sheep (Perez *et al,* 1996) and goats (Manning *et al,* 2003) with Map bacteraemia and clinical Johne's disease. One murine study suggested the liver was the second best organ, after ileum, to assess the effect of Map infection in that species (Hines *et al,* 2007). It is notable that no further comments have been recorded as to their significance, even though these hepatic lesions appear to provide evidence of a systemic CMI response (CMIR) to Map infection outside the alimentary tract. Therefore liver lesion may have diagnostic potential as a proxy measure for identifying systemic Map infection in sheep. Chapter five describes hepatic epithelioid macrophage micro-granulomas (HEM) as a predictor of systemic infection.

The prevalence of HEM and their significance within naturally infected sheep are currently unknown.

Under natural farming conditions systemic Map infection and subsequent infection of skeletal muscle has only been identified in sheep with Johne's disease or those suddenly forced to

graze in close contact with clinically affected shedding cohorts (Reddacliff *et al*, 2010; Smith *et al*, 2011). However, it is proposed that this sudden period of close grazing with shedding cohorts is not representative of natural grazing, therefore it could be considered a period of 'artificial' challenge that provides a means for systemic Map infection to occur without ileal pathology as discussed previously. Under natural grazing management, sheep with systemic Map infection developed HEM and have Type 3b or 3c ileal lesions (Perez *et al,* 1996; Smith *et al.* 2014). These hepatic lesions have not been identified in sheep without Johne's disease (Smith *et al,* 2014, Chapter seven). This suggests the dissemination of Map to organs outside the alimentary tract is a consequence of the development of advanced Type 3b or 3c ileal lesions and therefore occurs in the later stages of infection and disease.

The routes available for Map to leave the alimentary tract include haematogenous via the portal vein or lymphatic via the cisterna-chyli, with both supplying the blood system. The liver is the first organ to receive blood from the portal vein and as such is the most likely organ to become infected via the haematogenous route.

In New Zealand, HEM have been found only in sheep with Type 3b or 3c ileal lesions and as such only in animals with Johne's disease. Therefore it is proposed that the number of animals developing HEM annually equates to that of clinical disease which has been estimated to be between 0.13%-1% (West *et al,* 2000; Verdugo *et al,* 2014).

Chapter five proposed that the formation of HEM could be used as a surrogate measure, or proxy, for the presence of Type 3b or 3c ileal lesions, and to have diagnostic potential for identifying sheep with a high probability of Map bacteraemia and therefore presence in muscle (meat). This study identified HEM only in ewes with advanced Johne's disease with a high level of agreement between this and ileal pathology (Cohen's kappa = 0.82, $p<0.001$). The diagnostic sensitivity and specificity for identifying sheep with Johne's disease were

96% and 100%, respectively (Smith *et al,* 2014). As such, there is potential to utilise this diagnostic test to identify animals in which systemic Map infection may be occurring, at time of slaughter, although prior to commercial application, further research is now required to validate these results.

Despite the diagnostic efficacy for this diagnostic test, the suggested potential for commercial application is unlikely to eventuate as liver histopathology, although being quicker and cheaper than Map culture or PCR, is not a real time test and even though sampling could be targeted towards suspect carcases, as determined by ante-mortem inspection for body condition, and manual post mortem inspection, it would be unlikely to be adopted by the meat industry as a mass screening tool.

8.5 Alimentary Tropism

In both natural and artificial infection studies Map has an affinity for the alimentary tract. This tropism has been identified in studies where artificial parenteral routes of infection have been used. These include the intra-peritoneal (Tripathi *et al,* 2007; Stabel *et al,* 2009), intra-tonsilar (Gwodz *et al,* 2000; Begg *et al,* 2005), respiratory (Kluge *et al,* 1968; Merkal *et al,* 1968; Robbe-Austerman 2007; Eisenberg 2013), and intravenous (Kluge *et al,* 1968; Merkal *et al,* 1968; Larsen *et al*, 1977; Tripathi and Kolhe 2007). In these artificially infected animals, Map 'migrated' in the opposite direction than that following oral infection, from outside the alimentary tract to tissues in the alimentary tract, such as the ileum. No residual infection remained at the site of origin. In Chapter four, noting it was a small observational study from a single flock, 50% of healthy sheep were infected, with Map only identified in the alimentary tract and not in skeletal muscle. Alimentary tract tropism enables the removal of this organism through abattoir processing with tracts sent for rendering. This may provide the public some 'comfort' that despite the prevalence of Map infection there is a relatively

simple means to remove the risk to public health and that this process is already in place. However, this is not the case for sheep with Johne's disease with Map not restricted to the alimentary tract.

8.6 Developing a real time test – Hyperspectral imaging as a novel predictor for Map in muscle.

There is a need to develop an economically viable, real time diagnostic test that is capable of mass screening animal products so that this albeit small number of systemically infected carcases can be identified at the time of processing and prior to entry into the human food chain.

Anatomical changes within the liver with the formation of HEM have enabled the identification of sheep with Johne's disease to be achieved using visible light and histopathology. Spectroscopy and hyperspectral imaging have recently been utilised to identify changes in tissues with the development of algorithms that recognise, in real time, a hyperspectral signature that is unique for a disease.

It was proposed in Chapter seven that hepatic changes may also be able to be identified using hyperspectral imaging and that there may be a hepatic hyperspectral signature for Johne's disease. In this study the Johne's disease status of 95 mixed age cull ewes were histologically confirmed with 10 animals identified to have abundant ileal mucosa AFO, Type 3b ileal lesions, HEM and Johne's disease. A further 200 lamb livers were also sampled with histopathology performed. Hyperspectral imaging of the liver tissue, through the cranial serosal surface of the left liver lobe, produced a pattern of light wavelengths that was different for ewes with, compared to the lambs and those ewes without Johne's disease, enabling a support vector machine (SVM) to develop an algorithm that identified a hyperspectral signature differentiating these two groups. The aetiology for this hyperspectral

signature is currently unknown although HEM were present in all 10 ewes with Type 3b ileal lesions, but not present in the other 85 or the 200 lambs. This algorithm provides an opportunity to further develop a real time hyperspectral diagnostic test that could identify animals with Johne's disease and as such identify carcases that are a high risk of Map in muscle destined for human consumption at time of slaughter. This has potential to be applied commercially as an alternative to hepatic histopathology for identifying carcases with systemic Map infection. However, this was a pilot study and further research is now required to identify the structures within the liver tissue that provide this hyperspectral signature and to increase the overall sample size so that the sensitivity and specificity of the algorithm can be validated.

8.7 HEM formation and anti-mycobacterial therapeutic efficacy studies

The development of liver biopsy histopathology and the identification of HEM and the hyperspectral imaging study have potential, as diagnostic tests, to identify animals where systemic Map infection is occurring. Serial sampling post artificial challenge may provide opportunity to open up new fields of research, allowing animals with HEM or hepatic changes to be compared to those without, thus enabling production parameters or therapeutic efficacy to be assessed between animals with or without systemic Map infection.

In Chapter seven, hyperspectral imaging was undertaken on animals post mortem. Therapeutic efficacy studies require longitudinal serial sampling to enable comparisons to be made between treatment versus non-treatment groups and determine whether benefits, if any, occur. It is proposed that histologically prepared liver samples, as that described by Smith *et al,* (2014), may also provide adequate tissue for the SVM algorithm to identify a hepatic hyperspectral signature for systemic Map infection. Further research is now required to determine this and if confirmed provides another diagnostic test, alongside liver

histopathology, for quantifying therapeutic efficacy and the effects on production of systemic Map infection in sheep. This proposal is included in the future research proposals outlined below.

The liver is an organ readily accessed for sampling, with serial liver biopsy well tolerated in ruminants (West and Vermunt 1995). Chapter five determined that post mortem liver samples collected using a liver biopsy probe provided adequate tissue for the histological diagnosis of animals with Johne's disease through the identification of HEM. Therefore it was proposed that liver histopathology may provide a surrogate means to follow the development of Type 3b or 3c ileal lesions in naturally infected sheep and may also have potential to follow the development of HEM or other hepatic changes post artificial challenge.

In New Zealand, the number of new cases of Johne's disease occurring each year within an infected flock was estimated to be 0.13% (Verdugo *et al,* 2014) therefore performing therapeutic efficacy studies in naturally infected sheep would be logistically difficult, requiring a large number of animals to be sampled before any with clinical Johne's disease and HEM development were identified. This would be further exacerbated by possible different strains of Map affecting different animals within the same study, the stage of disease progression at time of initiating therapeutic treatment, the age of the animal at time of infection, onset of clinical signs and ultimately by the inability of any currently available diagnostic test to differentiate animals that are progressing towards clinical disease from those that are sub-clinically affected.

A solution to this is the use of longitudinal challenge studies where the strain type, initial challenge dose of Map, the route of infection, the age and timing of infection is known (Hines *et al,* 2007; Begg *et al,* 2005; Begg and Whittington 2008; Gardner *et al,* 2011). In these

studies, artificial oral Map dosing can increase the probability and speed of development of clinical disease with up to 20% of infected animals progressing onto clinical disease within a year of oral dosing (Begg and Whittington 2008). In these circumstances, the use of serial liver biopsy histopathology and the identification of HEM, may provide a means to follow Map infection in animals and therefore enable the therapeutic efficacy of anti-mycobacterial drugs or vaccines at limiting or preventing systemic Map infection to be tested. One such therapeutic agent is thalidomide, a potent anti-Tumour Necrosis Factor alpha (TNFα) drug that is used successfully in human medicine to treat patients affected with mycobacterial diseases such as Leprosy and Crohn's disease.

8.8 Thalidomide – a therapeutic candidate.

In human medicine, systemic Mycobacterial infection occurs in diseases such as leprosy and Crohn's disease (Chiodini *et al,* 1984, 1986; McFadden *et al,* 1987; Green *et al,* 1989; Sanderson *et al,* 1992; Naser *et al,* 2000, 2004; Schwartz *et al,* 2000; Sechi *et al,* 2001, 2004; Scanu *et al,* 2007; Uzwoige *et al,* 2007; Rocca *et al,* 2010). Therapeutic success has been achieved in patients with either of these diseases when potent anti-tumour necrosis factor alpha (TNFα) drugs are administered, with some chronic cases making full recoveries (Teo *et al,* 2002; Lazzerini *et al,* 2007; Leite *et al,* 2011; Zeng *et al,* 2011; Scribano *et al,* 2014).

In patients with Crohn's disease and sheep with clinical Johne's disease, the mRNA for the pro-inflammatory cytokine TNFα is up-regulated with increased genetic expression and elevated TNFα levels identified within the ileal tissue, suggesting this cytokine may play a role in these diseases (Alzuhurri *et al,* 1996; Aho *et al,* 2003; Juste *et al,* 2007). In humans, the response to therapy when treated with anti-TNFα drugs such as Infliximab (mono-clonal antibody), Adamamub, or thalidomide can be assessed from serial endoscopic biopsy of the

ileum with improvements in the ileal histology score over time indicative of therapeutic efficacy (Sehgal and Koltun 2010).

In sheep with clinical Johne's disease, serial endoscopic sampling of the ileum is also possible as a research tool (Dennis *et al,* 2011). However, work in Chapter five has demonstrated that HEM formation and ileal pathology are highly associated in naturally infected animals. Therefore ileal biopsy may be unnecessary since serial liver biopsy histopathology is easier, less invasive and may provide surrogate information for the assessment of therapeutic efficacy in sheep. There are no studies in humans regarding the identification of HEM in patients with systemic Map infection so it is proposed here that liver histopathology may also have a diagnostic role to play in patients with Crohn's disease. Further research could now be justified in this field.

The success of anti-TNFα therapy, especially the use of thalidomide, in humans with systemic Map infection, suggests that there may also be a potential therapeutic role for anti-TNFα drugs in sheep with similar systemic Map infection. The use of thalidomide in sheep is unlikely to ever be accepted due to the highly published teratogenic effects in humans and subsequent poor public perception. However, identifying the role TNFα may play in the pathogenesis of Johne's disease in sheep and potentially other ruminant species may help with future development of anti-Mycobacterial therapeutics and as such the use of thalidomide in these studies is justified. Research is now required to determine the role that this pro-inflammatory cytokine has to play in the establishment of clinical Johne's disease. The administration of thalidomide may provide opportunity to assess this.

Currently, there is no published information on the pharmacokinetics of thalidomide in sheep and as such establishing a dose regime for any clinical therapeutic study is difficult. This is addressed in Appendix 1, where the elimination half life ($t_{1/2}$) and time to reach maximum

plasma concentration (T_{max}) were determined. These values have been utilised to establish that a single 400mg oral dose of thalidomide, dissolved in dimethylsulphoxide (DMSO), is required in sheep to produce similar plasma concentrations (C_{max}) as seen in humans where successful treatments for Crohn's disease have been recorded.

It is proposed, that if artificial Map infection is representative of natural infection in sheep, as suggested by Begg *et al,* (2005), there may now be a relatively non-invasive means to assess the therapeutic efficacy of thalidomide and potentially other anti-TNFα drugs at preventing systemic Map infection and Johne's disease. Serial liver biopsy followed by histopathology or hyperspectral analysis of histopathological sections if validated for biopsy samples, may provide a means to determine whether these drugs have therapeutic efficacy and any role to play in the prevention or treatment of Johne's disease in sheep.

However, there was no data available quantifying the prevalence of systemic Map infection, or the time line for the formation or the duration of HEM lesions post artificial oral challenge. The aims in the longitudinal intervention study in Chapter six addresses these parameters and enables future protocols for therapeutic efficacy studies to be developed.

8.9 Longitudinal Intervention Study

The aetiology or trigger that culminates in the colonisation of the ileum with progressive development of lesions through to systemic Map infection and Johne's disease in naturally infected sheep is currently unknown. Farm management policies can affect the stress levels experienced by animals on farms where nutrition, parasite control and disease prevention practices affect growth rates and the overall animal health. It is currently unknown whether stress has a role to play as a trigger point for the formation of Johne's disease in sheep. Genetic resilience may also play a part in the development of Johne's disease. In recent work, it was found that artificial Map infection of the alimentary tract was similar for

different breeds of sheep. However, a genetic association was suggested for the development of disease progression, with different breeds having different levels of development of clinical disease (Begg *et al,* 2016). Further research is now required to determine the genetic influence on disease progression and the occurrence of systemic Map infection.

In the study by Smith *et al,* (2014, Chapter five) there may be an association with the presence of Type 3b or 3c ileal lesions and systemic Map infection. In naturally infected sheep, HEM only occur once ileal pathology has progressed to Type 3b or 3c ileal lesions (Smith *et al,* 2014, Chapter five). Liver biopsy and the presence of HEM provides a proxy measure for these Type 3 ileal lesions and therefore the occurrence of systemic Map infection in sheep. The longitudinal study in Chapter six followed the artificial infection guidelines established in a review by Begg and Whittington (2008). Serial liver biopsies and blood samples were collected over 820 days providing data to assess the occurrence of HEM formation, the time for their formation and resolution, sero-conversion and S/P ratio positivity and the cost on production of systemic Map infection in those animals that made recoveries after artificial oral exposure.

8.9.1 Artificial challenge versus natural Map infection

8.9.1.1 HEM prevalence

In Chapter five, observations were from naturally infected sheep in which HEM were recorded only in those with clinical Johne's disease (Smith *et al,* 2014). The aetiological mechanism for the development of systemic Map infection and subsequent HEM is currently unknown. It is likely that Map is causal because there was an almost perfect level of agreement between Type 3b or 3c ileal lesions in ileum and HEM. This supports that systemic infection occurs as a result of spill-over from advanced ileal pathology in naturally infected sheep (Smith *et al,* 2014).

In contrast, HEM occurred in all the artificially challenged lambs in the longitudinal study (Chapter 6). It was first identified in two lambs at 51days and all lambs at 195 days post oral exposure. Despite ileal histopathology not being undertaken in this study, it is unlikely that there was enough time post Map exposure for these artificially challenged lambs to develop Type 3b or 3c ileal lesions at those time intervals. This is supported in the review undertaken by Begg and Whittington (2008), of the 97 independent challenge studies examined, where necropsies of lambs were undertaken. None recorded Type 3b or 3c ileal lesions in the challenged lambs at a similar time frame to those in the present study, suggesting significant ileal pathology does not develop at this early stage post artificial oral challenge.

Further support is also found in the longitudinal study undertaken by Juste *et al,* (1994), where serial ileal histology was recorded after artificial challenge. In that study, eight lambs were orally administered exogenously sourced Map on two occasions, containing 1.36×10^6 organisms on each occasion, with necropsies performed on two lambs at each of 15, 45, 120 and 220 days after Map exposure. Ileal pathology increased over time with one, five and six granulomas recorded per histological section as the disease progressed from 45, 120 and 220 days, respectively. However, despite ileal lesions progressing, they did not advance to Type 3b or 3c lesions. Hepatic lymph node granulomas were also recorded in this study, providing evidence that systemic Map infection had occurred without Type 3b or 3c ileal pathology. Despite this first being mentioned by Juste *et al*, (1994), no further comment has been made as to their significance. Furthermore, Type 3a, 3b and 3c ileal lesions are considered to be irreversible, progressing to terminal clinical Johne's disease (Perez *et al,* 1996). In the longitudinal study in Chapter six, 14 of 18 lambs recovered, suggesting these irreversible Type 3 ileal lesions were not present at the time of HEM formation and that in artificially

challenged sheep, this event may occur independently from ileal pathology suggesting another aetiological mechanism is occurring.

8.9.1.2 Ileal CMI Capacity – Young versus Old

The contrasting result between artificial and natural exposure from the present study and other reports, suggest that the mechanism that allows Map to leave the alimentary tract and invade deeper tissues appears to be different for artificially or naturally exposed animals. Systemic Map infection in naturally exposed sheep occurs in older animals by which age Type 3b or 3c ileal pathology is present, whereas in lambs, artificially challenged with high doses of Map (>1 x 10^9 organisms), ileal pathology is absent.

In naturally infected sheep, oral uptake is considered to be the most likely route of infection for this organism, with tropism for the alimentary tract demonstrated in numerous studies (Gilmour *et al,* 1965; 1978; Kluge *et al,* 1968; Merkal *et al,* 1968; Larsen *et al,* 1977; Begara–McGorum *et al,* 1998; Gwozdz *et al,* 2000; Storset *et al,* 2001; Kurade *et al,* 2004; Reddacliff *et al,* 2003; Waters *et al,* 2003; Stewart *et al,* 2004; Begg *et al,* 2005; Sweeney *et al,* 2006; Robbe-Austerman *et al,* 2007; Stabel *et al,* 2009). The Ileal CMIR at the Peyer's patch and the mesenteric lymphatic chain are considered to be the primary means of defence against Map (Buergelt 1978, Perez *et al,* 1996), with infection of deeper tissue structures limited by the capacity of this CMI to withstand Map invasion (Juste *et al,* 1994). The invasion of Map into ileal tissues appears to occur readily and quickly (Wu *et al,* 2007), therefore prevention of deeper tissue invasion and subsequent systemic infection may require an ileal CMI that is pre-prepared and able to absorb or isolate the number of Map that it is exposed to and to do this with short notice. The robustness of the ileal CMI and its capacity to withstand Map invasion and therefore limit the occurrence of systemic infection may be

influenced by previous exposure to Map (Juste *et al,* 1994; Kurade *et al,* 2004; Dennis *et al,* 2011).

In sheep populations, where Map exposure has not occurred, there would be little requirement for the immune system to expend energy and protein to develop a competent and robust ileal CMI, with macrophage recruitment unlikely to occur without Map antigen stimulus (Juste *et al,* 1994; Begg and Whittington 2008). In these populations, young naïve animals with immature immune systems are likely to be more susceptible and have a lower ileal capacity to contain an event were they to be exposed to Map. For this reason, this class of stock is recommended for use in intervention studies and has been used to develop a model for Map infection (Begg *et al,* 2005; Hines *et al,* 2007; Begg and Whittington 2008; Nielsen and Toft 2007, Gardner *et al,* 2011).

In contrast, in older sheep, from farms where Map is present, previous chronic exposure to oral Map, through normal grazing management, stimulates an ileal CMIR with an associated up-regulation of the cytokine cascade including tumour necrosis factor α (TNFα), the interleukins (IL) and interferon gamma (Iγ). This results in the gradual recruitment of macrophages into the ileal tissues over a prolonged period of time that is proportional to the rate of exposure (Aho *et al,* 2003; Alzuhurri *et al,* 1996; Perez *et al,* 1996, 1997; Stabel *et al,* 2000). Older, previously exposed animals therefore have a greater ileal CMI capacity for responding to Map. Even though they are just as likely to become infected by Map as younger animals (Reddacliff *et al*, 2004), their ability to withstand Map invasion from a sudden influx of Map into the lumen of the ileum is much higher with little response to challenge likely to occur. This is one reason why this class of animal is not recommended for use in intervention studies (Shield 1983; Stanford 1983; Juste *et al,* 1994; Begg and Whittington 2008).

Further evidence of the enhancement of the ileal CMI capacity to Map due to previous exposure comes from a study where serial surgical biopsies of the ileum were undertaken in lambs naturally orally infected by Map with histology revealing an ileal mucosa with increasing macrophage recruitment over time providing a more robust ileal CMI (Dennis *et al,* 2011). Other studies also record this effect where lambs were artificially exposed to Map antigens via vaccination. This exposure pre-prepared the ileal mucosa with recruitment of macrophages enabling the vaccinated animals to prevent the diffuse invasion of Map into deeper ileal tissues when exposed to high numbers of oral Map as opposed to unvaccinated controls (Juste *et al,* 1994; Kurade *et al*, 2004).

It is proposed that the mechanism for the development of systemic Map infection comes from a process that overwhelms the ileal CMI enabling Map to spill over into the systemic circulation and infect organs distant to alimentary tract.

8.9.1.3 Overwhelming the CMI Capacity – HEM formation - exogenous or autogenous paths

It is hypothesised that for all sheep the aetiology of HEM formation and therefore systemic Map infection is the same, requiring the ileal CMI capacity to become overwhelmed by the number of Map presented to the ileum. Once the ileal CMI capacity is saturated, Map is free to invade the deeper tissues and pass into systemic circulation. However, it is proposed that the circumstances that supply adequate numbers of Map into the ileal lumen to produce an ileal CMI overwhelming event are different for natural and artificially infected sheep and that there may be different mechanisms that enable this event to occur.

One mechanism may occur when sheep are artificially dosed and the ileal lumen concentration of organisms suddenly exceeds the Peyer's patch capacity to sequester Map. At this saturation point systemic infection may occur with subsequent HEM formation, in the absence of Type 3 ileal lesions at this early stage. It is hypothesised that systemic infection

can occur when naïve lambs are suddenly artificially orally dosed on multiple occasions with > 5 x 10^8 exogenously sourced Map, as recommended in the model for artificial Map infection (Begg *et al,* 2005; Begg and Whittington 2008) or when adult sheep are forced to suddenly graze heavily contaminated paddocks immediately prior to slaughter as recorded from skeletal muscle culture in Chapter three. Both oral exposure events occur quickly with large numbers of Map presented to the Peyer's patch. With no time for the ileal CMI to recruit new macrophages or adapt to the sudden increase in bacterial challenge, Map saturation occurs enabling subsequent spill-over from the alimentary tract CMI. This proposal is supported in previous challenge studies, where an overwhelming event has been incidentally reported in naïve lambs orally challenged with > 5 x 10^8 organisms with the development of clinical disease and the formation of granulomas in lymph nodes outside the alimentary tract and liver (Juste *et al,* 1994; Gwozdz *et al,* 2000; Kurade *et al,* 2004; Begg and Whittington 2008; Bower *et al,* 2011). In the longitudinal study in Chapter six, an overwhelming event also occurred when oral doses > 1 x 10^9 organisms were administered on 10 occasions over a 30 day period resulting in HEM appearing in all the challenged lambs.

Another mechanism for systemic Map infection and the formation of HEM appears to occur in adult sheep under natural challenge circumstances. Here, chronic exposure to exogenously sourced Map from contaminated pasture is likely to occur on a regular basis in flocks where clinical Johne's disease has been diagnosed. This constant oral exposure is likely to produce a constant stimulation of the ileal CMI with recruitment proportional to exposure periods (Juste *et al,* 1994; Kurade *et al,* 2004; Dennis *et al,* 2011). In naturally grazed sheep, HEM have only been found when ileal pathology has progressed to Type 3b or 3c ileal lesions (Smith *et al,* 2014). To date, they have not been found in any other classes of stock including healthy four-month-old lambs at slaughter (Chapter seven, unpublished Smith *et al,* 2015) or ewes without clinical Johne's disease. This observation requires further validation but

suggests that the numbers of Map ingested from natural grazing is inadequate to overwhelm even naïve lambs and that the oral intake of exogenously sourced Map from naturally infected pasture may have little, if any role to play in producing an overwhelming event that leads to systemic Map infection.

For naturally infected sheep, it is hypothesised that following initial ingestion of Map a potentiating cause must exist to provide the requisite numbers of organisms necessary to overcome the capacity of an adult and mature ileal CMI. It is proposed that this potentiating source of Map comes autogenously, from sloughing of Map from the mucosa of the Type 3b or 3c ileal lesions with subsequent escalation of challenge due to continuous auto-re-infection at the Peyer's patch. This proposal supports and helps to explain the conclusions in Chapter five where HEM were only found in naturally infected adult sheep with advanced Type 3b (multibacillary) or 3c (paucibacillary) ileal lesions. In these animals the concentration of Map within the ileal tissues has been quantified, with up to 1×10^9 organisms per gram tissue (Reddacliff *et al,* 2003) and 8×10^8 organisms per gram faeces recorded (Whittington *et al,* 2000). It is therefore likely that sheep with Type 3b or 3c ileal lesions are continuously re-exposed to high numbers of Map, autogenously sourced from their own diseased ileal mucosa.

It is proposed that this continuous re-infection continuously stimulates the pro-inflammatory cytokine cascade with over-expression and up-regulation of TNFα, ILs and Ig mRNA, resulting in the further recruitment of macrophages, producing the terminal granulomatous enteritis associated with clinical Johne's disease (Alzuhurri *et al,* 1996; Burrells *et al,* 1998; Aho *et al,* 2003; Stewart *et al,* 2004; Shu *et al,* 2011). It is also proposed that over time the number of organisms sloughed from the diseased ileal mucosa eventually exceeds the ileal CMI capacity at the Peyer's patch and an overwhelming event occurs resulting in systemic

Map infection, the formation of HEM, cachexia and ultimately death from chronic weight loss.

In the longitudinal study in Chapter six, the histopathological identification of HEM was used, as a surrogate measure, to record the occurrence of the proposed 'overwhelming event' that produces systemic Map infection in sheep. The first overwhelming event was achieved by orally dosing naïve lambs with > 5 x 10^8 exogenously sourced Map, on 10 separate occasions over a 30 day period with HEM recorded in two lambs at 51 days and in all lambs by 195 days. After this time point, HEM numbers declined in 14 of 18 lambs until none were present at 482 days. It is proposed that this decline in HEM occurred because the supply of exogenous oral Map was limited to just 30 days, after which the progression to over whelming events ceased, the CMIR was successful in containing the infection, the subsequent Map bacteraemia was prohibited and the systemic CMI cleared the hepatic infection allowing animals to recover.

In contrast, four lambs went on to develop clinical Johne's disease with Map colonisation and subsequent multiplication occurring within the ileal tissues. In these animals a second overwhelming event was recorded at 233 days with HEM numbers increasing in excess of 20 lesions per liver biopsy sample. This "2nd wave" of HEM lesions occurred at 60 – 220 days prior to these animals being euthanised and diagnosed with Type 3b ileal lesions (Perez *et al,* 1996). It is proposed that this 2nd wave represents the occurrence of an overwhelming event originating from autogenously sourced Map from the Type 3b ileal lesions, and that the number of HEM lesions continued to increase up to point of no recovery and therefore euthanasia, due to the source being unlimited and continuous (Figure 8.1).

8.9.1.4 Sero-positivity

Sero-conversion and S/P ratio positivity in naturally infected animals often occurs in older animals when ileal pathology has developed and when clinical disease is occurring, suggesting ileal pathology may be associated with stimulating the humoral immune response (Hilbink *et al,* 1994; Burrells *et al,* 1998; Whittington and Sergeant 2001; Sergeant *et al,* 2003; Steadham *et al,* 2003; Robbe-Austerman *et al,* 2006). However, in previous artificial challenge studies, ELISA positive lambs have been recorded within 70 days of oral challenge when it is unlikely for there to be ileal pathology present (Begg *et al,* 2005; Nielsen *et al,* 2007; Begg and Whittington 2008). In the longitudinal study here, positive ELISA S/P ratios were recorded at 69 days, suggesting there may be a reason for sero-conversion unrelated to ileal pathology.

In Chapters five and six there was a substantial level of agreement between the number of lambs developing HEM and those with positive S/P ratios and in Chapter six as the number of HEM per sample increased, the S/P ratio for that period of time also increased. This suggests that systemic Map infection may be the initiator of sero-conversion irrespective of ileal pathology and that the stimulation of the humoral response requires an overwhelming event to occur. This occurs in artificial challenge studies with exogenous administration of oral Map and in natural infection when autogenous re-infection of Map sloughed from Type 3b or 3c ileal lesions occurs.

From Chapter six, it is tempting to conclude that a relatively simple solution exists for identifying sheep where systemic Map infection has occurred, with ELISA S/P ratios having diagnostic potential. However, this is not the case as sero-conversion did not occur in all of the challenged lambs despite all demonstrating HEM, and two of the four lambs that progressed to clinical Johne's disease remaining ELISA negative. In these two animals,

HEM were identified at 114 days suggesting an initial overwhelming event had occurred from the exogenous oral administration of the Map homogenate. A second wave of HEM formation was recorded from 233 days in one ewe and 364 days in the other suggesting autogenous re-infection was now occurring with systemic involvement. Both animals were euthanised with clinical Johne's disease, at 450 and 433 days, respectively. Despite increasing numbers of HEM over this period, no humoral immune response was recorded. The reason why this occurs is currently unknown and may contribute to the poor diagnostic efficacy associated with ELISA serology and Johne's disease (Nielsen & Toft 2007). Conversely, the diagnostic efficacy of liver histopathology for identifying sheep with Johne's disease and where systemic Map infection has occurred, that was previously recorded in Chapter five, was maintained in this longitudinal study (Smith *et al,* 2014).

8.9.1.5 Consequences of Map infection – quantifying non-systemic versus systemic periods of infection

It is proposed that to quantify the cost of Map infection on production, there are two different periods that require measurement, which are where infection is either non-systemic, with the organism localised in the alimentary tract or a period where infection is systemic and the organism has spilled over into the systemic circulation, infecting tissues distant to the alimentary tract.

In naturally infected sheep, many animals become exposed, with up to 50% of animals becoming sub-clinically, or to be more objective regarding the nomenclature of the type of infection present, non-systemically infected, with Map sequestered within the ileal or local lymphatic tissues (Smith *et al,* 2012). There are different categories of ileal response, with Types 1, 2 and 3a being non-systemic lesions, where systemic Map invasion is prevented by the ileal CMI (Perez *et al,* 1996). This sequestration requires proteins and energy to be

invested by the immune system and therefore would have a physiological cost. However, in naturally infected animals these different categories of immune response progress over a prolonged period of time with the chronic recruitment of macrophages occurring, as and when required, with the ileal CMIR being proportionate to the exposure rate (Juste *et al*, 1994; Kurade *et al*, 2004; Dennis *et al*, 2010). Quantifying the cost on production of these subtle adjustments within the ileal CMI may be impossible as there are no currently available commercial diagnostic tests that can identify non-systemically infected animals and therefore none that can make comparison between these animals and those that are uninfected. Additionally, the cost of this non-systemic immune stimulation may be masked by changing feeding levels throughout the year and as such trying to quantify the costs of this on production may be akin to looking for the 'holy grail'.

Currently, in naturally infected sheep, there have been no studies published where the cost of non-systemic Map infection have been successfully assessed. Production losses have been reported only in animals that are clinically affected with Type 3b or 3c ileal lesions and therefore where clinical Johne's disease is developing with systemic Map infection occurring (Thompson *et al*, 2002; De Lisle 2002; Morris *et al*, 2006; Bush *et al*, 2006, 2008; Begg and Whittington 2008; Nielson *et al*, 2008; McGregor *et al*, 2015). In the McGregor *et al*, (2015) study, decreases in live weight and albumin were recorded up to 10 months prior to the development of clinical Johne's disease. In these animals Type 3b or 3c ileal lesions were also developing at this time. It is proposed that these reported losses may not have been due to non-systemic infection but may have been from systemic infection, and had liver histopathology been undertaken then HEM may have been identified, confirming this event. A similar claim may also be made for Chapter six data where production losses were recorded in artificially infected animals only when systemic infection was occurring. In this study the formation of HEM was used as a proxy measure to identify animals where systemic

Map infection had occurred and, as such, enabled comparison of the effects of systemic Map infection on live weight and body condition score to be made between challenged and unchallenged groups of sheep.

Following oral challenge with Map, all the challenged lambs in the longitudinal study had a period of systemic Map infection with HEM formation recorded at 51 days and peaking at 195 days. Over this period live weight and body condition score losses occurred compared to the similarly managed control or unchallenged lambs and mean ELISA S/P ratios increased. After 195 days all challenged sheep gained weight at a similar rate to the unchallenged sheep with mean HEM numbers and S/P ratios declining, suggesting systemic Map infection was also declining. Four challenged lambs went on to develop Type 3b ileal lesions with a 2nd wave of HEM formation recorded at 233 days with a subsequent 2nd period of decreasing live weights and body condition scores. In these animals, HEM lesions continued to increase, along with increasing weight loss and decreasing body condition score, until all four were diagnosed with clinical Johne's disease and euthanised. In the remaining 14 sheep, the mean number of HEM lesions continued to decrease with corresponding increase in live weight and body condition score until 482 days when there were no HEM present and the growth rates were equal to the unchallenged sheep for this later period. The mean ELISA S/P ratios also declined over this period of time, with none of the challenged sheep remaining at the end of the study being ELISA positive.

In this study, artificial oral infection produced two periods of systemic infection. The first occurred in all the challenged sheep after oral dosing and the 2nd occurred from autogenously sourced Map in the four sheep that developed Type 3b ileal lesions and clinical disease. When HEM formation occurred, production losses in liveweight and body condition scores also occurred in all the challenged sheep and vice versa as HEM lesions resolved.

In periods of non-systemic infection, there were no differences in production detected between the challenged or unchallenged sheep. This suggests that in naturally and artificially infected sheep any loss of production from non-systemic Map infection may be insignificant and that losses only occur once systemic Map infection begins.

Production losses from systemic Map infection were recorded well before the onset of clinical Johne's disease, with decreases in liveweight and body condition score occurring up to 220 days prior. A similar result was recorded in the study by McGregor *et al,* (2015), suggesting the cost of systemic infection needs to not only take into account losses from animals dying from clinical Johne's disease but also all the losses of production that can occur for up to 10 months prior to the full onset of clinical signs. The longitudinal study concluded that in 18 artificially infected sheep, systemic Map infection resulted in production losses from 4 animals dying with clinical Johne's disease, with the remaining 14 surviving animals growing smaller skeletal sizes with a subsequent 11kg liveweight difference at 820 days. Losses due to non-systemic infection were unable to be quantified.

8.9.1.6 Survival and self-cure – Outcomes from Systemic Map infection.

In the review by Begg and Whittington (2008), one conclusion about the artificial challenge studies reviewed was that many ended when the desired research outcome had been achieved. However, the result for the animals involved may have been different had the study been allowed to go on for a longer period of time. Many of these studies failed to assess resilience or the ability of an animal to survive and continue to be productive despite being infected by Map, and none addressed resilience to systemic Map infection. Yet in all of these studies the source of Map was exogenous and the challenge period was finite, with animals only challenged for a limited period of time, providing ideal conditions for resilience to occur. In many of these studies, this event may have incidentally been recorded, as a majority of

challenged lambs failed to develop clinical Johne's disease, despite the administration of the recommended numbers of oral Map, and went on to recover, with few signs of ileal pathology at necropsy.

In Chapter five, one conclusion was that systemic Map infection in naturally infected sheep is always terminal and that animals with HEM always die from clinical Johne's disease. Yet, the result in the longitudinal study does not support this, with 14 artificially infected animals showing self-cure and recovery after HEM formation and systemic Map infection. It is proposed that the aetiology of HEM is the same for both naturally and artificially infected sheep and is dependent on an overwhelming event, allowing Map to spill-over from the alimentary tract with subsequent systemic infection. It is also proposed that the outcome for an animal and whether or not it will develop clinical Johne's disease after an overwhelming event is dependent on how the event occurred.

It is hypothesised that if the source of Map is limited, as in the longitudinal study (Chapter six) or the intermittent grazing of heavily contaminated pasture from shedding cohorts (Chapter three), then the overwhelming event is not dependent on ileal colonisation, with requisite numbers of Map for this event to occur supplied directly from the exogenous oral source. In these circumstances the period of systemic infection is limited, and as such animals lose weight and body condition for a limited period of time, systemic immunity is able to clear the infection and animals eventually recover. Conversely, if Map colonisation of the ileum occurs and lesions progress to Type 3b or 3c then there is no recovery as the source of Map is continuous, through sloughing from the Type 3b or 3c ileal lesions. With autogenous re-infection, a continuous overwhelming event occurs with animals continuously losing weight and body condition until they are 'consumed' by systemic infection and die

from cachexia and Johne's disease (Fig 8.1).

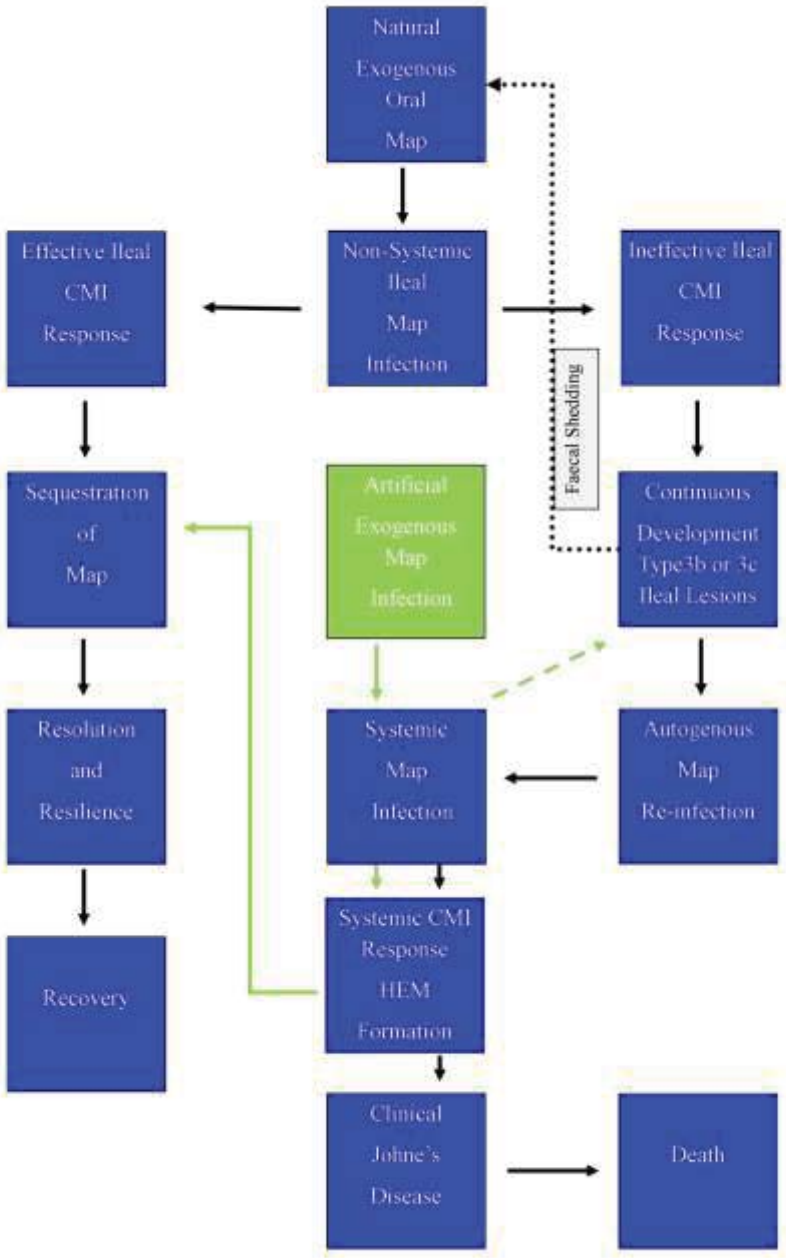

Figure 8.1 Flow Chart for Map infection including the exogenous and autogenous routes for both natural and artificially infected sheep.

8.10 Clinical Johne's disease – a new proposed pathogenesis

The current belief that animals with clinical Johne's disease die from cachexia and emaciation due to a protein losing enteropathy (PLE) from ileal lesions and subsequent malabsorption (Petterson and Berret 1969) needs to be questioned, as this is not supported in animals with Type 3c lymphocytic dominant paucibacillary enteritis, where there is mild enteritis and often no diarrhoea (Perez *et al,* 1996; Dennis *et al,* 2011). These animals do develop emaciation and terminal wasting yet have no signs of a PLE. It has been suggested by Dennis *et al,* (2011) and Juste *et al,* (1994) that an alternative mechanism exists for the development of the clinical signs of Johne's disease and that there may be either an active role for cytokine mediated-cachexia, or another source of Map. However, no evidence for these proposals was provided.

In the Australian study by McGregor *et al,* (2015), weight loss and decreasing serum albumin levels were recorded in naturally infected sheep from 21 months after exposure. However, up to 18 months the mean growth rates for animals in both groups were equal, with similar blood parameters. After 18 months it was proposed that a 'tipping point' had been reached, with differences in these parameters recorded between the two groups with an increased rate of catabolism suggested as a possible aetiology. It was postulated that the live weight losses up to this point may have come from a protein malabsorption syndrome through these changes in the ileal tissue, with an under supplied liver unable to produce adequate albumin. It was also suggested that a secondary protein-losing type enteropathy may have also been occurring, although neither of these suggestions were supported by evidence.

In Chapter six, weight loss and signs of systemic Map infection were first recorded some 220 days prior to the onset of clinical Johne's disease, suggesting that a 'tipping point' event also occurs, over a similar period, in artificial infection. It is proposed that an alternative explanation for this weight loss and decreasing serum albumin could be that systemic Map infection was occurring as a result of ileal pathology progressing to Type 3b or 3c ileal lesions and that what McGregor *et al,* (2015) had recorded some 10 months prior to clinical Johne's disease was the beginning of the systemic period of losses that were also recorded in the longitudinal study in Chapter six.

It is hypothesised that the clinical signs of wasting, weight loss and ultimately death do not come from PLE alone, and that the systemic CMIR that occurs from continuous autogenous re-infection plays a leading role in the pathogenesis of this disease in sheep.

It is proposed that the pathogenesis of Johne's disease should now be considered to be multi factorial, with a simultaneous and cumulative loss of energy and proteins resulting from the following progression;

- **a period of non-systemic infection and ileal CMI stimulation** as Map colonisation and multiplication occurs within the ileal tissues, with an initial immeasurable cost to production. A chronic ileal CMIR occurs, with continued cytokine expression and subsequent ileal macrophage recruitment occurring. This period develops over time, with Type 1 lesions progressing to Type 2 and onto Type 3a ileal lesions. These progress to gradually produce the ileal granulomas and lymphadenitis / lymphangitis and Type 3b or 3c ileal lesions that are associated with the gross pathology of clinical Johne's disease, followed by;
- **a period of systemic infection and systemic CMI stimulation** and cytokine over expression in response to Map sloughing from the diseased ileal mucosa, with autogenous re-infection, and subsequent bacteraemia with Map cultured in organs distal to the alimentary tract

including the skeletal muscles and continuously showering the liver stimulating it to produce HEM and;

- **a period of systemic infection with the physical loss of proteins** through the currently accepted pathways of protein losing enteropathy and malabsorption in some animals with formation of a granulomatous enteritis, and;
- **a period of humoral immunity stimulation** which occurs at the end stage of the disease and may be associated with systemic Map infection and HEM production.

8.11 Study Limitations

Larger study groups are always beneficial when trying to improve the robustness of results. Some of the studies in this thesis have limited data sets and require further investigation with larger numbers of animals investigated from more farms across New Zealand. Whilst, the observational study in Chapter three identified Map in muscle of sheep, providing evidence that meat is a source of Map for humans, this study was from a single farm, from a single class of stock using cull mixed age ewes. The collection of samples was part of an annual illthrift ewe investigation, undertaken as a veterinary undergraduate teaching exercise, at Massey University and as such, was repeated on two occasions, in July, over two years. Incorporating research with teaching is mutually beneficial between researchers and the undergraduate teaching programme, regarding both financial savings in labour costs and access to animals being used for teaching. This relationship was undertaken throughout the thesis although it is recognised that limitations do exist, such as the timing of sample collection with teaching schedules. As such, sample collection may have occurred over a prolonged period of time, such as in Chapter three, where ewes were sampled from the same farm on two different occasions over two years, and this may add potential for inter-year confounding variables to occur such as lab processing and differences in farm management

practices that could influence these results. Improvements could be made by increasing the number of animals sampled, sourcing sheep from more properties and completing the study over a shorter time frame. Likewise, the prevalence of Map infection in healthy sheep (Chapter four) provided information that systemic Map infection could not be found in healthy animals (BCS>3), which is fundamentally important to the meat industry. These ewes were from the same farm as the poor body condition sheep examined in Chapter three and provided insight into the prevalence of Map infection in sheep that are considered to be healthy on an infected property. However, only twenty four healthy ewes were examined.

Chapter five developed a new diagnostic test using histopathology and the presence of HEM to identify sheep with Johne's disease. The presence of HEM may also be used as a surrogate measure to determine the occurrence of systemic Map infection and therefore, by proxy, identify carcases infected by Map. This study again requires additional animals for validation, with potential to improve assessment of the diagnostic efficacy for both identifying Johne's disease and systemic infection, if tissue culture or PCR analysis was also undertaken. The use of Immunohistochemistry to further identify whether the HEM lesion is Map specific, as despite having AFO present within the HEM lesions and being confirmed by culture and PCR, other infectious agents, such as M avium, may also result in similar pathology.

The longitudinal intervention study followed the recommendations set out in Begg *et al,* (2005) model of artificial infection in sheep and could have been further improved had the two groups been replicated, although any advantages that this may have gained may be offset by the potential that natural infection in sheep is not represented when animals are artificially challenged. Nevertheless, replication may have removed any confounding variables that may have arisen through grazing management, although ad lib feeding was maintained for both

groups throughout the 820 days with the exception of the period of drought experienced in the last three months of the study where both groups had equally restrictive intakes.

The hyperspectral pattern that the SVM algorithm identified for sheep with Johne's disease in Chapter seven, comprises data from only 10 ewes with this disease, and 85 ewes and 200 lambs without. The number of animals in this pilot study is inadequate to establish diagnostic efficacy. Although from the current results, there appears to be potential for hyperspectral imaging to provide a real time diagnostic test that can identify sheep where systemic Map infection is occurring at time of slaughter.

8.12 Conclusion

The public perception of food safety can be fragile, with little publicity required for consumers to lose trust should there be a potential health risk associated with a food product. The now accepted association between Map and Crohn's disease could be perceived by the public as causation and therefore a risk to their health. As such, establishment of this association now poses a real threat to the meat industry world wide. This is despite that the actual risks could be considered to be quite low, due to meat from healthy sheep being unlikely to be infected by Map, the low number of sheep with Johne's disease being presented to the slaughter premises, public health inspections before slaughter with the culling of emaciated animals, the low risk of coliform contamination of carcases from processing (Biss and Hathaway 1995, 1996; Meadus *et al,* 2008) and current food preparation guidelines.

This thesis has provided insight into the occurrence of systemic Map infection in naturally infected sheep and demonstrated that meat from some sheep with Johne's disease may be

infected with this organism and may provide a potential source of Map for humans. Systemic infection was not recorded in healthy mixed age ewes or finished lambs and although further validation is required, meat sourced from healthy animals may pose little risk.

Preventing human exposure to meat infected by Map should be a high priority for the meat industry. The findings in Chapters three and five suggest poor body condition cull ewes are the most likely class of stock to have systemic Map infection and pose the greatest risk for human exposure. This study has determined that the risk to the consumer can therefore be reduced by a) the exclusion of poor body condition ewes from processing plants, b) preventing the mixing of healthy sheep with clinically affected sheep immediately prior to slaughter and c) preventing the grazing of healthy sheep on heavily contaminated holding paddocks immediately prior to slaughter. Further validation is now required for these recommendations.

These findings may indirectly help the meat industry decrease the risk of human exposure to Map as they provide guidelines for sheep producers to follow. However, applying these findings into current farm management practices and then ensuring that they are met on all farms where Map infection is known to occur would be difficult.

Alongside these guidelines, this study has also developed two new diagnostic tests, HEM histopathology and hepatic hyperspectral analysis, which enable animals with systemic Map infection to be identified, providing the meat industry with a direct means to mitigate this risk. There is now a need to further validate these tests for diagnostic efficacy in sheep and other ruminants. If they are sufficiently sensitive and specific, there may be commercial application of this technology into abattoirs, with the targeted screening of cull mixed aged animals, at time of slaughter, providing a cost effective means of achieving food safety assurances.

This study has proposed the term sub-clinical infection be changed to the more objective and measurable term of non-systemic infection and that there are two periods of Map infection in sheep - the non-systemic period which currently can only be identified by ileal biopsy histopathology and a systemic period of infection. In periods of non-systemic Map infection, no losses in live weight or body condition were observed in this study. In this period, it appears that meat is unlikely to be infected with Map and as such may pose little risk for public health. It is proposed that quantifying the cost on production during the non-systemic period may be difficult if not unattainable and may be reason why currently all costs on production have been attributable to the period of systemic infection.

Systemic infection can be identified using expensive and cumbersome diagnostic techniques such as blood culture or PCR, or via ELISA serology with poor diagnostic efficacy or with liver biopsy histopathology and the identification of HEM. The use of liver histopathology as a surrogate measure of systemic Map infection has enabled this study to compare animals with and without systemic infection and therefore identify production differences between the two groups. This ability to differentiate now also provides opportunity to assess therapeutic efficacy of new drugs and vaccines for the prevention of systemic Map infection in sheep.

The longitudinal study in Chapter six has enabled systemic infection to be followed with recovery or self-cure occurring and when combined with other results in this thesis and previous published studies has enabled a new hypothesis to be developed on the pathogenesis of systemic Map infection in sheep. It is proposed that the aetiology for systemic Map infection is similar for both artificial and naturally infected sheep, requiring an overwhelming event to occur where the number of Map presented to the ileum is greater than the ileal CMI capacity. This enables Map to overspill into systemic circulation and infect organs distant to the alimentary tract such as the liver and possibly skeletal muscle. In periods of systemic infection, negative effects on live weight and body condition were observed. It is

hypothesised that the mechanism that provides the requisite number of Map, that leads to an overwhelming event, comes from two sources and these are either exogenous or autogenous, with exogenous overspill occurring in artificial infection studies or when sheep are exposed to heavily contaminated pasture, and autogenous overspill occurring in naturally infected sheep when Map infection has progressed to Type 3b or 3c ileal lesions. The outcome from systemic Map infection after an overwhelming event has occurred is dependent on the duration of this event. If the duration is finite and the supply of Map stops, as is the case in artificial challenge studies, sheep lose weight during the systemic event and then recover. However, if the event is continuous due to ileal pathology and autogenous re-infection, then animals lose weight continuously, which is unsustainable, and die from clinical Johne's disease.

The above proposed pathogenesis for systemic Map infection suggests that the model for artificial infection may not be representative for natural infection and that interpretation of results from artificial studies may need to be done with caution.

Lastly, this thesis has provided insight into the pathogenesis of systemic Map infection and has proposed a new hypothesis for the pathogenesis of Johne's disease in sheep.

8.13 Future Research opportunities

Questions directly arising from this thesis prompt the recommendation of the following research;

- There is potential risk to the meat industry from how the public may react when informed that a potential zoonotic bacterium is present in meat. Currently there is little data available on the prevalence of Map infection in meat (Chapter three), making it difficult to establish procedures to mitigate this perceived risk. Addressing this shortcoming should be a priority for the meat industry and could be achieved by increasing the number of sheep sampled from flocks throughout New Zealand.

- The observation from Chapter four that meat from healthy sheep, that have not been exposed to clinically affected animals, immediately prior to slaughter, is not infected by Map needs to be validated.
- Should this be proven that meat from healthy sheep is uninfected, the proposal in Chapter four that short term grazing of healthy sheep with clinically affected shedding cohorts immediately prior to slaughter may result in systemic Map infection and subsequent contamination of meat needs to be validated. This could be achieved in a controlled and replicated study by grazing naïve lambs / naïve adults with mixed age cull ewes that were either shedding or not shedding Map in confined spaces for one week prior to slaughter to determine if a sudden increase in oral intake leads to an acute systemic infection and that this can result with meat becoming infected by Map and occur without the formation of ileal lesions in naturally infected sheep (Chapter three).
- To determine whether naïve lambs grazed on naturally infected pasture develop systemic infection with subsequent HEM formation and whether this practice increases the risk of human exposure to Map from young healthy animals. The practice of finishing store lambs and lamb trading provides an example where naïve animals could be suddenly exposed to Map from ingesting contaminated pasture. This may have potential to produce a transient period of systemic infection, infecting the meat, which after 6-8 weeks finishing, is processed for human consumption. It is also necessary to determine whether carcase contamination with Map occurs at time of processing in sheep and what factors contribute to this event.
- Pathophysiological study, based on the hypothesis proposed in this thesis, to determine whether continuous oral dosing of high numbers of Map (>1 x 10^9 organisms) to naïve lambs mimics the continuous autogenous shedding and continuous re-infection of Map, that is proposed to occur in naturally infected ewes with Type 3b or 3c ileal lesions, and

that this continuous artificial oral dosing leads to unsustainable weight loss and terminal cachexia in these lambs without the formation of Type 3b or 3c ileal lesions.

- Further validation of the use of liver biopsy histopathology and the identification of HEM to identify sheep with Johne's disease and where systemic Map infection may be occurring.
- Determine that systemic Map infection is causal for the formation of HEM and muscle infection using serial liver biopsy, histopathology, IHC, culture and PCR methods of diagnosis.
- Determine whether HEM occur in other ruminant species as a result of Map infection and if so, can they be identified using liver biopsy.
- Determine whether humans with Crohn's disease also develop HEM lesions and whether liver biopsy histopathology has a role to play in replacing the more surgically invasive current technique of laparotomy and intestinal biopsy for the diagnosis of this disease.
- Further research is now justified to establish the sensitivity and specificity of hyperspectral imaging as a means of predicting that a carcase is or is not contaminated with Map. Improvement of the SVM algorithm could be achieved by;
    - Expanding Chapter seven, with inclusion of not only more ill-thrift cull ewes, but also healthy ewes and lambs examined from more farms across New Zealand.
    - Addition of Map culture, IHC and PCR analysis on tissues collected such as ileum, liver and skeletal muscle to confirm the presence of Map and systemic infection and the contamination of meat.
- Determining the aetiology of the liver hyperspectral signature – biochemical, cellular or some other factor that produces a hyperspectral data set recognised by the algorithm for Johne's disease in sheep.
- Determine whether hyperspectral imaging can be performed on fresh or fixed liver.

- Determine whether fixed sections of liver, both trans-sectional and those sourced from liver biopsy can be used to identify sheep with clinical ovine Johne's disease using hyperspectral analysis. If so this would enable hyperspectral imaging to be used as a diagnostic tool in longitudinal studies for the assessment of systemic Map infection and in therapeutic efficacy studies.
- Perform a longitudinal intervention study to determine the therapeutic efficacy of thalidomide on the formation of HEM in artificially challenged sheep and whether anti-TNFα drugs have a role to play in the prevention of clinical Johne's disease in this species.
- In the longitudinal study round unidentified acid fast organisms (RUAFO) were identified within the HEM at 148 days post oral exposure. These acid fast organisms were not bacilli and may represent the spheroplastic form of Map described in human patients with Crohn's disease or may be the remnants of Map post phagolysosomal removal within HEM. Undertaking an Immunohistochemistry (IHC) investigation may enable these forms to be further identified (see Appendix A2.13 - RUAFO).

Appendix 1: Thalidomide pharmacokinetics in sheep

A1.1 Abstract

AIM: To determine the half life ($T_{1/2}$), time taken to reach maximum plasma concentration (T_{max}) and maximum plasma concentration (C_{max}) of thalidomide in sheep following I/V, oral and topical treatment with a single dose of thalidomide.

METHOD: Three groups of 4–6-month-old ram lambs were treated with thalidomide dissolved in dimethylsulphoxide (DMSO). The first group (n=10) was treated I/V with 100 mg thalidomide in 2 mL DMSO; the second group (n=8) received 400 mg thalidomide in 2 mL DMSO orally, and the third group (n=8) had 400 mg thalidomide in 4 mL DMSO applied topically. Plasma samples were collected up to 36 hours, snap-frozen at −80˚C and analysed for concentrations of thalidomide using high performance liquid chromatography.

RESULTS: Following I/V administration, $T_{1/2}$ was 5.0 hours, volume of distribution was 3,372 mL/kg and clearance was 487.1 mL/hour.kg. Topical application of 400 mg thalidomide did not increase plasma concentrations. Following oral administration, thalidomide bioavailability was 89%, with $T_{1/2}$, T_{max}, and C_{max} being 7.2 hours, 3.0 hours and 1,767.3 ng/mL, respectively.

CONCLUSION: Topical administration using DMSO as a solvent did not increase concentrations of thalidomide in plasma. The pharmacokinetic parameters determined following oral treatment with 400 mg of thalidomide were similar to those reported in humans receiving a single 400 mg oral dose ($T_{1/2}$ 7.3 hours; T_{max} 4.3 hours and C_{max} 2,820 ng/mL). There is potential for thalidomide to be used as a model for the treatment of chronic inflammatory conditions in sheep, such as Johne's disease, where tumour necrosis factor alpha plays a pathogenic role.

KEY WORDS: *Thalidomide, sheep, Johne's disease, pharmacokinetics, tumour necrosis factor alpha, TNF-α.*

C_{max}	Maximum plasma concentration
DMSO	Dimethylsulphoxide
HPLC	High performance liquid chromatography
LLQ-	Lower limit of quantification
$T_{1/2}$	Elimination half-life
T_{max}	Time to reach maximum plasma concentration
TNF-α	Tumour necrosis factor alpha

A1.2 Introduction

Thalidomide (α-phthalimide-glutarimide) has been used in human medicine since 1957 when it was initially prescribed as a sleeping pill and for the prevention of morning sickness in pregnant women. The teratogenic effects in humans first appeared in the late 1950s and it was withdrawn from sale in 1961. Despite this chequered history, interest in thalidomide was renewed in 1964 when it was shown to have efficacy for the treatment of patients with leprosy (Tseng *et al,* 1996; Teo *et al,* 2002; Walker *et al,* 2007) and more recently multiple myeloma (Singhal *et al,* 1999; Palumbo *et al*, 2008). The exact mechanism of action for teratogenesis or therapeutic success for patients with these medical conditions is not fully understood.

Thalidomide is an immune-modulatory agent with potent anti-angiogenic and anti-inflammatory properties (D'amato *et al,* 1994). For patients with multiple myeloma, the

prevention of the formation of blood vessels (angiogenesis) supplying the tumour may limit the tumour's progression (Rajkumar and Witzig 2000; Kumar *et al,* 2004). Patients with leprosy are infected with the multibacillary form of *Mycobacterium leprae* and have elevated concentrations of the pro-inflammatory cytokine tumour necrosis factor alpha (TNF-α) (Barnes *et al*, 1992). Angiodsyplastic conditions in humans, such as small intestinal bleeding disorders and Crohn's disease, are also associated with elevated pro-inflammatory cytokines including TNF-α (Clancy *et al,* 2007; Nakase *et al,* 2011). Although thalidomide does not have antimicrobial effects, it has anti-inflammatory properties by increasing the degradation rate of the mRNA encoding TNF-α (Sampaio *et al,* 1991; Moreira *et al,* 1993). In human clinical studies, anti-TNF-α drugs such as thalidomide (Zheng *et al,* 2011; Scribano *et al,* 2014) and the monoclonal antibodies infliximab (Cohen 2000) and adalimumab (Hanauer *et al,* 2006) have been used in the treatment of these diseases suggesting that targeting TNF-α may have a beneficial therapeutic effect. In studies where patients were given a single daily 400 mg oral dose of thalidomide, the mean terminal half life ($T_{1/2}$) was 7.3 hours, time taken to reach maximum plasma concentration (T_{max}) was 4.3 hours, and the maximum plasma concentration (C_{max}) was 2,820 ng/mL, and treatment with thalidomide was associated with decreased production of TNF-α (Teo *et al,* 1999; Matthews and McCoy 2003).

Increased expression of TNF-α occurs in the ileal tissue of cattle and sheep with clinical Johne's disease, however it is currently unknown whether this cytokine plays a pathogenic role in the progressive formation of the enteric granulomatous lesions characteristic of this disease (Alzuherri *et al,* 1996; Aho *et al,* 2003). It is proposed here that anti-TNF-α drugs such as thalidomide may provide further information on the role this cytokine has to play in ruminants with Johne's disease and, if a therapeutic benefit is identified, then opportunity exists for future research to develop new anti-TNF-α drugs and therapy programmes for ruminants.

Currently there are no pharmacokinetic data available for thalidomide in sheep. The aims of this study were to determine the $T_{1/2}$, T_{max} and C_{max} for sheep treated I/V, orally and topically with a single dose of thalidomide.

A1.3 Materials and methods

Ten 4–6-month-old, healthy Texel/Romney/Finnish Landrace composite cross (Highlander) ram lambs were selected from a group of 400 ram lambs and were housed on concrete, with free access to water and hay. This group of lambs had a mean body weight of 45.5 (min 30.7, max 57.8) kg and was treated I/V with 100 mg thalidomide dissolved in 2 mL of 99.5% dimethylsulphoxide (DMSO). An additional 16, four month old lambs of the same breed, were selected from another mob of 400 lambs from the same farm and housed similarly; the mean body weight of these lambs was 27.4 (min 22.6, max 31.5) kg. These lambs were randomly separated into two groups of eight lambs using odds and evens as they were run up a race and presented to the drafting gate. One group of eight lambs was treated orally with 400 mg thalidomide dissolved in 2 mL DMSO. The final group of animals had the wool clipped from the back midline and 400 mg thalidomide dissolved in 4 mL DMSO was applied as a single, 5 cm diameter spot onto the skin. Blood samples were collected from the jugular vein into sterile 10 mL lithium heparinised vacutainers (Becton Dickinson Co., Franklin Lakes, NJ, USA) before and 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours after I/V injection, and 0.5, 1, 4, 8, 12, 24, 32 and 36 hours after oral and topical administration. The blood samples were immediately centrifuged at 3000 rpm for 5 minutes. Plasma was collected in 1.5 mL micro-centrifuge tubes, snap frozen and stored in liquid CO_2 (−80˚C).

The study was approved by Massey University Animal Ethics Committee (Palmerston North, NZ).

A1.3.1 Drugs and reagents

Thalidomide was prepared at Massey University using the two step process described by Muller *et al,* (1999) with nuclear magnetic resonance 600 MHz analysis confirming 99.7% purity.

Methanol and acetonitrile were obtained from Sigma-Aldrich (Auckland, New Zealand). Acetate buffer stock solution (0.1 M) was prepared by mixing 1.03 mL acetic acid and 7.7 g ammonium acetate (Merck, Darmstadt, Germany) in purified water (Milli-Q PFplus system, Millipore Corporation, Billerica, MA, USA) and the volume made up to 1 L. Acetate buffer working solution (10 mM) was prepared daily by mixing 100 mL of buffer stock solution with 900 mL of purified water. The buffer stock solution was kept refrigerated and used for 1 week only.

A1.3.2 Sample Preparation

The plasma samples were prepared for analysis using a liquid-liquid extraction procedure; 400 µL of plasma was vortexed with 800 µL chilled 2% acetic acid solution in methanol and centrifuged at 2,400g for 15 minutes. The supernatant was separated and dried at 20°C under a gentle stream of air. The dried samples were reconstituted in 400 µL of mobile phase and again were centrifuged at 2,400g for 10 minutes. A 50 µL aliquot of the resulting supernatant was injected into the high performance liquid chromatography (HPLC) machine. Each sample was injected three times. The standard curve for thalidomide was prepared by spiking blank plasma (obtained from the lambs prior to thalidomide administration) with thalidomide to produce final concentrations between 6.25–4,000 ng/mL.

A1.3.3 High Performance Liquid Chromatography

The snap-frozen plasma samples were analysed by HPLC with diode array detection (Zhou *et al,* 2003). The system consisted of LC-20AD pumps, SIL-20AC HT auto-injector, diode array detector SPD-M20A, CTO-20A column oven, DGU-20A3 degasser (Shimadzu, Kyoto, Japan).

Thalidomide was separated with a Phenomenex (Auckland, New Zealand) C18A (150 x 4.6 mm i.d, 5 μm particle size) column at 32°C. The mobile phase consisted of 10 mM acetate buffer, pH 5.3 (80%) and acetonitrile (20%) with 1 mL/min flow rate. The detector was set at 235 nm, and the auto-injector was set at 4°C.

All chromatograms were analysed for peak height, area, width and concentration for the unknowns using LC Solutions software (Shimadzu, Kyoto, Japan).

HPLC Method Validation

The sample preparation method was validated by standard validation protocol as described by Masson (2007). The lower limit of quantification (LLQ) was measured by running series of low concentration thalidomide standards (5.75, 11.5, 23 and 46 ng/mL) spiked into drug-free plasma. The LLQ was set at the lowest concentration showing a signal to noise ratio of 10. Intra-day and inter-day accuracy was determined by spiking thalidomide into drug-free plasma at four different concentrations (23, 46, 92 and 184 ng/mL) every day for three consecutive days. The linearity of the measurements was checked by running 10 different concentrations from 6.25 to 4,000 ng/mL three times in spiked plasma. The recovery of thalidomide after liquid-liquid extraction was determined by comparing areas of the peaks for

46, 92 and 184 ng/mL thalidomide concentrations in the mobile phase with same concentrations spiked in the drug-free plasma.

A1.4 Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using non-compartmental analysis, as fewer assumptions are required compared with compartmental analysis (Gabrielsson and Weiner 2012). These included $T_{1/2}$, the area under the curve extrapolated from time zero to infinity ($AUC_{0-\infty}$), area under the moment curve extrapolated from time zero to infinity ($AUMC_{0-\infty}$), clearance, volume of distribution (V_D) and volume of distribution at steady state ($V_{D\,(ss)}$), and bioavailability (F). Parameters were calculated using the following equations in Microsoft Excel (Microsoft Corporation, Redmond, WA, USA), where K_{el} is the elimination rate constant:

$$AUC_{0-\infty} = AUC_{0-t} + (C_{last})/K_{el}$$

$$AUMC_{0-\infty} = AUMC_{0-t} + (C_{last})/K_{el}^2 + (t_{last})(C_{last})/K_{el}$$

$$V_D = (Dose/AUC_{0-\infty} \times K_{el})/ \text{ body weight}$$

$$V_{D\,(ss)} = Dose \times AUMC_{0-\infty}/AUC^2_{0-\infty}$$

$$F = AUC_{(oral)} \times Dose_{(I/V)}/AUC_{(I/V)} \times Dose_{(oral)}$$

A1.5 Results

The mean recovery of thalidomide in sheep plasma after sample preparation was 91.22%. The LLQ for this method was 11.5 ng/mL, being the lowest concentration of thalidomide that could be detected (Figure A1.1). The intra-day variations were between 0.5–2.5% and inter-day variations between 2.1–2.7%. The standard curve was linear from 10 - 2,000 ng/mL. The correlation coefficient for the standard curve thus obtained was 0.995. The specificity of this extraction method was confirmed using 10 different blank plasma samples and the absence of any interfering peaks at the same HPLC retention time.

Mean concentrations of thalidomide after I/V and oral administration are shown in Figure A1.2.

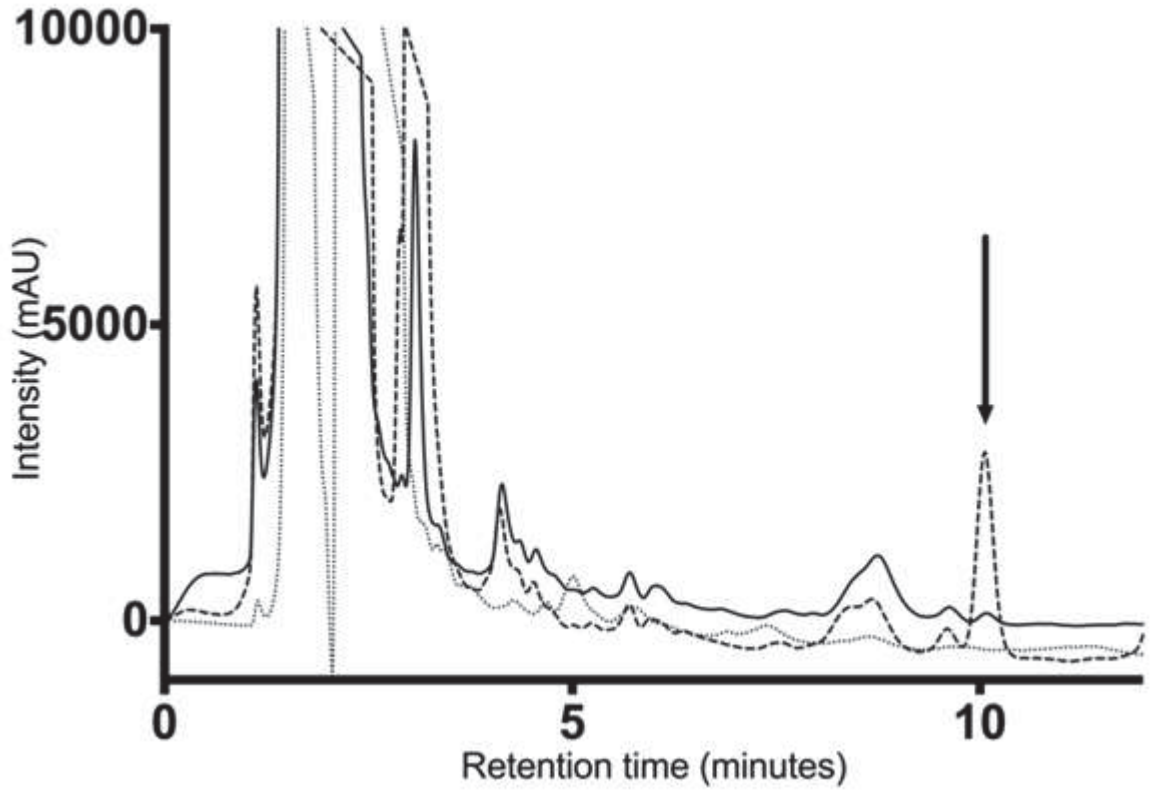

Figure A1.1 Chromatogram produced during validation of a high performance liquid chromatography assay for thalidomide showing the lower limit of quantification (arrow) determined using blank plasma (dotted line) spiked with 184 ng/mL (dashed line) and 11.5 ng/mL (solid line) thalidomide.

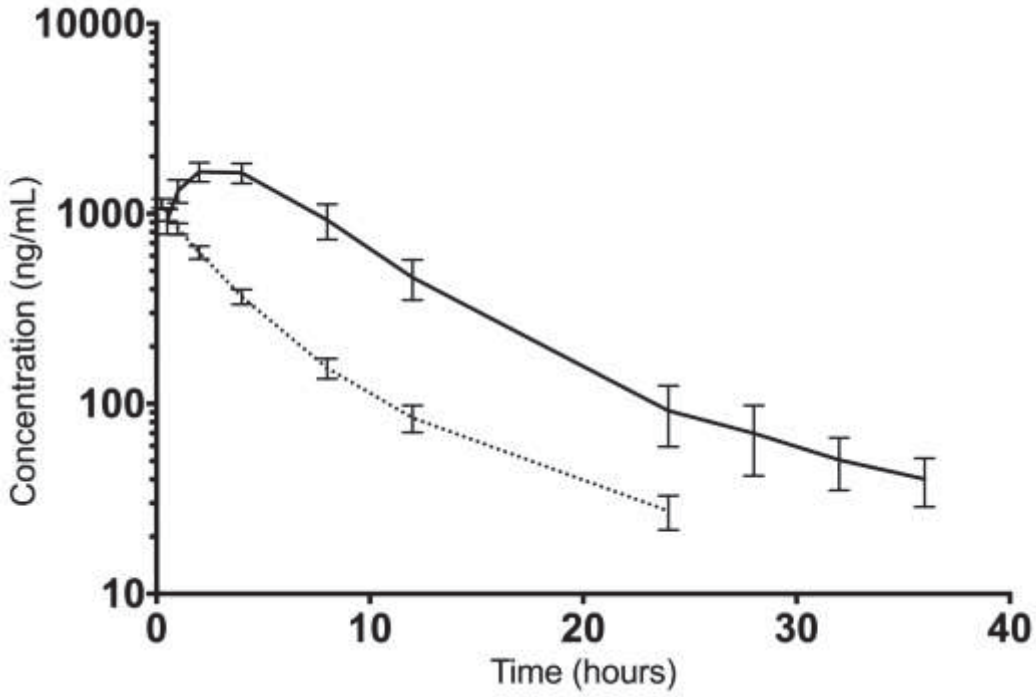

Figure A1.2 Mean (± SEM) concentration (ng/mL) of thalidomide in serum of ram lambs following administration of 100 mg (I/V, dotted line; n=10) or 400 mg (oral, solid line; n=8) of thalidomide.

The pharmacokinetics parameters are shown in Table A1.1 and A1.2. The mean C_{max} following a single 400 mg oral dose of thalidomide was 1,767 (SEM 178.1) ng/mL at 3 (SEM 0.4) hours. The bioavailability after oral administration was 89%. The mean minimum plasma concentration at the final sampling was 40.17 (SEM 11.53) ng/mL at 36 hours after oral administration and 27.29 (SEM 5.569) ng/mL at 24 hours after intravenous administration.

After topical administration, crystals of thalidomide were detected on the skin surface 1 hour after application, with no thalidomide detected in plasma except in two sheep where 30 ng/mL and 28 ng/mL were recorded at 32 and 48 hours, suggesting DMSO may not be a good solvent for transdermal delivery.

Table A1.1: Individual and mean pharmacokinetic parameters determined using a non-compartmental model following I/V administration of 100 mg thalidomide to ram lambs.

Lamb ID	$T_{1/2\beta}$ [a]	$AUC_{0-\infty}$ [b]	$AUMC_{0-\infty}$ [c]	Cl [d]	$V_{D\ (area)}$ [e]	V_{Dss} [f]	K_{el} [g]
1	3.7	2,364.4	2,532.9	856.4	4,586.1	151,581.4	0.2
2	6.1	4,751.4	4,965.2	427.6	3,739.0	122,933.6	0.1
A	5.9	6,199.0	6,344.3	513.4	4,369.7	74,479.2	0.1
B	4.7	6,833.2	6,987.7	393.2	2,671.3	81,013.1	0.1
C	3.6	3,545.5	3,914.6	540.5	2,767.4	104,678.6	0.2
D	2.7	2,983.3	3,116.8	555.1	2,152.3	115,627.0	0.3
E	5.4	4,582.7	5,313.9	425.8	3,294.4	104,777.9	0.1
F	6.4	5,361.8	5,574.8	372.9	3,424.7	108,094.3	0.1
G	6.9	6,137.3	6,625.8	382.1	3,810.5	122,346.5	0.1
H	5.0	3,814.3	4,322.5	404.5	2,902.5	117,281.4	0.1
Mean	5.0	4,657.0	4,970.0	487.1	3,372.0	110,281.3	0.1
SEM	0.4	469.3	472.5	46.1	244.3	6,907.0	0.0

[a] Elimination half-life (hours)

[b] Area under the curve extrapolated from time zero to infinity (ng.hours/mL)

[c] Area under the moment curve extrapolated from time zero to infinity ($ng.hours^2/mL$)

[d] Clearance (mL/hour.kg)

[e] Volume of distribution (mL/kg)

[f] Volume of distribution at steady state (mL)

[g] Elimination rate constant (1/h)

Table A1.2: Individual and mean pharmacokinetic parameters determined using a non-compartmental model following oral administration of 400 mg thalidomide to ram lambs.

Lamb	C_{max} [a]	T_{max} [b]	$T_{1/2\beta}$ [c]	$AUC_{0-\infty}$ [d]	$AUMC_{0-\infty}$ [e]
1	1,484.6	4.0	7.8	13,930.7	14,166.3
2	1,359.0	2.0	6.1	15,112.5	15,310.4
3	1,273.3	4.0	6.0	11,708.6	11,916.3
4	2,882.3	2.0	9.6	37,149.5	37,897.2
5	1,892.3	4.0	5.6	20,594.6	20,810.2
6	1,625.9	2.0	6.2	9,894.8	10,134.4
7	1,852.4	4.0	11.7	19,247.8	19,901.0
8	1,768.3	2.0	4.6	11,959.8	12,154.1
Mean	1,767.3	3.0	7.2	17,449.8	17,786.2
SEM	178.1	0.4	0.8	3,103.0	3,168.0

[a] Maximum plasma concentration (ng/mL)

[b] Time to reach maximum plasma concentration (hours)

[c] Elimination half-life (hours)

[d] Area under the curve extrapolated from time zero to infinity (ng.hours/mL)

[e] Area under the moment curve extrapolated from time zero to infinity ($ng.hours^2/mL$)

A1.6 Discussion

In human medicine, anti-TNF-α drugs are currently used to treat diseases characterised by chronic inflammation. Sheep with clinical Johne's disease have increased expression of TNF-α within ileal tissues (Alzuherri *et al,* 1996). There have been no studies undertaken using anti-TNF-α drugs in sheep and whilst the commercial use of thalidomide in food-producing animals is unlikely, this could be a useful model to test whether there is any therapeutic benefit from anti-TNF-α drugs. The anti-TNF-α effect in humans differs between *in vitro* and *in vivo* studies. *In vitro* studies using human mononuclear cells exposed to serum containing >4 μg/mL thalidomide recorded a decrease in the production of TNF-α of 40–75% (Shannon *et al,* 1997). This concentration can be achieved in serum of patients treated with 200 mg thalidomide four times daily (Shannon *et al,* 1997), but it may be unnecessary as total daily doses of 100 or 150 mg thalidomide have recently been shown to reduce circulating TNF-α (Lazzerini *et al,* 2013; Scribano *et al,* 2014).

In one study in healthy human subjects, oral dosing with 400 mg thalidomide resulted in mean $T_{1/2}$, T_{max} and C_{max} of 7.3 hours, 4.3 hours and 2,820 ng/mL, respectively (Teo *et al,* 1999), and treatment with thalidomide was associated with decreased production of TNF-α in human patients (Matthews and McCoy 2003). In the current study, the $T_{1/2}$, T_{max} and C_{max} were 7.2 hours, 3.0 hours and 1,767 ng/mL, respectively, following oral administration with 400 mg thalidomide, suggesting that this dose could be used to investigate anti- TNF-α properties in sheep.

In human studies, hydrolysis and depletion of thalidomide occurs in blood samples and appears to be temperature and pH dependent. This loss can be prevented by acidification or rapid, snap-freezing of samples after collection (Erickson *et al,* 1992; Boughton *et al,* 1995; Lyon *et al,* 1995). In this study, snap-frozen (−80˚C) samples were used in the analysis as a

similar depletion had been recorded; concentrations of thalidomide in plasma samples collected at 1 and 8 hours and left to chill in slushy ice for 1 hour prior to freezing were 9.4% and 16.9% lower, respectively, than snap-frozen samples.

The pharmacokinetic parameters of thalidomide in sheep are similar to those shown to have a therapeutic effect in humans when given the same oral daily dose. Further research is required to determine whether the administration of oral thalidomide has an anti-TNF-α effect in sheep with chronic inflammatory conditions such as Johne's disease, and whether there is a role for other, more commercially acceptable, anti-TNF-α products in the prevention or treatment of this disease in ruminants.

A1.7 Acknowledgements

Dean and Debbie Sparkes for aiding with sampling, use of their facility and their animal husbandry skills.

Appendix 2: General Photographs

1 

2 

3 

Figure A2.1 1. 'Delia' with clinical Johne's disease, 2. 'Delia' three weeks later, 3 Gross necropsy.

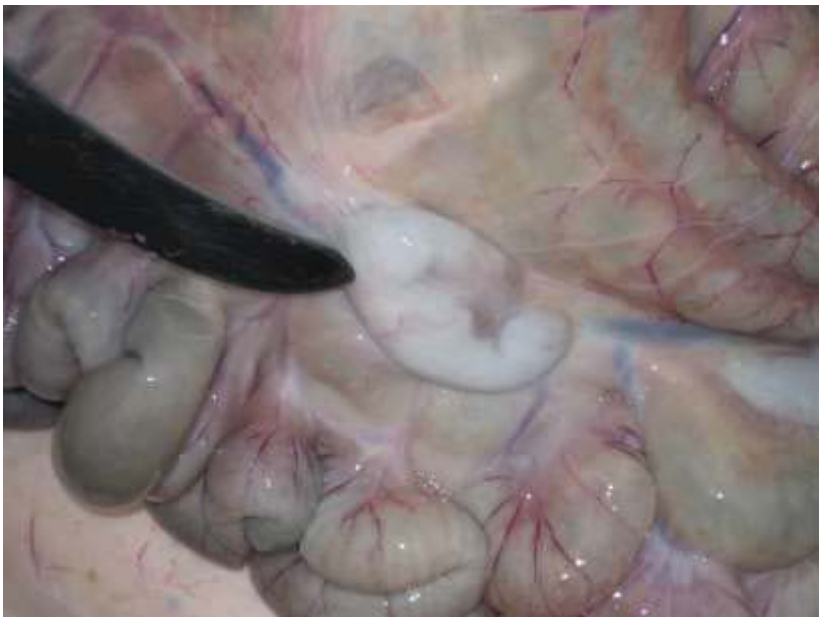

Figure A2.2 Lymphadenitis – enlarged 1st mesenteric lymph node

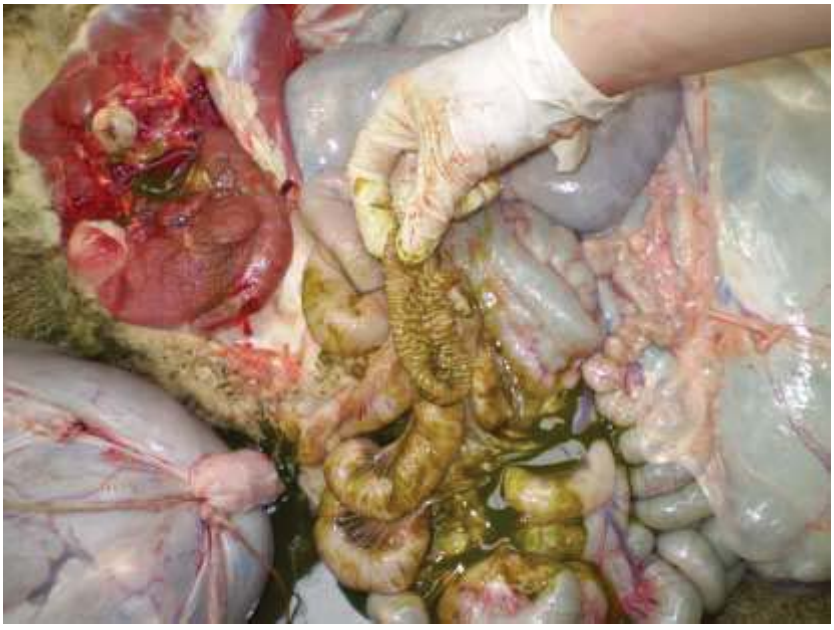

Figure A2.3 Chronic, corrugated mucosal thickening of the terminal ileum with epithelioid granulomatous enteritis.

1

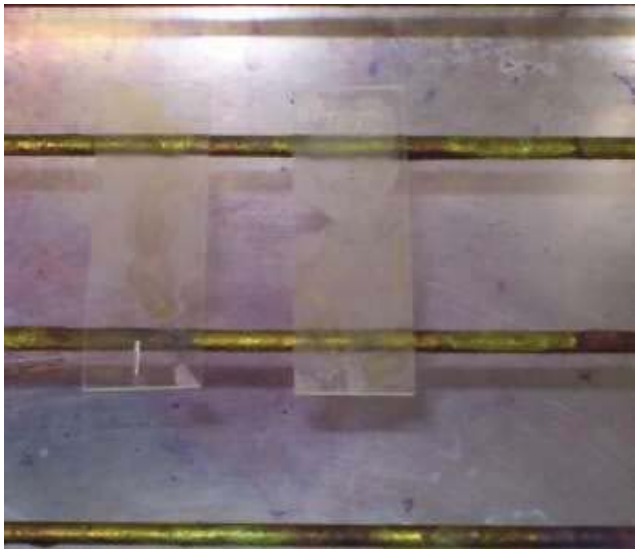

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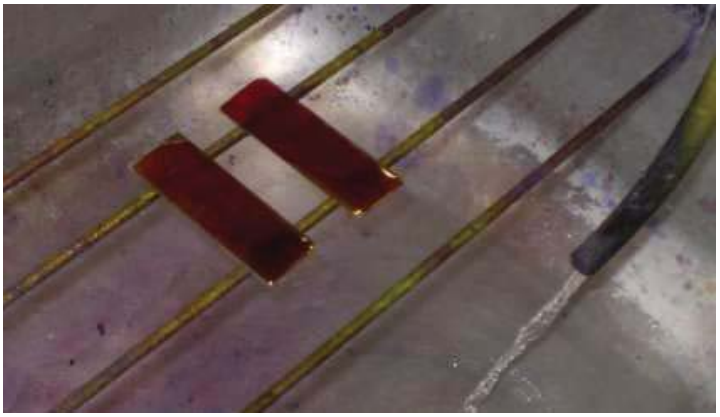
Figure A2.4 Method for Ziehl Neelsen(ZN) stain. 1. Heat fixed ileal mucosal smear, 2. Carbolfuscin stain, 3. Heat Carbolfuscin stain to 60'C for 15 minutes.

4



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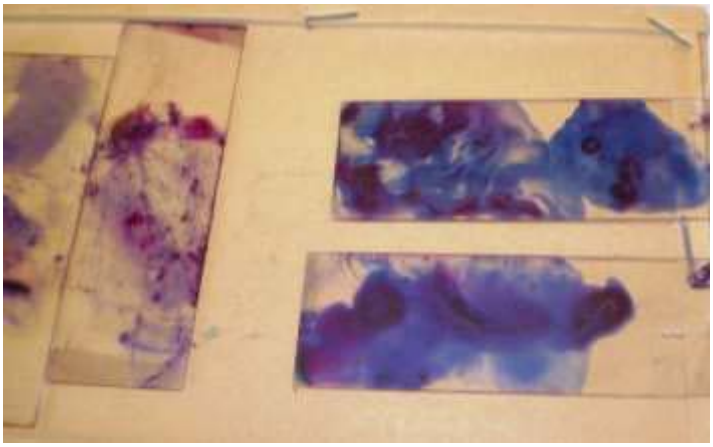

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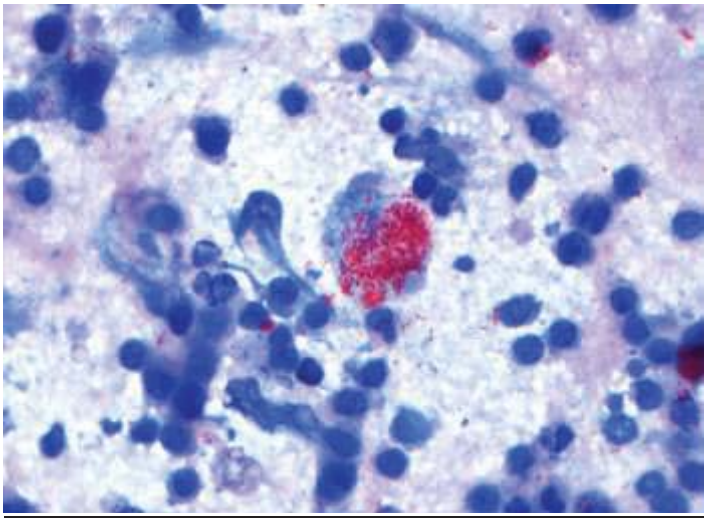


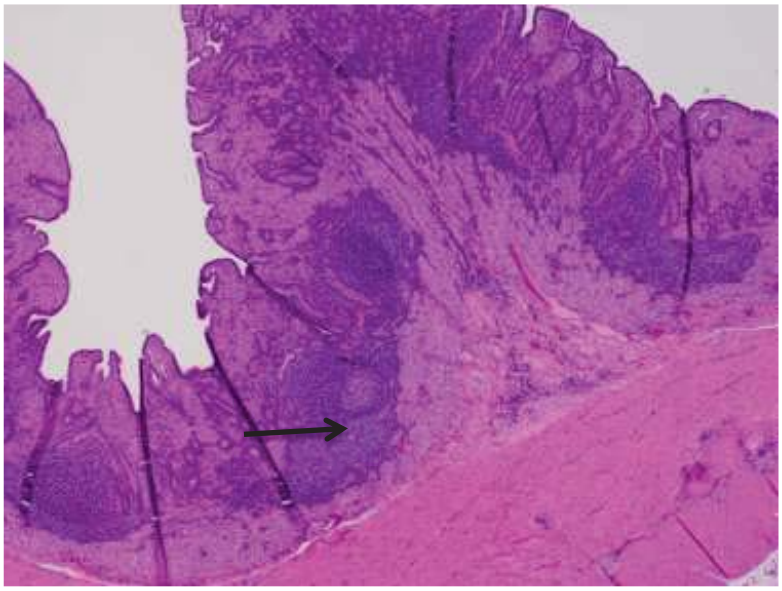
Figure A2.5 4. Rinse Carbolfuscin with 50:50 ethanol : HCl acid until clear,

5. Add methylene blue for 30 seconds and rinse with distilled water.

Air dry,

6. Oil immersion(x100), ZN positive with acid fast organisms.

1



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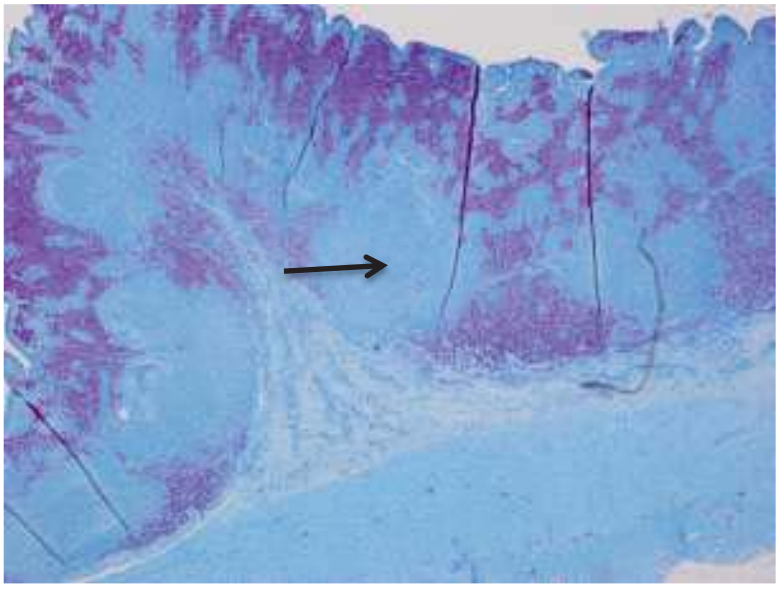
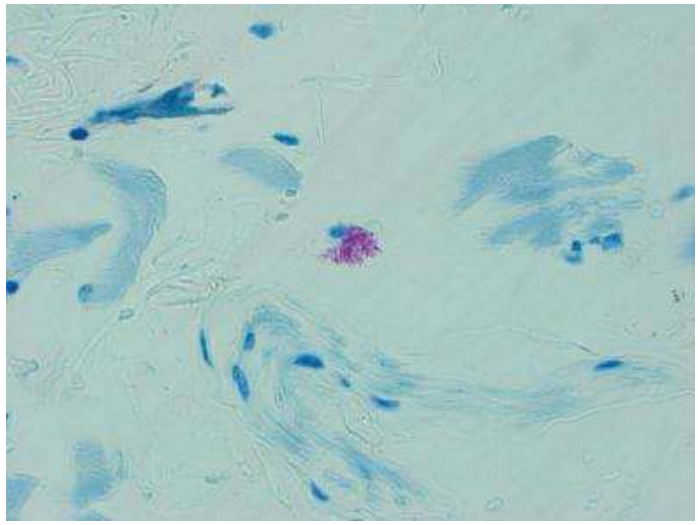


Figure A2.6 Type 3b Multibacillary terminal ileal lesions with Peyers patches

1. HE stain, 2. ZN stain.

1



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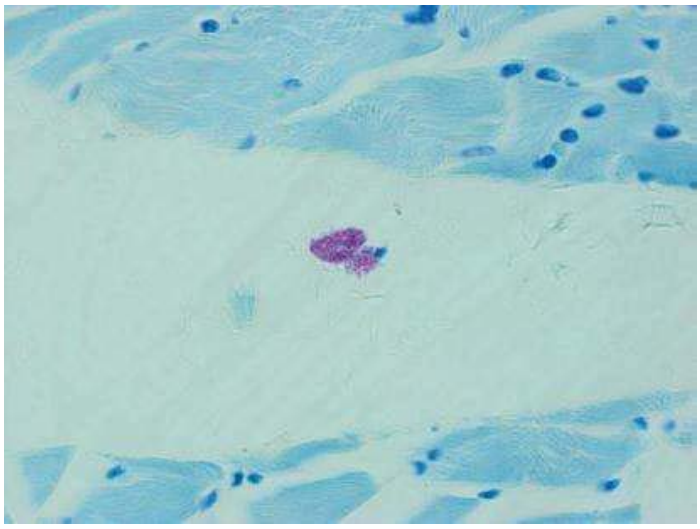

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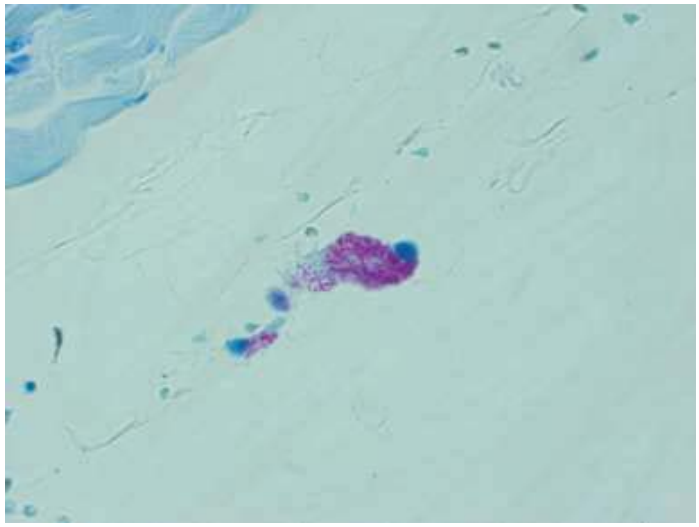
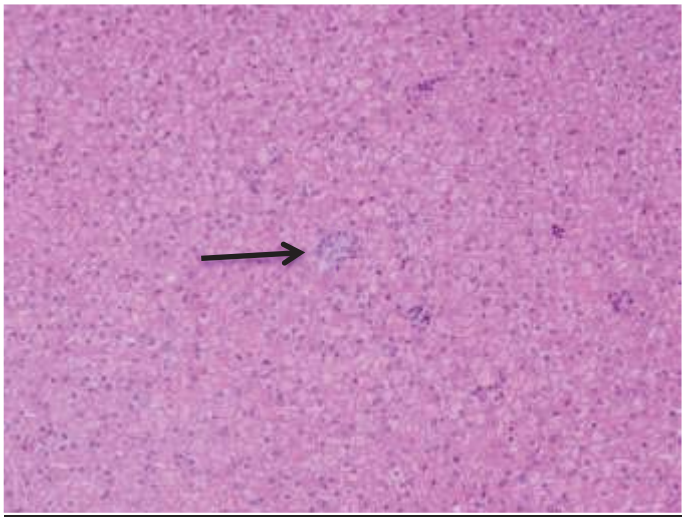


Figure A2.7 1,2,3. Macrophages with Map AFO from a single histological section of ovine biceps femoris muscle from a ewe with clinical JD, ZN (x100).



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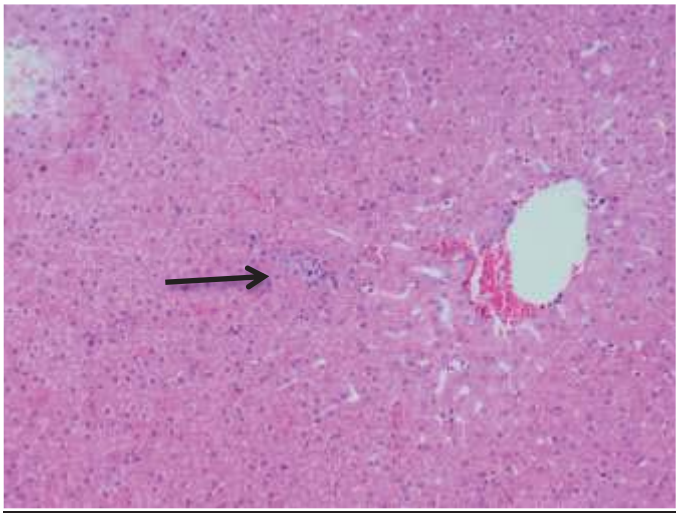

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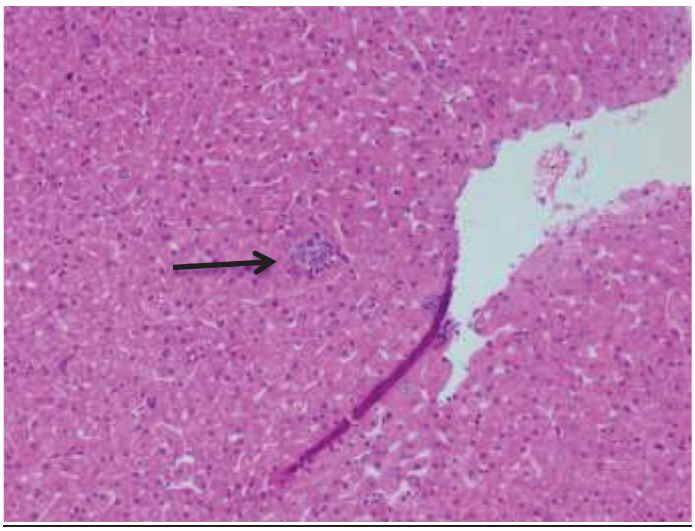


3

Figure A2.8 1, 2, 3. HEM, HE (x20), T = 51 days.

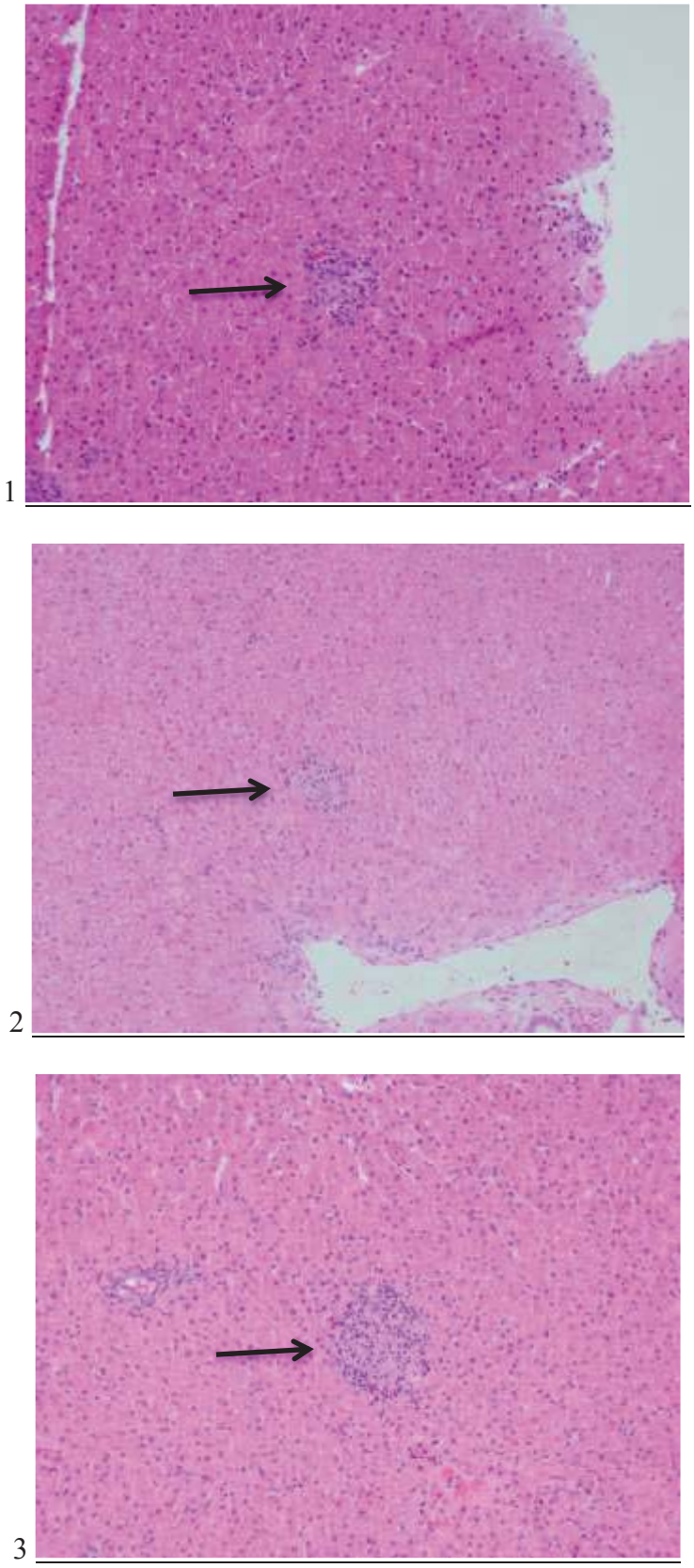


Figure A2.9 1, 2. HEM, HE(x20), 3. HEM, HE (x40), T = 114 days.

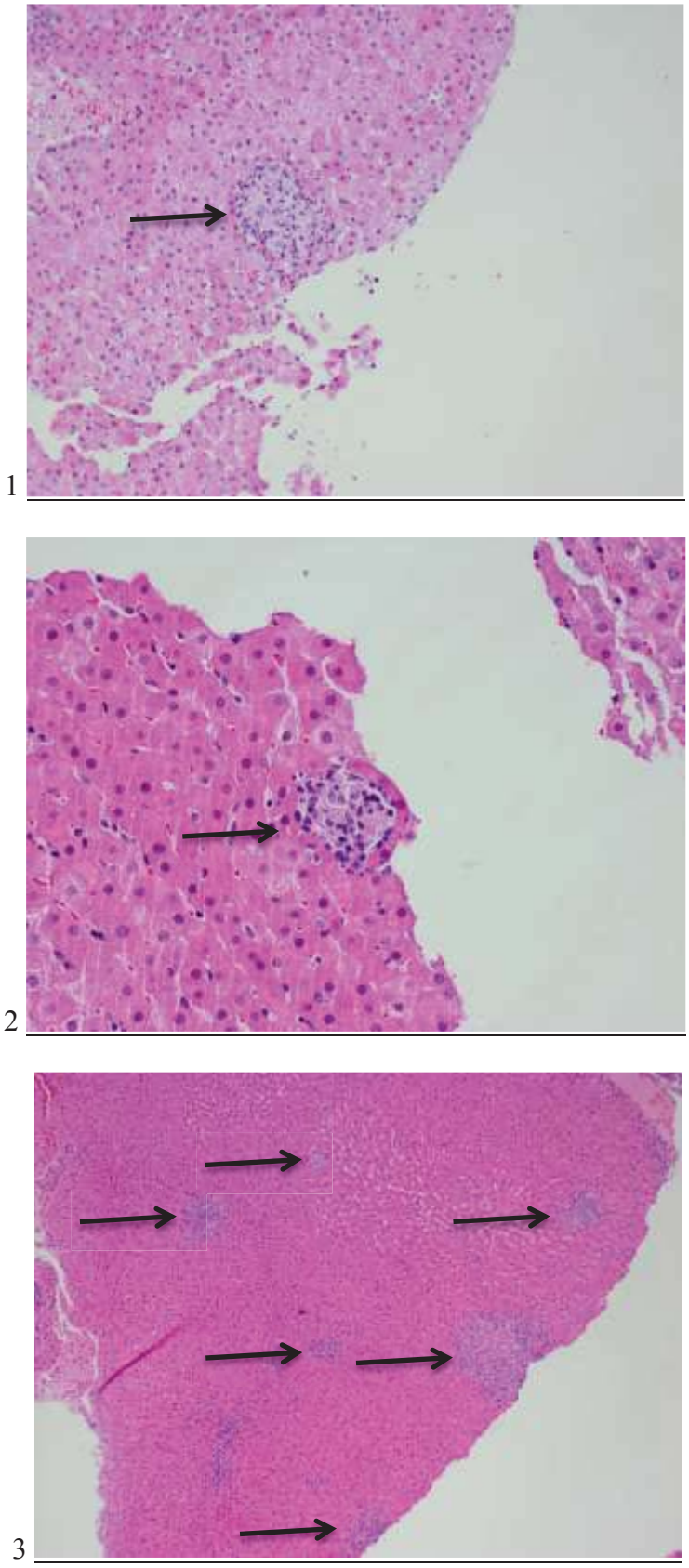
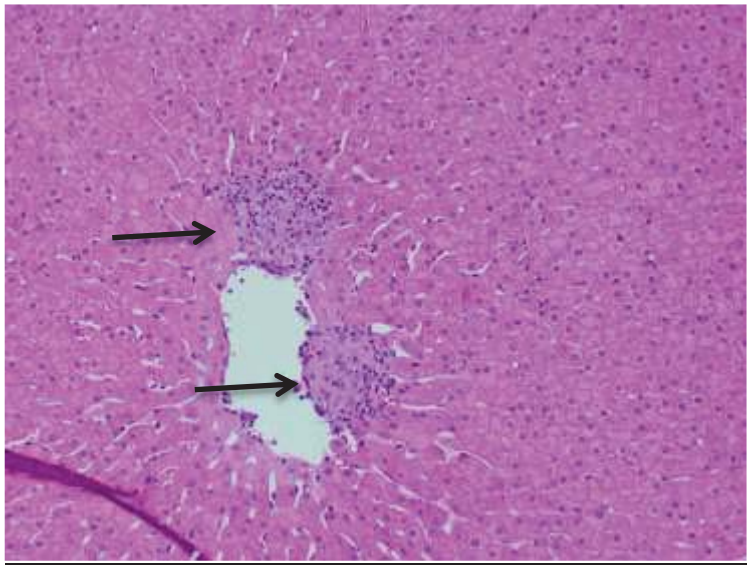
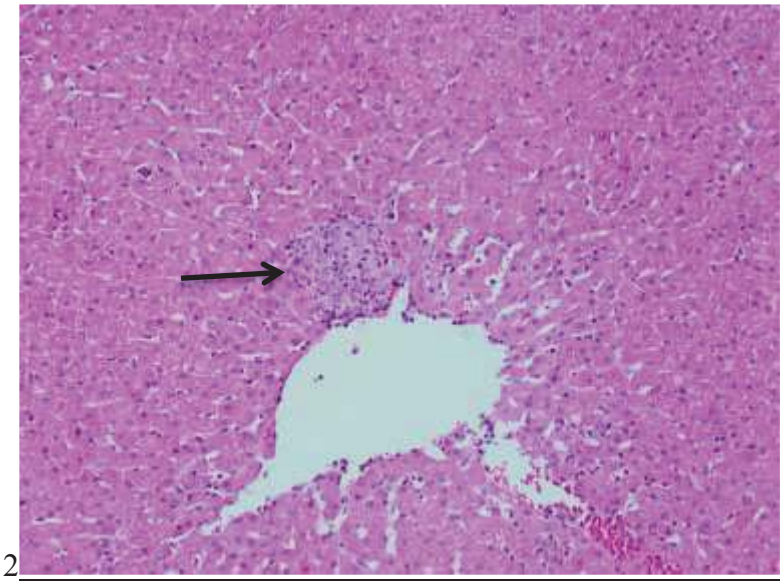


Figure A2.10 1. HEM, HE (x20), 2. HEM, HE (x40), 3. HEM, HE (x10), T = 154 days.



1



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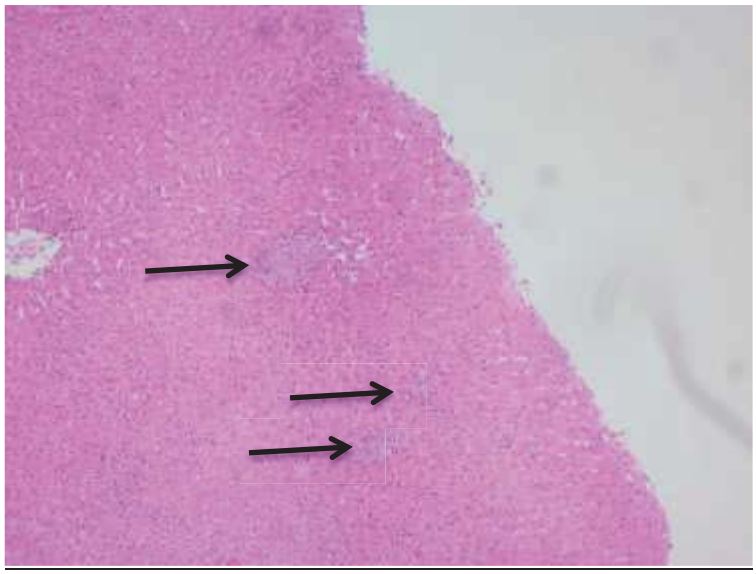

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Figure A2.11 1, 2. HEM, HE (x20), 3. HEM, HE (x10), T = 195 days.

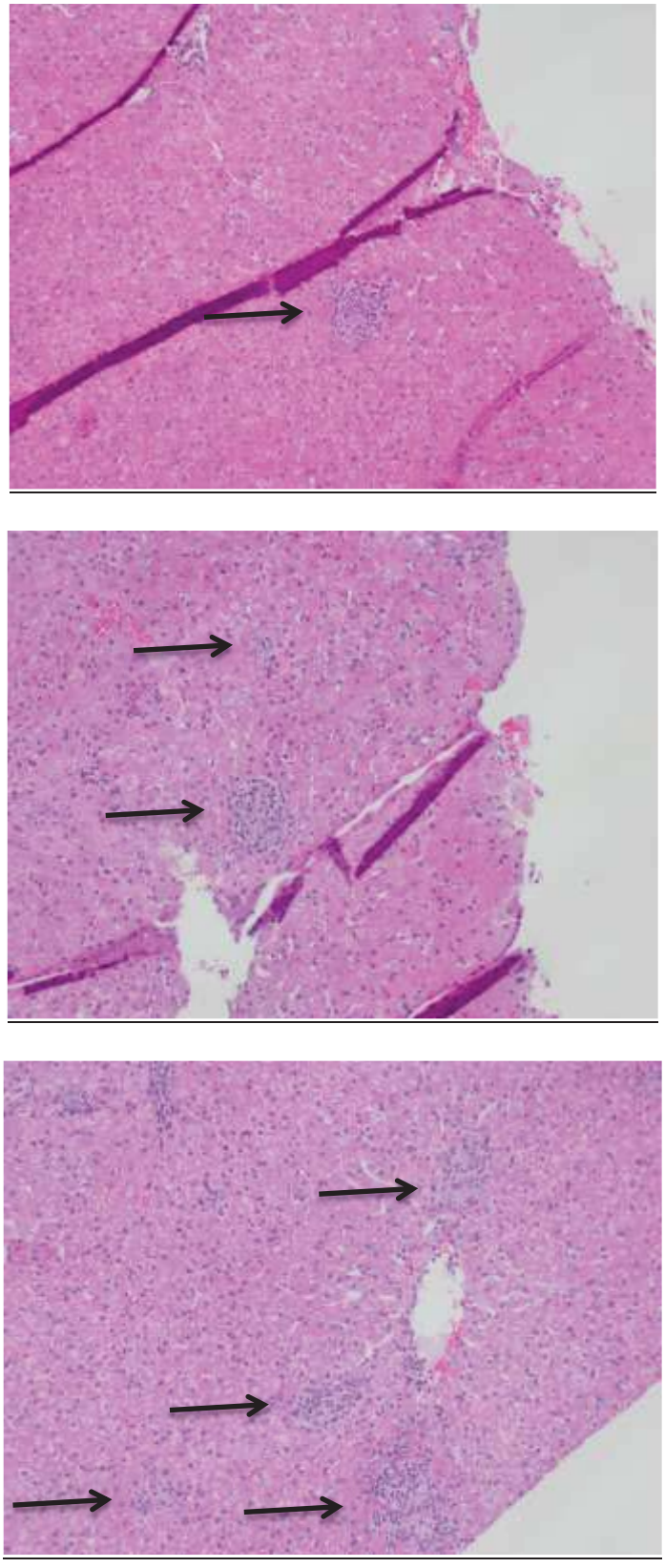
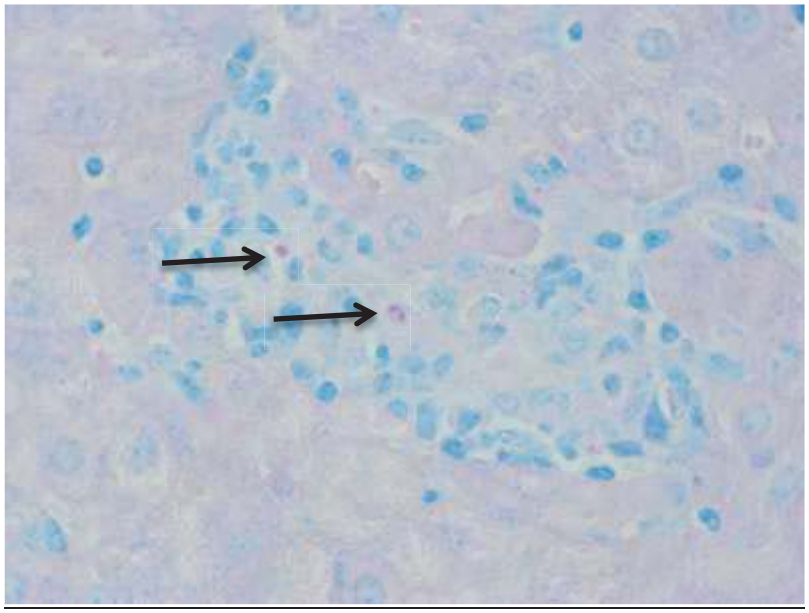


Figure A2.12 HEM, HE (x20), T = 233 days.



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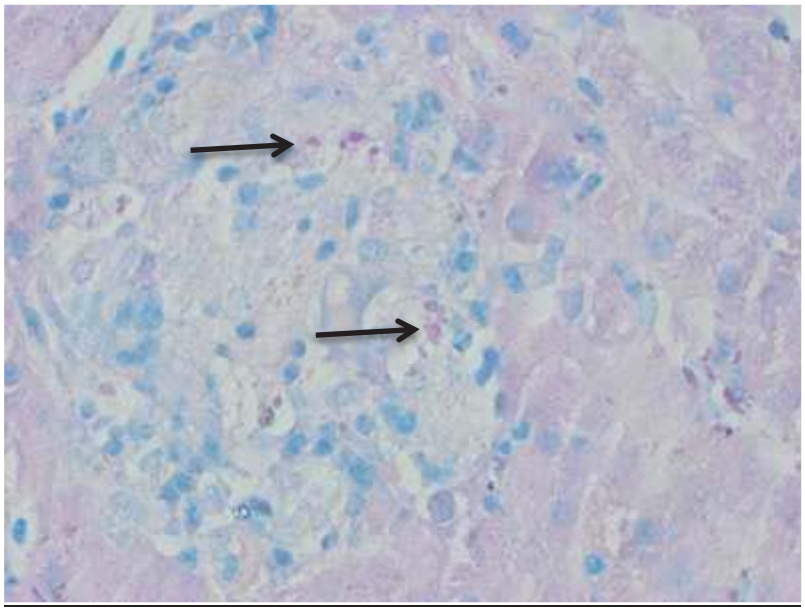


2

Figure A2.13 1, 2. HEM with round unidentified acid fast organisms (RUAFO), T = 148 days.

Appendix 3: The number of positive *Mycobacterium avium* subsp. *paratuberculosis* cultures from blood, biceps femoris muscle, and ileum tissue in ewes with (n=21) and without (n=30) clinical Johne's disease. (Data for 2008 and 2009 combined.) (Chapter Three - Map in Muscle study)

Number of Ewes in Study	Ear Tag	Johne's	Ileum	Mesenteric	Ileum	Mesenteric	Muscle	Blood
		Status	Histology	Lymph Node	Culture	Lymph Node	Culture	Culture
				Histology		Culture		
1	1	+	+	+	+	ND	-	+
2	2	+	+	+	+	ND	-	+
3	12	+	+	+	+	ND	+	+
4	18	+	+	+	+	ND	-	+
5	21	+	+	+	+	ND	+	+
6	22	+	+	+	+	ND	+	+
7	24	+	+	+	+	ND	+	+
8	26	+	+	+	+	ND	+	+
9	27	+	+	+	+	ND	+	+
10	37	+	+	+	+	ND	-	-
11	41	+	+	+	+	ND	+	-
12	49	+	+	+	+ 5	+ 5	+ 25	+ 29
13	80	+	+	-	+ 14	+ 14	+ 42	-
14	83	+	+	+	+ 14	+ 14	+ 29	+ 22
15	91	+	+	+	+ 14	+ 14	-	-
16	96	+	+	+	+ 14	+ 14	+ 15	-
17	100	+	+	+	+ 14	+ 21	-	-
18	210	+	+	+	+ 7	+ 7	+ 28	-
19	217	+	+	+	+ 7	+ 7	+ 28	-
20	227	+	+	+	+ 7	+ 7	+ 28	+
21	232	+	+	+	+ 14	+ 14	+ 36	+ 29
22	28	-	-	-	+	ND	-	-
23	29	-	-	-	+	ND	-	-
24	32	-	-	-	+	ND	-	-
25	34	-	-	-	+	ND	-	-
26	36	-	-	-	+	ND	-	-
27	38	-	-	-	+	ND	-	-

28	40	-	-	-	+	ND	-	-
29	46	-	-	-	+	ND	-	-
30	47	-	-	-	+	ND	+ 23	-
31	86	-	-	-	+ 21	+ 21	+ 29	-
32	90	-	-	-	+ 14	+ 14	-	-
33	94	-	-	-	+ 28	+ 28	-	-
34	95	-	-	-	+ 28	+ 28	-	-
35	201	-	-	-	+ (Cont)	+ 28	-	-
36	202	-	-	-	+ 14	+ 21	+ 28	-
37	203	-	-	-	+ 14	+ 28	+ 41	-
38	204	-	-	-	+ 28	+ 28	-	-
39	205	-	-	-	- (Cont)	-	-	-
40	206	-	-	-	- (Cont)	-	-	-
41	208	-	-	-	+ 14	-	-	-
42	209	-	-	-	+	+	-	-
43	211	-	-	-	+	+	-	-
44	213	-	-	-	+	+	-	-
45	214	-	-	-	+	+	-	-
46	215	-	-	-	+	+	-	-
47	222	-	-	-	+	-	-	-
48	224	-	-	-	-	+	-	-
49	225	-	-	-	+	+	-	-
50	226	-	-	-	+	-	-	+
51	228	-	-	-	+	+	-	-

1 – 21 Ewes with Clinical Johne's disease (n=21)

22 – 51 Ewes without clinical Johne's disease (n=30)

+ Positive test result days to reach dCGI1000

- Negative test result

Cont Contaminated Culture

Appendix 4: Twenty four healthy ewes; necropsy, culture and ELISA S/P ratio results (Chapter Four - Map Prevalence Study).

Johne's Status	Faecal Culture	Ileum Histology	Mesenteric Lymph Node Histology	Ileum Culture	Mesenteric Lymph Node Culture	Muscle Culture	Blood Culture	ELISA
-	-	-	-	-	+ 39	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	+ 22	-	-	-	-
-	-	-	-	+ 24	+ 35	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	+ 47	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	+ 45	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	+ 43	-	-	-
-	-	-	-	+ 28	-	-	-	+
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	+ 27	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	+ 60	-	-	-
-	-	-	-	+ 28	+ 39	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-
-	-	-	-	+ 25	-	-	-	-

+ / - Positive / negative test result days to reach Cumulative Growth Index 1000

Appendix 5: Liver Biopsy Histopathology, ELISA and BACTEC radiometric culture results for ileum, mesenteric lymph node, blood, muscle and liver.

Ewe ID	Gross Path (1-3)	BACTEC Culture / (Tissue) I,LN,B,M,L	Ileum Gran Hist / (AFO)	Mes LN Gran Hist / (AFO)	JD Status	ELISA + OD / SP% Ratio (>55)	Liver Gran Hist / (AFO)	Liver Biopsy Gran Hist / (AFO)	Perez Class OJD
Sparkes Dehlia	3	I,LN,B,M,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Riv. Deidre	3	I,LN,-,M,L	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Riv. #1	3	ND	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Green	3	I,LN,ND,M,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Doris White	3	I,LN,B,M,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Danna White	3	I,LN,ND,M,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Green Trudy	3	I,LN,ND,M,L	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Green #1	3	I,LNND,-,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love Green #2	3	I,LN,ND,-,-	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
LD 83	3	I,LN,B,M,ND	+ / +	+ / +	+	ND	+ / -	+ / -	OJD3b
LD 84	1	I,LN,-,-,ND	+ / -	+ / -	+	ND	- / -	- / -	OJDT1
LD 91	3	I,LN,-,-,ND	+ / +	+ / -	+	ND	+ / -	+ / -	OJD3b
LD 96	3	I,LN,-,M,ND	+ / +	+ / -	+	ND	+ / -	+ / -	OJD3b
LD 100	1	I,LN,-,-,ND	+ / -	+ / -	+	ND	- / -	- / -	OJDT1
LD 210	3	I,LN,-,M,ND	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
LD217	3	I,LN,ND,M,-	+ / +	+ / +	+ / +	+	+ / +	+ / +	OJD3b
LD 218	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJD3b
LD 227	3	I,LN,B,M,ND	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love 1	3	ND	+ / +	+ / +	+	ND	+ / -	+ / -	OJD3b
Love 2	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJD3b
45027	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJD3b
LD 86	2	I,LN,-,M,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 90	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 94	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 95	2	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 201	1	-,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 202	1	I,LN,B,M,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 203	1	I,LN,-,M,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 204	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 205	1	-,-,-,-	- / -	- / -	-	ND	- / -	- / -	-

LD 206	1	-,-,-,-	- / -	- / -	-	ND	- / -	- / -	-
Ewe ID	Gross Path (1-3)	BACTEC Culture / (Tissue)	Ileum Gran Hist / (AFO)	Mes LN Gran Hist / (AFO)	JD Status	ELISA + OD / SP% ratio	Liver Gran Hist / (AFO)	Liver Biopsy Gran Hist / (AFO)	Perez Class OJD
LD 208	1	I,-,-,-	- / -	- / -	-	ND	- / -	- / -	-
LD 209	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 211	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 213	2	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 214	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 215	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 222	1	I,-,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD224	1	-,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 225	2	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD 226	1	I,-,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD227	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJD3b
LD 228	1	I,LN,-,-,ND	- / -	- / -	-	ND	- / -	- / -	-
LD Ill-thrifty Ewes									
1	1	ND	- / -	- / -	-	-	- / -	- / -	-
2	1	ND	- / -	- / -	-	-	- / -	- / -	-
4	1	ND	- / -	- / -	-	-	- / -	- / -	-
5	1	ND	+ / -	+ / -	T1	-	- / -	- / -	OJDT1
6	1	ND	+ / -	+ / -	+	-	- / -	- / -	OJDT2
7	1	ND	- / -	- / -	-	-	- / -	- / -	-
8	1	ND	- / -	- / -	-	-	- / -	- / -	-
9	1	ND	- / -	- / -	-	-	- / -	- / -	-
10	3	ND	+ / +	+ / +	+	-	+/ -	+ / -	OJDT3c
11	1	ND	- / -	- / -	-	-	- / -	- / -	-
12	1	ND	+ / +	+ / -	+	+	+ / -	+ / -	OJDT3c
13	3	ND	+ / +	+ / +	+	-	+ / -	+ / -	OJDT3b
14	3	ND	+ / -	+ / -	+	-	- / -	- / -	OJDT2
15	1	ND	- / -	- / -	-	-	- / -	- / -	-
16	2	ND	+ / -	+ / -	+	-	- / -	- / -	OJDT2
17	1	ND	- / -	- / -	-	-	- / -	- / -	-
18	2	ND	- / -	- / -	-	-	- / -	- / -	-
19	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3c
20	1	ND	- / -	- / -	-	-	- / -	- / -	-
21	3	ND	+ / +	+ / +	+	+	+ / +	+ / +	OJDT3b
22	2	ND	+ / +	+ / -	+	+	+ / +	+ / +	OJDT3c
23	1	ND	- / -	- / -	-	-	- / -	- / -	-
24	1	ND	- / -	- / -	-	-	- / -	- / -	-
25	1	ND	- / -	- / -	-	-	- / -	- / -	-
26	1	ND	- / -	- / -	-	-	- / -	- / -	-
27	1	ND	- / -	- / -	-	-	- / -	- / -	-
28	1	ND	- / -	- / -	-	-	- / -	- / -	-
29	1	ND	- / -	- / -	-	-	- / -	- / -	-

30	1	ND	- / -	- / -	-	-	- / -	- / -	-
Ewe ID	Gross Path (1-3)	BACTEC Culture / (Tissue)	Ileum Gran Hist / (AFO)	Mes LN Gran Hist / (AFO)	JD Status	ELISA + OD / SP% ratio	Liver Gran Hist / (AFO)	Liver Biopsy Gran Hist / (AFO)	Perez Class OJD
31	1	ND	+ / +	+ / -	+	-	- / -	- / -	OJDT3c
32	1	ND	- / -	- / -	-	-	- / -	- / -	-
33	1	ND	- / -	- / -	-	+	- / -	- / -	-
34	1	ND	+ / -	- / -	T1	-	- / -	- / -	OJDT1
35	2	ND	- / -	- / -	-	-	- / -	- / -	-
36	1	ND	- / -	- / -	-	-	- / -	- / -	-
37	3	ND	+ / +	+ / +	+	+	+ / +	+ / +	OJDT3b
38	1	ND	- / -	- / -	-	-	- / -	- / -	-
39	1	ND	+ / -	+ / -	+	+	+ / -	+ / -	OJDT3c
40	1	ND	- / -	- / -	-	-	- / -	- / -	-
41	1	ND	- / -	- / -	-	-	- / -	- / -	-
42	1	ND	- / -	- / -	-	-	- / -	- / -	-
Waters 43	1	ND	- / -	- / -	-	-	- / -	- / -	-
Waters 44	1	ND	- / -	- / -	-	-	- / -	- / -	-
Waters 45	1	ND	- / -	- / -	-	-	- / -	- / -	-
Waters 46	1	ND	+ / -	- / -	T1	-	- / -	- / -	OJDT1
Waters 47	2	ND	- / -	- / -	-	-	- / -	- / -	-
Waters 48	3	ND	+ / +	+ / -	+	+	+ / -	+ / -	OJDT3b
Waters 49	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3b
Waters 53	3	ND	+ / -	+ / -	+	+	+ / -	+ / -	OJDT3c
Waters 60	1	ND	- / -	- / -	-	-	- / -	- / -	-
LD		I,LN,B,M,L,F							
E 78 LD 1	1	-,LN-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E79 LD 2	1	-,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E80 LD 3	1	I,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E81 LD 4	1	I,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E82 LD 5	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E83 LD 6	1	-,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E84 LD 7	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E85 LD 8	1	-,LN,-,-,ND,-	+	+	-	+	+ / -	+ / -	OJD3c
E86 LD 9	1	-,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E87 LD 10	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E88 LD 11	1	-,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E89 LD 12	1	I,-,-,-,ND,-	- / -	- / -	-	+	- / -	- / -	-
E90 LD 13	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E91 LD 14	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E92 LD 15	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E93 LD 16	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E94 LD 17	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E95 LD 18	1	I,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E96 LD 19	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E97 LD 20	1	-,LN,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E98 LD 21	1	I,LN,-,-,ND,-	- / -	- / -	-	Equiv	- / -	- / -	-

E99 LD 22	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
Ewe ID	Gross Path (1-3)	BACTEC Culture / (Tissue)	Ileum Gran Hist / (AFO)	Mes LN Gran Hist / (AFO)	JD Status	ELISA + OD / SP% ratio	Liver Gran Hist / (AFO)	Liver Biopsy Gran Hist / (AFO)	Perez Class OJD
E100 LD 23	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E101 LD 24	1	-,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E102 LD 25	1	I,-,-,-,ND,-	- / -	- / -	-	-	- / -	- / -	-
E105 45120	1	ND	- / -	- / -	-	ND	- / -	- / -	-
E106 45119	1	ND	+ / -	+ / -	+	ND	- / -	- / -	OJDT3c
E107 45121	1	ND	- / -	- / -	-	ND	- / -	- / -	-
248101	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJD3b
258101	1	ND	- / -	- / -	-	ND	- / -	- / -	-
258102	2	ND	+ /+	+ / -	+	ND	+ / -	+ / -	OJD3c
45090	1	ND	- / -	- / -	-	-	- / -	- / -	-
Love114710	3	ND	+ / +	+ / +	+	ND	+ / +	+ / +	OJDT3b
Bart1311010	1	ND	- / -	- / -	-	ND	- / -	- / -	-
Bart12310101	1	ND	- / -	- / -	-	ND	- / -	- / -	-
Bart12310102	1	ND	- / -	- / -	-	ND	- / -	- / -	-
Bart2310103	1	ND	- / -	- / -	-	ND	- / -	- / -	-
Bart1110101	1	ND	- / -	- / -	-	ND	- / -	- / -	-
Bart2110101	1	ND	- / -	- / -	-	ND	- / -	- / -	-
E201 Crystal 1	2	ND	+ / -	+ / -	+	+	- / -	- / -	OJDT3a
Crystal 2	1	ND	- / -	- / -	-	-	- / -	- / -	-
Crystal 3	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3b
Crystal 4	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3b
Love1230311	3	ND	+ / +	+ / -	+	+	+ / -	+ / -	OJDT3c
Riv1 45992	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3b
Riv2 45993	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJDT3b
45980	3	ND	+ / +	+ / +	+	+	+ / -	+ / -	OJD3b
45027	3	ND	+ / +	+ / +	+	ND	+ / -	+ / -	OJD3b
44931	1	ND	- / -	- / -	-	ND	- / -	- / -	-
46901	3	ND	+ / +	+ / +	+	+	+ / +	+ / +	OJD3b
Love201121	3	I +	+/+	+/+	+	+	+ / +	+ / +	OJD3b
Love 47247	3	ND	+/+	+/+	+	+	+ / +	+ / +	OJD3b
Love47265	3	ND	+/+	+/+	+	+	+ / +	+ / +	OJD3b
Love233121 Orange	3	ND	+/+	+/+	+	+	+ / +	+ / +	OJD3b
Love233121 Green	3	ND	+/+	+/+	+	+	+ / +	+ / +	OJD3b
Love233121 Purple	1	ND	- / -	- / -	-	-	- / -	- / -	AdCarc
Stewart 701312	3	I +	+/+	+/+	+	+	+ / +	+ / +	OJD3b

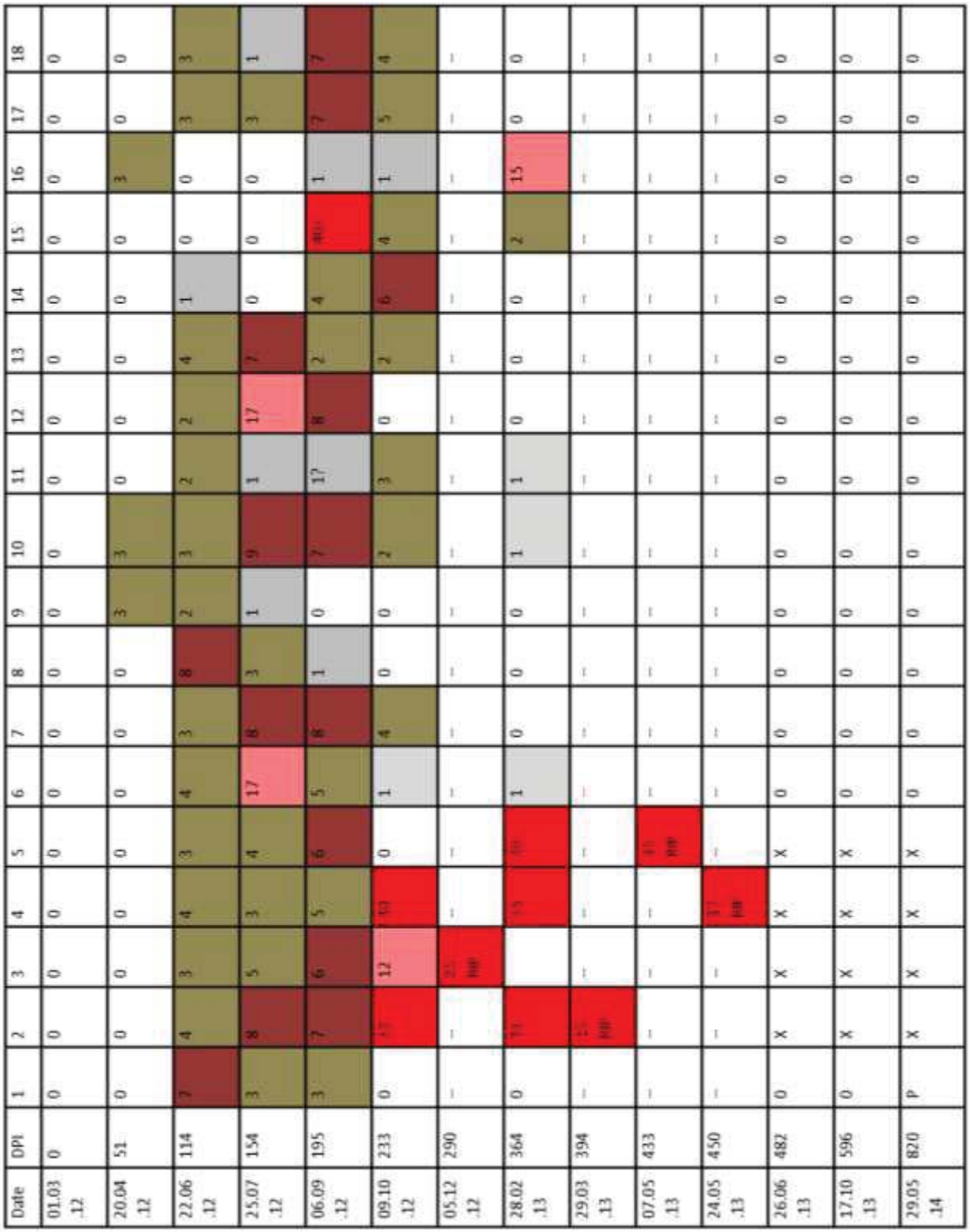
Gross Signs at Necropsy: 1 NAD 2 equivocal 3 positive OJD

BACTEC Map Culture Key I Ileum LN Mesenteric Lymph Node B Blood M Muscle L Liver F faeces ND Not Done OJD1, 2, 3a, 3b, 3c Perez Classification of ileal lesions

Appendix 6.1a: Number of HEM from nine histologically prepared sections for each liver biopsy sample collected from unchallenged sheep at time points t = 0 – 820 days.

Date	DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
01.03.12	0	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
20.04.12	51	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
22.06.12	114	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
25.07.12	154	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
06.09.12	195	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
09.10.12	233	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
28.02.13	364	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
26.06.13	482	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
17.10.13	596	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:
29.05.14	820	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:	:

Appendix 6.1b Number of HEM from nine histologically prepared sections for each liver biopsy sample collected from challenged sheep at time points t = 0 – 820 days.



Date	DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
01.03.12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20.04.12	51	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	3	0	0
22.06.12	114	7	4	3	4	3	4	3	8	2	3	2	2	4	1	0	0	3	3
25.07.12	154	3	8	5	3	4	17	8	3	1	9	1	17	7	0	0	0	3	1
06.09.12	195	3	7	6	5	6	5	8	1	0	7	17	8	2	4	41	1	7	7
09.10.12	233	0	17	12	13	0	1	4	0	0	2	3	0	2	6	4	1	5	4
05.12.12	290	-	-	21 HEM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28.02.13	364	0	11	-	15	21 HEM	1	0	0	0	1	1	0	0	0	2	15	0	0
29.03.13	394	-	21 HEM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
07.05.13	433	-	-	-	-	11 HEM	-	-	-	-	-	-	-	-	-	-	-	-	-
24.05.13	450	-	-	-	17 HEM	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26.06.13	482	0	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0
17.10.13	596	0	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0
29.05.14	820	P	X	X	X	X	0	0	0	0	0	0	0	0	0	0	0	0	0

Appendix 6.2: ELISA S/P ratios for sheep sampled at t = 0 – 820 days.

Unchallenged

Date	DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
01.03.12	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
04.03.12	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
07.03.12	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22.03.12	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29.03.12	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.04.12	43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08.05.12	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
01.06.12	93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21.06.12	114	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25.07.12	154	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
06.09.12	195	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
09.10.12	233	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
04.12.12	279	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27.02.13	364	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26.06.13	482	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17.10.13	596	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29.05.14	820	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Challenged – ELISA S/P ratio > 50 positive.

Date	DPI	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
01.03.12	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
04.03.12	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
07.03.12	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22.03.12	22	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29.03.12	29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12.04.12	43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20.04.12	51	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
08.05.12	69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
01.06.12	93	-	-	-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	-
21.06.12	114	-	-	-	-	-	+	+	-	-	+	-	+	+	-	+	-	+	-
25.07.12	154	-	-	-	-	-	+	-	+	-	+	-	+	+	-	+	-	-	+
06.09.12	195	+	-	-	-	-	+	-	+	-	+	-	+	+	-	+	-	+	+
09.10.12	233	+	+	+	-	-	+	-	+	-	+	+	+	+	-	+	-	+	+
04.12.12	279	+	+	X+	-	-	+	-	+	-	+	+	+	+	-	+	-	-	+
27.02.13	364	-	+	X	eq	-	-	-	+	-	eq	+	eq	eq	-	-	-	-	+
29.03.13	394		X	X															
07.05.13	433		X	X		-X													
24.05.13	450		X	X	Xeq	X													
26.06.13	482	-	X	X	x	X	-	-	Eq	-	-	Eq	-	Eq	-	-	-	-	+
17.10.13	596	-	X	X	X	X	-	-	Eq	-	-	-	-	-	-	-	-	-	+
29.05.14	820		X	x	X	x	-	-		-	-				-	-	-		

Appendix 6.2a: ELISA S/P ratios for unchallenged sheep (ID 1 – 18). Blood samples collected at time points t = 0 – 820 days.

Time	T=0	T=4	T=6	T=22	T=29	T=43	T51	T=69	T=93	T114	T154	T195	T233	T279	T364	T482	T596	T820
1	0.55	0.47	0.25	0.56	0.48	0.19	2.09	1.12	-1.5	0.25	0.22	0.30	0.39	0.11	-1.4	0.90	0.22	0.32
2	0.77	0.66	0.45	0.93	0.41	0.19	0.10	0.28	-0.9	0.45	0.45	0.60	0.26	0.11	-1.2	0.99	0.66	3.06
3	1.21	1.91	2.27	2.61	1.79	1.073	1.57	1.03	-1.0	4.71	0.80	1.11	1.56	0.33	-1.0	0.90	1.10	1.74
4	-0.1	0.38	0.55	0.56	0.72	0.29	0.52	0.56	-1.3	0.55	0.80	1.10	0.911	0.78	-1.1	0.45	1.68	2.62
5	0.77	0.28	0.65	0.84	0.49	0.19	0	0.56	-1.5	0.55	0.45	0.40	0.78	-0.1	-1.1	0.45	0.51	2.07
6	0.88	0.47	0.75	0.74	0.87	0.58	0.62	0.84	-1.0	0.86	0.57	0.80	1.30	0.44	-1.2	1.08	0.515	6.45
7	2.20	1.53	1.77	0.65	0.34	0.19	0.73	1.21	-1.4	0.05	0.45	0.30	1.30	0	-1.1	0.99	0.58	0.54
8	0.99	0.57	0.45	0.46	0.41	0.68	2.09	1.21	-1.4	0.96	0.45	0.70	2.08	0.55	-1.0	1.26	1.46	0.98
9	2.86	0.66	0.96	1.12	0.49	0	0.31	0.74	-1.5	0.35	0.42	0.30	1.30	0.44	-0.9	0.45	0.29	0.54
10	1.76	0.66	0.65	0.56	0.41	0.48	1.57	4.03	-1.1	1.46	0.34	0.90	2.08	1.67	3.16	18.94	4.77	2.62
11	1.54	1.24	1.06	0.74	0.41	0.39	0.62	0.84	-0.1	0.65	0.45	1.40	1.17	1.0	-1.1	0.63	1.02	1.74
12	0.66	0.76	0.96	0.65	0.49	0.48	1.04	1.03	-1.4	1.26	0.57	1.71	3.65	1.45	-0.1	0.99	1.17	1.85
13	0.66	0.66	1.06	0.65	0.57	0.19	0.62	2.24	-1.1	0.65	0.68	0.70	0.91	0.22	-1.1	0.72	0.58	0.76
14	0.77	0.76	0.75	0.65	0.41	0.87	0.62	0.65	-1.3	0.65	0	0.40	1.82	0.44	-1.2	1.54	0.58	22.74
15	0.66	0.19	0.55	0.37	0.19	0.29	0.73	0.93	-1.1	6.33	0.57	3.20	2.08	0.66	-1.0	1.087	2.12	2.84
16	1.54	1.33	1.26	2.24	2.09	1.65	1.78	1.12	6.54	3.09	0.68	0.60	1.95	0.66	-1.1	2.17	0.80	2.51
17	0.77	0.47	0.25	0.56	0.19	0.09	0.52	0.37	-1.6	0.55	0.68	0.30	1.95	0.11	-1.2	1.45	0.36	0.65
18	0.88	0.38	0.65	0.56	0.64	0.48	0.52	0.18	-1.3	0.65	0.34	0.50	2.21	0.55	0	2.08	0.95	1.96
Mean	1.07	0.74	0.85	0.86	0.64	0.46	0.89	1.05	-0.8	1.33	0.50	0.85	1.54	0.52	-0.8	2.06	1.08	3.11

Appendix 6.2b ELISA S/P ratios for challenged sheep (ID 1 – 18). Blood samples collected at time points t = 0 – 820 days.

Time	T=0	T=4	T=6	T=22	T=29	T=43	T51	T=69	T=93	T114	T154	T195	T233	T279	T364	T482	T596	T820
1	0.88	0.57	0.86	0.65	0.41	0.29	0.52	1.09	30.35	37.63	30.26	50.14	57.73	54.91	27.17	19.75	13.7	5.53
2	0.33	0.28	0.65	0.37	0.34	0.19	1.78	0.65	24.08	36.62	16.45	44.27	101.9	142.2	189			
3	0.33	0.38	0.65	0.46	0.49	0.29	0.73	0.84	10.64	14.23	8.51	44.95	76.97	90.06				
4	0.77	1.052	1.46	1.21	0.95	0.78	0.20	0.18	-0.85	9.67	24.85	38.29	43.71	49.44	44.95			
5	0.44	0.66	0.75	1.12	1.41	0.58	0.94	0.74	-1.65	1.06	7.36	42.21	32.70	26.22	28.78			
6	0.22	0.38	0.35	2.33	1.56	0.58	0.20	0.28	91.17	86.27	84.92	70.42	63.73	55.02	43.23	39.24	32.4	13.95
7	0.22	0.28	0.55	0.84	0.34	0.48	0.10	17.5	117.4	68.84	24.97	32.71	42.93	34.71	25.45	18.34	11.4	14.21
8	0.55	0.38	0.96	1.59	0.72	0.48	-0.20	16.8	21.12	41.69	59.60	76.88	89.09	73.88	59.28	52.76	45.2	19.13
9	0.66	0.28	0.65	2.5	2.49	1.46	0.52	2.06	1.19	6.63	14.61	9.20	9.45	5.92	2.86	3.772	3.25	18.97
10	0.55	0.38	0.86	0.65	0.49	0.58	0.62	0.56	57.68	69.14	112.5	98.13	84.65	70.98	53.09	41.87	35.9	20.53
11	1.32	1.72	2.07	1.21	0.87	0.48	0.20	0.84	10.87	32.47	18.06	29.87	57.95	86.49	75.45	53.55	41.9	44.42
12	1.32	1.33	2.48	1.12	0.72	0.68	0.94	1.49	30.92	85.86	108.5	86.19	57.39	54.35	46.78	31.69	33.7	21.75
13	0.44	-4.45	0.75	0.56	0.57	0	0.10	0	2.10	55.87	56.50	62.39	68.85	59.71	53.44	45.56	40.1	12.33
14	0.88	1.14	0.65	0.93	0.72	0.62	0.94	1.12	-1.19	2.68	19.33	38.29	37.04	41.40	26.37	23.35	10.7	14.99
15	0.44	0.095	0.45	0.28	0.26	0.68	0	16.8	82.63	83.84	106.3	76.29	80.2	65.40	42.20	35.20	21.7	22.70
16	0.55	0.38	0.86	0.84	1.03	3.51	2.623	6.18	3.70	7.34	4.94	6.26	4.22	6.25	19.15	3.336	3.42	2.513
17	1.76	0.95	1.46	2.15	1.10	0.68	0.83	20.9	27.05	54.15	41.08	60.43	62.62	44.64	19.03	17.47	15.1	5.802
18	0.88	0.57	1.26	0.84	0.49	0.68	0.10	-0.4	27.50	35.81	64.21	87.75	100.1	97.43	81.53	67.34	60.3	31.73
Mean	0.69	0.60	0.99	1.09	0.83	0.73	0.62	4.87	29.70	40.54	44.61	53.04	59.51	58.83	49.28	32.37	26.38	18.69

Appendix 6.3 Body condition scores for unchallenged and challenged sheep sampled at time points t = 0 – 820 days. (Score out of 5).

Unchallenged

	T=0	51	113	154	195	233	279	364	394	433	450	482	596	820
1	3	3	3.5	3.5	4	5	5	5	5	5	5	5	5	5
2	3	3	3	3	4	5	5	5	5	5	5	5	5	5
3	2.5	2.5	2.5	2.5	3.5	4	4	4.5	5	5	5	5	5	5
4	3	3	3	3.5	4	5	4.5	5	5	5	5	5	5	5
5	3	3	3	2.5	4	4.5	5	5	5	5	5	5	5	5
6	3	3	3	3.5	4	5	4	5	5	5	5	5	5	5
7	2.5	3	3	3.5	4	5	5	5	5	5	5	5	5	5
8	3	3	3	2.5	4	3.5	4	5	5	5	5	5	5	5
9	3	3	3	3.5	4	4	5	5	5	5	5	5	5	5
10	3	3	3	3	3.5	4.5	4.5	5	5	5	5	5	5	5
11	2.5	3	3	4	4	5	5	5	5	5	5	5	5	5
12	2.5	3	3	3.5	4.5	5	5	5	5	5	5	5	5	5
13	3	3	3	3	4	4.5	5	5	5	5	5	5	5	5
14	3	3	3	3	4	5	5	5	5	5	5	5	5	5
15	3	3	2.5	2.5	3.5	4.5	4.5	5	5	5	5	5	5	5
16	3	3	3	3.5	4	5	5	5	5	5	5	5	5	5
17	3	3	3	3.5	4	4.5	5	5	5	5	5	5	5	5
18	3	3	3	3.5	4	4.5	5	5	5	5	5	5	5	5

Challenged

	T=0	51	113	154	195	233	279	364	394	433	450	482	596	820
1	3	3	3	3.5	2	3.5	4.5	5	5	5	5	5	5	5
2	3	3	3.5	4.0	3.0	4	4	1.5	OJD	Dead	Dead	Dead	Dead	Dead
3	2.5	3	3	3.5	2.0	3	1.75	OJD	Dead	Dead	Dead	Dead	Dead	Dead
4	3	3	3	2.5	4	4	5	1.5	1.5	1.5	OJD	Dead	Dead	Dead
5	3	3	3	3.5	3	3	3.5	1.5	1.5	OJD	Dead	Dead	Dead	Dead
6	3	3	3	3.5	3.5	3.5	4	5	5	5	5	5	5	5
7	2.5	3	3	3.0	3.5	4.5	5	5	5	5	5	5	5	5
8	2.5	3	3	3.5	4.5	4.5	5	5	5	5	5	5	5	5
9	3	3	3	3.5	4	3	4.5	5	5	5	5	5	5	5
10	2.5	3	3	2.0	2.0	3	3.5	3	4.5	4.5	4.5	4.5	5	5
11	3	3	3.5	4.0	2.0	2.5	1.75	3.5	4.5	4.5	4.5	4.5	5	5
12	3	3	3	3.5	3.5	3	4.5	5	5	5	5	5	5	5
13	3	3	3	3.5	4	4	5	4	5	5	5	5	5	5
14	2.5	3	3.5	4.0	4.0	3	5	5	5	5	5	5	5	5
15	2.5	3	2.5	2.5	2	2	3.5	4.5	4.5	4.5	4.5	4.5	5	5
16	3	3	3	3.5	3.5	3	4.5	5	4.5	4.5	4.5	4.5	5	5
17	3	3	2.5	2.5	2	3	4	4	4.5	4.5	4.5	4.5	5	5
18	2.5	3	2.5	2.5	2	2.5	4	4.5	4.5	4.5	4.5	4.5	5	5

Appendix 6.4a: Live weights (kg) for unchallenged sheep recorded at time points 0 – 820 days.

Time (Days)	0	51	114	154	195	233	338	364	394	450	482	519	596	729	820
ID 1	36	48.6	51.4	53.6	56.2	56.4	81.5	79	80.5	81	78	83.5	83	99	89
2	36.5	45.9	49.5	54.8	53	54	75.5	75	75.5	77	75	80	78	97	87
3	35	44.4	45.2	46.9	49.2	50.2	72.5	68	69.5	70	69	77.5	80	97	87.5
4	34	44.4	45.5	47	50.6	52	79	77	77.5	80	79	85.5	81.5	101	91
5	37	43	45.4	47	51.6	52.8	73.5	74	75.5	76	74	80.5	86	100	91
6	36.5	49.2	53.2	54	58	60	85.5	85	85.5	90	90	94	98	113	104
7	36.5	41.4	44	47.7	51	53	73.5	75	76	78	75	81	79	96	87.5
8	36	45.5	47.5	48.5	48.3	49.4	70	70	71.5	69	69	75	75	91	77.5
9	34	47.2	49	50.6	52.4	52	77.5	77	77	81	81	87	87	105	92
10	38	42.3	46.4	49	47.8	50	77	76	80	82	85	89	88	107	96
11	37	47.5	49.5	52.4	53.6	54.6	79	78	80.5	81.5	78	83.5	80	98	86
12	36.5	46.8	50.4	53.6	57.4	58.6	82	82	83.5	81	81	89.5	86	103	89.5
13	34	48.7	48.4	47.8	49.6	51.4	74	73	73.5	72	70	75	77	89	79.5
14	37	45.4	47.4	48.5	50.2	50	72	71	73.5	72	69	78	80	91	84.5
15	36.5	44.9	46	47.6	51	51	74.5	73	73.5	74	72	80	81.5	95	85
16	36.5	46.5	48	49.5	51.6	53	75	76	77	78	76	82	82.5	96	89
17	35	45.1	48.5	50.6	53	54	74	73	73	72.5	76	80	78	87	81
18	36	48.1	50.5	52.8	54.6	53	79	79	79.5	78	73	81	82.5	101	90
Mean	36	45.8	48.1	50.1	52.2	53.1	76.4	75.6	76.8	77.4	76.1	82.3	82.4	98.1	88.2

Appendix 6.4b Live weights (kg) for Challenged lambs recorded at time points 0 – 820 days.

Time	0	51	114	154	195	233	338	364	394	450	482	519	596	729	820
ID 1	34	40.2	41	41.3	44	44.6	67	69	66	69	69	74	74	84	75
2	36	44.5	48.5	51.3	44	43.2	45.2	42	-	-	-	-	-	-	-
3	36.5	42.3	42.6	43.4	43	44.3	-	-	-	-	-	-	-	-	-
4	37	46.6	43.5	39	46.4	46.1	58.5	50	49	46.4	-	-	-	-	-
5	35	50.4	49	48.4	45.4	46.4	57.5	52	42	-	-	-	-	-	-
6	36.5	46	43.5	42.1	44	46.4	73	74	74.5	76	76	84	79	95	81
7	36.5	45.3	44.4	43.6	47.2	47.4	70	72	69.5	73	71	77.5	71.5	81	71
8	35	46.4	46	45.2	52.4	53.8	76.5	76	75.5	79	80	87	87	96	83
9	38	40.7	41.4	42	44.5	45.9	68.5	68	71	73	72	76	71.5	81	75
10	36	44.6	43.4	41	42.9	43.3	68.5	68	64.5	70	69	72.5	73	85	71
11	36.5	46.2	44	43.8	40.7	38.3	57.5	59	58	65	64	70.5	74	87	76
12	35	43.7	43.4	42	44	44	74.5	74	73.5	75.5	76	79.5	85	97	82
13	36.5	46.4	45.5	45.1	47.6	49.5	75	76	71.5	78	75	87	85	94	81
14	36.5	44.8	44	43.4	45	46.8	68	69	69	70	70	77.5	80.5	85	74
15	37	46.7	43.4	41.2	42.9	43.3	63	64	63.5	68	69	76	78	91	78
16	35	45.5	44.5	43.5	45.6	46.1	65.5	66	64	69	68	76	75	86	74
17	35	42.3	39.4	38	39.3	42	71.5	73	70.5	68	69	76.5	78.5	95	81
18	36.5	45.4	42.4	39.9	37.1	42	70	67	65.5	72	69	78	80	96	82
Mean	36	45	43.9	43	44.2	45.2	66.4	65.8	65.5	70.1	71.2	78	78	89.5	77.4

Appendix 6.5a Skeletal measurements (cm) for unchallenged sheep at 820 days.

Unchallenged	Poll/Rump	Right Metacarpal 3 Length	Right Metacarpal 3 Diameter (Medial – Lateral)	Tibial Crest To Medial Calcaneus	Calcaneus Length	Right Metatarsus Length	Right Metatarsus Diameter (Cranio-Caudal)	Right ulna Length Olecranon to 1st Metacarpal Joint
1	113.5	14.8	2.7	29	7.3	21.6	2.6	22
2	118	15.5	2.7	27	7.6	20.7	2.3	24
3	113.8	16.7	2.6	26	7.4	21.5	2.3	23.1
4	118.5	14.8	2.7	26	7.3	21.5	2.6	22
5	112	15	2.9	21.5	7.4	18.2	2.3	21.2
6	111	15.7	2.7	26.5	7.8	22.3	2.3	24
7	114.5	13.8	2.8	23.5	7.3	21	2	20.2
8	120	16.8	2.9	28	7.1	21.2	2.3	21.3
9	121.4	15	2.7	26.8	7.4	21	2.2	22.5
10	118	17.5	2.8	26.8	7.4	21.5	2.4	23.5
11	120.5	15.5	2.6	25	7.6	21.6	2.3	21.3
12	116.5	15.4	2.7	22	7.5	20	2.4	21
13	113	15.3	2.9	25.5	7.4	20.6	2.3	22.2
14	112.5	13.3	2.5	24	6.9	20	2	22.5
15	121.5	16.8	2.7	28.8	7.3	22.5	2.3	23.8
16	117	15.5	2.5	26.2	7.6	21	2.2	23.5
17	114.5	15.8	2.6	27.5	7.5	21.8	2.2	23.8
18	114.5	16.2	3.1	26	6.8	21.5	2.5	21.8
Average	116.15	15.5222222	2.72777778	25.8944444	7.36666667	21.0833333	2.30555556	22.4277778

Appendix 6.5b Skeletal measurements (cm) for challenged sheep at 820 days.

Challenged	Poll/Rump	Right Metacarpal 3 Length	Right Metacarpal 3 Diameter (Medial – Lateral)	Tibial Crest To Medial Calcaneus	Calcaneus Length	Right Metatarsus Length	Right Metatarsus Diameter (Cranio-Caudal)	Right ulna Length Olecranon to 1st Metacarpal Joint
1	111	15.3	2.6	25.3	6.5	19.5	2	22.8
6	108.5	14.5	2.7	23.8	7.2	18	2.2	19.8
7	111	13.5	2.6	25.8	7	20.5	2.1	22.3
8	112.5	14.2	2.5	24.2	6.7	20.5	2	23.3
9	106.5	14.5	2.4	24.5	6.6	20.5	2.2	22.3
10	116.5	13.5	2.7	25.5	6.8	20.5	2.2	22.2
11	108.5	14	2.6	25	6.9	19.5	2.1	20.8
12	112	14.5	2.7	25.5	6.9	21	2.2	22.5
13	115	14.5	2.9	24.5	7.3	21	2.3	22
14	113.5	14	2.7	25	6.6	20.2	2	22
15	113.8	13.7	2.8	25	6.8	19.8	2.1	22.3
16	104.1	15	2.7	25	7.3	21.5	2	22.5
17	110	15.5	2.8	27.5	6.9	20.7	2.2	22.2
18	111	13.3	2.7	24.5	7	18.5	2.2	22.2
Average	110.992857	14.2857143	2.67142857	25.0785714	6.89285714	20.1214286	2.12857143	22.0857143

Appendix 7 Statement of Contribution for Publications

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Stefan Lindsay Smith

Name/Title of Principal Supervisor: Professor David West

Name of Published Research Output and full reference:

SL Smith , DM West , PR Wilson , GW de Lisle , MG Collett , C Heuer, JP Chambers. Detection of Mycobacterium avium subsp.paratuberculosis in skeletal muscle and blood of ewes from a sheep farm in New Zealand. New Zealand Veterinary Journal 59(5), 240–243, 2011

In which Chapter is the Published Work: Appendix Seven

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Designing study, sourcing ewes from farms, collection of all samples, histological preparation, reading and interpretation, writing article for publication, dealing with reviewing process and submission for publication.

Candidate's Signature — 3rd February 2017 Date

Principal Supervisor's signature — 13 April 2017 Date

GRS Version 3– 16 September 2011

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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Name/Title of Principal Supervisor: Professor David West

Name of Published Research Output and full reference:

SL Smith , DM West , PR Wilson , GW de Lisle , MG Collett , C Heuer, JP Chambers. The prevalence of disseminated Mycobacterium avium subsp. paratuberculosis infection in tissues of healthy ewes from a New Zealand farm with Johne's disease present. New Zealand Veterinary Journal, 61(1), 41–44, 2013

In which Chapter is the Published Work: Appendix Seven

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

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Name/Title of Principal Supervisor: Professor David West

Name of Published Research Output and full reference:

SL Smith, P. R. Wilson, M. G. Collett, C. Heuer, D. M. West, M. Stevenson, and J. P. Chambers. Liver Biopsy Histopathology for Diagnosis of Johne's Disease in Sheep. Veterinary Pathology, 51 (5), 915-918, 2014

In which Chapter is the Published Work: Appendix Seven

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 95%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Designing study, sourcing ewes from farms, collection of all samples, design of histological preparation of slides, reading and interpretation, writing article for publication, dealing with reviewing process and submission for publication.

Candidate's Signature — Date: 3rd February 2017

Principal Supervisor's signature — Date: 13 April 2017

GRS Version 3– 16 September 2011

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Stefan Lindsay Smith

Name/Title of Principal Supervisor: Professor David West

Name of Published Research Output and full reference:

SL Smith, P Singh, D Harding, D Lun & JP Chambers. Thalidomide pharmacokinetics in sheep. New Zealand Veterinary Journal, 64(4), 238–242, 2016

In which Chapter is the Published Work: Appendix Seven

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 90%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Designing study, identifying and helping with the process of manufacturing pure Thalidomide in house, confirming the manufactured product was Thalidomide, sourcing lambs for dosing, administration of product and collection of all blood samples, storage and sample preparation prior to HPLC use, writing article for publication, dealing with reviewing process and submission for publication.

Candidate's Signature — 3rd February 2017 (Date)

Principal Supervisor's signature — 13 April 2017 (Date)

GRS Version 3– 16 September 2011

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Publications