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# **Epidemiological studies to inform control strategies for paratuberculosis in farmed deer**

A thesis presented  
in partial fulfilment of the requirements  
for the degree of Doctor of Philosophy  
at Massey University

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# Abstract

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Paratuberculosis, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), occurs in a range of ruminant species, and has been diagnosed in wild and domesticated deer worldwide. The disease process in other ruminants is chronic and fatal, with the highest clinical disease incidence generally seen in older animals. However, in farmed deer, disease incidence is highest in young animals, occurring as an acute syndrome in deer as young as eight months of age. The deer industry in New Zealand is concerned about the on-farm impact of paratuberculosis, and the consequences for the venison market should MAP be classified as a zoonosis. Research is thus directed at investigating tools for paratuberculosis control, to reduce the threat to the industry.

The aim of the research presented in this thesis was to provide epidemiological evidence that can be used to inform strategy, at industry and farm-level, for control of paratuberculosis in deer.

A survey of the deer slaughter population established a baseline prevalence of MAP infection, against which the effects of control initiatives can be measured. Infection was widespread in individuals (45%) and herds (59%), suggesting control rather than eradication as the goal of any industry programme.

On-farm disease control was investigated in a randomised controlled trial of vaccine efficacy in young naturally-infected deer. Vaccination reduced the incidence of clinical disease and subclinical pathology; no significant effect on mean production parameters was seen. There was no effect of vaccination on faecal MAP excretion, indicating vaccination may not reduce infection prevalence. Vaccinated deer had an increased risk of testing positively to diagnostic screening tests for bovine tuberculosis. Non-specificity was resolved by ancillary testing, but such tests come at an increased financial and test sensitivity cost.

Paratuberculosis control at the industry level may involve schemes to classify herd infection status. For this purpose, the sensitivity and specificity of individual faecal culture and

an IgG1 ELISA (Paralisa) to detect young deer infected with MAP was estimated using Bayesian latent class analysis. Paralisa and faecal culture had sensitivity of 19% and 77%, and specificity of 94% and 99%, respectively. Improved diagnostics are therefore needed if herd infection status is to be classified in a sensitive, specific, cost-effective and timely system.

The studies contribute to knowledge on different aspects of paratuberculosis control in the New Zealand farmed deer population, providing an evidence base for informed decision-making at farm and industry level.

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# Acknowledgements

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I arrived at Massey University with high hopes of expanding and enhancing my epidemiological skills while doing useful applied animal health research. On both counts my expectations were met in full and I sincerely thank my three supervisors Peter Wilson, Cord Heuer and Colin Mackintosh for guiding me, and for being so generous with their time and in sharing their knowledge and expertise. I particularly appreciated the diversity of opinion that would come out of our group discussions and felt I benefited hugely from the different perspective that each of you brought to scientific debate.

It has been a privilege to work with and for New Zealand deer farmers in some of the most beautiful places on earth, and I thank all who gave freely of their time, labour and patience during the vaccine trial. Thanks, too, to the vets who helped with recruitment and on-farm diagnosis. Noel Beatson, especially, put a huge personal effort into the field work of the trial, giving this deer-farming novice the benefit of his experience and knowledge of deer and (even more importantly) deer farmers. Thanks also to Geoff de Lisle and Gary Yates at Wallaceville for advice and diagnostic support that helped me get the best results from the projects and to all at DRL, Otago University for getting me started on PCR methodology.

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Finally, I thank the most important people- my parents and partner, David, who have supported me in every way in my various endeavours. To the relief of you all, my student days are finally now over.

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# Nomenclature

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AHB	Animal Health Board
CCT	Comparative cervical test
CI	Confidence/credible interval
DINZ	Deer Industry New Zealand
ETB	ELISA for bovine tuberculosis
GLM	Generalised linear model
IFC	Individual faecal culture
JD	Johne's disease
JML	Johne's Management Limited
LSS	Lesion severity score
MAP	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>
MCMC	Markov chain Monte Carlo
MCT	Mid-cervical test
OD	Optical density
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
TB	Tuberculosis



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## List of Publications

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**Goodwin-Ray K, Stringer LA, Wilson PR, Heuer C, Glossop JC.** Vaccination of deer for Johne's disease with Silirum: Safety, dose and tuberculosis test cross - reactivity. In: *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. Pp 44-9, 2008.

**Stringer LA, Heuer C, Mackintosh CG, Wilson PR.** Silirum vaccine in the control of Johne's disease in young farmed deer, preliminary results from a field efficacy trial. In: *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. Pp 57-61, 2009.

**Stringer LA, Wilson PR, Heuer C, Mackintosh CG, Glossop JC, Verdugo C.** Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from grossly normal mesenteric lymph nodes of slaughter deer, preliminary results from a nationwide prevalence study. In: *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. Pp 67-71, 2009.

**Wilson PR, Mackintosh CG, Heuer C, Hunnam JC, Stringer LA.** Paratuberculosis in deer: what we know, what we think we know and what we don't know. In: *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. Pp 45-50, 2009.

**Stringer LA, Wilson PR, Heuer C, Mackintosh CG.** The effect of Silirum vaccine on subclinical Johne's Disease in young farmed deer (*Cervus elpahus*) - preliminary results from a randomised controlled trial. In: *Proceedings of a Deer Course for Veterinarians, Deer Branch of the New Zealand Veterinary Association*. Pp 53-5, 2010.



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# Contents

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<b>Abstract</b>	<b>iii</b>
<b>Acknowledgements</b>	<b>v</b>
<b>Nomenclature</b>	<b>vii</b>
<b>List of Publications</b>	<b>ix</b>
<b>Preface</b>	<b>xix</b>
<b>1 Introduction</b>	<b>1</b>
1.1 The New Zealand Deer Industry . . . . .	1
1.2 Paratuberculosis in deer . . . . .	2
1.3 Thesis aim and structure . . . . .	4
<b>2 Literature review</b>	<b>7</b>
2.1 Introduction . . . . .	7
2.2 Transmission and pathogenesis . . . . .	8
2.3 Control measures . . . . .	11
2.3.1 Test and cull . . . . .	11
2.3.2 Management interventions . . . . .	16
2.3.3 Vaccination . . . . .	24
2.4 Effectiveness of control measures . . . . .	26
2.4.1 Test-and-cull and management interventions . . . . .	26
2.4.2 Vaccination . . . . .	30
2.5 Cost-effectiveness of control measures . . . . .	34
2.5.1 Cattle . . . . .	34
2.5.2 Sheep . . . . .	36

2.5.3	Deer . . . . .	36
2.6	Conclusions . . . . .	36
<b>3</b>	<b>MAP prevalence study</b>	<b>39</b>
3.1	Abstract . . . . .	39
3.2	Introduction . . . . .	40
3.3	Materials and methods . . . . .	41
3.4	Results . . . . .	43
3.5	Discussion . . . . .	46
3.6	Acknowledgements . . . . .	50
<b>4</b>	<b>Vaccine efficacy trial</b>	<b>53</b>
4.1	Abstract . . . . .	53
4.2	Introduction . . . . .	54
4.3	Materials and Methods . . . . .	55
4.4	Results . . . . .	60
4.5	Discussion . . . . .	67
4.6	Acknowledgements . . . . .	71
<b>5</b>	<b>TB test reactivity</b>	<b>73</b>
5.1	Abstract . . . . .	73
5.2	Introduction . . . . .	74
5.3	Materials and Methods . . . . .	76
5.3.1	Study 1: Effect of vaccination on tests for bovine TB . . . . .	76
5.3.2	Study 2: Risk factors for MCT reactivity . . . . .	78
5.4	Results . . . . .	80
5.4.1	Study 1 . . . . .	80
5.4.2	Study 2 . . . . .	81
5.5	Discussion . . . . .	84
5.6	Acknowledgements . . . . .	88
<b>6</b>	<b>Test validation study</b>	<b>91</b>
6.1	Abstract . . . . .	91
6.2	Introduction . . . . .	92

	<b>xiii</b>
6.3 Materials and Methods . . . . .	94
6.4 Results . . . . .	99
6.5 Discussion . . . . .	102
6.6 Acknowledgements . . . . .	107
<b>7 General Discussion</b>	<b>109</b>
7.1 Introduction . . . . .	109
7.2 Review of the individual epidemiological studies . . . . .	110
7.3 Implication of thesis findings for paratuberculosis control . . . . .	120
7.4 Future work . . . . .	123
7.5 Conclusion . . . . .	125
<b>Bibliography</b>	<b>127</b>
<b>A Appendix 1: WinBUGS Bayesian model code</b>	<b>A-1</b>



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# List of Figures

---

4.1	Boxplot of average daily liveweight gain of vaccine trial deer . . . . .	63
4.2	Proportion of vaccinates and controls shedding MAP in faeces . . . . .	64
4.3	Kaplan-Meier survival function of time to slaughter . . . . .	66
4.4	Proportion of carcasses with gross lymph node pathology . . . . .	67
5.1	Spatial distribution of TB reactivity study herds . . . . .	79
5.2	Study 1: TB test results . . . . .	80
6.1	Spatial distribution of test validation study herds . . . . .	95
6.2	Distribution of test positive deer . . . . .	100
6.3	Prior and posterior distributions from latent class analysis . . . . .	101



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## List of Tables

---

3.1	Isolation of MAP from normal mesenteric lymph nodes . . . . .	44
3.2	Univariate associations between MAP infection and predictor variables .	45
3.3	Logistic regression model output . . . . .	46
4.1	Summary of statistical methods used to assess vaccine efficacy . . . . .	60
4.2	Clinical disease incidence in vaccinates and controls . . . . .	62
5.1	Herd-level MCT and MAP results . . . . .	81
5.2	Univariate associations between risk factors and MCT reactivity . . . .	83
5.3	Logistic regression model output . . . . .	84
6.1	Prior information for Bayesian analysis . . . . .	98
6.2	IFC and Paralisa positive deer by herd and island . . . . .	99
6.3	Posterior estimates of test sensitivity and specificity . . . . .	102



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# Preface

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There is a theory which states that if ever anybody discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.

**Douglas Adams**



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## Introduction

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Epidemiology provides the evidence base for successful biological and cost-effective control of disease in farmed livestock populations. This thesis presents a series of epidemiological studies related to different aspects of control of paratuberculosis in farmed deer in New Zealand.

### 1.1 The New Zealand Deer Industry

The deer industry in New Zealand is relatively young, having formed in the early 1970s when feral deer, originally introduced to the wild from England and Scotland, were captured and domesticated (Pollard 1993). The industry has since developed to include breeding, venison and velvet production sectors and is an important contributor to the economy, generating export earnings of over NZ\$380m in 2009 (DINZ 2010). The number of deer herds in New Zealand is estimated at 3,400 and animal numbers at around 1.7 million. Approximately 40% of the farmed deer population is in the North Island of New Zealand, with 60% in the South Island.

Red deer (*Cervus elaphus*) is the predominant breed in New Zealand, representing 85% of the national herd. Red deer hinds are often crossbred with wapiti (*Cervus elaphus canadensis*) for faster growth and hybrid vigour, particularly for commercial venison production, and there is also a small population of farmed fallow deer.

Deer in New Zealand are grazed outside all year round, and on lowland deer farms are often grazed intensively on improved pasture. They are generally not the only livestock species within a farming enterprise; often sheep and cattle are also managed on the same farm, and they may be co-grazed with deer. Approximately 85% of deer enterprises also farm other species (Wilson 2007). Breeding hinds calve in November - December each

year, and as the risk of dystocia is low (0.5%) (Audige et al 2001), there is generally minimal management at this time. Calves stay with their dams until 4 to 5 months of age. Weaning may take place before the rut, but on some enterprises post-rut weaning is carried out. The venison production cycle is short, with the best-performing calves, born in early summer, reaching slaughter weights (>92kg) as early as the following spring, i.e. within 10 months.

New Zealand is a major supplier of venison globally. Eighty per cent of local production is exported to Europe, and the US is another important market at around 12%. Velvet is sold mainly to Asian markets, primarily China and South Korea, where it is utilised in traditional medicine. However, using velvet as a nutritional supplement has also been receiving increasing interest in Western cultures around the world, so there may be potential to further increase export earnings.

The deer industry has generally been progressive in funding and applying research directed at improving welfare, health and production of farmed deer. Research findings in these areas have been incorporated into on-farm quality assurance programmes, accreditation schemes and codes of conduct. In the last number of years, one disease that has had an increasing profile within the industry is paratuberculosis, or Johnes disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The key features of paratuberculosis in deer, and the implications for individual farms and for the industry are outlined below.

## 1.2 Paratuberculosis in deer

Paratuberculosis has been diagnosed in farmed deer in a number of countries including Argentina (Moreira et al 1999), Ireland (Power et al 1993), the UK (Fawcett et al 1995) and the USA (Manning et al 1998). The first clinical diagnosis in farmed red deer in New Zealand was in 1979 (Gumbrell 1986). The term paratuberculosis is applied generally to infection or disease caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). MAP-infected animals may have an inapparent infection, including a state in which the animal may not be infectious, or they may progress to subclinical or clinical disease. The disease process is essentially a granulomatous enteropathy, with inflammation of the small intestine leading to the clinical signs of wasting and diarrhoea and resulting ultimately in

death from cachexia. Clinical disease in cattle and sheep is generally a chronic progressive condition, affecting older animals at a low annual incidence risk (1% to 2%), and the clinical syndrome in adult deer may follow this pattern. However, the key difference between disease in deer and in other ruminant species is that deer as young as 8 months of age may be affected (Mackintosh et al 2004). Symptoms of weight loss and diarrhoea can progress to emaciation and death within a few weeks, and there have been reports of outbreaks of clinical disease in weaner deer with a mortality rate of up to 22% (Glossop et al 2008, Mackintosh et al 2004).

Affected farms may suffer economic losses associated with clinical and possibly subclinical disease, as well as loss of genetic potential (Bell 2005). The granulomatous visceral lymph node lesions associated with MAP infection are grossly similar to those caused by *M. bovis* and *M. avium*, causing difficulty for the meat industry at post mortem abattoir inspection (Campbell 1995). Affected carcasses are detained, pending laboratory investigation to exclude *M. bovis* infection as a diagnosis, incurring costs for the processor, farmer and the TB control scheme.

Although no structured herd prevalence survey has been completed, there have been indications, from anecdotal reports and from laboratory surveillance (de Lisle et al 2003, Griffin et al 2006), that the prevalence of MAP-infected New Zealand deer herds is increasing. To monitor prevalence trends, an industry-led national-level surveillance system for paratuberculosis in deer (Lynch 2007) was developed. The system collects data from each deer carcass processed at deer slaughter premises (DSP), making use of the gross pathology, typical of MAP infection, seen at post-mortem inspection of visceral lymph nodes of deer. This pathology, i.e. enlarged and/or granulomatous visceral lymph nodes is highly predictive (95%) of MAP infection (J.C Hunnam, unpublished data).

However, the significance of paratuberculosis to the deer industry has the potential to reach beyond on-farm and processing losses. The hypothesis that MAP is the causal agent of Crohns disease, a chronic inflammatory bowel condition of humans, has been under investigation for a number of years (Feller et al 2007). If public health concerns are substantiated, this may have major implications for ruminant meat processing and trade, and consequently for the whole deer industry.

Progress in on-farm control of paratuberculosis in deer is hampered by incomplete knowledge of the epidemiology of the disease, limited data on the characteristics of diagnostic

tests in the live animal and lack of information on the effectiveness and cost-effectiveness of control measures that could be applied practically in deer farming systems.

### 1.3 Thesis aim and structure

The aim of this thesis is to provide epidemiological evidence that can be used to inform strategy, at industry and farm-level, for control of disease and infection due to *Mycobacterium avium* subsp. *paratuberculosis* in deer.

Four field epidemiological studies were conducted to achieve this aim. The studies are presented in the thesis in the form of manuscripts prepared for publication in peer-reviewed journals. There is thus some repetition of background information, and presentation has been influenced by journal formatting style, and comments from co-authors, reviewers and editors. However, the reference style has been standardised throughout the thesis as that of the New Zealand Veterinary Journal.

Chapter 2 is a review of the published evidence that forms the basis of current herd-level control measures for paratuberculosis in farmed livestock, and how these may be applied to farmed deer. Evidence of biological effectiveness and cost-effectiveness of specific control strategies, in deer and other ruminant species, is critiqued with reference to the desired outcomes of control. For example, control measures may be directed at reduction of clinical disease or at reducing infection prevalence. The review concludes with a summary of the key areas where more evidence is needed to validate the use of specific control strategies in deer farming systems.

The first manuscript (Chapter 3) is a nationwide cross-sectional prevalence study undertaken with two main objectives: to estimate the prevalence of MAP infection in sub-clinically normal slaughtered deer, and to provide data to assess the significance of gross lymph node pathology as a predictor of MAP infection. The study provides baseline estimates which may be used by the industry when considering national-level control options, or to monitor the impact of interventions.

Chapter 4 presents a randomised controlled trial to assess the efficacy of a candidate vaccine in naturally infected young farmed deer. This field study was conducted in six commercial venison production herds in the South Island of New Zealand and involved 3335 weaner deer. The primary outcome was incidence of clinical disease, although other

outcomes such as liveweight gain, faecal MAP shedding and gross lymph node pathology were also analysed.

Chapter 5 reports a cohort study to assess the potential impact of vaccination on diagnostic tests for bovine tuberculosis (TB) in deer in the same population as the Chapter 4 study. Additional data from a previous case control study of risk factors for clinical disease were used to quantify the effect that MAP infection *per se* has on the specificity of diagnostic tests for TB. An additional study objective was to generate hypotheses on other potential risk factors affecting TB test specificity in deer.

The fourth manuscript (Chapter 6) presents the results of a cross-sectional study designed to estimate the characteristics of two diagnostic tests, individual faecal culture and an IgG1ELISA test, to detect MAP infection in young deer. There is no gold standard test available to detect MAP infection in live deer, therefore Bayesian latent class analysis was applied to estimate the sensitivity and specificity of each test, using a zero-inflated random effect logistic regression model.

The thesis concludes in Chapter 7, which considers the findings from the field studies in the context of current knowledge and in the wider perspective of disease control. The chapter includes an examination of experimental design and analysis, reviews the study limitations and offers suggestions on how the work could have been enhanced and for the direction of further research. Finally, the contribution the thesis has made to understanding key aspects of control of paratuberculosis in deer is discussed.



# **Review of the evidence for the effectiveness and cost-effectiveness of herd-level control measures for paratuberculosis of potential relevance to farmed deer**

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## **2.1 Introduction**

Eradication may be defined as the “regional extinction of an infectious agent” (Thrusfield 1995). Eradication of *Mycobacterium avium* subsp. *paratuberculosis*, by this definition, has not been considered an achievable aim in countries in which paratuberculosis is prevalent in domestic livestock. The epidemiology of MAP infection is not fully understood, the organism can persist for a long time in the environment (Whittington et al 2004) and is present in wildlife populations (Pavlik et al 2000, Beard et al 2001). Additionally, there is a lack of highly sensitive diagnostic tools to detect infection and limited practical interventions to reduce MAP transmission in all species. All of these factors mean that eradication of MAP is not a goal for the majority of international programmes.

Disease control, on the other hand, may be described generally as “any effort directed toward reducing the frequency of existing disease to levels biologically and/or economically justifiable or otherwise of little consequence” (Martin et al 1987). In considering herd-level control of paratuberculosis in particular, there are a number of specific end-points or outcomes that may be the aim of control. A control strategy may be applied, for example, to:

- Reduce clinical disease incidence
- Reduce infection prevalence
- Minimise subclinical losses
- Preserve genetic potential
- Minimise risk of infected or test-positive sale animals

Although voluntary herd-level control programmes are often assumed to be driven by economic cost-effectiveness, there are outcomes other than profit maximisation that influence decision-making by farmers (Edwards-Jones 2006). Lifestyle, cultural and social goals and values (Gasson 1973) contribute to outcomes for individual farmers, and attitudes to animal welfare are often particularly relevant when considering animal health measures. Although recognised as important, further consideration of these outcomes is beyond the scope of this review.

Whatever the specific aim of herd-level control, the measures applied on-farm are directed at reducing transmission of MAP to uninfected individuals using three main approaches: (1) identifying and removing infected or infectious animals (test-and-cull) (2) implementing specific management practices to reduce transmission or minimise the impact of disease and (3) vaccination.

This chapter reviews the evidence forming the basis of each element of these approaches for control of paratuberculosis in farmed livestock, and how they may be applied to farmed deer. The evidence underlying each approach is first reviewed, including pathogenesis where this explains the basis of the measure. The published epidemiological evidence of effectiveness and cost-effectiveness of the outlined interventions is subsequently examined, with reference to the main target outcomes of herd-level control where possible.

## **2.2 Transmission and pathogenesis**

Knowledge of transmission and pathogenesis is needed to identify the measures that may be effective in controlling infection and disease. An initial brief overview of transmission and pathogenesis of paratuberculosis is given in this section. The published evidence is discussed in more detail in the individual control measure sections, directly related to

specific aspects of control.

Transmission of MAP is thought to occur primarily by the faecal-oral route from contaminated pasture, feedstuffs, soil, water or during suckling (Whittington and Sergeant 2001). Bacteria are shed in faeces, and although excretion is greatest in the clinical phase of disease, shedding also occurs while the animal is subclinically affected (Harris and Barletta 2001). Infection can thus be spreading in a population before obvious clinical signs become evident. Passive shedding, in which ingested bacteria are shed in the faeces without infecting the host may be important in environmental contamination and has been reported in sheep, goats, cattle and deer (Whittington and Sergeant 2001). Although MAP is a facultative intracellular pathogen, growing and multiplying only within macrophages of susceptible hosts, it can remain viable for long periods in a range of environmental conditions. Survival within macrophages is thought to be related to the resistance of the mycobacterial cell wall to destruction or penetration and to factors that neutralise the antibacterial chemicals within macrophages (Collins 2003).

The organism has been recovered from the milk of cattle (Streeter et al 1995), sheep (Lambeth et al 2004), the mammary lymph node of deer (Thompson et al 2007), and from the semen of bulls (Larsen and Kopecky 1970) and rams (Eppleston and Whittington 2001). In-utero infection has been demonstrated in cattle (Seitz 1989), sheep (Lambeth et al 2004), goats (de Juan 2005) and deer (van Kooten et al 2006). Although these are all clearly potential transmission routes, the relative importance of each has not been established.

MAP has been isolated from a wide range of ruminant and non-ruminant wildlife associated with clinically affected farms (Beard et al 2001). Studies in Scotland have found a positive correlation between infection in the wild rabbit (*Oryctolagus cuniculus*) and domestic livestock on clinically affected farms (Greig et al 1999). Long-term persistence of infection in the rabbit population (Judge et al 2007), the high (17%) prevalence of infection in rabbits and the infectivity and quantity of rabbit faeces deposited on pasture suggest that rabbits may be important in maintaining infection in the environment (Daniels et al 2003). In New Zealand, MAP has been isolated from the tissues of hedgehogs, rabbits, possums, cats, hares, ferrets and birds and from faeces of rabbits and hedgehogs (Nugent et al 2007). However, the relative importance of wildlife in transmission of MAP to domestic livestock is not fully understood.

Pathogenesis has been studied most closely in cattle but is believed to be broadly similar in different ruminant species. Following ingestion, bacteria selectively adhere to M-cells overlying Peyer patches in the small intestine and penetrate the mucosa, where they are phagocytosed by macrophages (Momotani et al 1988). There they survive and replicate, and the inflammatory changes result in cell infiltration of the intestinal mucosa, decreased absorption and ultimately a protein-losing enteropathy leading to death of the animal. It is thought that the low concentration of T-cells within the ileal Peyer lymphoid tissue and thus the limited cellular immune response may favour survival of the mycobacteria at this location (Clarke 1997).

Histopathological changes may take a multibacillary or lepromatous form with diffuse large numbers of macrophages and epithelioid cells and acid-fast bacteria, or a paucibacillary or tuberculoid form in which there is a predominantly lymphocytic reaction and few or no acid-fast bacteria visible by light microscopy (Clarke 1997). Culture or PCR techniques can, however, demonstrate the organism in such lesions (Whittington and Sergeant 2001). The variation in response is thought to be associated with host immunity, with the CMI response predominating in animals with paucibacillary lesions, and a stronger humoral response and weaker cellular immunity in those with multibacillary lesions (Clarke 1997). The cell-mediated immune response is thus considered an important determinant of the outcome of MAP infections, with IFN- $\gamma$  the major cytokine involved in macrophage activation and resistance to Map (Reddacliff et al 2005). The inflammatory changes also involve the lymph nodes draining the intestine and give rise to the thickened, corrugated intestine, enlarged and oedematous mesenteric lymph nodes and dilated serosal lymphatic vessels seen at post-mortem of clinically affected cattle. Species differences have, however, been recognised in the gross pathological features of Johne's disease. In sheep gross intestinal lesions may be mild, and thickening of the intestinal wall is not always a feature. In deer, there may be no visible thickening of the ileum, but thickened lymphatic drainage vessels and enlarged mesenteric lymph nodes are a common feature. A distinctive gross finding is caseous necrosis within the jejunal and ileo-caecal or retropharyngeal lymph nodes of infected deer, causing difficulty at routine meat inspection due to a close resemblance both grossly and microscopically to lesions of tuberculosis (Mackintosh et al 2004).

The key clinical features of paratuberculosis - diarrhoea and weight loss - thus occur as

a result of protein malabsorption and loss. In cattle and sheep, a long incubation period means clinical disease is chronic and progressive and seen only in adult animals. Although disease is seen in adult deer, clinical signs may be seen in deer as young as 8 months of age, with rapid progression to death in a period of weeks (Mackintosh et al 2004). However, the factors that influence progression from infection to clinical disease are not fully understood in any species. The evidence underlying the role of host susceptibility and organism pathogenicity in infection and disease are discussed in more detail, in relation to control of paratuberculosis, in the following section.

## 2.3 Control measures

### 2.3.1 Test and cull

Herd-level test-and-cull programmes are directed at identifying and removing infected or infectious animals, thus reducing potential sources of infection for other susceptible livestock. This applies whether the overall aim of control is to reduce clinical disease incidence, reduce infection prevalence or to minimise sub-clinical losses. The term ‘paratuberculosis’ is applied in the literature to a range of conditions including infected, infectious, or clinically diseased. When assessing the performance of diagnostic tests for use in test and cull programmes, it is important to be clear which of these is the target condition, as the sensitivity of diagnostic tests varies with the stage of infection and thus the target condition (Nielsen and Toft 2008).

Dependent on the aim of control, specific objectives of test and cull may include identifying animals likely to progress to clinical disease, identifying animals that are infected or infectious, or identifying animals that excrete a high concentration (e.g. 1000 cfu/g) of MAP in faeces. Clinically affected animals are likely to be shedding the organism in faeces (Whitlock and Buergelt 1996) and are a source of transmission via the intrauterine route (Whittington and Windsor 2009), so removing them from the herd will remove one identifiable source of infection. However, identifying sub-clinically infected but infectious animals is more challenging. Such animals may be shedding MAP before they are detectable by diagnostic tests (Collins 2003) and they thus represent one of the major limitations to the success of test-and-cull strategies that aim to reduce prevalence of infection.

The diagnostic tests in current use in the live animal are based either on detection of the agent, the cell-mediated immune (CMI) response, or the humoral immune response, and are considered in general below. The section concludes with a review of diagnostic tests specific to deer.

### **Detection of MAP**

Isolation of the organism has been used as a diagnostic tool for almost 100 years. Recent advancements have included the use of radiometric culture and liquid culture systems (Collins et al 1990) to detect lower numbers of bacteria more rapidly and these techniques have also increased the sensitivity of detection of the slower-growing ovine-strain of MAP (Whittington 2010).

Intestinal tissue culture is regarded as the earliest and most sensitive means of detecting MAP infection, as it may identify non-shedders and animals with paucibacillary intestinal lesions (Perez et al 1996). Tissue culture is generally limited to necropsy specimens, but biopsy in the live animal is possible and has been used as a diagnostic technique in cattle (Pemberton 1979) and as a research tool (Mackintosh et al 2010a). However, biopsy does not have a practical application in screening programmes, so faecal culture or PCR methods are those generally applied to detect the organism in the live animal.

Sensitivity of faecal culture varies with factors such as age, culture technique and strain of organism. In dairy cattle, for example, the sensitivity of faecal culture has been shown to increase with age and lactational stress (Norton et al 2010). Liquid culture methods, such as BACTEC are more sensitive than solid culture to detect MAP, but modified BACTEC 12B is the only liquid culture medium that supports growth of all common MAP strains (Whittington 2010). Estimates of the sensitivity of faecal culture by all methods range from 23% to detect infected cattle, to 74% to detect infectious cattle (Nielsen and Toft 2008).

PCR techniques were initially considered poorly sensitive in biological samples (Grant et al 1998), but recent advances in methodology mean they are now considered to be possibly as sensitive as culture (Bolske and Herthnek 2010). PCR may be routinely applied to milk, tissue or faecal samples, and has the advantage of rapid turnaround time and MAP cell enumeration using real-time methods. It may therefore have particular application in identifying heavily shedding animals. In contrast to culture methods, there is no loss of

sensitivity when frozen faecal samples are tested (Khare et al 2008).

### **Detection of the cell-mediated immune response**

The cell-mediated immune (CMI) response is considered to be a key determinant of immunity to all mycobacterial infections, with the humoral response giving little or no protection (Chiodini 1996, Stabel 2000). The CMI response occurs early in infection (Stabel 2000), therefore tests to detect it have the greatest potential to detect infected animals before shedding of MAP occurs.

The intradermal skin test, using avian or johnin purified protein derivative (PPD), has been used in cattle to detect CMI response to the injected antigen. Assays to detect gamma-interferon (IFN- $\gamma$ ) release following antigen stimulation of whole blood have also been developed (Billmanjacobe et al 1992). There has been some discussion in the literature about the value of using IFN- $\gamma$ . Some authors (Jungersen et al 2002) advocate that in young animals it is a measure of exposure rather than infection, and should be used to assess the effect of control interventions, rather than to identify candidates for culling. The positive relationship in sheep between a strong CMI response and reduced intestinal pathology (Burrells et al 1998, Gwozdz et al 2000), and evidence of a correlation between negative IFN- $\gamma$  test and higher ELISA OD values in cattle (Mikkelsen et al 2009), give support to that suggestion. However, others assert that in an infected herd or flock, identifying and removing exposed and thus potentially infected animals will ultimately reduce MAP transmission and assist control (Bosward et al 2010). The value of using IFN- $\gamma$  therefore, may be dependent on the specific aim of diagnosis and its use may be indicated more in infection eradication than disease control programmes.

### **Detection of the humoral immune response**

While serum antibody may be detected by complement fixation (CF) or agar-gel immunodiffusion (AGID) techniques, the indirect antibody enzyme-linked immunosorbent assay (ELISA) is the most widely used test for detection of the humoral immune response to MAP in serum or milk, and a variety of ELISA tests have been developed for use in different ruminant species (Nielsen and Toft 2008). ELISA tests may be affected by the variability of the immune response of the individual, the stage of disease and the type of histopathological lesion i.e. paucibacillary or multi-bacillary (Clarke and Little 1996).

They have low (5-30%) sensitivity for detection of MAP-infected animals (Nielsen 2010). However, they have the advantage that they may detect infected animals before they become infectious, as antibody may be detectable before shedding of MAP; an important feature when the aim of test and cull is reduction of infection prevalence. Sensitivity of ELISA tests to detect infected cattle increases with increasing age, but sensitivity to detect infectious cattle does not appear to be age-dependent (Nielsen and Toft 2006). ELISA tests have the advantage that results can be obtained rapidly and relatively inexpensively and they are generally considered to have good sensitivity (75%) for detection of animals shedding high levels of MAP in faeces, although are less sensitive (15%) at detecting low shedders (Whitlock et al 2000).

In a recent comprehensive review of ante-mortem diagnosis of MAP, Nielsen and Toft (2008) summarised and critically reviewed ELISA and other diagnostic tests in a range of species. Target conditions were classified as 'infected', 'infectious' and 'affected', with 'affected' defined as clinically diseased or showing reduced production performance. The review identified many reports of ELISA test performance in the literature, but poor reporting of target condition and study populations. The authors concluded that for all species there was a "profound lack of reliable test evaluations".

### **Diagnostic tests relevant to deer**

Limited information is available on the characteristics of diagnostic tests in deer. A study to evaluate a modified ELISA and individual faecal culture in deer (Schroen et al 2003) reported that IFC detected 67.5% (112/166) of deer with confirmed JD. However, the case definition of 'confirmed' appears to include faecal and tissue culture and histopathological examination and this figure is thus not a sensitivity estimate. When assessed against tissue culture as a gold standard, IFC detected 47% (44/93) of tissue culture positive deer, but identified six further tissue culture negative deer as MAP infected. However, faecal samples were frozen prior to culture, which may have reduced the subsequent recovery of bacteria (Richards 1981).

The same study found maximum sensitivity of a deer-conjugate ELISA was 51% when specificity was 59%, while maximum specificity was 99.5% when sensitivity was 36%. However, only 110 of the 172 serum samples originated from deer that were tissue culture positive; the remainder were from animals classified as infected based on the results

of histopathology or faecal culture. The source of samples was recorded as “previous diagnostic submissions”, abattoirs and whole-herd on-farm testing. There were no data presented on the age of sampled deer, nor was there information on whether deer were clinically or sub-clinically affected for all analyses. All of these factors suggest that there is a possibility of bias towards samples from animals in more advanced stages of infection. This, together with the small sample size for test evaluation (172 “infected” animals and 210 “non-infected”) indicates that the results should be interpreted with caution.

An ELISA test used in farmed and wild deer populations in Spain (Reyes-Garcia et al 2008) appears to have been evaluated using PCR on MLN from 17 ELISA positive deer and “microbiological data” from nine ELISA negative deer and it is difficult from the information provided to assess the test accuracy.

The development and estimated performance of an IgG1 ELISA test, the Paralisa<sup>TM</sup>, for deer has been described by Griffin et al. (2005). Specificity of 99.5% and sensitivity of 85% to detect infected animals was reported when the two antigens under study (PPDj and PpAg) were used in series. However, the selection process for samples for analysis of test sensitivity was not fully described. Samples of serum (number not reported) from 10 farms with a history of clinical paratuberculosis were originally submitted for ELISA testing, and the infection status of the corresponding deer was established by tissue culture and histopathology. Preliminary estimates of test sensitivity were based on samples from 102 animals in which infection was confirmed. However, it is not clear whether the 102 selected comprised all confirmed cases or only a proportion, or whether selection bias may have been present, possibly influenced by knowledge of serum reactivity. There was no description of how the individual infection status of the controls or their source herds was established, other than that serum samples were sourced from herds with “no prior history or ongoing evidence of *M. paratuberculosis* infection”. Additionally, specificity estimation is described as using data from 508 “test-negative” animals. An estimate of sensitivity (77%) was given for detection of sub-clinically infected animals (n=250), using tissue culture status as the ‘gold standard’ reference. The ELISA cut point used for this part of the analysis was not stated. A figure of 90% for sensitivity of the assay to identify infected deer with detectable pathology was based on histopathological status, although 75/150 (50%) of the histologically positive panel were tissue culture negative. The estimate of sensitivity may thus be an overestimate, as histopathology is not perfectly

sensitive and specific.

Recent advances in the application of PCR methods to New Zealand deer samples include the development of a quantitative PCR method for faecal samples (O'Brien et al 2010). The technique has the potential to identify deer shedding high numbers of MAP organisms for culling and thus may have direct application to control programmes which aim to reduce clinical disease and infection prevalence.

There has been no formal evaluation of the performance of individual faecal or tissue culture to detect MAP infection in the New Zealand deer population. The sensitivity and specificity of the Paralisa has not been independently validated, and the performance of the test in young naturally infected deer has not been estimated. Data from an experimental infection study in young deer (Mackintosh et al 2007a) found 19/68 (28%) of sub-clinically infected tissue culture positive deer to be Paralisa positive. The proportion positive was related to disease severity: 20% of deer with no visible histopathology, or “very mild non-specific lesions” were Paralisa positive compared to 100% of the clinically affected deer.

The effectiveness and cost-effectiveness of test-and cull strategies as a control measure for paratuberculosis are examined in more detail later in this review.

### **2.3.2 Management interventions**

On-farm management control interventions are directed at reducing exposure of susceptible animals to infection, or to the factors that may precipitate progression from infection to disease. While identifying and removing infected and infectious individuals by testing and culling is one way of reducing transmission (Collins 2004), other control measures can be implemented, often with little financial cost. Management programmes for intensive systems, particularly for dairy stock, vary in detail and complexity. A typical strategy applying management principles is the “three-step calf plan” outlined for dairy herds in the Australian Bovine Johnes Disease (BJD) programme (Anonymous 2003). This advises removal of calves from dams within 12 hours of birth, separation from adults and their effluent, and not rearing calves up to 12 months of age on pastures that have had adult stock or stock that are known to carry BJD on them during the previous 12 months. The scientific evidence forming the basis of this type of advice is outlined below.

### Role of age resistance

Many management strategies, particularly for dairy cattle, are based on the principle that neonatal infection is an important factor in the development of clinical paratuberculosis. Young animals have long been considered to be more susceptible to infection following exposure to the organism via colostrum or via infected faecal material from the dam or environment (Manning and Collins 2010). Evidence for age resistance in cattle has been examined in a recent meta-analysis (Windsor and Whittington 2010), which concluded that there is a significant age-related difference in susceptibility to MAP infection and disease: calves less than 12 months old are more susceptible to infection than adults, and calves older than 6 months are less likely to develop clinical disease following exposure to MAP than younger calves. Age resistance is not absolute, though, and Rankin (1962) showed infection of adult cattle from a contaminated environment to be possible, with clinical disease developing in some animals.

In a review of pathogenesis in ruminants, Clarke (1997) suggested that the mechanism for age resistance is related to the large area of mucosal lymphoid tissue or Peyers patch present in young ruminants. The ileal patch has a low proportion of T-cells and so there is a limited cellular immune response, favouring survival of intracellular mycobacteria. Based on the work of Larsen (1975), Nisbet et al.(1962) and Reynolds and Morris (1983), Clarke proposed that “the progressive decrease in susceptibility to *M. a.* paratuberculosis with increasing age is related to the concomitant involution of the ileal patch and the disappearance of this favoured site for mycobacterial persistence”. Developing this work further, Chiodini (1996) proposed that the difference in clinical progression between calves and adult cattle could be explained by the difference in the particular T-cell population that promotes the cellular immune response. This T-cell population suppresses macrophage activation, and is larger in newborn calves than in adult cattle. Another suggested mechanism to explain age susceptibility is that the ‘open gut’ of the bovine neonate allows MAP to penetrate the mucosa in the first 24 hours of life (Sweeney 1996). Others propose that MAP concentrations may be affected by dilution or the biological effects of a functional rumen (Windsor and Whittington 2010).

Research by Sergeant (2005) has suggested that there is some age effect in sheep, possibly dependent on level of exposure, and showed that under conditions of heavy natural exposure adult sheep may be as susceptible as young stock. The findings of a recent age

resistance study in sheep (Delgado et al 2010) found 100% of lambs and 92% of adult sheep challenged with a high dose of MAP developed histological lesions which were different in each age group. Lambs had larger and more numerous granulomata and more acid-fast organisms in intestinal lymphoid tissue than adults. Immune responses also differed, with significantly higher antibody response and an earlier and higher CMI response found in adults. Although the study examined only experimental infection, the findings are consistent with the hypothesis that a strong and early CMI response limits disease progression. The difference in this response between adult sheep and lambs may thus be the underlying basis of age resistance to disease in sheep.

An age susceptibility study in deer (Mackintosh et al 2010b) compared the incidence of clinical and subclinical disease in deer challenged with MAP as 3-month old weaners (n=30), yearlings (n=20) and adults (n=20). A 50-week observation period followed. Clinical disease (33%) occurred only in the group challenged as weaners, despite MAP infection being confirmed by mesenteric lymph node culture in 94% of weaners, all yearlings and 90% of adults. A similar pattern was seen in histopathology, with a higher lesion severity score (LSS), a measure of the degree of disease progression as described by Clark et al.(2010), seen in weaners (including clinical cases), than in yearlings or adults. A higher proportion of weaners (68%) were shedding MAP in faeces at week 24, compared to yearlings (32%) or adults (5%). A major limitation of the trial was the lack of a control group, and while deer were sourced from herds with no history of clinical disease or typical pathology and were sero-negative to the Paralisa test before the trial, this may be insufficient evidence to be confident that animals were not infected prior to the trial. Glossop et al. (2006), for example, observed approximately 28% of confirmed infected herds experienced no evidence of clinical disease. Furthermore, adult and weaner deer were sourced from a different farm than the yearlings, so there is the possibility of a herd effect, either genetic or from other exposures, confounding the results. The deer were observed for 50 weeks so, although it was shown that infected weaners are more likely to develop disease than older deer within that time span, the short time-scale of the trial means that an absolute resistance to disease in the older deer in the longer term was not demonstrated.

### Role of host genetic resistance

The role of host genetics in resistance or susceptibility to paratuberculosis has been investigated by a number of studies. The Channel Islands cattle breed in the UK has been associated with a higher incidence of clinical disease than other dairy breeds (Cetinkaya et al 1997). However, the finding came from an observational study, in which it was difficult to separate breed from herd effects. In Dutch dairy cattle (Koets et al 2000) and US Holsteins (Gonda et al 2006), evidence of genetic variation in susceptibility to MAP infection has been demonstrated, although heritability estimates were low (0.06 and 0.1 respectively).

A study in naturally infected Merino sheep in Australia (Reddacliff et al 2005) examined associations between specific host alleles (NRAMP and MHC) and disease susceptibility or resistance. Although the study was limited to 2 flocks, and involved small numbers of sheep (n=200), there were positive associations identified between specific genotypes and clinical disease as well as multibacillary pathology. Negative associations were also found between genotype and absence of infection and disease. The findings suggested that there may be a role for breeding for resistance in control of paratuberculosis in sheep. In a further study in naturally infected Merino sheep in Australia, Dukkipati et al. (2010) investigated immune responses to a killed MAP vaccine. Five specific genetic polymorphisms were found to be positively associated with IFN- $\gamma$  response to vaccination. If these genetic associations can also be demonstrated when the sheep are exposed to natural infection, the polymorphisms described may have a role as markers of resistance to disease.

In red deer, resistance to tuberculosis has been shown to have a genetic component and to be highly heritable (Mackintosh et al 2000). The prospect of a similar genetic component to paratuberculosis in deer led to a study comparing the course of disease in young deer bred from susceptible (S) and resistant (R) sires (Mackintosh et al 2010a). Nine calves from each sire type were orally challenged with MAP at 4 months of age and monitored for clinical disease and pathology at slaughter. Mesenteric lymph node biopsies were taken at week 4 and week 13 post-challenge and scored for degree of pathological change, measured by LSS. Preliminary results showed that the mean severity of histopathological lesions for offspring of S sires was significantly higher than that of the R sire progeny at necropsy. While histopathological lesions were seen in the jejunal lymph node of all 18

subjects at 13 weeks, the LSSs of the R sire progeny reduced between week 13 and week 49. One R progeny showed no signs of pathology or infection at week 49. Clinical disease was diagnosed in one of the R and two of the S progeny over the study period. The study was an important preliminary assessment of the possible effect of host resistance on the pathogenesis of paratuberculosis in deer. However, small numbers of deer (n=18) were involved, and were the progeny of only two sire stags. Longer-term studies in a wider range of naturally infected deer herds are needed to confirm the observations and to assess the role of host resistance as a viable and practical control measure in farmed deer.

### **Effect of MAP pathogenicity**

Molecular characterisation by a variety of methods has identified two major groups or strain types of *Mycobacterium avium* subsp. *paratuberculosis*. Type I (sheep or s-strain) has predominantly been isolated from sheep, and type II (cattle or c-strain), has been isolated from cattle and a range of wildlife species (Motiwala et al 2006). However, although a number of subtypes are recognised within the broad type I and II classification, the full nature of the relationship between organism genotype, virulence and host preference is not yet fully understood. Although there appears to be strain host preference, no absolute host specificity has been demonstrated. Type II isolates have been found in sheep and non-ruminant hosts as well as cattle in Europe (Stevenson et al 2002), while type I isolates have been identified in clinically diseased cattle in Australia and Iceland (Whittington et al 2001). Different strain subtypes have even been isolated from the same animal (Pavlik et al 1995).

Experimental infection of lambs with type I and II isolates (Verna et al 2007) found differences in immunopathological responses. Type I isolates induced the most severe pathology with multifocal diffuse intestinal lesions, while small granulomatous focal lesions, mainly in lymph nodes, resulted from challenge with the type II isolate. Only one type I isolate was investigated, but differences in virulence and immune response were seen between the subtypes of the type II isolates.

An in vitro study (Gollnick et al 2007) investigated the cytotoxicity and intracellular survival in bovine macrophages of 4 different MAP subtypes of both type I and type II isolates. Differences in survival and phagocytosis were found between the sub-types, suggesting there may be an effect of MAP subtype on virulence. Examination of the im-

fact of different subtypes on pathogenesis in the host would, however, need further in vivo studies.

Red deer are susceptible to infection with both type I and type II strains of MAP, and both can cause clinical disease (de Lisle et al 1993). Type II isolates have been more commonly found than type I in deer (de Lisle et al 2006), being cultured from samples from 91/95 (96%) of herds with type I isolated from the remainder. However, these findings were based on diagnostic samples of TB-suspect granulomata submitted from abattoirs. A structured prevalence study to examine strain type distribution in deer is necessary before firm conclusions can be drawn, as it is possible that type II is simply more prevalent in TB-suspect pathological lesions.

In an experimental infection study in deer (O'Brien et al 2006), the type II isolate used had a higher infection rate and triggered a stronger CMI response in young red deer when compared with the same challenge dose of a type I isolate, suggesting that it was more pathogenic. However, no study examining the effect on pathogenicity of a wider range of molecular sub-types has been reported, and more research is needed before firm conclusions about pathogenicity can be drawn from the results. The same study found exclusively type II isolates in field clinical cases. The findings were based on a sample of 74 deer from 10 herds, which may not be representative of all deer herds, but they support the hypothesis of an association between type II isolates and clinical disease in deer.

With more research into the distribution of MAP isolates in deer and other species in New Zealand, and as advances are made in applying molecular sub-typing methods, it will be possible to investigate the relationships between MAP strain, host species and MAP pathogenicity in farmed deer. There is a wide range of typing methods now available, and increasing degrees of differentiation possible (Stevenson et al 2009). Applying the information at farm level to control MAP transmission or clinical disease in deer may mean, for example, avoiding direct or indirect contact between deer and potentially infected cattle, if doing that is shown to be effective and cost-effective for specific target outcomes of control.

### **Culling offspring of clinical cases**

Intrauterine transmission is recognised in cattle, with a meta-analysis (Whittington and Windsor 2009) concluding that approximately 9% of foetuses from sub-clinically affected

cows and 39% of foetuses from clinically affected cows were infected with MAP. Although there is no evidence currently available on the consequences of infection, e.g. whether calves infected *in utero* progress to disease, the authors recommended that offspring of clinically diseased and sub-clinically infected cows should be removed from the herd. This recommendation may be relevant where the aim of control is to reduce the prevalence of infection. However, more information is needed to assess the impact on clinical disease incidence and the biological and cost-effectiveness of the measure.

In a study of sheep from two heavily infected flocks in Australia, 5/5 clinically affected sheep were found to have tissue culture positive foetuses while 1/54 (1.9%) of foetuses from sub-clinically infected dams were MAP positive (Lambeth et al 2004). There is again no evidence to show the effect of *in utero* infection on the risk of developing clinical disease in sheep, but the authors recommended removing clinically affected ewes and their offspring from flocks with a high infection prevalence. The low foetal infection risk in sub-clinically infected ewes suggests that it is unlikely to be economically viable to identify such animals and remove their offspring.

Intrauterine infection also occurs in deer. In one field study, MAP was cultured from 9/10 foetuses from clinically affected hinds (van Kooten et al 2006), while in a study of foetuses from sub-clinically affected hinds, 14/18 (78%) were culture positive (Thompson et al 2007). The high risk of foetal infection may be one of the factors explaining rapid clinical progression in some deer, although longitudinal studies examining the outcome of foetal infection are required to test this hypothesis. Additionally, the relative importance of intrauterine infection as a transmission route has not been established. The impact of culling the offspring of clinical cases on the prevalence of disease or infection in the herd has not been formally assessed in deer, nor has the cost-effectiveness of the measure.

### **Reduction of stress**

There is a hypothesis that stress has an effect on disease susceptibility, due to the effect of immunosuppressive glucocorticoid release on immune function (Griffin and Thomson 1998). The hypothesis is supported by a field study of deer, in which a group subjected to increased social stress had lower T-lymphocyte reactivity than a control group (Hanlon et al 1995). Deer are exposed to stress during weaning, transport and social mixing, as well as when experiencing unfavourable nutritional and environmental conditions. However,

the effect of stress on the pathogenesis of paratuberculosis has not been quantified. While minimising stress is recognised as a tenet of good stock management, there are no data identifying which practices may have the greatest impact on disease progression or how they may be applied practically in deer.

### **Grazing management**

Although unable to grow and multiply in the environment, the highly impermeable thick waxy cell wall of MAP wall means that it can remain viable for long periods in a range of environmental conditions. Survival up to 55 weeks in shaded soil was reported in a study (Whittington et al 2004) that also found evidence suggestive of dormancy of the organism. Survival time was shortest (2 weeks) when faecal material and soil were fully exposed to sunlight and where vegetation was absent. MAP also survives well in biofilms on concrete and galvanised water troughs (Cook et al 2010), with recovery of the organism possible for up to 365 days.

A case-control study of risk factors for clinical JD in weaner deer identified an association between farmer-diagnosed disease and other species grazing on the deer-fenced area (Glossop et al 2007b). Grazing beef yearling cattle was positively associated, while grazing sheep was negatively associated with a disease prevalence of more than 0.4% in weaners. Analysis of data from the national surveillance database for deer and the database of agricultural premises (Agribase) found a similar effect. Deer farms which also had beef stock had a higher risk (RR 1.33, 95%CI:1.28-1.4) of such pathology, i.e. enlarged and/or granulomatous visceral lymph nodes, while deer farms with sheep had a reduced risk (RR 0.76, 95%CI:0.73-0.77) when compared to deer-only farms (Verdugo et al 2009). The nature of the data sources meant that the degree of contact or infection status of the beef or sheep stock was unknown, so further field work is needed to confirm these findings. However, there are possible biological explanations. Sheep graze close to the soil, so that any mycobacterial contamination on pasture is exposed to direct sunlight and shade has been shown to have a protective effect on the survival of type I MAP under Australian conditions (Whittington et al 2004), possibly due to the effects of temperature flux. The presence of sheep, therefore, may reduce the ability of MAP to survive on pasture. Another hypothesis is that exposure to a less pathogenic type I strain, commonly associated with sheep, may have a protective effect in deer.

A direct application of the above findings to deer management would involve young stock avoiding pasture previously used by MAP-infected livestock, or by cutting silage from such pasture and applying effective disinfection to water troughs. The specific impact of grazing management practices on disease or infection prevalence has not been assessed in deer or other species.

### **Depopulation**

Culling, or complete removal of all stock from an infected premises, accompanied by disinfection of buildings and equipment, and restocking with uninfected stock is one possible herd-level control method for paratuberculosis. In a study in sheep flocks in Australia (Taylor and Webster 2005), farms were depopulated of all susceptible stock for 15 to 21 months and restocked with sheep from flocks designated at low risk of MAP infection on the basis of a Market Assurance Programme standard. Within three years of restocking, 28/41 (68%) of monitored flocks were found to be infected. The study authors concluded that depopulation was neither biologically effective nor cost-effective as a measure to eradicate infection. The reasons for failure of the measure were reported as re-infection from neighbouring flocks (14/28) or from restocking with sheep from an infected source flock (4/28). The cause of the remaining 10 failures was not determined.

In contrast, culling all susceptible stock and maintaining a period of depopulation is the national policy applied in Swedish cattle herds when infection is identified (Sternberg et al 2003). This measure has been successful, with no new infections reported in cattle since 2005 (Sternberg et al 2007). However, it forms part of a national-level programme carried out in a country with a very low prevalence of infection which is not comparable to a situation in which disease is endemic.

### **2.3.3 Vaccination**

Vaccination as a control method for Johnes disease has been used worldwide since 1926 (Vallee and Rinjard 1926). Early vaccines were prepared from live attenuated MAP strains, generally suspended in mineral oil, but concerns surrounding shelf life, vaccinator safety and the possibility of environmental spread of live vaccine strains led to the development of vaccines containing killed bacteria (Emery and Whittington 2004).

Local granulomatous reactions are common at the injection site of oil-adjuvant vaccines

(Windsor and Eppleston 2006). Tissue reactions to early vaccines could be extensive, affecting market value of the carcass. Accidental self-inoculation by the vaccine administrator is a serious concern, as it can result in severe inflammation requiring surgical intervention (Richardson et al 2005, Windsor et al 2005). One of the long-recognised concerns in using vaccination in cattle has been the sensitisation of the animal to tests for bovine tuberculosis (TB) (Doyle 1964, Kohler et al 2001), due to the close antigenic similarity between mycobacterium species. This similarity, together with the stimulation of the CMI and humoral response by the oil adjuvant (Mackintosh et al 2005), may affect the specificity of TB tests. As a result, cattle vaccination has generally only been permitted in herds at low risk of TB (Wilesmith 1982). Conversely, it has been shown in cattle that intradermal tests for TB may interfere with diagnosis of Johnes disease by ELISA for up to 90 days (Varges et al 2009), and that TB infection itself may reduce the specificity of ELISA diagnosis (Lilenbaum et al 2009).

Subunit vaccines, prepared from immunodominant protein antigens, have potential to overcome the TB test cross-reactivity associated with whole-cell vaccines (Huygen et al 2010). They contain recombinant proteins, or DNA cocktails encoding specific antigens, which induce an immune response. Identification of suitable antigens has been aided by the sequencing of the MAP genome, which has been completed for the K10 strain, a bovine clinical isolate (Li et al 2005). Initial work on candidate antigens showed a reduced quantity of MAP in the spleen and liver of mice given a DNA vaccine and challenged with MAP, compared to unvaccinated challenged controls (Park et al 2008). Similarly, a goat challenge model demonstrated a significant CMI response and lower numbers of MAP bacteria in goats vaccinated with a multi-component subunit vaccine compared to unvaccinated controls (Kathaperumal et al 2009).

The development of novel live attenuated MAP strains suitable for vaccines is also in progress at a number of research centres. Research is directed at developing strains of MAP which are avirulent but which confer strong immunity. Although little of this work has been published to date, a study using macrophage, mouse and goat models to assess virulence of three candidate vaccine strains, developed using transposon mutagenesis, has been reported (Scandurra et al 2010). The study found different degrees of attenuation of some candidate strains in mouse and goat challenge models, suggesting that trials in specific target species will be a particularly important aspect of vaccine evaluation.

Evidence for the effectiveness of vaccination as a method of control in the main ruminant species and in deer is reviewed in the following section.

## **2.4 Effectiveness of control measures**

Ideally, the effectiveness of a specific control intervention should be assessed without changing any exposures in the study population, other than the intervention under investigation. However, epidemiologically robust longitudinal field studies to quantify the effect of control interventions can be complex to design, and are time-consuming and expensive. Consequently, published field studies assessing control interventions are often limited to ‘before and after’ descriptions of effect. Frequently, different interventions have been applied simultaneously. For example test-and-cull has been used along with calf management to reduce transmission of infection, making it difficult to separate the effects of individual interventions. For this reason, the evidence for the effectiveness of test-and-cull strategies is reviewed below alongside that for management interventions.

Simulation modelling is one method to assess or compare the effect of control strategies. A number of studies use this approach and some are reviewed here. Reduction of clinical disease is rarely an outcome in such models, most likely because there is little epidemiological information on the factors that influence progression from infection to clinical disease (Marce et al 2010). Nevertheless, where possible the effectiveness of each control intervention is reviewed with reference to specific aims of control.

### **2.4.1 Test-and-cull and management interventions**

#### **Field studies**

Early reports concluded that test-and-cull strategies could reduce the incidence of clinical disease (Moyle 1975, Pearson and Ogg 1967). More recently, a descriptive study (Jubb and Galvin 2004) reported on 18 beef herds in Australia participating in the Victorian Johnes disease test and control programme (TCP). The study assessed the effect on clinical disease incidence of applying annual test and cull of all ELISA positives, combined with grazing management and culling of high risk groups. Clinical disease incidence in the participating herds was reduced: 20 cases were diagnosed in the 7 years before the

TCP started and two cases were diagnosed in the 10 years following implementation of the TCP.

A six-year observational study in 9 Wisconsin dairy herds applying a calf-rearing management and test-and-cull control programme (Collins et al 2010), assessed the effect of the programme on prevalence of infection. A low-cost diagnostic test was used, only the cows with “strong-positive” ELISA results were culled, and analysis was limited to ‘before-and-after’ comparison of age-matched cohorts. A reduction in sero-prevalence, based on whole-herd testing, from 11.6 to 5.6% over the study period was found, representing a significant reduction in 8 of the 9 study herds. There is no discussion, though, on why there was no significant reduction in sero-prevalence in one of the herds, nor on the data showing that, in two further herds, the proportion of first-lactation cattle testing ELISA or faecal culture positive increased over the study period.

There have been few formal assessments of the relative effectiveness of the individual elements of management interventions. Ridge et al. (2010) used survival analysis of field data to assess the impact of the three-step calf rearing practices (Anonymous 2003) implemented on 137 infected dairy farms in Victoria, Australia. The outcome event of interest was the date of birth of a clinical case or an ELISA test positive animal born after two whole-herd tests had been completed and test positive animals had been removed. It appears the study was assessing the effect of the intervention on infection transmission, although the abstract states the outcome as “the occurrence of bovine Johnes disease”. The authors were unable to find an association between implementing the recommended calf-rearing practices and subsequent evidence of transmission. However, it seems the herds were also implementing a test and cull programme throughout the study period so it is difficult to draw clear conclusions from the study.

While the above studies give an indication of a possible impact of the programmes assessed, in the absence of clear control groups and with concomitant interventions, it is not possible to separate the effects of each intervention or to assess the influence of temporal factors. Effectively, no conclusions can be drawn on the merit of the specific interventions from these studies.

There have been no epidemiologically robust field studies examining the effect of test-and-cull or management interventions in deer. However, case studies from individual farms have been reported. Bell (2005) described the results of applying control measures

on one deer property from 2001 to 2005. Infection and disease were first recognised in 2001 and control measures subsequently applied included test-and-cull using the Paralisa, separating management groups from different sources, and culling clinically affected deer. A “decrease in JD clinically affected animals” and the study reported a reduction in the number of slaughter deer in 2005 with post-mortem pathology. The 2005 clinical disease incidence was 4%, but there is no annual denominator data presented for other years, and for some years no numerator data either. Comparison of the annual disease incidence or proportion of deer identified with gross pathology over the observation period is thus not possible.

A case study examining the effect of culling adult and juvenile deer on clinical disease incidence in young deer was reported by Griffin et al. (2005). The Paralisa was used to identify animals for removal in a “heavily infected” deer herd over a four-year period, from 2002-2005. There were no data presented on clinical disease incidence before the intervention, other than that there were “significant clinical losses” on the farm. The proportion of mixed-age hinds that were test positive decreased from 40% to less than 3% over the study period, and there were no calves with clinical disease reported in 2004. However, there are no data on annual clinical disease incidence in calves over the observation period, and no description of case definition or how clinical disease occurrence was monitored.

Both of the above studies are descriptive, and it is difficult to quantify the effect of specific measures from the data presented. Other management changes and time-varying effects may have influenced the outcome either positively or negatively, and the conclusions that can be drawn from the above reports of control interventions in single deer herds are therefore limited.

### **Modelling studies**

Collins and Morgan (1992) used a deterministic simulation model, based on the Reed-Frost method, to assess the effect of test-and-cull and management interventions on control of paratuberculosis following introduction into a dairy herd. The outcome of control was not well defined, being described variously as “spread of disease”, “infection rate” and “prevalence of paratuberculosis”, although prevalence of infection appears to be the main outcome modelled. Limitations included modelling only one route of infection

(cow-calf contact) and the assumption that all infected cows were infectious. Diagnostic test sensitivity was found to be the sole determinant of the effectiveness of the test-and-cull strategy modelled. Test sensitivity needed to be greater than 70% to reduce infection prevalence to less than 1% within 10 years, and control of transmission was more quickly achieved when both calf management and test-and-cull methods were applied together.

The assumption that all test positive animals need to be removed as part of a control strategy was considered in more detail by Lu et al. (2008). Their study used a mathematical model to assess the effect of culling only high shedders on control of MAP transmission in dairy herds. The strategy was found to be effective only in herds with good hygiene management; in herds with poor hygiene management culling of low shedding animals was also needed to control MAP transmission. Further mathematical modelling of the dynamics of infection and the effect of interventions (Lu et al 2010) similarly concluded that combining test-based culling together with hygiene management was more effective in eliminating infection than either alone.

The Dutch JD control programme for dairy herds was developed using a stochastic simulation model 'JohneSSim' to assess the effectiveness of control tools, including test-and-cull and calf management (Groenendaal et al 2002). Test-and-cull did not significantly affect infection prevalence and effective control of infection transmission was only possible with improved calf management. One of the JohneSSim model assumptions was that calves under 12 months do not shed MAP. However Weber et al. (2010) concluded, using a proportional hazards model, that the age at onset of faecal shedding was related to within-herd infection prevalence. In dairy herds with an apparent infection prevalence greater than 20%, 20% of cattle were estimated to shed MAP in faeces before 2 years of age. The JohneSSim model assumption may thus have resulted in overestimation of the role of management strategies.

The various studies modelling paratuberculosis control, reviewed in detail by Marce et al. (2010), incorporate different assumptions and have a number of limitations. These are generally related to the lack of available epidemiological information when the models were developed. For example none consider genetic variation in susceptibility, or the relative importance of infection transmission routes. The review authors suggested that incorporating indirect transmission of MAP via the environment, between-animal contact structure and transmission between calves in the models may allow more precise mod-

elling of infection transmission.

To date, no modelling studies of the effectiveness of any control interventions in farmed deer herds have been reported.

## 2.4.2 Vaccination

### Cattle

Although a variety of studies have reported on the results of implementing vaccination in ruminant livestock, again many have used a 'before and after' analysis to describe vaccine effectiveness. As a result, it is difficult to separate effects of vaccination from other measures implemented concurrently, such as improved hygiene or temporal effects such as variation in environmental conditions. For example, the use of the live 'Weybridge' vaccine in 231 cattle herds in the UK was reviewed by Wilesmith (1982). Although clinical disease was eliminated in herds that used the vaccine for at least four years, vaccination was accompanied by implementation of additional control measures, such as culling of clinical cases and their offspring and improved hygiene practices. The contribution of vaccination itself to the reduction in clinical disease incidence is thus difficult to assess. Vaccination in cattle herds in the Netherlands, similarly assessed in a before-and-after study (Wentink et al 1994), found a reduction in clinical disease incidence from 7.8% to 1.8%.

There have been contrasting reports of the effect of vaccination on faecal MAP shedding in cattle. A study using a killed vaccine in two Hungarian dairy herds, conducted over a period of 5 years (Kormendy 1994), reported that the prevalence of faecal culture positive animals reduced from 48% to 1.4% in vaccinates while remaining at around 30% in controls. However a larger cross-sectional study of 58 commercial Dutch dairy herds (Kalis et al 2001) concluded that there was not a significant difference between the proportion of faecal culture positive cattle in vaccinated and non-vaccinated herds (4.4% and 6.7%, respectively).

A comprehensive review of existing MAP vaccines and their effectiveness (Rosseels and Huygen 2008) provides a good synopsis of each vaccine and target species, so further detail is not given here. The review authors concluded that while currently available vaccines were effective in reducing clinical disease incidence, they did not reduce infection

prevalence or prevent shedding of MAP in faeces.

In general, the uptake of vaccination as a disease control measure in cattle herds has been poor (de Lisle 2010). The limited uptake is attributed to a failure of vaccine to prevent transmission of infection, as well as concerns about cross-reactivity to TB tests and inoculation site lesions.

### **Sheep**

Vaccination of sheep with a killed preparation reduced clinical disease incidence and post mortem pathology in vaccinates compared to controls when applied on affected farms in Iceland (Sigurdsson 1952). The first account of vaccination in a commercial sheep flock in England (Cranwell 1993) reported that after 3 years clinical cases of disease had “virtually ceased”; numerical data were not presented. However, in the latter study, implementation of vaccination was accompanied by a selective breeding programme for disease resistance, and culling of clinical cases and their offspring. Moreover, the study reported a ‘before-and-after’ comparison so it is difficult to estimate the contribution made by vaccination.

A five-year field trial of Gudair (Pfizer Animal Health), in sheep in Australia (Reddacliff et al 2006) demonstrated a 90% reduction in clinical disease in vaccinated compared to control sheep. The prevalence of faecal culture positives was reduced by 90% and the onset of shedding was delayed for 12 months post-vaccination in vaccinates compared to controls. The total excretion of MAP in each cohort was estimated, based on the concentration of MAP per gram of faeces, from individual or pooled faecal culture data. The number of bacteria excreted daily by vaccinates was at least one log lower than the number excreted by controls, for up to 30 months post-vaccination. However, the seven clinically diseased vaccinates observed all had multibacillary intestinal lesions, representing a potentially important source of infection for other animals. The study used only 3 flocks and one breed type (Merino) thus may not have been fully representative of the Australian sheep population and endemic strains of the organism. Nevertheless, the trial enabled registration of the vaccine in Australia and vaccination now plays an important role in the Australian Ovine Johnes Disease control programmes, with infected flocks in some states given financial support to implement it as a control measure (Animal Health Australia 2010).

## Deer

Reports of vaccination for paratuberculosis in deer outside of New Zealand are rare. One vaccination programme in a herd of farmed red deer in Scotland (Fawcett et al 1995) used the Weybridge vaccine in conjunction with a serological screening and slaughter policy. A reduction in clinical disease incidence in yearlings from 16/221 in 1985 to nil in 1989 was reported. However, the relative contribution of each control measure cannot be determined from the study.

Although studies to assess candidate vaccines in deer have been under way over recent years in New Zealand, prior to the research reported in Chapter 4 of this thesis there were no vaccines licensed for use in the species. The Neoparasec vaccine (Merial NZ Ltd, vaccine now discontinued) was initially assessed for its effect on diagnostic tests for TB in deer (Mackintosh et al 2005). TB test reactivity in vaccinated deer was compared to test reactivity in unvaccinated controls. The study found that vaccination with Neoparasec did elicit a cross-reactive immune response, with 15/15 vaccinates positive to the mid-cervical test (MCT) 12 weeks after vaccination. However, 11/14 controls were also positive to the MCT at that time. When the comparative cervical test (CCT) was used at 36 weeks post-vaccination, all vaccinates and controls were negative. Antibody to bovine and Johnin antigen persisted in vaccinates, resulting in positive reactions (13/15) to the ELISA test for TB (ETB) at 39 weeks post-vaccination, while there were no positive results in the control group. Although there were moderate injection site lesions, these were minimal by the time of slaughter and easily trimmed from the carcass.

An efficacy trial of Gudair vaccine (Pfizer Animal Health) used experimental oral challenge with MAP (Mackintosh et al 2008a), finding a significantly different proportion of vaccinates (0/30) with gross intestinal pathology compared to controls (6/30). No significant effect of vaccination on liveweight gain was found. The proportion of vaccinates and controls that were faecal culture positive at week 59 was 26% and 21% respectively. Twenty seven vaccinates and 27 (90%) controls were positive to the MCT at 23 weeks, and one control and two vaccinates were positive to the CCT at week 37, but by week 57 all animals were negative to the CCT. When tested with the ETB at week 59, 27/28 of vaccinates (96%) were positive. However, 23/30 controls (77%) were also ETB positive then, demonstrating the cross-reactivity caused by MAP infection itself. It was not possible to assess vaccine efficacy against clinical disease, as the experimental infection model

did not result in clinical disease in any of the trial animals.

Another experimental challenge study (Mackintosh and Thompson, 2007) involved Silirum (Pfizer Animal Health), a vaccine licensed for cattle in New Zealand, but not marketed due to concerns about cross-reactivity to diagnostic tests for TB. The vaccine is a whole-cell killed preparation of MAP strain 316F in a novel oil adjuvant. The trial involved 40 vaccinated and 40 unvaccinated red deer calves, each challenged with MAP. One vaccinate and 4 controls developed clinical disease and at slaughter there were significantly more gross pathological lesions observed in the MLN and intestines of the controls compared to the vaccinates ( $p < 0.05$ ). No significant effect of vaccination on liveweight gain was shown in the study, and little difference between the proportion of vaccinates (15/37) and controls (17/39) which were faecal culture positive (C. Mackintosh, pers. comm.). There was similar reactivity (95% positive) to the MCT at 20 weeks post-vaccination in both vaccinated and non-vaccinated groups. No deer were positive to the CCT at week 52. At week 22 post-vaccination, 82% of the vaccinated deer were positive to the ETB, compared to 35% of the controls.

The studies described above evaluated the effects of vaccination on clinical and sub-clinical disease in deer. They also focused heavily on the effect of vaccination on specificity of diagnostic tests for TB, as this has been shown to be an issue in cattle vaccinated against paratuberculosis. However, there is also potential for vaccination to have an effect on the sensitivity of tests for TB. A study to investigate the effect of vaccinating deer on the sensitivity of TB tests to diagnose infection with *M. bovis* was therefore conducted (Mackintosh et al 2008b). The study involved three-month old red deer calves vaccinated with Silirum ( $n=30$ ) or unvaccinated controls ( $n=30$ ). Half of each treatment group was experimentally challenged with *M. bovis* and responses to the MCT, CCT, ETB and a modified interpretation of the ETB (mod-ETB) were monitored. MCT sensitivity was not affected by vaccination, as all vaccinated and TB-challenged deer were MCT positive. However, CCT sensitivity was reduced in TB-challenged vaccinated deer, with 5/14 (36%) positive compared to 15/15 challenged controls. Sensitivity of the ETB was also slightly reduced in challenged vaccinates, with 12/13 (93%) positive at week 46 while all of the challenged controls were ETB positive. The nature of the trial meant that small numbers of deer were used, and there was no assessment of the additional effect of MAP infection on the outcome. It is thus not known whether concomitant MAP and *M. bovis*

infection would improve or reduce TB test sensitivity in the field situation. The results from this trial suggest that screening with the MCT may be one way to ensure optimal TB test sensitivity. However, the previous experimental work in deer has indicated that TB test specificity may be an issue when the MCT is used in vaccinated deer.

The experimental challenge trials have shown the potential beneficial effect of vaccination on clinical and sub-clinical disease, while experimental studies assessing the effect of vaccination on sensitivity and specificity of TB tests have indicated the possible limitations of vaccination as a control measure for paratuberculosis in deer. However, whether these results reflect the outcome in a natural challenge situation, or how long any effects may persist, is unknown. New Zealand is still implementing a national TB eradication scheme in cattle and deer herds, although at the time of writing there were only six deer herds under restriction for TB infection in the South Island, and none in the North Island. In the future therefore, the effect of vaccination on TB test performance may become less of an issue.

The experimental work has provided critical inputs to the design of a field study to assess the effectiveness of vaccination in naturally challenged deer under normal management conditions, reported later in this thesis.

## **2.5 Cost-effectiveness of control measures**

Establishing that a control programme reduces, for example, the prevalence of MAP infection is only one aspect of its application. More relevant to farmers is the economic benefit of such a programme. In the following section, analyses of the economic benefit of control strategies are reviewed.

### **2.5.1 Cattle**

An early economic decision-tree model to assess a test-and-cull programme in US dairy cattle was developed by Collins and Morgan (1991). The analysis aimed to assess the impact of different factors affecting the economics of control, rather than to describe the specific cost-benefit. The threshold initial infection prevalence, at which cost minus benefit was zero, was described for factors such as herd size, test performance, and test cost. The target outcome of the control programme was not clearly described, but was assumed

to be to reduce infection prevalence. A single diagnostic test, applied annually, was used and all test positive animals were culled. The model showed that test-and-cull was not cost-effective unless the initial prevalence of infection was higher than 5%. Increasing test sensitivity from 20% to 70% did not affect the cost-benefit threshold, although more sensitive tests increased profitability of the programme linearly as initial infection prevalence increased beyond the threshold. An increase in test specificity from 90% to 98% was inversely related to the cost-benefit threshold, which was lower for less expensive tests. The authors concluded that the optimal test was the one with the highest specificity and lowest cost.

The model described above has since been enhanced (Dorshorst et al 2006) to incorporate new information on disease epidemiology and the performance characteristics of five different diagnostic tests. The enhanced model also incorporated the effect of different management practices on economic efficiency. It was thus able to identify the most cost-effective control programme for a combination of herd-level factors, and to consider test-and-management (of test positives) as well as test-and-cull. The best strategy for control was found to require a farm-specific approach, was dependent on herd prevalence and hygiene levels and favoured low-cost diagnostics. The authors concluded that “improving herd management practices to control infection spread (hygiene) is often more cost-effective than testing”.

Kudahl et al. (2008) used a herd-simulation model to assess the economics of control strategies for typical Danish dairy herd management systems. Seven scenarios were modelled, including no control, test-and-cull, test-and-cull with improved calf management and different risk-based test-and-management strategies. Risk-based approaches were found to be more cost-effective than other approaches in reducing infection prevalence. Test-and-cull alone was neither cost-effective, nor did it reduce infection prevalence. Immediate culling of high shedding cows was only cost-effective if transmission routes from them could not be closed.

Vaccination as a control measure to reduce clinical disease incidence was evaluated in a partial budget analysis of field data from Dutch dairy herds (van Schaik et al 2001). The measure was shown to be profitable, with returns less costs estimated at US\$142 per cow over the 8-year trial period. Similarly, simulation modelling of the economics of vaccination in US dairy herds (Groenendaal and Galligan 2003) found vaccination cost-effective

in reducing economic losses associated with milk production, early culling and reduced slaughter value. The minimum annual net present value of vaccination for a 100-cow herd was estimated at US\$959.

### 2.5.2 Sheep

In infected sheep flocks, the cost of testing individual sheep makes test-and-cull an economically prohibitive option (Whittington and Sergeant 2001). A simulation model (Juste and Casal 1993) was developed to assess both strategies in Spanish sheep flocks. Control of infection, based on vaccinating replacement ewes, took longer than test and cull but was the most cost-effective option at low (8%) and high (24%) initial prevalence of infection. The limits of the approach included using a fixed flock size and modelling test sensitivity at 95%. Even if tests with a sensitivity of 95% were available, test and cull was not economic over a 10-year period.

### 2.5.3 Deer

There has been no formal economic analysis of the cost-effectiveness of test-and-cull, vaccination or specific management interventions for control of paratuberculosis in deer herds.

## 2.6 Conclusions

This review has focused on potential control measures and on assessing the evidence for the effectiveness and cost-effectiveness of control strategies for paratuberculosis considering, where possible, the different measurable outcomes that may be the aim of control. Studies evaluating herd-level test-and-cull programmes in cattle conclude that they are not effective in the absence of other management measures, and that even with 'ideal' tests, they are not cost-effective. However, outcomes other than reducing infection prevalence are rarely reported by studies modelling paratuberculosis control. Defining the outcome as reducing the incidence of clinical disease or minimising economic losses *per se*, rather than eradication of infection would be more useful, as these may represent more realistic aims of control interventions for deer. The effectiveness and cost-effectiveness of test-

and-cull as a control measure has not been robustly quantified for any target outcomes in deer.

There is some preliminary evidence that breeding for resistance to paratuberculosis in deer may be possible, although much more work is needed to assess whether it may be a practical and cost-effective method of control. However, there are no quantitative measures of the effectiveness of other individual management interventions in deer. Hygiene measures effective in dairy systems are limited in their practical application to deer farming. Calf and dam separation, for example, is not a viable management approach for the majority of deer breeding herds. Nonetheless, pasture management, stress minimisation, early culling of clinical cases and their offspring and breeding for resistance are all control measures that could be applied in deer herds if shown to be biologically and cost-effective. It is necessary to determine the relative importance of transmission routes, and to source more robust epidemiological data on the effect of these measures, so that input parameters to transmission and economic modelling work can be more precisely defined.

The outcome of experimental trials of vaccination in deer has indicated its potential value as a tool in the control of clinical and sub-clinical paratuberculosis in the species. As in cattle, vaccination has not been shown to reduce faecal MAP shedding, suggesting it may not have an effect on transmission and thus prevalence of infection. The experimental work has also highlighted possible issues of interference with diagnostic tests for tuberculosis. The effectiveness of vaccination, and the effect and duration of effect on diagnostic tests for TB when vaccination is applied in naturally infected deer herds has not been assessed.

The current state of knowledge is not sufficient to make firm recommendations on cost-effective control strategies to control clinical disease, reduce infection prevalence or to minimise economic losses from paratuberculosis in deer herds. This review has identified a number of key areas where robust data are needed, before evidence-based recommendations on control strategies at the herd and industry level can be made. The next four chapters of this thesis present the results of epidemiological studies designed to address a number of the current knowledge gaps.

Chapter 3 is prepared for submission to *Epidemiology and Infection*. It is intended for publication alongside a study entitled ‘Association between *Mycobacterium avium* subspecies *paratuberculosis* and lymph node size in New Zealand farmed deer (*Cervus elaphus*)’ by J Hunnam, PR Wilson, C Heuer, L Stringer, RG Clark and CG Mackintosh, which assesses MAP infection in grossly abnormal mesenteric lymph nodes of deer. At the time of writing, permission from the funders to submit the manuscript was awaited.

**Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in grossly normal mesenteric lymph nodes of New Zealand farmed red deer (*Cervus elaphus*)**

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LA Stringer, PR Wilson, C Heuer, JC Hunnam, C Verdugo, CG Mackintosh

### **3.1 Abstract**

This study estimated the prevalence of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in grossly normal mesenteric lymph nodes (MLN) in farmed deer slaughtered in New Zealand, and assessed predictors of infection. MLN samples (n=251) collected from two North and two South Island slaughterhouses were cultured for MAP. Age, gender, and the presence of other carcasses with enlarged and/or granulomatous MLN in the same line (line status) were assessed as predictors of infection using multivariable logistic regression. A national cluster-adjusted individual prevalence of 45% (95% CI 30%-60%) was estimated, with a North and South Island prevalence of 29% (95% CI 16-45%) and 51% (95% CI 36-66%) respectively. Line status was a strong predictor of infection in young deer (OR 7.1, 95% CI 2.4-21.5), but not in older deer. The prevalence of infected herds was 44% (95% CI 24-64%) in the North Island and 67% (95% CI 49-85%) in the South Island. Weighted adjustment resulted in a national herd-level prevalence estimate of 59% (95% CI 41-78%).

## 3.2 Introduction

The causal agent of paratuberculosis, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), was first confirmed by culture in a clinically-affected farmed red deer in New Zealand in 1986 (Gumbrell 1987). The disease results in mortality and subclinical losses on affected farms, while granulomatous visceral lymph node lesions, caused by MAP, confound post mortem inspection of slaughtered deer, because of similarity to those caused by *M. bovis* (Campbell 1995). Affected carcasses are detained pending laboratory investigation, as part of the national bovine tuberculosis control programme (AHB 2005). A national surveillance system is now in operation to record gross visceral lymph node pathology consistent with paratuberculosis, i.e. enlarged and/or granulomatous ('abnormal') lymph nodes, at routine meat inspection. Data from the inspection of every deer slaughtered at deer slaughter premises (DSP) in New Zealand are recorded to a database and information is relayed back to herd-owners to alert them of likely infection status and to encourage implementation of disease control measures on affected farms. The database is managed by a company established by the deer industry, Johnes Management Limited (JML) (Lynch 2007).

Gross lymph node abnormality as described above is highly associated with the presence of MAP, with 92% culture positive, and a further 3% histologically typical of MAP infection (JC Hunnam et al, unpublished data). However, the true animal and herd-level prevalence of infection is likely to be higher than that estimated by clinical or pathological evidence alone. Additionally, the application of the 'abnormal' lymph node classification as the primary diagnostic criterion for a national surveillance programme requires, as a point of reference, the prevalence of MAP in apparently 'normal' lymph nodes. A pilot study conducted in DSPs in one region of the South Island of New Zealand in 2007 (JC Hunnam, unpublished data) found MAP in 69% of grossly normal MLN.

The present study was undertaken to estimate the national prevalence of MAP infection in grossly normal MLN of deer slaughtered in New Zealand, and to provide data to assess the significance of abnormal MLN as a predictor of MAP infection. Factors associated with infection at the animal-level were investigated as a secondary objective, using a multi-level regression modelling approach.

### 3.3 Materials and methods

#### Study design

A cross-sectional study was carried out in four DSPs, two in each island of New Zealand, with samples collected between October 2008 and January 2009. The study population comprised deer slaughtered at DSPs in the southern and northern regions of the South and North Islands of New Zealand. Sample size calculations were based on estimating an individual-level MAP prevalence of 40% , with 10% absolute precision and 95% level of confidence. Sample size was increased by a design effect or variance inflation factor of 2.5, since four samples were to be collected from each line of slaughter deer and the observations were thus clustered within herds. This resulted in a required sample size of 230 lymph nodes.

#### Sample collection

A line was defined as a consignment of deer from the same herd presented for slaughter on the same day. In each DSP, 15 consecutive lines of deer were sampled over a number of days. Sample collectors were instructed to select four intestinal tracts with grossly normal MLN from each line by systematic randomisation. The number of samples per line was selected to minimise the effect on the analysis of clustering of observations within herds. Classification of MLN as grossly normal was carried out by the plant meat inspectors as part of routine post-mortem inspection procedures. This inspection includes specific criteria for MLN classification whereby a ‘normal’ node is defined as having a circumference of less than 55 mm and no gross lesions, while an ‘abnormal’ node is defined as having a circumference of 55mm or more and/or granulomatous lesions. A sample of mid-jejunal lymph node from each tract was collected into an individual pottle, using new gloves and sterile instruments for each sample, and kept chilled prior to despatch to the laboratory in an insulated container. Samples were cultured for MAP, using a decontamination step with cetylpyridinium chloride and BACTEC 12B liquid culture medium containing egg yolk and mycobactin, as described by Whittington et al. (1999).

Information on the age, gender and carcass weight of the animal, and presence of abnormal MLN in other carcasses in the line was obtained and all data were recorded to Microsoft Excel ( Microsoft Corporation). Due to variation between DSPs in the preci-

sion of age recording, age was coded as a dichotomous variable,  $<27$  or  $\geq 27$  months of age, i.e. ‘young’ or ‘older’.

## Data analysis

### Herd prevalence

A herd was classified MAP positive if MAP was isolated from at least one MLN from the herd, and herd-level prevalence was estimated as the proportion of herds in each island that were MAP positive. The proportion of infected herds was compared between islands with the Pearson chi-square test.

The national herd prevalence estimate was calculated as a weighted mean, with weights being the proportion of herds from which deer were slaughtered at North and South Island DSPs among all herds slaughtering deer throughout NZ (source: JML Database). These proportions were multiplied with the herd prevalence for each island, and the products were added to derive the weighted national estimate.

### Individual prevalence

Analyses were carried out using STATA 10 (StataCorp LP). Estimates of individual prevalence for each island were adjusted to account for the clustering of individual observations within herds using a logistic regression model with generalised estimating equations (GEE), with herd as a subject effect (Diggle 2002). The cluster-adjusted odds ratio and p-value were used to compare individual-level prevalence between the islands.

A true prevalence (TP) estimate at national level was derived from the apparent prevalence (AP), using the formula below (Rogan and Gladden 1978), and published values for the sensitivity (92.7%) and specificity (100%) of tissue culture in deer (Schroen et al 2003).

$$TP = \frac{AP + specificity - 1}{sensitivity + specificity + 1}$$

### Factors associated with MAP positive MLN

Covariate analysis applied the Pearson chi-square or Student's t-test of univariate associations between the outcome (isolation of MAP from individual normal MLN) and each of the predictor variables i.e. age, gender, carcass weight and line status. Line status was classified as 'suspect' if at least one carcass within the line was identified by meat inspection staff as having an abnormal MLN. Line rather than herd status was used as the unit of analysis for this factor as one of the herds from which 3 lines were sampled had two lines classified normal and one 'suspect'.

A multivariable logistic regression model was developed using a forward stepwise selection procedure. Improvement in the model by addition of variables was assessed with the likelihood ratio test, using a retention criteria of  $p < 0.1$ . Estimates were adjusted to account for clustering of subjects within lines using GEE. The effect of individual infection status on carcass weights was examined in a multivariable linear regression GEE model adjusting for clustering by herd as a subject effect and using the same variable selection method and retention criteria.

## 3.4 Results

### Herd-level MAP prevalence

Samples were collected from 60 lines from 57 individual herds, since in the North Island one herd presented two lines and another three lines during the sampling period. The herds sampled were geographically representative of the regions in which the DSP were located, and broadly representative of the underlying national deer farm distribution. Twelve of the 27 herds sampled in the North Island and 20/30 South Island herds were MAP positive. The North and South Island herd-level prevalence estimates were 44% (95% CI 24-64%) and 67% (95% CI 49-85%) respectively, a difference that was not statistically significant ( $p=0.09$ ). The crude national prevalence estimate of infected herds was 56% (95% CI 43-69%). The proportion of the deer kill, by herd, slaughtered in the North Island was 33% and in the South Island 67%. When adjusted to a weighted mean, the national herd-level prevalence estimate was thus 59% (95% CI 41-78%).

## Prevalence of MAP in individual MLN

In total 251 MLN samples were collected, with the number of samples collected from each line averaging 4 (range 3 to 6). Results are summarized by DSP and island in Table 3.1. MAP was isolated from 98 normal MLN, a crude individual prevalence of 39% (95% CI 33-45%). The North Island prevalence of 29% (95% CI 21-36%) and South Island prevalence of 51% (95% CI 42-60%) were significantly different ( $p < 0.001$ ). The cluster-adjusted prevalence estimate for the North Island was 29% (95% CI 16-45%) and for the South Island 51% (95% CI 36-66%), with the difference remaining significant ( $p = 0.047$ ).

**Table 3.1:** Isolation of MAP from grossly normal MLN of deer (n=251) sampled in New Zealand deer slaughter premises in 2008-2009

Deer slaughter plant	Nodes cultured (n)	MAP positive	MAP prevalence
South Island	1	34	58%
	2	26	44%
	Total	60	51%
North Island	3	21	29%
	4	17	28%
	Total	38	29%
Both islands	251	98	39%

The proportion of individual deer slaughtered in the North Island in 2008-2009 was 27% and in the South Island 73%. Applying these proportions to the prevalence values for each island, and adjusting for clustering, resulted in a national individual animal apparent prevalence estimate of 45% (95% CI 30-60%); this increased by 3 percentage points when adjusted to true prevalence.

## Factors associated with MAP positive MLN

Descriptive statistics and crude measures of association found in univariate analyses are presented in Table 3.2. Age and gender information was missing for two of the samples, so analysis was restricted to 249 observations. MAP was isolated from 35% of stags and from 49% of hinds, and from 39% of young and 41% of older animals. Overall, 21 of 60

lines sampled were classified 'suspect', nine of these in the North and 12 in the South Island. Nineteen of the 21 'suspect' lines and 16 of the 39 normal lines were MAP positive. Isolation of MAP from individual grossly normal MLN was associated in univariate analysis with 'suspect' line status ( $p < 0.001$ ) and a lower proportion of stags were MAP-infected ( $p = 0.06$ ).

**Table 3.2:** Univariate associations between isolation of MAP from normal MLN of slaughter deer ( $n = 249$ ) and predictor variables

Variable	n	MAP positive	OR	95% CI	p
Line status					
Normal	160	40	Ref		
Suspect	89	58	5.6	3.0-10.4	<0.001
Age					
< 27 months	212	83	Ref		
$\geq$ 27 months	37	15	1.1	0.5-2.2	0.9
Gender					
Hind	82	40	Ref		
Stag	167	58	0.6	0.3-0.96	0.03

Table 3.3 presents the output from the logistic regression model. An interaction between age and line status was identified, with MLN of young deer in 'suspect' lines more likely to be culture positive (OR 7.1,  $p < 0.001$ ) than those from young deer in normal lines. In the older age stratum, no statistically significant effect of line status was observed on the risk of culture positivity (OR 1.7,  $p = 0.4$ ).

### Carcass weight

The 2.9kg (95% CI -1.1-6.8kg) difference between mean carcass weight of MAP culture positive (mean 57.8kg, SEM 1.2) and culture-negative (mean 60.7kg, SEM 1.4) individuals in univariate analysis was not significant ( $p = 0.15$ ). When adjustment for covariates and clustering was applied in the multivariable model, carcasses with culture positive MLN were 1.2 kg (95% CI -5.2-2.8kg) lighter than those with culture negative MLN ( $p =$

0.5).

**Table 3.3:** Estimates from a logistic regression model of isolation of MAP from normal mesenteric lymph nodes of slaughter deer (n=249)

Variable	OR	95% CI	wald test p-value
<b>Line status*Age</b>			
Young			
Normal line	Ref		
Suspect line	7.1	2.4-21.5	<0.001
Older			
Normal line	Ref		
Suspect line	1.7	0.5-5.9	0.39
<b>DSP</b>			
South Island	1	Ref	
	2	1.4	0.3-5.5
			0.7
North Island	3	0.4	0.1-1.6
	4	0.4	0.1-1.4
			0.1
<b>Gender</b>			
Hind	Ref		
Stag	0.6	0.3-1.3	0.2

### 3.5 Discussion

This study is the first to provide evidence of the prevalence of MAP infection in farmed deer in New Zealand, based on sampling clinically normal deer with no evidence of gross MLN pathology. *Mycobacterium avium* subsp. *paratuberculosis* was cultured from 29% of MLN of deer in the North Island and 51% in the South Island. The estimated national apparent prevalence was 45% of individuals and 59% of herds.

The prevalence of MAP infection in domestic livestock has been investigated in a variety of studies in many other countries. In an abattoir survey in the USA, 34% of cull cows had MAP isolated from ileocaecal lymph nodes (Wells et al 2009), while in Canada and Maine the figure was 16% (McKenna et al 2004). A study in the UK found 3.5% of cull

cows were MAP-positive by PCR detection (Cetinkaya et al 1996). In a recent review of MAP prevalence in live farmed animals in Europe (Nielsen and Toft 2009), individual cattle MAP prevalence was estimated at around 20% and herd-level prevalence at over 50%, with flock-level prevalence for sheep and goats estimated at over 20%. However, conclusions about true prevalence were limited by the paucity of data on the sensitivity and specificity of the diagnostic tests used in those surveys.

Few MAP prevalence studies have been carried out in farmed deer, although a number have been done in free-ranging cervids. A seroprevalence of 31% has been estimated in wild deer in Spain (Reyes-Garcia et al 2008), while a study in free-ranging white-tailed deer populations in the south-eastern United States (Davidson et al 2004) found an animal-level prevalence of 0.3% by tissue culture. The range of different sampling techniques, diagnostic tests and case definitions for infection in the studies described above, though, makes any meaningful comparison between the populations difficult.

Although no structured prevalence survey has yet been carried out on New Zealand farmed deer, a study of case histories from 1986-2000 suggested that MAP infection and disease has been spreading in deer herds (de Lisle et al 2003). The majority of the cases in that study were detected at meat inspection as being ‘suspect’ bovine tuberculosis and submitted for further laboratory investigation, therefore could not be considered representative of the whole farmed deer population. However, in the next 5 years the number of deer herds with microbiologically confirmed infection in the same target population had doubled (de Lisle et al 2006). Pooled faecal culture in herds of unknown infection status investigated for a case-control study in 2005 (Glossop et al 2007b) found 44% of herds sampled to be MAP-infected. Herds were not randomly selected, and results were not weighted by island, so the study gives only a broad indication of possible herd prevalence.

The random selection of herds and individuals reported in this paper minimises the likelihood of selection bias in the resulting estimates of prevalence of infected herds (59%), and animals (45%). Our results do, however, represent only the slaughtered population of farmed deer, specifically those with no clinical or sub-clinical signs of disease, as animals presented at abattoirs must pass an ante-mortem veterinary inspection to be eligible for slaughter for human consumption. Sampling at abattoirs is thus recognised to have a bias analogous to the ‘healthy worker effect’ in human occupational studies (McMichael 1976), i.e. the working (or abattoir) population is likely to be healthier than the general

population. However, this study aimed to report infection prevalence in clinically and subclinically normal deer, and the bias described is unlikely to have a large effect on the population estimates reported.

A seasonal pattern has been recognised in cattle MAP infection in other abattoir studies, with McKenna et al. (2004) reporting a significantly higher proportion of MLN of cows in Canada to be MAP culture positive at slaughter in the spring months. The abattoir study of adult cattle in England (Cetinkaya et al 1996) similarly found a higher proportion of PCR-positive intestinal LN in April compared to other months, although this was not statistically significant. A seasonal effect has been seen in identification of gross lymph node pathology consistent with paratuberculosis in deer (Hunnam et al 2009) and in clinical disease incidence (Glossop et al 2008), with a higher prevalence of pathology recorded in summer although clinical disease incidence on farm is higher in winter. The explanation for this apparently paradoxical observation may lie in the effect of environmental and nutritional stress on disease expression in deer, as these factors are both associated with season (winter). The data source for lymph node pathology is the abattoir population, which explicitly excludes clinically diseased animals, and measuring the prevalence of this subclinical indicator may therefore be confounded by season, as in winter more of the subclinical cases may progress to disease and thus are not represented in the data. It is plausible therefore that there may also be a seasonal effect on infection prevalence, as measured by an abattoir study. One of the study limitations was thus the maximum one-week sampling period in each DSP during the spring and early summer, meaning that no potential seasonal variation could be examined.

Sampling was carried out using a sterile technique, but there remains the possibility of cross-contamination between intestinal tracts after removal from the carcasses by the slaughterman, the meat inspector or via the 'gut tray', before they reached the sampling point. The effect of this is difficult to quantify, but since these results are limited to grossly normal nodes, the tissues are less likely to have been extensively handled or incised during the dressing or inspection procedure, lowering the risk of contamination.

The accuracy of the true national prevalence estimate (48%) is sensitive to the accuracy of the parameters described for the tissue culture method. While a specificity of 100% is generally accepted for MAP culture procedures, the sensitivity of the test for deer lymph node samples in New Zealand has not been formally validated. The sensitivity estimate of

92.7% used in calculation of true prevalence was derived from a small non-peer reviewed study of 110 samples from an indeterminate number of Australian deer herds and the true prevalence estimate should therefore be interpreted considering that limitation.

A previous report suggested a difference in prevalence of infected herds between the North and South islands. Glossop et al. (2006) found 29% (15/51) of herds in the North Island to have MAP isolated from pooled faecal culture compared to 55% (35/64) of herds in the South Island, although the herd sample was not randomly selected. The sensitivity of pooled faecal culture is lower than that of individual tissue culture (Schroen et al 2003) but the herd-level prevalence of 44% in the North Island and 67% in the South estimated in the present study are consistent with those findings. The lack of statistical significance to this comparison at the herd-level is likely to be a function of the small sample size, as the present study was designed primarily to estimate individual-level rather than herd-level prevalence. An average of four samples was collected per line of deer, thus the sensitivity of detection at the herd level was not optimal and herd-level prevalence was thus likely to be underestimated. However, studies to investigate the herd and geographical risk factors underlying the observed difference would be worthwhile. One potentially valuable resource will be the use of molecular sub-typing, currently under development for New Zealand MAP isolates. Type I (sheep or s-strain), for example, has been less frequently isolated from deer lymph node lesions (de Lisle et al 2006) than type II (cattle or c-strain), and a type I isolate has been shown in experimental infection studies to be less pathogenic for deer than a type II (Mackintosh et al 2007). Application of more discriminatory sub-typing methods may help to gain insights into the epidemiology and pathogenesis of MAP infection in New Zealand by examining the regional distribution of sub-types and by analysing associations between sub-type, pathology and clinical Johnes disease.

Regional or DSP differences in sensitivity of detection of enlarged MLN by meat inspectors, i.e. the line status classification assessed in the multivariable analysis, have been previously recognised (Glossop et al 2007a) as an important element of the overall accuracy of the surveillance system to identify MAP-infected herds. It is possible that, in this study, the presence of researchers interacting with meat inspectors to record the occurrence of 'suspect' mesenteric lymph nodes may have increased the detection rates. Nevertheless, a strong association was found between isolation of MAP and 'suspect' line classification

in young deer. This provides further data to support the use of the ‘abnormal’ lymph node category as a predictor of MAP infection in deer herds. However, this appears to be more predictive in younger than older animals since this study failed to find a similar association in older animals. This may be a statistical effect of the small number of older animals in the sample, although there may be a biological basis, related to the consequences of infection itself. Deer presented as adults at the slaughterhouse are those that have not succumbed to clinical disease on farm and may represent a group that are more resistant to infection. Evidence from a recent trial suggested a resolution of MLN pathology and possibly elimination of infection in some experimentally-infected deer bred for resistance (Mackintosh et al 2010a). Additionally, older animals may be more likely to have MLN pathology as a result of other chronic conditions such as infection with other pathogens, parasites or neoplasia.

The isolation of MAP from 16/39 lines with no evidence of gross MLN pathology is a finding which merits further investigation. Most of the samples were taken in October/November and therefore may have originated from farms with infection, but have only included the better-performing animals, i.e. those reaching slaughter weight earlier in the season, and so possibly less likely to have evident MLN pathology. Longer-term studies of the association between MLN pathology and MAP infection at the herd-level would therefore be useful to validate the overall surveillance system as a herd classification tool. In summary, the data presented here provide a national baseline prevalence estimate for MAP infection at the individual and herd-level, and show a contrast between the North and South Islands. Industry-led surveillance is designed to reduce MAP infection levels through feedback and advice to farmers of affected herds, and progress towards this aim may be measured by reference to these data. More research to investigate the factors contributing to the difference in infection prevalence seen between the islands may help to identify the measures necessary to achieve control of MAP in deer herds.

### **3.6 Acknowledgements**

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Chapter 4 presents the results of a randomised controlled trial of vaccine efficacy, and has been prepared although not yet submitted for publication in the journal *Vaccine*.

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# Efficacy of Silirum<sup>®</sup> vaccine in control of paratuberculosis in naturally infected young farmed deer

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## 4.1 Abstract

A randomised controlled trial to assess the efficacy of Silirum<sup>®</sup> vaccine in the control of subclinical or clinical paratuberculosis (Johne's disease (JD)) in young farmed deer was carried out in 2008 in the South Island of New Zealand, in six commercial deer herds with a history of a high (>5%) annual incidence of clinical JD. Treatment allocation was randomly assigned and farmers were blinded to the vaccination status of the deer. Vaccination used 0.5ml Silirum given subcutaneously in the anterior neck in March/early April (around 4 months of age). Vaccinates (n=1,671) and controls (n=1,664) were grazed together and weighed at vaccination and in July and November. Faecal samples were collected from 125 vaccinates and 123 controls on five farms in November. All deer clinically affected by disease resembling JD were necropsied and diagnosis confirmed by culture for *Mycobacterium avium* subsp. *paratuberculosis* (MAP). All surviving deer were slaughtered between 11 and 20 months of age and the incidence of gross lymph node pathology typical of paratuberculosis in deer, i.e. enlarged and/or granulomatous visceral lymph nodes, was recorded. Clinical JD was confirmed in 18 control and seven vaccinated deer, resulting in a vaccine efficacy estimate of 60% (95% CI: 3-83%, p=0.04). Overall 127 (51%) of faecal samples were MAP positive; 47% (95% CI: 38-56%) of vaccinates

and 55% (95% CI: 46-64%) of controls ( $p=0.5$ ). Average daily liveweight gain did not differ between the cohorts. At slaughter 1.4% of vaccinates and 4.5% of controls had lymph node characteristics typical of paratuberculosis, a relative risk of 0.32 (95% CI: 0.19-0.54,  $p<0.001$ ).

These data indicate that vaccination may have a role in reducing the incidence of clinical and subclinical paratuberculosis in young deer.

## 4.2 Introduction

Paratuberculosis or Johnes disease (JD), a granulomatous enteropathy caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), occurs in a range of ruminant species on every continent (Clarke 1997). The disease was first recognised in deer in New Zealand in 1979 (Gumbrell 1986) and since then, MAP infection has been confirmed in an increasing number of farmed deer herds (de Lisle et al 2003). Young deer have a higher annual clinical disease incidence than adults, with a median of 2% in yearling, compared to 1.3% in adult stags on farms with clinical JD (Glossop et al 2008). The same study reported annual disease incidence up to 21.5% in weaner deer, thus on some farms JD can result in substantial economic losses (Bell 2006). Currently there are limited options for on-farm control due to incomplete knowledge of the epidemiology of infection and disease in deer or of the effectiveness of test and slaughter strategies in deer herds.

Vaccination as a control measure for JD has been used worldwide since 1926, primarily in young animals since effectiveness of vaccination appears best when used before or soon after infection with MAP (Emery and Whittington 2004). When vaccination was used in cattle in the UK (Stuart 1965), Hungary (Kormendy 1994) and Spain (Garcia-Pariente 2005), a reduction in clinical disease incidence resulted, while Wilesmith (1982) claimed that clinical JD had been eliminated in some UK cattle herds that used vaccine long-term. A recent field trial of Gudair<sup>®</sup>, a whole-cell killed vaccine, in Australia (Reddacliff et al 2006) demonstrated a 90% reduction in mortalities due to JD in vaccinated sheep.

Experimental studies with oil-adjuvant vaccines in deer have shown that some reduce the severity of subclinical JD (Mackintosh et al 2008a). An experimental challenge trial using Silirum, an oil-adjuvant vaccine licensed for use in cattle, found significantly less gross pathology and a non-significant reduction in clinical disease (1/40 vaccinates vs 4/40 con-

trols) in vaccinated deer (Mackintosh and Thompson 2007). Those observations justified investigation of vaccination on commercial farms, involving larger numbers of deer under natural challenge conditions.

This paper describes a randomised controlled trial of Silirum vaccine in commercially farmed deer under field management conditions in a naturally infected young deer population. The primary objective was to estimate vaccine efficacy against clinical JD. The effect of vaccination on average daily liveweight gain, faecal shedding of MAP and gross lymph node pathology at slaughter was also assessed. The trial was preceded by a study to determine safety of the vaccine, immunological responses and optimal dose (Goodwin-Ray et al 2008).

## 4.3 Materials and Methods

### Study design

The study was conducted between March 2008 and August 2009 and implemented a randomised controlled trial in commercial deer herds with a history of a high annual incidence of clinical JD in young stock.

Six study farms were selected on the basis of a history of clinical JD in weaner deer of at least 5% in the previous two years, and with no recent changes in control interventions or source of stock, i.e. they were anticipated to continue to have similar levels of disease. All were finishing farms which sourced weaners from breeding farms. Additional inclusion criteria were previous confirmation of JD by culture, and/or a history of typical JD pathology at post-mortem examination. This history was based on the incidence of gross lymph node pathology consistent with JD in deer, i.e. the presence of enlarged and/or granulomatous visceral lymph nodes, in all deer commercially slaughtered as recorded in a national deer JD database (Lynch 2007).

Regulatory compliance required that all study animals had to be destined for slaughter, at around 12 -15 months of age, with none retained for breeding or velvet antler production, to eliminate the potential for cross-reactivity in future testing for bovine tuberculosis (TB). Candidate farms were identified via previous participation in a nationwide case-control study (Glossop et al 2007b) or were nominated by deer veterinary practitioners. Any with an annual TB testing interval, i.e. were considered at risk of bovine tubercu-

losis, were excluded, due to concerns about cross-reaction with TB diagnostic tests. All farms selected were in the South Island, and were located in the Canterbury, Southland and Otago regions. They thus represented a reasonable geographic distribution of South Island deer finishing farms.

Vaccinated and control deer were grazed in the same management groups to minimise potentially confounding management and environmental effects and to ensure each cohort experienced similar levels of exposure to MAP during the trial. The deer were all venison production stock comprising red deer (*Cervus elaphus*), and wapiti (*C.e.canadensis*) x red deer crossbreeds, and were slaughtered, according to normal management practice, from 11 to 20 months of age. Farmers and their veterinary practitioners, who performed diagnostic investigations, were blinded to the vaccination status of the animals.

### **Interventions**

The vaccine (Silirum<sup>®</sup>, Batch No.06/001, Pfizer Animal Health) is a whole-cell inactivated vaccine of MAP strain 316F in an oil adjuvant and is licensed for use in cattle in New Zealand. Vaccination was carried out by the researchers between 15 March and 2 April 2008, when the animals were around 4 months old. Whole mobs were yarded, and deer were handled in holding pens in small groups of 6-10. Half of each small group was randomly selected for vaccination and were injected subcutaneously in the right upper neck with 0.5ml of Silirum, using a 1ml syringe and 18-G x 1/4 inch needle. Selection was based on vaccinating every other deer starting alternatively from the right or left, as they stood side by side in the pen. The dose rate was determined from an earlier study assessing immunological responses at dose rates of 0.5ml, 1ml and 2ml (Goodwin-Ray et al 2008). The control animals received no intervention.

### **Outcomes**

The primary objective of the study was to estimate vaccine efficacy against clinical Johnes disease in young farmed deer. The secondary objective was to examine the effects of vaccination on subclinical disease, including faecal shedding of MAP and liveweight gains. The primary outcome measure was clinical disease incidence, while secondary outcome measures were daily liveweight gain, faecal MAP shedding, gross lymph node pathology, carcass weight, carcass grade and time to reach slaughter weight. Data were also

collected on the occurrence of injection site lesions resulting from vaccination and their effect on carcass quality.

### **Case definition**

The definition of a suspected clinical case was 'loss of body condition and weight relative to herd-mates in animals that are otherwise eating well; weakness; poor coat appearance; and/or diarrhoea; unresponsiveness to treatment'. Farmers were asked to contact their veterinarian when they identified suspected cases of JD in trial animals. A clinical examination and on-farm euthanasia and post-mortem were then carried out if considered appropriate by the attending veterinarian. A standardised report on pathological findings was completed and samples were taken from grossly affected lymph nodes when present, or a pool of anterior, mid and posterior jejunal lymph node segments and ileocaecal lymph node. Samples were submitted for confirmation of MAP by culture. Deer with suspect clinical disease were defined as confirmed clinical cases if there was both typical gross pathology and MAP was isolated from tissue samples.

### **Measurements**

At vaccination in March 2008 the ear tag number, vaccination status and gender of each animal were manually recorded and each deer was weighed. Each animal was double tagged. On four farms where additional electronic tags had been applied, individual weights were automatically recorded to electronic file while the remainder were manually recorded. Data were recorded to a relational database (Microsoft Access), with double entry of manually recorded data to ensure accuracy of transcription.

All deer were individually weighed in July/August and again in November, when faecal samples were collected from the five herds retaining most of their animals at that time. Twenty five deer from each cohort were selected by systematic randomization and approximately 10g of faeces were collected per rectum using a fresh glove for each animal. Samples were immediately stored on ice packs in an insulated container and were sent by courier the same day to the bacteriology laboratory.

The culture procedure applied a decontamination step using cetylpyridinium chloride and BACTEC 12B liquid culture medium containing egg yolk and mycobactin (Whittington et al 1999). Growth indices (GI) were recorded as a semi-quantitative measure of the

number of viable organisms in the sample (Reddacliff et al 2003). Sample GIs were measured weekly and time to first detection of MAP in liquid culture media, at a cumulative growth index of 15, was recorded for MAP positive vials.

In November, the vaccination sites of trial deer on three farms were palpated and callipers were used to measure any reactions identified. The animals were sent for slaughter at a Deer Slaughter Premises (DSP) once they reached optimal slaughter weight, at the discretion of the farmer. Date of slaughter, carcass weights, carcass grade and gross lymph node pathology at post-mortem examination were accessed from central records maintained by the processing companies and from the national deer JD database. Time to slaughter was calculated by assigning a common date of birth of 01/11/2007 to all trial deer, according to accepted industry practice, as information was not available on actual birth dates. Analysis of carcass grades was limited to data from one processing company, to ensure standardised classification.

All procedures on live animals were approved by the Massey University Animal Ethics Committee.

### **Sample size**

The null hypothesis for the primary outcome was that the cumulative incidence of clinical Johnes disease during the trial was the same in the vaccinated and control groups. Sample size calculations were based on a confidence level of 95%, and a power of 80% to detect a 50% relative difference in disease incidence between the cohorts, postulating an incidence of 5% in the controls and 2.5% in the vaccinates. To examine the effect of vaccination on the shedding of MAP in faeces, sample size was estimated based on an anticipated prevalence of 50% of culture positive samples in controls, and 25% in vaccinates. A design effect of two was included, to take account of an expected moderate intra-class correlation due to grouping of animals within the study herds. The required sample size to estimate efficacy against clinical disease was 3,600 deer (1,800 in each cohort), and that for the faecal shedding was 232 deer. All trial deer were weighed.

### **Statistical analysis**

The unit of analysis was the individual deer. Table 4.1 summarises the objectives of analysis and the statistical methods applied for each outcome. Analyses were conducted using

Stata10 (StataCorp, College Station, TX). Crude associations between vaccination and dichotomous outcomes, i.e. confirmed clinical cases, faecal MAP shedding and pathology, were examined for significance with the chi-square or Fisher's exact test on 2 x 2 contingency tables. Continuous outcomes i.e. mean liveweight gain, carcass weight and time to slaughter, were tested for significance of association with vaccination in univariate analysis using the Student's t-test.

Multivariable analysis was carried out using general linear models to examine and control for the effects of covariates (gender, herd, and weight at vaccination) on the association between vaccination and each outcome. Binary outcomes were analysed with logistic regression and risk ratio estimates risk were then derived using modified Poisson regression with robust error variance (Zou 2004) to adjust for the effect of clustering of animals within herds. Average daily liveweight gain (ADG) was calculated using the difference between individual liveweights measured in March, July and November, divided by the number of intervening days using the actual weighing dates for each herd. Analysis of ADG used generalised estimating equations (GEE) to adjust for repeated measures on the same subject. The potential effect of clustering of the observations within herds on the variance of the estimates was assessed by calculation of the intra-class correlation coefficient (ICC).

The vaccine efficacy (VE) estimate was derived from the preventable or attributable fraction comparing the risk of disease (R) in vaccinated compared to unvaccinated individuals, assuming equal exposure to the infectious agent in each cohort (Halloran et al 1999):

$$VE = 1 - \frac{R(\text{vaccinates})}{R(\text{non - vaccinates})}$$

The denominators used were the number of individual animals in each cohort that were enrolled in the trial.

**Table 4.1:** Statistical methods applied to analyse the effect of vaccination on each outcome in a Silirum vaccine efficacy trial in young deer

<b>Response variable</b>	<b>Measure of effect</b>	<b>Objective of analysis</b>	<b>Statistical technique</b>
Clinical disease	Risk ratio	Compare the incidence of clinical JD in each cohort	Logistic regression
Average daily liveweight gain (ADG)	Mean difference	Compare ADG in each cohort	General linear model with GEE
Faecal shedding	Risk ratio	Compare the proportion of deer shedding MAP in each cohort	Logistic regression
Time to detection	Hazard ratio	Semi-quantitative comparison of MAP concentration in faeces in each cohort	Cox regression
Carcass weight	Mean difference	Compare carcass weights between cohorts	Linear regression
Time to slaughter	Survivor function	Compare time to reach slaughter weight in each cohort	Log-rank test
Pathology	Risk ratio	Compare the incidence of lymph node pathology in each cohort	Logistic regression
Grade	Odds ratio	Compare carcass grades in each cohort	Multinomial logistic regression

## 4.4 Results

### Experimental population

A total of 3,335 deer were enrolled in March 2008, with 1,671 randomised to the vaccinated and 1,664 to the control cohort groups comprising 1,206 hinds and 2,127 stags; the gender of two animals was not recorded. Liveweights in March 2008 ranged from 25 to 76 kg (mean 53.7, SD 8.0) in vaccinates and from 22.4 to 77.5kg (mean 53.6, SD 8.5) in controls. The majority of the trial deer (n=2,107) were red/wapiti crossbred, while the remainder (n=1,228) were red deer. Management groups, or mobs, on each farm consisted of a single breed type. There was no statistically significant difference between the co-

horts in the proportion of each gender ( $p=0.5$ ), nor in the mean liveweights at vaccination ( $p=0.7$ ).

### Clinical cases

The incidence of confirmed clinical cases on each farm is presented in Table 4.2. All trial farms experienced a lower incidence of clinical disease in the 2008/2009 season than in previous years, evident in both the trial animals and other similar mobs on the farms. Twenty two controls and nine vaccinates were investigated as suspect cases of JD, while 18 controls and seven vaccinates were confirmed by necropsy and culture. There were no culture results available for two of the suspect cases (both vaccinates), due to severe autolysis of the carcass, two (both controls) showed no evidence of pathology and were negative at culture, one suspect (control) had typical pathology but MAP was not isolated, while at necropsy another suspect (control) was found to have a chronic leg injury and no pathological evidence of Johnes disease, despite being MAP culture positive. These six suspect cases were thus not confirmed as clinical cases.

Five of the confirmed clinical cases were hinds, 20 were stags, while 13 were red deer and 12 were wapiti hybrids. No further analysis was possible on the breed data as it could not be separated from herd effects.

The crude risk difference was 0.007 (95% CI: 0.0008- 0.012) with a risk ratio of 0.39 (95% CI: 0.16 - 0.92,  $p=0.03$ ). The risk ratio estimate and statistical significance of the effect of vaccination was altered only slightly by multivariable analysis, with the adjusted measure of vaccine efficacy estimated at 60% (95% CI: 3-83%,  $p=0.04$ ). The ICC for these data was 0.004, so no further adjustment for clustering was made.

While univariable analysis of the risk ratio of confirmed clinical disease in stags (0.9%) vs. hinds (0.4%) was not significant (RR =2.3, 95% CI: 0.85-6.0,  $p=0.09$ ), after adjusting for the effects of covariates, the RR for stags was 3.3 (95% CI: 1.2-9.2,  $p=0.02$ ).

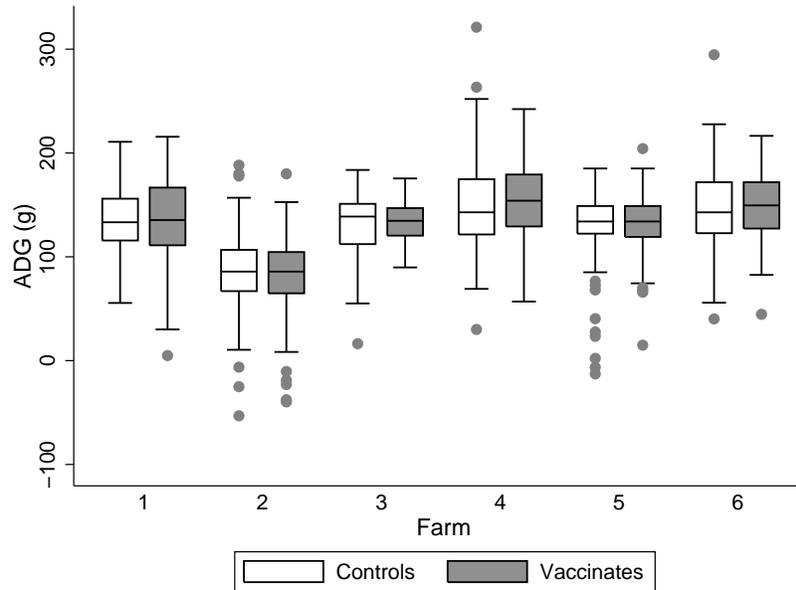
**Table 4.2:** Cases and cumulative incidence (CI) of clinical Johne's disease confirmed by pathology and culture of MAP, in vaccinated and control deer in a randomised controlled trial of Silirum vaccine

Farm	Vaccinates			Controls		
	n	Confirmed cases	CI1(%)	n	Confirmed cases	CI (%)
1	90	1	1.1	90	0	0
2	563	5	0.9	557	12	2
3	269	0	0	270	2	0.7
4	295	0	0	296	0	0
5	311	1	0.3	309	4	1.3
6	143	0	0	142	0	0
All	1671	7	0.4	1664	18	1.1

### Liveweight and daily liveweight gain

Liveweights measured at vaccination and mid-trial in July/August differed by less than 0.1kg between cohorts across all farms ( $p=0.4$ ). Average daily liveweight gain (ADG) was 119g/day for vaccinates and 118g/day for controls over the entire measurement period of March to November. No statistically significant differences in ADG were observed within or across farms ( $p=0.6$ ) (Figure 4.1).

Multivariable analysis found no significant association between vaccination and ADG, either over the whole measurement period or during the specific time periods of March to July or July to November.



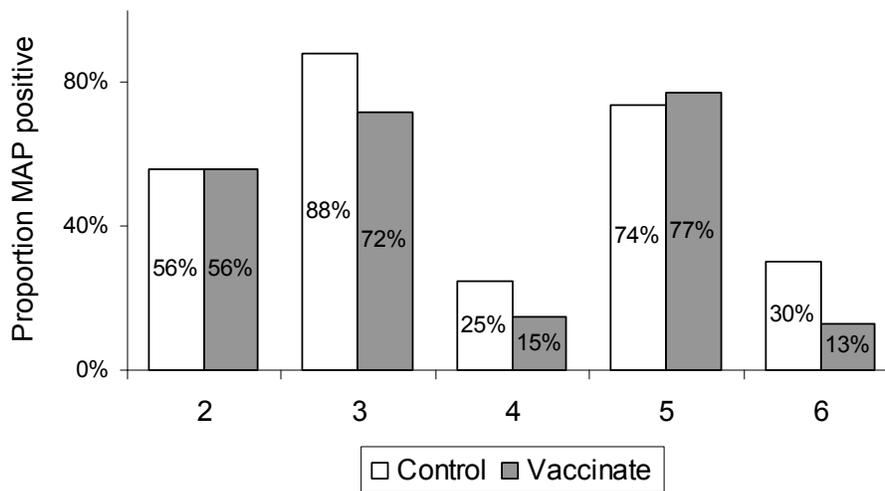
**Figure 4.1:** Boxplot of average daily liveweight gain (ADG) of vaccinated and control deer from March to November 2008 for each farm in a randomised controlled trial of Silrum vaccine

### Faecal culture

The proportion of faecal samples MAP positive for each farm is presented in Figure 4.2. MAP was isolated from 127 of 248 faecal samples (51%) comprising 47% (95% CI: 38-56%) of vaccinated and 55% (95% CI: 46-64%) of control animals. The prevalence on individual farms ranged from 20% to 80%. The adjusted effect of vaccination on faecal MAP positivity across all herds was not statistically significant (RR 0.9, 95% CI: 0.7-.1.1,  $p=0.5$ ).

Sixty one percent (94/155) of stags sampled were culture positive compared with 34% (33/92) of hinds (RR 1.4, 95% CI: 1.02 -1.9,  $p=0.04$ ).

The mean time to detection of MAP in culture was 5 weeks for each cohort ( $p=0.4$ ). The comparison remained non-significant in multivariable analysis.



**Figure 4.2:** Proportion of faecal samples from young deer which were MAP culture positive in a randomised controlled trial of Silirum vaccine

### Vaccination site lesions

Examination of vaccination sites of 486 deer in November revealed 181 (38%) with palpable subcutaneous lesions. Reaction size ranged from 5 to 39mm (mean 14.4, SD 5.93). Most were firm circumscribed reactions, although two discharging sinuses were found. No adverse events were reported following vaccination. At slaughter, meat inspection staff reported that although vaccination site reactions were evident, these were generally circumscribed and were removed with the hide or were easily trimmed. The recording of the occurrence of these was not consistent between plants so the data is not presented. No difficulties were experienced with access to export markets, nor was there any apparent loss of carcass value of vaccinated deer due to injection site lesions. The New Zealand Food Safety Authority (NZFSA) currently requires additional inspection of deer vaccinated against Johnes disease: “Palpation and deep incision of the muscles lateral and parallel to the ligamentum nuchae at or about the likely site of injection. Lengthen the incisions when suspicious lesions have migrated along the lymphatics of fascial planes”. No tracking lesions were identified in vaccinated animals as a result of these additional procedures. A granulomatous lesion within the prescapular lymph node of one vaccinated animal resulted in detention of the carcass in accordance with routine TB suspect procedures. Histological examination was unable to rule out TB and the lesion was cultured for

*M. bovis* with negative results, allowing release of the carcass.

### **Carcass weight**

Information on slaughter date, pathology and carcass parameters were available for 2,516 of the trial deer. On-farm mortalities including clinical suspects numbered 88, of which 37 were vaccinates and 51 were controls, but the majority of the missing data resulted from a failure to adequately record individual animal identification at slaughter due to problems with barcode scanning equipment in the lairage of one meat plant. The missing data were evenly distributed between vaccinates (n=367) and controls (n=364). Mean carcass weight for all vaccinates combined was 52.2kg (95%CI: 52-52.4) and for the controls 52kg (95%CI: 51.8 -52.2) (p=0.2). Carcass weight was higher (1.3 kg, p=0.02) in vaccinates only in herd 4.

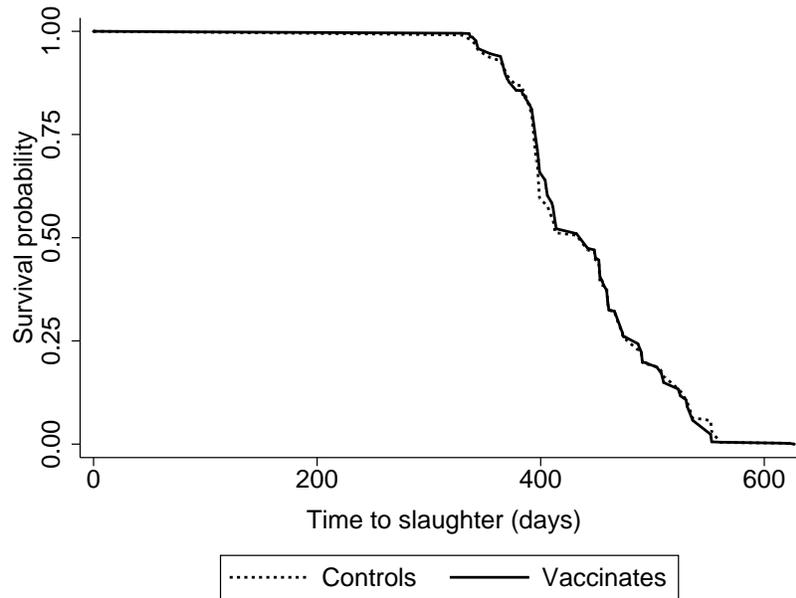
There was no difference found between the cohorts in carcass weight after controlling for the effect of covariates (adjusted mean difference 0.1kg, p=0.4).

### **Time to slaughter**

Survival curves for time to slaughter of vaccinates and controls are presented in Figure 4.3. The mean time to slaughter from birth was 441 days and was the same for both cohorts. Mean time by farm ranged from 372-490 days, with no statistically significant difference found between the cohorts within or across all herds. The log-rank test for equality of survivor functions was non-significant (p=0.3) indicating no difference between the cohorts in distribution of time to slaughter.

### **Slaughter grade**

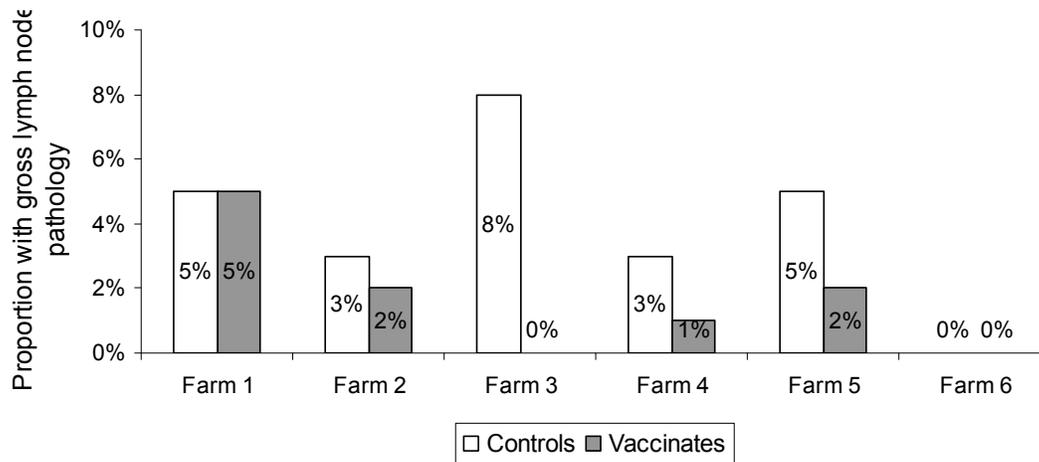
The distribution of carcass grades was examined for 2,079 slaughter deer comprising 1,040 controls and 1,039 vaccinates. No significant difference was found between vaccinates and controls overall (p=0.3) or within each herd in univariate analysis or following multinomial logistic regression analysis, controlling for the effect of gender and weaning weight. Two carcasses were condemned by meat inspection staff at post-mortem examination for reasons not associated with vaccination.



**Figure 4.3:** Kaplan-Meier survival function from birth to slaughter of control and vaccinated deer (n=2516) for each farm in a randomised controlled trial of vaccine efficacy

### Pathology at slaughter

Data on gross lymph node pathology of trial deer are presented in Figure 4.4. The overall cumulative incidence in the deer for which slaughter data was available was 2.9% (74/2516) comprising 1.4% (18/1267) of vaccinates and 4.5% (56/1249) of controls, ( $p < 0.0001$ ). Fifty stags (3.1%) and 24 hinds (2.6%) ( $p = 0.5$ ) were recorded with pathology. Two vaccinates and eight controls were recorded with granulomatous lymph node lesions ( $p = 0.02$ ). The adjusted risk ratio for gross lymph node pathology in vaccinates compared to controls was 0.32 (95% CI: 0.19-0.54,  $p < 0.001$ ). The ICC for these data was 0.004 so no further adjustment for clustering was applied. Gender was not a significant risk factor in multivariable analysis ( $p = 0.7$ ). Carcasses with gross lymph node pathology had lower mean carcass weight (1.3kg, 95% CI: 0.5-2.0,  $p = 0.001$ ).



**Figure 4.4:** Proportion of carcasses of vaccinated and control deer with gross lymph node pathology in a randomised controlled trial of Silirum vaccine

## 4.5 Discussion

This field trial found that vaccination with Silirum significantly reduced the incidence of clinical Johnes disease and gross lymph node pathology in young deer. There were no differences between vaccinates and controls in average daily liveweight gain, carcass weight or grade, time to slaughter, prevalence of faecal MAP shedders, or time to faecal culture positivity, a semi-quantitative measure of shedding.

The low incidence of clinical disease recorded during the study (1.1% in controls, compared with approximately 5% reported previously on the trial farms) limited the precision of the estimate of vaccine efficacy. Incidence of clinical JD in deer is known to vary between years, but one possible explanation is that incidence was influenced by the herd immunity effect. Direct protective effects of vaccination occur at the individual animal level and indirect effects at the herd level. Herd immunity thus refers to the protection of non-vaccinates due to the presence of immune individuals and the resultant reduction in sources of infection (Fine 1993). At the design stage of this study, the decision was made to maintain the vaccinated and control deer mixed together in the same management mobs. This was done to ensure that all trial animals had the same environmental, management and infection exposures. The effect of herd immunity in this type of design may bias the estimate of vaccine efficacy towards the null, if vaccination reduces transmission of

infection and controls have reduced exposure as a result. The estimate is similarly biased to the null if vaccinates are heavily exposed to the agent by the presence of infectious controls, thus overcoming their immunity (Dohoo et al 2003). However, in deer, transmission of MAP may be via the intra-uterine route (van Kooten et al 2006, Thompson et al 2007) and young deer on infected farms may also be exposed to MAP via infected colostrum or milk (Thompson et al 2007) or from the environment in the first few months of life. The finishing deer were brought on to the individual trial farms at around 4 months of age and were thus expected to have already been exposed to infection on their farms of origin, before the trial started. This was expected to reduce the influence of herd immunity.

In addition, the presence of a herd immunity effect is not supported by results of individual faecal sampling, since vaccination had no significant effect on the proportion of deer shedding MAP in faeces, or on the semi-quantitative measurement of MAP. This demonstrates the likelihood of continuing exposure of all trial deer to the organism. Furthermore, other (non-trial) mobs of deer on the farms experienced similarly low levels of clinical Johnes disease during the trial period. All of the deer herd managers considered that the mild winter and good spring pasture growth in 2008 contributed to lower than previous disease rates. This evidence combined suggests that herd immunity was not a significant factor in this trial.

Failure to find an effect of vaccination on faecal MAP shedding prevalence is consistent with other studies of JD vaccination in deer (Mackintosh et al 2008) and in dairy cattle (Kalis et al 2001). In contrast, Reddacliff et al (2006) reported a 90% reduction in faecal shedding in vaccinated sheep, although some vaccinates did demonstrate high levels of MAP excretion. The power of this study was designed to detect a 25% difference in prevalence of faecal shedding between the cohorts. A larger sample size may indeed have found statistical significance, but with 47% of vaccinates shedding MAP in faeces, it would not have altered the biological relevance in terms of continuing transmission of the agent. The isolation of MAP from the faeces of so many deer on farms with little clinical disease was notable. On farm 3, for example, MAP was isolated from 80% of all faecal samples, yet the incidence of clinical disease in trial deer was 0.4%, and this was the best-performing herd in terms of mean November liveweight. Farms with the lowest disease incidence had the lowest prevalence of faecal MAP positivity although on farms with no reported clinical disease, the proportion of culture positive control animals was

still up to 30%. These proportions of potentially infectious animals raise questions about the impact of infection *per se* and the specific factors that influence progression from infection to clinical disease in deer.

The quantitative measure for MAP concentration in faeces applied in this study was crude, with analysis limited to time to first evidence of MAP in culture media. Further work to derive a more precise quantification measure is required before robust conclusions can be made.

Gender was found to be a risk factor in this study, with stags having over three times the risk of clinical disease and a higher risk (RR 1.4) of shedding MAP in faeces. This confirms anecdotal reports by farmers and veterinary practitioners that males seem more susceptible to clinical disease. The explanation for this may lie in the effects of environmental and nutritional stress impacting more on faster-growing animals.

No effect of vaccination on average daily liveweight gain, carcass weights, carcass grades or time to slaughter was shown in this trial. The liveweight findings are similar to those of a previous experimental challenge study of Silirum vaccination in New Zealand deer (Mackintosh and Thompson 2007), while a sheep vaccine trial (Reddacliff et al 2006) recorded significantly higher liveweights (0.73kg,  $p < 0.05$ ) in young control sheep than vaccinates only at 12 months post-vaccination. The study herds were selected to represent herds with a high incidence of Johnes disease in young deer and results may thus not be generalisable to all infected herds. However, failure to find an effect in this population suggests that a production effect is even less likely to be observed in herds with lower infection prevalence.

The data on gross lymph node pathology at meat inspection should be interpreted with some caution. Although the sensitivity of meat inspection to detect enlarged and/or granulomatous mesenteric lymph nodes is estimated at 25% (J.C. Hunnam, unpublished data), the identification and recording of findings is known to be variable between individuals and deer slaughter plants. However, any misclassification is likely to be non-differential between the cohorts, thus biasing estimates of effect towards the null (Copeland et al 1977). The NZFSA requirement to carry out additional inspection on vaccinated animals meant that individual DSPs needed advance notification if vaccinated animals were to be presented for slaughter. In the majority of DSPs, though, it was logistically simpler to treat the whole consignment as vaccinated rather than separate the cohorts in the lairage

and meat inspectors were thus effectively blinded to the individual carcass status. However, one DSP limited the number of vaccinates that could be presented each day and inspected them separately. Although the status of the carcasses would then have been known, differential bias in reporting was considered unlikely but, if present, was more likely to bias the estimate of effect to the null since carcasses of vaccinates were subjected to a higher degree of inspection. The finding of an effect of vaccination on the incidence of gross lymph node pathology is thus considered robust. It is also important in terms of efficiency of carcass processing. At present, the similarity of granulomatous lymph node lesions associated with MAP infection to those caused by *M. bovis* in deer causes difficulty for the meat industry at post mortem inspection (Campbell 1995), as carcasses in which these lesions have been identified require detention pending laboratory investigation to exclude *M. bovis* as a diagnosis. There are logistical implications and increased costs for the meat plant and the authorities in sample transport and laboratory procedures and detained carcasses may miss the opportunity of sale to export markets. A reduction in levels of such pathology therefore benefits both the producer and the processor.

The deer in this trial were vaccinated around weaning, close to 4 months of age, as this was the first opportunity to do so on most of the trial farms. It is possible that vaccination may be more effective if administered earlier in life, but on many breeding farms deer calves are not handled until this stage. Further research to assess the effect of the vaccine when used at a younger age may be useful to inform those deer managers who are able or willing to implement vaccination earlier.

In conclusion, Silirum vaccine was effective in reducing the incidence of clinical Johnes disease and gross lymph node pathology in young deer vaccinated at approximately 4 months of age. The low incidence of clinical disease reported during the study means that further data are required to more precisely quantify vaccine efficacy. No impact on excretion of MAP bacteria in faeces was demonstrated, although a more precise measure for quantification of shed organisms is needed for fuller evaluation. Vaccination had no negative impact on carcass quality or on acceptability of venison to export markets. These data therefore suggest that Silirum may be useful as an aid to control losses associated with clinical JD in young deer.

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## 4.6 Acknowledgements

The study was funded by Pfizer Animal Health, Massey University and supported by Massey University and International Doctoral Research Scholarships. Pfizer Animal Health approved the design of the study and supplied the vaccine but was not involved in the collection, analysis or interpretation of data. We would also like to thank a long list of students who helped with the trial field work as well as staff at AgResearch Wallaceville and the Disease Research Laboratory, Otago University, and veterinary practitioners for diagnostic support. Special thanks go to the farmers for contributing their animals, time and enthusiasm to the study.

Chapter 5 considers the effect of vaccination and infection on diagnostic tests for bovine tuberculosis. The manuscript is prepared in the style of the New Zealand Veterinary Journal and has been accepted for publication.

# Specificity of diagnostic tests for bovine tuberculosis in paratuberculosis-vaccinated and naturally infected farmed red deer (*Cervus elaphus*)

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## 5.1 Abstract

**AIMS:** To assess reactivity to diagnostic tests for bovine tuberculosis (TB) in deer vaccinated against paratuberculosis (Johnes disease) and exposed to natural challenge with *Mycobacterium avium* subsp. *paratuberculosis* (MAP), compared with naturally challenged control deer, and to investigate MAP infection as a factor in TB test cross-reactivity at the herd level.

**METHODS:** Study 1. Yearling deer (n=180 vaccinates and 181 controls) were randomly selected from three commercial deer herds participating in a randomised controlled trial of Silirum<sup>®</sup> vaccine. The deer were subjected to the comparative cervical skin test (CCT) for TB at 44 weeks post-vaccination. Interpretation as a mid-cervical skin test (MCT) was also recorded. Serum from deer positive to the CCT was collected 3-4 weeks after tuberculin injection and tested with the ELISA TB test (ETB) with both standard and the modified-ETB interpretations.

Study 2. The association between herd-level MAP infection and MCT reactivity was investigated in deer herds which completed a herd MCT test in 2005 and participated in a national case-control study of clinical paratuberculosis. Herds (n=102) were assigned

as outcome positive if one or more deer gave a positive reaction to the MCT, and were categorized MAP positive or negative based on results of pooled faecal culture. Information on a range of other potential risk factors was collected by personal interview of the farmer with a standardised questionnaire. The data were analysed in a multivariable logistic regression model.

**RESULTS:** In study 1, 79/180 vaccinates (44%) and 42/181 controls (23%) were positive to the MCT ( $p < 0.001$ ). Two vaccinates (1.1%) and three controls (1.7%) were CCT positive. The two CCT positive vaccinates were both positive to the standard ETB and negative to the modified-ETB. One of the three CCT positive controls was negative to the standard ETB while two were positive; both controls were modified-ETB positive.

In study 2, 58/102 herds tested were MCT positive. Significantly more MCT positive herds (71%) than MCT negative herds (41%) were infected with MAP ( $p = 0.003$ ). The OR for MCT reactivity in MAP positive compared to MAP negative herds was 3.1 (95%CI=1.3-7.5). Herd size was positively correlated with TB test positivity ( $p = 0.004$ ).

**CONCLUSIONS:** Infection with MAP and vaccination with Silirum increases the risk of non-specificity of the MCT in deer. The CCT and modified-ETB used in series are effective tools to resolve the reduced specificity of the MCT. However, where use of these tests is not permitted, non-specificity related to infection and vaccination will be more difficult to address.

## 5.2 Introduction

Infection with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) and vaccination for paratuberculosis are both potential causes of cross-reactivity with tuberculosis tests. A randomised controlled trial to evaluate the field efficacy of Silirum<sup>®</sup> vaccine in the control of paratuberculosis in young deer reared for venison production in New Zealand has recently been reported (Stringer et al unpublished data). The vaccine contains a whole-cell inactivated strain (316F) of MAP, delivered in a mineral oil adjuvant. A long-recognised concern in the use of such vaccines is the sensitisation of the animal to tests for bovine TB (Doyle 1964, Kohler et al 2001) due to the close antigenic similarity between mycobacteria species and stimulation of the cell-mediated and humoral immune response by the oil adjuvant. Experimental studies in deer have suggested that cross-reactivity wanes with

time and that the comparative cervical test (CCT) or ELISA test for TB (ETB) are effective as ancillary tests to resolve false positive diagnoses to the mid-cervical test (MCT) (Mackintosh et al 2005). The MCT is used routinely as a herd screening test for bovine TB in deer in New Zealand, and involves a single intradermal injection of bovine purified protein derivative (PPD) while in the CCT, both avian and bovine PPD are administered at separate sites.

Mycobacteria within the *M. avium-intracellulare* complex (MAC) including *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* have a close antigenic similarity to *M. bovis* and are considered important factors affecting the specificity of diagnostic tests for TB. Although clinical disease due to infection with MAC organisms other than MAP occurs only occasionally in New Zealand farmed deer, gross lymph node lesions associated with MAC infection but similar in appearance to those of *M. bovis* are relatively common (Mackintosh and Carter 1999). Mycobacteria other than *M. bovis* have been cultured from the tissues of TB test reactors (de Lisle and Havill 1985, de Lisle et al 1995), suggesting that they may play a role in reducing test specificity. Exposure of animals to saprophytic mycobacteria is a well-recognised cause of sensitisation to tuberculin skin tests (Monaghan et al 1991). Moreover, mycobacteria persisting in pond water and in sphagnum moss vegetation in New Zealand have been shown to sensitise guinea pigs to bovine tuberculin (Kazda and Cook 1988). Deer are likely to be exposed to a range of these organisms in their natural environment.

Factors other than paratuberculosis vaccination that may affect the specificity of TB skin tests in deer in New Zealand have not previously been formally investigated quantitatively. The hypothesis that an increasing prevalence of MAP infection in deer is a factor in TB test cross-reactivity has been proposed by field veterinarians and is supported by culture results from TB test reactors (de Lisle et al 2003). This paper presents two studies of cross-reactivity with TB testing in farmed deer in New Zealand. The first assesses reactivity to diagnostic tests for TB in paratuberculosis-vaccinated and control deer while the second explores the association between potential risk factors and diagnostic test results, with the objective of quantifying the effect of MAP infection on MCT skin test reactivity at the herd level.

## **5.3 Materials and Methods**

### **5.3.1 Study 1: Effect of vaccination on diagnostic tests for bovine tuberculosis**

#### **Study design**

A full description of the selection process for the vaccine efficacy trial is outlined in Chapter 4. Six farms in the South Island of New Zealand with a history of at least a 5% annual incidence of clinical paratuberculosis in weaner (3-12 month old) deer were recruited. Weaners were randomly assigned to receive vaccine or to the untreated control cohort within each mob on each farm. Vaccinates and controls were managed together in the same mobs to ensure that exposures other than vaccination were similar for each cohort. All trial deer were located on commercial finishing farms and intended for venison production. The study farms were all considered low risk herds for TB, being either in areas of low risk, as defined by the Animal Health Board (AHB) under the National Pest Management Strategy (AHB 2005), or subject to abattoir surveillance only i.e. exempt from routine on-farm diagnostic testing. Herds at higher risk of TB infection are normally required to complete regular testing of all deer over 8 months of age (AHB 2005).

Sample size calculations for the Tb cross-reactivity study used information from a preliminary safety study (Goodwin-Ray et al 2008) that found 50% of deer vaccinated with 0.5ml of Silirum were positive to the MCT. An absolute difference of 20% in the MCT positive rate between vaccinated and control deer was postulated. Sample size was increased by a factor of two, as observations were clustered within herds, therefore 60 vaccinated and 60 control deer were required on each of three farms. The three farms were selected as those with the most trial deer remaining in January 2009. The deer were selected by systematic randomisation from those remaining on-farm at around 44 weeks post-vaccination, i.e. those that had not reached finishing weight and been slaughtered.

#### **Interventions and measurements**

Vaccination with Silirum (batch 06/001, Pfizer Animal Health Ltd) was carried out between 15 March and 2 April 2008. A 0.5ml dose was delivered by subcutaneous injection in the right upper neck using a syringe and sterile 18-G x needle. The gender of all deer

was recorded.

In January 2009 there were sufficient numbers of deer left on three of the vaccine trial farms to carry out the study reported here. The CCT was conducted by one operator (LAS), in accordance with the test procedure and interpretation as defined by the AHB. Briefly, hair was clipped to 2mm over two injection sites of dimension 100mm x 100mm in the mid-cervical region. The difference in double skin fold thickness was measured with vernier calipers before and 72 hours after intradermal injection with 0.1ml (2500 IU) of avian and 0.1ml (5000 IU) of bovine PPD. The test was interpreted as both a MCT, for which a positive test was defined as “any palpable or visible reaction (excluding 2mm nodular or ‘rice-grain reactions’)”, and as a CCT for which a positive test was defined as “any reaction at the site of the bovine tuberculin which is 2 mm or more and this reaction is equal to or greater than any reaction at the site of the avian tuberculin”.

The ETB is an ELISA test which may be used as an ancillary test 13 to 33 days after a positive reaction to the MCT. Applying the standard interpretation, if bovine PPD minus avian PPD = 20 ELISA units, then the ETB is positive. At the discretion of the AHB, a modified interpretation of the ETB may be used in herds at low risk of TB infection. The modified-ETB adapts the standard ETB to include interpretation of antibody responses to Johne’s protoplasmic antigen (PPA) and bovine specific antigen MBP70. If the MBP70  $\geq$  50 ELISA units, then the modified-ETB is positive. If the MBP70 < 50 ELISA units and the PPA > bovine PPD and PPA > 50 units, then the modified-ETB is negative (Mackintosh et al 2008b).

All deer positive to the CCT were sampled by jugular venepuncture 3-4 weeks after tuberculin injection and serum was submitted to the Disease Research Laboratory (Otago University, Dunedin) for ELISA testing. The standard and the modified interpretation of the ETB were both reported.

All interventions and measurements involving live animals were approved by the Massey University Animal Ethics Committee (protocol 07/166).

### **Statistical analysis**

The association between TB test reactivity and vaccination status was assessed at the individual animal level. The chi-square test was used to compare the proportion of positive MCT results in vaccinated and control deer. Possible interaction and the extent of con-

founding of this association by gender was assessed using the Mantel-Haenszel technique to derive a pooled relative risk estimate (Rothman 2002). The potential effect of clustering of individual observations within herds on the variance estimates was examined by calculation of the intra-class correlation coefficient (ICC) (Dohoo et al 2003). Values of the ICC range from 0 to 1, with a low ( $<0.1$ ) value indicating little clustering.

### **5.3.2 Study 2: Risk factors for MCT reactivity**

#### **Study design**

This was a cross-sectional study using data collected during a nationwide case control study. Herds were selected from those participating in the case-control study, which assessed risk factors for the presence of MAP at the herd level (Glossop et al 2007). In that study, 174 deer herds were selected from an initial population of 315 responding to a postal questionnaire. In 2004/2005, six pools of faeces from 10 adult breeding hinds on each farm were cultured on BACTEC media (Becton Dickinson, Sparks, Maryland, USA) and herds were classified as MAP positive or negative accordingly. Herd characteristics and risk factor data were collected at the same time by personal interview of the farmer with a standardised questionnaire and, with farmer permission, the TB test history of the property was obtained from AHB records. Herds for inclusion were those that had carried out a whole or partial herd MCT between 1 January 2005 and 31 December 2005 ( $n=111$ ). Nine herds that had been classified as TB-infected by the AHB within three years of this test were excluded from analysis. The study population thus numbered 102 deer herds. The spatial distribution of study herds is illustrated in Figure 5.1 The original questionnaire collected data on 300 risk factors; only those ( $n=14$ ) biologically plausibly associated with TB test specificity and possible confounding factors were selected for inclusion in this study.

#### **Statistical analysis**

The unit of analysis was the herd. A binary outcome was MCT positive or negative based on one or more deer being MCT positive, or all being negative, respectively. The chi-square test was used to examine associations between the outcome and herd MAP infection, along with each of the other risk factors. Fisher's exact test was applied if the value

of any of the cells of the contingency table was less than 5. Factors associated with the outcome at probability values  $<0.20$  in univariate analysis were included in a multivariable logistic regression model, using a forward stepwise selection method. Improvement in the model by addition of variables was assessed using the likelihood ratio test, with the criteria for retention set at  $p < 0.1$ . First order interaction terms were tested and considered for inclusion if significant ( $p < 0.05$ ), although none were found. The Hosmer-Lemeshow goodness-of-fit test was used to assess model fit. All statistical analyses were carried out using STATA10 (StataCorp, College Station, TX).

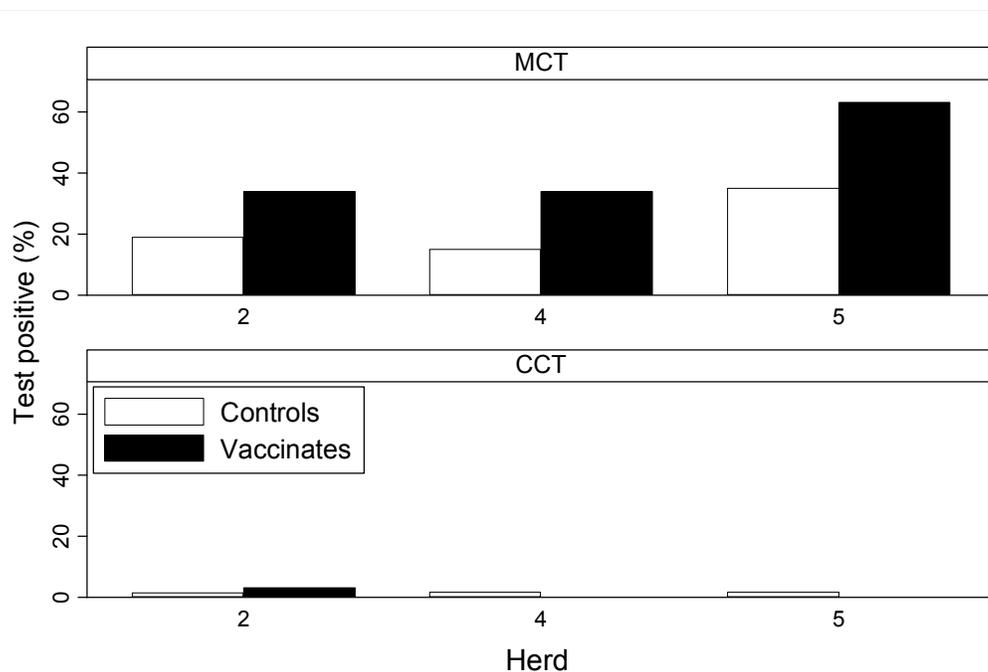


**Figure 5.1:** Study 2: Spatial distribution of New Zealand deer farms ( $n=102$ ) participating in a case control study of risk factors for TB test reactivity

## 5.4 Results

### 5.4.1 Study 1

Intradermal tests results are summarized by herd in Figure 5.2. One hundred and eighty vaccinates and 181 controls were tested and, across all herds, 79 vaccinates (44%, 95%CI=37-51%) and 42 controls (23%, 95%CI=17-29%) were MCT positive ( $p < 0.001$ ). Five CCT positive deer were identified, of which two were vaccinates and three were controls. Both CCT positive vaccinates were positive to the standard ETB interpretation and negative to the modified-ETB. One of the three CCT positive controls was negative to the standard ETB interpretation while the two remaining controls were both modified-ETB positive and were slaughtered as TB reactors, under AHB regulations. There were no macroscopic lesions identified at post-mortem inspection, and the presence of *M. bovis* was not confirmed by culture in either of the animals.



**Figure 5.2:** Study 1: proportion of vaccinated (n=180) and control (n=181) deer positive to the mid-cervical (MCT) and comparative cervical (CCT) tests for bovine tuberculosis applied during a randomised controlled trial of Silirum vaccine

Stratified analysis revealed no evidence of confounding by gender of the relationship between MCT result and vaccination. The ICC was calculated at 0.07, so no adjustment for

clustering of observations within herds was made. The risk ratio for MCT reactivity comparing vaccinated with control deer was therefore the crude estimate of 1.9 (95% CI=1.4-2.6). The risk difference was estimated at 20% (95% CI=10%-30%). The association between vaccination and CCT positivity was not significant in univariate analysis (Fisher's exact  $p=1$ ). The low numbers involved precluded further statistical analysis of covariate effects.

### 5.4.2 Study 2

The deer herds in Study 2 included 30 herds in the North Island and 72 in the South Island. The relationship between herd-level MAP infection and TB test positivity is presented in Table 5.1. The majority of herds completed a herd MCT test in 2005, although two herds carried out a partial test. The proportion of MCT positive deer ranged from 0.07% to 12.8%. MAP was the only mycobacteria isolated from pooled faecal samples from the study farms.

**Table 5.1:** Study 2: Herd-level results of the mid-cervical test (MCT) for bovine tuberculosis and presence of MAP in New Zealand deer herds (n=102)

	Herd MCT result		Total
	+	-	
Herd MAP+	41	18	59
Herd MAP -	17	26	43
Total	58	44	102

The summary and results of univariate analysis of the other risk factors for TB test positivity that were investigated are detailed in Table 5.2. The crude association between herd MCT result and MAP infection status was significant ( $p=0.003$ ), with an OR of 3.5 (95% CI=1.5-8.0) comparing MAP infected and culture negative herds. The Mantel-Haenszel pooled OR for herd MAP status stratified by herd size was 3.1 (95% CI=1.5-8.3). Grazing deer on hill or high country was a marginally significant factor (OR 2.3,  $p=0.048$ ). The multivariable logistic regression model output is summarised in Table 5.3. Factors retained in the model were herd-level MAP infection status and herd size. After adjustment, the odds ratio for MCT positivity comparing MAP positive to MAP negative herds,

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was 3.1 (95%CI:1.31-7.49,  $p < 0.01$ ).

**Table 5.2:** Study 2: Univariate associations between postulated risk factors and reactivity to the mid-cervical test for bovine tuberculosis in 102 New Zealand deer herds surveyed with a questionnaire in 2005

Risk factor	Category	Control herds	Case herds	OR (95% CI)	p
		(n=44)	(n=58)		
Deer herd size	1(1-630)	21	13	Ref	
	2(636-1499)	15	17	1.8(0.7-4.9)	0.23
	3(1500-9370)	8	28	5.7(2.0-16.1)	0.001
Season of test	1(summer)	5	4	Ref	
	2(autumn)	1	5	6.3(0.5-77.5)	0.15
	3(winter)	22	39	2.2(0.5-9.1)	0.27
	4(spring)	16	10	0.8(0.2-3.6)	0.07
Hill or high country	0 No	32	31	Ref	
	1 Yes	12	27	2.3(0.98-5.5)	0.048
Island	1 South	28	44	Ref	
	2 North	16	14	0.6(0.2-1.3)	0.18
Downlands	0 No	20	20	Ref	
	1 Yes	24	38	1.6(0.7-3.6)	0.26
Ducks	0 Non/rare	8	7	Ref	
	1 abundant	36	51	1.6(0.7-3.6)	0.39
Still water	0 No	15	24	Ref	
	1 Yes	29	34	0.7(0.3-1.7)	0.46
Grain fed	0 No	25	31	Ref	
	1 Yes	19	27	1.1(0.5-2.5)	0.74
Flat pasture	0 No	18	22	Ref	
	1 Yes	26	36	1.1(0.5-2.5)	0.76
Irrigation	0 No	38	49	Ref	
	1 Yes	6	9	1.2(0.4-3.6)	0.79
Sheep on DFA	0 No	17	21	Ref	
	1 Yes	27	37	1.1(0.5-2.5)	0.80
Closed deer herd	1 Yes	13	16	Ref	
	2 Sire stags	9	15	1.4(0.4-4.1)	0.59
	3 No	22	27	1.0(0.4-2.5)	0.99
Cattle on DFA	0 No	10	14	Ref	
	1 Yes	34	44	0.9(0.4-2.3)	0.87
Gulls	0 none/rare	23	31	Ref	
	1 abundant	21	27	0.9(0.4-2.1)	0.91

**Table 5.3:** Study 2: Output of a logistic regression model of factors associated with reactivity to the mid-cervical test for bovine tuberculosis in deer herds (n=102) in New Zealand (Hosmer-Lemeshow  $p=0.7$ )

Variable	OR	95% CI	p-value
Herd MAP status	3.13	1.31-7.49	0.01
Herd size category 1	Ref		
Herd size category 2	1.54	0.55-4.29	0.41
Herd size category 3	4.9	1.66-14.47	0.004

## 5.5 Discussion

The results presented here showed that 44% of young deer vaccinated with Silirum in the study were positive to the mid-cervical tuberculin test for bovine tuberculosis 44 weeks later and that 23% of control deer were also MCT positive. Two vaccinated and three control deer were positive to the CCT and, while the vaccinates were subsequently negative to the modified-ETB, two of the controls were positive. This indicates that non-specificity due to the vaccine alone can be distinguished by ancillary tests. The study also found that MAP positive herds are 3.1 times as likely to experience false positive MCT results than MAP negative herds.

A herd selection criterion for the vaccine efficacy trial was a history of at least 5% annual incidence of clinical paratuberculosis in weaner deer, so these herds represent a population believed to have significant exposure to MAP infection. Since this is the type of herd most likely to use vaccination as a means of controlling clinical disease, they are considered representative of the target population. Testing with the MCT and CCT during a preliminary safety study (Goodwin-Ray et al 2008), in a herd with no clinical history of MAP infection, found similarly high levels of MCT reactivity (50%) in vaccinated young deer after 48 weeks, although no CCT positives. However, in that study there were no MCT positive control animals. An experimental challenge trial (Mackintosh and Thompson 2007), in which young deer were vaccinated with Silirum and then vaccinates (n=39) and controls (n=40) were experimentally challenged with MAP, found a high proportion (95%) of both vaccinates and controls to be MCT positive 20 weeks post-vaccination.

All subjects were negative to a CCT at week 52 post-vaccination. Although that study was not directly comparable since it used a higher dose (1ml) of vaccine and artificial challenge with MAP, the findings are consistent with those presented here, i.e. that MAP infection itself is a cause of MCT positivity. It also indicated that the CCT may resolve false positive reactions resulting from either vaccination or MAP infection.

The herds in this field trial were considered to be at low risk of tuberculosis infection, being either exempt from on-farm testing or in triennial testing areas. The skin test reactions are thus considered likely to be due to non-specificity. This assumption was endorsed by the Animal Health Board, as none of the herds were classified as infected with TB as a result of the tests carried out during the trial. Furthermore, none have been classified as infected during the last 10 years.

However, test sensitivity is also a potentially important issue in vaccinated animals. The CCT and ETB ancillary tests are used in deer to increase the specificity of diagnostic screening, rather than to enhance detection of *M. bovis*. Therefore, AHB policy currently prescribes that the CCT may not be approved as a stand-alone screening test in TB risk areas due to a lower sensitivity to bovine TB than the MCT. Similarly the modified interpretation of the ETB may only be used at the discretion of the AHB, in herds at low risk of TB infection. This is an important issue, since the vaccinated deer in this study were determined negative only by the modified-ETB interpretation, suggesting that diagnostic specificity could be a problem if vaccination was used on farms on which use of the modified-ETB was excluded.

Preliminary data from an experimental infection study examining the effect of paratuberculosis vaccination on the sensitivity of diagnostic tests for *M. bovis* infection in deer suggest that both MCT and CCT sensitivity is reduced in vaccinated animals (Mackintosh et al 2008b). However, investigation of two novel serological tests for TB diagnosis in deer experimentally infected with *M. bovis* (Buddle et al 2010), found a higher proportion of test positives in TB-infected deer vaccinated with MAP compared to unvaccinated deer. Although sensitivity of both novel tests to detect vaccinated deer infected with *M. bovis* was 100% (13/13) two weeks following the CCT, specificity of the novel tests was low at 64% (9/14) in the vaccinated deer compared to 100% in controls. Test specificity was also low (60%) in deer naturally infected with MAP, illustrating again the effects of MAP infection *per se* on test specificity.

At present, Silirum is recommended for use in New Zealand only in finishing deer destined for slaughter at a young age, prior to becoming eligible for TB testing. These deer are monitored at post-mortem inspection at the abattoir and are not normally included in on-farm TB surveillance tests. In addition, an official earmarking system to identify vaccinated animals has been made compulsory. Both of these measures exist to assist interpretation of the results of diagnostic tests in vaccinated deer that are required to be or are inadvertently TB tested. As the number of deer herds infected with TB has fallen from 74 in December 2003 to 7 in June 2010 (M. Bosson, AHB, pers. comm.), continued successful progress with the national TB control programme may further reduce restrictions on use of the CCT and other ancillary tests and thus permit vaccination in deer intended for breeding.

Study 1 examined the effect on diagnostic testing at a single time interval post-vaccination (approximately 44 weeks). Previous experimental studies have suggested that skin test reactivity may reduce as the time interval from vaccination increases. When tested at week 24 and week 36 post-vaccination a reduction in reactivity, measured by the average difference in skin thickness at the bovine tuberculin injection site, was recorded in deer treated with oil-adjuvant MAP vaccine (Mackintosh et al 2005). A separate study involving a different oil-adjuvant MAP vaccine (Mackintosh et al 2008a) also found a reduction in the skin thickness difference at the bovine tuberculin injection site between weeks 37 and 57. There would therefore be value in further studies to examine the longer-term effect of vaccination on the MCT. If the vaccine is used in deer to be retained on farms for breeding or velvet antler production, they would normally become test-eligible only from 15 months of age, and some may not be first tested until they are several years old.

In this study, the CCT was applied as a screening rather than as an ancillary test, for logistical reasons. However, depending on the outcome of further studies on the persistence of cross-reactivity, and approval from the AHB, the CCT may be the most appropriate screening test for TB surveillance for vaccinated commercial deer herds. Although the CCT is more expensive initially, this would largely obviate the additional cost and inconvenience of using ancillary tests on MCT positive vaccinated deer. However, in addressing cross-reactivity due to vaccination, the effect of natural infection with MAP *per se* on TB testing should not be overlooked. Two of the controls failed to clear ancillary tests and had to be slaughtered as TB reactors, indicating that infection alone can reduce the speci-

ficity of TB tests.

The results of the second study presented here demonstrate an association between MAP infection and MCT reactivity at the herd level, with MAP-infected herds having 3.1 times the odds of a positive MCT test result. There were limitations within the data available, as no record of the identity of individual animals is maintained in the AHB database if the MCT result is negative. This meant that no analysis could be carried out at the individual animal level. However, the herd-level approach is reasonable in a study designed to generate hypotheses on risk factors for TB test reactivity. The information on exposures and outcome at the herd level related to the same time period (2005), thus addressing the issue of temporality. Classification of herds as MCT positive or negative using the test records kept by the AHB was objective. In practice, though, the ability to detect a positive MCT reaction may be influenced by factors such as adequate lighting and restraint of stock, and misclassification of the outcome was thus possible. However, any misclassification was unlikely to be biased by the exposures under study. The categorisation of herds as MAP-infected was also a possible source of misclassification. Although faecal culture is considered 100% specific for presence of MAP, sensitivity at the individual animal level is estimated at 47% when compared against tissue culture as a gold standard (Schroen et al 2003). The sampling protocol applied for the classification of herds for this study was estimated to have a herd-level sensitivity of 81% (Glossop et al 2007b). It is therefore possible that some MAP-infected herds were incorrectly classified as MAP negative. This could bias the observed estimate of the effect of MAP status, the direction of bias being dependent on results of the MCT test of wrongly classified herds.

The original case-control study from which the data for Study 2 were sourced investigated over 300 exposures as potential risk factors for clinical paratuberculosis, but for this study only those plausibly biologically associated with TB test reactivity were collated. Exposure to avian wildlife, for example, was included as a potential source of *M. avium* exposure, as this organism has been recognised as a possible cause of cross-reactivity to bovine tuberculin (Griffin and Buchan 1991). Preliminary reports of risk factor studies for clinical paratuberculosis (Glossop et al 2007b) have suggested that the presence of sheep on the deer fenced area is negatively associated with clinical disease in weaner deer, while the presence of cattle is positively associated. These exposures were investigated as independent risk factors in this study, as it is possible they may have an

effect on MCT specificity through the introduction of other cross-reactive antigens to the deer population. However, no statistical association was found between either of these factors and MCT positivity at the herd level.

The association demonstrated between increasing herd size and a positive herd-level MCT result is explained by the probability of at least one false-positive test result increasing with the number of animals tested with an imperfectly specific test (Christensen and Gardner 2000), thus a herd size effect could be anticipated. This was shown in analysis to have a slight positive confounding effect on MAP infection as a risk factor. Failure to find significant associations with the other risk factors must be interpreted in the light of the relatively small sample size and therefore power of the study. Those factors that appeared marginally significant in univariate analysis, i.e. season of test, topography (hill or high country) and island, may be subjected to more detailed investigation in a study using a larger, national dataset of TB test results, post-mortem indicators of MAP infection and spatial information that is currently being planned.

In conclusion, infection with MAP and vaccination with Silirum increases the risk of non-specificity of the MCT in deer. The CCT and modified-ETB used in series are effective tools to resolve the reduced specificity of the MCT. However, where use of these tests is not permitted, non-specificity related to infection and vaccination will be more difficult to address.

## 5.6 Acknowledgements

We would like to thank the property owners/managers and veterinarians involved in the studies and in sample collection, staff at the Disease Research Laboratory, University of Otago and the AgResearch bacteriology laboratory, Wallaceville for diagnostic support and Caryl Lockhart for help with the field testing. The vaccine trial was funded by Pfizer Animal Health. The work was financially supported by Johnes Research Group, DEEResearch Ltd, FRST, and Massey University and the New Zealand Government International Doctoral Research Scholarships.

The study which follows in Chapter 6 has been prepared for submission to the Journal of Veterinary Diagnostic Investigation. At the time of writing, approval from the funders of the study to submit the manuscript was awaited.



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# Bayesian estimation of the sensitivity and specificity of individual faecal culture and Paralisa<sup>TM</sup> to detect *Mycobacteria avium* subsp. *paratuberculosis* infection in young farmed deer

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## 6.1 Abstract

A Bayesian latent class model was used to estimate the sensitivity and specificity of an IgG1 serum enzyme-linked immunosorbent assay (Paralisa) and individual faecal culture (IFC) to detect young deer infected with *Mycobacterium avium* subsp. *paratuberculosis*. A zero-inflated random effect logistic model was developed to allow for zero-infection herd status, as well as capturing between-herd heterogeneity conditional on a positive infection state. Paired faecal and serum samples were collected, between July 2009 and April 2010, from 20 individual yearling (12-24 month old) deer in each of 20 South Island (SI) and 17 North Island (NI) herds and subjected to culture and the Paralisa test, respectively. Two faecal samples and 15 serum samples from 336 NI deer, and 55 faecal and 37 serum samples from 401 South Island deer, were positive. Estimates and 95% credible intervals (CI) are reported, based on the median and 2.5% and 97.5% quantiles of the posterior distribution. The estimate of IFC sensitivity was 77% (95% CI 61-92%) with specificity 99% (95% CI 98-99.6%). The Paralisa sensitivity estimate was 19% (95% CI 10-30%), with specificity 94% (95% CI 93-96%). All estimates were robust to variation of priors and assumptions tested in a sensitivity analysis. These data inform the use of the tests in determining infection status at the individual and herd level. They

may therefore be applied to developing herd classification programmes and to monitor the effects of control interventions in New Zealand deer herds.

## 6.2 Introduction

Deer farmers in New Zealand have been seeking to reduce the on-farm impact of paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In contrast to the chronic disease syndrome seen in adult sheep and cattle, the highest incidence of clinical paratuberculosis or Johnes disease is recorded in young (< 2-year-old) farmed deer, which succumb rapidly to emaciation and death, often within weeks (Mackintosh et al 2004). Industry-funded research has been directed at investigating risk factors for disease (Glossop et al 2007b), the influence of age at exposure on disease outcome (Mackintosh et al 2010b) and the efficacy of candidate vaccines (Mackintosh et al 2008a, Stringer et al 2009a). A national surveillance system to monitor the incidence of gross pathology typical of paratuberculosis, i.e. enlarged and/or granulomatous visceral lymph nodes, in the deer slaughter population has been established (Lynch 2007). The aim of slaughterhouse surveillance is to feed back information to affected herds and to encourage disease control, ultimately reducing the impact of paratuberculosis at farm level and the prevalence of infection and gross pathology in deer presented for slaughter.

A baseline estimate of individual animal (45%) and farm (59%) infection prevalence in slaughtered deer has been established (Stringer et al 2009b), to allow the effect of this and other interventions to be monitored. However, the factors influencing progression from infection to disease in deer are still poorly understood, and on-farm management measures, other than test-and-slaughter, are generally aimed at reducing exposure to the organism and minimising environmental and nutritional stress (Wilson and Castillo-Alcala 2004). On farms believed to be free of infection, ensuring that replacements are sourced from herds of similar infection status is an important factor in maintaining that status. It is also an expectation that stud deer presented for sale should not be infected. Herd classification programmes may be set up to assist such herds, using diagnostic testing to support absence of infection in putatively uninfected herds. Evaluation of herd classification and on-farm control programmes for deer requires an estimate of the accuracy of the currently available diagnostic tests, namely faecal culture and an IgG1 serum enzyme-linked im-

munosorbent assay, commercially marketed as the 'Paralisa'. Knowledge of diagnostic test sensitivity allows herd classification programmes to be developed using an accurate estimate of the number of animals that require to be tested in a herd for a given level of confidence in freedom from infection. The threshold number of positive results that can be found while still concluding that the herd is free from infection at the given confidence level requires knowledge of the test specificity.

Where a 'gold standard' reference test exists that perfectly identifies true infection status, sensitivity and specificity of a second test may be directly estimated. Although intestinal tissue culture is considered an early and sensitive method of classifying true MAP infection status (Whittington and Sergeant 2001), there is no gold standard test available for MAP infection in the live animal. The true infection status of the live individual is therefore unknown ('latent'). In this situation, latent class methods (Hui and Walter 1980) may be used to evaluate two conditionally independent tests when applied to two populations of differing infection prevalence. Modelling may use maximum likelihood methods (Pouillot et al 2002) or Bayesian inference (Johnson et al 2001). The Bayesian approach incorporates prior information on test accuracy and infection prevalence, and has the advantage that point estimates and probability intervals for test parameters can be estimated without the need for a large sample size (Enoe et al 2000).

The test characteristics of individual faecal culture (IFC) have not previously been established in the NZ deer population. While estimates of Paralisa sensitivity and specificity have been published for clinically affected deer (Griffin et al 2005), there is limited data on the performance of the Paralisa in young and sub-clinically infected deer.

The aim of this study was to estimate the sensitivity and specificity of IFC and the Paralisa to identify young deer infected with *Mycobacterium avium* subsp. *paratuberculosis*. The unit of analysis was the individual, and the primary objective was to assess test accuracy for the purpose of herd classification or 'freedom from infection' type sampling. Estimates of test sensitivity and specificity may also be used to adjust for misclassification in surveillance studies of the target population, i.e. inferring true from apparent prevalence, therefore the accuracy of the tests for use in prevalence estimation was also assessed.

## 6.3 Materials and Methods

### Study populations

Two populations of deer with differing infection prevalence were required for the analysis. Previous studies have suggested that the prevalence of clinical disease and MAP infection in deer is higher in the South Island (SI) of New Zealand compared to the North Island (NI). A national questionnaire-based study (Verdugo et al 2010) of deer farms carried out in 2008 found 25% reported suspect or laboratory-confirmed paratuberculosis in the NI compared to 39% in the SI, while a prevalence study of grossly normal mesenteric lymph nodes of slaughter deer in 2008/2009 (Stringer et al 2009b) found MAP in 29% of samples from the North Island and 51% from the South Island. These data supported the use of North and South Island deer as the two populations. Cross-sectional sampling of these populations was carried out between July 2009 and April 2010.

### Herds

Samples were sourced from deer herds participating in a national multi-species paratuberculosis and leptospirosis prevalence study involving a postal survey and on-farm sampling of a sub-set of responding farms. The survey is described in detail by Verdugo et al. (2010). The deer herds used for on-farm sampling were selected from a sampling frame of farmers with at least 40 deer ( $n=241$ ) who responded to a postal questionnaire providing information on clinical incidence of paratuberculosis over the previous three years. Sample size for the test validation study reported here was limited to 750 paired samples for logistical and resource reasons. A subset of 20 herds from the South Island and 18 from the North Island were selected for blood and faecal sample collection, based on maintaining the same proportions of clinically affected and putatively infection-free herds as calculated from the postal questionnaire data. Twenty five percent of North and 40% of South Island herds reported clinical paratuberculosis in the survey, therefore samples were processed from 5 on-farm survey herds that reported suspect or confirmed disease from the North Island and 8 from the South Island. Sampled herds were distributed throughout the deer farming regions of New Zealand (Figure 6.1).



**Figure 6.1:** Distribution of deer herds (n=37) used for a test validation study of individual faecal culture and Paralisa in yearling deer

## Tests

Samples were collected on-farm by veterinary practitioners or technicians supplied with comprehensive written instructions. Whole management groups of deer 12-24 months of age were gathered and samples of faeces (10g) and blood (10ml) were collected from twenty randomly selected clinically normal individual deer in each herd. Faecal samples were taken per rectum using a fresh glove for each sample and blood samples were collected by jugular venipuncture into 10ml vacutainer tubes, using a new sterile needle for each animal. The sampling instructions were field-tested with three veterinary practitioners at the beginning of the survey to ensure they were clear to understand and practical to implement. Confirmation was sought from each veterinarian that fresh gloves had been used to collect each faecal sample before the samples were used for this study.

Fresh faecal samples for MAP culture were sent in chilled insulated containers to the mycobacteriology laboratory at the National Centre for Biosecurity and Infectious Diseases in Wellington, New Zealand. The culture procedure applied a decontamination step using cetylpyridinium chloride and BACTEC 12B liquid culture medium containing egg yolk, mycobactin and antibiotics as described by (Whittington et al 1999). All positive cultures were confirmed for presence of the IS900 sequence by PCR.

Blood samples were centrifuged at 3000rpm (1512xg) for 15 minutes and serum samples were drawn and tested fresh with the Paralisa at the Disease Research Laboratory, Otago University, Dunedin. The Paralisa is a customised IgG1 antibody ELISA test (Griffin et al 2005) that uses two antigens, MAP protoplasmic antigen (PPAg) and MAP purified protein derivative (PPDj), and an in-house anti-deer antibody. Optical density (OD) values were converted to ELISA units by subtracting the OD of a known negative and multiplying by 100 and samples were scored positive or negative if the cut point of 50 ELISA units was reached for either antigen.

## Statistical analysis

A Bayesian latent class model, based on the two-test, two-population model described by (Johnson et al 2001), was developed to estimate the sensitivity and specificity of the IFC and Paralisa tests. The estimates were derived from the median of the posterior distributions of the parameters and associated 95% credible intervals. The model was

fitted using WinBUGS (Spiegelhalter et al 1996) and was run for 100,000 iterations, after discarding a burn-in period of 5,000.

Let  $a \in \{0, 1\}$  be the outcome of test  $a$ , and  $b \in \{0, 1\}$  be the outcome of test  $b$ , with 1 denoting test positive, and 0 the converse. Then let  $p_{ab}$  be the probability of observing the respective test combinations. For example,  $p_{11}$  is the probability that both tests are positive.  $Se$  and  $Sp$  denotes test sensitivity and specificity of test  $a$  (Paralisa) or test  $b$  (IFC). Independent multinomial distributions of the data  $y_{ik} = (y_{11ik}, y_{10ik}, y_{01ik}, y_{00ik},)$  within each herd ( $k$ ) in island ( $i$ ) were modelled such that:

$$y_{ik} \sim \text{Multinomial}(n_{ik}, p_{11ik}, p_{10ik}, p_{01ik}, p_{00ik})$$

and

$$p_{11ik} = \pi_{ik} Se_a Se_b + (1 - \pi_{ik})(1 - Sp_a)(1 - Sp_b)$$

$$p_{10ik} = \pi_{ik} Se_a(1 - Se_b) + (1 - \pi_{ik})(1 - Sp_a)Sp_b$$

$$p_{01ik} = \pi_{ik}(1 - Se_a)Se_b + (1 - \pi_{ik})Sp_a(1 - Sp_b)$$

$$p_{00ik} = \pi_{ik}(1 - Se_a)(1 - Se_b) + (1 - \pi_{ik})Sp_aSp_b$$

The true prevalence  $\pi_{ik}$  in the  $k$ th herd on the  $i$ th island is a zero-inflated logit-normal random variable such that

$$\pi_{ik} = z_{ik}\pi_{ik}^*$$

where

$$z_{ik} \sim \text{Bernouilli}(\varphi)$$

and

$$\text{logit}(\pi_{ik}^*) = \alpha + \mu_{ik}$$

where

$$\alpha \sim \text{Normal}(-0.26, 0.42)$$

and

$$\mu_{ik} \sim \text{Normal}(0, 1/\tau)$$

where

$$\tau \sim \text{Gamma}(1, 1)$$

Thus  $z_{ik}$  takes a value of 0 or 1, allowing herds to have a non-infected status, informed by  $\phi_i$ , which takes a beta prior distribution of the proportion of infected herds in each island. Then, conditional on  $z_{ik}=1$ , the within-herd prevalence is modelled using a normal linear model with a logit link function. The logit-transformed within-herd prevalence for both islands is represented by  $\alpha$  with  $\tau$  the precision of  $\alpha$ , while the random effect  $\mu_{ik}$  captures herd-level heterogeneity. The model assumes that the two tests are independent conditional on true infection status, with constant test accuracy across populations. Expert opinion and data from previous field studies provided the prior information on test sensitivity, test specificity and prevalence of infected herds in each island. Uncertainty about these values was modelled using independent beta prior distributions (Table 6.1), derived from the modal (most likely) values and 5th or 95th percentiles using the Beta Buster software programme (Chun-Lung 2010). For example, the most likely value for IFC specificity was considered to be 98% and the expert was 95% certain that the value was greater than 95%, corresponding to beta (151.8, 4.08).

Sensitivity analyses were conducted to assess the influence of the prior distributions on parameter estimates, using a Uniform (0, 1) distribution for within-island herd prevalence and optimistic and pessimistic priors for test accuracy.

Model convergence was assessed in WinBUGS by examining time series plots and by specifying three sets of initial parameter values and examining the corresponding Brooks-Gelman-Rubin plots and convergence statistic.

**Table 6.1:** Prior information on test sensitivity and specificity, herd prevalence and within-herd prevalence for Bayesian latent class analysis

Parameter	Prior estimate	5th/95th percentile	Distribution
Herd prevalence North Island	0.50	>0.1	Beta(1.53, 1.53)
Herd prevalence South Island	0.70	>0.5	Beta (6.33, 3.28)
Sensitivity faecal culture	0.50	>0.2	Beta (3.26, 3.26)
Sensitivity Paralisa	0.77	>0.1	Beta (1.35, 1.10)
Specificity faecal culture	0.98	>0.95	Beta (151.8, 4.08)
Specificity Paralisa	0.995	>0.95	Beta (70.9, 1.35)
Within-herd prevalence	0.30	<0.9	Logit-normal (-0.24, 0.42)

Examination of the MCMC mixing indicated dependence between the random effect and the precision of the within-herd prevalence ( $\tau$ ). A partially non-centred MCMC algorithm was hand-coded to test for the effect of mixing quality on the results (Papaspiliopoulos et al 2007). This technique helps to reduce the dependence between  $\tau$  and the vector  $\mu$ , thereby improving algorithmic efficiency. However, the results obtained were very close to those obtained using the less efficient, centred algorithm as implemented by WinBUGS indicating that the dependence between  $\mu_{ik}$  and  $\tau$  did not bias the estimates. Modelling and sensitivity analysis was therefore carried out in WinBUGS, due to its operational convenience.

## 6.4 Results

### Descriptive

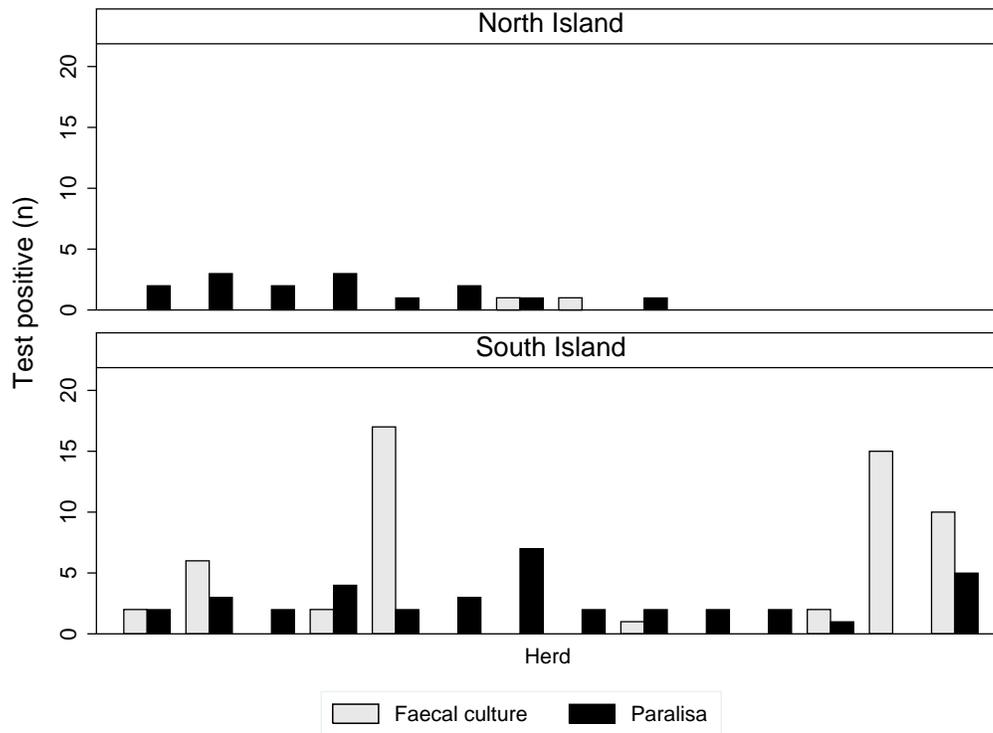
Data were available for 737 paired samples from 37 herds, 17 located in the North and 20 in the South Island and are presented in Table 6.2. Contamination of 7/20 of the faecal samples from the 18th herd sampled in the North Island meant that re-culturing on solid media was necessary and results were not available at the time of writing. Overall, 57 faecal samples were culture positive and 52 serum samples were Paralisa positive.

**Table 6.2:** Individual faecal culture (IFC) and Paralisa results by island

	IFC +	IFC -
North Island		
Paralisa +	1	14
Paralisa -	1	320
South Island		
Paralisa +	10	27
Paralisa -	45	319

The herd-level results are summarised in Figure 6.2. The number of culture positive samples in each herd ranged from 0-17, while the number of Paralisa positives ranged from 0-7. In samples (n=259) originating from herds reporting at least one suspected or

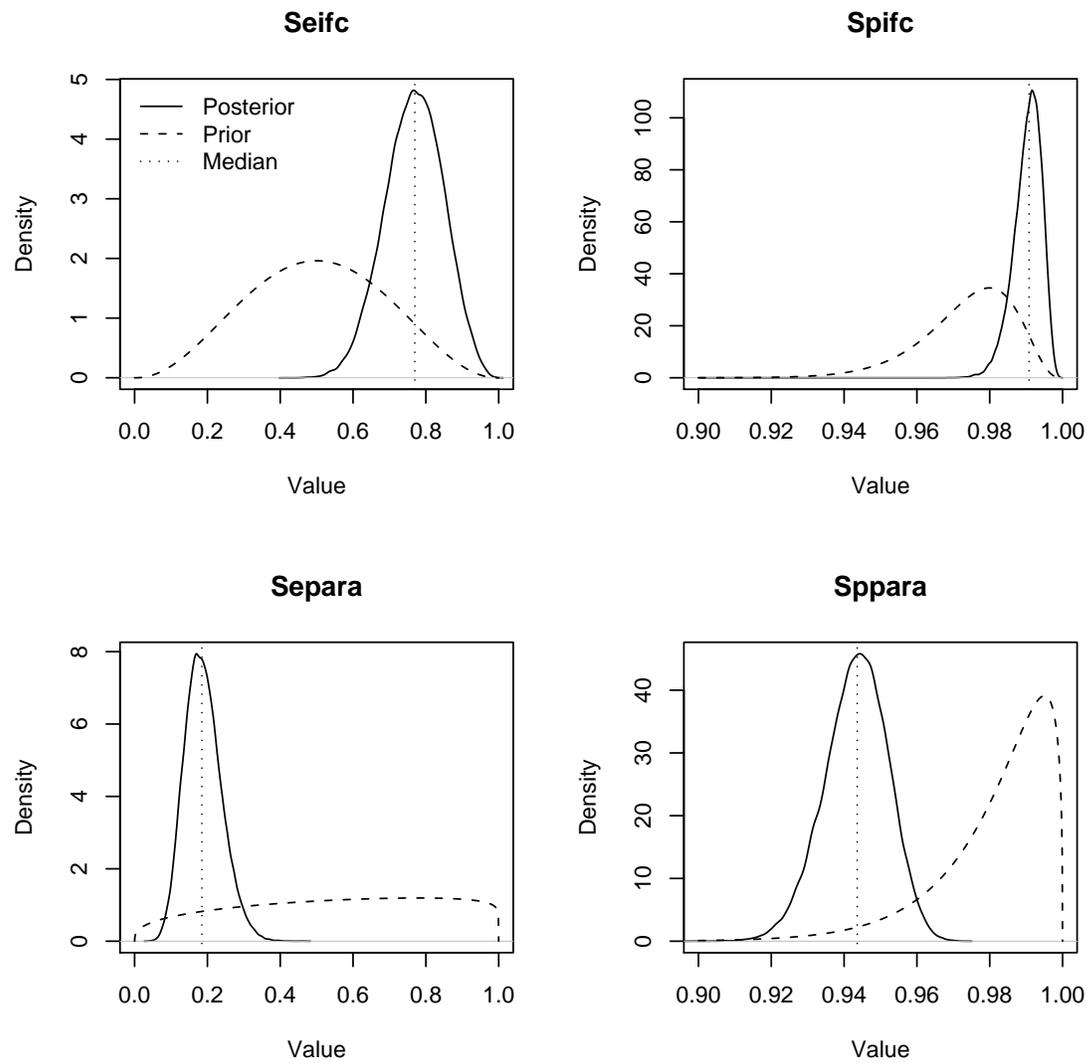
confirmed case of clinical paratuberculosis in 2006-08, there were 23 IFC and 25 Paralisa positives. There were 34 IFC and 27 Paralisa positives in samples (n=478) from herds that did not report suspicion of disease.



**Figure 6.2:** Number of faecal culture and Paralisa test positive deer in 9/17 herds sampled in the North Island and 14/20 herds sampled in the South Island of New Zealand

## Test sensitivity and specificity

The sensitivity of faecal culture to identify young deer infected with MAP was estimated at 77% and that of the Paralisa was 19%. The specificity estimates for IFC and Paralisa were 99% and 94% respectively. The estimates and 95% credible intervals for the test parameters are summarised in Table 6.3, while the associated posterior distributions are illustrated in Figure 6.3.



**Figure 6.3:** Prior and posterior distributions, and posterior median values of sensitivity (Se) and specificity (Sp) of individual faecal culture (IFC) and the Paralisa from Bayesian analysis

## Sensitivity analysis

Applying weakly informative priors for herd prevalence, or more pessimistic priors for test specificity, did not affect the estimates. Amending the test sensitivity priors influenced the median of the posterior distributions of the amended parameter, although the change was small in relation to the credible interval around the point estimate. In one herd, 15 faecal samples were IFC positive, but all serum samples were Paralisa negative. The model was run excluding all data from this herd to assess the corresponding influence

on the estimates. The estimates remained similar for all parameters except the Paralisa sensitivity estimate, which increased to 27% and had a wider credible interval (15-41%). There was no evidence of lack of convergence of the model when initiated from different starting values

**Table 6.3:** Posterior median estimates, SD and 95% credible intervals, obtained from Bayesian analysis, of sensitivity and specificity of individual faecal culture (IFC) and Paralisa to identify young deer infected with *Mycobacterium avium* subsp. *paratuberculosis*

Parameter	Median	SD	2.5% quantile	97.5% quantile
Sensitivity IFC	0.77	0.08	0.61	0.92
Sensitivity Paralisa	0.19	0.05	0.10	0.30
Specificity IFC	0.99	0.004	0.99	1.00
Specificity Paralisa	0.94	0.009	0.93	0.96

## 6.5 Discussion

This study used a cross-sectional sampling design and Bayesian latent class analysis to estimate the performance of individual faecal culture (IFC) and Paralisa to identify MAP-infected yearling deer. Sensitivity of IFC and Paralisa was estimated at 77% (95% CI 61-92%) and 19% (95% CI 10-30%) and specificity at 99% (95% CI 99-100%) and 94% (95% CI 93-96%), respectively.

Direct estimation of test performance is carried out using gold standard methods, with samples from animals of known infection status. As these are necessarily classified by one or more reference tests, they may have an inherent ‘spectrum of disease’ bias, as they often represent the more severe cases or most heavily infected subjects in a population. Internal validity, i.e. the generation of unbiased estimates, is more important than precision in test evaluation, since biases of estimates at the individual animal level may have a significant impact when applied to herd-level interpretation (Christensen and Gardner 2000). Latent class methodology has the advantage that estimates of test accuracy can be obtained without the need for a ‘perfect’ reference test, and it is thus less prone to bias introduced by the reference test. In addition, establishing a panel of samples of known status for gold standard methods can be logistically challenging and costly for the required

sample size, even if partial verification methods are used, i.e. the population is screened with the new test and only a proportion are followed up with the gold standard (Begg and Greenes 1983). A further advantage of the Bayesian approach is thus the reduced requirement for a large sample size. The selection of the Bayesian latent class method for test evaluation was therefore not only related to the lack of a gold standard test for MAP infection in the live animal, but also to consideration of potential bias and cost. Bayesian methods also allow complete blinding at all levels of sample collection, processing, testing and analysis. No herds or animals for which test results were available were excluded, so another possible source of selection bias was eliminated.

A key assumption of the model used in this type of study is that the candidate tests are conditionally independent, i.e. that the outcome of one test does not depend on the results of another for infected and non-infected animals. Tests which measure different biological processes are likely to be independent of each other (Gardner et al 2000). Since faecal culture identifies the presence of the MAP bacteria and Paralisa detects serum antibody, the assumption of conditional independence is therefore reasonable.

Variation in test sensitivity and specificity between and within populations is recognised (Branscum et al 2005), and it was expected that there would be differences in infection prevalence between the herds. Individual within-herd prevalence was therefore modelled using random effect logistic regression. The estimates derived from this analysis were population-averaged sensitivity and specificity values. However, it is recognised that if there is a difference in test performance between the two main populations, in this case the North and South Islands of New Zealand, there may be a corresponding effect on the estimates of test sensitivity. They may be biased towards the population with highest disease prevalence, since most of the data supports that estimate (Toft et al 2005). The sensitivity of the tests may therefore be overestimated, as the majority of the data on test positive animals (92/109) originated from South Island herds.

The target population was clearly defined as clinically normal yearling deer and the primary test purpose was ‘freedom from infection’ sampling, as this may be used in developing herd classification schemes. While the specificity of culture methods to identify the presence of MAP is considered to be 100%, it is thought that animals may ingest MAP which is then detectable in faeces although the animal is not infected, referred to as ‘passive shedding’. The frequency of occurrence and the relevance of this in disease

transmission have not been established. It is difficult to know with certainty that faecal culture positive animals are not infected, given the lack of absolute sensitivity of tissue culture and histopathology methods. If passive shedding does occur, the specificity of IFC to classify deer as infected may be less than 100%. The distinction is not an important one when herd infection status is the ultimate aim of testing, but it may be relevant when the test purpose is animal-level infection prevalence estimation.

Accordingly, the prior value used for specificity of faecal culture was 98%, and a more pessimistic lower bound of IFC specificity was modelled during sensitivity analysis. The posterior distributions for the test parameters remained similar using the adjusted prior, and it is thus reasonable to use the summary values reported here as test accuracy for prevalence estimation.

The source population for the study was based on response to a postal questionnaire and active participation in a nationwide on-farm sampling survey for paratuberculosis and leptospirosis. The resulting sampling frame can introduce bias, as farmers with experience of these diseases may be more likely to respond, and thus animals may be less representative of the target population. However, the postal survey participants may have responded due to an interest in either leptospirosis or paratuberculosis, hence lowering the risk that there was bias in response due solely to experience of paratuberculosis. As there was a similar distribution of positive results in samples from herds that reported suspect or confirmed paratuberculosis and those that didn't, it is unlikely that response bias, if present, had an effect on external validity.

The estimate for the sensitivity of the Paralisa was lower than the estimate of 77% previously reported in sub-clinically infected deer (Griffin et al 2005). However, that study was carried out in clinically affected herds, in older animals and in the context of a test-and-cull control programme. Sensitivity of ELISA tests for paratuberculosis is related to the stage of infection, being lower in the early stage, and higher in high faecal MAP shedders (Collins 1996, Whitlock et al 2000). An estimated sensitivity of 91% for the Paralisa in clinically affected deer has been reported (Griffin et al 2005), indicating the effect of stage of infection on test sensitivity. It is plausible that test sensitivity in heavily infected and diseased herds is higher, as such herds are likely to have a higher proportion of animals in more advanced stages of infection than herds with no clinical disease.

A recent study in cattle has shown age to be a determinant of ELISA test performance,

with test sensitivity higher in older than in young dairy cattle (Norton et al 2010). The application of control programmes themselves may affect test performance. For example, the removal of test-positive animals may increase specificity by removing both infected animals and false positives from the population (Greiner and Gardner 2000). As test validation should consider the target condition, test purpose and the distribution of covariates that may influence performance in the population in which the test will be applied (OIE, 2004), direct comparison of these estimates is difficult.

In an experimental challenge trial in young deer (Mackintosh et al 2007), the Paralisa identified 27% (19/68) of sub-clinically infected deer in which MAP infection was confirmed by tissue culture. The proportion of sub-clinically infected deer that were IFC positive was 4/68 (6%). The sensitivity of IFC in the study was lower than previous estimates and the estimate presented from our study. One possible explanation is that during the experimental trial, faeces were stored at -20C prior to culture. These storage conditions can lead to significantly reduced MAP viability and thus lower culture sensitivity (Khare et al 2008). Our point estimate for Paralisa sensitivity (19%) was lower than the 27% described by Mackintosh et al. (2007) but that observation falls within the bounds of the credible interval of our estimate (10-30%). Additionally, the range of different herd and environmental exposures, MAP sub-types, and host genetics included in our study means that the results are a population-averaged estimate of test performance, rather than limited to a single specific combination of conditions.

In an Australian study (Schroen et al 2003), a deer-conjugate ELISA had a sensitivity of 33% in sub-clinically infected deer, when compared to tissue culture as a gold standard, using a cut-point giving a specificity of 99.5%. In contrast, the sensitivity and specificity estimates obtained in our study are more comparable to the range of values for ELISA in the same target condition (infection) in cattle, as reported in a review by Nielsen and Toft (2008), i.e. sensitivity 7-22%, specificity 85-100%.

The sensitivity estimate for individual faecal culture (77%) is high but plausible. Quantitative studies have suggested that the concentration of MAP in faeces of infected deer is an order of magnitude greater than that of cattle (O'Brien et al 2010). Faecal samples from clinically normal 11-month-old deer tested with IFC in a previous study (Stringer et al 2009a) found animal-level apparent prevalence of up to 80% in herds where there was clinical disease.

Although a number of studies have used latent class methods to estimate sensitivity of IFC in cattle (van Schaik et al 2007, Norton et al 2010, Wells et al 2006), finding values ranging from 40% to 75%, there have been few reports of IFC performance in deer. In the Australian deer study, (Schroen et al 2003) IFC detected 67.5% of deer (112/166) with “confirmed JD” and 47% which were tissue culture positive. However, the case definition of “confirmed” includes faecal and tissue culture and histopathological examination and there is no information on age or clinical disease status, making comparison difficult.

Clinical disease is seen in yearling deer more frequently in winter and spring (Glossop et al 2008) and, as sensitivity of both tests is related to disease severity, season may have an influence on the resulting estimates. In yearling deer it is difficult to separate the effects of herd, age and season to examine seasonal effects on test performance. The sampling period covered all seasons and ages of deer from 12-24 months, thus had the advantage that average values for test performance in yearling deer were obtained.

The sensitivity analyses showed that the model outputs were robust to changes in prior values, being driven by the data rather than the prior values. The inherent difference in prevalence of infection between the islands was the key driver of the robustness of the estimates, although the relatively small number of positive samples meant that there were wide credible intervals around the sensitivity estimates.

The hypothesis that there is an effect of strain type on MAP pathogenicity in deer is supported by evidence from the previously described experimental infection study (O’Brien et al 2006, Mackintosh et al 2007), in which a type I MAP isolate used was found to be less pathogenic in young deer than a type II isolate. It is plausible that there could be a corresponding effect of MAP type on test sensitivity. The Paralisa was developed using samples from clinically affected herds, in which type II isolates may be more prevalent. The MAP pathogenicity study of Mackintosh et al. (2007) found 1/11 (9%) of the sub-clinically infected deer challenged with a medium dose of the type I isolate were Paralisa positive compared to 5/16 (31%) of those challenged with the same dose of a type II isolate. There are no robust data on the relative prevalence of type I and type II strains of MAP in deer in New Zealand either in clinically affected or sub-clinically infected herds. Investigation of the effect of strain type on test performance would therefore be a useful enhancement to this study.

In summary, this study has estimated the sensitivity and specificity of Paralisa and IFC to

identify yearling deer sub-clinically infected with MAP. The estimates may be applied in developing herd classification programmes and to monitor the effects of control interventions in New Zealand deer herds.

## **6.6 Acknowledgements**

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## General Discussion

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### 7.1 Introduction

The aim of this thesis was to provide epidemiological evidence that can be used to inform strategy, at industry and farm-level, for control of disease and infection due to *Mycobacterium avium* subsp. *paratuberculosis* in deer.

The work presented in the previous chapters has established a baseline individual animal and herd-level prevalence estimate for MAP infection in slaughter deer, assessed the efficacy of a candidate vaccine for control of clinical and subclinical disease in young deer, examined the effect of using the candidate vaccine as well as MAP infection *per se* on the specificity of diagnostic tests for bovine tuberculosis in deer and estimated the sensitivity and specificity of faecal culture and the Paralisa to detect MAP infection in clinically normal young deer.

The thesis began with a review of the evidence base for the biological and cost effectiveness of interventions that may be applied for herd-level control of paratuberculosis in deer. The individual epidemiological studies conducted to achieve this aim were then presented as papers prepared for submission to refereed scientific journals, and referenced existing knowledge in each discussion section. That format allows only limited critique of the science, relevant literature and implications. Therefore, in this concluding chapter, the methodology and results of the thesis studies are considered individually in more detail, with suggestions for how they could have been enhanced, followed by recommendations for future work. The chapter concludes with a discussion of the implications of the thesis findings for control of paratuberculosis in deer at the national and herd-level.

## 7.2 Review of the individual epidemiological studies

The individual studies are considered in more depth in this section, which includes a discussion of design issues, collaborative work and additional data collection or analysis which was originally planned or now recommended. The implications of the results of each study in the wider context of paratuberculosis control are discussed in section 7.3.

### **Nationwide MAP prevalence study**

The aim of the prevalence study (Chapter 3) was to estimate the prevalence and describe the geographical distribution of MAP infection in the New Zealand deer population. The study was the first to establish national MAP infection prevalence in slaughter deer. There had been no previous work to determine the national prevalence of infection or incidence of clinical disease in New Zealand deer or deer herds. Some published articles refer to a high clinical disease incidence (>20% in weaners, i.e. up to 1-year of age) in some New Zealand deer herds (Griffin et al 2005, Mackintosh et al 2004, Glossop et al 2008). However, the reports are based on information from individual affected herds; the national prevalence of herds experiencing such levels of disease has not been established. A study by our research group is currently under way to estimate the national prevalence of deer herds with MAP infection and clinical disease (Verdugo et al 2010). Although the Chapter 3 study provided an estimate of infected herd prevalence, it was designed for individual animal rather than herd level infection prevalence estimation; four samples per consignment of slaughter deer were collected, which was not sufficient to determine infection status at the herd level, and only 57 herds were sampled. The prevalence of infected herds was thus likely to be underestimated.

The abattoir population was chosen for several reasons: sample collection was logistically fairly straightforward and could include a larger number of herds in a short period of time than on-farm sampling; a highly sensitive and specific test (tissue culture) could be applied to samples; and random selection of carcasses and herds minimised the selection bias that may result from voluntary participation in studies such as that of Glossop et al. (2007b). However, abattoirs are not a sterile environment in which to take bacteriological samples and although sample collection was carried out with a sterile technique, the risk of cross-contamination remained. This is less of an issue where the data are used as a

baseline against which to measure future abattoir-based prevalence estimates. However, as a stand-alone measure the prevalence may be over-estimated if cross-contamination did occur.

Concern about the possible public health implications if MAP should be shown to be a causal factor in Crohns disease may make interpretation of the prevalence data more important. Therefore in the paper prepared for publication, careful reference was made to sampling of nodes rather than carcasses, as no inference on the presence of MAP in muscle tissue was intended.

At the design stage of the prevalence study, a specific initial objective was to characterise the resulting MAP isolates. This work would have been a pilot study to describe the distribution of molecular sub-types in the population, as no such study had previously been carried out in the New Zealand deer population. I was particularly interested to compare sub-type distributions between the North and South Islands. Previous work (Glossop et al 2008) had suggested a higher prevalence of infected deer herds in the South Island, and this was also found in the nationwide prevalence study. I intended to test the hypothesis that molecular sub-type is a factor explaining this observed difference, and also intended to use the data to examine the association between the presence or absence of MLN gross pathology and sub-type. Although there is limited information on the molecular sub-types of MAP found in cases of Crohns disease, it appears that a particular group may be associated with human infection (Motiwala et al 2006). Knowing which strains affect deer, and whether infection with these may be detected at post-mortem examination may have an impact on how human health risks are managed, should MAP become a public health issue.

Accordingly, I initiated a collaborative project with the Moredun Research Institute in Scotland and undertook training in pulse-field gel electrophoresis and MIRU-VNTR methods. The work was intended as a pilot study to help determine the most suitable method for characterising the New Zealand MAP isolates. However, despite obtaining an import licence and setting up export certification, we were unable to get agreement to access the stored isolates from the laboratory at Wallaceville. Subsequently, the development of a sub-typing system for New Zealand MAP isolates was funded by the national Johnes Disease Research Consortium. Unfortunately the system under development for the NZ population of MAP isolates was not finalised at the time of writing, but tissue and isolates

have been stored so that the work described above can be done in the future.

The prevalence data from the Chapter 3 study may be used to inform decisions about control at the industry level, and provides a baseline against which to measure industry-level interventions. The data may also be used to validate the national deer surveillance system operated by Johnes Management Limited (JML). The culture data represent one input to estimating sensitivity and specificity of the whole system to detect a MAP-infected herd, using for example scenario tree modelling, and have already been used as part of a validation study of MLN size as a predictor of MAP infection (JC Hunnam, unpublished data). The recently developed mission statement of JML is to “develop and maintain a database for the monitoring of Johne’s Disease in deer as a cost-effective tool for reducing the risk and cost of this disease to the New Zealand deer industry” (S. Norton, JML, pers. comm.). JML feeds back information to deer farmers on post-mortem pathology typical of paratuberculosis, and supports them in controlling disease on-farm, using a network of veterinarians trained as advisors in on-farm management of paratuberculosis. The impact of the initiative on infection prevalence in slaughtered deer can now be measured, by comparing the results from similarly conducted prevalence studies against the baseline data from Chapter 3. Providing data to assist in validating the surveillance system, as well as a means to measure progress, is important in motivating and maintaining action. The work presented in Chapter 3 thus contributes to control of paratuberculosis in a broader context.

### **Vaccine efficacy trial**

The vaccine study was conducted as a randomised controlled trial (RCT), long recognised as the most rigorous way of determining the effect of an intervention. Sir Austin Bradford Hill is credited with introduction of this methodology into medicine in the UK in the mid-1940s, to test the efficacy of the pertussis vaccine, and the RCT has since become the foundation of evidence-based medicine (Stolberg et al 2004).

The initial experimental design for the study was a rather ambitious effort. It included plans to test a proportion of all trial deer with the Paralisa before vaccination, and to continue to serially test the controls with the Paralisa and both cohorts with faecal culture. The aims were to: (1) estimate the proportion of deer Paralisa positive in each cohort at the beginning of the trial; (2) assess the association between Paralisa positivity and

clinical and subclinical disease outcome; (3) compare vaccine efficacy in deer that were Paralisa positive and negative at the beginning of the trial; (4) describe the time of onset of faecal MAP shedding; and (5) assess the relationship between onset of faecal MAP shedding and Paralisa titre. In an ideal situation, with unlimited financial and manpower resources, and farmers with limitless time and patience, the additional data collected may well have expanded our knowledge. However, the cost and logistics of the extra work was substantial, and with the benefit of hindsight (particularly the small number (n=25) of clinical cases which occurred), the decision not to proceed was fortuitous. Furthermore, the results of the Chapter 6 test validation study suggest that misclassification of infection status by the Paralisa may have affected some of the inferences from the additional analyses.

I was very keen that the field work of the vaccine trial would also benefit other paratuberculosis researchers, and held discussions with other groups, including AgResearch and the Pathobiology group at the Hopkirk Institute, Massey University, to see what other useful data or samples could be collected. An initial suggestion to collect blood samples from all trial deer for host genomic research again proved too ambitious logistically, but we were able to provide lymph node samples from clinical cases to Alan Crawford at AgResearch for that purpose. Serum samples from clinical cases were also provided to Made Sriasih in the Hopkirk Institute, where they were used in a PhD project to identify proteins secreted by MAP strain 316F (the vaccine strain) for application in diagnostic tests.

The decision to manage vaccinated and control deer in the same management groups was taken only after much consideration of the practical advantages and the potential effects on the outcome measures. Ensuring that vaccinates and controls had the same exposures other than the intervention was high priority, and the design also helped to blind the farmer and clinician to the treatment group. An additional consideration was ensuring farmer compliance with the trial protocol by keeping the management of vaccinates and controls as simple as possible, i.e. within already established mobs. However, vaccine interventions are generally applied at the population level. As discussed in Chapter 4, the disease challenge represented by the presence of unvaccinated individuals in the design used can result in an underestimate of the effectiveness of vaccination when compared to applying it to the whole population. There are methods available which use simulation

modelling to extrapolate the effect of vaccination in a whole population from a study in which partial vaccination has been carried out (Carpenter 2001). Applying such modelling to the data from the vaccine trial may be useful additional work. The results could then be used as an input to evaluating the cost-effectiveness of whole-herd vaccination for a range of population sizes.

Although the vaccine study found that vaccination reduced the incidence of clinical disease and subclinical pathology, it showed no effect on production parameters such as liveweight gain, or time to reach slaughter weight. There is little evidence in the published literature of the effect of infection *per se* on deer production measures. One study (Thompson et al 2007) reported a lower pregnancy rate in subclinically infected hinds selected for a study examining intrauterine MAP transmission compared to “unaffected animals” from the farms (69% vs 85-90% pregnant). The findings were essentially anecdotal, as only the infection status of the 24 deer selected for the trial was established; there were no data presented on infection of the remainder of hinds on the farms. Production losses, particularly of milk production, associated with MAP in dairy cows have been well documented (Benedictus et al 1987) but in deer there are, as yet, no robust data on production losses associated with subclinical infection in the live animal.

There are, though, data on the effect of lymph node pathology on carcass weights in young deer. Carcasses with MLN pathology, as recorded to the JML database, were 2.6-4.7kg ( $p < 0.001$ ) lighter than those with no recorded pathology in the period January to June 2010 (Goodwin-Ray 2010), while the proportion of carcasses with MLN pathology was 0.36%.

It was not possible to source data from the culture process to derive a robust quantitative measure of the amount of MAP excreted by vaccinates. However, the crude analysis of time to culture positivity data did not find a difference between vaccinates and controls, suggesting that vaccination may not lead to a reduction in transmission or consequently within-herd infection prevalence. The benefits, therefore, of using Silirum vaccine may be limited to the effect on clinical losses. A detailed cost-benefit analysis of vaccination is recommended, but using the crude data from the herds which experienced clinical disease during the trial, the cost of vaccine (\$5.25 a dose) was \$6,500 while the benefits, in terms of venison weight at 2008 market prices (\$9/kg) was \$5,100. The clinical disease incidence of 1.5% recorded in the vaccination study was too low, therefore, for vaccination

to be an economic option. Benefits do not exceed costs until there is a disease incidence in excess of 3%. The vaccine trial provided the first estimate of the field efficacy of a vaccine for paratuberculosis in naturally infected young deer under normal management conditions. Consequently, Silirum has been licensed and is now marketed for control of clinical paratuberculosis in deer in New Zealand.

### **Effect of vaccination and infection on TB test specificity**

The original design for the Chapter 5 (TB test specificity) study involved carrying out TB testing and faecal sampling, at the same time and on the same animals, during the vaccine efficacy trial. The effect of MAP infection *per se* on the specificity of TB tests at the individual level could have been assessed using the data collected. However, there was a conflict between the timing of the field work for each purpose. The faecal sampling was done in November 2008, when the final liveweight measurements were being taken, as by then finished deer were already being slaughtered and the population was becoming less representative. However, deer are not normally TB tested until they are 15 months of age, so the optimal time for TB testing was in February 2009. In addition, the logistics of weighing and faecal sample collection and despatch meant that TB testing at the same time was not feasible. In the end, the TB testing was carried out in January 2009, as by February there were likely to be few trial deer left on farm.

Another initial aim of the TB test specificity study was to assess how long after vaccination animals would still react positively to the TB tests. Although most venison deer are slaughtered at 10-15 months, some specialist supplier groups finish venison production deer over a 24-month period. Such a group was identified in the North Island, but it proved difficult to recruit individual farmers and this element of the study was not pursued. A study to evaluate the persistence of MCT reactivity in vaccinated deer is therefore still recommended, to provide further data to farmers and policy-makers on the longevity of the effect of vaccination on TB test specificity.

A proposed extension of the TB test specificity study aimed to assess and quantify, at national level, the association between deer farms with MCT positivity and those identified as having slaughtered deer with gross MLN pathology typical of MAP infection, using data from the JML database. A relative risk map was planned, to describe the spatial distribution of the relationship and to examine any variation in risk between the North and

South Islands. I planned to use spatial regression techniques to analyse the data further, incorporating information on potential environmental factors such as soil type, rainfall, and humidity. The planned study was essentially a hypothesis-generating one: to identify geographical and environmental factors for more detailed investigation, and to explore possible factors to explain the apparent difference in MAP infection and disease prevalence between the islands. Both the Animal Health Board (AHB) and JML were keen to collaborate and provide data, but limited resources at the AHB meant that the TB data could not be provided in time for the study to be included in the thesis. However, I still hope to obtain the data and carry out the study, and it may soon be possible to additionally incorporate MAP molecular subtype information in the analysis.

The results of the TB test specificity study were considered in the context of those from other researchers in Chapter 5. However, only the specificity aspect of test performance was assessed, as that was a significant concern of farmers. The effect of vaccination on sensitivity of TB diagnosis is clearly the more important issue for the national TB control scheme. Recent research on novel serological tests (Buddle et al 2010), discussed in Chapter 5, suggested they may have high sensitivity for detecting TB-infected vaccinated deer. If this proves to be the case, it may be appropriate to use the CCT as a screening test in vaccinated herds. Further data are needed, but herd-level sensitivity may be sufficient to detect a TB-infected herd and following confirmation of infection, the novel serological tests may then have an application in within-herd TB control.

The work presented in Chapter 5 was important in providing empirical data to address concerns about reduced TB test specificity in vaccinated deer. The data informs both individual farmers and the national authorities about the possible consequences of using vaccination to control paratuberculosis, and thus contributes to the wider evidence base underpinning decision-making on control options.

### **Latent class analysis to evaluate diagnostic test performance**

The Chapter 6 study was undertaken to establish some of the key information needed to design a paratuberculosis herd classification scheme for deer farms. The estimates of diagnostic test sensitivity and specificity may also be used to interpret the data from infection prevalence studies applied at farm or national level. The test characteristics of IFC had not previously been established in the NZ deer population, and there was limited data

on the performance of the Paralisa in young and sub-clinically infected deer. The original study design applied a gold standard approach, using samples collected at slaughter. Blood samples were to be collected from stunned deer at the sticking point in the abattoir, and MLN and faecal samples were to be collected from the same deer. Lines of slaughter deer in which there were carcasses with MLN pathology were to be targeted as a potential source of infected deer for sensitivity estimation, while lines in which all carcasses had normal MLN were a possible source of uninfected deer. The historical data on gross MLN pathology would also have been sourced from the national JML database for herds presenting normal lines.

However, the original study design was not used, as data (S.Liggett, pers.comm) showed the sensitivity of the Paralisa was reduced in serum collected post-mortem compared to ante-mortem sampling. The logistics and cost of sourcing faecal and serum samples on farm from tissue culture positive and negative deer in order to apply a ‘gold standard’ analysis were prohibitive. It was also possible that a population of tissue culture positive deer may have been biased towards animals in the more advanced stages of infection. These factors, combined with the lower sample size required for Bayesian methods, made the study design and statistical analysis described in Chapter 6 the method of choice for the study. Latent class analysis is a well established method, and is recognised by the OIE as part of the validation process for diagnostic tests (OIE 2004).

The estimates of Paralisa performance (sensitivity 19%, specificity 94%) differed substantially from the results (sensitivity 77%, specificity 99.5%) previously published by the Disease Research Laboratory (DRL) at Otago University (Griffin et al 2005), where the test was developed. The sensitivity analyses carried out showed the model outputs to be robust to changes in the priors for herd prevalence, within-herd prevalence and test specificity and only slightly affected (by a maximum of 5 percentage points) by adjusting the priors for test sensitivity. The small number of test positives in the dataset accounted for the effect of the priors on test sensitivity, as there were fewer data than for the other parameters. There is a view among some Bayesian statisticians that adjusting the prior estimates should not be part of the modelling process, i.e. that the prior belief should not change. However, in this particular analysis it was important that the process was as transparent as possible, and ultimately the robustness of the model was supported by the sensitivity analyses.

As discussed in Chapter 6, there were limited independent data against which to compare the Paralisa sensitivity estimate. Experimental challenge trials are one possible source of data, but may not represent the natural challenge field situation, as there is often a high proportion of experimentally infected animals in a management group. For example, in a trial to assess the effect of different MAP strain types on clinical disease and pathology (Mackintosh et al 2007), one group of control deer were grazed with a group treated with a high dose of a type II MAP isolate. The control group became infected naturally, with 16/17 deer tissue culture positive at slaughter, although there were no clinical cases. Paralisa testing the control group found 11/16 (69%) to be test positive. However, the incidence of disease in the co-grazing challenged group was 31% (5/16), and the natural challenge to the control group was thus likely to be higher than that in a field situation. Additionally, Paralisa testing was carried out two weeks after intradermal TB testing. Intradermal TB tests involve injecting avian or bovine tuberculin, and may increase ELISA sensitivity (Mackintosh et al 2008), while reducing specificity (Varges et al 2009). In experimental trials, deer are often sourced from a single farm, and challenged with a single MAP subtype. All of these factors may explain the variation in Paralisa results from experimental trials of vaccine efficacy (Mackintosh et al 2008a, Mackintosh and Thompson 2007), age susceptibility (Mackintosh et al 2010b), MAP pathogenicity (Mackintosh et al 2007) and genetic resistance (Mackintosh et al 2010a). The proportion of sub-clinically infected young trial deer that have been Paralisa positive in these experimental trials ranges from 28% to 69%.

Faecal samples collected during the test validation study were also subjected to quantitative PCR analysis, as part of a collaborative project with DRL aimed at validating the PCR to detect deer shedding a high number of MAP organisms in faeces (specific data not presented). Only one of the 33 faecal culture positive samples that were scored non-nil by the PCR was classified as a 'moderate' shedder. The remainder of the faecal samples were classified 'low' or 'suspect' on PCR, suggesting that the majority of deer sampled on farm for the study were shedding at a low level. These data may also explain the relatively low sensitivity of the Paralisa estimated in this study compared to the published sensitivity estimate (77%). The latter was achieved in two herds with "significant losses due to JD". There may have been more high-shedding deer in those herds and correspondingly increased test sensitivity, although there were no quantitative data on concentration

of MAP in faeces presented by Griffin et al. (2005) to support or refute this explanation. The test validation study was designed to estimate population-level sensitivity and specificity for the purpose of herd classification or prevalence estimation. The study population thus comprised herds of unknown infection status. To validate the test for use in a test-and-cull programme would have involved a different study population of herds, for example those with a history of a high (e.g. >5%) annual clinical disease incidence, as these are the herds most likely to implement a test-and-cull programme. Test sensitivity may be higher in those herds than in the Chapter 6 study herds, as they are likely to have a higher proportion of animals in more advanced stages of infection, both clinical and subclinical. Sensitivity of ELISA tests is related to infection stage: test sensitivity can be considered as a “direct function of the distribution of the infection stages in the test population” (Collins and Sockett 1993), therefore it cannot be assumed that the results of the Chapter 6 study are directly applicable to use of the test in the initial stages of a control programme.

It was useful to estimate the sensitivity of IFC to detect individually infected deer. However, it has the disadvantage of expense (\$55 per sample compared to \$15 for the Paralisa) and results take up to three months to be confirmed. During the validation study, faecal samples (n=10) from individuals were also pooled and cultured. Further work, combining the data from individual and pooled cultures is planned, so that the sensitivity of pooled faecal culture can be estimated. Pooled samples represent a more cost-effective way of establishing herd infection status, but there is currently no published data for the technique in the New Zealand deer population.

There was, however, a significant disadvantage to using the latent class modelling approach. The statistical analysis underlying the method is complex, and is not easily explained or understood. Although Bayes theorem has been applied in statistics over hundreds of years, statisticians and epidemiologists working in the veterinary field have only recently begun to use Bayesian analysis. The number of veterinary epidemiologists experienced in the method is therefore limited. Communicating the results of the study in a clear and transparent way, and having them understood and accepted by other scientists was already difficult. An even greater challenge was to have the study results accepted as robust by industry leaders and decision-makers. Indeed, at the time of writing, the funders of the study (DEEResearch) have refused permission for the work to be submitted for

peer-reviewed publication, and have prevented the results being communicated to the deer industry or field veterinarians. I recognise that there are other issues including political and commercial sensitivity surrounding these decisions. However, given the controversial nature of the results, I believe a straightforward ‘gold standard’ analysis producing the same results would have been more easily understood and have had a greater chance of acceptance.

### **7.3 Implication of thesis findings for paratuberculosis control**

Eradication may be considered as the ultimate achievement of control (Schukken et al 2007), but it is not feasible, or possibly desirable, to eradicate every disease that can be controlled. One definition of eradication is “termination of all transmission of infection by extermination of the infectious agent through surveillance and containment” (Last 2001). However, international initiatives to eradicate human pathogens have been successful in achieving eradication, according to that definition, only in the case of smallpox (Fenner 1988). A definition of eradication possibly more relevant to veterinary medicine refers to the “reduction of prevalence in a specified area to a level at which transmission does not occur” (Andrews and Langmuir 1963). Eradication is considered to be a “time-limited capital investment” (Yekutiel 1980). Conversely, control implies an on-going process, and therefore continuing financial support, to “reduce morbidity and mortality from disease. . . embracing all measures intended to interfere with the unrestrained occurrence of disease, whatever its cause” (Thrusfield 1995).

The lessons learned from public health campaigns have led to the development of three types of criteria to take into account when considering an eradication programme: “(1) biological and technical feasibility, (2) costs and benefits and (3) societal and political considerations” (Aylward et al 2000). These criteria may similarly be applied when considering eradication or control of infectious disease in livestock populations. The first two criteria described are relevant to voluntary action on livestock disease at the farm level, while the third is also pertinent to national level programmes. For example, risk to public health is one obvious driver for a compulsory livestock disease eradication campaign such as the one for BSE in Europe.

Considering paratuberculosis specifically, there have been few national-level attempts at eradication. Few countries consider eradication of MAP infection in domestic livestock to be a realistic goal. The ‘biological and technical feasibility’ criterion against which to consider eradication has not been satisfied in many regions, due to the complexity of the host-pathogen interaction, persistence of MAP in the environment and in wildlife populations, lack of highly sensitive diagnostic tools to detect infection and limited evidence of the effectiveness of individual interventions to reduce transmission in all species. The focus of efforts internationally, therefore, has been on control of paratuberculosis infection and disease rather than eradication of infection. Multidisciplinary collaborations such as the US Johnes Disease Integrated Programme (JDIP) and the European ParaTBTools initiative have been established to research diagnostic methods and other aspects of control of paratuberculosis in domestic livestock.

In New Zealand, the Johnes Disease Research Consortium (JDRC) has been established to direct research that may provide tools for control strategies for paratuberculosis within the sheep, cattle and deer industries. National-level control programmes have not yet been implemented, as “current methods are not considered economic or capable of delivering significant benefit to farmers if used as the basis of a national control programme” (Burton, 2007).

One of the objectives of JDRC is “a reduction in human exposure to *Mycobacterium paratuberculosis* from animals or products” (Burton, 2007). The studies presented in this thesis have provided evidence to inform strategies directed towards that objective, but they have also raised issues that have implications for successful control of infection. The high prevalence of infected slaughter deer (45%) and herds (59%) found in the Chapter 3 study indicated the extent of the task of reducing infection faced at national level. That clinical disease is the exception in MAP infection was aptly demonstrated by the proportion of deer that were faecal culture positive in the vaccine efficacy study; 80% in one herd which had a clinical disease incidence of 0.7%. The vaccine study and test validation study both found infection in herds with no concurrently observed clinical disease.

These findings raise questions about the benefits that individual farmers will realise from attempting to control infection *per se*. While the Silirum vaccine was shown to be effective in reducing the incidence of clinical disease and sub-clinical pathology, there was no demonstrable effect on infection prevalence (as measured by faecal culture), or on pro-

duction parameters.

However, whether reducing sub-clinical infection is an economically desirable goal for individual herds, in the absence of losses from clinical disease, needs closer examination. There is very little data quantifying the impact of subclinical infection in deer, and data from both the vaccine trial and the prevalence study did not show an effect of infection on production performance in terms of mean liveweight gain or carcass weight. The JML database recorded 0.36% of carcasses with MLN pathology. When this figure is compared to the carcass infection prevalence estimate (39%) from Chapter 3, even taking into account the sensitivity of detection and temporal difference, detectable pathology appears a relatively uncommon outcome of infection. Therefore, the case for MAP infection *per se* being a source of economically significant losses in deer has not been made.

The upper CI limit of the estimate of Paralisa sensitivity in sub-clinically infected deer was 30%, suggesting limited usefulness as a tool for herd classification. However, the Paralisa may be more effective in identifying high faecal MAP shedders, or deer most likely to progress to clinical disease. The sensitivity and specificity of the Paralisa to detect these target conditions, and the effectiveness and cost-effectiveness of the approach when applied at the herd-level, has still to be quantified.

The challenge for decision-making on herd-level control of paratuberculosis, therefore, lies in defining the aim of control and therefore the level of intervention needed for individual herds. It may be that for some finishing herds with high clinical disease incidence, for example, control of clinical losses is the most practical and cost-effective outcome. Reducing infection prevalence *per se* may not be an outcome which, in the absence of public health or market access considerations, is economically attractive. Even in finishing herds with clinical losses, the losses may not be sufficient to warrant the costs of control. Despite reports of annual clinical disease incidence of up to 20% in young deer, farmers experiencing such large losses proved difficult to identify when I was trying to recruit herds for the vaccine efficacy trial. Deer veterinarians were able to suggest clients with maximum JD-related mortality losses of 5-7%, and even these were few in number. It may be, therefore, that a small number of widely publicised high incidence outbreaks have created an impression that clinical disease is a more frequent outcome than is the case in the deer herd population. Data is needed to describe the distribution of clinical disease incidence, as an input to economic analysis of the impact of paratuberculosis in

New Zealand deer herds. Such work is currently under way within our research group. Indeed, it may be that rather than defining the target outcome as control of disease or reduction of infection prevalence, a better approach at the individual farm level would be to examine what level of intervention is economically optimal. Previously described modelling studies (Dorshorst et al 2006) measured net cost-benefit outcomes for a combination of control strategies as an outcome, and it may be useful to apply this decision analysis approach to paratuberculosis control in deer.

Assuming that control of infection or disease caused by *Mycobacterium avium* subsp. *paratuberculosis* will remain a voluntary commercial decision for individual livestock enterprises overlooks the potential impact of MAP being classified a zoonotic pathogen. The deer industry is therefore wise to prepare for such an eventuality, but needs much more robust data (summarised in the following section) with which to make informed decisions. Evidence of a link between MAP infection and Crohns disease has been accumulating over the last number of years, with a meta-analysis of 28 human case-control studies (Feller et al 2007) finding a statistically significant association between the presence of MAP and Crohns disease. The organism has not yet been shown to play a causal role in the aetiology of Crohns disease, but research is continuing. Although there have been no studies to confirm the presence or absence of MAP in venison, the deer industries aim to reduce the prevalence of MAP infection in the deer slaughter population thus addresses risks to public health and to export market access should MAP be declared zoonotic. The challenge facing deer farming is to identify effective and cost-effective means of achieving that aim.

## 7.4 Future work

The studies presented in this thesis do not provide all of the information needed to recommend biologically effective and cost-effective control strategies for minimising economic losses from paratuberculosis in deer at herd or at national level. The following suggestions for further research identify some of the key areas where more detailed data is required:

1. A more accurate estimate of the prevalence and impact of MAP infection on clinical disease and subclinical losses within and between herds is needed, as inputs to assessing the economic impact of paratuberculosis at the industry and farm level. The inputs are also necessary for modelling and comparison of the biological and cost-effectiveness of different control strategies. Current research at Massey University is directed at estimating between-herd prevalence and will provide some data on the proportion of deer herds with reported clinical disease.

2. Longitudinal field studies are required to provide data to quantify the biological effectiveness and cost-effectiveness of test-and-cull strategies or of applying other specific interventions such as breeding for resistance in New Zealand deer herds. While simulation modelling can be used to evaluate control strategies, robust field data is needed to provide accurate input data to such modelling.

3. The effect of MAP molecular sub-type on pathogenicity in deer has not been explored beyond experimental infection work using single type I and type II isolates, and the observation that type II is the predominant isolate from clinical cases of paratuberculosis in deer. Once the sub-typing system for the NZ population of MAP isolates has been finalised, there are a number of epidemiological studies that should be implemented:

- The distribution of MAP sub-types in sheep, cattle and deer should be examined on multi-species farms. Molecular epidemiology of MAP isolates from different livestock sources on such farms may help to define the role of inter-species transmission in introducing and maintaining MAP in deer herds.
- The geographical distribution of sub-types should be analysed to test the hypothesis that the difference in prevalence of infection and incidence of clinical paratuberculosis between the islands is explained by differences in MAP molecular sub-type.
- Isolates from normal and abnormal MLN should be typed to analyse the effect of sub-type on MLN pathology.
- Typing the MAP isolates from the test validation study would allow assessment of the effect of sub-type on diagnostic test performance as well as describing the range of isolates found in individual herds.

4. The relative importance of vertical, pseudo-vertical and horizontal transmission routes for MAP infection in deer is not known. A longitudinal study to quantify the relative risk of infection and clinical disease in calves born to infected dams would be a key input to assessing the effectiveness and cost-effectiveness of culling the offspring of clinically diseased or infected hinds.

5. A survey on three MAP-infected deer farms found hedgehogs, rabbits, possums, cats, hares, ferrets and birds to be MAP tissue culture positive (Nugent et al 2007). The importance of wildlife in the epidemiology of MAP infection in deer, though, is not known. Wildlife may be spillover hosts or may be important vectors representing a risk to programmes to reduce of infection prevalence on deer premises. Again, molecular epidemiology may give insights to the role of wildlife in introducing and maintaining paratuberculosis in deer herds. If indicated, longitudinal studies could be used to compare the effect of wildlife control on the success of strategies to reduce within-herd MAP prevalence.

## 7.5 Conclusion

This thesis has provided epidemiological evidence that can be used to inform strategy, at industry and farm level, for control of paratuberculosis in deer. The baseline prevalence estimate of MAP infection in the subclinically normal slaughter deer population gives the industry robust data on which to base decisions about eradication or control. Considering the experience of eradication attempts in other countries, the current state of knowledge of the epidemiology of paratuberculosis in deer, and the lack of quantitative data on the impact of test and cull and other management interventions, the conclusion is that eradication of infection is not a realistic national goal for the deer industry.

While vaccination was shown to be effective in reducing clinical disease incidence in young deer, it appears unlikely to reduce infection prevalence. In high disease incidence herds, it may be a tool to minimise economic losses associated with clinical disease, but appears unlikely to have an effect on infection prevalence. However, the consequences of vaccination for the sensitivity and specificity of screening tests for TB is an issue which has still to be resolved; the optimal test strategy to maximise diagnostic test sensitivity while maintaining acceptable specificity has not yet been identified.

Investigating the characteristics of diagnostic tests showed that individual faecal culture was more sensitive than Paralisa for use in herd classification schemes. An estimate of the sensitivity of pooled faecal culture is under way, as IFC is prohibitively expensive. The industry is still considering whether to implement a herd classification scheme and these data provide some of the inputs to developing testing strategies for that purpose.

The research presented in this thesis contributes to knowledge on different aspects of paratuberculosis control in the New Zealand farmed deer population, as well as highlighting to farmers, scientists and industry the areas where more epidemiological information is needed.

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## Appendix 1: WinBUGS Bayesian model code

---

```
model
{

# Population 1
for (i in 1:ny1) {
myN1[i] <- y1[i,1] + y1[i,2] + y1[i,3] + y1[i,4]
y1[i,1:4] ~ dmulti(p1[i,1:4], myN1[i])

p1[i,1] <- pi1[i]*Separa*Seifc + (1-pi1[i])*(1-Sppara)*(1-Spifc)
p1[i,2] <- pi1[i]*Separa*(1-Seifc) + (1-pi1[i])*(1-Sppara)*Spifc
p1[i,3] <- pi1[i]*(1-Separa)*Seifc + (1-pi1[i])*Sppara*(1-Spifc)
p1[i,4] <- pi1[i]*(1-Separa)*(1-Seifc) + (1-pi1[i])*Sppara*Spifc

pi1[i] <- z1[i] * pistar1[i]
z1[i] ~ dbern(phi1)
logit(pistar1[i]) <- alpha + U1[i]
U1[i] ~ dnorm(0, tau)
}

# Population 2
for (i in 1:ny2) {
myN2[i] <- y2[i,1] + y2[i,2] + y2[i,3] + y2[i,4]
y2[i,1:4] ~ dmulti(p2[i,1:4], myN2[i])

p2[i,1] <- pi2[i]*Separa*Seifc + (1-pi2[i])*(1-Sppara)*(1-Spifc)
p2[i,2] <- pi2[i]*Separa*(1-Seifc) + (1-pi2[i])*(1-Sppara)*Spifc
p2[i,3] <- pi2[i]*(1-Separa)*Seifc + (1-pi2[i])*Sppara*(1-Spifc)
p2[i,4] <- pi2[i]*(1-Separa)*(1-Seifc) + (1-pi2[i])*Sppara*Spifc
```

```
pi2[i] <- z2[i] * pistar2[i]
z2[i] ~ dbern(phi2)
logit(pistar2[i]) <- alpha + U2[i]
U2[i] ~ dnorm(0, tau)
}

## Priors

# Sensitivity and specificity
Seifc ~ dbeta(3.26, 3.26) ## Mode=0.50, 95% sure Seifc >0.2
Spifc ~ dbeta(151.77,4.08) ## Mode=0.98, 95% sure >0.95
Separa ~ dbeta(1.35,1.1) ## Mode=0.77, 95% sure Separa >0.1
Sppara ~ dbeta(70.9, 1.35) ## Mode=0.995, 95% sure Sppara >0.95

#herd prevalence given infected
alpha ~ dnorm(-0.26,0.42) ## Mode=0.3, 95% sure <0.9

# precision (1/variance) of within-herd prevalence (alpha)
tau ~ dgamma(1,1)

#proportion of infected herds each island
phi1 ~ dbeta(1.53,1.53)#NI mode 0.5, 95%sure >0.1
phi2 ~ dbeta(6.33,3.28)#SI mode 0.7, 95% sure >0.5

}
```