Epidemiology, diagnosis and vaccination control of leptospirosis in farmed deer in New Zealand

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Abstract

Leptospirosis is a bacterial zoonotic disease of global importance. It is caused by infection with pathogenic *Leptopsira* species. Leptospirosis encompasses a wide spectrum of clinical or subclinical disease in both humans and animals. In New Zealand (NZ), leptospirosis is considered to be the most important occupational zoonosis. Livestock farming plays an important role as a major occupational risk factor for human leptospirosis and farmed deer is one of the contributing factors.

Commercial farming of deer began in NZ in the early 1970s. It remains the world's largest and most advanced deer farming industry. Leptospirosis in farmed deer can cause illness and possibly sub-clinical production losses. Farmed deer also play an important role in the transmission of leptospirosis by shedding the organisms in their urine, putting both other animals and humans at risk. *Leptospira* serovars Hardjobovis and Pomona are the most commonly found serovars in this stock group. The first substantial case report of leptospirosis in farmed deer was in the 1980s but it was not until 2006 that a substantial epidemiological study of this disease in farmed deer has been reported. The purpose of this research was to improve and extend current knowledge on the epidemiology of leptospirosis on mixed-species deer farms, to develop and validate a novel molecular diagnostic tool and to enhance understanding of control measures and their outcomes by means of vaccination.

A pilot longitudinal seroprevalence survey of leptospirosis on mixed-species deer farms was conducted. Results from this study revealed that leptospiral infection averaged 70% in all species on mixed-species farm in the lower North Island of NZ. Co-grazing with infected sheep and/or cattle was positively associated with deer herd serological status to both serovars Hardjobovis and Pomona which suggests the possibility of inter-species transmission. Results from this study justify further investigation of leptospirosis on mixed-species farm at the national level.

A collaborative study between Massey University and the WHO/FAO/OIE reference laboratory for leptospirosis in Brisbane to investigate for exotic serovars in farmed deer revealed seropositivity to Arborea which has never been found in NZ. Attempts to isolate Arborea from kidney samples of farmed deer were unsuccessful and require further investigation.

Real-time PCR assay was developed and validated against culture as the gold standard for use on deer kidney tissue and urine as a research and diagnostic tool for determining infection, carrier and shedding status of deer. This research revealed that the real-time PCR assay was highly sensitive (sensitivity; 85% for kidney and 96.7% for urine) and specific (specificity; 98.8% for kidney and 100% for urine). It is a useful tool for the rapid and cost-effective detection of pathogenic leptospires in clinical samples. It can also be used to quantify the concentration of leptospires from clinical samples and identify the likely infecting serovar in NZ when adjunct with a DNA sequencing technique.

Vaccination control for leptospirosis has proven to be efficacious and likely to be cost-effective. Present research has determined the effect of a commercial bivalent leptospiral vaccine (Leptavoid-2, Intervet/Schering-Plough Animal Health Limited, NZ) on leptospiral shedding, growth and reproduction of farmed deer under NZ pastoral conditions. The study was designed to simulate an infection-free herd scenario followed by exposure to natural challenge. Results have shown the potential of vaccine to improve mean weight gain (up to 6.5 kg) and weaning rate (average 6.9%) in infected herds and prevent urinary shedding after natural challenge with Hardjobovis. It also provides the first evidence of adverse subclinical effects on deer production by Hardjobovis alone.

A pilot study to investigate the presence and localisation of pathogenic *Leptospira* in the uterus and foetus of female deer revealed evidence of a foetal infection using real-time PCR. This finding suggests a possible explanation for effects of leptospiral infection on NZ farmed deer reproduction. However, further study is required to justify this proposition.

This research has contributed significantly to understanding of epidemiology of leptospirosis in NZ farmed deer, providing objective data to assist producers in decision-making on leptospirosis control. Furthermore, this study has made available a valuable diagnostic resource for future leptospirosis studies, and has provided direction for future research into leptospirosis on farmed deer and mixed species farms.

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Declaration

Each chapter in this thesis is set out as a paper in the style and format required of the journal to which it is in the process of being submitted to at the date of submitting this thesis. As a result, there is some repetition, particularly in the methods, and there are inconsistencies with the style and format between the chapters. Contributions to the research have been made by co-authors indicated in each chapter. However, my input was the greatest as I designed the study, undertook all the fieldwork and laboratory work, analysed the data and wrote the manuscripts.

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Publications

Heuer C, Dreyfus A, Wilson PR, Benschop J, Subharat S, Ayanegui-Alcérreca MA, Fang F, Collins-Emerson JM, Midwinter AC. Epidemiology and control of leptospirosis in New Zealand. *Proceeding of the SVEPM conference*, 2010 in press

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*** Speaker**

Chapter 1

Literature Review

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This thesis chapter is prepared in the style format of

Zooneses and Public Health

1.1 Introduction

Leptospirosis is considered to be a serious zoonotic disease of increasing prevalence and worldwide distribution (Bharti *et al.*, 2003, Levett, 2001). Awareness of the disease has increased internationally over the past decade for both developing and industrialized countries. Leptospirosis is caused by infection with pathogenic leptospires: helical shaped motile spirochetes which belong to the family *Leptospiracae,* genus *Leptospira*. Globally, it is claimed to affect millions of people annually and cause substantial domestic livestock loss. It is widely accepted that the incidence of leptospirosis is under-reported due to lack of clinical suspicion and barriers to diagnostic capacity. Case findings and reporting in both human and veterinary medicine have been limited and biased (Cachay & Vinetz, 2005).

Traditionally, leptospires are classified into two species: *L. interrogans*, comprising all pathogenic strains; and *L. biflexa*, containing the saprophytic strains isolated from the environment. Based on a surface antigenic classification system, there has been over 200 serovars grouped into 23 serogroups of *Leptospira* that have been isolated and described in various parts of the world (Bharti et al., 2003) proving that these organisms are ubiquitous, diverse and complex. Distribution of leptospirosis is largely dependent on the presence of local carrier animals and environmental conditions that enable leptospires to be maintained outside the hosts (Torten & Marshall, 1994).

Leptospirosis is considered to be the most important occupational zoonosis in developed countries (Holk *et al.*, 2000, Jansen *et al.*, 2005). In New Zealand, the epidemiology of leptospirosis follows the pattern that occurs in temperate regions involving relatively few serovars, and dominated by exposure to domestic livestock. Livestock farming plays an important role as a major occupational risk factor for human leptospirosis in New Zealand (Thornley *et al.*, 2002) unlike in other countries where the primary exposure to humans is from vectors such as rodents and wildlife. The major economic impact of leptospirosis in New Zealand involves livestock industries. There are costs associated with prevention for both humans and animals, medical and veterinary care and loss in animal production and human productive working time.

This review describes the history of leptospirosis, taxonomy and classification, epidemiology, pathogenesis, immune response, clinical signs and pathology. Current information on diagnostic and typing are discussed along with animal production issues, treatment and vaccination. In particular the epidemiology of leptospirosis for both human and New Zealand livestock, with particular reference to farmed deer, is reviewed. In this review, the term “leptospirosis” is defined as both clinical and subclinical disease while “infection” refers to the invasion and multiplication of leptospires in host tissues, manifest either clinically or subclinically.

1.2 History of leptospirosis

In humans, a disease resembling leptospirosis was recognized as an occupational hazard of rice harvesting in ancient China, and the Japanese name "Akiyami" or autumn fever still persists (Levett, 2001). It was identified elsewhere more than one century ago as a disease causing severe icterus and renal failure in humans. Initially, it was named "Weil's disease" after Adolf Weil who first reported it in 1886 (Weil, 1886). Twenty years later, a spirochete in a patient with Weil's disease that died from "yellow fever" was isolated and named "*Spirocheta interrogans*" as the organism with its hook ends resembles a question mark (Stimson, 1907). This same spirochete was later isolated independently in both Japan and Germany and named as "*Spirocheta icterohaemorrhagiae*" (Hubener & Reiter, 1915, Inada *et al.*, 1916, Uhlenhuth & Fromme, 1915). Two years later, this organism was shown to differ from other spirochetes and reclassified into a new genus called "*Leptospira*" (Noguchi, 1917). Rats were initially considered to be the primary source of this organism (Ido, 1917) but animals now known to be involved in human occupational exposure include livestock, rodents, pets and wild animals. The importance of occupation as a risk factor for leptospirosis was first described in soldiers (Johnston *et al.*, 1983), miners, sewer workers and rice planters (Padre *et al.*, 1988) all of whom work in wet conditions (Faine *et al.*, 1999).

In animals leptospirosis was identified as a clinical entity in 1850, approximately 30 years before Adolf Weil described the disease in humans. In 1898, an epidemic in dogs was recorded in Stuttgart, Germany, but its cause was not confirmed until the aetiology of Weil's disease was recognised (Torten & Marshall, 1994). The importance of leptospirosis in domesticated animals was increasingly realised in the 1920s when dogs were found to be infected by *L. icterohaemorrahgiae* (Alston & Broom, 1958). During the 1950s to 1960s, the significance of domesticated animals other than dogs in leptospirosis was highlighted by the development of vaccines for veterinary uses. Accumulated evidence showed that leptospires could infect all known mammals and possibly some lower vertebrates such as reptiles, amphibians, fish and birds (Torten & Marshall, 1994, Farr, 1995). In the 1970s and 1980s the ubiquitous distribution of Hardjo infections in cattle and humans who handled them was recognised. Redefinition of the occupational risks to humans, clarification of the means of transmission and improved diagnostic methods were the main advances in those periods. Basic understanding of the microbiology of leptospires and host immune responses provided an improvement in risk management of leptospirosis in animals (Faine et al., 1999).

1.3 The organism

Leptospires are spirochetes, a group of bacteria that developed in early bacterial evolution. They belong to the order Spirochaetales family *Leptospiraceae* which includes three genera, namely *Leptospira*, *Leptonema* and *Turneria* (Torten & Marshall, 1994). Their cells have pointed ends with either one or both sides bent into a hook (Figure 1.1). Leptospires are actively motile, exhibiting both translational and non-translational movement (Berg *et al.*,

1978, Cox & Twigg, 1974). They have a typical double membrane structure similar to other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane (Haake, 2000). Leptospiral lipopolysaccharide (LPS) has a composition similar to other gram-negative bacteria (Vinh *et al.*, 1986) but with less endotoxic activity (Shimizu *et al.*, 1987).

Leptospires are about 0.25 x 6.25μm and pass through a 0.45μm filter. For direct visualization, dark-field or phase-contrast microscopy of wet preparations is required (Bharti et al., 2003). Leptospires are catalase and oxidase positive. The genome of *Leptospira* consists of two circular chromosomes and is large compared with that of other spirochetes such as *Treponema* or *Borrelia*. This may contribute to its ability to live in various environments (Bharti et al., 2003).

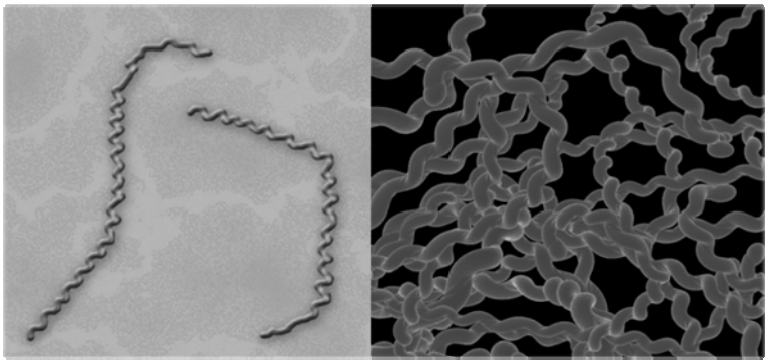

Figure 1.1 Leptospires under electron microscope and computerised graphic model (adapted from www.leptospirosis.org)

1.4 Isolation

Leptospires are obligate aerobes with an optimal growth temperature of 28 to 30°C. They grow in media enriched with vitamins (B2 and B12), long-chain fatty acids, and ammonium salts (Ellinghausen & McCullough, 1965). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by β-oxidation. Isolation of leptospires is time-consuming but with pure subculture into liquid medium they will usually grow in 10 to 14 days. The culture should be retained for at least four months before being discarded as negative. The optimal pH for growth and survival of leptospires is between 7.2 to 7.6 (Faine et al., 1999).

Various kinds of media for leptospires including liquid (EMJH, Korthof, Fletcher and Stuart), semi-solid (media with 0.2% agar) and solid (media with 1% agar) have been developed. Liquid media are necessary for culture and typing. Semisolid media are generally used for isolating strains and for the maintenance of stock cultures, whereas solid media are used for isolating leptospires from contaminated sources, separating mixed culture of leptospires and for detecting haemolysin production (Roth *et al.*, 1961, Stamm & Charon, 1979). Media are made selective by adding several antibiotics, the most common being 5-

fluorouracil and neomycin sulphate (Bharti et al., 2003). Culture from clinical samples is usually a definitive diagnosis of leptospiral infection but its drawback is that it is time-consuming, labour-intensive and easy to contaminate (Ellis, 1986)

1.5 Taxonomy and Classification

Up to the late 1970s, the genus *Leptospira* comprised two species, *Leptospira interrogans* and *Leptospira biflexa*. *L. interrogans* contained 23 serogroups whose strains were either parasitic or pathogenic to humans or animals. *L. biflexa* contained 28 serogroups whose strains were usually found in fresh surface water or moist soil and rarely isolated from man or animals (Kmety & Dikken, 1993, Johnson & Faine, 1984). A new family *Leptospiraceae*, which comprised the genera *Leptospira* and *Leptonema*, was proposed at that time (Hovind-Hougen, 1979). In 1981, an additional species, *Leptospira parva* was proposed (Hovind-Hougen *et al.*, 1981). However, according to analysis of G+C content and 16s rRNA gene sequencing showing its extreme heterogeneity, it has been decided that it is sufficiently different from the genus *Leptospira* or *Leptonema* to be ascribed a new genus in the family *Leptospiraceae* called "*Turneriella"* (Figure 1.2) (Levett *et al.*, 2005a).

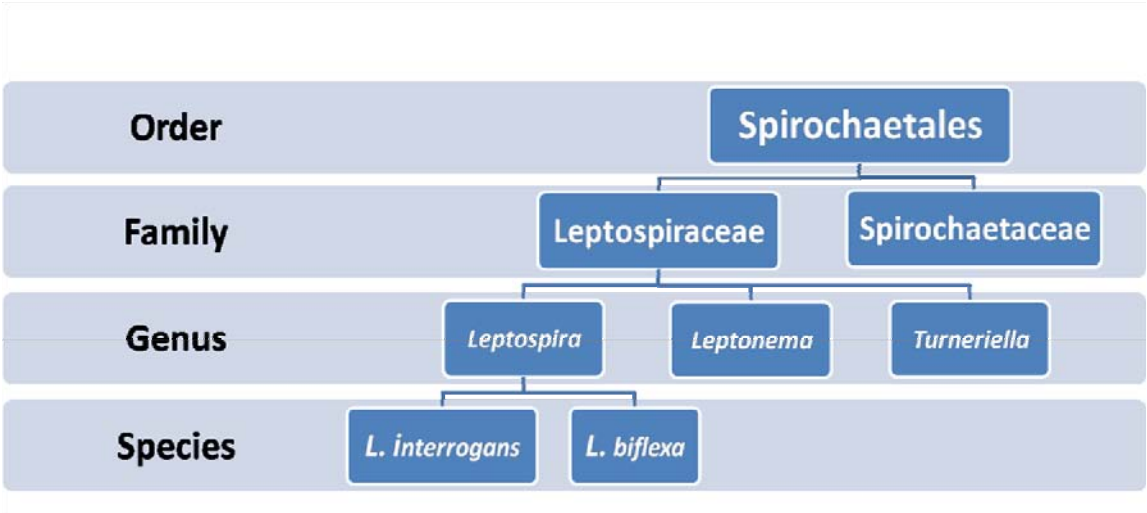

Figure 1.2 Taxonomy of *Leptospira sensu lato*

Currently, the reclassification of leptospires on genotypic grounds is proven to be correct and provides a strong foundation for future classification. However, the genomospecies of *Leptospira* do not correspond to the previous serological classification because pathogenic and non-pathogenic *Leptospira* serovars occur within the same genomospecies. Furthermore, molecular classification is complicated for the clinical microbiologist. Until simple DNA-based methods are developed and validated, serological classification methods need to be retained.

1.5.1 Serological classification

A serological taxonomy system (*sensu lato*) is used to divide *Leptospira* into serovars on the basis of surface antigen patterns. Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by the Cross Agglutination Absorption Test (CAAT) with homologous antigen. If more than 10% of the homologous titre remains in at least one of the antisera after repeating absorption, two strains are said to belong to different serovars (Dikken & Kmety, 1978). Serovars that are antigenically related are grouped into serogroups (Kmety & Dikken, 1993). While serogroups have no taxonomic standing, they have proved useful for epidemiological understanding (Bharti et al., 2003). There have been over 200 serovars grouped into 23 serogroups of pathogenic *Leptospira* that have been isolated and described (Levett, 2001, Bharti et al., 2003).

1.5.2 Genotypic classification

The current classification of *Leptospira* genomospecies are summarised in Table 1.1. Genomic species of leptospires are determined by the group of *Leptospira* whose DNA show 70% or more homology at the optimal re-association temperature of 55°C or 60% or more homology at a stringent re-association temperature of 70°C and in which the related DNA contains fewer than 5% of unpaired bases (Yasuda *et al.*, 1987). The genotypic classification of leptospires is supported by Multi-locus Enzyme Electrophoresis (MLEE) data (Letocart *et al.*, 1999). DNA hybridisation studies have also confirmed the taxonomic status of the monospecific genus *Leptonema* (Brenner *et al.*, 1999). According to the genetic taxonomy of *Leptospira* (*sensu stricto*), DNA-DNA hybridisation techniques have classified the genus of *Leptospira* into 18 genomospecies divided into pathogenic, non-pathogenic and opportunistic pathogenic *Leptospira.* Pathogenic *Leptospira include L. interrogans, L. alexanderi, L. kirschneri, L. meyeri, L. borgpetersenii, L. weilii, L. noguchii, L. santarosai, L. genomospecies 1, L. genomospecies 4* and *L. genomospecies 5*. Non pathogenic *Leptospira* include *L. biflexa, L.wolbachii and L. genomospecies 3.* Opportunistic pathogenic *Leptospira* include *L. fainei, L. inadai L. broomii,* and *L. licerasiae* (Levett *et al.*, 2006, Brenner et al., 1999, Matthias *et al.*, 2008). The DNA sequence of the entire genome of *Leptospira* has been recently established (Nascimento *et al.*, 2004, Bulach *et al.*, 2006, Ren *et al.*, 2003) and this has opened a new era of molecular diagnosis using PCR-based methods (Merien *et al.*, 2005, Branger *et al.*, 2005a) and genotyping of leptospires (Majed *et al.*, 2005, Slack *et al.*, 2005).

1.6 Epidemiology

Leptospirosis is a worldwide zoonosis. The source of infection in humans is usually either by direct or indirect contact with urine of infected animals. The core determinants of transmission are the presence of carriers, suitable environment for survival of leptospires and interaction between humans, animals and the environment (Figure 1.3) (Sehgal, 2006). After leptospires are excreted in urine, they may survive for weeks or months in optimal conditions. The disease is maintained in the environment by persistent renal colonization and urinary

shedding from maintenance hosts. Human infections may be acquired through occupational, recreational or non-vocational exposure. The extent to which infection is transmitted depends on many factors such as climate, population density and the degree of contact between maintenance and incidental hosts (Levett, 2001).

Table 1.1 Classification of *Leptospira* genomospecies

Genomospecies	**Serovar**	**Serogroup**	**Pathogenicity**
L. interrogans	Australis	Australis	yes
	Bratislava	Australis	yes
	Bataviae	Bataviae	yes
	Canicola	Canicola	yes
	Copenhageni	Icterohaemorrhagiae	yes
	Hardjoprajitno	Sejroe	yes
	Hebdomadis	Hebdomadis	yes
	Icterohaemorrhagiae	Icterohaemorrhagiae	yes
	Lai	Icterohaemorrhagiae	yes
	Pomona	Pomona	yes
	Pyrogenase	Pyrogenase	yes
L. alexanderi	Manhao3	Manhao	yes
L. kirschneri	Bim	Autumnalis	yes
	Cynopteri	Cynopteri	yes
	Grippotyphosa	Grippotyphosa	yes
	Mozdok	Pomona	yes
	Panama	Panama	yes
L. meyeri	Semaranga	Semaranga	yes
L. borgpetersenii	Arborea	Ballum	yes
	Ballum	Ballum	yes
	Castellonis	Ballum	yes
	Hardjobovis	Sejroe	yes
	Javanica	Javanica	yes
	Sejroe	Sejroe	yes
	Tarassovi	Tarassovi	yes
L. weilii	Celledoni	Celledoni	yes
L. noguchii	Fortbragg	Autumnalis	yes
L. santarosai	Brasiliensis	Bataviae	yes
	Georgia	Mini	yes
L. genomospecies 1	Pingchang	Ranarum	yes
L. genomospecies 4	Hualin	Icterohaemorrhagiae	yes
L. genomospecies 5	Saopaulo	Semaranga	yes
L. fainei	Hurstbridge	Hurstbridge	yes/no
L. inadai	Lyme	Lyme	yes/no
L. broomii	n/a	n/a	yes/no
L. licerasiae	Varillal	Iquitos	yes/no
L. biflexa	Patoc	Semaranga	no
L. wolbachii	Codice	n/a	no
L. genomospecies 3	Holland	Holland	no

Adapted from (Bharti et al., 2003, Levett et al., 2006, Matthias et al., 2008)
n/a = not available

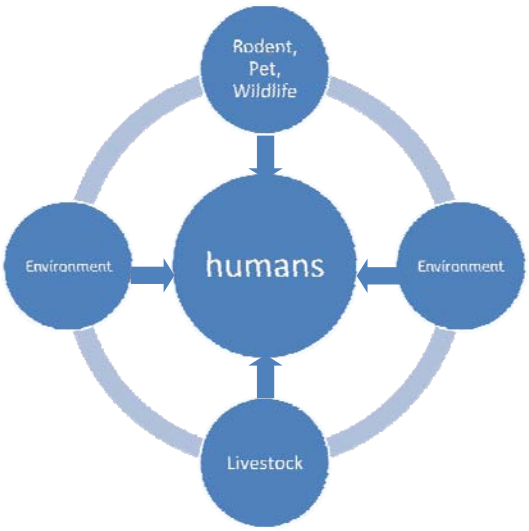

Figure 1.3 Transmission of leptospirosis

Occupation is a significant risk factor for human leptospirosis. Direct contact with infected animals accounts for most infections in farmers, meat workers, meat inspectors, veterinarians, rodent control workers and other occupations that require contact with animals (Demers *et al.*, 1985, Terry *et al.*, 2000, Thornley et al., 2002). Indirect contact is important for sewer workers, miners, soldiers, fish famers, rice planters and banana farmers (Johnston et al., 1983, Padre et al., 1988, Robertson *et al.*, 1981, Smythe *et al.*, 2000). Tradition and religion may determine the degree of contact between humans and animals such as pigs, dogs and cattle. Perception of illness, tolerance of pain and suffering, expectation and understanding of medical care, availability and type of medical care all contribute to whether or not a person will be recognised or reported as having leptospirosis (Faine et al., 1999).

Leptospires are able to survive in alkaline soils, mud, swamps, streams, rivers, organs and tissues of live or dead animals and diluted milk. Survival of pathogenic leptospires in the environment is dependent on several factors including pH, temperature, and the presence of inhibitory compounds. In general, they are sensitive to dryness, heat, acids and basic disinfectants (Alston & Broom, 1958, Faine et al., 1999). Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8.0 (Crawford *et al.*, 1971). When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 14 days (Karaseva *et al.*, 1973). Survival of serovar Pomona has been documented for 42 days in soil under simulated winter conditions, 183 days in wet soil and 94 days in river water (Miller *et al.*, 1991).

The presence of a maintenance host is crucial to the endemic existence of leptospirosis. A maintenance host is defined as a species in which infection is persistent and shedding of viable organisms in the urine lasts for months, and may even last for a lifetime (Marshall & Manktelow, 2002). Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of animal. Animals may be a maintenance host for some serovars but incidental host for others that may cause severe or

fatal disease (Hathaway *et al.*, 1983). Specific leptospiral serovars have been associated with different mammalian hosts (Farr, 1995).

The epidemiology of leptospirosis has been described in three patterns. The first occurs in temperate climates where few serovars (mainly Hardjo, Pomona, Tarasovi and Grippotyphosa) are involved and human infection mostly occurs by direct contact with infected animals through farming. Under these conditions, it is likely that leptospirosis could be controlled by a vaccination programme. The second pattern occurs in tropical wet areas, where there are many more serovars infecting humans and animals and also with a larger number of reservoir species, including rodents, farm animals and pets. The incidence is much higher than in a temperate climate due to longer survival of leptospires in warm and humid conditions (Faine et al., 1999). The disease is seasonal with the likelihood of infection increasing during periods of high rainfall. Poor drainage, high water-holding capacity of soil, flooding and high density of carrier and susceptible animals are risk factors that correlate with a high incidence of leptospirosis (Heath & Johnson, 1994). Control of rodent populations, drainage of wet areas and occupational hygiene are all necessary for the prevention of human leptospirosis. The third pattern comprises rodent-borne infection in urban environments, most commonly associated with poverty and poor housing and sanitary conditions. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods, hurricanes or other disasters (Faine et al., 1999).

1.7 Pathogenesis

Pathogenesis of leptospirosis is claimed to be similar for every animal species including humans. Leptospires enter the host via small abrasions, breaches of the surface integument, conjunctiva, mucous membrane and genital tract (Faine et al., 1999). They do not localise at the site of entry but spread rapidly through the blood stream and lymphatics to target tissues. Motility of leptospires may play an important role at the beginning of infection and spreading of organisms to various organs (Bharti et al., 2003). Leptospires do not cause an acute inflammatory response to their presence in tissue. Non-pathogenic strains are cleared rapidly by reticulo-enthothelial phagocytosis whereas pathogenic strains can evade this process and multiply exponentially both in bloodstream and tissues.

The incubation period depends on infective dose, growth rate of organisms, their toxicity, and host immunity. In a carrier state, leptospires may persist in certain tissues that are immunologically privileged sites such as proximal renal tubules, the brain, anterior chamber of the eye and the genital tract. A study of renal colonization in a rat model have shown that leptospires initially disseminate throughout various organs, followed by clearance except from the kidneys, suggesting that kidneys are the most immune privileged site (Athanazio *et al.*, 2008). In the host renal tubule, leptospires migrate through the interstitial space to settle on the brush border and later excrete in host urine which is the common source of infection to other animals and humans (Faine et al., 1999).

The mechanisms by which leptospires cause disease are not well understood. Attachment to cell surface and toxicity seem to be important properties of virulent leptospires. A number of putative virulence factors have been suggested, but with few exceptions, their role in pathogenesis remains unclear (Levett, 2001). However, the advent of whole-genome sequencing of leptospires has greatly impacted on development of bioinformatics. This helps to reveal proteins independent of their abundance without the need of culturing organisms. Functional genomics studies, including transcription profile, gene cloning and protein expression complement *in silico* analysis have helped the understanding of pathogenesis of leptospires (Atzingen *et al.*, 2008).

1.7.1 Attachment

Several attachment *in vitro* assays have shown that virulent leptospires adhere to fibroblastic cells (Vinh *et al.*, 1984), renal epithelial cells (Ballard *et al.*, 1986) and human endothelial cells (Thomas & Higbie, 1990). Leptospires have been shown to be phagocytosed by macrophages only in the presence of specific immune serum and complement (Vinh *et al.*, 1982, Cinco *et al.*, 1981, Pereira *et al.*, 1998, McGrath *et al.*, 1984). This suggests that the outer envelope of pathogenic leptospires possesses an antiphagocytic activity (Levett, 2001). In one study, it has been shown that pathogenic leptospires attached to neutrophils but were not killed by them (Wang *et al.*, 1984). Leptospiral lipopolysaccharide (LPS) is claimed to stimulate adherence of neutrophils which is mediated by platelet-activating-factor (PAF) (Isogai *et al.*, 1997). This suggests a role of LPS in the development of thrombocytopenia (Levett, 2001).

In the past decade, there has been a belief that interaction by leptospires with extracellular matrix (ECM) of host cells plays an important role in the colonization (Barbosa *et al.*, 2006). The first step in adhesion of bacteria to the target cell membrane involves molecular recognition between surface proteins of both cell types (Merien *et al.*, 2000). Normally, pathogens may gain access to ECM component after tissue trauma by cuts and abrasions, chemical injury or bacterial toxin and lytic enzymes (Ljungh & Wadstrom, 1996). In mammals, ECM is composed of two main macromolecules i.e. glycosaminoglycans and fibrous protein such as collagen, elastin, laminin and fibronectin (Barbosa et al., 2006). Fibronectin, a type of glycoprotein that is distributed widely in host cells was proposed to be the primary target forleptospires to adhere. Several fibronectin-binding proteins such as 36-kDa (Merien et al., 2000), LigA, LigB (Choy *et al.*, 2007), Lsa21 (Atzingen et al., 2008) and Lsa 24 (Barbosa et al., 2006) have been studied and claimed as potential virulence factors of leptospires. Recently, *Leptospira* lipoprotein Loa22 was also proposed as an essential for *in vivo* infection of pathogenic leptospires (Nally *et al.*, 2007, Ristow *et al.*, 2007).

1.7.2 Toxins

Endotoxin activity has been reported in several serovars of leptospires. Leptospiral LPS preparations exhibit activity in biological assays for endotoxin similar to other Gram-negative bacteria. Some studies have shown that leptospiral LPS has less lethal potency than *E. coli* (De-Souza & Koury, 1992, Isogai *et al.*, 1990). This may be due to the fact that leptospiral LPS stimulates innate immunity via a TLR2-dependent mechanism whereas other Gram-negative LPS stimulates innate immunity via TLR4-dependent mechanism (Werts *et al.*, 2001).

Haemolysins have been suggested to be phospholipases, that act on erythrocytes (Thompson & Manktelow, 1986) and other cell membranes containing the substrate phospholipids, leading to cytolysis (Lee *et al.*, 2002). Haemolysins from several serovars have been characterized. For example, serovar Pomona is known for production of haemolytic disease in cattle while serovar Ballum creates similar symptoms in hamsters (Levett, 2001). The haemolysins of serovars Hardjo and Pomona are claimed to be have sphingomyelinase C activity (Bernheimer & Bey, 1986, del Real *et al.*, 1989). Also, phospholipase C activity has been reported in serovar Canicola (Yanagihara *et al.*, 1982). However, a haemolysin gene (SphH) from serovar Lai is not associated with sphingomyelinase or phospholipase C and is clearly demonstrated to form pores in mammalian cells (Lee *et al.*, 2000, Lee et al., 2002). Recently, haemolysins were further divided into 2 groups, sphingomyelinase and non-sphingomyelinase hemolysins (Zhang *et al.*, 2005). The spingomyelinase haemolysin gene Sph2 was also claimed to induce apoptosis in human liver cells (Zhang *et al.*, 2008).

Cytotoxic activity of leptospires was first observed in supernatant extract from serovars Pomona and Copenhageni (Cinco *et al.*, 1980). It has also been detected in the plasma of infected animals (Knight *et al.*, 1973). This toxin elicited a typical cytopathic effect, with infiltration of macrophages and polymorphonuclear cells (Yam *et al.*, 1970, Diament *et al.*, 2002). Later, a glycolipoprotein (GLP) fraction of leptospires was claimed to be the cause and shown to be toxic to cultured fibroblasts displaying endotoxic properties (Vinh et al., 1986). A similar fraction from serovar Canicola inhibits Na^+, K^+-ATPase activity either in purified preparation or in isolated nephron segments from rabbit kidney (Younes-Ibrahim *et al.*, 1995). The hypothesis is that GLP will be released after lysis of leptospires by the host immune response. Then, it will cause an impairment of Na^+ and K^+-ATPase which increase nonesterified unsaturated fatty acids (NEUFAs) and bilirubin serum level and decrease albumin concentrations leading to hepatic icterus and renal dysfunction (renal failure) of the host (Burth *et al.*, 2005).

1.8 Clinical signs

1.8.1 Humans

Human infection with leptospirosis may present variable clinical manifestations ranging from subclinical disease with undifferentiated febrile illness, to jaundice, renal failure and potentially lethal pulmonary disease (Bharti et al., 2003). Severe leptospirosis is frequently reported with infection by serovars Icterohaemorrhagiae, Copenhageni and Lai (Cachay & Vinetz, 2005). However, disease severity depends largely on geographic location, ecology of local maintenance host (Levett, 2001), human host genetics and dose of infection (Cachay & Vinetz, 2005).

Most people infected by *Leptospira* will develop mild fever with flu-like symptoms. People usually do not seek medical attention or may be misdiagnosed by unaware practitioners resulting in under-reporting of the true leptospirosis incidence (Heuer *et al.*, 2008). In more severe cases, the disease will begin with moderate fever with a combination of symptoms such as general malaise, chills, headache, muscle pain and weakness, pneumonia, anaemia, conjunctival suffusion and photophobia. Aseptic meningitis may also occur in young patients. The disease usually last two to three weeks and a long recovery period is common (Levett, 2001, Torten & Marshall, 1994). Weil's syndrome represents the most severe form of leptospirosis characterised by jaundice and acute renal failure that occurs in the second phase of the illness. The case fatality rate may reach 15% despite supportive care (Ko *et al.*, 1999). However, the incidence of Weil's syndrome is low. Pulmonary and cardiac involvements have also been reported in human leptospirosis (Chakurkar *et al.*, 2008, Dolhnikoff *et al.*, 2007).

1.8.2 Animals

In animals, clinical features of leptospirosis are similar across species (Faine et al., 1999). Various signs of disease can occur because of interactions between host adaptation, virulence of the serovars and current immune status of the host (Heath & Johnson, 1994). Clinical signs are usually mild or subclinical when infected by host-adapted serovars. However, when infected by non-host-adapted serovars, the clinical signs can range from mild to severe icterohaemorrhagic disease depending on age and host immunity. Normally, severe cases are observed more commonly in young than in older animals (Ellis, 1994). Clinical signs of acute leptospirosis usually start with elevated temperatures accompanied by anorexia, dull appearance, agalactia, haemolytic anaemia, haematuria, haemoglobinuria, jaundice, renal failure, all of which may progress to death. Animals that recover may have poor growth rates and significant renal lesions at slaughter (Faine et al., 1999). Chronic leptospirosis usually involves infection by host-adapted serovars which can take the form of abortion, stillbirth, weak offspring, chronic interstitial nephritis, poor reproductive performance and poor growth (Ellis, 1994, Ayanegui-Alcérreca, 2006). However, it should be noted that abortion caused by host-adapted serovars tends to occur sporadically compared with abortion storms that occur

with non-host-adapted serovars (Grooms, 2006). Due to the diverse symptomatology of leptospirosis, it is difficult to diagnose animal leptospirosis based on clinical signs alone (Torten & Marshall, 1994).

1.9 Pathology

The main pathologic lesions of leptospirosis in animals and humans are similar, commonly marked in the kidney, liver, lung and heart except in peracute cases which only show signs of acute septicaemia. The differences are determined by serovar, the host immune responses and chronic-carrier state where leptospires survive, grow and pass on infection (Faine et al., 1999). The primary lesion of leptospirosis in both animals and humans is damage of the endothelial cell membrane of the capillaries which is believed to be caused by leptospiral toxin. It is characterised by development of vasculitis and inflammatory infiltrates composed of monocytic cells, plasma cells, histiocytes and neutrophils. With gross lesions, petechial haemorrhages are common and organs are discoloured if an icteric status is present (Levett, 2001). The vascular changes predispose ischemic damage, leading to necrosis of the target organs i.e. kidney, liver, lung, brain, placenta and muscle (Faine et al., 1999). These result in the common findings of leptospirosis which are nephritis, hepatitis and jaundice, pulmonary haemorrhage and oedema, encephalopathy and meningitis, placentitis and abortion or stillbirth, interstitial myocarditis and acute muscle tenderness (Chakurkar et al., 2008, Dolhnikoff et al., 2007, Faine et al., 1999). However, the severity of the disease depends on each individual.

The main histopathological lesions considered to be typical of leptospirosis are in the kidney. Cortical cellular necrosis, petechiae and ecchymotic haemorrhages particularly in the glomeruli and the proximal convoluted tubule are observed along with infiltration of mononuclear cells, insterstitial nephritis and fibrosis. Leptospires may be seen within renal tubular epithelium on microscopic examination using Warthin-Starry stain (Langston & Heuter, 2003, Faine et al., 1999). Hepatic lesions included centrilobular necrosis, hepatocellular vacuolation and sometimes retention of bile (Fairley *et al.*, 1986) . Pulmonary congestion and haemorrhage are common histological findings in human pulmonary involvement in leptospirosis (Dolhnikoff et al., 2007).

1.10 Immune response

Immunity to leptospires includes type-1 or cell mediated immunity (CMI) and type-2 or humoral mediated immunity (HMI). Formerly, it was thought that protective immunity to leptospiral infection was entirely humoral because it is highly serovar-specific (Adler & Faine, 1977). It has been proposed that immunity is linked to antibodies directed against leptospiral polysaccharide (LPS) which is a complex antigenic structure (Adler & Faine, 1977, Faine et al., 1999, Sonrier *et al.*, 2001). Also, LPS has been shown to stimulate the innate immune system which comprises the cells and mechanisms that defend the host in a

non-specific manner via the TLR2-dependent mechanism, unlike other Gram-negative organisms that stimulate antibody production via the TLR4-dependent mechanism (Werts et al., 2001). The antibody response is classic with peak Immunoglobulin M (IgM) antibodies appearing first, quickly followed by Immunoglobulin G (IgG) antibodies. Anamnestic responses develop after re-exposure to organisms of the same serovar (Heath & Johnson, 1994).

Recently, the role of CMI in leptospiral infection has been realised. Insight into the protective response by CMI has been gained from study of a monovalent killed *L. borgpetersenii* vaccine in cattle. Those studies have demonstrated high level of interferon-γ (IFN-γ) produced by CD4+ T cells and gamma delta (γδ) T cells which correspond to CMI (Naiman *et al.*, 2001, Naiman *et al.*, 2002, Brown *et al.*, 2003). Another study has demonstrated that leptospiral glycolipoprotein (GLP) extracted from pathogenic leptospires induced cellular activation of peripheral blood mononuclear cells (PBMC) through the secretion of tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10) whereas leptospiral GLP extracted from non-pathogenic leptospires did not (Diament et al., 2002). Naive human PMBCs from healthy individuals have also been shown to produce a large quantity of IFN-γ-producing γδ T cells after *in vitro* stimulation by *L. interrogans*. Furthermore, in patients with acute leptospirosis, there was a significant increase of γδ T cells in peripheral blood (Klimpel *et al.*, 2003). Thus, much remains to be established to fully understand the mechanisms of host immunity to leptopiral infection and to develop novel efficient leptospiral vaccines (Bharti et al., 2003, Koizumi & Watanabe, 2005).

1.11 Diagnosis

The diverse clinical signs of leptospirosis make laboratory confirmation essential. However, interpretation of results must be cautious since they only represent the evidence of leptospiral infection, not necessarily disease causation. Consistent criteria are required in combination with clinical or pathology findings to confirm the diagnosis of leptospirosis. In animal populations, especially livestock, it is appropriate to diagnose leptospiral infection on a herd basis to provide morbidity or prevalence statistics that determine the magnitude of problem and determine control programs.

There are two main imperatives of leptospirosis diagnostic tests, namely detection and identification with the approach depending on the intention of diagnosis. On an individual animal basis, methods for detection *per se* are sufficient to confirm the infection and initiate a treatment. However, to deal with leptospirosis in a broader perspective, methods for serovar identification will provide much more information especially for understanding its epidemiology and determining control programmes. Isolation of *Leptospira* organisms from clinical samples is the definitive method since it can both detect and identify the organism, using serological (CAAT) or molecular typing. However, isolation is uncommonly used because it is technically demanding, tediousness, complex, time consuming (e.g. 16 weeks

before declaring a sample negative) and expensive. Thus, a variety of alternative methods have been developed in an attempt to provide more rapid and less expensive diagnosis of leptospirosis. These include methods that demonstrate organisms or antigens, that detect host antibody, and that detect genomic materials or DNA. Each method has its own purposes, advantages and disadvantages as summarised in Table 1.2.

1.11.1 Direct examination and Antigen detection

Leptospires may be visualized in urine by dark-field microscopy because of their characteristic shape and movement but this method suffers from poor sensitivity and specificity (Vijayachari *et al.*, 2001b). It requires a high degree of operator skill since the organisms can be mistaken by other cell debris. Approximately 10^4 to 10^5 leptospires/ml are necessary for one cell per field to be visible under dark-field microscopy of urine (Turner, 1970, Langston & Heuter, 2003). A variety of staining methods for histological sections such as silver staining (Skilbeck & Chappel, 1987), Warthin-Starry staining (Elliott, 1988), immunofluorescent staining (Skilbeck, 1986) and immunohistochemical staining (Yener & Keles, 2001) have been applied to increase the sensitivity of microscopic examination of leptospires on histological sections. Recently, a monoclonal antibody-based method has been evaluated to detect leptospiral antigens in cattle urine, claimed to detect as few as 10^3 leptospires/ml of urine (Suwimonteerabutr *et al.*, 2005).

1.11.2 Antibody detection

Since *Leptospira* require several weeks to culture, laboratory diagnosis often depends on the demonstration of antibodies against *Leptospira* in sera. Serological tests are either serogroup-specific or genus-specific (Levett, 2004). Formerly, agglutination tests were developed such as the microscopic agglutination test (MAT) which is read under dark-field microscope, and macroscopic slide agglutination test (MSAT) which is read by the naked eye (Galton *et al.*, 1958). The MAT is the most widely used standard reference test because of its high sensitivity and specificity and the ability to identify to serogroup or serovar level (O'Keefe, 2002, Vijayachari *et al.*, 2001a). By comparison, the MSAT is a low performance test with poor reproducibility (Marin-Leon *et al.*, 1997). Recent study has reported MAT sensitivity ranging from 91.1% to 100.0% and specificity ranging from 94.3% to 100% (McBride *et al.*, 2007b). However, the MAT has some limitations including the requirement for maintenance of a large number of live *Leptospira* cultures for antigens and complexity of methodology and interpretation which limits its use in the standard laboratory (Myers, 1976, Palmer, 1988).

Interpretation of MAT results is complicated by a number of factors including cross-reactivity of antibodies, antibodies induced by vaccination, and lack of consensus about what antibody tires are indicative of infection. An agglutinating titre of $\geq$ 1:200 is considered significant in humans, in combination with present clinically illness, whereas $\geq$ 1:100 is considered significant in animals as evidence of previous exposure (Levett, 2001). However,

the diagnostic cut point titre at ≥ 1:100 may be exceeded in vaccinated animals or may not be reached in host-adapted serovar infection (Grooms & Bolin, 2005). Different studies using different cut points make it difficult for comparison between studies. Paired sera are required to confirm the stage of infection. If the titre remains low, it may be assumed that it was due to previous infection but if the titre increases, it may be assumed due to recent infection. A fourfold or greater rise in titre between paired sera taken 7 to 10 days apart is considered diagnostic in both humans and animals (Faine et al., 1999).

Due to the complexity of the MAT, many rapid screening immunological tests have been developed and evaluated for determination of specific leptospiral IgM or IgG antibodies such as the complement fixation test (CF) (Terzin, 1956), Microcapsule Agglutination test (MCAT) (Arimitsu *et al.*, 1982, Sehgal *et al.*, 1997), haemolysin test (HL) (Cox, 1957), indirect immunofluoresccent antibody test (IFA) (Appassakij *et al.*, 1995), indirect haemagglutination test (IHA) (Imamura *et al.*, 1974, Sulzer *et al.*, 1975), dipstick assay (Smits *et al.*, 1999, Smits *et al.*, 2000) and enzyme-linked immunosorbent assay (ELISA) (Adler *et al.*, 1980, Terpstra *et al.*, 1985). Several specific monoclonal antibodies have been developed and combined with ELISA format in an attempt to further identify antibodies to serovar level (Surujballi *et al.*, 1997, Yan *et al.*, 1999).

Whole-Cell *Leptospira*-based serologic assays are the most widely used methods for laboratory diagnosis of leptospirosis. Several commercial kits have been developed based on ELISA and immunochromatrographic methods such as IgM ELISA, Dip-S-Tick (PanBio Inc.) and LeptoTek Dri-Dot (Biomerieux). These tests were extensively validated for their sensitivity and specificity against the MAT (Effler *et al.*, 2002, McBride et al., 2007b, Ooteman *et al.*, 2006). In general, the sensitivity of these tests were low in the acute phase (33% to 67%) but improved in the convalescent phase (84% to 100%) of the disease with high specificity (88% to 100%) (McBride *et al.*, 2007a, McBride et al., 2007b, Ooteman et al., 2006). However, some studies reported that whole-cell *Leptopira* assays had low specificity (Blacksell *et al.*, 2006, Vijayachari *et al.*, 2002).

Recently, recombinant protein-based serologic tests have been developed because of their specificity and reproducibility. A recombinant flagella antigen immunocapture assay was described for serodiagnosis of bovine leptospirosis (Bughio *et al.*, 1999). *Leptospira* immunoglobin-like (Lig) proteins were evaluated as a serodiagnostic marker for human leptospirosis using an immunoblot assay. It has resulted in an improvement in detecting acute leptospiral infection, with sensitivity of 85% to 96% and specificity of 90% to 100% (Croda *et al.*, 2007). There has also been an attempt to use the conserved regions of the Lig proteins as the serodiagnostic marker in differentiating between vaccinated animals and naturally infected animals (Palaniappan *et al.*, 2004).

1.11.3 Molecular detection

Diagnosis of leptospirosis requires an assay able to detect a small number of leptospires in blood or urine and that can be performed rapidly, so infection can be detected and treated at an early stage. For epidemiological studies such an assay should be sensitive and allow many samples to be processed simultaneously. DNA-based techniques have been introduced into the field of leptospirosis research. Formerly, leptospiral DNA in clinical samples has been detected by DNA-hybridisation with labelled radioactive (^{32}P) or non-radioactive (biotin) probes. The principle is that if the nucleotide sequence of the samples match with those of the probes, DNA-hybridisation will occur and this can be detected by autoradiographic or colourimetric methods depending on the type of probes. However, these techniques are probably no more sensitive than immunological or immunohistochemical methods (Terpstra *et al.*, 1987, Terpstra *et al.*, 1986). Polymerase Chain Reaction (PCR) assay has been demonstrated to be a sensitive and rapid technique. It involves *in vitro* enzymatic amplification of a target DNA sequence through a series of polymerisations carried out by a thermostable DNA polymerase. The specificity of the assay can be adjusted by the choice of short DNA fragment called "Primer". First designed primers were developed from the serovar Hardjo DNA library in order to detect DNA of leptospires in cattle urine. It is also claimed to be more sensitive than culture from urine (Van Eys *et al.*, 1989). This technique was later improved by modification of the DNA extraction method and was claimed to detect as few as 5 to 10 leptospires/ml (Gerritsen *et al.*, 1991). Another study has designed primers from 16S rRNA gene of serovar Canicola and claimed to detect as few as 10 cells of leptospires in urine, CSF and blood when combined with the DNA hybridisation technique (Merien *et al.*, 1992). However, primers designed from previous two studies were unable to differentiate between pathogenic and non-pathogenic *Leptospira*.

Thus, another study has developed combined primer sets of G1/G2 and B64-I/B64-II that can detect only pathogenic *Leptospira* by assessing them with a large number of reference strains belonging to all pathogenic and non-pahogenic *Leptospira* species (Gravekamp *et al.*, 1993). These primers were widely used and validated in later studies for detection of leptospiral DNA in clinical samples from both animals and humans (Bal *et al.*, 1994, Fonseca Cde *et al.*, 2006, Brown *et al.*, 1995, Ooteman et al., 2006, Parma *et al.*, 1997, Wagenaar *et al.*, 2000). The limit of detection of these PCR techniques varied from 1 cell/ml to 1,000 cells/ml depending on type of sample tested and DNA preparation methods. One study reported a sensitivity of 91% and a specificity of 100% when using these primers on cattle urine compare with culture (Wagenaar et al., 2000). In addition, several studies have more recently reported newly designed primers (Branger et al., 2005a, Woo *et al.*, 1997b, Fearnley *et al.*, 2008, Slack *et al.*, 2006, Shukla, 2003). Others employ a multiplex PCR technique (Tansuphasiri *et al.*, 2006, Kositanont *et al.*, 2007) which uses more than one primer pair in a single reaction in an attempt to distinguish between pathogenic and non-pathogenic *Leptospira*.

PCR was claimed to be a promising method for the quick and early detection of leptospires in clinical samples in the period before antibodies become detectable (Brown et al., 1995, Fonseca Cde et al., 2006, Kee *et al.*, 1994). The nested PCR technique which requires two rounds of PCR reaction with outer primer and inner primer pairs was developed to increase sensitivity and specificity of PCR in order to detect pathogenic leptospires (Bomfim *et al.*, 2008, Nassi *et al.*, 2003).

Recently, an extension of PCR that has significant application to the diagnosis of leptospirosis is the real-time PCR. These assays are rapid and sensitive and can quantify the amount of DNA in clinical samples without the use of conventional agarose gel electrophoresis. Most of the real-time PCR techniques that have been reported for detection of leptospiral DNA involve the use of double-stranded DNA intercalating dye, such as SyBr Green I (Levett *et al.*, 2005b, Merien et al., 2005, Slack et al., 2006) or the use of fluorescent labelled probes such as Taqman (Cox *et al.*, 2005, Woo *et al.*, 1998, Smythe *et al.*, 2002, Slack *et al.*, 2007) or fluorescent resonance energy transfer (FRET) (Fearnley et al., 2008) to emit a signal at a specific wavelength for detection of amplified PCR product. All reported real-time PCR assays were able to differentiate between pathogenic and non-pathogenic *Leptospira*. Moreover, DNA sequencing of PCR products combined with a nucleotide Basic Local Alignment Search Tool (BLASTn) on the National Centre for Biotechnology Information (NCBI) database were able to identify the genomospecies of *Leptospira* DNA sample (Fearnley et al., 2008, Slack et al., 2006). Primer pairs Lepto F/ Lepto R designed from 16s rRNA gene were able to detect leptospiral DNA in serum and seeded urine samples (Smythe et al., 2002). This same technique was modified and reported with a sensitivity of 96.4% and a specificity of 99.5% on patient serum samples compared with bacterial culture as the gold standard. The detection limit is reported at 10 cells/reaction (Slack et al., 2007). This technique has also been used as a tool to predict a carrier state of the flying fox from kidney and urine sample (Cox et al., 2005). Another study has used a real-time PCR assay to measure the concentration of leptopiral DNA in patient's sera and reported the range of 80 to 39,000 cells/ml which can be associated with the prognosis of leptospirosis (Merien et al., 2005).

1.11.4 Molecular typing

Due to the cumbersomeness of serology techniques for identification of leptospires, there has been interest in developing molecular techniques for this purpose. DNA-DNA hybridisation is considered to be the gold standard technique for species-level identification of leptospires (Brendle *et al.*, 1974, Brenner et al., 1999, Levett et al., 2006, Ramadass *et al.*, 1992, Yasuda et al., 1987). However, it is seldom used because of its complexity. Assays involving digestion of chromosomal DNA by several restriction enzymes such as restriction endonuclease analysis (REA) (Marshall *et al.*, 1981, Thiermann *et al.*, 1986, Brown & Levett, 1997, Savio *et al.*, 1994, Djordjevic *et al.*, 1993, Skilbeck & Davies, 1989), restriction fragment length polymorphism (RFLP) (Corney & Colley, 1996, Zuerner *et al.*, 1993), pulsed-field gel electrophoresis (PFGE) (Herrmann *et al.*, 1992), ribotyping (Perolat *et al.*,

1993, Perolat *et al.*, 1994) and multilocus enzyme electrophoresis (MEE) (Letocart et al., 1999) have also been used for identification of leptospires. All these techniques however, suffer from disadvantages such as requirement for special expensive equipment, laborious and time-consuming procedure, poor reproducibility, ambiguous interpretation of data and most importantly, requirement of a large quantity of purified DNA. As a result, several methods based on analysis of amplified PCR product from leptospiral DNA have been developed.

PCR-based methods for identification of leptospires are rapid and require only small amounts of DNA. RFLP has been improved by performing the technique on amplified PCR product known as PCR-RFLP (Woo *et al.*, 1997a). This technique has been reported in the detection and differentiation of *Leptospira* in clinical samples such as bovine semen (Heinemann *et al.*, 2000) and human blood (Kawabata *et al.*, 2001). DNA fingerprinting using arbitrary primers with low stringency known as arbitrary primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) has been studied extensively (Corney *et al.*, 1993, Gerritsen *et al.*, 1995, Letocart *et al.*, 1997, Perolat et al., 1994, Ralph *et al.*, 1993, Ramadass *et al.*, 1997, de Caballero *et al.*, 1994). This technique is simple, rapid and can be performed without previous information of leptospiral DNA base sequences. However, the use of non-specific primers in this technique results in low specificity and reproducibility.

Thus, the low-stringency single specific primer PCR (LSSP-PCR) technique was subsequently developed to solve these issues (Bomfim & Koury, 2006). Another DNA fingerprinting technique using repetitive extragenic palindrome (REP) elements from the leptospiral genome has been developed. This REP-PCR technique has been reported in identification of *Leptospira interrogans* (Zuerner *et al.*, 1995). Fluorescent amplified fragment length polymorphism (FAFLP) has been developed for a molecular epidemiology study of leptospirosis in India. This technique combines the power of RFLP with the flexibility of PCR. The main principle is that the leptospiral genomic DNA will be cut into small fragments by restriction enzymes and bound to fluorescent labelled adapters that are designed to be complementary to PCR primers. Then, the PCR amplified fragments will be detected by fluorescent reading equipment such as DNA sequencers (Vijayachari *et al.*, 2004).

Recently, investigation of multi-locus variable number of tandem repeat methodology (MLVA) has been described for identification of *Leptospira interrogans* (*sensu stricto*) at the serovar level (Majed et al., 2005, Slack et al., 2005). This technique detects the differences in copy number of tandem repeats from selected VNTR loci which are polymorphic and allows for genotyping. It has proven to be a simple and reproducible method with highly discriminatory power. Moreover, it can also be performed under standard laboratory conditions without sophisticated equipment (Slack et al., 2005). However, this technique may suffer from an inaccuracy of agarose gel electrophoresis and problems associated with transfer of data between different laboratories. These issues can be resolved by DNA sequence-based identification methods.

Table 1.2 Summary of laboratory diagnostic tests for leptospirosis, including sample required and advantages and disadvantages

Test	Sample	Advantage	Disadvantage
Isolation			
Bacterial culture	Blood, urine, kidney, CSF	Gold standard, Ready for typing	Easy to contaminate, Cumbersome
Direct and Antigen detection			
Dark field Microscopy	Urine	Simple	Low sensitivity
Histological staining	Kidney	Simple	Low sensitivity
Immunological staining	Kidney	Increased sensitivity	Requires special reagent or equipment
Monoclonal antibody	Urine	Increased sensitivity	Complicated, expensive
Antibody detection			
MAT	Serum	Gold standard, Serogroup specific	Biohazard, Laborious
MSAT		Simple	Low sensitivity
CF		Non Biohazard	Genus specific
MCAT		Early detection	Genus specific
HL		Non Biohazard	Genus specific
IFA		Non Biohazard	Requires fluorescent microscope
IH		Non Biohazard	Genus specific
Dipstick		Simple and rapid	Genus specific
ELISA		Early detection (IgM), Can combine with modern technology	Genus specific
Molecular detection			
DNA-hybridisation	DNA from isolates or clinical samples	High specificity	Limitation to probe
PCR		High sensitivity/specificity	Easy to contaminate
Real-time PCR		Not rely on gel picture	Expensive equipment
Serological typing			
CAAT	Viable isolates	Gold standard	Cumbersome, Biohazard
Molecular Typing			
DNA hybridisation	DNA from isolates	Gold standard	Cumbersome
REA		High discrimination	Complicated
RFLP		Rapid identification	Ambiguous data
PFGE		High discrimination	Expensive equipment, Complicated
MEE		High discrimination	Complicated
PCR-RFLP		Ability to perform directly on clinical sample	Ambiguous data
AP-PCR or RAPD		Rapid identification	Poor reproducibility Ambiguous data
LSSP-PCR		Increased reproducibility	Ambiguous data
REP-PCR		High reproducibility	Ambiguous data
FAFLP		High discrimination	Requires special equipment
MLVA		High discrimination	Rely on gel picture
Single gene sequencing		High reproducibility, Unambiguous data	One gene may not be polymorphic enough
MLST		High discrimination, Unambiguous data	Require special equipment

Analysis of the nucleotide arrangement of the nucleic acid strand forms a powerful tool to compare multiple leptospiral isolates. The sequencing of a gene at the same locus helps to characterize different species of *Leptospira.* The usual target for DNA-sequence based identification of *Leptospira* species is the 16S rRNA gene as previously described (Fearnley et al., 2008, Hookey *et al.*, 1993, Postic *et al.*, 2000, Levett et al., 2005a). However, the 16S rRNA gene is claimed to be not polymorphic enough due to its conserved nature. Several genes such as *rpoB* gene (La Scola *et al.*, 2006) and *gyrB* gene (Slack et al., 2006) were proposed as alternatives for identification of *Leptospira* species. Multilocus sequence typing (MLST) is the most recent method that has been developed for genotypic classification of *Leptospira* (Ahmed et al., 2006, Thaipadungpanit et al., 2007). It generates the DNA sequence data of *Leptospira*, which is unambiguous and suitable for creating electronically accessible databases (Ahmed et al., 2006). In general, MLST requires the amplification of several housekeeping genes which evolve slowly by PCR and then compare the amplified product by DNA sequencing method. MLST has a high discriminatory power, reproducibility and robustness. The only limitation of MLST is that it requires highly skilled personnel and expensive equipment which will limit its use to sophisticated laboratories.

1.12 Treatment

Several studies have evaluated *in vitro* susceptibility of leptospires to antimicrobial agents (Ellinghausen, 1983, Hospenthal & Murray, 2003, Oie *et al.*, 1983, Shalit *et al.*, 1989). They show a high degree of efficacy of a broad range of antibiotics such as β-lactams, fluoroquinolones, macrolides, ketolides, tetracyclines, cephalosporins and streptomycin but not for chloramphenicol and sulphonamides. However, the relevance of the *in vitro* findings to clinical outcome is yet to be reported. Several studies have shown that ampicillin, ofloxacin and oxytetracycline fail to eliminate leptospires from the kidneys despite being effective *in vitro* (Ketterer & Dunster, 1985, Truccolo *et al.*, 2002). Laboratory animals have been used to assess the *in vivo* efficacy of antibiotics to experimentally-induced leptospirosis (Alt & Bolin, 1996, Alexander & Rule, 1986, Moon *et al.*, 2006, Moon *et al.*, 2007). One study has employed real-time PCR to measure the density of leptospires in organs of experimentally infected hamsters after treatment with antibiotics (Truccolo et al., 2002).

Currently, penicillin and doxycycline are the drugs of choice for treatment of human leptospirosis because they have been studied in randomised controlled clinical trials (Edwards *et al.*, 1988, McClain *et al.*, 1984, Watt *et al.*, 1988, Prescott, 1991). The treatment is most effective when started early during acute illness. For patients who develop jaundice, pulmonary symptoms and acute renal failure, supportive treatments such as fluid therapy and dialysis are required (Levett, 2001). Data from a randomised double-blind controlled field trial in United States army have shown the potential of low dose doxycycline (200mg/week) administered orally as a short-term chemoprophylaxis for leptospirosis (Takafuji *et al.*, 1984).

In animals, especially pets, the treatment for acute leptospirosis is generally similar to humans (Langston & Heuter, 2003). However, animals are usually treated with dihydrostreptomycin or oxytetracycline (Thiermann, 1984). In case of an outbreak, prophylactic antibiotic treatment of all animals in the mob or herd will markedly decrease the incidence of disease. Combination of prophylactic treatment with whole-herd vaccination and animal isolation is effective (Dean *et al.*, 2005).

Another perspective in the treatment of leptospirosis in animals is to eliminate the carrier state in chronic leptospirosis. Several studies have assessed the ability of antibiotics to clear leptospiral organisms from cattle (Alt *et al.*, 2001, Ellis *et al.*, 1985, Gerritsen *et al.*, 1994, Gerritsen *et al.*, 1993, Smith *et al.*, 1997). Single dihydrostreptomycin injection at a dose rate of 25mg/kg was claimed to eliminate the shedding status of both experimentally-infected and naturally-infected cows with serovar Hardjobovis (Gerritsen et al., 1994, Gerritsen et al., 1993). However, this is in contrast to another study which reported the failure of dihydrostreptomycin at the same dose rate to remove *Leptospira* serovar Hardjoprajitno from the genital tract and kidney (Ellis et al., 1985). The variation between these studies may result from the serovar susceptibility to antibiotics and detection methods of infection. There is an attempt to find an alternative drug to dihydrostreptomycin because of concerns over drug residues and safety, since it can be ototoxic in some animals. Amoxycillin is proposed to be efficacious in eliminating the carrier status of cattle infected with serovar Hardjobovis and also is removed rapidly from the body (Smith et al., 1997). Recently, newer antibiotics such as tulathromycin and ceftiofur crystalline free acids suspension have been shown to clear serovar Hardjobovis organisms from the urine and kidney tissue of experimentally infected cattle (Cortese *et al.*, 2007).

1.13 Control

Control of leptospirosis requires sophisticated and holistic approaches that incorporate a range of strategies. The arguments go beyond the obvious need to prevent clinical illness and economic losses in domestic animals. There are also needs to minimise the risk of human infection by controlling exposure from animals (Ellis, 1994). Control of sporadic leptospirosis (animals infected with non-host-adapted serovars) requires identification and reduction of the maintenance hosts and/or immunisation of the incidental host whereas control of endemic leptospirosis (animal infected with host-adapted serovars) requires different strategies. The key factor is to limit the direct and indirect transmission of the organisms between susceptible hosts, carriers and contaminated environment (Heath & Johnson, 1994). This can be achieved through elimination of carriers in the maintenance host populations by antibiotic treatment, reducing transmission risk factors by management and through prevention by vaccination.

Leptospiral vaccination has been shown to reduce clinical and subclinical disease and bacterial shedding in dairy cattle and pigs, resulting in a significant reduction of animal and human cases of leptospirosis in New Zealand (Thornley et al., 2002). Currently, all licensed leptospiral vaccines are killed whole cell vaccines containing local serovars since the immunity is serovar-specific (Koizumi & Watanabe, 2005). Leptospires are grown in protein medium and inactivated by mechanical (heat, pressure) or chemical (formalin, phenol, methiolate) means. Vaccines are designed to induce the production of antibodies against the LPS found on the surface of leptospires (Brown et al., 2003). Vaccines should be given to young animals when maternal immunity wanes and a booster should be given four to six weeks later and thereafter at one to two year intervals (Torten & Marshall, 1994). In cattle and deer, commercially available vaccines containing serovar Hardjobovis alone or in combination with serovar Pomona have been shown to decrease the incidence of infection and the duration and intensity of urinary shedding (Bolin & Alt, 2001, Mackintosh *et al.*, 1980a, Marshall *et al.*, 1979, Allen *et al.*, 1982, Ayanegui-Alcérreca, 2006). Vaccination has also shown the ability to induce CMI by production of IFN-γ-producing cells (Brown et al., 2003, Naiman et al., 2001, Naiman et al., 2002). Clearing of *Leptospira* from an endemically infected herd by combining the use of an aggressive vaccination program e.g. whole-herd vaccination (Little *et al.*, 1992) with antibiotic treatment, may be possible (Alt et al., 2001) provided external sources of infection are eliminated.

There were attempts to develop leptospiral vaccine candidates from LPS and protein antigens (Koizumi & Watanabe, 2005, Wang *et al.*, 2007). It has been found from one study that LPS vaccine prepared from non-pathogenic *L. bifexa* serovar Patoc can effectively protect hamsters against *L. interrogans* serovar Manilae (Matsuo *et al.*, 2000). Another study demonstrated cross-protective immunity induced by protein extracts administered to gerbils (Sonrier et al., 2001). Results from those studies suggest the possibility of developing new vaccines that protect against various serovars. Until now, several leptospiral protein antigens such as OmpL1 and LipL41 (Haake *et al.*, 1999), LipL32 (also known as Hap-1) (Branger *et al.*, 2001) and Lig proteins (Koizumi & Watanabe, 2004) have been shown to elicit protective immunity in animal models . Moreover, whole genome analysis of available *Leptospira* DNA sequences has been used to identify candidate antigens for development of leptospiral vaccine (Gamberini *et al.*, 2005). Recently, a DNA vaccine has been developed and shown to induce protection against a lethal challenge of *L. interrogans* serovar Canicola in gerbils (Branger *et al.*, 2005b). DNA vaccine technology is claimed to be a promising new approach for vaccination against leptospirosis due to several advantages such as easy construction, low cost mass production, simple administration, temperature stability and ability to induce both HMI and CMI responses (Branger et al., 2005b, Wang et al., 2007).

1.14 Effects of leptospirosis on animal production

Farm animals infected with non-host-adapted leptospiral serovars may develop a significant illness leading to loss of production (reproduction or growth) or death, e.g. infection of cattle with serovar Pomona. While measuring clinical disease is easy, to measure the animal production costs associated with subclinical leptospirosis is a more complex task because it is determined by interactions between management factors and factors intrinsic to animals in any given environment which influence prevalence, and effects are often difficult to detect without systematic recording of management outcomes such as pregnancy, calving and weaning rates, and growth rate. Reproductive efficiency is a major contributing factor to the economic viability of any livestock industry. *Leptospira* organisms are proven to infect the reproductive organs and affect reproductive performance of livestock such as cattle (Ellis *et al.*, 1986c, Ellis & Thiermann, 1986b, Thiermann, 1982), pigs (Ellis *et al.*, 1986a, Ellis *et al.*, 1986b, Ellis & Thiermann, 1986a), sheep and goats (Lilenbaum *et al.*, 2008) and deer (Ayanegui-Alcérreca *et al.*, 2007). Several studies have attempted to find and explain the association between subclinical leptospirosis and reproductive performance especially in cattle and pig industries (Dhaliwal *et al.*, 1996c, Boqvist *et al.*, 2002, Kazami *et al.*, 2002, Kasimanickam *et al.*, 2007, Grooms, 2006). One study analysed fertility data and demonstrated the reduction in conception rates of cows infected with *Leptospira* serovar Hardjo (Dhaliwal *et al.*, 1996b). Another study from the same author also demonstrated that vaccination against *Leptospira* serovar Hardjo improved fertility in cows (Dhaliwal *et al.*, 1996a). This is consistent with reports in other species such as pigs in Brazil where seroreactivity to *Leptospira* is proven to be associated with reproductive losses (Ramos *et al.*, 2006). In New Zealand farmed deer, the report has shown an improvement in weaning rate to 97% in vaccinates, compared with 88% in controls in the presence of evidence of leptospiral infection (Ayanegui-Alcérreca *et al.*, 2007).

The effects of leptospiral infection on growth of livestock animals have not been studied widely. One study reported that seropositivity for *Leptospira* did not affect daily weight gain of beef cattle (Fava *et al.*, 2004). This is contrary to the data from a study in farmed deer in New Zealand which reported that yearling deer with evidence of serological infection during the previous nine months were 3.7 kg lighter at 12 months of age than those without sero-converting (Ayanegui-Alcérreca et al., 2007). More research is needed to understand the role of leptospiral infection in growth response of livestock.

1.15 Leptospirosis in New Zealand

Leptospirosis outbreaks in New Zealand livestock were first reported in the 1950s (Bruere, 1952, Hartley, 1952, Kirschner *et al.*, 1952). It has now been recognised in humans, domestic animals and wildlife (Blackmore *et al.*, 1976, Hathaway, 1981). Until now, eight serovars in two genomospecies were isolated in New Zealand as summarised in Table 1.3. Cattle and farmed deer are recognised as a maintenance host for serovar Hardjo (Note: serovar Hardjo in

New Zealand is now known as Hardjobovis), pigs for serovar Pomona and Tarassovi, Norway rats (Rattus norvegicus) for serovar Copenhageni, black rats (Rattus rattus) for serovar Ballum, brush tail possums (*Trichosurus vulpecula*) for serovar Balcanica. Serovar Australis and Canicola have been isolated from human patients and are not considered to be endemic (Midwinter & Fairley, 1999). All other species reported with leptospirosis were considered to be accidental hosts (Hathaway, 1981, Marshall & Manktelow, 2002).

1.15.1 Humans

Leptospirosis is the most common occupationally-acquired infectious disease in New Zealand. The major source of infection in humans is from domestic animals, with clearly definable high risk occupational groups, consisting of farmers, meat workers, veterinarians and forestry related personnel (Schollum & Blackmore, 1982, Thornley et al., 2002). After the first report of leptospirosis in a human in the 1950s (Kirschner & Gray, 1951), leptospirosis was made notifiable under the Health Act of 1956 and all human cases must be recorded (Thornley et al., 2002) and risk factor data from the notifying doctor are entered into the surveillance database (EpiSurv). The disease is also under laboratory surveillance and testing is done by ESR (Environmental Science and Research). These laboratory and notification data are brought together nationally by ESR for the New Zealand Ministry of Health (Keenan, 2007).

Table 1.3 Classification of *Leptospira* species in New Zealand and their maintenance host

Species		**Serovar**	**Serogroup**	**Reservoir host**
Sensu lato	**Sensu stricto**			
L. interrogans	*L. borgpetersenii*	Hardjobovis	Sejroe	Cattle, Deer
L. interrogans	*L. interrogans*	Pomona	Pomona	Pig
L. interrogans	*L. interrogans*	Copenhageni	Icterohaemorrhagiae	Norway rat
L. interrogans	*L. borgpetersenii*	Ballum	Ballum	Black rat Mouse Hedgehog
L. interrogans	*L. borgpetersenii*	Balcanica	Sejroe	Possum
L. interrogans	*L. borgpetersenii*	Tarassovi	Tarassovi	Pigs
L. interrogans	*L. interrogans*	Australis	Australis	n/a
L. interrogans	*L. interrogans*	Canicola	Canicola	n/a

Adatped from (Ayanegui-Alcérreca, 2006, Marshall & Manktelow, 2002)
n/a = not available

In the early 1980s, the reported annual incidence of leptospirosis in humans in New Zealand was one of the highest in the world and the highest rates of infection were from dairy farming areas (Mackintosh *et al.*, 1980b). After cattle and pig vaccination became widespread, the annual incidence of human cases declined from 5.7 to 2.9 cases per 100,000 (Thornley et al., 2002). The average annual incidence of human leptospirosis from 2001 to 2003 was 4.0 cases per 100,000 based on laboratory-identified cases. However, this is likely

to be an underestimate because many individuals with leptospirosis may not seek medical attention because the symptoms may have been mild and short-lived, and possible mis-diagnosis by general practitioners (Baker & Lopez, 2004, Heuer et al., 2008). Recent cross-sectional serological survey of meat workers in a lamb-only abattoir in the Hawkes Bay area revealed a 9.5% seroprevalence of either serovar hardjobovis or Pomona (Benschop *et al.*, 2008). Infection with serovar Hardjobovis in humans in New Zealand has been associated with contact with cattle, sheep and deer whilst infection with serovar Pomona has been associated with pigs, cattle, sheep and deer (Thornley et al., 2002, Baker & Lopez, 2004), (Brown, 2005, Bell, 2005).

1.15.2 Deer

Previous reports have shown that leptospirosis is a well recognised clinical disease in New Zealand farmed deer (Ayanegui-Alcérreca *et al.*, 2003, Wilson *et al.*, 1998, Ayanegui-Alcérreca et al., 2007). The first substantial case report of leptospirosis in farmed deer was in the 1980s (Fairley et al., 1986) but recently a substantial epidemiological study of this disease in farmed deer has been reported (Ayanegui-Alcérreca, 2006). *Leptospira borgpetersenii* serovar Hardjobovis and *Leptospira interrogans* serovar Pomona are the most commonly detected serovars. *Leptospira borgpetersenii* serovar Tarassovi and *Leptospira interrogans* serovar Copenhageni were also reported (Flint *et al.*, 1988, Wilson et al., 1998) but it is likely that positive titres to those serovars resulted from cross-reactivity (Ayanegui-Alcérreca, 2006). Young animals are generally more susceptible to disease than adults and may suffer severe outbreaks and high mortality rates (Ayanegui-Alcérreca et al., 2007). It is suggested that the transmission of the infection and disease usually occurs before 1 year of age (Wilson & McGhie, 1993, Ayanegui-Alcérreca, 2006).

Data from a regional seroprevalence survey of 110 farms (Ayanegui-Alcérreca *et al.*, 2010 in press) showed evidence that 81% of farmed deer herds in New Zealand were infected with *Leptospira*. Serovar Hardjobovis was found in 61% of herds, Pomona in 4% and a combination of both serovars in 16%. At the individual level, serovar Hardjobovis was found in 54% of deer, Pomona in 2% and a combination of both serovars in 7%. No differences were found between regions. It is proposed that farmed deer in New Zealand are maintenance hosts for serovar Hardjobovis and an accidental host for serovar Pomona and that deer play an important role in the infection cycle of leptospirosis on New Zealand multi-species livestock farms (Ayanegui-Alcérreca, 2006).

Clinical cases of leptospirosis in farmed deer were associated with serovar Pomona with exception of two associated with serovar Hardjobovis (Wilson & McGhie, 1993). A recent case report of Pomona outbreak on a Southland deer farm described clinical signs such as weakness, lethargy, red urine and sudden death (Dean *et al.*, 2005). The necropsy findings of deer leptospirosis include enlarged kidney with small to large numbers of white spots and fibrotic scarring, jaundice with swollen liver and red urine in the bladder (Fairley et al., 1986, Wilson et al., 1998). Histopathological lesions in kidney reveal infiltration of mononuclear

cells, insterstitial nephritis and fibrosis. Hepatic lesions included hepatitis, centrilobular necrosis, hepatocellular vacuolation and haemosiderin pigmentation (Fairley et al., 1986).

Research has shown immunological response to commercially available leptospiral vaccine in farmed deer with no interference with colostrum-derived antibody (Ayanegui-Alcérreca, 2006). Little was known about the effect of leptospiral vaccine on deer growth and reproduction, but this has been the subject of present research (Subharat *et al.*, 2008).

1.15.3 Cattle

In the late 1970s and early 1980s, several studies were conducted to investigate the seroprevalence of leptospiral infection in cattle, particularly dairy cattle, in New Zealand. The data from those studies confirmed that Hardjo infection was endemic and that cattle were a maintenance host for this serovar but not for serovar Pomona (Hathaway, 1981, Bahaman *et al.*, 1984). A serological survey of dairy cattle in the Taranaki district showed that 62% of the animals had a titre to serovar Hardjo and 4% had a titre to serovar Pomona. It was suggested that leptospiral infection in cattle occurs during the first two years of age (Bahaman et al., 1984). Data from a serological survey in beef herds in the Hawkes Bay area showed that 100% of 50 herds had a positive titres to serovar Hardjo with 64% of individuals having a titre of ≥1:96, and 44% a titre of ≥1:384 (Matthews *et al.*, 1999).

1.15.4 Sheep

An early epidemiological study of leptospirosis in sheep from 45 lines at a slaughterhouse revealed evidence of serovar Hardjo infection in 20% of animals and serovar Pomona infection in 3.8% (Blackmore *et al.*, 1982). That study also suggested that sheep were unlikely to be a maintenance host and not a significant source of infection of other species with serovar Hardjo (Blackmore et al., 1982). More recent data from an abattoir study during 2004-5 (Dorjee *et al.*, 2005) showed that 85.7% and 23.4% of lines of hoggets and lambs, respectively, were seropositive for Hardjobovis and 28.6% and 10.6% of lines of hoggets and lambs, respectively, were seropositive for Pomona. Seroprevalences for Hardjobovis and Pomona at the individual animal level were 6.1% and 1.6%, respectively. The within-line prevalence of Hardjobovis ranged from 3.3% to 15.4% for lambs and from 3.3% to 60% for hoggets while the within-line prevalence of serovar Pomona ranged from 3.3% to 6.7% for lambs and 3.3% to 40.0% for hoggets. A low but persistent seroprevalence of Hardjobovis in sheep throughout the two years of study indicates low endemicity to this serovars, whereas Pomona infections appear to be sporadic (Dorjee *et al.*, 2008). The data from this study also suggests that approximately 13 of every 1,000 sheep slaughtered are potentially shedding live leptospires and pose a zoonotic risk to meat workers in the sheep abattoir (Dorjee et al., 2008).

1.16 Conclusion

This review has summarised current knowledge on important aspects of leptospirosis with particular relevance to the research presented in this thesis. The history of leptospirosis along with its aetiology, current taxonomy and classification, as well as its general epidemiology, clinical signs and pathology has been described. Current information on pathogenesis mechanisms, immune response and advances in diagnostic methods has been clarified and the treatment and control of leptospirosis has been discussed with the focus on vaccination and vaccines.

The ability to rapidly determine whole genome sequences of *Leptospira* has been a breakthrough in leptospirosis research during the past few years. The strategy of reverse genetics in which the leptospiral genome is analysed by computer programs has been applied for identification of leptospiral virulence factors. This has helped understanding of the pathogenesis and immunity-induced mechanisms of leptospires that will also lead to the development of novel diagnostic tests and identification of leptospiral candidate antigens for vaccines.

To our knowledge, this review provides the most current information on animal production related to leptospirosis. It is clear that information available in this area is limited. More study is needed to establish an understanding of the role of leptospiral infection in terms of production response in livestock, since this may be an important stimulus for farmers to control leptospirosis with dual animal production and health and human health implications.

Lastly, the epidemiology of leptospirosis in both humans and New Zealand livestock has been reviewed. Because livestock farming in New Zealand is becoming increasingly multi-species including deer, cattle and sheep, stimulated by potential for financial stability and biological benefits, there is an increasing risk of diseases such as leptospirosis that can be transferred between species. Thus, understanding the role of each potential host on mixed-species farms is essential to the overall understanding of the epidemiology of leptospirosis, and has particular relevance to implementation of control measures on-farm aimed at reducing the clinical and subclinical incidence of disease, and similarly, reduction of risk to humans in New Zealand.

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Chapter 2

Growth response and bacterial shedding in urine following vaccination for leptospirosis in young farmed deer

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(Raw data of this chapter are in Appendix 2a, 2b and 2c)

Abstract

AIM: To investigate growth response and shedding of leptospires in urine following serovar Hardjobovis and Pomona leptospiral vaccination in rising-one-year-old farmed red deer.

METHODS: Five previously *Leptospira* seropositive commercial deer farms were recruited. Between 2 - 20 March 2007, 435 mixed-sex, 3-month-old deer received a single dose of streptomycin (25 mg/kg) to minimise leptospiral infection. They were randomly allocated to streptomycin treated vaccinate (SV) and streptomycin treated control (SC) groups. Half (n=217) received a 2ml subcutaneous injection of a bivalent whole-cell killed leptospiral vaccine (Leptavoid-2) followed by a booster four weeks later. They were grazed with unvaccinated controls (SC) (n=218). These animals were isolated from other 3-month-old deer on each property which constituted a no-streptomycin control (NSC) group. In May, after 51 to 55 days, SV and SC deer were amalgamated with NSC deer on each property to be exposed to natural leptospiral challenge. Natural challenge of *Leptospira* was monitored in SC deer by serology against serovars *L. borgpetersenii* serovar Hardjobovis and *L. interrogans* serovar Pomona using a standard microscopic agglutination test (MAT), in May, August and November and in NSC deer in November. Urine was collected in November from females in all groups for detection of leptospiral shedding by using bacterial culture and real-time PCR. Male SV and SC deer were weighed in March, May, August and November.

RESULTS: Vaccination induced antibody to both Hardjobovis and Pomona in most animals in May, with a range of titres between 1:24 to 1:96 and 1:24 to 1:1536, respectively. Serologically, three of the five farms had evidence of Hardjobovis infection in male SC deer (seroprevalence range 6.7 – 87.5%) and four had evidence in female SC deer (seroprevalence range 4.3 – 77.8%) with titres up to 1:768. All were seronegative to Pomona. Vaccinated male deer (SV group) on the three farms with evidence of Hardjobovis infection had a higher growth rate (12.4 – 26.5g/d) than controls (SC group). Between August and November, the period corresponding to the time of seroconversion, growth was on average 49g/day ($p=0.0004$) higher in the SV group. On two farms with evidence of urinary shedding by real-time PCR, no shedding was found in vaccinated deer (SV) but the proportion shedding in SC and NSC deer was 23.5 and 52.6%, respectively. Shedding was reduced by vaccination from 56% (5/9) to 0% (0/11) on Farm 1 and from 12% (3/25) to 0% (0/25) on Farm 4, the overall difference of 24.5% being highly significant ($p = 0.0012$).

CONCLUSION: The bivalent leptospiral vaccine used significantly improved growth and prevented urinary shedding of young deer exposed to Hardjobovis providing evidence of vaccine efficacy and that Hardjobovis can cause adverse effects on productivity of young deer.

KEY WORDS: *Leptospirosis, farmed deer, vaccine, growth, urine shedding, Hardjobovis, Pomona, New Zealand*

List of abbreviations

ADG = Average daily gain
CMI = Cell-mediated immunity
EMJH = Ellinghausen-McCullough-Johnson-Harris medium
MAT = Microscopic agglutination test
NSC = No-streptomycin control
PBS = Phosphate-buffered saline
RD = Risk difference
SC = Streptomycin treated control
SV = Streptomycin treated vaccine

2.1 Introduction

Leptospirosis is a widespread zoonotic disease that affects most mammals and is an important cause of production loss in livestock throughout the world. Infection occurs via skin abrasions or mucous membranes followed by primary localisation in the kidney from where leptospires are voided in urine to expose other animals or humans (Faine *et al.* 1999).

In New Zealand (NZ) farmed deer, *Leptospira borgpetersenii* serovar Hardjobovis infection is endemic whilst *Leptospira interrogans* serovar Pomona is sporadic (Ayanegui-Alcérreca *et al.* 2007). A regional leptospirosis sero-prevalence survey of 110 deer farms in NZ showed evidence that 81% were seropositive, with Hardjobovis alone in 61% of herds, Pomona alone in 4% and a combination of both in 16% of herds (Ayanegui-Alcérreca *et al.* 2010 in press). Infection and disease in farmed deer usually occurs before 1 year of age (Ayanegui-Alcérreca 2006) and evidence suggests that most if not all clinical cases were associated with serovar Pomona (Wilson and McGhie 1993). Subclinical effects of these serovars on farmed deer have not been investigated.

Control of leptospirosis on deer farms should be aimed not only at preventing clinical illness and economic loss in animals, but also at minimising the risk of exposure to humans. Human leptospirosis incidence in New Zealand is among the highest in developed countries and is largely associated with livestock including farmed deer (Baker and Lopez 2004; Bell 2005). Farmed deer shed leptospires in urine for seven weeks on average (Ayanegui-Alcérreca 2006) but shedding may extend up to a year as reported in cattle (Ellis and Michna 1977; Hellstrom and Marshall 1978; Leonard *et al.* 1992). Vaccination is likely to be the most effective tool for reducing shedding rates in deer. Several commercial vaccines have been reported to decrease the incidence of infection, renal colonisation and duration and intensity of urinary shedding after experimental or natural challenge of serovar Hardjobovis and Pomona in cattle (Marshall *et al.* 1979; Mackintosh *et al.* 1980; Marshall *et al.* 1982; Bolin and Alt 2001) and pigs (Hodges 1977; Hodges *et al.* 1985).

Preliminary research into a leptospiral vaccine in farmed deer has shown reduced urine shedding, shedding duration, and positive kidney culture incidence in currently infected herds (Ayanegui-Alcérreca 2006). The vaccine has not been evaluated in deer which were leptospira-free prior to vaccination. Additionally, that author showed that in one herd, yearling deer, with evidence of infection during the growth period, were on average 3.7 kg lighter at 12 months of age than those without evidence of infection. By contrast, a study in beef cattle showed that seropositivity for *Leptospira* did not affect daily weight gain of (Fava *et al.* 2004). Thus, the growth response shown by Ayanegui-Alcérreca (2006) in deer requires replication, and if replicated, more data are needed on the relationship between prevalence and production responses at the herd level to assist in determination of likely cost-benefits of vaccination.

The purpose of this study was to replicate and further evaluate the effect of a bivalent leptospiral vaccine on growth and leptospiral shedding in young farmed deer under pastoral conditions typical of New Zealand, using different methodology to previous studies.

2.2 Materials and methods

2.2.1 Ethical approval

All procedures involving the use of animals in this project were approved by the Massey University Animal Ethics Committee under protocols 06/68 and 06/149.

2.2.2 Farms and animals

During August to November 2006, 20 commercial red deer (*Cervus elaphus*) farms, but possibly containing some wapiti (*C.e. canadensis)* genes, in the Manawatu and Hawkes Bay regions were recruited for a cross-sectional survey of leptospirosis to identify *Leptospira* seroprevalence. In early 2007, based on farmer willingness to participate and suitable numbers of animals, 435 mixed-sex, 3-month-old deer from five candidate farms with positive *Leptospira* serological status were enrolled. The location of farm, number of animals and sex and date of sampling are summarised in Table 2.1.

Table 2.1 Location, number of deer in each group, sex (F = Female, M = Male) and vaccination and sampling dates for each farm.

Farm	Location	No. of animals					Vaccination		Blood and urine sampling		
		SC		SV		NSC	Sensitiser* Mar	Booster Apr	May	Aug	Nov
		F	M	F	M	F+M					
1	Hawkes Bay	15	15	15	15	60	20	19	10	3	21
2	Manawatu	27	22	27	21	80	15	17	8	8	6
3	Hawkes Bay	21	20	20	21	110	2	5	26	1	16
4	Hawkes Bay	28	20	28	20	170	13	13	3	6	8
5	Hawkes Bay	24	26	25	25	120	8	5	1	10	13

SC = Streptomycin treated control; SV = Streptomycin treated and vaccinated; NSC = No streptromycin control

* Also blood and urine sampled

2.2.3 Trial design

A summary of the study design is shown in Figure 2.1. The study commenced with newly weaned 3-4 month-old deer in March 2007. Males (n=205) were used to evaluate growth response (average daily gain in grams (ADG)) to vaccination. Females (n=230) were used to evaluate shedding of leptospires in urine using real-time PCR and bacterial culture. These deer were randomly selected from the total available (Day 1), separated from their cohorts, ear tagged and treated with streptomycin (Vibrostrep, Stockguard Animal Health Limited, NZ) at 25 mg/kg intramuscularly to attempt to eliminate any residual leptospiral infection

(Ellis *et al.* 1985; Mackintosh 1993). Half of the streptomycin treated animals were vaccinated with a bivalent leptospiral vaccine (group SV) whereas the other half were left as unvaccinated controls (group SC). In the SV group, males were weighed whereas females were blood and urine sampled. In the SC group, males were weighed and blood sampled whereas females were urine and blood sampled. These two groups were kept isolated from other animals in a "low risk" area of the farm to reduce risk of re-exposure to leptospires while immunity developed to the vaccine. In April (Day 28), the SV group received a booster vaccination.

In May (Day 56), these two groups (SV and SC) were re-united with their original cohort group that did not receive antibiotic treatment (no streptomycin control (NSC)) to become exposed to natural infection. The weighing and blood and urine sampling regimes for these deer were repeated in August and November. For the NSC group, 20 females were randomly urine sampled at joining (May) and again in November.

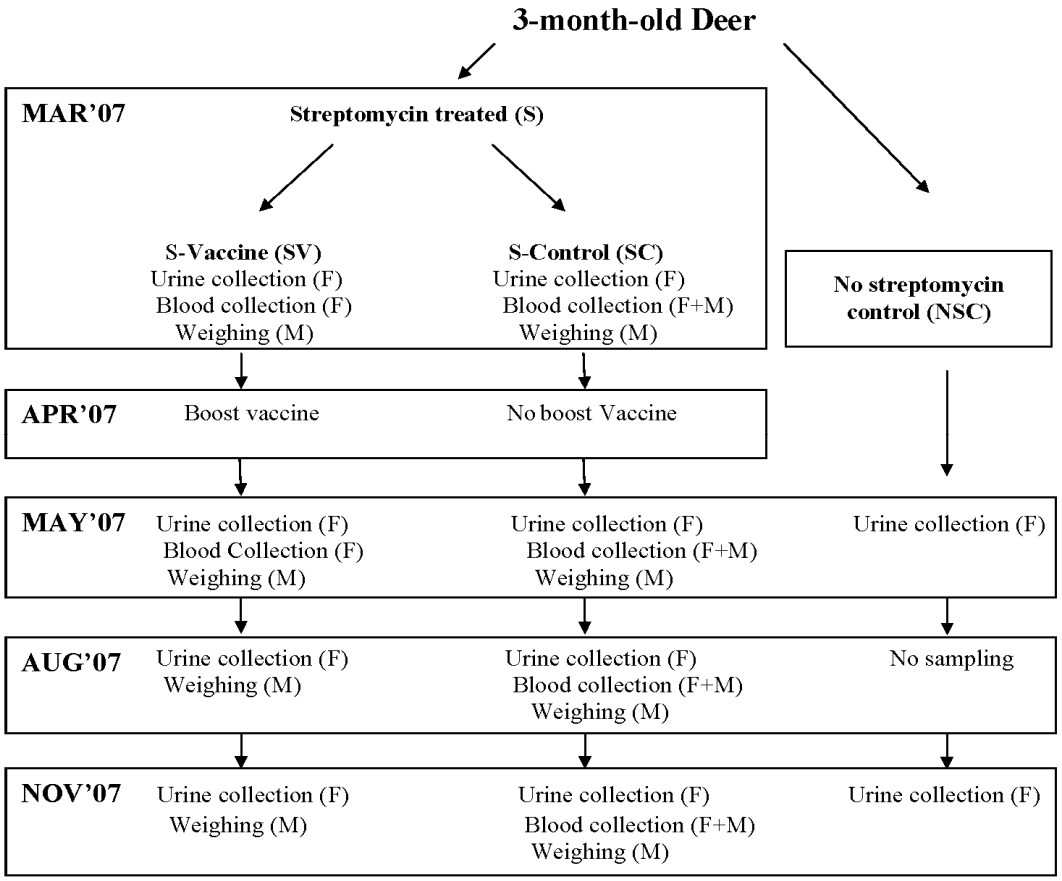

Figure 2.1 Vaccination study design (Groups within the same boxes grazed together) (M = male; F = female).

2.2.4 Vaccination

A commercial bivalent leptospiral vaccine (Leptavoid-2, Intervet/Schering-Plough Animal Health Limited, NZ) was administered subcutaneously and by inserting the needle into a hand-held skin fold.

2.2.5 Blood sampling

Deer were physically restrained for blood collection from the jugular vein using a new 20 gauge needle and a 10 ml evacuated blood tube with no anticoagulant. Blood samples were left to clot at room temperature before centrifugation at 3,000 rpm (1512 x *g*) for 15 minutes and storage of serum at -20°C.

2.2.6 Serology

The microscopic agglutination test (MAT) was used to test serum reactivity to laboratory standardized *Leptospira* serovars Hardjobovis and Pomona. The method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the Control of Leptospirosis" (Faine 1982). An initial serum dilution of 1:12 and two-fold serial dilution of serum covering the range of 1:24 to 1:3072 were tested. Titre was recorded as the reciprocal of the highest dilution at which ≥ 50% of leptospires were agglutinated. The cut-off point at ≥1:48 was used for both serovars to determine positivity (Blackmore *et al.* 1982; Dorjee *et al.* 2008).

2.2.7 Growth

2.2.7.1 Sample size: The number of samples required to evaluate leptospiral vaccine efficacy in terms of growth response to leptospirosis infection was calculated assuming that at least 4 kg difference in total weight gain from March to September between vaccinated and control deer was to be shown significant. This critical difference was based on previous results (Ayanegui-Alcérreca 2006). Thirty-six deer per group (vaccinated and control) were required to achieve 95% confidence and 80% power.

2.2.7.2 Weight: Male deer were weighed at intervals individually by digital scale. The animal identification number was recorded and matched with weight. Average daily gain (g/d) was calculated for periods March to May, May to August, August to November and overall between March and November.

2.2.7.3 Statistical analysis: Data were recorded and maintained using Microsoft Excel (Microsoft Corp, Redmond WA, USA) and checked for data entry errors against written records and by descriptive analysis (min/max values, data plots) before performing analysis. Statistical analyses were performed using SAS v9.1 (SAS Institute, Cary NC, USA). Linear regression analysis was used to determine the effect of vaccination on weight gain only on farms with serological evidence of leptospiral infection. Univariate and bivariate analyses were performed prior to multivariable linear regression analysis. Biologically sensible interactions between vaccination status effects, period effects and farm effects were tested in the model. A value of $p<0.05$ denoted statistical significance.

2.2.8 Urine shedding of leptospires

2.2.8.1 Sample size: The number of samples required to evaluate leptospiral vaccine efficacy in terms of urine shedding of leptospires was calculated by assuming a shedding rate of 2% in vaccinated deer and 10% in controls (Ayanegui-Alcérreca 2006). One hundred and eight deer per group (vaccinated and control) were needed to achieve 95% confidence and 80% power.

2.2.8.2 Urine collection and processing: Urination was induced by administration of furosemide ("Salix", Intervet, NZ) at 1-1.5 mg/kg intramuscularly (Warren and Whelan 1981; Fairley *et al.* 1984). A new 70 ml plastic collector was held beneath the vulva after urination began (Figure 2.2). As much middle-stream urine as possible was collected and immediately held at 4°C. After transport to the laboratory, urine was centrifuged at 3,000 rpm (1512 × *g*) for 10 minutes to provide sediments which were re-suspended with 400 µl of phosphate-buffered saline (PBS) to neutralise the pH (Levett 2001).

Figure 2.2 Urine collection from female R1yo deer.

2.2.8.3 Culture and serotyping: The culture method used was developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the Control of Leptospirosis" (Faine 1982). Half of re-suspended urine sediments (200 µl) were inoculated into 5 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium with an addition of antibiotic (5'-fluouracil) for contamination inhibition. From this inoculated medium, a 100 µl aliquot was transferred to another 5 ml of medium, followed by further transfer of 100 µl to another 5ml of medium. All three inoculated media were incubated aerobically at 28-30°C and examined every two weeks under dark-field microscopy. Cultures that showed no growth within four months were discarded and declared as negative. Isolates of leptospires were serotyped against standardised antisera of *Leptospira* serovars Hardjobovis and Pomona.

2.2.8.4 DNA extraction and Real-time PCR: Half of the re-suspended urine sediment (200 µl) was extracted for leptospiral DNA using a High Pure Template Kit (Roche, Germany) as per manufacturer's instructions. The real-time PCR technique was a modification of the method described by Slack et al (2006). SYTO9 (Invitrogen, Oregon, USA) was used as fluorescent double-stranded DNA specific intercalating dye (Monis *et al.* 2005) for real-time PCR assays. The assay was performed in a Rotor-Gene 6000 machine (Corbett Research, Mortlake, Australia) using primers 2For 5'-tgagccaagaagaaacaagctaca-3' and 504Rev 5'-matggttccrctttccgaaga-3' (Slack *et al.* 2006). Each 25µl reaction contained 2µl of DNA extracted from samples, 1.5µM SYTO9, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 12.5pmol of 2For primer, 12.5pmol of 504Rev primer, 0.1% bovine serum albumin (BSA), 1 unit of Taq DNA polymerase and double distilled water. Thermal cycling consisted of initial denaturation at 95°C for 10min followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 20 sec. Melting temperature (Tm) of PCR product was determined by melting curve analysis. It was performed by heating the PCR product from 70°C to 90°C and monitoring fluorescence change every 0.1°C. Confirmation of positive samples was determined by melting temperature (Tm) of the PCR product compared with the positive control. The Tm of positive samples was found to be between 83°C and 84°C. The positive control used for the real-time PCR assay was field isolates of *Leptospira* serovar Hardjobovis and the negative control was double distilled water.

Statistical analysis: A stratified analysis was performed only on farms with evidence of urinary shedding to adjust for farm effects. The risk differences (RD) between SV and SC deer shedding, with 95% confidence intervals, were computed using the Mantel-Haenszel procedure. A value of $P<0.05$ was selected to confirm that the RD was not equal to zero.

2.3 Results

Twenty deer (2 male and 5 female SV, 8 male and 5 female SC) were lost to follow-up during the study because of accidental death and disappearance. There were 100 SV and 95 SC male deer included in growth response analyses and 110 SV and 110 SC female deer included in shedding response analyses.

2.3.1 Serology

Serology results for females are shown in Table 2.2. Prior to vaccination (March), some deer on Farms 1 and 2 were seropositive to Hardjobovis but non-vaccinates were seronegative in May, suggesting that the initial titres were to maternal antibody. In May, 21 - 26 days after booster vaccination, 62/110 (59.6%) female SV deer were seropositive to Hardjobovis with titres ranging from 1:24 to 1:96, and 101/110 (97.1%) were seropositive to Pomona with titres ranging from 1:24 to 1:1536. In the female SC group, seroprevalence to Hardjobovis in November varied from 0 to 77.8%. Serology results for male deer are presented in Table 2.3. Male deer in the SC group on Farms 1-3 were seropositive for Hardjobovis by November,

with seroprevalence ranging from 5 to 87.5%. Farm 4 was seropositive for Hardjobovis only in female deer (n=2, 7.8%), with titres 1:192 and 1:768. No samples were seropositive for Pomona.

2.3.2 Growth

Weight data for each farm are shown in Table 2.4, and means (± 95% CI) for vaccinates and controls are presented in Figure 2.3. The ADG of SV deer from three farms with evidence of Hardjobovis infection was 48.7 g/day (95%CI = 22.4 – 73.6) higher than the ADG of SC between August and November (Figure 2.3) (p=0.0004), the period when most seroconversions occurred. On Farms 1, 2 and 3, seropositive to Hardjobovis, the overall mean differences in ADG between vaccinates and controls were 26.5 g/day (p<0.05), 12.4 g/day (p>0.05) and 16.3 g/day (p>0.05), respectively. This resulted in a mean live weight difference in November of 6.5 kg, 3.1 kg, and 4.0 kg on Farms 1 – 3 respectively, that were seropositive, and of 0 kg and -1.2 kg on Farms 4 and 5, respectively, that were seronegative.

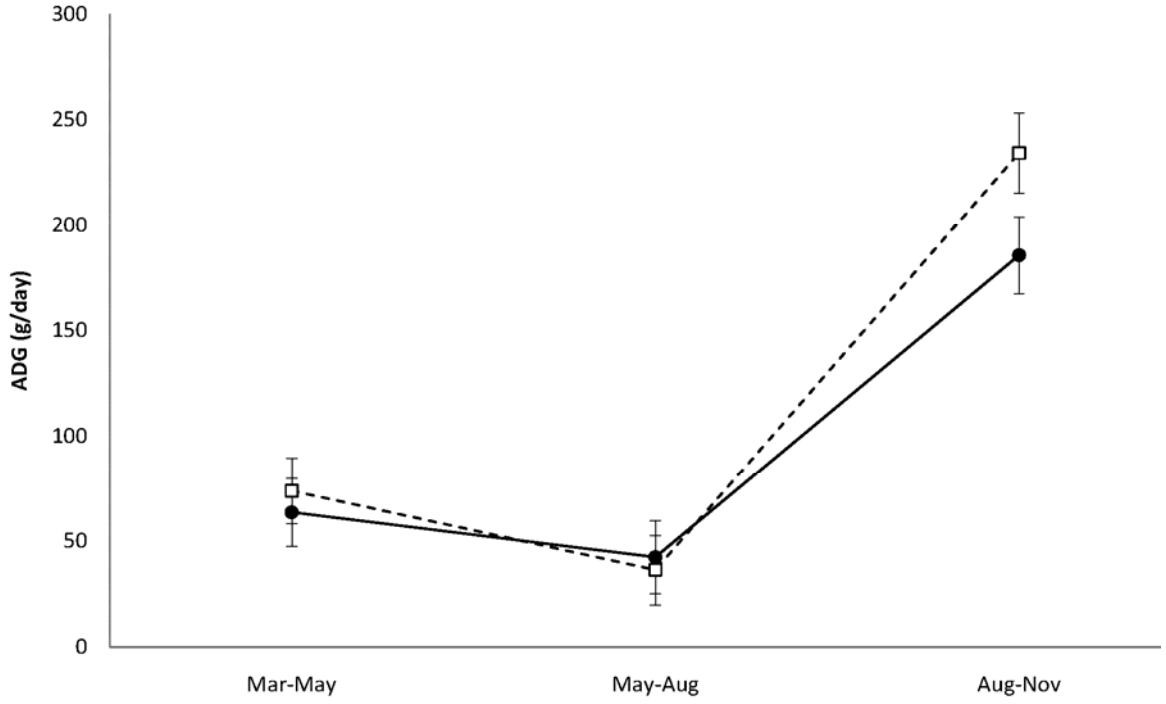

Figure 2.3 Mean ADG (±95% CI) at each weighing period in vaccinates (SV (--□--)) and controls (SC (–●–)) on farms with evidence of Hardjobovis infection (Note: seroconversion occurred principally between August and November).

Table 2.2 Seroprevalence (%) and (reciprocal titre range) for Hardjobovis (H) and Pomona (P) in female deer on each farm from March (pre-vaccination) to November. (Deer were grazed in contact with untreated cohorts after the May sampling).

Farm	No. of animals		March (pre-vaccination)				May				August		November	
	SC	SV	SC		SV		SC		SV		SC		SC	
			H	P	H	P	H	P	H	P	H	P	H	P
1	14	14	7.1 (48)	0 (-)	14.3 (48)	0 (-)	0 (-)	0 (-)	64.3 (24-96)	100 (24-768)	0 (-)	0 (-)	77.8 (48-192)	0 (-)
2	25	26	4.0 (48)	0 (-)	11.5 (48)	0 (-)	0 (-)	0 (-)	50.0 (24-96)	92.3 (24-1536)	8.3 (96)	0 (-)	4.3 (48)	0 (-)
3	21	18	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	38.9 (24-96)	77.8 (24-384)	0 (-)	0 (-)	12.5 (48-96)	0 (-)
4	26	27	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	76.9 (24-96)	88.5 (24-384)	0 (-)	0 (-)	7.8 (192-768)	0 (-)
5	24	25	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	40.9 (24-96)	100 (24-384)	0 (-)	0 (-)	0 (-)	0 (-)

Table 2.3 Seroprevalence (%) and (reciprocal titre range) for Hardjobovis (H) and Pomona (P) in male deer on each farm from March (pre-vaccination) to November. (Deer were grazed in contact with untreated cohorts after the May sampling).

Farm	No. of animals	March (pre-vaccination)		May		August		November	
		SC		SC		SC		SC	
		H	P	H	P	H	P	H	P
1	15	13.3 (48)	0 (-)	6.7 (48)	6.7 (48)	0 (-)	0 (-)	87.5 (48-192)	0 (-)
2	20	0 (-)	0 (-)	0 (-)	0 (-)	11.1 (96-192)	0 (-)	5.0 (48)	0 (-)
3	18	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	6.7 (48)	0 (-)
4	18	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
5	24	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)

Table 2.4 Mean ADG (and 95%CI) of vaccinated (SV) and control (SC) male deer on each farm for each period (Deer were grazed in contact with untreated cohorts after the May weighing).

Farm	Period								ADG difference (Mar – Nov)
	Mar - May		May - Aug		Aug - Nov		Overall (Mar - Nov)		
	SC	SV	SC	SV	SC	SV	SC	SV	SV - SC
1	119.0 (90.5 – 147.4)	169.9 (126.9 – 213.0)	46.5 (14.9 – 78.2)	21.7 (6.2 – 37.3)	204.6* (181.4 – 227.8)	254.3* (230.8 – 277.7)	126.8* (112.9 – 140.6)	153.3* (144.0 – 162.6)	26.5*
2	45.3 (23.0 – 67.7)	65.3 (50.3 – 80.3)	67.1 (49.5 – 84.7)	71.6 (59.9 – 83.4)	166.2 (142.7 – 189.8)	183.1 (169.8 – 196.5)	101.4 (89.3 – 113.4)	113.8 (106.4 – 121.2)	12.4
3	31.4 (18.1 – 43.9)	4.7 (-14.9 – 24.2)	2.4 (-14.6 – 19.4)	3.5 (-6.8 – 13.8)	173.7 (121.1 – 226.3)	273.2 (225.3 – 321.1)	95.1 (76.2 – 113.9)	111.4 (100.7 – 122.1)	16.3
4	125.1 (88.6 – 161.7)	105.9 (63.3 – 148.5)	34.6 (13.0 – 56.3)	43.6 (22.8 – 64.4)	190.9 (152.4 – 229.4)	185.6 (152.8 – 213.1)	113.0 (95.7 – 130.2)	113.0 (99.5 – 125.9)	0
5	61.3 (41.8 – 80.7)	79.5 (59.1 – 100.0)	32.0 (19.6 – 44.3)	26.1 (14.1 – 38.2)	235.2 (208.8 – 261.6)	231.6 (205.9 – 257.4)	114.3 (101.5 – 127.1)	109.5 (95.1 – 123.8)	-4.8

*Statistically significant at P<0.05

2.3.3 Urine culture/PCR for *Leptospira*

Urine culture and PCR data from samples collect in November are summarised in Table 2.5. There was no evidence of urinary shedding of *Leptospira* in vaccinated deer. Urinary shedding was identified by culture and/or real-time PCR on Farms 1 and 4 and only in control (both SC and NSC) groups. The prevalence of urinary shedding determined by real-time PCR in SC and NSC groups in Farm 1 was 55.6% and 83.3%, respectively, whereas on Farm 4 prevalence was 12.0% and 25.0%, respectively. All animals shedding on Farm 1 had titres to Hardjobovis in the range 1:48 to 1:192 whereas only one of three PCR positive animals on Farm 4 had a titre to Hardjobovis (1:192). All *Leptospira* isolates derived from urine culture from Farm 1 were identified as serovar Hardjobovis.

Shedding was reduced by vaccination from 56% (5/9) to 0% (0/11) on Farm 1 and from 12% (3/25) to 0% (0/25) on Farm 4. Stratified analysis of shedding results from farms with evidence of urinary shedding (Farms 1 and 4) revealed an average risk difference (RD_{mh}) of -24.5% (95%CI -38.9% – -10.1%) between the SV and SC deer (p=0.0012), indicating a difference in shedding between vaccinates and controls.

Table 2.5 Number of deer (and %) urine culture and real-time PCR *Leptospira* positive in SC, SV and NSC groups in November.

Farm	Group					
	SC		SV		NSC	
	Culture	PCR	Culture	PCR	Culture	PCR
1	1/9 (11.1)	5/9 (56%)	0/11 (0)	0/11 (0)	6/18 (33%)	15/18 (83%)
2	0/24 (0)	0/24 (0)	0/24 (0)	0/24 (0)	0/20 (0)	0/20 (0)
3	0/19 (0)	0/19 (0)	0/16 (0)	0/16 (0)	0/19 (0)	0/19 (0)
4	0/25 (0)	3/25 (12%)	0/25 (0)	0/25 (0)	0/20 (0)	5/20 (25%)
5	0/21 (0)	0/21 (0)	0/22 (0)	0/22 (0)	0/20 (0)	0/20 (0)

2.4 Discussion

This study has confirmed data of Ayanegui-Alcérreca (2006) showing both a reduction of growth attributable to exposure to leptospires, and that vaccination was effective in reducing the urinary shedding rate of leptospires. This paper also confirmed that serovar Hardjobovis has caused sub-clinical production loss on deer farms, and that this can be controlled by vaccination.

The present study was designed to mimic in a research context, the situation in which a whole infection-free herd was vaccinated and then challenged. It was intended to better evaluate the effect of a vaccine on growth and shedding outcomes than possible by vaccination in the face of endemic infection. Rising-one-year-old deer were selected for this study since a previous study has demonstrated that infection was more common in this than other age groups (Ayanegui-Alcérreca 2006). Female deer were selected for the urine shedding study for ease of urine sampling and male deer were selected for the growth study since they are more likely to be slaughtered at one year of age. Urine shedding observed by both culture and real-time PCR in November, in the NSC group along with the evidence of serology in SC group, confirmed that natural challenge by Hardjobovis was present in at least two herds.

The weak MAT antibody response to vaccination against Hardjobovis in this study was similar to that described in cattle (Marshall *et al.* 1979; Mackintosh *et al.* 1980; Allen *et al.* 1982) and deer (Ayanegui-Alcérreca 2006). However, it has been proposed that the efficacy of killed leptospiral vaccine in terms of protection is derived from cell-mediated immunity (CMI) which is not detected by MAT. A similar monovalent vaccine (Sv. Hardjobovis) used in cattle studies was claimed to induce CMI by production of IFN-γ-producing cells (Naiman *et al.* 2001; Naiman *et al.* 2002; Brown *et al.* 2003) and prevented renal colonisation and urinary shedding after experimental challenge of serovar Hardjobovis (Bolin and Alt 2001).

Evidence of MAT response from control and vaccinated deer at the start of the trial from Farms 1 and 2 could be due to maternally derived antibodies as reported by Ayanegui-Alcérreca (2006), since titre prevalence declined at the May and August samplings. Subsequent increasing MAT titres to Hardjobovis after joining with NSC deer and evidence of *Leptospira* shedding from SC and NSC deer have demonstrated the existence of natural challenge by Hardjobovis on four of five farms. This supports that our study model was effective in terms of natural challenge of leptospires and mimicking the effect of the vaccine in previously un-infected animals. However, since seroprevalence was low, it would appear that exposure to leptospires was limited on all but Farm 1. A severe drought occurring in the study area in 2007 may have played an important role in limiting the transmission and survival of leptospires during the year of study (Hellstrom and Marshall 1978).

In New Zealand, farmed deer are believed to be a maintenance host for Hardjobovis with high susceptibility to infection but low pathogenicity (Ayanegui-Alcérreca *et al.* 2007). However, while the subclinical effect of this serovar on growth response has been preliminarily investigated demonstrating a negative effect, that observation required replication since such response challenges conventional beliefs about subclinical effects of leptospiral infection in host-adapted species. Farm 1 which experienced a high seroprevalence of Hardjobovis and confirmed infection using urine PCR, showed a statistically significant greater weight gain in vaccinated animals. The difference of ADG was approximately 27 g/day, which resulted in a mean of 6.5 kg in November, after a growing period of 241 days following vaccination.

On Farms 2 and 3 which also showed evidence of Hardjobovis infection, the differences in mean live weight in November of 3.1 kg and 4.0 kg respectively, were not statistically significant. On Farm 4 and 5 with no evidence of Hardjobovis, no evidence of mean live weight difference was observed. This finding is consistent with the previous report of Ayanegui-Alcérreca (2006) that deer with evidence of leptospiral infection during the growth period were 3.7 kg lighter at 12 months of age than those without evidence of infection. That data, combined with the observation of a significant difference in this study were only on the farm with the highest seroprevalence and urine shedding ri ate, supports that herd mean responses, and therefore economic responses to vaccination are seroprevalence-related.

When the ADG of deer on the three farms with Hardjobovis infection was combined and analysed by period, a significantly higher weight gain (49 g/day) was found in vaccinated deer during August to November (94 days) when almost all of the seroconversion occurred. This corresponded to a 4.6 kg mean difference between vaccinates and controls in November.

An important argument for control of leptospirosis is its potential impact upon humans. Zoonotic infections of human with *Leptospira* represent a significant public health problem in New Zealand. This study indicates that a suitable vaccine is capable of reducing the public health hazard of *Leptospira* shedding in deer urine as in dairy cattle (Thornley *et al.* 2002). The detection of leptospiruria was based on real-time PCR rather than culture since a recent study has shown that culture of field-sampled deer urine suffered high contamination rates and real-time PCR was claimed to be more sensitive and 100% specific, and detects only pathogenic *Leptospira* spp. (Slack *et al.* 2006; Subharat 2010 in press).

The prevalence of urine shedding in NSC deer was high (83.3%) on Farm 1 where seroprevalence to Hardjobovis was 87.5% in non-vaccinates, suggesting strong natural challenge. The prevalence of urinary shedding in NSC deer was lower (25.0%) on Farm 4, and only one of three control deer with evidence of urine shedding had a titre to Hardjobovis of 1:192. This suggests a scenario of recent infection with low spreading rate during the sampling period of this farm. There was no evidence of urine shedding from vaccinated deer on both two farms suggesting vaccine protection. The calculated Mantel-Haenszel RD has shown that vaccination could reduce the risk of deer shedding leptospires by 24.5%

(p=0.0012). This finding is consistent with previous reports in cattle (Bolin and Alt 2001) and deer, albeit in already infected animals (Ayanegui-Alcérreca 2006).

Prevention of urinary shedding of leptospires in farmed deer could reduce the risk of leptospirosis for other animals and humans. As the majority of notified human leptospirosis cases in farmers and meat workers is associated with Hardjobovis (Thornley *et al.* 2002; Baker and Lopez 2004), it should be possible to formulate a vaccination programme for farmed deer which will reduce the occupational exposure to humans while also providing positive economic returns if challenge is sufficiently high.

This study demonstrated that vaccination of young deer with a bivalent leptospiral vaccine has the potential to significantly improve mean weight gain in infected herds and prevent urinary shedding after natural challenge with Hardjobovis. It also provides the first evidence of adverse subclinical effects on deer production by serovar Hardjobovis alone.

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Chapter 3

Reproduction response following vaccination for leptospirosis in New Zealand farmed deer

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(Raw data of this chapter are in Appendix 3a and 3b)

Abstract

AIM: To investigate reproduction response in terms of weaning rate, following leptospiral vaccination against serovars Hardjobovis and Pomona in rising-two-year-old (R2yo) farmed red deer hinds.

METHODS: Six previously *Leptospira* positive commercial deer farms were recruited. In Mid-February 2007, 382 R2yo hinds on six farms received a single dose of streptomycin (25 mg/kg) to minimise leptospiral infection. They were randomly allocated to streptromycin treated vaccinate (SV) and streptomycin treated control (SC) groups. SV hinds (n=191) received a 2ml S/C injection of a bivalent whole-cell killed leptospiral vaccine (Leptavoid-2) followed by a booster four weeks later and were grazed with SC hinds (n=191). These animals were isolated from other no-streptomycin control (NSC) hinds on each property. After the rut (June), all SV and SC hinds were amalgamated with all NSC hinds on the property for maximum exposure to natural leptospiral challenge. Natural challenge of *Leptospira* was evidenced in SC hinds by serology against serovars (*L. borgpetersenii* serovar Hardjobovis and *L. interrogans* serovar Pomona) using the microscopic agglutination test (MAT) and in NSC hinds by detection of organism shedding in urine through bacterial culture and real-time PCR. Pregnancy scanning was done in May/June 2007 by rectal ultrasound to determine conception rate. Only pregnant vaccinated and control hinds were included for analyses. In late October, prior to calving, the pregnant SV and SC hinds were checked for carriage of foetus to term by abdominal ballotment and udder palpation to determine calving rate. In March 2008, at weaning, SV and SC hinds were examined for lactation status by udder observation and palpation and the calves were counted for determination of weaning rate.

RESULTS: One hundred and thirty Hinds (n=130) from two properties were excluded for failure to conceive or because they were sold for management reasons. Exclusion rates were similar for SV and SC hinds. Of 252 remaining hinds, 203 (80.6%) were scanned pregnant and remained in the study. Serology revealed evidence of Hardjobovis infection on all four remaining farms, and a single hind was serologically positive for Pomona between October and March of the following year. Real-time PCR from urine samples confirmed urine shedding in the NSC group on two farms. The calving rate of the SV and SC groups averaged 97.9% (range 94.7 – 100%) and 97.2% (range 94.1 – 100%), respectively ($p>0.05$). The weaning rates of SV and SC groups were 88.7% (range 77.8 – 94.7%) and 83.0% (range 76.5 – 87.5%), respectively ($p=0.015$).

CONCLUSIONS: Vaccination for leptospirosis resulted in no difference in calving rate, but a significantly higher weaning rate than unvaccinated controls suggesting that vaccination reduced pre-weaning mortality. This reduced reproductive performance was attributable to serovar Hardjobovis.

KEY WORDS: *Leptospirosis, farmed deer, vaccine, reproduction, calving rate, weaning percentage, Hardjobovis, Pomona, New Zealand*

List of abbreviations

EMJH = Ellinghausen-McCullough-Johnson-Harris medium
MAT = Microscopic agglutination test
NSC = No-streptomycin control
PBS = Polyphosphate-buffered saline
PCR = Polymerase chain reaction
Rpm = Revolutions per minute
R2yo = Rising-two-year-old
SC = Streptomycin treated control
SV = Streptomycin treated vaccinate

3.1 Introduction

Leptospirosis is a worldwide disease of livestock with potential major economic impact on reproductive efficiency (Grooms 2006). *Leptospira* infection occurs via skin abrasions or mucous membranes. In livestock, following leptospiremia, leptospires localise and persist primarily in the kidney and genital tract. Leptospires are shed in urine which serves as a source of infection to other animals (Faine *et al.* 1999). Reproductive efficiency is a major factor contributing to the economic viability of livestock farming. Leptospirosis can reduce reproductive outcomes and therefore have a substantial economic impact (Grooms 2006).

Considerable data have been published about the effect of leptospirosis on reproduction in cattle (Ellis and Thiermann 1986; Ellis 1994; Dhaliwal *et al.* 1996a, 1996b; Dhaliwal *et al.* 1996d) and pigs (Kazami *et al.* 2002; Ramos *et al.* 2006). Chronic leptospirosis causes impaired fertility, neonatal death, abortions and decreased milk production (Lilenbaum *et al.* 2008). However, little is known of the effect of leptospirosis on reproduction of pastoral farmed deer since this is a relatively new industry and largely limited to New Zealand.

In New Zealand (NZ) farmed deer, *Leptospira borgpetersenii* serovar Hardjobovis infection is endemic whereas *Leptospira interrogans* serovar Pomona is sporadic (Ayanegui-Alcérreca *et al.* 2007). A NZ regional leptospirosis prevalence survey of 110 farms showed evidence that 81% of farmed deer herds in New Zealand were seropositive with serovar Hardjobovis in 61% of herds, Pomona in 4% and a combination of both in 16% of herds (Ayanegui-Alcérreca *et al.* 2010 in press). Infection and disease in farmed deer usually occurs before 1 year of age (Ayanegui-Alcérreca 2006) and most clinical cases have been associated with serovar Pomona (Wilson and McGhie 1993).

Vaccination is one of the most effective tools for controlling leptospirosis. Several commercial monovalent and bivalent vaccines have been reported to prevent renal colonisation and urinary shedding after experimental or natural challenge in cattle (Marshall *et al.* 1979; Mackintosh *et al.* 1980; Bolin and Alt 2001) and pigs (Hodges *et al.* 1985). One study in vaccinated and non-vaccinated cows showed a correlation between renal and foetal infection in cows that became infected with serovar Hardjobovis after experimental challenge (Bolin *et al.* 1989). Vaccination improved the fertility of cattle (Dhaliwal *et al.* 1996c). Preliminary research into a leptospiral vaccine in farmed deer in New Zealand has shown a nine percentage point (88 vs. 97%) improvement in weaning rate in vaccinated deer on a farm with evidence of serovar Hardjobovis and Pomona infection (Ayanegui-Alcérreca *et al.* 2007). However, that observation requires replication to fully investigate and validate reproductive effects of leptospiral infection.

The purpose of this study was to determine the effect of a bivalent leptospiral vaccine on reproductive performance, measured as weaning rate of confirmed pregnant hinds, in leptospiral infected farmed deer herds under pastoral conditions in New Zealand.

3.2 Materials and methods

3.2.1 Ethical approval

All procedures involving manipulations on animals in this project have been approved by the Massey University Animal Ethics Committee under protocols 06/68 and 06/149.

3.2.2 Farms, animals and sample size

From August to November 2006, 20 commercial red deer (*Cervus elaphus*) farms but possibly containing some wapiti (*C.e. canadensis)* genes in the Manawatu and Hawkes Bay region were recruited for a cross-sectional survey of leptospirosis to identify *Leptospira* seroprevalence. Based on *Leptospira* seropositivity, farmer's willingness to participate, and presence of suitable number of animals, six candidate farms were enrolled in early 2007. R2yo hinds were chosen. The number required to evaluate leptospiral vaccine efficacy in terms of reproduction outcomes was calculated by assuming a nine percentage point difference of weaning rate between vaccinates and controls based on previously collected data (Ayanegui-Alcérreca *et al.* 2007). An estimated 105 hinds per group were required to achieve 95% confidence and 80% power for demonstrating significance of a difference of nine percentage points or more. Thus, it was proposed to use a minimum of 30 hinds per group on each of six farms assuming 70% of R2yo hinds would conceive and allowing for some loss to follow up. The farm location, final number of animals and vaccination and sampling dates for each farm are presented in Table 3.1.

Table 3.1 Location, number of deer in each group, vaccination and sampling date for each farm recruited for the study.

Farm	Location	No. animals			Vaccination		Pregnancy determination		
		SC	SV	NSC	Sensitiser*	Booster	Post-rut	Pre-calving*	Weaning*
1	Manawatu	20	20	25	19 Feb	19 Mar	21 May	1 Nov	5 Mar
2	Manawatu	42	42	60	12 Feb	15 Mar	12 Jun	6 Nov	3 Mar
3	Hawkes Bay	32	32	50	16 Feb	22 Mar	6 Jun	26 Oct	20 Mar
4	Hawkes Bay	31	33	40	23 Feb	3 Apr	31 May	25 Oct	13 Mar
5	Hawkes Bay	34	36	43	21 Feb	22 Mar	15 Apr	n/a	n/a
6	Hawkes Bay	30	30	40	14 Feb	22 Mar	29 May	n/a	n/a

SC = Streptomycin treated control; SV = Streptomycin treated and vaccinated; NSC = No streptomycin control; n/a = not available as these farms were lost to follow up

* Also blood sampled

3.2.3 Trial design

The study design for each farm is shown in Figure 3.1. Hinds (n=382) were randomly selected from the total number available in February 2007 (Day 1), and separated from their cohorts which remained on-farm. They were treated with streptomycin (Vibrostrep, Stockguard Animal Health Limited, NZ) at 25mg/kg intramuscularly to attempt to eliminate any residual leptospiral infection (Mackintosh 1993). Half were simultaneously vaccinated with a bivalent vaccine ("Leptavoid-2" Intervet-Shering Plough) (SV group) whereas the other half were blooded sampled and acted as unvaccinated control (SC group). These two groups were grazed together but isolated from other deer (constituting a no streptomycin or vaccine control group (NSC)) on the property to reduce risk of re-exposure to leptospires while vaccine-induced immunity developed. On March 2007 (Day 28), the vaccinated animals received a booster vaccination. Natural mating was undertaken.

Late May to early June 2007 the SV and SC groups were joined with naturally mated cohort hinds (NSC group), to become exposed to natural infection. All trial hinds (SV, SC and NSC) on each property were scanned for pregnancy by rectal ultrasound 28 to 60 days post-mating and the SC group was blood sampled. The pregnancy status shortly prior to calving was examined in late October to early November 2007 either by udder palpation, abdominal palpation or scanning to determine carriage of foetus to term. The SC hinds were blood sampled again on that occasion. At weaning in March 2008, all hinds were examined for lactation status by udder examination to determine whether they reared a calf to weaning. Calves were counted to establish weaning rate (calves weaned/hinds scanned pregnant). The SC hinds were blood sampled again on that date. For the NSC group, 20 were randomly urine sampled when joined with the SV and SC groups (June) and at weaning (March) to determine leptospiral shedding status.

3.2.4 Vaccination

A commercial bivalent leptospiral vaccine (Leptavoid-2, Intervet/Schering-Plough Animal Health Limited, NZ) was administered subcutaneously and by inserting the needle into a hand-held skin fold.

3.2.5 Blood sampling

Deer were physically restrained for blood collection from the jugular vein using a sterile 20 gauge needle and a 10 ml evacuated blood tube with no anticoagulant. Blood samples were left to clot at room temperature before centrifugation at 3,000 rpm (1512 x g) for 15 minutes and sera stored at -20°C.

3.2.6 Serology

The microscopic agglutination test (MAT) was used to test serum reactivity to laboratory standardized *Leptospira* serovars Hardjobovis and Pomona. The method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the

Control of Leptospirosis" (Faine 1982). An initial serum dilution of 1:12 and two-fold serial dilution of serum covering the range of 1:24 to 1:3072 was tested. Titre was recorded as the reciprocal of the highest dilution at which ≥ 50% of leptospires had agglutinated. A cut-off point of ≥1:48 was used for both serovars to determine serological positivity (Blackmore *et al.* 1982; Dorjee *et al.* 2008).

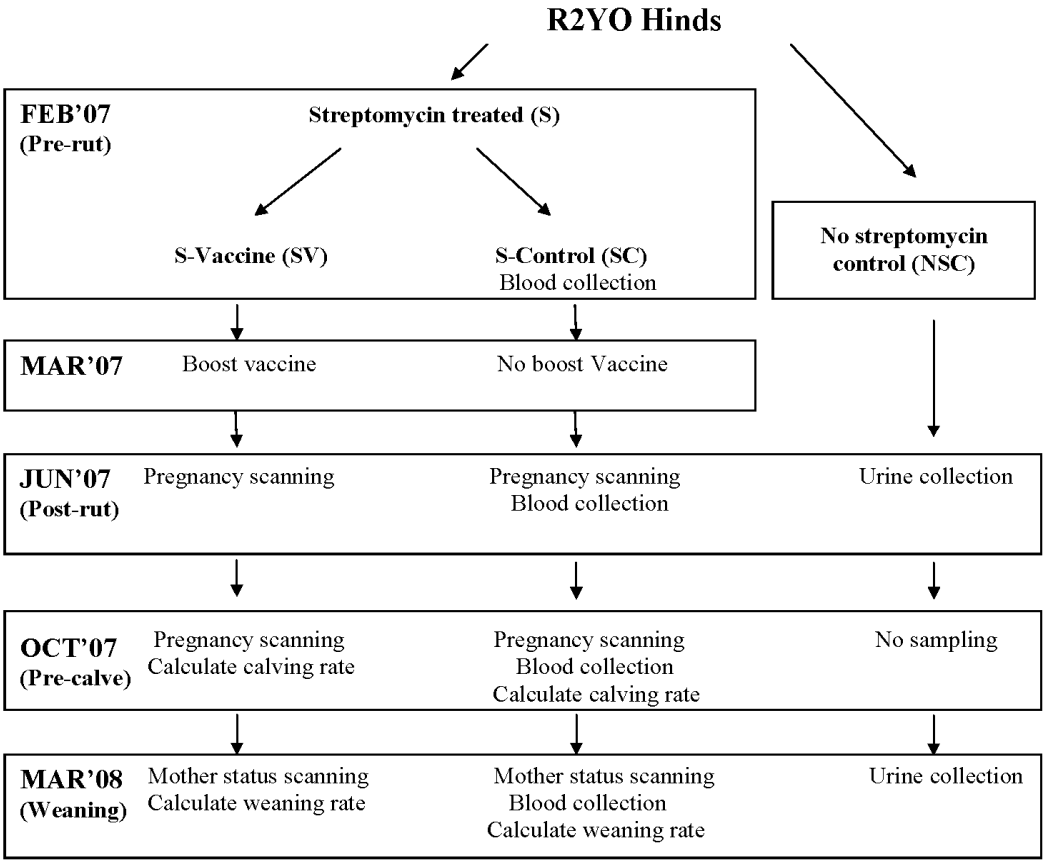

Figure 3.1 Vaccination study design (Groups within the same box were grazing together).

3.2.7 Urine shedding

3.2.7.1 Urine collection and processing: Urination was induced by administration of furosemide ("Salix", Intervet, NZ) at 1-1.5 mg/kg intramuscularly (Warren and Whelan 1981; Fairley *et al.* 1984). A sterile 70 ml plastic collector was held beneath the vulva after urination began. As much middle-stream urine as possible was collected and immediately held at 4°C. After transport to the laboratory, urine was centrifuged at 3,000 rpm (1512 × g) for 10 minutes to provide sediment which was re-suspended with 400 µl of polyphosphate-buffered saline (PBS) to neutralise the pH (Levett 2001).

3.2.7.2 Culture and serotyping: The culture method used was developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the Control of Leptospirosis" (Faine 1982). Half of the re-suspended urine sediments (200 µl) were inoculated into 5 ml of Ellinghausen-McCullough-Johnson-Harris (EMJH) medium

with an addition of antibiotic (5'-Fluouracil) for contamination inhibition. From this inoculated medium, a 100 µl aliquot was transferred to another 5 ml of medium, followed by further transfer of 100 µl to another 5ml of medium. All three inoculated media were incubated aerobically at 28-30°C and examined every two weeks under dark-field microscopy. Cultures that showed no growth within four months were discarded and declared as negative. Isolates of leptospires were serotyped against standardised antisera of *Leptospira* serovars Hardjobovis and Pomona.

3.2.7.3 DNA extraction and Real-time PCR: Half of re-suspended urine sediments (200 µl) were extracted for leptospiral DNA using a High Pure Template Kit (Roche, Germany) as per manufacturer's instructions. The real-time PCR technique was a modification of the method described by Slack *et al.* (2006). SYTO9 (Invitrogen, Oregon, USA) was used as fluorescent double-stranded DNA specific intercalating dye (Monis *et al.* 2005) for real-time PCR assays. The assay was performed in a Rotor-Gene 6000 machine (Corbett Research, Mortlake, Australia) using primers 2For 5'-tgagccaagaagaaacaagctaca-3' and 504Rev 5'-matggttccrctttccgaaga-3' (Slack *et al.* 2006). Each 25µl reaction contained 2µl of DNA extracted from samples, 1.5µM SYTO9, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 12.5pmol of 2For primer, 12.5pmol of 504Rev primer, 0.1% bovine serum albumin (BSA), 1 unit of Taq DNA polymerase and double distilled water. Thermal cycling consisted of initial denaturation at 95°C for 10min followed by 40 cycles of denaturation at 95°C for 10sec, annealing at 60°C for 20sec and extension at 72°C for 20 sec. Melting temperature (Tm) of the PCR product was determined by melting curve analysis. It was performed by heating the PCR product from 70°C to 90°C and monitoring fluorescence change every 0.1°C. Confirmation of positive samples was determined by melting temperature (Tm) of the PCR product compared with the positive control. The Tm of positive samples was found to be between 83°C and 84°C. The positive control used for the real-time PCR assay was field isolates of *Leptospira* serovar Hardjobovis and the negative control was double distilled water.

3.2.8 Pregnancy determination and calf rearing

Rectal ultrasound scanning for pregnancy was done in SV and SC hinds in late May to early June (Figure 3.2). During late gestation, late October to early November, pregnancy was confirmed using one or more of udder and/or abdominal palpation, visual assessment and lower abdominal flank ultrasound scanning to determine calf loss during pregnancy. At weaning in March, udder palpation and calf count were used to determine calf loss from birth to weaning (Beatson *et al.* 2000).

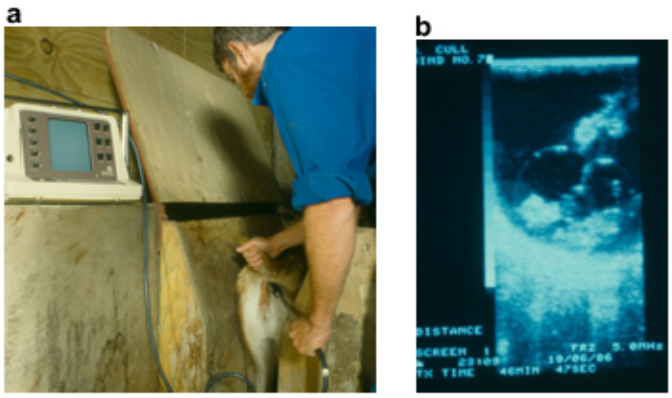

Figure 3.2 (a) Rectal ultrasound scanning of a hind (b) Ultrasound image of a deer foetus

3.2.9 Statistical analysis

Data were recorded and maintained using Microsoft Excel (Microsoft Corp, Redmond WA, USA) and checked for data entry errors against written records before performing analysis. All statistical analyses were performed using SAS v9.1 (SAS institute, Cary NC, USA). Mantel-Haenszel chi-square analysis was used to explore a possible interaction between farm and the effect of vaccination on calving and weaning outcomes. In addition, logistic regression was employed to adjust the effect of vaccination on calving and weaning for the potential confounding effect of deer breed. The interaction between vaccination status and farm was tested again. A value of $p<0.05$ was selected for statistical significance. The risk difference (RD) was calculated as a measure of vaccine efficacy.

3.3 Results

3.3.1 Pregnant hinds included in study analysis

Of 382 R2yo hinds, 130 from Farms 5 and 6 were removed from the study because there were no hinds on Farm 5 scanned pregnant due to undiagnosed reasons, whereas on Farm 6, the owner sold all the hinds in the study because of drought conditions. Of 252 hinds on the remaining four farms (Farms 1-4), 203 (80.6%) conceived and remained in the study. They comprised 97 vaccinated and 106 control hinds. Data are presented from the four remaining farms.

3.3.2 Serology

Serology results are presented in Table 3.2. At the start of the trial (February), SC hinds from Farms 2, 3 and 4 were seropositive to serovar Hardjobovis. All hinds on Farm 1 were seronegative despite a previously established infection status of deer on that farm. The seroprevalence for Hardjobovis was lower in June on Farms 2-4 and increased through to March. Farm 1 developed a high seroprevalence between June and October and maintained it until March. For Pomona, there was only a single seropositive hind on Farm 1.

3.3.3 Urine culture/PCR

No *Leptospira* organisms were isolated by culture from any urine samples of NSC hinds in June, when joined with SC and SV deer. There was PCR evidence of *Leptospira* shedding: in 4/20 and 2/20 of NSC hinds on Farm 1 when groups were joined and at weaning, respectively. On Farm 4, 6/20 NSC hinds were urine PCR positive at weaning only. No shedding was observed in deer on Farms 2 and 3.

Table 3.2 Seroprevalence (%) and (reciprocal titre range) for Hardjobovis (H) and Pomona (P) in streptomycin treated control hinds (group SC) on each farm from February (pre-vaccination of SV group) to March the following year. (Deer were grazed in contact with untreated cohorts from the June sampling).

Farm	No. of animals	Feb (pre-vaccination)		Jun		Oct		Mar	
		H	P	H	P	H	P	H	P
1	17	0 (–)	0 (–)	0 (–)	0 (–)	43.8 (96 – 384)	0 (–)	46.7 (48 – 384)	6.7 (48)
2	24	87.5 (48 – 1536)	0 (–)	8.3 (48)	0 (–)	14.3 (48 – 96)	0 (–)	28.6 (48 – 96)	0 (–)
3	32	55.0 (48 – 192)	0 (–)	9.1 (48 – 96)	0 (–)	40.0 (48 – 96)	0 (–)	41.4 (48)	0 (–)
4	33	32.0 (48 – 192)	0 (–)	9.6 (48 – 96)	0 (–)	12.9 (48 – 192)	0 (–)	21.2 (48 – 96)	0 (–)

3.3.4 Calving rate

Calving rates were similar in the two groups SV and SC (Table 3.3). The average calving rate (i.e. carriage of foetus to term) of the scanned pregnant vaccinated hinds was 97.9% (range 94.7 – 100%) and in the control hinds was 97.2% (range 94.1 – 100%; $p>0.05$). There was no interaction between vaccination and farm, thus vaccination had no measurable impact in calving rates on all farms at a statistically significant level.

Table 3.3 Calving rate (number of hinds carrying foetus to term/number scanned pregnant (%)) of hinds in control (SC) and vaccinated (SV) groups, measured in November, and the difference.

Farm	SC	SV	Difference (SV – SC)
1	16/17 (94.1)	18/18 (100.0)	5.9
2	24/24 (100.0)	18/19 (94.7)	-5.3
3	31/32 (96.9)	31/31 (100.0)	3.1
4	32/33 (97.0)	28/29 (96.6)	-0.4
All farms	**103/106 (97.2)**	**95/97 (97.9)**	**0.7**

3.3.5 Weaning rate

The weaning data are summarised in Table 3.4. The mean weaning rate of the scanned pregnant vaccinated hinds (SV) was 88.7% whereas the mean weaning rate of the scanned pregnant control hinds (SC) was 83%. Logistic regression analysis showed that the 5.7% (95%CI = 1.2% − 8.9%) higher weaning rate of the vaccinated group was significant (p=0.015).

Table 3.4 Weaning rate (number weaned/number scanned pregnant (%)) of hinds in control (SC) and vaccinated (SV) groups in March 2008, and the difference.

Farm	SC	SV	Difference (SV – SC)
1	13/17 (76.5)	14/18 (77.8)	1.3
2	21/24 (87.5)	18/19 (94.7)	7.2
3	27/32 (84.3)	29/31 (93.5)	9.2
4	27/33 (81.8)	25/29 (86.2)	4.4
All farms	**88/106 (83.0)**	**86/97 (88.7)**	**5.7**

3.4 Discussion

This study has complemented and is consistent with a previous finding by Ayanegui-Alcérreca (2006) that vaccination of deer against leptospirosis can improve the weaning rate on farms that have evidence of leptospiral infection. This study found a significant 5.7 percentage point increase in weaning rates whereas the study of (Ayanegui-Alcérreca 2006) reported a nine percentage points increase in weaning rates due to vaccination. That study was on multiparous hinds. Our study was designed to mimic in a research context and as far as practical, a situation in which a whole infection-free herd was vaccinated and then challenged by leptospires. This was intended to better evaluate the efficacy of a vaccine on reproduction outcomes than possible with vaccination in the face of endemic infection. R2yo hinds were selected for this study since this is the reproductively mature age group most likely to be seronegative when exposed to leptospires, and hence most likely to demonstrate a response to vaccination should leptospira infection occur and affect reproductive performance.

Increasing titres and prevalence to serovar Hardjobovis on all four remaining study farms after joining with NSC hinds confirmed that there was natural challenge in those animals, and observation of urine shedding by PCR confirmed active exposure to the SV and SC groups. Seropositivity to Pomona during this study was sparse, suggesting that the reproduction response observed was due primarily, if not in full, to serovar Hardjobovis.

Despite no *Leptospira* organisms being isolated from urine samples of NSC hinds, real-time PCR revealed evidence of *Leptospira* shedding on Farms 1 and 4. The sensitivity of PCR for identification of leptospires in urine is higher than that of isolation, but is likely to be less than 100%, particularly when shedding rates are low (Subharat 2010 in press). Real-time PCR evidence combined with seroconversion of SC deer on all four farms suggest that the study model was effective in terms of providing natural challenge by leptospires.

In New Zealand, farmed deer are believed to be a maintenance host for serovar Hardjobovis with high susceptibility to infection but low pathogenicity (Ayanegui-Alcérreca *et al.* 2007). Ayanegui-Alcérreca (2006) was the first to investigate potential subclinical effects of this serovar on reproduction of farmed deer demonstrating a weaning rate of 97% in vaccinated hinds and 88% in non-vaccinated in-contact controls. However, that study did not differentiate whether losses were pre-, peri- or post-natal, whereas the present study included observations on calf survival peri- or post-partum. In dairy cattle, persistent infection by Hardjobovis was reported to cause reduced fertility, reduced conception rates (Dhaliwal *et al.* 1996b), increased number of services per conception and prolonged calving interval. It is also a cause of early embryonic death, abortion, stillbirth and weak calf syndrome (Smyth *et al.* 1999). Reports about serovar Pomona suggest that it can cause more severe clinical effects such as abortion storm (Knott and Dadswell 1970; Gilmour 2007), but the sporadic occurrence renders Pomona less economically important than Hardjobovis (Givens 2006). This may equally apply to farmed deer.

There is paucity of information about effects of leptospiral vaccination on animal reproduction. One study in Queensland, Australia reported a reduction of abortion rates in beef cows treated with antibiotics and vaccinated against Hardjobovis (Holroyd 1980). However, another study in the USA failed to observe a difference in calving rate between vaccinated and unvaccinated beef cows despite the finding that beef cows positive to Hardjobovis as determined by increasing titre and urine shedding achieved lower calving rates (Kasimanickam *et al.* 2007). The authors postulated that inefficacy of vaccine and low level of challenge may be the contributing factors in failure to observe the difference between vaccinated and control cows.

In the present study, there was no difference in calving rate, i.e. carriage of foetus to term, between vaccinated and control hinds suggesting no evidence of prenatal losses. However, the weaning rate increased by 5.7 percentage points (6.9% improvement, range 1.5 – 10.7% between farms) due to vaccination. Therefore, the adverse effect of leptospiral infection was limited to the interval from calving to weaning, e.g. stillbirth, mis-mothering and/or birth of weak non-viable calves. The apparent absence of foetal loss may be explained by the initial streptomycin treatment which happened pre-mating and appeared to reduce re-infection rates until after June.

The reason for a lower weaning rate in our study compared with that of (Ayanegui-Alcérreca 2006) may be a relatively low challenge of the four trial farms and possibly the absence of

Pomona as deer in that report were infected by both serovars at higher prevalence and higher titres to both Hardjobovis (seroprevalence 85%; titres up to 1:384) and Pomona (seroprevalence 100%; titres up to 1:1536). In addition, Pomona is believed to be more pathogenic and to cause more severe effects than Hardjobovis in farmed deer (Ayanegui-Alcérreca *et al.* 2007), although there is limited robust data. Furthermore, infection with Hardjobovis may interfere with milk production of hinds. Several studies have shown that Hardjobovis infection can cause mastitis and milk drop syndrome in cattle (Hoare and Claxton 1972; Ellis *et al.* 1976) and agalactia in sheep (McKeown and Ellis 1986). This effect on milk production, if happen, could potentially decrease the success of calf rearing in farmed deer.

Findings from Farms 2, 3 and 4, but not Farm 1, suggest that the reproduction response to vaccination correlated with seroprevalence. Since the weaning rates of both SC and SV hinds were lower on Farm 1 (76.5 and 77.8%) than on the other farms (81.8 – 94.7%) whereas the calving rates were not, we speculate that specific events had occurred on Farm 1 between birth and weaning that impaired calf rearing. Such events could be related to general management or level of nutrition, which in total could have negated any effect of *Leptospira* vaccination on calf rearing.

We conclude that this study concurred with a previous report of improvement of reproductive performance associated with leptospirosis vaccination. It also confirmed that *Leptospira* serovar Hardjobovis can reduce reproductive performance on deer farms and that vaccination with a bivalent *Leptospira* vaccine has the potential to significantly improve weaning rates after natural challenge with this serovar. Further research is needed to determine the mechanism of this response and the relative importance of serovars Hardjobovis and Pomona in causing reproductive loss on deer farms.

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Chapter 4

Longitudinal serological survey and herd-level risk factors for *Leptospira* serovars Hardjobovis and Pomona on deer farms with sheep and/or beef cattle

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(Farm questionnaire and raw data of this chapter are in Appendix 4a and 4b)

Abstract

AIM: To investigate serology of *Leptospira* serovars Hardjobovis and Pomona on deer farms with sheep and/or beef cattle in the lower North Island of New Zealand and identify risk factors influencing serological status of each livestock species.

METHODS: Serological screening was conducted on commercial deer farms with sheep and/or beef cattle between August and October each year in 2006 to 2008 to determine seroprevalence of two leptospiral serovars. Serum samples were obtained from species farmed on deer only (n=3), deer and beef cattle (n=3), deer and sheep (n=2) and deer, beef cattle and sheep (n=12) farms in the Manawatu, Hawkes Bay and Wairarapa regions. On each farm every year, 20 or more rising 2-year-old replacement animals were randomly sampled from each species (1,173 deer, 817 beef cattle, 1,300 sheep). The microscopic agglutination test (MAT) was used to detect leptospiral antibodies against *Leptospira borgpetersenii* serovar Hardjobovis and *Leptospira interrogans* serovar Pomona. A cut point titre of ≥1:48 determined a positive sample. A questionnaire was completed by the farm owner or manager. Bivariate analysis was used to test for simple associations between potential risk factors and seropositive status of *Leptospira*, at one or more of the sampling dates, in deer herds for each serovar, using chi-square or Fisher's exact test. Prevalence Ratio and 95% confidence intervals were computed to determine the strength of association between risk factors and the risk for seropositivity to *Leptospira* in deer.

RESULTS: One farm having deer and sheep was lost to follow-up after the first year. The proportion of deer, cattle and sheep herds/flocks seropositive for Hardjobovis over three samplings averaged 64.9%, 69.2% and 64.1%, respectively. Equivalent results for serovar Pomona were 29.80%, 23.0% and 10.3%, respectively. At the individual animal level, 19.9% of deer, 39.1% of cattle and 30.1% of sheep were Hardjobovis positive, 8.8% of deer, 6.2% of cattle and 1.9% of sheep were Pomona positive and 4.3% of deer, 4.8% of cattle and 1.5% of sheep were positive for both serovars. Deer herds were more likely to be Hardjobovis positive when large (n=>560) (PR=1.46, p=0.036), deer were on farms with hilly topography (PR=4.67, p<0.001) and deer were co-grazing with Hardjobovis positive cattle herds (PR=1.93, p=0.022) or sheep flocks (PR=1.70, p=0.007). Deer herds were more likely to be Pomona positive when deer were co-grazing with Pomona positive cattle herd (RR=7.50, p=0.050) and Pomona positive deer herds. Deer herd were less likely to be Pomona positive when the farm had an open herd replacement policy (PR=0.28, p=0.007).

CONCLUSIONS: Exposure to *Leptospira* was widely distributed in deer, sheep and beef cattle in the lower North Island of New Zealand and concurrent infection of more than one species was common. Co-grazing of deer with other leptospiral positive species on farm tended to increase the risk of seropositivity to both Hardjobovis and Pomona in deer herds.

KEY WORDS: *Leptospirosis, deer, cattle, sheep, serology, Hardjobovis, Pomona, epidemiology, risk factors, mixed-species grazing, New Zealand*

List of abbreviations

MAT = Microscopic agglutination test

4.1 Introduction

Livestock farming in New Zealand has become increasingly multi-species including deer, beef cattle and sheep on the same property (Hilson 2007; Wilson 2007). Previous reports have shown that leptospirosis is a well recognised clinical disease in New Zealand livestock including farmed deer (Dean *et al.* 2005; Ayanegui-Alcérreca *et al.* 2007), cattle (Mackintosh 1981) and sheep (Thornton 1994; Vermunt *et al.* 1994). Leptospirosis has been shown to cause mortality, reproductive failure and production losses in deer (Ayanegui-Alcérreca 2006; Subharat 2010 in press), cattle (Smyth *et al.* 1999; Gilmour 2007) and sheep (Lilenbaum *et al.* 2008).

Several studies have been conducted to establish the epidemiology of leptospirosis separately in deer (Wilson *et al.* 1998; Ayanegui-Alcérreca 2006), cattle (Bahaman *et al.* 1984; Matthews *et al.* 1999) and sheep (Dorjee *et al.* 2005). Data from a regional seroprevalence survey of 110 deer farms (Ayanegui-Alcérreca *et al.* 2010 in press) showed 81% of farmed deer herds in New Zealand were seropositive, with *Leptospira* serovar Hardjobovis alone in 61% of herds, Pomona in 4% and a combination of both serovars in 16%. At the individual animal level, serovar Hardjobovis was found in 54% of deer, Pomona in 2% and a combination of both serovars in 7%. No differences were found between regions.

In the late 1970s and early 1980s, several studies were conducted to investigate the seroprevalence of leptospirosis in cattle, particularly dairy cattle, in New Zealand. A serological survey in Taranaki showed that 62% of dairy cattle were positive for serovar Hardjo and 4% for serovar Pomona and that infection usually occurred during the first two years of life (Bahaman *et al.* 1984). Data from a serological survey of leptospirosis in beef herds in the Hawke's Bay area showed that all of 50 herds had positive titres for serovar Hardjo whereas 64% of the individuals had a titre of ≥1:96, and 44% had a titre of ≥1:384 (Matthews *et al.* 1999).

An early epidemiological study of leptospirosis in sheep from 45 lines at a slaughterhouse revealed Hardjo (now Hardjobovis) infection in 20% of animals and serovar Pomona in 3.8% (Blackmore *et al.* 1982). More recent abattoir data from 68 lines comprising 65 different properties showed that 85.7% and 23.4% of lines of hoggets and lambs respectively, were seropositive for Hardjobovis and 28.6% and 10.6% respectively, were seropositive for Pomona. Seroprevalence for Hardjobovis and Pomona at the individual animal level was 6.1% and 1.6% respectively. The within-line prevalences of Hardjobovis ranged from 3.3% to 15.4% for lambs and from 3.3% to 60% for hoggets while that for serovar Pomona ranged from 3.3% to 6.7% for lambs and 3.3% to 40.0% for hoggets (Dorjee *et al.* 2005).

Despite these individual species surveys and epidemiological studies of leptospirosis in pastoral farming in New Zealand, no mixed-species surveys or epidemiological studies have been carried out. Understanding the role of each potential host on mixed-species farms is important to understanding of the disease, and has particular relevance to choice and

implementation of control measures on-farm aimed at reducing the clinical and subclinical incidence of disease, and reduction of risk to humans.

This paper describes a preliminary study conducted in the lower North Island of New Zealand designed to provide baseline data for future serological and molecular epidemiological study of leptospirosis and to evaluate potential risk factors for seropositive status of deer herds on mixed-species farms.

4.2 Materials and methods

4.2.1 Ethical approval

All procedures involving the use of animals in this project have been approved by the Massey University Animal Ethics Committee under protocol No. 06/68.

4.2.2 Farms and animals

Twenty commercial farms, three with deer only and 17 with deer and sheep and/or cattle in the Manawatu, Hawkes Bay and Wairarapa regions in the lower North Island of New Zealand were recruited as detailed in Table 4.1. Candidate farms were selected based on the farmer's willingness to participate, history of no previous leptospirosis vaccination, and the presence of suitable animals and handling facilities. The farms recruited were deer only (n=3), deer and beef cattle (n=3), deer and sheep (n=2) and deer, beef cattle and sheep (n=12). A herd or flock was defined as animals of the same species (*i.e.* deer herd, cattle herd and sheep flock) grazed on the same farming property. Two farms had a closed herd policy, breeding their own replacements for all classes of stock.

4.2.3 Study design

A longitudinal seroprevalence survey design was chosen to determine the *Leptospira* status of both individual animals and herds/flocks from 2006 to 2008. Between August and October each year, jugular (sheep and deer) or coccygeal vein (cattle) (Figure 4.1) blood samples were taken using a 20 gauge needle and 10mL evacuated glass tubes without anticoagulant from a randomly selected sub-sample of replacement mobs that were more than one year old of each species on farm. The required numbers of samples per farm were 20 deer, 20 cattle and 60 sheep, as calculated by FreeCalc software. Estimates were based on a MAT sensitivity of 85% and specificity of 95%, a minimum within-farm seroprevalence of 30% to be detected in deer and cattle and 5% in sheep based on previous observations, and an average herd size of 350, to provide 95% confidence and 80% power. To categorise deer and cattle herds as positive, at least 3 of 20 serum samples had to be positive at the titre of 1:48. To categorise sheep flocks as positive, initially at least 5 of 60 serum samples had to be positive based on a MAT sensitivity of 85% and specificity of 95%, a minimum within-farm seroprevalence of 12% to be detected in sheep based on previous observations, and an average herd size of 1000, to provide 95% confidence and 80% power. However, based on higher than expected

within flock seroprevalence of 42% in the first year (2006), the number of samples required from sheep was reduced to 20 per farm in 2007 and 2008 with the same criteria to consider flock positive as for deer and cattle. In total, 3,290 animals were sampled including 1,173 deer, 817 beef cattle and 1,300 sheep. Farm owners or managers were asked to complete a questionnaire at both the 2007 and 2008 samplings to obtain information on farm demographics, geography, animal replacement policy, neighbours, grazing management, vaccination programme and history of clinical leptospirosis since the previous sampling as described in Table 4.2.

Figure 4.1 Blood collection from cattle (a), sheep (b) and deer (c).

4.2.4 Serology

Blood samples were left to clot at ambient temperature. Subsequently, samples were centrifuged at 3,000 rpm (1512 x *g*) for 15 minutes to extract serum for the microscopic agglutination test (MAT). MAT was used to test serum reactivity to laboratory standardised *Leptospira* serovar Hardjobovis and Pomona to determine the *Leptospira* status of the individual animals and herds. The method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the Control of Leptospirosis" (Faine 1982). An initial serum dilution of 1:12 and two-fold serial dilution of serum covering the range of 1:24 to 1:3072 were tested. Titre was recorded as the reciprocal of the highest dilution at which ≥ 50% of leptospires were agglutinated. To consider a sample positive, the cut-off point at 1:48 was used for both serovars (Blackmore *et al.* 1982; Dorjee *et al.* 2008).

4.2.5 Statistical analysis

Data collected were recorded and maintained using Microsoft Excel (Microsoft Corp, Redmond WA, USA). Data entries were checked against the written records for errors before performing analysis. The proportion of leptospiral antibody-positive deer, cattle herds and sheep flocks were calculated for both *Leptospira* serovars Hardjobovis and Pomona for descriptive analysis. Seroprevalence for each species was calculated as: (number of antibody positive animals/number of sampled animals) x 100 for each herd. Initially, it was evaluated whether the serological status of a herd correlated in subsequent years using the intra-class-correlation criterion. Where correlation was present, statistical hypothesis tests were adjusted by a variance inflation factor (McDermott and Schukken 1994). Due to the small number of herds, only simple bivariate analysis was used to test for associations between herd level variables shown in Table 4.2 and serological status of herds in each year for each serovar. Statistical inferences were based on Fisher's Exact Test. For variables with more than two levels that implied an increase of exposure, associations were tested for the presence of an increasing or decreasing trend (Rothman and Greenland 1998a). Associations were calculated as prevalence ratios (PR) and 95% confidence interval (Rothman and Greenland 1998b) to express the strength of association between risk factors and the risk for seropositivity to *Leptospira* in deer herds. A value of $p<0.05$ denoted statistical significance.

Table 4.1 Summary of farm characteristics based on 2007 and 2008 questionnaire data.

Farm	Location	Mean herd/flock size			Farm geography*	Replacement policy*	Neighbour waterway*	Co-graze with cattle	Co-graze with sheep	Past clinical lepto
		Deer*	Cattle	Sheep						
1	Palmerston Nth	71	-	-	Flat	Open	Yes	No	No	No
2	Masterton	191	-	-	Flat	Open	Yes	No	No	No
3	Waipawa	425	-	-	Flat	Open	Yes	No	No	No
4	Palmerston Nth	99	9	-	Flat	Open	Yes	Yes	No	No
5	Dannevirke	652	826	-	Hilly	Open	Yes	Yes	No	No
6	Waipukurau	820	162	-	Hilly	Open	Yes	Yes	No	Yes
7	Pohangina	751	-	360	Hilly	Open	Yes	No	Yes	Yes
8	Pohangina	304	155	1783	Hilly	Open	Yes	Yes	Yes	Yes
9	Apiti	813	76	1260	Hilly	Open	No	Yes	Yes	No
10	Pohangina	900	91	630	Hilly	Open	Yes	Yes	Yes	No
11	Rangiwahia	559	60	10120	Hilly	Open	Yes	Yes	Yes	No
12	Dannevirke	210	12	560	Hilly	Open	Yes	No	Yes	No
13	Takapau	1201	270	2660	Hilly	Closed	Yes	Yes	Yes	Yes
14	Takapau	652	150	1170	Hilly	Open	Yes	Yes	No	No
15	Waipawa	824	1020	3900	Hilly	Open	Yes	Yes	No	No
16	Pohangina	824	36	232	Hilly	Open	Yes	Yes	No	No
17	Pohangina	234	250	2400	Hilly	Closed	No	Yes	Yes	No
18	Takapau	667	90	950	Hilly	Open	Yes	Yes	Yes	No
19	Colyton	305	161	900	Hilly	Open	Yes	Yes	No	No

* Variables for statistical analysis described in Table 4.2

Table 4.2 Description of variables used for statistical analysis.

Variable	Description
Deer herd size	Total number of deer on farm including all classes
	Large = deer herd size > median (n=560)
	Small = deer herd size < median
Farm geography	Hilly = >50% of area of farms are hilly
	Flat = >50% of area of farms are flat
Replacement policy	Open = introduce animals on a regular basis from other farms
	Closed = do not introduce animals from other farms
Neighbour waterway	Yes = waterway from neighbour passes through farm
	No = no waterway from neighbour passes through farm
Graze with Hardjobovis positive cattle	4 = co-grazed with Hardjobovis positive cattle
	3 = not co-grazed but Hardjobovis positive cattle present on farm
	2 = co-grazed with Hardjobovis negative cattle
	1 = not co-grazed, no Hardjobovis positive cattle present on farm
	0 = no cattle on the property
Graze with Hardjobovis positive sheep	4 = co-grazed with Hardjobovis positive sheep
	3 = not co-grazed but Hardjobovis positive sheep present on farm
	2 = co-grazed with Hardjobovis negative sheep
	1 = not co-grazed, no Hardjobovis positive sheep present on farm
	0 = no sheep on the property
Graze with Pomona positive cattle	4 = co-grazed with Pomona positive cattle
	3 = not co-grazed but Pomona positive cattle present on farm
	2 = co-grazed with Pomona negative cattle
	1 = not co-grazed, no Pomona positive cattle present on farm
	0 = no cattle on the property
Graze with Pomona positive sheep	4 = co-grazed with Pomona positive sheep
	3 = not co-grazed but Pomona positive sheep present on farm
	2 = co-grazed with Pomona negative sheep
	1 = not co-grazed, no Pomona positive sheep present on farm
	0 = no sheep on the property

4.3 Results

4.3.1 Farm characteristics

Farm characteristics are summarised in Table 4.1. During the study one farm (deer and sheep) was lost to follow-up in 2007 and 2008 since the farmer withdrew, thus data from 19 farms are presented and analysed. The median deer herd size was 560 animals (range 71 – 1,201) and used to differentiate the size of farm as large or small in study analyses. The median herd/flock size of cattle and sheep were 150 (range 9 – 1020) and 1170 (range 232 – 10120), respectively. Four farms were described as flat whereas the remainder were hilly. Effective area of farms ranged between 10 and 1376 hectares. Deer fenced areas ranged between 10 and 350 hectares. Sixteen farms (84%) had at least one waterway from neighbouring, stock carrying properties that passed through farm. All the mixed and multi-species farms co- and/or cross-grazed other stock with deer herds during both years studied. No leptospiral vaccination was recorded from these farms despite four having clinical leptospirosis confirmed by a veterinarian in the five years prior to this study. Farms 6, 7 and 10 had records of clinical leptospirosis in hinds, weaner deer and sheep, respectively and Farm 13 in both weaner deer and cows within the same year.

4.3.2 Herd level infection

Summary of within-herd seroprevalence, reciprocal titre range and herd/flock serological status for Hardjobovis and Pomona are presented in Tables 4.3 and 4.4, respectively. The proportion of seropositive deer, cattle and sheep herds/flocks (both serovars) over three samplings averaged 72%, 74% and 64%, respectively. The proportion of Hardjobovis seropositive deer, cattle and sheep herds/flocks over three samplings was 42.1%, 52.8% and 53.9%, respectively. The proportion of Pomona seropositive deer, cattle and sheep herds/flocks over three samplings was 7.0%, 4.8% and 0%, respectively. Dual infections with serovars Hardjobovis and Pomona were found in 22.5% of deer herds, 16.3% of cattle herds and 10.3% of sheep flocks (Table 4.5). Persistence and non-persistence of *Leptospira* status for Hardjobovis and Pomona in deer, cattle and sheep herds/ flocks is shown in Table 4.6. For Hardjobovis positive deer, cattle and sheep herds/flocks, 42.1%, 42.9% and 46.2% remained positive for at least two consecutive years, respectively. For Pomona positive deer, cattle and sheep herds/flocks, 26% of deer herds remained positive for at least two consecutive years.

4.3.3. Individual animal level infection

Individual animal level seroprevalence data are shown in Table 4.7. Overall, 19.9% of deer (n = 1154) were positive for Hardjobovis, 8.8% were positive for Pomona and 4.3% were positive for both. For cattle (n = 817), 39.1% were positive for Hardjobovis, 6.2% were positive for Pomona and 4.8% were positive for both. For sheep (n = 1244) 30.1% were positive for Hardjobovis, 1.9% were positive for Pomona and 1.5% were positive for both serovars.

Table 4.3 Within-Herd/flock seroprevalence (No +/No sampled) (herd/flock status in parenthesis) and positive reciprocal titre range to Hardjobovis in each species on farms each year.

Farm	Deer						Cattle						Sheep					
	06		07		08		06		07		08		06		07		08	
	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre
1	1/19 (–)	48	1/19 (–)	96	1/20 (–)	96	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
2	0/20 (–)	0	5/19 (+)	48	2/19 (–)	48	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
3	1/20 (–)	48	0/20 (–)	0	1/20 (–)	48	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
4	1/21 (–)	96	4/16 (+)	48	0/20 (–)	0	3/5 (+)	96-192	0/8 (–)	0	0/7 (–)	0	NP	NP	NP	NP	NP	NP
5	3/21 (+)	48-96	1/20 (–)	384	0/20 (–)	0	13/20 (+)	48-384	0/10 (–)	0	2/20 (–)	48-192	NP	NP	NP	NP	NP	NP
6	8/20 (+)	48-96	12/20 (+)	48-192	13/20 (+)	48-768	16/20 (+)	48-384	17/20 (+)	48-192	1/20 (–)	192	NP	NP	NP	NP	NP	NP
7	6/20 (+)	48-96	6/20 (+)	48-96	6/15 (+)	48-384	NP	NP	NP	NP	NP	NP	0/30 (–)	0	0/19 (–)	0	0/19 (–)	0
9	6/20 (+)	48-96	0/19 (–)	0	4/18 (+)	48-192	10/17 (+)	48-96	7/19 (+)	48-192	5/20 (+)	48-96	18/60 (+)	48-384	10/17 (+)	48-96	12/20 (+)	48-192
10	0/20 (–)	0	6/19 (+)	48-96	10/20 (+)	48-96	16/20 (+)	48-192	5/18 (+)	48-192	11/20 (+)	48-768	3/60 (–)	48-192	7/20 (+)	48-192	12/20 (+)	96-768
11	3/19 (+)	48-96	17/20 (+)	48-96	9/20 (+)	48-96	0/20 (–)	0	5/20 (+)	48-192	1/20 (–)	48	20/60 (+)	48-384	9/20 (+)	96-768	7/20 (+)	48-192
12	3/20 (+)	48	4/20 (+)	48-96	11/15 (+)	48-96	3/20 (+)	48-384	7/20 (+)	48-192	13/20 (+)	48-1536	1/60 (–)	96	13/20 (+)	48-96	17/19 (+)	96-768
13	9/14 (+)	48-96	9/20 (+)	48-96	0/20 (–)	0	17/19 (+)	48-384	NP	NP	NP	NP	0/60 (–)	0	0/20 (–)	0	0/20 (–)	0
14	9/18 (+)	48-384	4/20 (+)	48-96	1/20 (–)	48	20/20 (+)	48-768	0/20 (–)	0	0/19 (–)	0	41/60 (+)	48-768	6/20 (+)	48-192	0/18 (+)	0
15	5/19 (+)	48-96	2/19 (–)	48-384	7/18 (+)	48-96	13/19 (+)	48-192	11/18 (+)	48-384	0/19 (–)	0	55/59 (+)	48-384	13/19 (+)	48-96	10/20 (+)	48-96
16	8/20 (+)	48-192	3/20 (+)	48	5/20 (+)	48-192	13/20 (+)	48-384	0/20 (–)	0	0/19 (–)	0	5/60 (+)	192-384	0/20 (–)	0	9/20 (+)	48-384
17	0/20 (–)	0	13/19 (+)	48-192	1/20 (–)	96	6/15 (+)	48-384	7/7 (+)	48-768	18/20 (+)	48-384	38/60 (+)	48-768	4/20 (+)	48-96	7/20 (+)	48-192
18	4/20 (+)	48-96	8/20 (+)	48-192	0/20 (–)	0	7/20 (+)	192-768	17/20 (+)	48-192	11/20 (+)	38-96	8/60 (+)	48-1536	1/19 (–)	384	0/20 (–)	0
19	6/20 (+)	48-384	9/40 (+)	48-192	5/39 (+)	48	20/20 (+)	96-768	12/40 (+)	48-96	34/39 (+)	48-384	37/60 (+)	48-768	9/20 (+)	48-768	9/20 (+)	48-192
20	7/21 (+)	48-192	10/20 (+)	48-96	10/20 (+)	48-384	5/18 (+)	48-768	4/20 (+)	48-96	0/10 (–)	0	4/46 (+)	48-384	0/19 (–)	0	2/20 (–)	96-192
Mean prev (%) (min-max)	**31.5 (15.0-64.0)**		**39.0 (15.0-85.0)**		**45.0 (25.0-68.8)**		**63.1 (15.0-100.0)**		**53.3 (20.- 100.0)**		**58.6 (25.0-100.0)**		**41.9 (8.3-95.0)**		**45.9 (20.0-68.4)**		**56.2 (35.0-89.5)**	

Prev = Prevalence, + = Herd/flock positive, – = Herd/flock negative, NP = Not present

Table 4.4 Within-Herd/flock seroprevalence (No +/No sampled) (herd/flock status in parenthesis) and positive reciprocal titre range to Pomona in each species on farms each year.

Farm	Deer						Cattle						Sheep					
	06		07		08		06		07		08		06		07		08	
	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre	Prev	Titre
1	0/19 (–)	0	0/19 (–)	0	0/20 (–)	0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
2	0/20 (–)	0	0/19 (–)	0	0/19 (–)	0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
3	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP	NP
4	7/21 (+)	96-384	4/16 (+)	48-192	9/20 (+)	48-1536	2/5 (+)	48-96	0/8 (–)	0	0/7 (–)	0	NP	NP	NP	NP	NP	NP
5	10/21 (+)	48-384	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	0/19 (–)	0	19/20 (+)	96-1536	NP	NP	NP	NP	NP	NP
6	1/20 (–)	192	0/20 (–)	0	0/20 (–)	0	1/20 (–)	48	0/20 (–)	0	0/20 (–)	0	NP	NP	NP	NP	NP	NP
7	1/20 (–)	192	1/20 (–)	96	1/15 (–)	48	NP	NP	NP	NP	NP	NP	0/30 (–)	0	0/19 (–)	0	0/19 (–)	0
9	2/20 (–)	48-192	3/19 (+)	48-192	11/18 (+)	48-192	2/17 (–)	48-192	8/19 (+)	48-96	7/20 (+)	48-1536	19/60 (–)	96-384	1/17 (–)	48	6/20 (+)	48-384
10	1/20 (–)	192	12/19 (+)	48-384	4/20 (+)	48-96	2/20 (–)	48-192	2/18 (–)	48-96	2/20 (–)	96	1/60 (–)	192	3/20 (+)	48-96	0/20 (–)	0
11	0/19 (–)	0	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	2/20 (–)	192-384	0/60 (–)	0	0/20 (–)	0	0/20 (–)	0
12	0/20 (–)	0	0/20 (–)	0	0/15 (–)	0	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	0/60 (–)	0	1/20 (–)	96	0/19 (–)	0
13	0/14 (–)	0	0/20 (–)	0	1/20 (–)	48	0/19 (–)	0	NP	NP	NP	NP	0/60 (–)	0	0/20 (–)	0	0/20 (–)	0
14	6/18 (+)	48-384	10/20 (+)	48-384	2/20 (–)	48	5/20 (+)	96-384	1/20 (–)	96	0/19 (–)	0	2/60 (–)	96-768	1/20 (–)	48	0/18 (–)	0
15	0/19 (–)	0	0/19 (–)	0	0/18 (–)	0	1/19 (–)	96	0/18 (–)	0	0/19 (–)	0	0/59 (–)	0	0/19 (–)	0	0/20 (–)	0
16	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	0/20 (–)	0	4/19 (+)	48-192	0/60 (–)	0	0/20 (–)	0	0/20 (–)	0
17	0/20 (–)	0	1/19 (–)	48	1/20 (–)	192	0/15 (–)	0	0/7 (–)	0	0/20 (–)	0	1/60 (–)	48	0/20 (–)	0	0/20 (–)	0
18	7/20 (+)	48-1536	12/20 (+)	48-384	12/20 (+)	48-192	2/20 (–)	96-192	0/20 (–)	0	6/20 (+)	48-192	0/60 (–)	0	0/19 (–)	0	0/20 (–)	0
19	0/20 (–)	0	4/40 (+)	48-96	0/39 (–)	0	0/20 (–)	0	0/40 (–)	0	10/39 (+)	48-384	0/60 (–)	0	15/20 (+)	48-384	5/20 (+)	48-192
20	8/21 (+)	48-192	11/20 (+)	48-192	9/20 (+)	48-384	0/18 (–)	0	3/20 (+)	96-192	0/10 (–)	0	0/46 (–)	0	1/19 (–)	96	2/20 (–)	48-384
Mean prev (%) (min-max)	**37.9 (33.3-50.0)**		**41.3 (15.8-63.2)**		**46.2 (20.0-60.1)**		**32.5 (25.0-40.0)**		**32.5 (15.0-50.0)**		**46.9 (21.1-95.0)**		**0 (0)**		**45 (15.0-75.0)**		**23.3 (15.0-30.0)**	

Prev = Prevalence, + = Herd/flock positive, – = herd/flock negative, NP = Not present

Table 4.5 Number (and %) of herds/flocks serologically positive for Hardjobovis and Pomona each year.

Species	No. of herds/flocks sampled			No. of positive herds/flocks and (%)											
				Hardjobovis			Pomona			Both			Overall		
	06	07	08	06	07	08	06	07	08	06	07	08	06	07	08
Deer	19	19	19	9 (47.4)	8 (42.1)	7 (36.8)	1 (5.3)	1 (5.3)	2 (10.5)	4 (20.0)	6 (31.6)	3 (15.8)	14 (73.6)	15 (78.9)	12 (63.2)
Cattle	15	14	14	12 (80.0)	8 (57.1)	3 (21.4)	0 (0)	0 (0)	2 (14.3)	2 (13.3)	2 (14.2)	3 (21.4)	14 (93.3)	10 (71.4)	8 (57.1)
Sheep	13	13	13	9 (69.2)	6 (46.2)	6 (46.2)	0 (0)	0 (0)	0 (0)	0 (0)	2 (15.4)	2 (15.4)	9 (69.2)	8 (61.5)	8 (61.5)

Table 4.6 Number (and %) of herds/flocks persistently positive or negative for Hardjobovis and Pomona or change status between years.

Serovar	Species	No. of herds/ flocks	Herd/ flock *Leptospira* status		
			Consistently positive	Consistently negative	Change between years
Hardjobovis	Deer	19	8 (42.1)	2 (10.5)	10 (52.6)
	Cattle	14	6 (42.9)	0 (0)	8 (57.1)
	Sheep	13	6 (46.2)	2 (15.4)	5 (38.5)
Pomona	Deer	19	5 (26.3)	11 (57.9)	3 (15.8)
	Cattle	14	0 (0)	6 (42.9)	8 (57.1)
	Sheep	13	0 (0)	10 (76.9)	3 (23.1)

Table 4.7 Number (%) of animals seropositive to Hardjobovis and Pomona alone or combined each year.

Species	No. tested			No. of positive animals and (%)											
				Hardjobovis			Pomona			Both			Overall		
	06	07	08	06	07	08	06	07	08	06	07	08	06	07	08
Deer	372	398	384	67 (18.0)	90 (22.6)	73 (19.0)	30 (8.1)	35 (8.8)	37 (9.6)	13 (3.3)	23 (5.8)	13 (3.4)	110 (29.6)	148 (37.2)	123 (32.0)
Cattle	273	271	273	151 (55.3)	88 (32.5)	80 (29.3)	4 (1.5)	12 (4.4)	35 (12.8)	11 (4.0)	3 (1.1)	15 (5.5)	166 (60.8)	103 (38.0)	130 (47.6)
Sheep	735	253	256	225 (30.6)	63 (24.9)	86 (33.6)	1 (0.1)	14 (5.2)	8 (3.1)	5 (0.6)	8 (3.2)	5 (1.9)	231 (31.4)	85 (33.6)	99 (38.7)
All species	1,380	922	913	443 (32.1)	241 (26.1)	239 (26.2)	35 (2.5)	61 (6.6)	80 (8.8)	29 (2.0)	34 (3.7)	33 (3.6)	507 (36.7)	336 (36.4)	352 (38.6)

4.3.4 Association between leptospiral positive deer herds and risk factors

There were no correlations between serological status of a herd sampling in subsequent years using the intra-class-correlation criterion. Repeated tests of animals from the same herd were therefore regarded as independent (n = 3 years * 19 herds = 57 herd-years). The prevalence ratio for factors associated with a deer herd being leptospiral seropositive for Hardjobovis and Pomona are summarised in Tables 4.8 and 4.9, respectively.

For Hardjobovis, there was a higher PR of a deer herd being seropositive when the deer herd size was large, the farm was hilly and when deer were co-grazing with Hardjobovis positive cattle or sheep. There were trends for increases in the proportion of Hardjobovis positive deer herds when exposed to Hardjobovis positive cattle herds or sheep flocks at different level of co-grazing. No association was found between replacement policy and joint water sources with neighbouring farms.

For Pomona, there was a significantly higher PR of a deer herd being seropositive when co-grazing with Pomona positive cattle herds. There were trends for increases in seropositivity to Pomona in deer herds when exposed to Pomona positive cattle herds but not sheep flocks at different level of co-grazing. There was a lower PR of a deer herd being seropositive if the farm brought in stock from other farms. No association was found between deer herd size, farm geography and a waterway from a neighbour and seropositivity of deer herds for Pomona.

Table 4.8 Prevalence Ratio (PR), 95% confidence interval (CI) and p-value for risk factors associated with a deer herd being seropositive for Hardjobovis (Hardjo).

Risk factors	Herds Hardjo+	Herds Hardjo-	PR	95% CI	p-value
Deer herd size					
- Large (> 560 animals)	21	6	1.46	1.00 – 2.16	0.036
- Small (< 560 animals)	16	14	Ref		
Farm geography					
- Hill	35	10	4.67	1.30 – 16.67	<0.001
- Flat	2	10	Ref		
Replacement policy					
- Open herd (Buy in stock)	33	18	0.97	0.53 – 1.77	0.346
- Closed herd	4	2	Ref		
Neighbour waterway					
- Yes	32	16	1.20	0.65 – 2.23	0.235
- No	5	4	Ref		
Co-grazing with Hardjobovis + cattle*					
Co-grazed with Hardjobovis positive cattle	17	5	1.93	1.00 – 3.74	0.022
Not co-grazed but Hardjobovis positive cattle present on farm	5	2	1.79	0.82 – 3.88	0.149
Co-grazed with Hardjobovis negative cattle	6	2	1.88	0.90 – 3.92	0.104
Not co-grazed, no Hardjobovis positive cattle present on farm	3	2	1.50	0.58 – 3.82	0.298
No cattle on the property	6	9	Ref		
Co- grazing with Hardjobovis + sheep*					
Co-grazed with Hardjobovis positive sheep	12	1	2.77	1.41 – 5.42	<0.001
Not co-grazed but Hardjobovis positive sheep present on farm	9	3	2.25	1.08 – 4.67	0.026
Co-grazed with Hardjobovis negative sheep	1	1	1.50	0.32 – 6.94	0.479
Not co-grazed, no Hardjobovis positive sheep present on farm	9	3	2.25	1.08 – 4.67	0.026
No sheep on the property	6	12	Ref		

Ref = Reference category, + = positive
*significant for increased trend at $p \leq 0.05$

Table 4.9 Prevalence Ratio (PR), 95% confidence interval (CI) and p-value for risk factors associated with a deer herd being seropositive for Pomona (Pom).

Risk factors	Herds Pom+	Herds Pom-	PR	95% CI	p-value
Deer herd size					
- Large (> 560 animals)	6	21	0.61	0.26 – 1.42	0.116
- Small (< 560 animals)	11	19	Ref		
Farm geography					
- Hilly	14	31	1.24	0.43 – 3.64	0.263
- Flat	3	9	Ref		
Replacement policy					
- Open herd (Buy in stock)	12	39	0.28	0.15 – 0.52	0.007
- Closed herd	5	1	Ref		
Neighbour waterway					
- Yes	12	36	0.45	0.21 – 1.01	0.063
- No	5	4	Ref		
Co-grazing with Pomona + cattle*					
Co-grazed with Pomona positive cattle	3	3	7.50	1.00 – 58.59	0.050
Not co-grazed but Pomona positive cattle present on farm	3	0	15.00	2.26 – 99.64	0.005
Co-grazed with Pomona negative cattle	6	18	3.75	0.50 – 28.17	0.131
Not co-grazed, no Pomona positive cattle present on farm	5	4	8.30	1.15 – 60.46	0.014
No cattle on the property	1	14	Ref		
Co-grazing with Pomona + sheep					
Co-grazed with Pomona positive sheep	3	1	3.38	1.20 – 9.48	0.072
Not co-grazed but Pomona positive sheep present on farm	-	-	-	-	-
Co-grazed with Pomona negative sheep	2	9	0.82	0.18 – 3.75	0.354
Not co-grazed, no Pomona positive sheep present on farm	8	19	1.33	0.47 – 3.78	0.236
No sheep on the property	4	14	Ref		

Ref = Reference category, + = positive
*significant for increased trend at $p \leq 0.05$

4.4 Discussion

This study provided preliminary, regionally-based data on leptospirosis on mixed-species farms. Data will be used to inform more substantial studies of leptospirosis on mixed-species farms in New Zealand in the future. It demonstrated that infection by *Leptospira* serovars Hardjobovis and Pomona was common in mixed species commercial farms in the lower North Island of New Zealand, and has described some risk factors for deer seropositivity. Deer in larger herds, grazing hill country and grazing with Hardjobovis seropositive cattle

and sheep were at higher risk of being seropositive to this serovar than deer grazed alone or on flat land. Similarly, deer on properties with cattle seropositive for Pomona were more likely to be seropositive to that serovar. Deer on farms that had an open replacement policy were less likely to be seropositive for Pomona.

The predominant serovar observed in this study was Hardjobovis which is consistent with previous findings in deer (Wilson *et al.* 1998; Ayanegui-Alcérreca *et al.* 2003), cattle (Bahaman *et al.* 1984; Matthews *et al.* 1999) and sheep (Blackmore *et al.* 1982; Dorjee *et al.* 2005) in New Zealand. Sampling was conducted between August and October in each year which is the wet season in the study region. Wet conditions are suitable for survival and transmission of leptospires (Hellstrom and Marshall 1978) optimising the chance of observing active infections on farms in this study. The target sampling group was young replacements of each species in order to observe transmission to the next generation and persistence of the herd/flock leptospiral status, as demonstrated to occur in deer herds (Ayanegui-Alcérreca 2006).

The 63 – 79% seroprevalence of leptospirosis in deer at the herd level presented here is lower than the 82% national seroprevalence demonstrated in a New Zealand-wide prevalence survey but was similar to data for the Manawatu/Hawkes Bay regions (Ayanegui-Alcérreca *et al.* 2010 in press). Moreover, this was similar to that described in an earlier survey from the Manawatu area (Wilson et al 1998). These data support the assertion that farmed deer in New Zealand are a maintenance host for Hardjobovis and accidental host for Pomona. However, the seroprevalence of Hardjobovis alone presented here, at the herd and individual animals levels, (42% and 20%, respectively) were lower than reported by Ayanegui-Alcérreca *et al.* (2010 in press) (61% and 54%, respectively). The likely reason is the difference of titre cut point used for each study since Ayanegui-Alcérreca *et al.* (2010 in press) used a titre cut point at 1:24 for serovar Hardjobovis, while the present study used a titre cut point at 1:48. The latter cut point has been shown to result in less non-specific agglutination and cross reaction (Blackmore *et al.* 1982). Climatic and other factors may alter seroprevalence between years.

Data for beef cattle from this study identifying that serovar Hardjobovis infections were endemic and serovar Pomona infections were sporadic agrees with previously data from dairy cattle (Bahaman *et al.* 1984) which showed the seroprevalence of Hardjobovis in cattle herds was 70% whereas Pomona infections were 21%. The 43% seroprevalence of Hardjobovis in individual cattle in this study was lower than 68% reported in a survey in beef herds in Hawke's Bay when using the same titre cut points (Matthews *et al.* 1999). In a survey in dairy cattle herds in Taranaki, individual animal seroprevalence for Pomona was 58.5% (Bahaman *et al.* 1984).

During the first year of this study, 60 sheep per farm were sampled to determine the flock leptospiral status, based on estimated seroprevalence using published data. However, since the within-flock prevalence of leptospirosis in sheep at the first sampling was higher than

expected, the number of animals/farm required subsequently reduced to 20. The 64% seroprevalence of Hardjobovis in sheep at the flock level in this study was lower than 86% reported previously in hoggets ((Dorjee *et al.* 2005). The likely reason is the different criteria to determine the status of positive sheep flock since Dorjee *et al*. (2005) determined the flock status as 1 seropositive of 30 sheep while the present study determined the flock status by 5 seropositive of 60 in 2006 and 3 seropositive of 20 samples in 2007 and 2008. However, the 30% seroprevalence of Hardjobovis in adult sheep at the individual animal level in this study was similar to that in ewes (31%) but higher than in hoggets (23%) reported by Blackmore *et al.* (1982). Dorjee *et al.* (2005) reported a seroprevalence of 16.0% in hoggets at the same titre cut point as used in the present study (1:48). The reason that seroprevalence of Hardjobovis in sheep was higher than previous studies may be that this study was based on deer farms and deer is likely to be a maintenance host for this serovar (Ayanegui-Alcérreca *et al.* 2007), although climatic effects may have contributed.

The 10% seroprevalence of Pomona in sheep at the flock level in this study was lower than in a previous study that reported a seroprevalence of 28.6% in hogget lines (Dorjee *et al.* 2005). Nevertheless, the 3.4% seroprevalence of Pomona in sheep at the individual animal level in this study was similar to 3.8% reported by Blackmore *et al.* (1982) and 4% by Dorjee *et al*. (2005).

Blackmore *et al.* (1982) proposed that sheep were not a maintenance host for Hardjobovis but the seroprevalence of this serovar from this and other studies which are in the same range as in cattle and deer suggest that sheep may act as a maintenance host. This proposition is supported by other studies (Cousins *et al.* 1989; Gerritsen *et al.* 1994). The proposition by Blackmore et al (1982) was almost 30 years prior to this study, thus it could be that during the past years, livestock farming in New Zealand has become increasingly multi-species including deer, beef cattle and sheep on the same property (Hilson 2007; Wilson 2007) affecting the disease dynamic, potentially altering the host status of sheep in the epidemiology of infection. However, proof that sheep is a maintenance host for Hardjobovis requires evidence of long term leptospiruria by urine culture or PCR and strain typing, and epidemiological studies on sheep-only farms, which was beyond the scope of this study.

There was a higher proportion of farms (13/15) that had at least one livestock species persistently positive to Hardjobovis in two consecutive years than to Pomona (3/5). This further supports that all three livestock species may be maintenance hosts for Hardjobovis as proposed for deer (Ayanegui-Alcérreca *et al.* 2007), cattle (Hathaway 1981) and possibly sheep (Cousins *et al.* 1989; Gerritsen *et al.* 1994). Observations here were consistent with other observations of endemicity of this serovar in that more than 67% of deer herds and 50% of sheep flocks seronegative to Hardjobovis in 2006 had become seropositive in 2007 suggesting that *Leptospira* negative herds/flocks at a single sampling are likely to become positive on a later date. This finding is critical for risk evaluation in terms of farmer decision making to control or vaccinate their animals.

The persistence of seropositivity to Pomona was found particularly in deer but not in cattle and sheep on some farms, which demonstrates the potential for Pomona to be transferred to replacement stock on infected farms if the risk factors persist. This finding was consistent with previous longitudinal study in deer which reported persistence of this serovar over at least a two-year period (Ayanegui-Alcérreca 2006). It has been reported that Pomona can survive for at least 42 days in New Zealand soil under simulated winter conditions (Hellstrom and Marshall 1978) and is maintained in pigs (Hathaway 1981). Nevertheless, evidence that Pomona could persist for a long period in farmed deer justifies further investigation into the role of this species in maintaining this serovar at either individual or population levels. It may be possible that for Pomona, deer fall between a sporadic and maintenance host at the individual animal level and a maintenance host at the population level.

The Hardjobovis or Pomona status of repeated herd tests in subsequent years was not measurably correlated. Thus, statistical hypothesis tests did not have to be adjusted for correlation in the data. Given that initial analysis at the animal level revealed strong within-herd correlation, the animal level analyses that were appropriately adjusted for the correlation did not provide reliable estimates and all significant factors were at the herd level, therefore, all analysis was done on the herd basis and each herd-year could be assumed to be an independent observation.

Some environmental factors were tested for association with leptospiral status of deer herds, but only grazing hill pasture was significantly positively associated with deer herd status, and only for serovar Hardjobovis. This may be because most farms studied were hilly and there was high prevalence of Hardjobovis observed in deer herds. However, it is possible that hilly terrain can facilitate the organism spreading because the source of water for stock on hill pastures is more likely to be natural waterways than reticulated water. This risk factor will be investigated further in future studies.

Herd-level risk factors for seropositivity to *Leptospira* have been studied in other countries, mainly in dairy cattle, for serovar Hardjo. Larger herd size, co-grazing with infected livestock, access to contaminated water sources and introduction of other animals on farm have been reported to be associate with seroprevalence of leptospirosis (Lilenbaum and Santos 1996). By contrast, in the present study, an open herd replacement policy was associated with a 72% reduction in the risk of seropositivity of deer herds for serovar Pomona, although not for Hardjobovis. This apparent effect could, however, be spurious since the majority of studied farms were open herds and there was a low prevalence of Pomona in deer herds. Larger deer herd size (>560 animals) was positively associated with deer herd status with serovar Hardjobovis but not Pomona. Some reports suggest that seroprevalence is related to herd size in cattle (Ellis 1994; Lilenbaum and Santos 1996) although other studies in cattle reported that herd size was not a risk factor for leptospiral infection (Espi *et al.* 2000; Alonso-Andicoberry *et al.* 2001).

An increasing level of co-grazing exposure of deer with cattle or sheep (as described in Table 4.2) was associated with significant increasing trends of seropositivity to Hardjobovis and Pomona in deer herds. This finding was consistent with observations of infection in a cattle herd that co-grazing with infected cattle and sheep (Lilenbaum and Santos 1996). It suggests that inter-species transmission of leptospires may occur on farms in New Zealand. However, further studies of a greater number of mixed-species farms including bacterial isolation and importantly, strain typing, are needed to confirm that between-species transmission is occurring, since it is possible that host adaptation within serovars or different strains may occur.

There was variation in seroprevalence between species and between years within and between species observed in this study. Climatic changes between years of study could be one of the contributing factors. There was a correlation between humidity and seroprevalence of serovars Hardjobovis and Pomona in cattle reported in Queensland, Australia (Elder *et al.* 1986). A correlation between seroconversion for Hardjobovis in cattle and the wettest period of the year in New Zealand has also been reported (Hellstrom and Marshall 1978). However, it was not appropriate to analyse climatic variables in this study because there could be many confounding factors between farms which we did not observe. This will be subject to further study in a larger epidemiological study of leptospirosis on mixed-species farms.

In summary, this study demonstrates that leptospiral infection is prevalent across species on mixed-species farms in the lower North Island of New Zealand. The seroprevalence of leptospiral infection in deer, beef cattle and sheep was moderate to high at the herd/flock level, but lower at the individual animal level. Hardjobovis was the predominant serovar with Pomona at a lower seroprevalence. Co-grazing of farmed deer with leptospiral infected cattle and/or sheep is a significant risk for deer herds to be seropositive to *Leptospira*. This study provides baseline data for more intensive serological and molecular epidemiological study of leptospirosis on mixed-species farms in the future.

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Chapter 5

Evaluation of a SYTO9 real-time PCR to detect and identify pathogenic *Leptospira* species in kidney tissue and urine of New Zealand farmed deer

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Abstract

A SYTO9 real-time PCR assay for detection of pathogenic *Leptospira* species based on amplification of DNA gyrase subunit B (*gyrB*) gene has been optimized and evaluated for sensitivity and specificity on kidney and urine samples from New Zealand farmed deer. The detection limit was approximately 10^3 cells/ml or equivalent to 2 to 10 copies/reaction. Comparison of this PCR assay on deer kidneys (n = 268) using culture as the gold standard revealed a sensitivity and a specificity of 85.0% and 98.8%, respectively. For deer urine (n=113), the assay was compared with known inoculated samples as the gold standard and revealed a sensitivity and specificity of 96.7% and 100%, respectively. The detectable concentration of leptospires shed naturally in deer urine collected on-farm (n = 28) was 3.7 x 10^3 to 1.7 x 10^6 cells/ml. To assess the assay's capability for identifying pathogenic *Leptospira* species, 14 field isolates of serovars Hardjobovis and Pomona were amplified for PCR product, purified and sequenced. When compared with the NCBI database using a BLASTn search, sequence data matched with *L. borgpetersenii* serovar Hardjobovis in 13 samples and *L. interrogans* serovar Pomona in one sample which was consistent with the microscopic agglutination test (MAT). Sequence analysis of purified PCR product amplified directly from kidney and urine samples also yielded serovar- comparable MAT results. Results from this study suggest that this real-time PCR assay is a useful tool for rapid, economic, sensitive and specific detection of pathogenic leptospires in clinical samples from farmed deer for diagnostic purposes and epidemiological, vaccine and other research. It can also be used for estimating the concentration of leptospires in urine and identifying *Leptospira* species in combination with DNA sequencing.

5.1 Introduction

Leptospirosis is an emerging zoonotic disease with worldwide distribution. It is caused by infection with pathogenic *Leptospira*, helical shaped motile spirochetes that belong to the family *Leptospiracae* genus *Leptospira.* Humans and animals usually get infected by direct contact with blood, urine or kidney of infected animals or indirectly by contact with surface water, mud and soil that is contaminated with pathogenic leptospires from excretor animals (Levett, 2001). There have been over 200 serovars of pathogenic *Leptospira* within 13 species isolated and described (Bharti et al., 2003). Six serovars within two species of *L. borgpetersenii and L. interrogans* were isolated in animals in New Zealand (Hathaway, 1981, Midwinter et al., 1999).

In New Zealand, livestock, including farmed deer, play an important role in the spread of leptospirosis to both animals and humans because they excrete organisms into the environment via urine, and at slaughter, put meat workers at risk via urine and kidney tissue. Leptospirosis is a well recognised clinical disease and sub-clinical infection in New Zealand farmed deer (Ayanegui-Alcérreca et al., 2010 in press, Wilson et al., 1998). *Leptospira borgpetersenii* serovar Hardjobovis and *L. interrogans* serovar Pomona are the most commonly detected serovars (Ayanegui-Alcérreca et al., 2007). A regional leptospirosis sero-

prevalence survey of 110 deer farms in NZ showed evidence that 81% were seropositive, with Hardjobovis alone in 61% of herds, Pomona alone in 4% and a combination of both in 16% of herds (Ayanegui-Alcérreca et al., 2010 in press). It is proposed that farmed deer in New Zealand are maintenance hosts for Hardjobovis and an accidental host for Pomona and that deer play an important role in the infection cycle of leptospirosis on New Zealand livestock farms (Ayanegui-Alcérreca, 2006).

Culture of *Leptospira* organisms from clinical samples is the definitive method that allows identification of infecting serovars. However, culture is rarely used because it is technically demanding, tedious, complex, time consuming, prone to contamination and expensive. The Microscopic Agglutination Test (MAT) is most widely used as the standard serology test because of its high sensitivity and specificity and the ability to identify leptospires infection to serogroup or serovar level (O'Keefe, 2002, Vijayachari et al., 2001). However, the MAT does not reflect the carrier status of the host. It also has drawbacks including the maintenance of live *Leptospira* cultures for antigens and complexity of methods and interpretation that limits its use in the laboratory (Myers, 1976, Palmer, 1988). Conventional methods such as dark-field microscopy and Warthin-Starry staining to detect leptospires in clinical samples are unreliable because of their low sensitivity (O'Keefe, 2002).

With the introduction of Polymerase Chain Reaction (PCR), rapid detection of small numbers of leptospires in clinical samples has become practical due to specific amplification of leptospiral DNA. PCR can also determine the shedding and carrier status of leptospires, detecting leptospiral DNA in cattle urine (Bal et al., 1994, Bomfim et al., 2006), pig kidney (Fearnley et al., 2008), and cattle semen and vaginal fluids (Lilenbaum et al., 2008). There have also been applications of PCR for rapid diagnosis by detection of leptospires in blood (Fonseca Cde et al., 2006, Slack et al., 2007).

Real-time PCR (RT-PCR) has further improved the diagnosis of leptospirosis. A new double-stranded DNA intercalating dye from the SYTO family has been introduced as an alternative to conventional SyBr Green I. SYTO9 has been shown to produce robust and consistent DNA melting curves that are not affected by DNA concentration and can be used with a broad range of dye concentrations without causing PCR inhibition. It is easier to use than SyBr Green I, particularly for adapting conventional assays to a real-time format and for DNA melting curve analysis (Monis et al., 2005).

Recently, the DNA gyrase subunit B gene (*gyrB*) has been proposed as an alternative target to 16S rRNA gene for species identification. The *gyrB* gene is a single-copy gene, present in all bacteria, which encodes the ATPase domain of the enzyme DNA gyrase essential for DNA replication. The amino acid sequences of *gyrB* gene allow the comparison of bacterial taxonomy (Dauga, 2002). Recently, *gyrB* gene has been used as a target for identification of *Leptospira* species and is claimed as an alternate identification gene (Kawabata et al., 2006, Slack et al., 2006).

This paper reports the evaluation and validation of a quantitative real-time PCR using SYTO9 detection technology in combination with amplification and sequencing of a partial fragment of *gyrB* gene for detection and identification of pathogenic *Leptospira* species in urine and kidney samples from New Zealand farmed deer. We evaluated the detection limit, sensitivity and specificity in clinical contexts, examining kidney tissue and urine and comparing results with the gold standard, culture. We also report the range of concentration of leptospires shed naturally in deer urine, identification of pathogenic *Leptospira* species based on DNA sequence of *gyrB* gene PCR product from field isolates of deer kidney and urine, and phylogenetic analysis.

5.2 Materials and methods

5.2.1 Sample sources

Deer kidneys (n=268, range 8 – 25 per farm) paired with blood samples as available (n=209, range 0 – 25 per farm) were collected from 19 randomly selected slaughter lines from 18 farms at Deer Slaughter Premises (DSPs) in Feilding, Manawatu and Makarewa, Southland of New Zealand during the period of November 2006 to November 2008. Deer were rising-one-year-old or older of both sexes. There were no data on previous exposure to *Leptospira.* Additionally, urine (n=111, range 15 – 28 per farm) collected from female rising-one-year-old deer on five commercial deer farms in the Manawatu and Hawkes Bay regions of New Zealand in November 2007 as part of vaccine efficacy study (Subharat, 2010 in press) were used in this study. Sampled animals were phenotypically red deer (*Cervus elaphus*) but possibly containing some wapiti (*Cervus elaphus canadensis*) genes.

5.2.2 Sample collection and preparation

5.2.2.1 Blood

Blood samples collected at DSPs were by free flow into a new 10 ml plain blood tube after animal sticking, immediately after stunning, at the beginning of the slaughter line. On farms, blood samples were collected from the jugular vein of physically restrained deer using a new 20-gauge needle and a 10 ml evacuated blood tube with no anticoagulant. Blood was held at 4°C before transport to the laboratory for centrifugation at 3,000 rpm (1,512 x *g*) for 15 minutes after which serum was aliquoted into new, labeled 1.5ml microcentrifuge tubes and stored at -20°C.

5.2.2.2 Kidney

Whole kidneys were collected at the evisceration and inspection area of the slaughter board after capsule removal, put into labeled sterile plastic bags aseptically and held at 4°C before transport to the laboratory. Within 6-18 hours of collection, the kidney surface was swabbed with 70% alcohol and randomly aspirated from several sites over the entire kidney into a 5ml, sterile syringe using a sterile 16-gauge needle. Half of the extracted kidney tissue (approximately 50mg) was used for bacterial culture and the other half used for DNA

extraction for real-time PCR. Processed kidney sample was later split into two portions. One half was stored in freezer at -20°C whereas the other half was stored in 10% formalin.

5.2.2.3 Urine

Urination was induced by administration of furosemide ("Salix", Intervet, NZ) at 1-1.5 mg/kg intramuscularly (Fairley et al., 1984, Warren et al., 1981). A new 70 ml plastic collector was held beneath the vulva after urination began. As much middle-stream urine as possible was collected and immediately held at 4°C. After transport to the laboratory, urine was centrifuged at 3,000 rpm (1512 × *g*) for 10 minutes to provide sediment which was re-suspended with 400 µl of phosphate-buffered saline (PBS) to neutralize the pH (Levett, 2001). Half (200µl) was used for bacterial culture and half was used for DNA extraction for real-time PCR. Leptospire-free deer urine was determined by no presence of organism or its DNA in bacterial culture and real-time PCR along with no detectable antibodies against *Leptospira* serovars Hardjobovis and Pomona in blood from the same animals.

5.2.3 Serology

The MAT was used to test serum reactivity to laboratory standardized *Leptospira* serovar Hardjobovis and Pomona. The method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on "Guidelines for the Control of Leptospirosis" (Faine, 1982). A titer of ≥1:48 was considered positive for both serovars (Blackmore et al., 1982, Dorjee et al., 2008).

5.2.4 Bacterial culture

Ellinghausen-McCullough-Johnson-Harris (EMJH) was used as a selective medium with an addition of antibiotic (5'-fluouracil) for contamination inhibition. The processed samples (kidney and urine) were inoculated and sub-cultured into two consecutive tubes, incubated at 28-30°C and examined every two weeks under dark-field microscopy for four months. Isolates of leptospires were serotyped against the standardized antisera of *Leptospira* serovar Hardjobovis and Pomona. These methods were adapted from the standard protocols from the Massey University Leptospirosis Research Unit, based on "Guidelines for the Control of Leptospirosis" (Faine, 1982).

5.2.5 DNA extraction

DNA was extracted from 50mg of kidney tissue and 200µl of urine using the High Pure Template Kit (Roche, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 200µl.

5.2.6 PCR amplification

The real-time PCR assay was modified from an assay described by (Slack et al., 2006). Magnesium and primer titration was performed to determine the optimal concentration to give the lowest cycle threshold (CT) value. SYTO9 (Invitrogen, USA) was used as a fluorescent, double-stranded DNA-specific, intercalating dye (Monis et al., 2005) for all real-

time PCR assays. The assay was performed in a Rotor-Gene 6000 machine (Corbett Research, Australia) using primers 2For 5'-tgagccaagaagaaacaagctaca-3' and 504Rev 5'-matggttccrctttccgaaga-3' (Slack et al., 2006). Each 25µl reaction contained 2µl of DNA extracted from samples, 1.5µM SYTO9, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 12.5pmol of 2For primer, 12.5pmol of 504Rev primer, 0.1% Fraction V bovine serum albumin (BSA) (Sigma, USA), 1 unit of Taq DNA polymerase and double distilled water. Thermal cycling consisted of initial denaturation at 95°C for 10min followed by 40 cycles of denaturation at 95°C for 10sec, annealing at 60°C for 20sec and extension at 72°C for 20sec. Melting temperature (Tm) of PCR product was determined by melting curve analysis. It was performed by heating the PCR product from 70°C to 90°C and monitoring fluorescence change every 0.1°C. Confirmation of positive samples was determined by Tm of PCR product or performing electrophoresis of PCR product in a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The positive control used was *Leptospira* serovar Hardjobovis and the negative control was double distilled water.

5.2.7 PCR product purification

The amplified PCR product was purified to remove excess primers and dNTPs using the High Pure PCR Product Purification Kit (Roche, Germany) as per manufacturer's instructions. Purified PCR product was eluted in a final volume of 50µl.

5.2.8 DNA sequencing and phylogenetic analysis

The purified PCR products were forwarded to the Allan Wilson Centre Genome Service, Massey University, New Zealand for ABI sequencing using the ABI3730 DNA capillary instrument. Purified PCR products were sequenced in both orientations by the dideoxy-chain termination method using 2For and 504Rev specific primers. The sequence data were assembled and trimmed to a minimum of two contiguous sequences using the MT Navigator software (Perkin Elmer, USA). Unknown sequence data were submitted to a nucleotide Basic Local Alignment Search Tool (BLASTn) available at the National Center for Biotechnology Information (NCBI) website. Analysis of DNA sequences was performed using the Geneious Basic version 4.6.1 software. Multiple alignments of the DNA sequence were performed for phylogenetic comparison using the neighbor joining method.

5.2.9 Detection limit

The limit of leptospiral DNA detection by real-time PCR was evaluated using artificially inoculated, leptospire-free, deer urine that was negative by culture and was from MAT negative animals. Urine samples were prepared by mixing 180µl of leptospire-free deer urine with 20µl of *Leptospira* serovar Hardjobovis (field isolate suspensions at a concentration of 2 x 10^8 cells/ml measured in a Petroff-Hausser counting chamber (Faine, 1982). The next step was seven serial, ten-fold dilutions from 10^7-10^1 cells/ml of leptospire-free urine. The diluted samples were subsequently subjected to DNA extraction (see section 5.2.5). Two microliters of extracted DNA samples obtained from all seven dilutions were used as templates in the

PCR. The procedure was repeated with four different field isolates of *Leptospira* serovar Hardjobovis for confirmation of detection limit.

5.2.10 Diagnostic sensitivity and specificity

Two experiments were carried out to determine the sensitivity and specificity of real-time PCR on kidney and on urine samples.

For kidneys, fresh tissue samples from 268 deer were subjected directly to real-time PCR and compared with culture as the gold standard using the techniques described above. Blood samples from 209 of those animals were subjected to MAT for serovar identification. The suitability of long term storage of kidney tissue as either frozen or formalin-fixed samples for real-time PCR was compared with fresh samples from 11 kidneys. The agreement between results of the real-time PCR and kidney culture was tested by Kappa statistic.

For urine, the sensitivity and specificity of real-time PCR was determined by creating artificially inoculated urine samples at the lowest detection limit (as described in 5.2.9 above) to mimic a natural gold standard. Thirty aliquots of three different field isolates of *Leptospira* serovar Hardjobovis containing approximately 2 x 10^8 cells/ml were diluted in phosphate buffered saline (PBS) pH 7.4 to 10^4 cells/ml. Thereafter, 20µl of the diluted aliquot were inoculated with 180µl of 30 different samples of leptospire-free deer urine to achieve a concentration of 10^3 cells/ml. All samples were chilled at 4°C overnight to simulate the usual transport time for field-collected samples. All 30 inoculated urine samples and 83 leptospire-free deer urine samples were subjected to DNA extraction and real-time PCR as below to determine sensitivity and specificity.

5.2.11 Quantification of urinary shedding

Twenty-eight real-time PCR positive deer urine samples from an on-farm vaccine efficacy study (Subharat, 2010 in press) were used for estimating concentrations of leptospires shed naturally in deer urine. A standard curve was constructed for each PCR run from serial dilutions of leptospires inoculated in leptospire-free deer urine as described in 5.2.9 above. Each sample was tested in duplicate in each PCR run to estimate repeatability. The concentration of leptospires was calculated using Rotor-Gene 6000 series software (Corbett Research, Australia). The concentration of leptospires was compared with culture from the same animals to determine the lowest concentration of leptospires in field-collected deer urine that could be cultured.

5.2.12 Identification of pathogenic Leptospira species

To assess ability to identify pathogenic *Leptospira* species, 14 isolates of unknown serovar from kidney and urine within this study (as in 5.2.10 above), three real-time PCR positive deer kidney tissue samples and seven real-time PCR positive deer urine samples were amplified for the product of *gyrB* gene. The amplified PCR products were then purified and forwarded to the Allan Wilson Genome Service Centre, Massey University, Palmerston North, New Zealand, for DNA sequencing. The sequence data were BLASTn searched to

assess homologies with sequences in the NCBI database. Phylogenetic analysis was performed to compare the DNA sequence between isolates.

5.3 Results

5.3.1 Detection limit

The lowest detection limit was approximately 10^3 cells/ml or equivalent to 2-10 copies/reaction. This was shown by both visualization of specific 500bp DNA fragments in the gel (Fig. 5.1, Lane 5) and melting curve analysis by real-time PCR revealing that concentrations below 10^3 cells/ml could not be distinguished from the negative control (Fig. 5.2, Line 5). This pattern was identical for all 5 different field isolates that were subjected to serial dilutions.

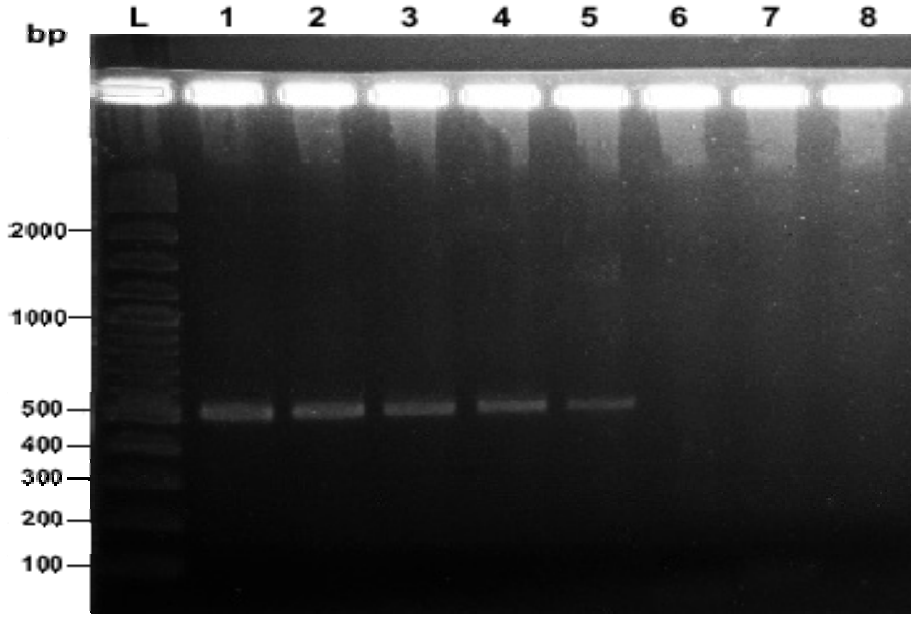

Fig. 5.1 Representative gel electrophoresis showing the limit of detection (Lane 5) of real-time PCR in deer urine dilution of field isolates of *Leptospira* serovar Hardjobovis. Lane L: 2-Log DNA ladder ranging from 100bp-10,000bp; Lanes 1-7: 10^7 - 10^1 cells/ml, respectively; Lane 8: negative control.

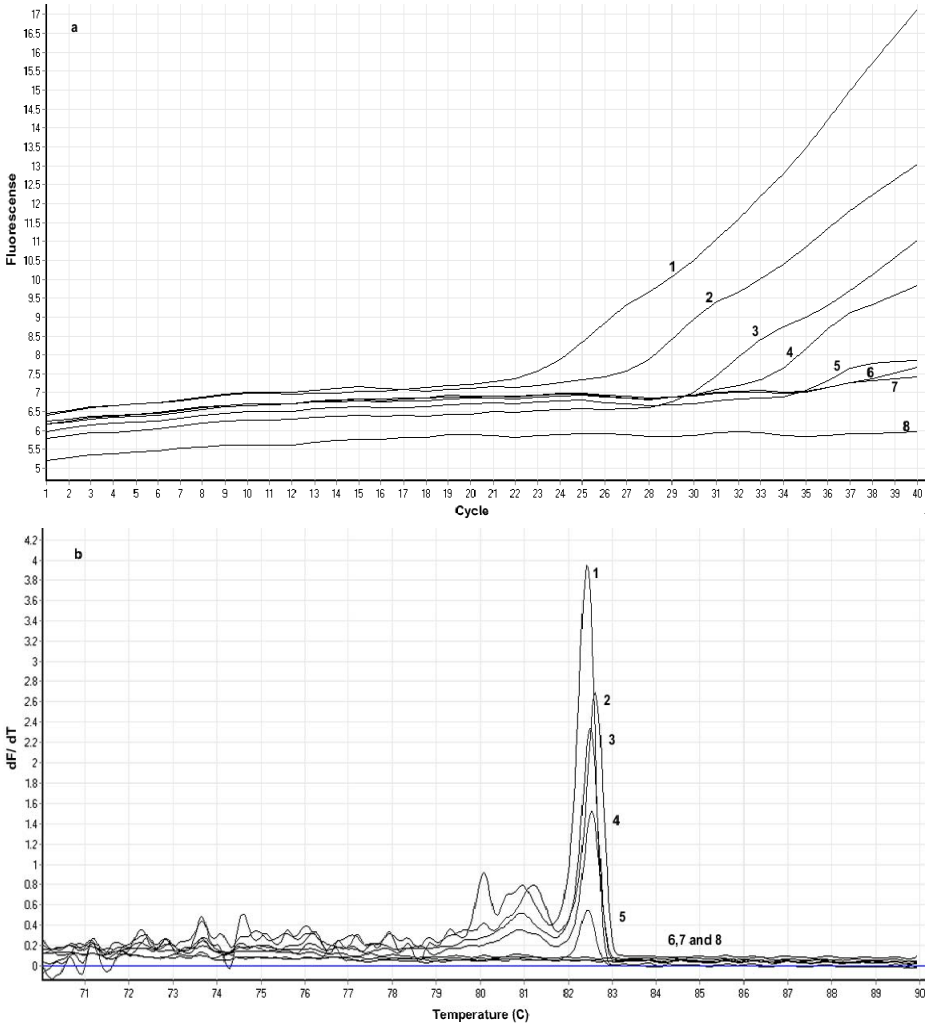

Fig. 5.2 Representative a) cycling and b) melt curve analysis showing the limit of detection of real-time PCR in deer urine dilutions of field isolates of *Leptospira* serovar Hardjobovis . Line 1: 10^7 cells/ml; Line 2: 10^6 cells/ml; Line 3: 10^5 cells/ml; Line 4: 10^4 cells/ml; Line 5: 10^3 cells/ml; Line 6: 10^2 cells/ml; Line 7: 10^1 cells/ml; Line 8: negative control.

5.3.2 Diagnostic sensitivity and specificity

Results of culture and real-time PCR for kidney samples corresponding to MAT are summarized in Table 5.1. Of 268 kidney samples, 17 were both culture and PCR positive. Two samples were culture negative but PCR positive and three were culture positive but PCR negative. MAT results were available for four of the five of those samples, all of which were positive.

Data of real-time PCR on kidney against culture and its performance are presented in Table 5.2. The sensitivity and specificity were 85.0% and 99.2%, respectively. The positive predictive value, negative predictive value and Kappa (agreement test) were 89.5%, 98.8% and 86.2%, respectively. Of 11 real-time PCR-positive, fresh kidney samples, seven (64%) were positive after being frozen at -20°C and none were positive after being fixed in 10% formalin.

Table 5.1 Kidney culture, real-time PCR and MAT results from 19 slaughter lines.

Line	No. tested	No. positive		No. MAT positive		Titer range	
		Culture	PCR	Hardjobovis	Pomona	Hardjobovis	Pomona
1	10	3	3	9	7	1:48-1:192	1:96-1:768
2	20	4	4	15	0	1:48-1:96	-
3	20	0	0	0	4	-	1:48
4	10	0	0	0	0	-	-
5	8	0	0	1	0	1:48	-
6	13	4	2	9	4	1:48-1:192	1:96-1:192
7	14	0	0	0	2	n/a	1:48-1:768
8	14	0	0	2	0	1:48	-
9	15	2	2	7	2	1:48-1:96	1:48-1:96
10	15	0	0	0	0	-	-
11	12	0	0	3	1	1:48-1:192	1:48
12	14	1	1	4	3	1:48-1:192	1:48-1:384
13	10	0	0	5	1	1:48-1:192	1:48
14	13	0	0	4	0	1:48	-
15	15	0	0	n/a	n/a	n/a	n/a
16	13	0	0	n/a	n/a	n/a	n/a
17	14	6	5	n/a	n/a	n/a	n/a
18	13	0	0	n/a	n/a	n/a	n/a
19	25	0	2	22	0	1:48-1:3072	n/a
All	268	20/268	19/268	81/209	24/209	1:48-1:3072	1:48-768

n/a = not available

Data of real-time PCR on urine against inoculated samples at the detection limit of 10^3cells/ml and its performance are presented in Table 5.3. Of 30 inoculated urine samples, 29 were positive by real-time PCR. None of 83 leptospire-free urine samples were positive by real-time PCR. The analytical sensitivity and specificity of the SYTO9 PCR assay in comparison to inoculated samples as the gold standard were 96.7% and 100.0%, respectively.

Table 5.2 Result of real-time PCR on kidney against culture and its performance (%) with 95% CI in brackets.

	Kidney	
	Culture positive	Culture negative
PCR positive	17	2
PCR negative	3	246
Sensitivity	85.0 (62.1-96.8)	
Specificity	99.2 (97.1-99.9)	
Positive predictive value	89.5 (66.8-98.7)	
Negative predictive value	98.8 (96.5-99.8)	
Kappa	86.2 (74.2-98.2)	

Table 5.3 Result of real-time PCR on urine against inoculated samples at the detection limit of 10^3 cells/ml and its performance (%) with 95% CI in brackets.

	Urine	
	Inoculated	Leptospire-free
PCR positive	29	0
PCR negative	1	83
Sensitivity	96.7 (82.8-99.9)	
Specificity	100 (96.5-100)	

5.3.3 Quantification of urinary shedding

The concentration of leptospires from 28 real-time PCR positive deer urine samples was between 3,690 and 1,714,450 cells/ml (Table 5.4). The lowest concentration of leptospires that was cultured from deer urine was 10,120 cells/ml (No culture result was available for sample 23, with a lower count, due to contamination). All seven isolates derived from urine culture were serotyped as serovar Hardjobovis using standard antisera typing method.

5.3.4 Identification of pathogenic Leptospira species

The *gyrB* gene sequences of 14 unknown isolates were compared to those deposited on the NCBI database with BLASTn search. Thirteen isolates were 100% homologous with the DNA sequence of *L. borgpetersenii* serovar Hardjobovis (NCBI accession no. CP000350.1 and CP000348.1) and one isolate was 100% homologous with three different *gyrB* gene sequences of *L. interrogans* serovar Pomona (NCBI accession no. AY896738.1), *L. interrogans* serovar Medanensis (AY896746.1) and *L. interrogans* serovar Canicola (AY896745.1). There was 100% agreement between the *gyrB* gene sequencing and conventional antisera typing of 13 isolates being serovar Hardjobovis and one isolate being serovar Pomona. Phylogenetic analysis has confirmed 100% similarity between 13 isolates of serovar Hardjobovis that all differed from the single Pomona isolate. In addition, DNA sequences of three amplicons derived directly from deer kidney and seven amplicons derived directly from deer urine were 100% homologous with the DNA sequence of *L. borgpetersenii* serovar Hardjobovis (NCBI accession no. CP000350.1 and CP000348.1) when a BLASTn search was performed.

Table 5.4 Concentration of leptospires in real-time PCR positive urine from deer on commercial farms, and corresponding culture results.

Sample	Farm region	Concentration (cells/ml)	Culture
1	Manawatu	11,213	−
2	Manawatu	1,205,170	−
3	Manawatu	270,179	+
4	Manawatu	59,575	C
5	Manawatu	48,237	−
6	Manawatu	1,071,519	+
7	Manawatu	859,663	+
8	Manawatu	10,120	+
9	Manawatu	167,652	−
10	Manawatu	1,011,509	−
11	Manawatu	78,673	−
12	Manawatu	38,646	C
13	Manawatu	165,583	C
14	Manawatu	703,169	+
15	Manawatu	325,092	C
16	Manawatu	12,207	C
17	Manawatu	32,026	C
18	Manawatu	305,271	C
19	Manawatu	1,618,695	+
20	Manawatu	1,714,450	+
21	Hawkes Bay	22,190	−
22	Hawkes Bay	5,092	−
23	Hawkes Bay	3,690	C
24	Hawkes Bay	136,343	−
25	Hawkes Bay	598,999	−
26	Hawkes Bay	565,415	−
27	Hawkes Bay	363,936	−
28	Hawkes Bay	5,849	−

+ = positive, − = negative, C = contaminated

5.4 Discussion

This study evaluated and validated the performance of a diagnostic PCR assay for use on deer kidney tissue and urine as a research and diagnostic tool for determinating infection, carrier and shedding status of New Zealand farmed deer. A SYTO9 real-time PCR was developed using primers previously designed for a conserved region of *gyrB* sequences of pathogenic *Leptopira* species (Slack et al., 2006) for use on the Corbett Rotor-Gene system. It was the first study using real-time PCR directly on kidney and urine samples from farmed deer with evaluation against culture as the gold standard. To our knowledge, it was also the first study to quantify pathogenic leptospires shed naturally in animal urine. The study also identified specific *Leptospira* species by DNA sequencing of PCR amplicons.

Based on the method of Slack et al (2006) using a Lightcycler system, we optimized PCR conditions to suit the Rotor-Gene instrument. SYTO9 ds-DNA intercalating dye was adopted as it has shown to have advantage over SyBr Green I (Monis et al., 2005) being easily adapted using standard conventional PCR reagents. That this assay does not need a specific probe like Taqman or FRET makes it cheaper and able to run on a larger scale than other assays.

The lowest detection limit of the assay was determined by DNA extracted from known concentrations of leptospires measured in a Petroff-Hausser counting chamber and dark field microscopy as described by (Faine, 1982). The detection limit of the PCR using the DNA extracted from dilution of leptospires mixed with leptospire-free deer urine was approximately 10^3 cells/ml or equivalent to 2 to 10 copies/reaction. This was consistent with another study which reported the detection limit of 10^3 cells/ml in inoculated human serum (Ooteman et al., 2006) and 3 to 10 copies/reaction in inoculated human serum and urine (Levett et al., 2005). However, this was in contrast to (Gerritsen et al., 1991) who reported the detection limit of 10 cells/ml in bovine urine.

The assay showed 85% sensitivity (95% CI 62.1%-96.8%), 99% specificity (95% CI 97.1%-99.9%) and "excellent" agreement (Kappa = 86.2%, 95%CI 74.2%-98.1%) with kidney culture as the gold standard. Failure of the PCR to detect leptospires in some culture positive kidney samples may be due to leptospire concentrations below detection limit. (Grooms et al., 2005) claimed that DNA extracted from tissue can contain inhibitors interfering with PCR reactions. A possible reason for two PCR positive kidney samples being culture negative may be lack of viable organisms at the time of processing since those samples were from a DSP in Southland, a long distance from the laboratory, requiring more than 24 hours before processing. Samples from the other DSP were placed in culture within approximately six hours of collection. It is notable that the source animals for those two samples were seroreactive to serovar Hardjobovis with high titers consistent with recent exposure and/or current infection with leptospires. Hence, we assert that there is a high probability that the real-time PCR result does indicate infection.

Long term storage of kidney samples for use in DNA extraction and real-time PCR was investigated on a limited number of specimens in this study. The lowered sensitivity from frozen compared with fresh samples and the inability to detect DNA in formalin fixed tissues suggest that kidney samples need to be processed fresh to achieve the highest sensitivity.

Approximately 30% of urine cultures from deer on farms were contamination whereas real-time PCR returned several positive results from such samples. Determination of the analytical sensitivity of real-time PCR was therefore undertaken using urine artificially inoculated with leptospires showing the lowest detection limit to be 10^3cells/ml. The analytical sensitivity at this concentration and above was 96.7% (95% CI 82.8%-99.9%) and the specificity was 100% (95% CI 90.5%-100%). These results were comparable with the findings of Slack et al (2007) who reported a diagnostic sensitivity of 96.4% and a specificity

of 99.5% on patient sera using primer pairs Lepto F/ Lepto R. Those primers which were designed from the 16s rRNA gene detected leptospiral DNA in both serum and seeded urine samples (Smythe et al., 2002).

In addition to detection of the organism *per se*, the ability of real-time PCR to quantify the concentration of leptospires in clinical samples provides diagnostic benefits, for example in evaluating antibiotic efficiency by post-treatment clearance of leptospires in urine. The technique may also be used for epidemiological studies involving *Leptospira* shedding and possibly even surface contamination when studying potential risk factors for disease transmission. One study has developed a real-time PCR assay to measure the concentration of leptopiral DNA in patient's sera and reported a range of 80 to 39,000 cells/ml that informed the prognosis for patients suffering from leptospirosis (Merien et al., 2005). To our knowledge, this study is the first reporting detectable concentrations of *Leptospira* serovar Hardjobovis shed in field samples of deer urine, finding a range from 3.7×10^3 to 1.7×10^6 cells/ml. This defined the detection limit of this PCR assay at 10^3 cells/ml although it is possible that deer may shed lower concentrations of leptospires in urine. The lowest concentration of leptospires (sv. Hardjobovis) that could be cultured from deer urine was 10^4 cells/ml which was higher than that from a previous report of culture of 10^2 cells/ml in water and 10^3 cell/ml in bovine semen (sv. Hardjoprajitno) (Heinemann et al., 2000). Considering that urine samples collected on-farm are prone to contamination, as shown in this study (approximately 30% of samples were contaminated), this detection limit of urine culture is plausible and likely explains the higher sensitivity of real-time PCR over urine culture. However, urine from other sources (e.g. humans) collected under aseptic conditions may yield a higher proportion of positive cultures.

A limitation of PCR-based diagnosis of leptospiral infection is the inability to identify the infecting leptospires at the species, serogroup or serovar level (Lilenbaum et al., 2008, Merien et al., 2005). The infecting serovar may be predicted by combining real-time PCR results with the MAT since data from our study have shown that approximately 85% of kidneys positive to real-time PCR were from animals positive to the MAT. However, caveats to this conjecture are in those cases of early infection when the host immunity has not been activated, or in the event of dual or multiple serovar infections.

Species identification can be addressed by amplicon sequencing of PCR product of *gyrB* gene (Slack et al., 2006). All 14 unknown isolates and 10 real-time positive samples subjected to PCR amplification and sequencing of *gyrB* gene resulted in 100% homology with sequences deposited on the NCBI database when a BLASTn search was performed. In addition, the BLASTn search results matched fully with standard antisera typing showing that 13 isolates were identified as serovar Hardjobovis and one as serovar Pomona. The BLASTn search results and phylogenetic analysis agreed with the findings of Slack et al. (2006) that sequencing of *gyrB* gene could differentiate pathogenic leptospires to species level but not to serovar level. However, since serovars Hardjobovis and Pomona are the most prevalent serovars in New Zealand livestock and that they belong to different species (*L. borgpetersenii*

and *L. interrogans*), these techniques represent a rapid tool for identifying pathogenic *Leptospira* species and the likely serovar from clinical samples. Similar deduction would be appropriate in regions where only a few species and serovars of pathogenic leptospires are encountered. For more accurate genotypic classification, Multilocus Sequence Typing (MLST) is the most up-to-date method that has been developed and claimed to have high discriminatory power, reproducibility and robustness (Ahmed et al., 2006, Thaipadungpanit et al., 2007).

In conclusion, we have validated a real-time PCR assay based on SYTO9 technology for the detection of pathogenic leptospires in kidney and urine samples of farmed deer in New Zealand. The assay was clinically evaluated against the gold standard (culture) and found to have comparable diagnostic sensitivity, specificity and predictive values for kidney samples, and better sensitivity for urine samples. This assay identifies pathogenic *Leptospira* species when combined with DNA sequencing method. This method is simple, rapid and easily adapted with conventional PCR reagents which make it cheaper than commercial real-time PCR reagents. It has potential for application as a research tool for the determination of carrier or shedding status of animal species for epidemiology studies and evaluation of vaccine efficacy, and as a clinical diagnostic tool on tissue and urine and potentially blood samples for rapid diagnosis in animals and humans.

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Chapter 6

Investigation of possible novel leptospiral serovars in farmed deer in New Zealand

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(Raw data of this chapter are in Appendix 6a, 6b and 6c)

Abstract

AIM: To investigate possible novel leptospiral infection in farmed deer in New Zealand.

METHODS: In September 2006, five serum samples from a serum bank from each of 70 farms sampled for a previous national seroprevalence survey were forwarded to the World Health Organisation/Food and Agriculture Organisation/World organisation for animal health (WHO/FAO/OIE) reference laboratory for leptospirosis in Brisbane, Australia, to test for 23 reference panel serovars, most believed to be exotic to New Zealand, using the microscopic agglutination test (MAT). In addition to apparent cross-reactivity to some serovars, there was evidence of Arborea, a serovar novel to New Zealand, on eleven farms. Subsequently 126 additional banked sera samples from nine out of those eleven farms (8 - 20/farm) were sent to the reference laboratory for similar serology in July, 2007. Two farms in the Southland region were considered seropositive for Arborea. Culture of deer kidneys (n=43) from these two farms collected at a Deer Slaughter Premise (DSP) in Makarewa was attempted in November 2007 and November 2008. Sera from those deer were sent to the Brisbane laboratory for serology confirmation by MAT.

RESULTS: From the initial 350 deer sera, 96 (27.4%) and 19 (5.4%) samples were seroreactive to Hardjobovis and Pomona, respectively. There was evidence of cross-reactivity between Hardjobovis with Medanensis and Szwajizak. Serology for Tarassovi, Grippotyphosa, Celledoni, Australis, Zanoni, Robinsoni, Canicola, Kremastos, Bulgarica, Cynopteri, Ballum, Bataviae, Djasiman, Javanica, Panama, Shermani and Topaz was negative or sporadic, generally with titres of 1:50 and therefore likely non-specific. Fourteen samples (4.0%) from eleven farms were seroreactive to serovar Arborea, justifying further investigation. The remaining 126 banked sera samples from nine out of those eleven farms were tested for serovar Arborea in July 2007 revealing a seroprevalence of 15.4% and 30.0% on two farms, respectively. None of 43 deer kidney and sera samples collected subsequently from those two farms were culture or serology positive to serovar Arborea.

CONCLUSIONS: While there was serological evidence for Arborea in deer, attempts to isolate the organism were unsuccessful. Since sample size for the follow-up investigation was insufficient to validate presence or absence of infection, further study should be undertaken to verify the status of this serovar of *Leptospira* in New Zealand in both deer and other livestock species.

KEY WORDS: *Leptospirosis, farmed deer, exotic, serology, culture, Arborea, New Zealand*

List of abbreviations

DSP = Deer slaughter premise
EMJH = Ellinghausen-McCullough-Johnson-Harris medium
MAT = Microscopic agglutination test

6.1 Introduction

Leptospirosis is considered to be an emerging zoonotic disease with worldwide distribution. It is caused by infection with pathogenic leptospires, helical shaped motile spirochaetes which belong to the family *Leptospiracae*, genus *Leptospira.* A serological taxonomy system (*sensu lato*) classifies *Leptospira* into serovars on the basis of surface antigen patterns has determined over 200 serovars (Bharti *et al.* 2003). In New Zealand, leptospirosis has been recognised in humans, domestic animals and wildlife (Blackmore *et al.* 1976; Hathaway 1981). To date, six serovars have been isolated from animals in New Zealand. Cattle and farmed deer are recognised as a maintenance host for *L. Borgpetersenii* serovar Hardjobovis, pigs for *L. interrogans* serovar Pomona and *L. borgpetersenii* serovar Tarassovi, Norway rats (*Rattus norvegicus*) for *L. interrogans* serovar Copenhageni, black rats (*Rattus rattus*) for *L. borgpetersenii* serovar Ballum, brush tail possums (*Trichosurus vulpecula*) for *L. borgpetersenii* serovar Balcanica (Midwinter and Fairley 1999).

Leptospirosis has been reported as a clinical disease in farmed deer since the 1980s (Fairley *et al.* 1986). A recent review confirmed that serovar Hardjobovis and Pomona are the most commonly detected serovars in this species (Ayanegui-Alcérreca *et al.* 2007) although serovar Copenhageni has also been isolated from deer. Serological evidence for Tarassovi, Ballum and Balcanica has been reported in deer (Flint *et al.* 1988; Wilson *et al.* 1998) but it is likely that positive titres to those serovars resulted from cross-reactivity (Ayanegui-Alcérreca 2006). A national prevalence survey of serovar Hardjobovis, Pomona and Copenhageni from 110 farms in nine regions of New Zealand was undertaken recently (Ayanegui-Alcérreca *et al.* 2010 in press) demonstrating widespread distribution and 82% seroprevalence. However, no screening for other serovars has been reported from deer or other animals in New Zealand, so it is possible that further serovars may be present in this country. Knowledge of the serovars present in livestock populations is important for both animal disease control and public health.

This paper reports a collaborative study between Massey University Deer Research Group and the WHO/FAO/OIE reference laboratory for leptospirosis in Brisbane to investigate potentially novel leptospiral serovars in New Zealand farmed deer based on a serum bank from an earlier national seroprevalence survey (Ayanegui-Alcérreca *et al.* 2010 in press). Based on serology results, attempts were initiated to isolate serovar Arborea which had previously not been found in New Zealand.

6.2 Materials and methods

6.2.1 Study design

Initially, a cross-sectional study design was chosen to determine the individual and herd seroprevalence to leptospiral serovars based on stored samples from a recent seroprevalence

study (Ayanegui-Alcérreca *et al.* 2010 in press). In August 2006, 350 stored sera from 70 farms were randomly selected (5/farm). The samples were couriered frozen to the WHO/FAO/OIE reference laboratory for leptospirosis in Brisbane for serological test (MAT) on a panel of 23 serovars. This sample number was based on MAT sensitivity of 90 %, specificity of 99% (McBride *et al.* 2007), within-herd prevalence of 50% and herd size of 350 to provide 95% confidence and 80% power. Based on serological results, further samples from nine of the farms were submitted for serological screening. Subsequently, further blood and kidney tissue samples were collected from two farms that presented with evidence of serovar Arborea.

6.2.2 Serum samples

6.2.2.1 Initial serological screening: Sera were selected from a serum bank from a recent national seroprevalence study of leptospirosis in farmed deer. The serum bank contained approximately 20 serum samples stored at -20^{o}C, from 9 - 30 month-old red or red x wapiti from each of 110 farms as described by (Ayanegui-Alcérreca *et al.* 2010 in press). Five sera were randomly selected from 70 farms based on completeness of data. The number of farms selected by region is presented in Table 6.1.

6.2.2.2 Follow-up serology: Based on initial screening that revealed seroreactivity to Arborea on nine farms, 126 additional serum samples from those farms (8 - 18/farm) were submitted for screening for Arborea in July 2007 to estimate within-herd prevalence. Serology was done concurrently with serovar Ballum which may cross-react with Arborea in the MAT to determine specificity.

6.2.2.3 Additional blood and tissue sampling: Follow-up serology confirmed seropositivity to Arborea, on two farms in Southland. Between November 2007 and November 2008, 43 deer (Farm 4 = 8, Farm 5 = 35) were blood and kidney sampled on two visits at a Deer Slaughter Premise (DSP) in Makarewa, Southland as shown in Table 6.4.

Table 6.1 Number of farms tested by region

Region	Number of farms
Otago	13
West coast	5
Northland	7
Canterbury	9
Bay of Plenty	10
Manawatu-Wanganui	7
Waikato	2
Southland	14
Hawkes Bay	3
Total	**70**

6.2.3 Additional sample collection and processing

6.2.3.1 Blood: Blood samples were collected by free flow into a new 10 ml plain blood tube after animal sticking, immediately after stunning. Blood samples were left to clot at room temperature. Samples were then centrifuged at 1512 *g* for 10 minutes after which serum was aliquoted into new, labelled 1.5ml microcentrifuge tubes and stored at -20°C. The sera were couriered frozen to WHO/FAO/OIE reference laboratory for leptospirosis in Brisbane for serology.

6.2.3.2 Kidney: Kidney samples were taken at the evisceration and inspection area of the slaughter line. Whole kidneys, without renal capsule, were put into labelled sterile plastic bags aseptically and held at 4°C before transport back to the laboratory. Later, the kidney surface was swabbed with 70% alcohol and randomly aspirated from several sites over the entire kidney into a 5ml sterile syringe using a sterile 16-gauge needle. Approximately 50 mg of kidney tissue was used for bacterial culture.

6.2.4 Laboratory procedures

6.2.4.1 Serology: Microscopic Agglutination Test (MAT) was used to test serum reactivity against a standardised reference leptospiral panel of *L. interrogans* serovars Pomona, Copenhageni, Canicola, Australis, Szwajizak, Medanensis, Zanoni, Robinsoni Bataviae, Djasiman and Kremastos, *L. borgpetersenii* serovars Hardjobovis, Tarassovi, Arborea, Ballum and Javanica, *L. kirschneri* serovars Grippotyphosa , Bulgarica, Cynopteri and Panama , *L. weilii* serovars Celledoni and Topaz and *L. santarosai* serovar Shermani to determine the leptospiral titre status of individual animals and herds. An initial serum dilution of 1:25 and two-fold serial dilution of serum covering the range of 1:50 to 1:6400 were tested. Titre was recorded as the reciprocal of the highest dilution at which $\geq$ 50% of leptospires were agglutinated (Stallman 1984). To consider a sample positive, the cut-off point at 1:50 was used for all serovars which was regarded as evidence of past or present exposure. To consider a herd positive, at least one serum sample had to be positive at the minimum dilution of 1:50.

6.2.4.2 Bacterial culture: The culture method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on “Guidelines for the Control of Leptospirosis” (Faine 1982). Ellinghausen-McCullough-Johnson-Harris (EMJH) was used as a selective medium with an addition of 5’-fluouracil for contamination inhibition. The medium was inoculated with processed kidney, incubated at 28-30°C and examined every two weeks for four months under dark-field microscopy.

6.3 Results

6.3.1 Initial serological screening

Serology data at herd and individual level of each serovar testing positive on at least one farm, with reciprocal titre range and evidence of cross-reaction are presented in Table 6.2. Forty herds (57.1%) and 96 individual samples (27.4%) were seroreactive to Hardjobovis with evidence of cross reactivity to Kremastos, Szwajizak, Medanensis, Panama and Djasiman. Nine herds (12.9%) and 19 individual samples (5.4%) were seroreactive to Pomona with evidence of cross reactivity to Grippotyphosa, Topaz, Cynopteri and Djasiman. Eleven herds (15.7%) and 14 individual samples (4.0%) were seroreactive to Arborea with evidence of cross reactivity to Ballum, Javanica, Panama and Topaz. One individual sample (0.3%) in one herd (1.5%) was seroreactive to Bataviae with no evidence of cross reactivity. There was no evidence of seroreactivity to Copenhageni, Tarassovi, Celledoni, Australis, Zanoni, Robinsoni, Canicola, Bulgarica and Shermani.

Table 6.2 Herd and individual seroprevalence for seroreactive serovars with range of number positive/herd, reciprocal titre range and potential evidence of cross-reaction, from initial screening of 70 herds (5 samples/herd).

Serovar	No. of herds positive (%)	Number of samples positive/herd	No. of individuals positive (%)	Reciprocal titre range	Potentially cross-reacting serovar (%)
Hardjobovis	40 (57.1)	1 – 5	96 (27.4)	50 – 400	Kremastos (6.3), Szwajizak (22.9), Medanensis (38.5), Panama (1.0), Djasiman (2.1)
Pomona	9 (12.9)	1 – 5	19 (5.4)	50 – 1600	Grippotyphosa (5.3), Topaz (15.8), Cynopteri (5.3), Djasiman (5.3)
Grippotyphosa	1 (1.5)	1	1 (0.3)	50	Pomona (100.0)
Kremastos	5 (7.1)	1 – 2	6 (1.7)	50	Hardjobovis (100.0)
Szwajizak	17 (24.3)	1 – 3	22 (6.3)	50 – 200	Hardjobovis (100.0)
Medanensis	23 (32.9)	1 – 3	37 (10.6)	50 – 200	Hardjobovis (100.0)
Cynopteri	1 (1.5)	1	1 (0.3)	100	Pomona (100.0)
Arborea	11 (15.7)	1 – 3	14 (4.0)	50 – 800	Ballum (7.1)
Ballum	1 (1.5)	1	1 (0.3)	50	Arborea (100.0)
Bataviae	1 (1.5)	1	1 (0.3)	50	None
Djasiman	4 (5.7)	1	4 (1.1)	50 – 100	Hardjobovis (50.0), Pomona (25.0)
Javanica	1 (1.5)	1	1 (0.3)	100	Arborea (100)
Panama	6 (8.6)	1 – 2	7 (2.0)	50 – 200	Arborea (57.1), Hardjobovis (14.3)
Topaz	2 (2.9)	1 – 3	4 (1.1)	50 – 200	Pomona (75.0), Arborea (25.0)

6.3.2 Follow-up serology

The within-herd prevalence and reciprocal titre range for Arborea and Ballum from and initial and follow-up serum band samples combined from nine farms initially positive for Arborea are presented in Table 6.3. Three farms were classified with evidence of Arborea with within-herd prevalence of 12.5%, 20.0% and 5.6% on Farms 4, 5 and 6, respectively. Evidence of cross reactivity with Ballum was found only on Farm 6.

6.3.3 Culture for Arborea

Bacterial culture and serology results of serum and kidney samples collected at a DSP from Farms 4 and 5, considered positive to Arborea after follow-up sampling, are summarised in Table 6.4. None of 43 kidney samples were culture positive whereas one sample from Farm 5 was seroreactive to both Arborea and Ballum.

Table 6.3 Seroprevalence and reciprocal titre range for Arborea and Ballum on each farm positive for Arborea at initial screening (Table 6.2) and follow-up, combined, and farm location.

Farm	Region	No. of samples	Arborea		Ballum	
			Seroprevalence (%)	Reciprocal titre range	Seroprevalence (%)	Reciprocal titre range
1	Canterbury	20	5.0	800	0	–
2	Bay of Plenty	14	7.1	50	0	–
3	Southland	20	5.0	50	0	–
4	Southland	13	15.4	50	0	–
5	Southland	20	30.0	50 – 100	0	–
6	Manawatu	23	13.0	50 – 100	4.3	50
7	Southland	16	6.3	50	0	–
8	Canterbury	25	4.0	50	0	–
9	Southland	20	5.0	50	0	–

Table 6.4. Kidney culture and seroprevalence with reciprocal titre range, for Arborea and Ballum on two farms most likely to be infected, based on follow-up serological screening.

Farm	No. of samples	Culture (%)	Arborea		Ballum	
			Seroprevalence (%)	Reciprocal titre range	Seroprevalence (%)	Reciprocal titre range
4	8	0	0	–	0	–
5	35	0	2.9	50	2.9	50

Discussion

This study provides data on potentially exotic leptospiral serovars in farmed deer in New Zealand. Initial screening suggested serovar Arborea in animals from eleven herds. Follow-up screening suggested infection was possible in two herds but subsequent tests failed to confirm infection with this serovar. There was no herd-level evidence for other serovars not already known to be present in New Zealand.

These results confirmed endemicity of Hardjobovis, sporadicity of Pomona as observed from a previous national seroprevalence study (Ayanegui-Alcérreca *et al.* 2010 in press). However, the seroprevalence of Hardjobovis at the herd and individual level from this study (57.1% and 27.4%, respectively) was lower than that of Ayanegui-Alcérreca *et al.* (2010 in press) (77.7% and 60.8%, respectively). The difference in individual animal seroprevalence is likely to be due to the difference of titre cut point used for each study since Ayanegui-Alcérreca *et al.* (2010 in press) used a titre cut point at 1:24 for Hardjobovis, while the present study used a titre cut point at 1:50. The difference in herd prevalence is likely to be due to the smaller number of animals sampled for initial screening (n=5) compared with approximately 20 for the study by Ayanegui-Alcérreca *et al.* (2010 in press). For Pomona, the seroprevalence at the herd and individual level from this study (12.9% and 5.5%, respectively) is consistent with those of Ayanegui-Alcérreca *et al.* (2010 in press) (20.0% and 8.4%, respectively), while the titre cut point used was similar (1:50 vs. 1:48). Additionally, differences in sensitivity of tests between laboratories could contribute to differences in individual animal seroprevalence observed between studies. However, a Kappa (agreement test) between results from the Massey University laboratory (Ayanegui-Alcérreca *et al.* 2010 in press) compared with those from the reference laboratory used in this study was 0.81 for Hardjobovis and 1.0 for Pomona at the herd level demonstrating excellent agreement.

A high degree of cross reaction between serovar Hardjobovis, Szwajizak and Medanensis was observed. This is consistent with previous reports in cattle (Black *et al.* 2001) and possums (Eymann *et al.* 2007). It is generally accepted that the serovar with the highest antibody titre likely represents the infective serovar (O'Keefe *et al.* 2002) but this may not be true in every circumstance (Levett 2001). For other serovars such as Tarassovi, Grippotyphosa, Celledoni, Australis, Zanoni, Robinsoni, Canicola, Kremastos, Bulgarica, Cynopteri, Ballum, Bataviae, Djasiman, Javanica, Panama, Shermani and Topaz, their serology was either negative, or sporadic, with titres of 1:50 and therefore likely to be due to non-specific reactivity.

Serovar Arborea has never been reported in New Zealand. Serovar Arborea was first isolated in Europe in the 1940s and belongs to the serogroup Ballum. It appears to be newly established in Queensland, Australia and is an emerging source of human leptospirosis (Slack *et al.* 2006) with cases in people involved with horticulture and livestock based occupations. Commonly, this serovar is maintained in rodents such as the house mouse (*Mus domesticus*) and black rats (*Rattus rattus*) (Vanasco *et al.* 2000; Slack *et al.* 2006). In addition, one study

in Australia suggested that the brush tail possum (*Trichosurus vulpeca*), which is widespread in New Zealand, can be an accidental host for Arborea (Eymann *et al.* 2007). Importation of goods from other countries and human travel are possible routes means of introduction of Arborea to New Zealand.

In this study Arborea seroprevalence at both herd and individual animal levels was as high as that recorded commonly for serovar Pomona. Initial screening found titres to Arborea in five herds in Southland, three in Manawatu, two in Canterbury, one in Bay of Plenty. The two herds (Farm 4 and 5) with the highest prevalence after follow-up screening, were chosen for kidney and further blood sampling (n=43) as they were farms most likely to be culture positive. However, we failed to isolate any leptospiral organism from those 43 kidney samples and MAT detected only one deer positive to Arborea which cross-reacted with Ballum at a low titre. We therefore considered this as being non-specific for both serovars. Based on MAT sensitivity of 90% and specificity of 99%, and the previous with-in herd prevalence of 15.4%, a result of 8 negative samples on Farm 4 could be expected by chance. It was therefore insufficient to conclude that the Arborea-prevalence was zero in this herd and we tried unsuccessfully to obtain more samples from this farm. By contrast, 34 negative samples from 35 tested on Farm 5 suggest that the probability of Arborea infection in this herd was likely to be absent. It appears that evidence for Arborea infection was either non-specific in the first instance or had cleared from this herd in the interval since it was initially detected.

Failure to isolate Arborea organisms from kidney samples is consistent with previous studies on the relationship between seropositivity and culture in sheep (Dorjee *et al.* 2008). Similarly, it is uncommon for seronegative deer to be kidney culture positive, with culture from only 5% of seropositive deer (Ayanegui-Alcérreca 2006). Theoretically, the probability for failure of growing an isolate from kidneys from 43 seropositive deer is 11% when the true prevalence is 10% and culture sensitivity is 50%. Thus, a sample of 43 was not large enough to provide substantial confidence for the detection of at least one isolate. Since Arborea belongs to the same serogroup as Ballum which is well-established in black rats (*Rattus rattus*) in New Zealand (Midwinter and Fairley 1999), positive serology for Arborea could potentially be confounded by cross reaction. However, evidence of serology from this study suggests Arborea titres were not due to cross-reaction since positive samples showed no or little evidence of cross reaction with Ballum.

In summary, this study provides serological data on a possible exotic leptospiral organism in farmed deer in New Zealand. The serological findings suggest that Arborea infection may have occurred on the two farms studied. It is possible that an accidental exposure to Arborea may have occurred via indirect contact with free-living rodents or wildlife that carried the organism. However, further serological screening and culture is required to be totally confident that this serovar is indeed present or not in livestock, rodents and wildlife in New Zealand.

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Chapter 7

Detection of *Leptospira* spp. in an early foetus but not uterine tissue of non-pregnant farmed deer

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(Raw data of this chapter are in Appendix 7)

Abstract

AIM: To find evidence for uterine localisation and foetal infection of *Leptospira* in New Zealand farmed deer during and shortly after the breeding season using culture and real-time PCR.

METHODS: Between February and July 2008, 120 kidney, 116 blood, 120 uterine samples and 27 foetuses were collected from 120 mixed-age hinds from lines from nine farms at a Deer Slaughter Premise at Fielding. Kidney, uterine and foetal renal/peri-renal samples were subjected to bacterial culture using Ellinghausen-McCullough-Johnson-Harris (EMJH) medium and real-time polymerase chain reaction (PCR) using DNA gyrase subunit B gene primers. Blood samples were tested for antibodies against *Leptospira borgpetersenii* serovar Hardjobovis and *Leptospira interrogans* serovar Pomona using the microscopic agglutination test (MAT).

RESULTS: Seven of 120 kidney samples were positive by culture and five of these, but no others, were positive by real-time PCR. Of 120 uterine samples, none were culture or PCR positive. None of 27 foetus samples were culture positive but one, collected on July 11, was positive by real-time PCR. Thirty four of 116 serum samples (29.3%) were positive to serovar Hardjobovis and 13 (11.2%) to serovar Pomona. The PCR positive foetus was from a dam that was kidney culture negative, but which had a titre of 1:192 for Hardjobovis.

CONCLUSIONS: Attempts to isolate *Leptospira* from the genital tracts and early foetuses of farmed deer were unsuccessful. However, molecular evidence suggested foetal infection in one case. This finding justifies further study of the role of leptospires in the genital tract and foetus as a cause of reproductive loss in farmed deer.

KEY WORDS: *Leptospirosis, farmed deer, uterus, foetus, culture, real-time PCR, New Zealand*

List of abbreviations

BSA = Bovine serum albumin
DNA = Deoxyribonucleic acid
EMJH = Ellinghausen-McCullough-Johnson-Harris medium
MAT = Microscopic agglutination test
PCR = Polymerase chain reaction
RPM = Revolutions per minute
Tm = Melting temperature

7.1 Introduction

Leptospira serovars Hardjobovis and Pomona are well-known pathogens causing reproductive losses in livestock worldwide (Grooms 2006). Pathogenic leptospires are helical-shaped bacteria which penetrate through skin abrasions or mucous membranes, localising and persisting primarily in the proximal renal tubules of kidney but have also been found in the genital tracts of sexually mature cattle (Ellis *et al.* 1986b; Ellis and Thiermann 1986) and pigs (Ellis *et al.* 1985; Ellis *et al.* 1986a). Leptospires localising in renal tubules are intermittently excreted in urine which serves as a source of infection to other animals (Faine *et al.* 1999; Levett 2001). Seven serovars have been isolated from various animal hosts in New Zealand (Hathaway 1981; Midwinter and Fairley 1999), including Hardjobovis, Pomona and Copenhageni from deer (Ayanegui-Alcérreca *et al.* 2007).

Leptospiral infection has been shown to affect fertility in several domestic animal species including cattle (Dhaliwal *et al.* 1996a), pigs (Ramos *et al.* 2006), goats and sheep (Lilenbaum *et al.* 2008). It increases the number of services required for conception and the calving interval in dairy cattle (Dhaliwal *et al.* 1996a). It also causes early embryonic death, abortion, stillbirth and weak newborn in cattle (Smyth *et al.* 1999) and pigs (Kazami *et al.* 2002). There have been attempts to determine the presence of leptospires in the genital tracts of these species to establish an association with reproductive loss. In cattle, leptospires have been isolated from placenta of experimentally infected heifers (Ellis and Michna 1977), oviduct and uterus of non-pregnant cows (Ellis *et al.* 1986b; Ellis and Thiermann 1986) and viable and aborted foetuses (Ellis *et al.* 1982a; Ellis *et al.* 1982b; Langoni *et al.* 1999). In pigs, leptospires have been isolated from the oviduct, uterus and foetus of aborted sows (Ellis *et al.* 1985; Ellis *et al.* 1986a). These data suggests that leptospires have the ability to cross the placenta and invade the foetus of those species.

Several techniques such as immunofluorescence and enzyme-linked immunosorbent assay (ELISA) have been applied to determine the presence of leptospiral antigen or antibody in genital discharges of cattle (Dhaliwal *et al.* 1996b; Dhaliwal *et al.* 1996c). Recently, polymerase chain reaction (PCR) has been used to detect leptospires in vaginal fluids of sheep and goats (Lilenbaum *et al.* 2008) and aborted cattle foetus (Richtzenhain *et al.* 2002). Real-time PCR assay has been developed based on conventional PCR and is rapid and sensitive, requiring no post-PCR manipulations (Slack *et al.* 2007). It has been used for detection of leptospires in pig kidney tissue, aborted foetus (Fearnley *et al.* 2008) and flying fox urine (Cox *et al.* 2005).

Leptospirosis is a well established infection and disease in New Zealand farmed deer (Wilson *et al.* 1998; Ayanegui-Alcérreca *et al.* 2007). A regional prevalence survey of 110 commercial deer herds in New Zealand showed that 81% had evidence of exposure to leptospires: 61% of the herds to *Leptospira borgpetersenii* serovar Hardjobovis and 4% to *Leptospira interrogans* serovar Pomona, and 16% to both serovars (Ayanegui-Alcérreca *et al.* 2010 in press). Thus serovar Hardjobovis is endemic in most herds whereas serovar Pomona

occurs sporadically (Ayanegui-Alcérreca *et al.* 2007). Transmission of the organism and disease commonly occur in deer before one year of age (Wilson and McGhie 1993; Ayanegui-Alcérreca 2006). Infection and clinical disease has been well described in New Zealand farmed deer (Ayanegui-Alcérreca *et al.* 2007) but abortion has not. Abortion has been demonstrated following artificial infection with serovar Pomona in white tailed deer in North America (Trainer *et al.* 1961). While Ayanegui-Alcérreca (2006) described a significant (10%) improvement in weaning rate in naturally infected primiparous hinds following vaccination in a herd with dual serovar Hardjobovis and Pomona infection, and Subharat et al (2008) showed an average of 5% greater weaning rate in this age-group in five other herds with Hardjobovis infection, nothing is known of the localisation of this organism in the genital tract or foetus of farmed deer.

This paper reports a study to explore evidence for vertical transmission by determining the presence of pathogenic *Leptospira* in the uterus and foetus of non-pregnant and early pregnant farmed deer in the North Island of New Zealand using bacterial culture and real-time PCR.

7.2 Materials and methods

7.2.1 Animals

From February to July 2008, 120 mixed age hinds, phenotypically red deer (*Cervus elaphus*), but possibly containing some wapiti (*C.e. canadensis)* genes, from randomly selected lines from nine commercial deer farms with no previous information on leptospiral infection were sampled at the Deer Slaughter Premise (DSP) in Feilding, Manawatu, New Zealand. In total, 116 blood, 120 kidney and 120 uterine samples were collected and 27 foetuses were available from samples collected in July. Based on sample size calculation using FreeCalc software, the number of reproductive tissue samples required for finding at least one carcass with evidence of leptospira in reproductive tissue was estimated to be 115 (95% confidence; 80% power) based on bacterial culture sensitivity of 50% and specificity of 100% and an assumed true infection prevalence of 5%.

7.2.2 Sample collection and processing

7.2.2.1 Blood: Blood samples were collected by free flow into a new 10 ml plain blood tube after animal sticking, immediately after stunning. Blood was held at 4°C before transport back to the laboratory where samples were centrifuged at 3,000 rpm (1,512 x g) for 15 minutes after which serum was aliquoted into new labelled 1.5ml microcentrifuge tubes and stored at -20°C for serology.

7.2.2.2 Kidney: Hind kidney samples were taken at the evisceration and inspection area of the slaughter line, put into labelled sterile plastic bags aseptically and held at 4°C before transport back to the laboratory. Later, the kidney surface was swabbed with 70%

alcohol and randomly aspirated from several sites over the entire kidney into a 5ml sterile syringe using a sterile 16-gauge needle. Half of the extracted kidney tissue (approximately 50mg) was used for bacterial culture and the other half used for DNA extraction for real-time PCR.

7.2.2.3 Uterus and foetus: Whole uteri with or without a foetus were collected at the same time as the kidney. They were placed into labelled sterile plastic bags aseptically and held at 4°C before transport to the laboratory where they were processed aseptically within a biohazard cabinet. The uterus was cut open and the uterine body and horn mucosal epithelium randomly excised using a scalpel. Foetuses, if available, were incised and tissue removed from the area of kidney and visceral organs (Figure 7.1). Approximately 50mg was used for each of bacterial culture and DNA extraction for real-time PCR.

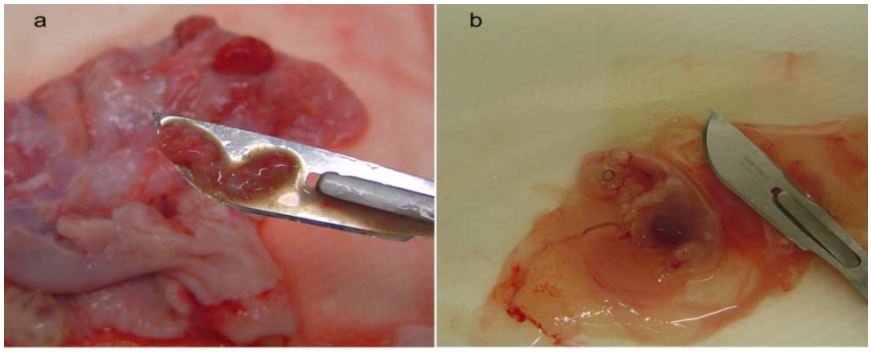

Figure 7.1 Processing of uterus and foetus samples. (a) Scraping of uterine epithelium (b) Deer foetus from pregnant uterus.

7.2.3 Laboratory procedures

7.2.3.1 Serology: The microscopic agglutination test (MAT) was used to test serum reactivity to laboratory standardised *Leptospira* serovars Hardjobovis and Pomona. The method has been developed by the Leptospirosis Research Unit, IVABS, Massey University based on the guidelines for the control of leptospirosis (Faine 1982). For both serovars a titre of ≥1:48 was considered positive (Blackmore *et al.* 1982; Dorjee *et al.* 2008).

7.2.3.2 Bacterial culture and serotyping: Ellinghausen-McCullough-Johnson-Harris (EMJH) was used as a selective medium with an addition of antibiotic (5-Fluouracil) for contamination inhibition. The medium was inoculated with processed kidney, uterus and foetus samples, incubated at 28-30°C and examined every two weeks for four months under dark-field microscopy for. Isolates of leptospires were serotyped against standardised antisera of *Leptospira* serovars Hardjobovis and Pomona. These methods were adapted from the Leptospirosis Laboratory SOP, IVABS, Massey University based on the guidelines for the control of leptospirosis (Faine 1982).

7.2.3.3 DNA extraction and real-time PCR: Leptospiral DNA was extracted from 50mg of processed samples (kidney, uterus and foetus) using the High Pure Template Kit (Roche, Germany) as per manufacturer's instructions. DNA was eluted in a final volume of 200µl. The real-time PCR technique was a modification of the method described by Slack et al (2006). SYTO9 (Invitrogen, Oregon, USA) was used as fluorescent double-stranded DNA specific intercalating dye (Monis *et al.* 2005) for all real-time PCR assays. The assay was performed in a Rotor-Gene 6000 machine (Corbett Research, Mortlake, Australia) using primers 2For 5'-tgagccaagaagaaacaagctaca-3' and 504Rev 5'-matggttccrctttccgaaga-3' (Slack *et al.* 2006). Each 25µl reaction contained 2µl of DNA extracted from samples, 1.5µM SYTO9, 1X PCR buffer, 1.5mM $MgCl_2$, 200µM dNTPs, 12.5pmol of 2For primer, 12.5pmol of 504Rev primer, 0.1%bovine serum albumin (BSA), 1 unit of Taq DNA polymerase and double distilled water. Thermal cycling consisted of initial denaturation at 95°C for 10min followed by 40 cycles of denaturation at 95°C for 10sec, annealing at 60°C for 20sec and extension at 72°C for 20sec. Melting temperature (Tm) of PCR product was determined by melting curve analysis. It was performed by heating the PCR product from 70°C to 90°C and monitoring fluorescence change every 0.1°C. Confirmation of positive samples was determined by melting temperature (Tm) of the PCR product compared with the positive control. The Tm of positive samples was found to be between 83°C and 84°C. The positive control used for the real-time PCR assay was field isolates of *Leptospira* serovar Hardjobovis and the negative control was double distilled water.

7.3 Results

7.3.1 Serology

There was evidence of seroreactivity to *Leptospira* serovar Hardjobovis in 34 of 116 serum samples (29.3%) and for serovar Pomona in 13 serum samples (11.2%). One of nine lines had no animals seropositive to either serovar. Results are summarised in Table 7.1.

Table 7.1 Details of blood samples, the number and percentage seropositive, and the positive reciprocal titre range for each line.

Farm	Sampling date	No. of samples	No. seropositive (%)		Positive reciprocal titre range	
			Hardjobovis	Pomona	Hardjobovis	Pomona
1	11 Feb	13	9 (69.2)	4 (30.8)	48 - 192	96 - 192
2	11 Feb	14	0 (0)	2 (14.3)	-	48 - 768
3	18 Feb	14	2 (14.3)	0 (0)	48	-
4	18 Feb	15	7 (46.7)	2 (13.3)	48 - 96	48 - 96
5	21 Feb	15	0 (0)	0 (0)	-	-
6	11 Jul	8	3 (37.5)	1 (12.5)	48 - 192	48
7	11 Jul	14	4 (28.6)	3 (21.4)	48 - 192	48 - 384
8	17 Jul	10	5 (50.0)	1 (10.0)	48 - 1:192	48
9	17 Jul	13	4 (30.8)	0 (0)	48	-
All		**116**	**34 (29.3)**	**13 (11.2)**	**48 - 192**	**48 - 768**

7.3.2 Bacterial culture

Seven of 120 kidney samples (5.8 %) from three farms were culture positive. All seven isolates were confirmed as serovar Hardjobovis. Five culture positive hinds were seropositive for hardjobovis. None of 120 uteri or 27 foetuses was culture positive. Bacterial culture and PCR results are summarised in Table 7.2.

7.3.3 Real-time PCR

Five of 120 kidney samples (4.2%) were positive by real-time PCR, all of which were culture positive (Table 7.2). None of 120 uteri were positive by real-time PCR. One of 27 foetal samples was positive by real-time PCR (Figure 7.2). While this foetus was from a kidney culture and PCR positive line the dam was culture and PCR negative but seropositive to Hardjobovis (titre 1:192).

Table 7.2 Results of bacterial culture and real-time PCR of kidney, uterus and foetus samples

Line	Sampling date	Kidney		Uterus		Foetus	
		Culture	PCR	Culture	PCR	Culture	PCR
1	11 Feb	4/13	2/13	0/13	0/13	n/a	n/a
2	11 Feb	0/14	0/14	0/14	0/14	n/a	n/a
3	18 Feb	0/14	0/14	0/14	0/14	n/a	n/a
4	18 Feb	2/15	2/15	0/15	0/15	n/a	n/a
5	21 Feb	0/15	0/15	0/15	0/15	n/a	n/a
6	11 Jul	0/12	0/12	0/12	0/12	0/8	0/8
7	11 Jul	1/14	1/14	0/14	0/14	0/11	1/11
8	17 Jul	0/10	0/10	0/10	0/10	0/1	0/1
9	17 Jul	0/13	0/13	0/13	0/13	0/7	0/7
All		**7/120**	**5/120**	**0/120**	**0/120**	**0/27**	**1/27**

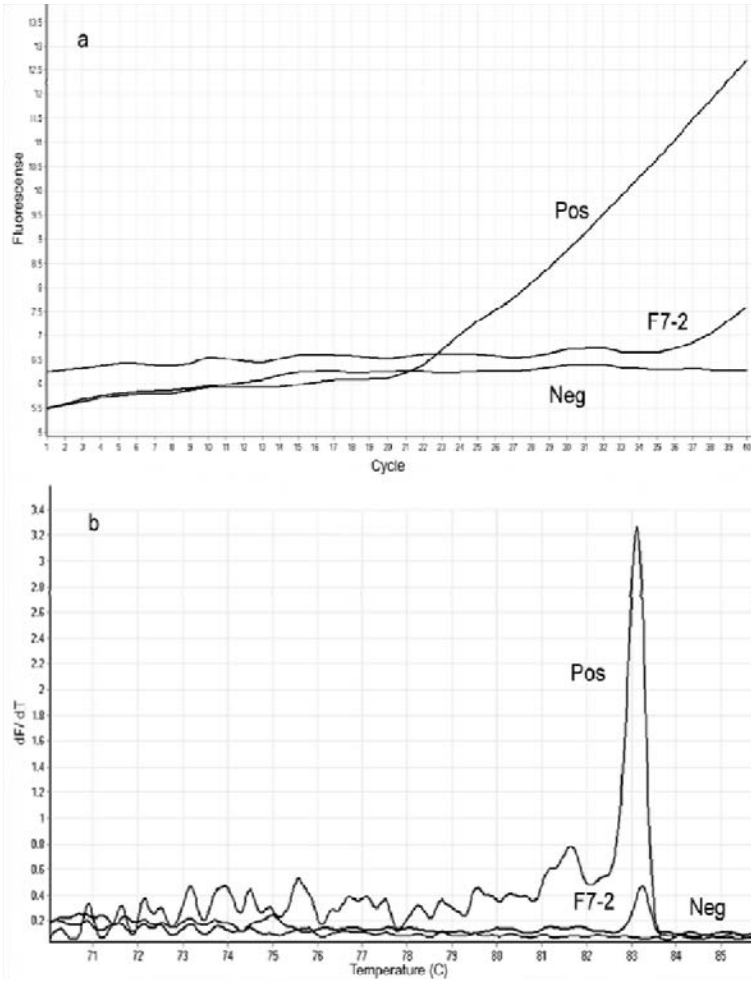

Figure 7.2 Real-time PCR results for foetus: (a) cycling of positive control (Pos), positive foetus (no.2 from line 7: F7-2) and negative control (Neg); (b) Melt curve of positive control (Pos), the positive foetus (F7-2) and negative control (Neg).

7.4 Discussion

Leptospirosis is well-recognised and prevalent in farmed deer in New Zealand with a recent survey (Ayanegui-Alcérreca *et al.* 2010 in press) estimating that 82% of herds were infected. Two studies have shown reduction of up to 10% in weaning percentage from hinds, associated with leptospirosis (Ayanegui-Alcérreca 2006; Subharat *et al.* 2008). Localisation of leptospires in the uterus may lead to foetal infection, with subsequent reproductive losses. The presence of leptospires in the genital tract has not been investigated. This is the first reported attempt to identify leptospires in uteri or foetuses of farmed deer. The study was designed to determine whether leptospiral infection could be detected in the uterus of non-pregnant deer in the late summer and in uterus and foetus (as available) from deer in the late autumn and early winter period corresponding to approximately the first three months of gestation.

Real-time PCR provided evidence of infection of one foetus, which was approximately three months of age, being sampled July 11. This tentatively indicates that leptospires may pass from dam to foetus in deer early in pregnancy. Uterine infection has been demonstrated in cattle from day 121 of gestation to term (Ellis *et al.* 1982a). Foetal infection with leptospires may contribute to reproductive wastage in deer as in other livestock species. While this observation is based on PCR evidence but not culture, validation of the PCR for leptospires in deer tissue used here has been shown it to be 100% specific (Subharat 2010 in press). Moreover, this foetal PCR evidence is consistent with positive MAT results of its dam with a titre of 1:192. Therefore, foetal infection is likely to be a valid inference.

The lines of hinds sampled for this study were randomly selected with no previous information of leptospiral infection. The chance of detecting leptospires could have been enhanced by selecting the lines from farms with a history of leptospiral infection or abortion as described in previous studies in cattle (Ellis *et al.* 1982b; Langoni *et al.* 1999) and pigs (Ellis *et al.* 1985; Ellis *et al.* 1986a) or the lines with kidney lesions. However, this is difficult logistically, and costly. Further, if only yearlings were sampled, the possibility of detecting infection may be higher because it is the age group that is recently infected (Ayanegui-Alcérreca 2006) whereas the older deer are more likely to be immune and less likely to be shedding or harbouring the organism. An association between reproductive wastage and leptospirosis was observed in both adults and the younger age group (Ayanegui-Alcérreca 2006; Subharat *et al.* 2008). No studies of reproductive performance related to leptospirosis have been conducted in older deer.

The animal level seroprevalence of 29.3% to *Leptospira* serovar Hardjobovis and 11.2% to serovar Pomona in this study was consistent with a recent report (Subharat *et al.* 2007) on deer in the same catchment area, but was lower than 45.0% and 12.2% reported previously from this area at the same DSP despite using a higher titre cut-point at 1:96 (Wilson *et al.* 1998). As transmission of leptospires is favoured by warm and humid conditions, the low prevalence observed might be associated with climatic effects since the summer and autumn preceding sample collection experienced particularly low rainfall. Ayanegui-Alcérreca (2006) reported differences in seroprevalence associated with high and low rainfall summer and autumn seasons. This study therefore likely suffered from a lower than optimum number of infected deer. The predominance of serovar Hardjobovis was expected for this population based on earlier reports (Wilson *et al.* 1998; Ayanegui-Alcérreca 2006) confirming that the serological status for this study was representative of leptospiral infection in deer herds in the region.

Bacterial culture and real-time PCR from kidney samples was done concurrently with uterine and foetal samples to provide complementary evidence of the infection status of the dam, which is not available from serology alone. The kidney is the most immune privileged site for this organism (Athanazio *et al.* 2008). In this study, culture and/or real-time PCR failed to detect the organism from any uterus, or all but one foetus, despite some of those animals being kidney culture and/or real-time PCR positive. The animal from which the PCR positive

foetus was derived was seropositive for serovar Hardjobovis (titre 1:192) but was negative for kidney culture and real-time PCR. The reason the foetal tissue was PCR but not culture positive may be that culture has specific limitations, including fastidious growth of the organism and contamination (Faine *et al.* 1999) and that PCR does not depend on the microbiological viability of the organism.

Failure of culture and real-time PCR to detect leptospires in uterine tissue may be because the concentration of leptospires scraped from uterine epithelium was below the detection limit of both techniques, or alternatively, the uterus of deer is rarely, if ever, infected by leptospires. Although, there have been some studies demonstrating isolation of leptospires from the uterus in non-pregnant cattle (Ellis *et al.* 1986a; Ellis *et al.* 1986b) this may not be the case in farmed deer. Different leptospiral strains are reported to behave differently. Within serovar Hardjobovis, some strains have been shown to persist primarily in kidney whereas other strains have a predilection for the genital tract and yet others persist in both organs (Ellis 1994). The seropositive adult deer in this study may have been exposed some time previously, developed immunity and subsequently cleared the infection. It is also possible that infection of the uterus only occurs in the acute or early phase of infection during the period of haematogenous spread, but none of the studied animals were in this phase of the infection process at the time of sampling.

The PCR evidence of leptospires from the deer foetus in this study suggests foetal infection. Foetal infection is claimed to cause stillbirth, abortion and weak offspring in cattle. With serovar Pomona infections, abortion usually occurs in the last three months of gestation whereas for serovar Hardjobovis infections, abortion has been observed from early gestation through to term (Ellis 1994). Ellis et al (1982a) has demonstrated lower prevalence of leptospires isolated from normal foetuses when compared with aborted foetuses from a previous study (Ellis *et al.* 1982b). The difference in prevalence of leptospires isolated from normal and aborted foetuses suggest that leptospires, particularly serovar Hardjo, play an important role in bovine abortion (Ellis *et al.* 1982a). A more recent study has supported that theory by isolating Hardjo from aborted cattle foetuses (Langoni *et al.* 1999).

Real-time PCR assay may be a useful tool for detection of pathogenic leptospires in deer foetuses. The DNA gyrase subunit B gene primers used in this study are claimed to be genus-specific and detects only pathogenic *Leptospira* (Slack *et al.* 2006). It offers several advantages over the standard bacterial culture including being less time-consuming, inexpensive and detects leptospiral DNA from lysed or inactive organism. However, real-time PCR does not differentiate the serovar of causative leptospires which limits is use as a sole test for epidemiological study of infection (Lilenbaum *et al.* 2008) or diagnosis of serovar. However, this study has shown a 100% correlation between MAT and cultured serovar from the same animals (5 of 7) suggesting that PCR should also correspond to MAT serovar. Previous observations support this relationship (Ayanegui-Alcérreca 2006). This interpretation, however, needs to take account of the stage of infection when the immunity of the host has not been activated and in the event of dual or multiple serovar infections.

Techniques such as DNA sequencing (Slack *et al.* 2006) and restriction fragment length polymorphism (Heinemann *et al.* 2000) of the PCR product are under investigation to overcome this problem.

In conclusion, this study failed to isolate or show the molecular evidence of pathogenic leptospires from the uterus of deer in the early autumn and the period of early gestation. Nevertheless, we have demonstrated evidence of pathogenic leptospires in a deer foetus using a real-time PCR assay. Thus early infection of the foetus may play a role in adverse effects of leptospirosis on deer reproductive performance demonstrated earlier (Ayanegui-Alcérreca 2006; Subharat *et al.* 2008). This observation, while based on sound experimental design, is tentative, due to the small number of animals involved. However, it indicates that further research in both early and late pregnancy is warranted to fully investigate and confirm the presence of this organism and its impact on reduced reproductive performance in farmed deer.

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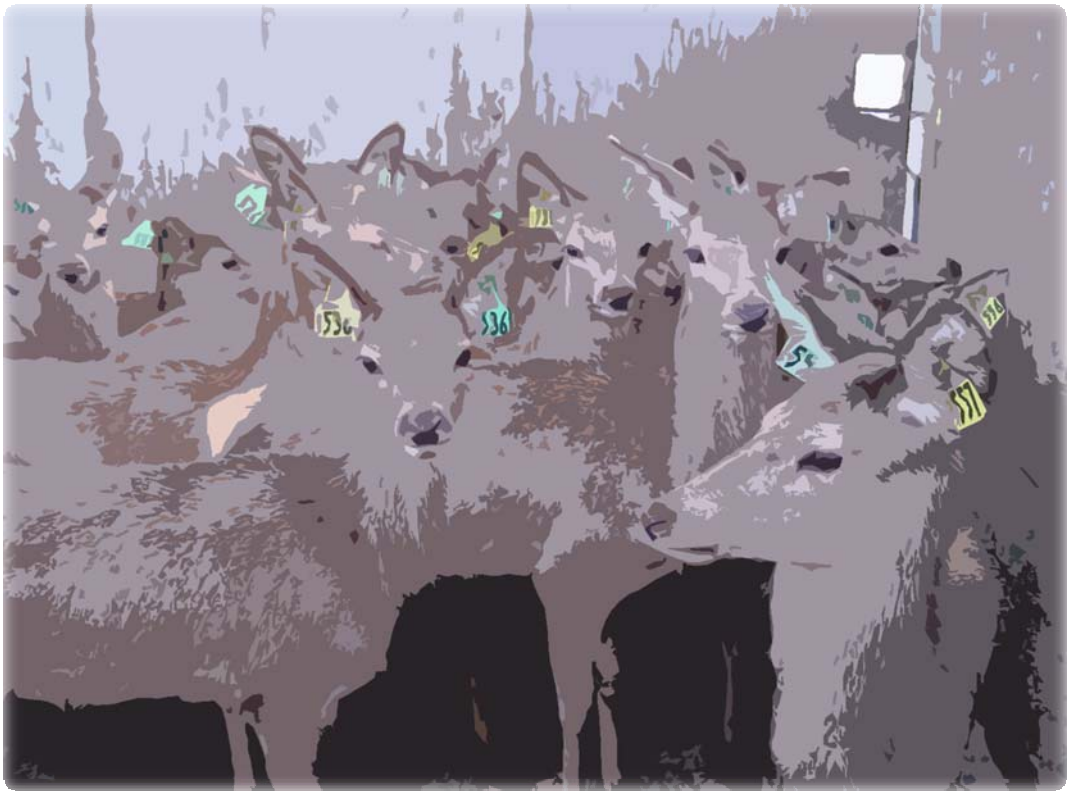
Chapter 8

General Discussion



8.1 Introduction

The studies presented in this thesis were designed to further investigate the epidemiology of leptospirosis in New Zealand farmed deer based on current knowledge (Ayanegui-Alcérreca 2006), to develop and validate a novel molecular technique for diagnostic purposes and to enhance understanding of control measures for leptospirosis by means of vaccination. The study employed serology, bacteriology and a newly developed real-time PCR to determine infection and vaccine efficacy under natural challenge conditions. It concentrated largely on *Leptospira* serovars Hardjobovis and Pomona since they are the two most common serovars reported in farmed deer in New Zealand (Ayanegui-Alcérreca *et al.* 2007).

This chapter discusses the overall research findings in this thesis summarised broadly as epidemiology, diagnosis and vaccination control. This chapter also critiques experimental design and methodology, and addresses limitations and suggested areas for future research. It also proposes action to control leptospirosis for the deer industry.

8.2 Epidemiology of leptospirosis

8.2.1 Leptospirosis on mixed-species farm

Recent data from a regional seroprevalence survey (Ayanegui-Alcérreca *et al.* 2010 in press) showed evidence that 81% of farmed deer herds in New Zealand were infected with *Leptospira* and no differences were found between regions. It was proposed that farmed deer in New Zealand are maintenance hosts for serovar Hardjobovis and accidental hosts for serovar Pomona and that deer may play an important role in the infection cycle of leptospirosis on multi-species livestock farms (Ayanegui-Alcérreca 2006). Livestock farming in New Zealand is becoming increasingly multi-species with approximately 85% of farms with deer also farming beef cattle and/or sheep on the same property (Hilson 2007; Wilson 2007). Despite several studies having been conducted to gain information on the epidemiology of leptospirosis separately in deer (Wilson *et al.* 1998; Ayanegui-Alcérreca 2006), cattle (Bahaman *et al.* 1984; Matthews *et al.* 1999) and sheep (Dorjee *et al.* 2005), epidemiological links between these species on farms are unclear. It is important to understand the role played by each potential host on mixed-species farms in the epidemiology of the disease to determine choice and implementation of appropriate control measures aimed at reducing the incidence of clinical and sub-clinical disease, and reduction of risk to humans.

Chapter 4 describes a pilot longitudinal seroprevalence study of leptospirosis on 19 deer farms with sheep and/or beef cattle in the lower North Island of New Zealand. This study had a dual purpose, with herd screening being for both identification of farms for vaccine studies of growth and reproduction (Chapters 2 and 3), and this

multi-species study *per se*. It is the first mixed-species epidemiological study carried out on-farm in New Zealand attempting to identify potential risk factors influencing *Leptospira* serological status of the deer herd.

Results from this study suggested that leptospirosis was prevalent on mixed-species farms in the lower North Island. The predominant serovar was Hardjobovis whereas Pomona was less common. Seroprevalence was consistent with that reported in single-species deer (Wilson *et al.* 1998; Ayanegui-Alcérreca *et al.* 2003), cattle (Bahaman *et al.* 1984; Matthews *et al.* 1999) and sheep studies (Blackmore *et al.* 1982; Dorjee *et al.* 2005).

Co-grazing with infected sheep and/or cattle was significantly positively associated with deer herd status to both serovar Hardjobovis and Pomona. This finding suggests inter-species transmission. Nevertheless, appropriate molecular typing of leptospiral organisms from each species on a farm is required to determine whether there are livestock-host adapted strains or not, and, to test the theory of cross-species infection. Multilocus Sequence Typing (MLST) is the candidate technique and is currently being validated within the Leptospirosis Research Unit, IVABS, Massey University (Subharat *et al.* 2009).

Another important finding from this study was the 64.1% flock prevalence of *Leptospira* serovar Hardjobobis in sheep. Blackmore *et al.* (1982) randomly sampled carcasses of lambs and proposed that sheep were not a maintenance host for Hardjobovis but the seroprevalence of Hardjobovis from this and other studies (Cousins *et al.* 1989; Gerritsen *et al.* 1994b) suggest that sheep may indeed act as a maintenance host. Lamb carcasses may not be a suitable source population as it tends to underestimate population prevalence. Conventionally, only cattle and deer in New Zealand are regarded as maintenance host for this serovar. Thus, if leptospiral infection can be controlled efficiently in those species, for example by vaccination, and sheep do not maintain the organism, theoretically, leptospiral infection could be indirectly eliminated from sheep. This would be attractive for sheep farmers since vaccination of this species would be more costly against returns at the individual animal level than for cattle and deer. This proposition needs further investigation since sheep comprise the largest population of livestock in New Zealand and it could prove significant for effective control strategies for leptospirosis on mixed-species farms.

Proof that sheep is a maintenance host for Hardjobovis requires evidence of long-term leptospiruria by urine culture or PCR and molecular typing to see whether they carry species-specific *Leptospira* organisms that are different from cattle and farmed deer with no evidence of continuous challenge from other species. One possible way to test whether sheep is the maintenance host for Hardjobovis could be to compare the flock prevalence of this serovar between sheep-only farms and sheep on mixed-species

farms. If the flock prevalence of Hardjobovis from sheep-only farms was lower than that of the sheep on multi-species farm, this may suggest that sheep are spill over host and may not maintain Hardjobovis. However, control for confounding factors for such study must be appropriate. Another possible way to test this could be to vaccinate deer and cattle on farms that have sheep on the property and monitor the seroprevalence of *Leptospira* in sheep for a long period. If the seroprevalence in sheep declined over time, it would suggest that sheep were not a maintenance host and deer and or cattle were necessary for maintenance in sheep.

The study in Chapter 4 provides a basis for future studies on mixed-species farms. Due to the limited number of farms and incomplete farm categories (no cattle-only, sheep-only and cattle and sheep farms), data from this study were not suitable for multivariable analysis. To permit such analyses, a study should be larger, involve all regions of New Zealand and include all categories of farms.

Sera stored in a serum bank from this study could contribute to a future analysis for possible novel serovars of *Leptospira* in cattle and sheep although it will only represent the herds/flocks from the lower North Island of New Zealand.

8.2.2 Possible novel serovar of *Leptospira* in NZ

The availability of the serum bank from a previous regional seroprevalence study (Ayanegui-Alcérreca *et al.* 2010 in press) provided a unique opportunity to further investigate possible novel serovars of *Leptospira* in farmed deer (Chapter 6). A collaborative study between Massey University Deer Research Group (MUDRG) and the World Health Organisation/Food and Agriculture Organisation/World organisation for animal health (WHO/FAO/OIE) reference laboratory for leptospirosis in Brisbane was initiated in 2006 to investigate exotic serovars, and allow inter-laboratory comparisons for serology for Hardjobovis and Pomona. To our knowledge, this study was the first in 30 years to investigate for possible novel leptospiral serovar against full reference panels in any animal species in New Zealand. Serology against 23 reference serovars confirmed the endemicity of Hardjobovis and sporadicity of Pomona. It also revealed seropositivity to Arborea which has never been found in New Zealand in 11/70 (15.7%) deer herds tested. This finding was reported to MAF-Biosecurity as a possible new organism according to requirements, although no follow up was undertaken.

The pattern of serological evidence suggested that Arborea titres were not due to cross-reaction since positive samples showed no or little evidence of being positive for Ballum, which is from the same serogroup and is found in New Zealand. However, attempts to isolate the organism from kidney samples of farmed deer from two seropositive farms in 2007 and 2008 were unsuccessful. Since follow-up was opportunistic, attempts to investigate for Arborea were limited by time and financial

resources. Thus, results cannot exclude the presence of this serovar from New Zealand. Further serological screening and culture are required to give greater confidence that this serovar is indeed present or not in deer, other livestock, rodents, wildlife or humans in New Zealand. This could be focused initially in the Southland region where the present serological evidence of Arborea was clustered. The sample size for deer could be estimated based on prevalence data from this study. However, such study will require significant resources.

8.3 Diagnosis

Highly sensitive and specific diagnostic tests are desirable for epidemiological study of infections such as leptospirosis. Chapter 5 describes development and validation of a molecular diagnostic tool for detection of leptospiral organisms in deer clinical samples. The rationale for developing this technique was to address shortcomings of conventional methods in detection of leptospires in clinical samples, such as dark-field microscopy and Warthin-Starry staining, neither of which are sufficiently sensitive (O'Keefe 2002). Furthermore, culture, which is usually used as the gold standard, is tedious, complex, time-consuming and prone to contamination. Real-time Polymerase Chain Reaction assay was chosen to overcome many of those shortcomings.

This study evaluated and validated the performance of a real-time PCR assay for use on deer kidney tissue and urine as a research and diagnostic tool for determining infection, carrier and shedding status of deer. It was the first study to use real-time PCR directly on deer kidney and urine samples with evaluation against culture as the gold standard. Furthermore, it was also the first study to quantify pathogenic leptospires shed naturally in deer urine. This technique is also able to identify specific *Leptospira* species by DNA sequencing of PCR amplicons. This research confirmed that the real-time PCR assay was a useful tool for rapid, cost-effective, sensitive and specific detection of pathogenic leptospires in clinical samples, particularly urine.

Urine samples for validation of this real-time PCR assay were from the vaccination validation study (Chapters 2 and 3) whereas kidney samples were from randomly selected lines at deer slaughter premises. Results have shown that the real-time PCR was highly sensitive and specific compared with culture. A limitation of this study was that urine samples were highly contaminated, thus it was not possible to robustly evaluate clinical sensitivity of real-time PCR on urine samples against culture. However, if deer urine samples could be collected more aseptically, for example, from urinary bladder puncture at slaughter, it would likely reduce the contamination rate in culture and make it possible to fully evaluate clinical sensitivity of urine real-time PCR.

The technique can be applied for future epidemiological studies involving *Leptospira* shedding in other species and possibly even environmental contamination when studying potential risk factors for disease transmission. It may also be applied for early diagnosis of human leptospirosis by detection of leptospiral DNA in blood during the leptospiremia phase as reported in other studies (Levett *et al.* 2005; Merien *et al.* 2005; Fonseca Cde *et al.* 2006).

The ability of real-time PCR to quantify the concentration of leptospires in clinical samples provides diagnostic benefits, for example in evaluating the concentration of the organisms shed in urine during different stages of infection, or even antibiotic efficiency evaluated by post-treatment clearance of leptospires in urine. Merien *et al.* (2005) used this assay to measure the concentration of leptopiral DNA in patient's sera, informing the prognosis for those suffering from leptospirosis.

This real-time PCR, in combination with DNA sequencing, provides a rapid tool for identifying pathogenic *Leptospira* species and the likely serovar from clinical samples. Since the two most common serovars found in New Zealand (Hardjobovis and Pomona) belong to different species, it is possible to conjecture the infection serovar by this technique. Nevertheless, this would be appropriate only in regions where few species and serovars of pathogenic leptospires were encountered. For more accurate genotypic classification where multiple serovars may be present, Multilocus Sequence Typing (MLST) is the most up-to-date method that has been developed for typing and is claimed to have high discriminatory power to strain level, reproducibility and robustness (Ahmed *et al.* 2006; Thaipadungpanit *et al.* 2007). This technique is currently being developed and validated at the Leptospirosis Research Unit, IVABS, Massey University (Platero 2009; Subharat *et al.* 2009). *Leptospira* isolates from this study including all DNA extracted samples from both urine and kidney have been stored as reference material for future development and validation of new techniques.

Research in Chapter 6 provided an opportunity to compare the results of Microscopic Agglutination Tests (MAT) for Hardjobovis and Pomona between the Leptospirosis Research Unit, IVABS, Massey University and the WHO/FAO/OIE reference laboratory for leptospirosis in Brisbane. The Kappa (K) agreement test has shown excellent agreement of MAT between the two laboratories for both serovars Hardjobovis (K=0.81) and Pomona (K=1.0) at the herd level and moderate agreement for Hardjobovis (K=0.41) and substantial agreement for Pomona (K=0.68) at individual animal level. Variation between laboratories at the individual animal level was expected due to the subjectivity of test interpretation, as is widely accepted and reported elsewhere (Faine 1982; Levett 2001). Because of the subjectivity of interpretation of a MAT titre, only one person should read positive MAT results for all samples of a given study to ensure consistency and validity of results. If this is not possible, appropriate training is required in combination with blind testing against

known results to ensure the greatest consistency between personnel. Serum samples from this study were stored in the deer serum bank and are currently available for such training.

8.4 Vaccination control of leptospirosis

Control for leptospirosis requires a range of strategies. The key is to limit direct and indirect transmission of the organisms between susceptible host, carrier and contaminated environment (Heath and Johnson 1994). Vaccination is likely to be effective and practical in reduction of transmission of *Leptospira* to both animal and human cases as has been shown earlier in cattle and pigs (Marshall and Manktelow 2002). It is recognised as a tool aimed at reducing the carrier state as a means of limiting the infection cycle. Little *et al.* (1992) claimed that vaccination is the best option in controlling for leptospirosis under pastoral systems.

The purpose of research in Chapters 2 and 3 was to determine the effect of a commercial bivalent leptospiral vaccine (Leptavoid-2, Intervet/Schering-Plough Animal Health Limited, NZ) on leptospiral shedding, growth and reproduction of farmed deer under New Zealand pastoral conditions involving natural challenge. Generally, the conventional method for evaluation of vaccine efficacy is to vaccinate animals and expose them to artificial challenge in a controlled environment (Bolin *et al.* 1989). However, this may not reflect the natural challenge of *Leptospira* as the infective dose and route of administration may be different from artificial challenge. Further, challenge models for testing vaccine efficacy for infectious disease agents that can cause potentially serious clinical disease in experimental subjects present animal welfare concerns. Alternatives to challenge models for vaccine efficacy studies, such as serological equivalence and field studies are becoming accepted.

This study attempted to investigate the efficacy of vaccine under natural challenge to address those issues. However, in a natural challenge situation, it is likely that some animals may be infected prior to vaccination, confounding the apparent effect of the vaccine. The study was therefore designed to simulate an infection-free herd scenario, as much as possible, created by the use of streptomycin to attempt to eliminate the subclinical kidney infection and shedding state, prior to vaccination. This was followed by exposure to natural challenge. Streptomycin treated deer initially showed no evidence of increasing titres or *Leptospira* shedding. This changed once they were joined with no-streptomycin treated control animal; their subsequent increase in titres and shedding demonstrating that the study model was effective in terms of natural challenge with *Leptospira* and mimicked the effect of vaccine in previously uninfected animals. It is proposed that this design better evaluated the effect of a vaccine on growth and shedding outcomes than vaccination in the face of endemic infection. The latter model would likely under-estimate the true effect of the vaccine in a situation of low or zero challenge, which is what would be expected over time in

a herd with a prolonged vaccination programme. The detection of leptospiruria in this trial was based on real-time PCR since high contamination rates in urine culture from earlier studies thwarted vaccine efficacy evaluation in terms of urine shedding (Ayanegui-Alcérreca *et al.* 2010 in press).

The major limitation of this study was the severe drought season which resulted in the deer on studied farms being underfed. Moreover, dry conditions likely reduced organism survival and transmission, contributing to a low natural challenge that resulted in the low observed seroprevalence in most herds. Further, one farm had to drop out from the study because the farmer sold most of their stock due to insufficient feed. Severe drought also delayed the time taken for deer to reach slaughter weight. Thus, the relevance of kidney samples to observe carrier status and kidney lesions at slaughter was reduced. The collection of kidney samples from those deer in the following year was not an option since it would have been beyond the study period and the possible expected efficacy of the vaccine as shown in other species (Hancock *et al.* 1984). In addition, animals in this study only experienced Hardjobovis infection unlike a previous study by (Ayanegui-Alcérreca 2006) that reported some dual infections of both Hardjobovis and Pomona. Evidence suggests that serovar Hardjobovis is rarely if ever associated with clinical disease (Wilson and McGhee, 1993), thus it is assumed that Pomona is more pathogenic in deer as it appears to be in sheep and cattle. Thus, if this study had Pomona infection, the effect observed for both growth and reproduction response could have been greater. Nevertheless, importantly, the presence of only one serovar allowed this study to confirm for the first time that serovar Hardjobovis alone appeared to be capable of causing sub-clinical production loss, both growth and reproduction, on deer farms.

8.4.1 Growth response

Not much was known of the effect of leptospiral infection on growth of farmed deer prior to this study. Preliminary studies on individual animal data in one herd, showed that yearling deer with evidence of infection during the previous 8-month growth period were 3.7 kg lighter than those without the evidence of infection (Ayanegui-Alcérreca 2006). However, that observation needed replication before robust claims of the effect of leptospirosis on growth could be made. The present study was designed to evaluate growth response due to vaccination at the herd level. A significant growth response was confirmed. This was despite the drought conditions likely predisposing to lower prevalence and poor feed quality and quantity, thus it may be that the magnitude of the difference may have been greater if optimum feeding was available, and had the climate been less extreme.

Data from this study suggested that the magnitude of the vaccination response at the herd level, and hence the economics of vaccination, will depend on the prevalence of infection, and serovar. No response would be expected in uninfected herds but

significant responses are likely in high prevalence situations. Data from Farm 1 in Chapter 2 which experienced a 87% seroprevalence of Hardjobovis and confirmed infection using urine PCR, showed a difference of average daily gain (ADG) at 27 g/day, which resulted in a mean liveweight difference of 6.5 kg from March to November, after a growth period of 241 days. This was a result of sufficient magnitude to be statistically significant on that farm alone. Based on this data, the marginal return on investment would be $29 per deer (assuming $8.0/kg carcass weight, 56% dressing of liveweight), for an investment of $3.20 ($2.60 for vaccine and $0.60 for labour and sundry costs), a 9.1 times return on vaccination cost (Wilson *et al.* 2009). Chapter 2 presents data on seroprevalence cut-points for a cost-effective response to vaccination.

Analysing growth response due to vaccination at the herd level, rather than at individual animal level as reported by Ayanegui-Alcérreca (2006), may have underestimated the true effect at animal level because some animals in the control group had no serological evidence of being infected. However, data from this study did not withstand such analysis since the prevalence on each of infected farms (Farm 1, 2 and 3 in Chapter 2) were either too high or low. Thus, the number of animals in the control group representing infected vs. non-infected was too small to draw any conclusion. Furthermore, to combine those data from each infected farm together was not appropriate since it would be confounded by factors such as management, feeding and genetics. The strong causal animal-level argument is that these deer were allocated to vaccine and control groups at random and kept under identical conditions on each farm.

The greatest effect of vaccine on growth in Chapter 2 was on Farm 1 with the highest liveweight observed in November (Table 8.1). It suggests that other than herd seroprevalence and management and climatic factors, the magnitude of vaccination response may also be influenced by the genetic growth potential on individual farms because deer with higher growth potential may lose more growth than deer with a low growth potential. However, this proposition needs to be tested to fully understand the magnitude of the effect of leptospirosis on growth in a range of circumstances.

The mechanism for leptospirosis causing reduction in growth is unknown. It is possible that localisation of leptospires in the kidney caused alteration of kidney function, pain that influenced food intake, or continued immune challenge on the cost of energy in feed. It is common to observe kidney lesions in deer associated with leptospiral infection, with the worst-affected lines generally having the highest seroprevalence and titres (Wilson et al 1998). Thus, metabolic function could have been affected particularly during the establishment phase of infection, resulting in reduction of growth rate and food conversion efficiency. It was notable that the greatest reduction in ADG occurred in the period corresponding with the period of greatest seroconversion providing further evidence of a causal relationship. Activation

of the immune system during infection and stress may change the priority of partitioning nutrients from growth to host defence (Colditz 2002).

Table 8.1 Mean liveweight (kg) (and range) of control and vaccinated male deer on each farm in November and the difference (from Chapter 2).

Farm	Control	Vaccinate	Difference (Vaccinate – control)
1*	89.7 (76.0 – 101.0)	99.1 (89.0 – 107.0)	9.4
2*	70.8 (48.0 – 86.5)	72.6 (58.0 -86.5)	1.8
3*	71.0 (48.4 – 87.6)	77.0 (65.6 – 91.2)	6.0
4	82.1 (57.5 – 96.5)	80.8 (61.5 – 91.0)	-1.3
5	81.7 (66.5 – 104.0)	78.7 (58.5 – 97.5)	-3.0

* Farms with evidence of leptospiral infection in male deer

8.4.2 Shedding response

In order to control leptospirosis, consideration goes beyond the obvious need to prevent clinical illness and economic loss in deer. There is also a need to minimise the risk of human infection by controlling exposure from them. It is clear from reported statistics and farmer and veterinary anecdotes that there is a risk of transmission from deer to humans with an apparently relatively higher risk to those at Deer Slaughter Premises (Bell 2005; Brown 2005). A recent cross-sectional survey of 1,895 farmers in 2008-09 has shown that human leptospirosis incidence was 8-fold higher in farmers reporting that their deer were affected by leptospirosis than farmers where leptospirosis was not observed in deer (Verdugo and Heuer 2009). This confirms the specific public health implication of leptospirosis in deer. Furthermore, there is also risk of transmission to other in contact animals of the same or different species. Transmission of *Leptospira* is usually via urine containing organisms. The study in Chapter 2 has investigated whether leptospiral vaccination prevented urinary shedding if the animal received the vaccine, therefore developing immunity before exposure to the organisms.

That study has shown 100% reduction of shedding, based on urine PCR. This was in deer given streptomycin to eliminate the carrier state and vaccinated, followed by a period of reduced risk of exposure to infection while immune responses developed, followed by natural challenge. That there were no vaccinated deer shedding leptospires in urine provides strong evidence that vaccine is behaving in a similar manner to that reported in cattle and pigs (Marshall *et al.* 1982; Hodges *et al.* 1985). This complements a previous report using the same type of vaccine (Leptavoid-3, Intervet/Schering-Plough Animal Health Limited, NZ) which was shown to reduce

the incidence of urine shedding, based on dark field microscopy, by 44% in young deer herds already infected and continuously exposed to infection (Ayanegui-Alcérreca 2006). Hancock *et al.* (1984) suggested it is unlikely that vaccination will prevent shedding in all animals already infected at the time of vaccination. The present study demonstrated that vaccine did prevent leptospiral shedding when immuno-naïve deer received vaccine prior to infection. On a whole farm basis, elimination of shedding is likely to be possible over a prolonged period of whole herd vaccination as persistently infected animals are replaced by vaccinated un-infected animals (Little *et al.* 1992). To date, there are no data for the efficacy of streptomycin in eliminating *Leptospira* shedding in farmed deer. However, there is evidence that naturally infected cows stop shedding leptospires after a single treatment with dihydrostreptomycin at the same dose used in this study (Gerritsen *et al.* 1994a). Given the similarity of infection in farmed deer it is likely that streptomycin has the same effect in this species.

Since human leptospirosis incidence in New Zealand is among the highest in developed countries and is largely associated with livestock including farmed deer (Baker and Lopez 2004; Bell 2005), results from this study suggest that leptospiral vaccination in farmed deer should reduce this risk to humans. This could potentially reduce the cost for potential medical expenses or the need to employ replacement labour during illness and recuperation for the farmer and meat worker due to leptospirosis. It could also reduce the hidden national cost of this disease since almost 50% of leptospirosis patients require hospitalisation (Wilson *et al.* 2009).

8.4.3 Reproduction response

It is well known that *Leptospira* serovars Hardjobovis and Pomona are pathogens causing reproductive losses in livestock worldwide (Grooms 2006). Reports about Pomona suggest that it can cause more severe clinical effects such as abortion storms (Knott and Dadswell 1970; Gilmour 2007), but the sporadic occurrence renders Pomona less economically important than Hardjobovis (Givens 2006). This may also apply to farmed deer. Chronic leptospirosis causes impaired fertility, neonatal death, abortions and decreased milk production (Lilenbaum *et al.* 2008). Previous study in adult hinds reported a nine percentage point higher weaning rate (10.2% improvement) in vaccinated compared with control hinds in a herd with dual Hardjobovis and Pomona infection (Ayanegui-Alcérreca 2006). The present study has complemented and is consistent with that study, replicating that vaccination of deer against leptospirosis can improve the weaning rate on farms with evidence of leptospiral infection. This study found an overall significant 5.7 percentage point increase in weaning rates (6.9% improvement) due to vaccination. However, it was not appropriate to estimate the odds ratio of seropositive vs. seronegative hinds rearing calf in the control group due to the low level of challenge. Therefore, better evidence was provided by comparing weaning rates of vaccinated and control deer

allocated at random and kept under identical conditions. Nevertheless, this should be subjected to further study with a larger sample size, and target the farms with a high level of challenge, although the latter is difficult to achieve reliably because it is difficult to predict seroprevalence because of large seasonal differences.

Based on weaner deer value in March at $225/animal, the average gain of 6.9% reported in Chapter 3 would result in a return on vaccination of $15.5 per hind, and the highest response of 10.7% would result in a return of $24.1 per hind. The break-even point for economic return on vaccination in terms of reproduction is an improvement in weaning rate of 1.4%. Wilson *et al.* (2009) suggested that more than 50% of farmers might expect an economic return for vaccination of yearling hinds based on surveys of seroprevalence in deer herds, and this financial value of progeny.

The pilot study in Chapter 7 which investigated the presence of pathogenic *Leptospira* in the uterus and foetus of non-pregnant and early pregnant farmed deer could establish an association with reproductive loss. Leptospires have been isolated from several reproductive organs in cattle such as placenta (Ellis and Michna 1977), oviduct and uterus of (Ellis *et al.* 1986; Ellis and Thiermann 1986) and viable and aborted foetuses (Ellis *et al.* 1982a; Ellis *et al.* 1982b; Langoni *et al.* 1999). However, nothing is known of the localisation of this organism in the genital tract or foetus of farmed deer. This study has demonstrated evidence of pathogenic leptospires in a deer foetus, using a real-time PCR assay, but it failed to isolate or show molecular evidence of pathogenic leptospires from the uterus of deer. Early infection of the foetus may play a role in the adverse effects of leptospirosis on deer reproductive performance demonstrated in this study and elsewhere (Ayanegui-Alcérreca 2006; Subharat *et al.* 2008).

Data from Chapter 3 suggested that reproductive losses due to leptospiral infection were peri- or post-natal, but pre-natal losses should not be ruled out since abortion has been demonstrated following artificial infection with serovar Pomona in white tailed deer (Trainer *et al.* 1961). This could be serovar-related since only Hardjobovis infection was found in the present study. In dairy cattle, reproductive losses have been shown to be pre-, peri and post-natal. Persistent infection by Hardjobovis was reported to cause reduced conception rates and fertility (Dhaliwal *et al.* 1996), increased number of services per conception and prolonged calving interval. It is also a cause of early embryonic death, abortion, stillbirth and weak calf syndrome (Smyth *et al.* 1999).

Since this study was done only in the early autumn, when most hinds are in early gestation, and found evidence in only one deer, further research in both early and later pregnancy and in non-pregnant hinds during the corresponding period is needed to fully investigate and confirm the presence of this organism and its role in reducing reproductive performance. Primiparous hinds should be the target group since they are

more likely to be recently infected. The lower weaning rate in this class of animal rather than adults is consistent with the hypothesis of an infectious pathogen being a cause of the reproductive loss. Thus, adoption of vaccination may have an important positive impact on the reproductive efficiency of farmed deer by improving the retention rate of young hinds, hence increasing the average breeding life, and improving production efficiency by requiring fewer replacement hinds to be retained.

8.4.4 Implementation of vaccination and control

The production (growth and reproduction) increase through vaccination in deer shown from this study suggested a likely economical effect on most farms over a period of time. It is estimated that about 67% of deer farms could achieve economic response (Wilson *et al.* 2009) in any one year, and since the status of farms is not static, it is likely that almost all farms will experience infection and therefore risk negative effects of leptospirosis on a long-term basis. In the absence of public subsidies for the control of human leptospirosis, vaccination against leptospirosis in livestock for the purpose of protection of people needs to be economically attractive for farmers to motivate the investment.

There are three commercial vaccines labelled for use in deer in New Zealand, namely Leptavoid-2, Leptavoid-3 (Intervet/Schering-Plough Animal Health Limited, NZ) and Leptoshield (Pfizer Animal Health Limited, NZ). However, only Leptavoid-2 and Leptavoid-3 have been tested for vaccine efficacy on farmed deer. While there is no evidence that other vaccines are not efficacious it should not be assumed that all vaccines will have the same effect until supported by scientifically sound evidence (Wilson *et al.* 2009).

It is likely that continued whole herd vaccination would reduce the incidence of disease and infection, as shown in dairy cattle. Evidence from a longitudinal study of cattle in Luing Island, Scotland demonstrated that when whole herd immunity was sustained by vaccination for at least five years, Hardjobovis infection could be eliminated (Little *et al.* 1992). However, elimination could be challenged by potential sources of re-infection such as introduction of infected and shedding stock, and contaminated waterways from neighbouring properties and wildlife vectors. The principles of risk evaluation in relation to animal health decision-making, including vaccination should be considered (Wilson *et al.* 2009).

Prior to consideration of vaccination, serological testing can determine the leptospiral infection status of a herd. Animals to target for sampling are those 1-2 years of age since the seroprevalence in that group has been shown generally to be higher than in other age classes (Ayanegui-Alcérreca 2006). A minimum of 20 samples should be collected to enable the presence or absence of infection to be evaluated with reasonable confidence as determined in Chapter 4. However, if a herd is believed on

the basis of this testing to be uninfected it therefore lacks herd-level immunity. It may be that if the organism is introduced, the morbidity and mortality rate may be higher than in an endemically infected herd where natural immunity exists. For a vaccination regime, Ayanegui-Alcérreca (2006) recommended that young deer should be sensitised at 3-months old followed by a booster a month later. All classes of hinds should be boosted one month prior to calving to enhance maternal derived antibody. Stags should be boosted annually at a convenient time that fits normal management practice. While this proposed programme is based on a range of epidemiological and vaccine studies, it is recommended that it should be tested in longitudinal studies on a number of deer farms for its effectiveness.

Maintaining farmed deer in a closed herd would reduce the risk of introducing infection. A quarantine program is an option when introducing new stock to the herd in order to minimise potential transmission. This applies particularly to amalgamation of young weaner deer from different sources when they are most susceptible. Wilson and McGhee (1993) noted that transport and amalgamation of deer from several sources, particularly of young deer in the autumn would appear to be the most significant risk factor for disease outbreaks in that age group. If replacement deer are introduced to the herd, their serological status could be tested. If seropositive, infection could be reduced by treatment with Streptomycin at 25 mg/kg (Mackintosh 1993) immediately prior to shipping and quarantined when arriving at farms. Deer introduced to the herd should be vaccinated and boosted prior to introduction. Optimum nutrition and animal health (e.g. trace elements and internal parasites) could improve resilience to clinical disease, but would unlikely affect infection *per se* (Wilson *et al.* 2009). Transmission risks such as waterways on farm could be fenced out from grazing. Drainage, reducing the risk of surface water contaminated with urine would reduce risk of transmission.

Recent data from 237 deer herds surveyed in 2008-09 revealed that 11 herds (4.6%) had clinical leptospirosis confirmed or suspected in the past three years (Verdugo and Heuer 2009). This figure could well be an under-estimate due to unawareness of the disease by farming and veterinary sectors. The risk of clinical disease depends on management, environment, climate, immuno-competence, and exposure to various sources of infection, thus making it impossible to predict even with reasonable confidence the magnitude of herd, mob or individual animal level losses. A clinical outbreak can be controlled by the use of an antibiotic and vaccination, the former providing short-term effect and the latter providing longer term protection. However, this would rely on early detection since some outbreaks have been explosive, with large losses before detection and diagnosis (Wilson *et al.* 2009).

Cessation of a vaccination programme after a period of whole herd vaccination will result in an increased in risk of clinical disease unless a total prevention of future exposure can be assured. This is because the unvaccinated generation will be

immuno-naïve and therefore at higher risk of disease, should they be exposed to the organism, than a naturally infected herd that has developed herd-level immunity by continuous natural challenge, although disease can still occur in this circumstance. There is no information on the efficacy or duration that vaccine protects leptospiral infection and/or disease in farmed deer. However, a study in cattle (Hancock *et al.* 1984) suggested that the immunity could persist for one year then an annual booster is needed. Thus, a decision to vaccinate has long-term implications (Wilson *et al.* 2009).

Since most deer farming properties are mixed-species, vaccination control programmes need to consider all species on-farm. However, the need to vaccinate all species will depend on establishment of whether sheep are maintenance hosts for Hardjobovis or not. For example, if sheep are not maintenance hosts, vaccination of cattle and deer may indirectly reduce or eliminate the risk of infection in sheep. Research into this question needs to be commenced as soon as possible in order to provide evidence for appropriate control measures for leptospirosis on mixed-species farms.

8.5 Proposed industry action

Research undertaken within this thesis is clearly relevant to the 2007 Deer Industry New Zealand (DINZ) productivity strategy which is "*to improve deer farmer profitability and industry sustainability through improved growth and reproductive productivity over the next 5 years to 2012*" (Pearse and Fung 2007). Epidemiological studies provide valuable information on disease distribution and risk factors associated with leptospirosis, whereas the newly developed real-time PCR will be a useful tool for diagnosis and the case definition for future research. Vaccination has proven to be an efficacious means of control and likely to return a cost-effective production response on as many as 67% of deer farms in a single production cycle. The demonstrated effect on growth of up to 6.5 kg contributed approximately 60% of the targeted improvement proposed by the productivity strategy (increase of 5.4 kg carcass weight equivalent to 10 kg live weight at the point of sale) (Pearse and Fung 2008). Furthermore, increasing the weaning rate by 5.7 percentage points contributed to 50% of the strategic productivity target (improving weaning 8 percentage points in first calving hinds) (Pearse and Fung 2008). This suggests that the targeted increase in productivity set by the strategy could be achieved by controlling subclinical leptospirosis alone. Thus the achievable gains in productivity are likely larger than those defined in the industry's productivity strategy.

Deer farmers need to be well informed about the risk of leptospirosis, production losses and their relationships to human infection, its epidemiology and control options that are provided and discussed in this thesis, which builds upon recent research within the Massey University Deer Research Group and Leptospirosis Research Unit. The deer industry organisation, DINZ, should highlight these findings to producers to

ensure that deer farmers make well-informed and therefore best-practice decisions tailored to individual circumstances. The New Zealand Veterinary Association (NZVA) should take part in providing up-to-date information for farmers about disease risks in both human and animal health perspectives, production losses associated with leptospirosis and available options for disease control. The best option for control is vaccination and leptospiral vaccines in New Zealand are Prescription Animal Remedies (P.A.R.) class I, that is, under prescription by a veterinarian (IVS 2010). Thus the veterinarian has a dual incentive to promote awareness of leptospirosis to their deer farmer clients: improvement of productivity on client's farms, and business revenue. This will, in the long run, help increase the productivity of deer farming in terms of growth and reproduction.

Last but not least, due to the nature of farming in New Zealand which involves multi-species including deer, cattle and sheep, on the same property, DINZ should coordinate with the sheep and beef industries to control leptospirosis. Data of disease dynamics between each species on farms are still lacking and need further investigation. In addition, there is no research into subclinical production losses in sheep and beef cattle in New Zealand similar to that studied in this thesis. Research into these aspects should be undertaken to determine how these effects can be built into an overall extension strategy by the industry. It will be ideal that all industries take part in sharing information and provide funding together for further research since it is unlikely that control for leptospirosis in one species of interest on farms will be efficient.

8.6 Areas for further research

This study has provided significant data on epidemiology of leptospirosis on mixed-species deer farms, a recent molecular diagnostic technique for *Leptospira* and control by means of vaccination. However, it has also raised more research questions and identified several areas that should be addressed in further research. These are summarised below:

- A nationwide epidemiological study of leptospirosis on mixed-species farm that include all farm categories should be conducted to fully understand disease dynamics, production effects and identify potential risk factors for leptospiral infection. Data available from the present study could be used for study design and sample size calculation.
- A study to test whether sheep act as a maintenance host for *Leptospira* should be conducted. This could be done by longitudinal observations on seroprevalence of sheep on farms with or without presence of other species or by vaccinating other species on farms and seeing whether seroprevalence of sheep declined over time. However, appropriate control for other sources of infection is critical.

- Inter-species transmission of *Leptospira* between species on farm should be investigated using MLST and/or other genetic analyses on isolates arising from this study and the mixed-species study proposed above. It should also compare within and between regions of New Zealand.
- Investigation of the possible presence of Arborea should be continued and expanded to other livestock species including wildlife vectors and humans to confirm or refute its identity. Southland should be the first region to be targeted for sampling based on data from the study.
- Sheep and beef cattle should be investigated for exotic serovars in the same manner as performed in farmed deer from this study. Serum samples of beef cattle and sheep stored in the serum bank from this study could be randomly selected and used as a preliminary resource that represents Manawatu region.
- The recently developed real-time PCR should be validated, for example by comparison with other reported PCRs, culture and/or MAT using Bayesian methods. Stored DNA samples from this study are available for this purpose.
- A study involving a whole-herd vaccination programme should be conducted. Serology and urinary shedding should be monitored longitudinally to observe the duration of immunity. The study should include all species present on farm. In addition, testing whether sheep are maintenance host as mentioned above could be adjunct with this study.
- Growth and reproduction response in deer observed from this study should be replicated by using the same study design or long term vaccination over at least two successive seasons without prior antibiotic treatment. However, it should try to enrol large number of animals and farms that are high risk to experience natural challenge of *Leptospira*.
- A study to confirm the presence of leptospiral organisms in the female reproductive tract and foetus should be conducted. It should aim for both early and late pregnancy periods. While primiparous hinds should be the primary target group since they are more likely to be recently infected, adult hinds should also be evaluated.

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Appendix

Appendix 2a. Raw data of average daily gain (ADG) and serology in male deer.

(Period: 1 = March-May, 2 = May-August, 3 = August-November)

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
1	1	control	1	39.2	0	0
2	1	control	1	176.5	0	0
3	1	control	1	137.3	0	0
4	1	control	1	137.3	0	0
5	1	control	1	98.0	0	0
6	1	control	1	176.5	0	0
7	1	control	1	156.9	0	0
8	1	control	1	117.6	24	0
9	1	control	1	176.5	0	0
10	1	control	1	137.3	0	0
11	1	control	1	0	0	0
12	1	control	1	176.5	48	48
13	1	control	1	137.3	0	0
14	1	control	1	58.8	0	0
15	1	control	1	58.8	0	0
16	1	vaccine	1	176.5	-	-
17	1	vaccine	1	137.3	-	-
18	1	vaccine	1	196.1	-	-
19	1	vaccine	1	196.1	-	-
20	1	vaccine	1	215.7	-	-
21	1	vaccine	1	117.6	-	-
22	1	vaccine	1	176.5	-	-
23	1	vaccine	1	372.5	-	-
24	1	vaccine	1	19.6	-	-
25	1	vaccine	1	137.3	-	-
26	1	vaccine	1	19.6	-	-
27	1	vaccine	1	176.5	-	-
28	1	vaccine	1	235.3	-	-
29	1	vaccine	1	156.9	-	-
30	1	vaccine	1	215.7	-	-
1	1	control	2	162.8	0	0
2	1	control	2	46.5	0	0
3	1	control	2	81.4	0	0
4	1	control	2	11.6	0	0
5	1	control	2	34.9	0	0
6	1	control	2	11.6	0	0
7	1	control	2	-23.3	0	0
8	1	control	2	-	-	-
9	1	control	2	93.0	0	0
10	1	control	2	11.6	0	0
11	1	control	2	34.9	0	0
12	1	control	2	-	0	24
13	1	control	2	-	0	0
14	1	control	2	0	0	0
15	1	control	2	93.0	-	-
16	1	vaccine	2	104.7	-	-
17	1	vaccine	2	0	-	-
18	1	vaccine	2	0	-	-
19	1	vaccine	2	23.3	-	-
20	1	vaccine	2	11.6	-	-
21	1	vaccine	2	11.6	-	-
22	1	vaccine	2	11.6	-	-
23	1	vaccine	2	-11.6	-	-
24	1	vaccine	2	23.3	-	-
25	1	vaccine	2	-23.3	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
26	1	vaccine	2	58.1	-	-
27	1	vaccine	2	23.3	-	-
28	1	vaccine	2	23.3	-	-
29	1	vaccine	2	23.3	-	-
30	1	vaccine	2	46.5	-	-
1	1	control	3	-	-	-
2	1	control	3	238.5	-	-
3	1	control	3	211.0	192	0
4	1	control	3	174.3	96	0
5	1	control	3	192.7	96	0
6	1	control	3	201.8	96	0
7	1	control	3	119.3	192	0
8	1	control	3	-	-	-
9	1	control	3	211.0	192	0
10	1	control	3	238.5	48	0
11	1	control	3	247.7	-	-
12	1	control	3	-	-	-
13	1	control	3	-	-	-
14	1	control	3	211.0	24	0
15	1	control	3	-	-	-
16	1	vaccine	3	-	-	-
17	1	vaccine	3	247.7	-	-
18	1	vaccine	3	247.7	-	-
19	1	vaccine	3	-	-	-
20	1	vaccine	3	275.2	-	-
21	1	vaccine	3	-	-	-
22	1	vaccine	3	284.4	-	-
23	1	vaccine	3	-	-	-
24	1	vaccine	3	-	-	-
25	1	vaccine	3	-	-	-
26	1	vaccine	3	293.6	-	-
27	1	vaccine	3	211.0	-	-
28	1	vaccine	3	220.2	-	-
29	1	vaccine	3	-	-	-
30	1	vaccine	3	-	-	-
31	2	control	1	74.1	0	0
32	2	control	1	-37.0	0	0
33	2	control	1	120.4	0	0
34	2	control	1	-46.3	0	0
35	2	control	1	-9.3	0	0
36	2	control	1	74.1	0	0
37	2	control	1	46.3	0	0
38	2	control	1	83.3	0	0
39	2	control	1	37.0	0	0
40	2	control	1	37.0	0	0
41	2	control	1	46.3	0	0
42	2	control	1	18.5	0	0
43	2	control	1	64.8	0	0
44	2	control	1	111.1	0	0
45	2	control	1	0	0	0
46	2	control	1	-18.5	0	0
47	2	control	1	64.8	0	0
48	2	control	1	-	-	-
49	2	control	1	101.9	0	0
50	2	control	1	92.6	0	0
51	2	vaccine	1	74.1	-	-
52	2	vaccine	1	55.6	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
53	2	vaccine	1	0	-	-
54	2	vaccine	1	46.3	-	-
55	2	vaccine	1	64.8	-	-
56	2	vaccine	1	37.0	-	-
57	2	vaccine	1	0	-	-
58	2	vaccine	1	111.1	-	-
59	2	vaccine	1	74.1	-	-
60	2	vaccine	1	27.8	-	-
61	2	vaccine	1	37.0	-	-
62	2	vaccine	1	92.6	-	-
63	2	vaccine	1	83.3	-	-
64	2	vaccine	1	92.6	-	-
65	2	vaccine	1	92.6	-	-
66	2	vaccine	1	74.1	-	-
67	2	vaccine	1	120.4	-	-
68	2	vaccine	1	55.6	-	-
69	2	vaccine	1	111.1	-	-
70	2	vaccine	1	55.6	-	-
31	2	control	2	37.6	0	0
32	2	control	2	96.8	0	0
33	2	control	2	48.4	0	0
34	2	control	2	53.8	0	0
35	2	control	2	129.0	0	0
36	2	control	2	64.5	0	0
37	2	control	2	129.0	0	0
38	2	control	2	59.1	0	0
39	2	control	2	80.6	0	0
40	2	control	2	139.8	192	0
41	2	control	2	48.4	0	0
42	2	control	2	37.6	0	0
43	2	control	2	0.0	0	0
44	2	control	2	80.6	96	0
45	2	control	2	102.2	0	0
46	2	control	2	0	-	-
47	2	control	2	53.8	0	0
48	2	control	2	-	-	-
49	2	control	2	59.1	0	0
50	2	control	2	53.8	0	0
51	2	vaccine	2	75.3	-	-
52	2	vaccine	2	75.3	-	-
53	2	vaccine	2	91.4	-	-
54	2	vaccine	2	48.4	-	-
55	2	vaccine	2	43.0	-	-
56	2	vaccine	2	53.8	-	-
57	2	vaccine	2	-	-	-
58	2	vaccine	2	64.5	-	-
59	2	vaccine	2	60.2	-	-
60	2	vaccine	2	80.6	-	-
61	2	vaccine	2	145.2	-	-
62	2	vaccine	2	53.8	-	-
63	2	vaccine	2	64.5	-	-
64	2	vaccine	2	107.5	-	-
65	2	vaccine	2	91.4	-	-
66	2	vaccine	2	64.5	-	-
67	2	vaccine	2	37.6	-	-
68	2	vaccine	2	43.0	-	-
69	2	vaccine	2	91.4	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
70	2	vaccine	2	69.9	-	-
31	2	control	3	89.9	0	0
32	2	control	3	44.9	0	0
33	2	control	3	118.0	0	0
34	2	control	3	146.1	0	0
35	2	control	3	185.4	0	0
36	2	control	3	118.0	0	0
37	2	control	3	224.7	0	0
38	2	control	3	207.9	0	0
39	2	control	3	179.8	0	0
40	2	control	3	146.1	48	0
41	2	control	3	213.5	0	0
42	2	control	3	202.2	0	0
43	2	control	3	-	0	0
44	2	control	3	174.2	0	0
45	2	control	3	185.4	0	0
46	2	control	3	-	0	0
47	2	control	3	174.2	0	0
48	2	control	3	-	0	0
49	2	control	3	207.9	0	0
50	2	control	3	207.9	0	0
51	2	vaccine	3	112.4	-	-
52	2	vaccine	3	213.5	-	-
53	2	vaccine	3	179.8	-	-
54	2	vaccine	3	157.3	-	-
55	2	vaccine	3	191.0	-	-
56	2	vaccine	3	-	-	-
57	2	vaccine	3	-	-	-
58	2	vaccine	3	219.1	-	-
59	2	vaccine	3	189.9	-	-
60	2	vaccine	3	196.6	-	-
61	2	vaccine	3	174.2	-	-
62	2	vaccine	3	162.9	-	-
63	2	vaccine	3	157.3	-	-
64	2	vaccine	3	168.5	-	-
65	2	vaccine	3	241.6	-	-
66	2	vaccine	3	168.5	-	-
67	2	vaccine	3	196.6	-	-
68	2	vaccine	3	207.9	-	-
69	2	vaccine	3	191.0	-	-
70	2	vaccine	3	168.5	-	-
71	3	control	1	50.9	0	0
72	3	control	1	9.1	0	0
73	3	control	1	21.8	0	0
74	3	control	1	98.2	0	0
75	3	control	1	10.9	0	0
76	3	control	1	21.8	24	0
77	3	control	1	27.3	0	0
78	3	control	1	54.5	0	0
79	3	control	1	50.9	0	0
80	3	control	1	43.6	0	0
81	3	control	1	-36.4	0	0
82	3	control	1	21.8	0	0
83	3	control	1	29.1	0	0
84	3	control	1	18.2	0	0
85	3	control	1	18.2	0	0
86	3	control	1	36.4	0	0

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
87	3	control	1	45.5	0	0
88	3	control	1	43.6	0	0
89	3	vaccine	1	27.3	-	-
90	3	vaccine	1	43.6	-	-
91	3	vaccine	1	29.1	-	-
92	3	vaccine	1	-10.9	-	-
93	3	vaccine	1	0	-	-
94	3	vaccine	1	-21.8	-	-
95	3	vaccine	1	-134.5	-	-
96	3	vaccine	1	18.2	-	-
97	3	vaccine	1	-14.5	-	-
98	3	vaccine	1	87.3	-	-
99	3	vaccine	1	-50.9	-	-
100	3	vaccine	1	18.2	-	-
101	3	vaccine	1	-9.1	-	-
102	3	vaccine	1	-50.9	-	-
103	3	vaccine	1	3.6	-	-
104	3	vaccine	1	36.4	-	-
105	3	vaccine	1	36.4	-	-
106	3	vaccine	1	27.3	-	-
107	3	vaccine	1	-9.1	-	-
108	3	vaccine	1	43.6	-	-
109	3	vaccine	1	29.1	-	-
71	3	control	2	-	-	-
72	3	control	2	0	0	0
73	3	control	2	28.9	0	0
74	3	control	2	-26.8	0	0
75	3	control	2	-	-	-
76	3	control	2	-4.1	0	0
77	3	control	2	-25.8	0	0
78	3	control	2	-16.5	0	0
79	3	control	2	-5.2	0	0
80	3	control	2	30.9	0	0
81	3	control	2	-56.7	0	0
82	3	control	2	6.2	0	0
83	3	control	2	-	-	-
84	3	control	2	67.0	0	0
85	3	control	2	61.9	0	0
86	3	control	2	-28.9	0	0
87	3	control	2	5.2	0	0
88	3	control	2	0	0	0
89	3	vaccine	2	-24.7	-	-
90	3	vaccine	2	10.3	-	-
91	3	vaccine	2	6.2	-	-
92	3	vaccine	2	28.9	-	-
93	3	vaccine	2	15.5	-	-
94	3	vaccine	2	-6.2	-	-
95	3	vaccine	2	35.1	-	-
96	3	vaccine	2	-	-	-
97	3	vaccine	2	-8.2	-	-
98	3	vaccine	2	4.1	-	-
99	3	vaccine	2	2.1	-	-
100	3	vaccine	2	-16.5	-	-
101	3	vaccine	2	-32.0	-	-
102	3	vaccine	2	-	-	-
103	3	vaccine	2	15.5	-	-
104	3	vaccine	2	-	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
105	3	vaccine	2	-6.2	-	-
106	3	vaccine	2	-15.5	-	-
107	3	vaccine	2	56.7	-	-
108	3	vaccine	2	14.4	-	-
109	3	vaccine	2	-16.5	-	-
71	3	control	3	-	-	-
72	3	control	3	300.0	0	0
73	3	control	3	237.4	0	0
74	3	control	3	276.6	0	0
75	3	control	3	-	0	0
76	3	control	3	65.4	0	0
77	3	control	3	287.9	48	0
78	3	control	3	39.3	0	0
79	3	control	3	172.9	0	0
80	3	control	3	246.7	0	0
81	3	control	3	-33.6	0	0
82	3	control	3	175.7	24	0
83	3	control	3	-	-	-
84	3	control	3	276.6	0	0
85	3	control	3	200.0	0	0
86	3	control	3	170.1	0	0
87	3	control	3	190.7	0	0
88	3	control	3	-	-	-
89	3	vaccine	3	284.1	-	-
90	3	vaccine	3	375.7	-	-
91	3	vaccine	3	244.9	-	-
92	3	vaccine	3	250.5	-	-
93	3	vaccine	3	296.3	-	-
94	3	vaccine	3	-	-	-
95	3	vaccine	3	373.8	-	-
96	3	vaccine	3	-	-	-
97	3	vaccine	3	196.3	-	-
98	3	vaccine	3	207.5	-	-
99	3	vaccine	3	388.8	-	-
100	3	vaccine	3	-	-	-
101	3	vaccine	3	-	-	-
102	3	vaccine	3	-	-	-
103	3	vaccine	3	-	-	-
104	3	vaccine	3	-	-	-
105	3	vaccine	3	343.9	-	-
106	3	vaccine	3	314.0	-	-
107	3	vaccine	3	94.4	-	-
108	3	vaccine	3	181.3	-	-
109	3	vaccine	3	-	-	-
110	4	control	1	166.7	0	0
111	4	control	1	107.8	0	0
112	4	control	1	166.7	0	0
113	4	control	1	137.3	0	0
114	4	control	1	156.9	0	0
115	4	control	1	49.0	0	0
116	4	control	1	137.3	0	0
117	4	control	1	-	-	-
118	4	control	1	166.7	0	0
119	4	control	1	-117.6	0	0
120	4	control	1	107.8	0	0
121	4	control	1	127.5	0	0
122	4	control	1	186.3	0	0

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
123	4	control	1	254.9	0	0
124	4	control	1	107.8	0	0
125	4	control	1	98.0	0	0
126	4	control	1	117.6	0	0
127	4	control	1	156.9	0	0
128	4	vaccine	1	156.9	-	-
129	4	vaccine	1	284.3	-	-
130	4	vaccine	1	88.2	-	-
131	4	vaccine	1	19.6	-	-
132	4	vaccine	1	98.0	-	-
133	4	vaccine	1	186.3	-	-
134	4	vaccine	1	215.7	-	-
135	4	vaccine	1	205.9	-	-
136	4	vaccine	1	39.2	-	-
137	4	vaccine	1	107.8	-	-
138	4	vaccine	1	127.5	-	-
139	4	vaccine	1	29.4	-	-
140	4	vaccine	1	147.1	-	-
141	4	vaccine	1	19.6	-	-
142	4	vaccine	1	205.9	-	-
143	4	vaccine	1	-156.9	-	-
144	4	vaccine	1	58.8	-	-
145	4	vaccine	1	39.2	-	-
146	4	vaccine	1	88.2	-	-
147	4	vaccine	1	156.9	-	-
110	4	control	2	41.7	0	0
111	4	control	2	41.7	0	0
112	4	control	2	36.5	0	0
113	4	control	2	-15.6	0	0
114	4	control	2	-10.4	0	0
115	4	control	2	83.3	0	0
116	4	control	2	31.3	0	0
117	4	control	2	-	0	0
118	4	control	2	0	0	0
119	4	control	2	36.5	0	0
120	4	control	2	78.1	0	0
121	4	control	2	-10.4	0	0
122	4	control	2	-62.5	0	0
123	4	control	2	10.4	0	0
124	4	control	2	78.1	0	0
125	4	control	2	104.2	0	0
126	4	control	2	99.0	0	0
127	4	control	2	46.9	0	0
128	4	vaccine	2	41.7	-	-
129	4	vaccine	2	41.7	-	-
130	4	vaccine	2	-5.2	-	-
131	4	vaccine	2	161.5	-	-
132	4	vaccine	2	41.7	-	-
133	4	vaccine	2	72.9	-	-
134	4	vaccine	2	67.7	-	-
135	4	vaccine	2	-57.3	-	-
136	4	vaccine	2	5.2	-	-
137	4	vaccine	2	72.9	-	-
138	4	vaccine	2	0	-	-
139	4	vaccine	2	-	-	-
140	4	vaccine	2	62.5	-	-
141	4	vaccine	2	36.5	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
142	4	vaccine	2	52.1	-	-
143	4	vaccine	2	78.1	-	-
144	4	vaccine	2	72.9	-	-
145	4	vaccine	2	10.4	-	-
146	4	vaccine	2	72.9	-	-
147	4	vaccine	2	0	-	-
110	4	control	3	198.9	0	0
111	4	control	3	182.8	0	0
112	4	control	3	193.5	0	0
113	4	control	3	215.1	0	0
114	4	control	3	279.6	0	0
115	4	control	3	247.3	0	0
116	4	control	3	215.1	0	0
117	4	control	3	-21.5	0	0
118	4	control	3	204.3	0	0
119	4	control	3	129.0	0	0
120	4	control	3	241.9	0	0
121	4	control	3	274.2	0	0
122	4	control	3	317.2	0	0
123	4	control	3	220.4	0	0
124	4	control	3	188.2	0	0
125	4	control	3	172.0	0	0
126	4	control	3	21.5	0	0
127	4	control	3	155.9	0	0
128	4	vaccine	3	209.7	-	-
129	4	vaccine	3	86.0	-	-
130	4	vaccine	3	182.8	-	-
131	4	vaccine	3	204.3	-	-
132	4	vaccine	3	166.7	-	-
133	4	vaccine	3	182.8	-	-
134	4	vaccine	3	134.4	-	-
135	4	vaccine	3	263.4	-	-
136	4	vaccine	3	166.7	-	-
137	4	vaccine	3	263.4	-	-
138	4	vaccine	3	220.4	-	-
139	4	vaccine	3	-	-	-
140	4	vaccine	3	177.4	-	-
141	4	vaccine	3	215.1	-	-
142	4	vaccine	3	220.4	-	-
143	4	vaccine	3	177.4	-	-
144	4	vaccine	3	215.1	-	-
145	4	vaccine	3	274.2	-	-
146	4	vaccine	3	145.2	-	-
147	4	vaccine	3	21.5	-	-
148	5	control	1	-18.5	0	0
149	5	control	1	18.5	0	0
150	5	control	1	-	0	0
151	5	control	1	37.0	0	0
152	5	control	1	46.3	0	0
153	5	control	1	37.0	0	0
154	5	control	1	-	-	-
155	5	control	1	64.8	0	0
156	5	control	1	64.8	0	0
157	5	control	1	74.1	0	0
158	5	control	1	64.8	0	0
159	5	control	1	-9.3	0	0
160	5	control	1	-	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
161	5	control	1	101.9	0	0
162	5	control	1	83.3	0	0
163	5	control	1	-18.5	0	0
164	5	control	1	74.1	0	0
165	5	control	1	148.1	0	0
166	5	control	1	64.8	0	0
167	5	control	1	129.6	-	-
168	5	control	1	83.3	0	0
169	5	control	1	111.1	0	0
170	5	control	1	27.8	0	0
171	5	control	1	101.9	0	0
172	5	vaccine	1	37.0	-	-
173	5	vaccine	1	83.3	-	-
174	5	vaccine	1	74.1	-	-
175	5	vaccine	1	-	-	-
176	5	vaccine	1	55.6	-	-
177	5	vaccine	1	129.6	-	-
178	5	vaccine	1	-9.3	-	-
179	5	vaccine	1	9.3	-	-
180	5	vaccine	1	129.6	-	-
181	5	vaccine	1	120.4	-	-
182	5	vaccine	1	120.4	-	-
183	5	vaccine	1	55.6	-	-
184	5	vaccine	1	-	-	-
185	5	vaccine	1	101.9	-	-
186	5	vaccine	1	64.8	-	-
187	5	vaccine	1	120.4	-	-
188	5	vaccine	1	64.8	-	-
189	5	vaccine	1	92.6	-	-
190	5	vaccine	1	55.6	-	-
191	5	vaccine	1	166.7	-	-
192	5	vaccine	1	0	-	-
193	5	vaccine	1	157.4	-	-
194	5	vaccine	1	92.6	-	-
195	5	vaccine	1	27.8	-	-
148	5	control	2	0	0	0
149	5	control	2	-	0	0
150	5	control	2	-	-	-
151	5	control	2	19.6	0	0
152	5	control	2	9.8	0	0
153	5	control	2	49.0	0	0
154	5	control	2	-	0	0
155	5	control	2	49.0	0	0
156	5	control	2	24.5	0	0
157	5	control	2	93.1	0	0
158	5	control	2	19.6	0	0
159	5	control	2	-24.5	0	0
160	5	control	2	-	0	0
161	5	control	2	14.7	0	0
162	5	control	2	49.0	0	0
163	5	control	2	29.4	0	0
164	5	control	2	-4.9	0	0
165	5	control	2	53.9	0	0
166	5	control	2	-	-	-
167	5	control	2	19.6	0	0
168	5	control	2	53.9	0	0
169	5	control	2	53.9	0	0

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
170	5	control	2	29.4	0	0
171	5	control	2	68.6	0	0
172	5	vaccine	2	29.4	-	-
173	5	vaccine	2	0	-	-
174	5	vaccine	2	88.2	-	-
175	5	vaccine	2	-	-	-
176	5	vaccine	2	4.9	-	-
177	5	vaccine	2	0	-	-
178	5	vaccine	2	-19.6	-	-
179	5	vaccine	2	49.0	-	-
180	5	vaccine	2	14.7	-	-
181	5	vaccine	2	34.3	-	-
182	5	vaccine	2	29.4	-	-
183	5	vaccine	2	44.1	-	-
184	5	vaccine	2	-	-	-
185	5	vaccine	2	49.0	-	-
186	5	vaccine	2	58.8	-	-
187	5	vaccine	2	-	-	-
188	5	vaccine	2	53.9	-	-
189	5	vaccine	2	34.3	-	-
190	5	vaccine	2	44.1	-	-
191	5	vaccine	2	9.8	-	-
192	5	vaccine	2	29.4	-	-
193	5	vaccine	2	-29.4	-	-
194	5	vaccine	2	-4.9	-	-
195	5	vaccine	2	29.4	-	-
148	5	control	3	260.6	0	0
149	5	control	3	-	0	0
150	5	control	3	-	-	-
151	5	control	3	271.3	0	0
152	5	control	3	223.4	0	0
153	5	control	3	271.3	0	0
154	5	control	3	297.9	0	0
155	5	control	3	292.6	0	0
156	5	control	3	271.3	0	0
157	5	control	3	308.5	0	0
158	5	control	3	218.1	0	0
159	5	control	3	106.4	0	0
160	5	control	3	-	-	-
161	5	control	3	133.0	0	0
162	5	control	3	223.4	0	0
163	5	control	3	266.0	0	0
164	5	control	3	239.4	0	0
165	5	control	3	207.4	0	0
166	5	control	3	-	0	0
167	5	control	3	239.4	0	0
168	5	control	3	260.6	0	0
169	5	control	3	-	-	-
170	5	control	3	-	-	-
171	5	control	3	143.6	0	0
172	5	vaccine	3	281.9	-	-
173	5	vaccine	3	186.2	-	-
174	5	vaccine	3	223.4	-	-
175	5	vaccine	3	170.2	-	-
176	5	vaccine	3	79.8	-	-
177	5	vaccine	3	223.4	-	-
178	5	vaccine	3	138.3	-	-

Deer no.	Farm no.	Status	Period	ADG (g/day)	Hardjobovis	Pomona
179	5	vaccine	3	260.6	-	-
180	5	vaccine	3	297.9	-	-
181	5	vaccine	3	287.2	-	-
182	5	vaccine	3	212.8	-	-
183	5	vaccine	3	-	-	-
184	5	vaccine	3	159.6	-	-
185	5	vaccine	3	260.6	-	-
186	5	vaccine	3	239.4	-	-
187	5	vaccine	3	-	-	-
188	5	vaccine	3	-	-	-
189	5	vaccine	3	202.1	-	-
190	5	vaccine	3	276.6	-	-
191	5	vaccine	3	255.3	-	-
192	5	vaccine	3	191.5	-	-
193	5	vaccine	3	324.5	-	-
194	5	vaccine	3	271.3	-	-
195	5	vaccine	3	250.0	-	-

Appendix 2b. Raw data of urine shedding and serology in female deer.

(Period: 1 = March, 2 = May, 3 = August, 4 = November)(Culture and PCR: 0 = negative, 1 = positive)

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
1	1	control	1	0	0	0	0
2	1	control	1	0	0	0	0
3	1	control	1	0	0	24	0
4	1	control	1	0	0	48	0
5	1	control	1	0	0	0	0
6	1	control	1	0	0	0	0
7	1	control	1	0	0	0	0
8	1	control	1	0	0	0	0
9	1	control	1	0	0	0	0
10	1	control	1	0	0	0	0
11	1	control	1	0	0	0	0
12	1	control	1	0	0	0	0
13	1	control	1	0	0	0	0
14	1	control	1	0	0	0	0
15	1	vaccine	1	0	0	0	0
16	1	vaccine	1	0	0	48	0
17	1	vaccine	1	0	0	48	0
18	1	vaccine	1	0	0	0	0
19	1	vaccine	1	0	0	24	0
20	1	vaccine	1	0	0	24	0
21	1	vaccine	1	0	0	0	0
22	1	vaccine	1	0	0	0	0
23	1	vaccine	1	0	0	0	0
24	1	vaccine	1	0	0	24	0
25	1	vaccine	1	0	0	0	0
26	1	vaccine	1	0	0	0	0
27	1	vaccine	1	0	0	0	0
28	1	vaccine	1	0	0	0	0
1	1	control	2	0	0	0	0
2	1	control	2	0	0	0	0
3	1	control	2	0	0	0	0
4	1	control	2	0	0	0	0
5	1	control	2	0	0	0	0
6	1	control	2	0	0	0	0
7	1	control	2	0	0	0	0
8	1	control	2	0	0	0	0
9	1	control	2	0	0	0	0
10	1	control	2	-	-	0	0
11	1	control	2	0	0	0	0
12	1	control	2	0	0	0	0
13	1	control	2	0	0	0	0
14	1	control	2	0	0	0	0
15	1	vaccine	2	0	0	24	48
16	1	vaccine	2	0	0	48	48
17	1	vaccine	2	0	0	24	48
18	1	vaccine	2	0	0	24	768
19	1	vaccine	2	0	0	24	96
20	1	vaccine	2	0	0	0	48
21	1	vaccine	2	0	0	0	96
22	1	vaccine	2	0	0	24	96
23	1	vaccine	2	0	0	96	192
24	1	vaccine	2	0	0	0	24
25	1	vaccine	2	0	0	0	96
26	1	vaccine	2	0	0	24	96
27	1	vaccine	2	0	0	48	24

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
28	1	vaccine	2	0	0	0	192
1	1	control	3	0	0	0	0
2	1	control	3	0	0	0	0
3	1	control	3	0	0	0	0
4	1	control	3	0	0	0	0
5	1	control	3	-	-	0	0
6	1	control	3	0	0	0	0
7	1	control	3	0	0	0	0
8	1	control	3	0	0	0	0
9	1	control	3	0	0	0	0
10	1	control	3	0	0	0	0
11	1	control	3	0	0	0	0
12	1	control	3	0	0	0	0
13	1	control	3	0	0	0	0
14	1	control	3	0	0	0	0
15	1	vaccine	3	0	0	-	-
16	1	vaccine	3	0	0	-	-
17	1	vaccine	3	0	0	-	-
18	1	vaccine	3	0	0	-	-
19	1	vaccine	3	0	0	-	-
20	1	vaccine	3	0	0	-	-
21	1	vaccine	3	0	0	-	-
22	1	vaccine	3	0	0	-	-
23	1	vaccine	3	0	0	-	-
24	1	vaccine	3	0	0	-	-
25	1	vaccine	3	0	0	-	-
26	1	vaccine	3	0	0	-	-
27	1	vaccine	3	0	0	-	-
28	1	vaccine	3	0	0	-	-
1	1	control	4	0	1	96	0
2	1	control	4	0	0	24	0
3	1	control	4	-	-	-	-
4	1	control	4	0	0	96	0
5	1	control	4	0	0	24	0
6	1	control	4	0	1	192	0
7	1	control	4	1	1	48	0
8	1	control	4	-	-	-	-
9	1	control	4	0	0	-	-
10	1	control	4	0	1	48	0
11	1	control	4	-	-	-	-
12	1	control	4	-	-	-	-
13	1	control	4	0	1	48	0
14	1	control	4	-	-	96	0
15	1	vaccine	4	0	0	-	-
16	1	vaccine	4	-	-	-	-
17	1	vaccine	4	0	0	-	-
18	1	vaccine	4	0	0	-	-
19	1	vaccine	4	-	-	-	-
20	1	vaccine	4	0	0	-	-
21	1	vaccine	4	0	0	-	-
22	1	vaccine	4	0	0	-	-
23	1	vaccine	4	-	-	-	-
24	1	vaccine	4	0	0	-	-
25	1	vaccine	4	0	0	-	-
26	1	vaccine	4	0	0	-	-
27	1	vaccine	4	0	0	-	-
28	1	vaccine	4	0	0	-	-

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
29	2	control	1	0	0	0	0
30	2	control	1	0	0	0	0
31	2	control	1	0	0	0	0
32	2	control	1	0	0	0	0
33	2	control	1	0	0	0	0
34	2	control	1	0	0	0	0
35	2	control	1	0	0	0	0
36	2	control	1	0	0	0	0
37	2	control	1	0	0	24	0
38	2	control	1	0	0	0	0
39	2	control	1	0	0	24	0
40	2	control	1	0	0	0	0
41	2	control	1	0	0	0	0
42	2	control	1	0	0	0	0
43	2	control	1	0	0	0	0
44	2	control	1	0	0	0	0
45	2	control	1	0	0	0	0
46	2	control	1	0	0	0	0
47	2	control	1	0	0	0	0
48	2	control	1	0	0	0	0
49	2	control	1	0	0	0	0
50	2	control	1	0	0	24	0
51	2	control	1	0	0	48	0
52	2	control	1	0	0	0	0
53	2	control	1	0	0	0	0
54	2	vaccine	1	0	0	0	0
55	2	vaccine	1	0	0	0	0
56	2	vaccine	1	0	0	0	0
57	2	vaccine	1	0	0	48	0
58	2	vaccine	1	0	0	0	0
59	2	vaccine	1	0	0	48	0
60	2	vaccine	1	0	0	0	0
61	2	vaccine	1	0	0	0	0
62	2	vaccine	1	0	0	0	0
63	2	vaccine	1	0	0	0	0
64	2	vaccine	1	0	0	0	0
65	2	vaccine	1	0	0	48	0
66	2	vaccine	1	0	0	0	0
67	2	vaccine	1	0	0	24	0
68	2	vaccine	1	0	0	0	0
69	2	vaccine	1	0	0	0	0
70	2	vaccine	1	0	0	0	0
71	2	vaccine	1	0	0	0	0
72	2	vaccine	1	0	0	0	0
73	2	vaccine	1	0	0	0	0
74	2	vaccine	1	0	0	0	0
75	2	vaccine	1	0	0	0	0
76	2	vaccine	1	0	0	0	0
77	2	vaccine	1	0	0	0	0
78	2	vaccine	1	0	0	24	0
79	2	vaccine	1	0	0	0	0
29	2	control	2	0	0	0	0
30	2	control	2	0	0	0	0
31	2	control	2	0	0	0	0
32	2	control	2	0	0	0	0
33	2	control	2	0	0	0	0
34	2	control	2	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
35	2	control	2	0	0	24	0
36	2	control	2	0	0	0	0
37	2	control	2	0	0	0	0
38	2	control	2	0	0	0	0
39	2	control	2	0	0	0	0
40	2	control	2	0	0	0	0
41	2	control	2	0	0	0	0
42	2	control	2	0	0	0	0
43	2	control	2	0	0	0	0
44	2	control	2	0	0	0	0
45	2	control	2	0	0	0	0
46	2	control	2	0	0	0	0
47	2	control	2	0	0	0	0
48	2	control	2	0	0	0	0
49	2	control	2	0	0	0	0
50	2	control	2	0	0	0	0
51	2	control	2	0	0	0	0
52	2	control	2	0	0	0	0
53	2	control	2	0	0	0	0
54	2	vaccine	2	0	0	48	96
55	2	vaccine	2	0	0	768	192
56	2	vaccine	2	0	0	0	48
57	2	vaccine	2	0	0	0	48
58	2	vaccine	2	0	0	48	96
59	2	vaccine	2	0	0	24	384
60	2	vaccine	2	0	0	0	96
61	2	vaccine	2	0	0	48	192
62	2	vaccine	2	0	0	0	24
63	2	vaccine	2	0	0	48	384
64	2	vaccine	2	0	0	0	24
65	2	vaccine	2	0	0	0	96
66	2	vaccine	2	0	0	0	0
67	2	vaccine	2	0	0	0	96
68	2	vaccine	2	0	0	96	1536
69	2	vaccine	2	0	0	0	96
70	2	vaccine	2	0	0	0	48
71	2	vaccine	2	0	0	24	192
72	2	vaccine	2	0	0	24	1536
73	2	vaccine	2	0	0	24	192
74	2	vaccine	2	0	0	96	384
75	2	vaccine	2	0	0	0	96
76	2	vaccine	2	0	0	48	192
77	2	vaccine	2	0	0	24	96
78	2	vaccine	2	0	0	0	0
79	2	vaccine	2	0	0	0	24
29	2	control	3	0	0	0	0
30	2	control	3	0	0	0	0
31	2	control	3	-	-	-	-
32	2	control	3	0	0	0	0
33	2	control	3	0	0	96	0
34	2	control	3	0	0	0	0
35	2	control	3	0	0	96	0
36	2	control	3	0	0	0	0
37	2	control	3	0	0	0	0
38	2	control	3	0	0	0	0
39	2	control	3	0	0	0	0
40	2	control	3	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
41	2	control	3	0	0	0	0
42	2	control	3	0	0	0	0
43	2	control	3	0	0	0	0
44	2	control	3	0	0	0	0
45	2	control	3	0	0	0	0
46	2	control	3	0	0	0	0
47	2	control	3	0	0	0	0
48	2	control	3	0	0	0	0
49	2	control	3	0	0	0	0
50	2	control	3	0	0	0	0
51	2	control	3	0	0	0	0
52	2	control	3	0	0	0	0
53	2	control	3	0	0	0	0
54	2	vaccine	3	0	0	-	-
55	2	vaccine	3	0	0	-	-
56	2	vaccine	3	0	0	-	-
57	2	vaccine	3	0	0	-	-
58	2	vaccine	3	0	0	-	-
59	2	vaccine	3	0	0	-	-
60	2	vaccine	3	0	0	-	-
61	2	vaccine	3	0	0	-	-
62	2	vaccine	3	0	0	-	-
63	2	vaccine	3	0	0	-	-
64	2	vaccine	3	0	0	-	-
65	2	vaccine	3	0	0	-	-
66	2	vaccine	3	0	0	-	-
67	2	vaccine	3	0	0	-	-
68	2	vaccine	3	-	-	-	-
69	2	vaccine	3	0	0	-	-
70	2	vaccine	3	0	0	-	-
71	2	vaccine	3	0	0	-	-
72	2	vaccine	3	0	0	-	-
73	2	vaccine	3	-	-	-	-
74	2	vaccine	3	0	0	-	-
75	2	vaccine	3	-	-	-	-
76	2	vaccine	3	0	0	-	-
77	2	vaccine	3	0	0	-	-
78	2	vaccine	3	0	0	-	-
79	2	vaccine	3	0	0	-	-
29	2	control	4	0	0	0	0
30	2	control	4	0	0	0	0
31	2	control	4	0	0	-	-
32	2	control	4	0	0	0	0
33	2	control	4	0	0	48	0
34	2	control	4	0	0	0	0
35	2	control	4	0	0	0	0
36	2	control	4	-	-	-	-
37	2	control	4	0	0	0	0
38	2	control	4	0	0	0	0
39	2	control	4	0	0	0	0
40	2	control	4	0	0	0	0
41	2	control	4	0	0	0	0
42	2	control	4	0	0	0	24
43	2	control	4	0	0	0	0
44	2	control	4	0	0	0	0
45	2	control	4	0	0	0	24
46	2	control	4	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
47	2	control	4	0	0	0	0
48	2	control	4	0	0	0	0
49	2	control	4	0	0	0	0
50	2	control	4	0	0	0	0
51	2	control	4	0	0	0	0
52	2	control	4	0	0	0	24
53	2	control	4	0	0	0	0
54	2	vaccine	4	0	0	-	-
55	2	vaccine	4	0	0	-	-
56	2	vaccine	4	0	0	-	-
57	2	vaccine	4	0	0	-	-
58	2	vaccine	4	0	0	-	-
59	2	vaccine	4	0	0	-	-
60	2	vaccine	4	0	0	-	-
61	2	vaccine	4	0	0	-	-
62	2	vaccine	4	0	0	-	-
63	2	vaccine	4	0	0	-	-
64	2	vaccine	4	0	0	-	-
65	2	vaccine	4	0	0	-	-
66	2	vaccine	4	0	0	-	-
67	2	vaccine	4	0	0	-	-
68	2	vaccine	4	0	0	-	-
69	2	vaccine	4	0	0	-	-
70	2	vaccine	4	-	-	-	-
71	2	vaccine	4	0	0	-	-
72	2	vaccine	4	0	0	-	-
73	2	vaccine	4	0	0	-	-
74	2	vaccine	4	0	0	-	-
75	2	vaccine	4	0	0	-	-
76	2	vaccine	4	0	0	-	-
77	2	vaccine	4	-	-	-	-
78	2	vaccine	4	0	0	-	-
79	2	vaccine	4	0	0	-	-
80	3	control	1	0	0	0	0
81	3	control	1	0	0	0	0
82	3	control	1	0	0	24	0
83	3	control	1	0	0	0	0
84	3	control	1	0	0	24	0
85	3	control	1	0	0	0	0
86	3	control	1	0	0	0	0
87	3	control	1	0	0	0	0
88	3	control	1	0	0	0	0
89	3	control	1	0	0	0	0
90	3	control	1	0	0	0	0
91	3	control	1	0	0	24	0
92	3	control	1	0	0	24	0
93	3	control	1	0	0	0	0
94	3	control	1	0	0	0	0
95	3	control	1	0	0	0	0
96	3	control	1	0	0	0	0
97	3	control	1	0	0	0	0
98	3	control	1	0	0	0	0
99	3	control	1	0	0	0	0
100	3	control	1	0	0	0	0
101	3	vaccine	1	0	0	0	0
102	3	vaccine	1	0	0	0	0
103	3	vaccine	1	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
104	3	vaccine	1	0	0	0	0
105	3	vaccine	1	0	0	0	0
106	3	vaccine	1	0	0	0	0
107	3	vaccine	1	0	0	0	0
108	3	vaccine	1	0	0	0	0
109	3	vaccine	1	0	0	0	0
110	3	vaccine	1	0	0	0	0
111	3	vaccine	1	0	0	0	0
112	3	vaccine	1	0	0	0	0
113	3	vaccine	1	0	0	0	0
114	3	vaccine	1	0	0	0	0
115	3	vaccine	1	0	0	0	0
116	3	vaccine	1	0	0	0	0
117	3	vaccine	1	0	0	0	0
118	3	vaccine	1	0	0	0	0
80	3	control	2	0	0	0	0
81	3	control	2	0	0	0	0
82	3	control	2	0	0	0	0
83	3	control	2	0	0	0	0
84	3	control	2	0	0	0	0
85	3	control	2	0	0	0	0
86	3	control	2	0	0	0	0
87	3	control	2	0	0	0	0
88	3	control	2	-	-	0	0
89	3	control	2	0	0	0	0
90	3	control	2	0	0	0	0
91	3	control	2	0	0	0	0
92	3	control	2	0	0	0	0
93	3	control	2	0	0	0	0
94	3	control	2	0	0	0	0
95	3	control	2	0	0	0	0
96	3	control	2	0	0	0	0
97	3	control	2	0	0	0	0
98	3	control	2	0	0	0	0
99	3	control	2	0	0	0	0
100	3	control	2	-	-	0	0
101	3	vaccine	2	0	0	0	96
102	3	vaccine	2	0	0	0	192
103	3	vaccine	2	0	0	24	96
104	3	vaccine	2	0	0	24	48
105	3	vaccine	2	0	0	0	96
106	3	vaccine	2	0	0	24	48
107	3	vaccine	2	0	0	0	48
108	3	vaccine	2	0	0	0	24
109	3	vaccine	2	0	0	0	24
110	3	vaccine	2	0	0	0	0
111	3	vaccine	2	0	0	48	0
112	3	vaccine	2	0	0	0	384
113	3	vaccine	2	0	0	24	0
114	3	vaccine	2	0	0	96	192
115	3	vaccine	2	0	0	0	96
116	3	vaccine	2	0	0	0	0
117	3	vaccine	2	0	0	96	24
118	3	vaccine	2	0	0	0	24
80	3	control	3	0	0	0	0
81	3	control	3	0	0	0	0
82	3	control	3	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
83	3	control	3	0	0	0	0
84	3	control	3	0	0	0	0
85	3	control	3	0	0	0	0
86	3	control	3	0	0	0	0
87	3	control	3	0	0	0	0
88	3	control	3	0	0	0	0
89	3	control	3	0	0	0	0
90	3	control	3	0	0	0	0
91	3	control	3	0	0	0	0
92	3	control	3	0	0	0	0
93	3	control	3	0	0	0	0
94	3	control	3	0	0	0	0
95	3	control	3	0	0	0	0
96	3	control	3	0	0	0	0
97	3	control	3	0	0	0	0
98	3	control	3	0	0	0	0
99	3	control	3	-	-	0	0
100	3	control	3	0	0	0	0
101	3	vaccine	3	0	0	-	-
102	3	vaccine	3	0	0	-	-
103	3	vaccine	3	0	0	-	-
104	3	vaccine	3	0	0	-	-
105	3	vaccine	3	0	0	-	-
106	3	vaccine	3	0	0	-	-
107	3	vaccine	3	0	0	-	-
108	3	vaccine	3	0	0	-	-
109	3	vaccine	3	0	0	-	-
110	3	vaccine	3	0	0	-	-
111	3	vaccine	3	0	0	-	-
112	3	vaccine	3	-	-	-	-
113	3	vaccine	3	0	0	-	-
114	3	vaccine	3	0	0	-	-
115	3	vaccine	3	0	0	-	-
116	3	vaccine	3	0	0	-	-
117	3	vaccine	3	0	0	-	-
118	3	vaccine	3	0	0	-	-
80	3	control	4	0	0	0	0
81	3	control	4	0	0	0	0
82	3	control	4	0	0	24	0
83	3	control	4	0	0	-	-
84	3	control	4	0	0	0	0
85	3	control	4	0	0	0	0
86	3	control	4	0	0	0	0
87	3	control	4	0	0	0	0
88	3	control	4	0	0	48	0
89	3	control	4	0	0	-	-
90	3	control	4	0	0	0	0
91	3	control	4	-	-	-	-
92	3	control	4	0	0	0	0
93	3	control	4	0	0	0	0
94	3	control	4	0	0	0	0
95	3	control	4	-	-	-	-
96	3	control	4	0	0	0	0
97	3	control	4	0	0	0	0
98	3	control	4	0	0	0	0
99	3	control	4	0	0	96	0
100	3	control	4	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
101	3	vaccine	4	0	0	-	-
102	3	vaccine	4	0	0	-	-
103	3	vaccine	4	0	0	-	-
104	3	vaccine	4	0	0	-	-
105	3	vaccine	4	0	0	-	-
106	3	vaccine	4	0	0	-	-
107	3	vaccine	4	0	0	-	-
108	3	vaccine	4	0	0	-	-
109	3	vaccine	4	0	0	-	-
110	3	vaccine	4	0	0	-	-
111	3	vaccine	4	0	0	-	-
112	3	vaccine	4	0	0	-	-
113	3	vaccine	4	0	0	-	-
114	3	vaccine	4	0	0	-	-
115	3	vaccine	4	0	0	-	-
116	3	vaccine	4	-	-	-	-
117	3	vaccine	4	-	-	-	-
118	3	vaccine	4	0	0	-	-
119	4	control	1	0	0	0	0
120	4	control	1	0	0	0	0
121	4	control	1	-	-	0	0
122	4	control	1	0	0	0	0
123	4	control	1	0	0	0	0
124	4	control	1	0	0	0	0
125	4	control	1	0	0	0	0
126	4	control	1	0	0	0	0
127	4	control	1	0	0	0	0
128	4	control	1	0	0	24	0
129	4	control	1	0	0	0	0
130	4	control	1	0	0	0	0
131	4	control	1	-	-	0	0
132	4	control	1	0	0	0	0
133	4	control	1	-	-	0	0
134	4	control	1	0	0	0	0
135	4	control	1	0	0	0	0
136	4	control	1	0	0	0	0
137	4	control	1	0	0	0	0
138	4	control	1	0	0	0	0
139	4	control	1	-	-	0	0
140	4	control	1	0	0	0	0
141	4	control	1	0	0	0	0
142	4	control	1	0	0	0	0
143	4	control	1	0	0	0	0
144	4	control	1	0	0	0	0
145	4	vaccine	1	0	0	0	0
146	4	vaccine	1	-	-	0	0
147	4	vaccine	1	0	0	0	0
148	4	vaccine	1	0	0	0	0
149	4	vaccine	1	0	0	0	0
150	4	vaccine	1	0	0	24	0
151	4	vaccine	1	0	0	24	0
152	4	vaccine	1	0	0	0	0
153	4	vaccine	1	0	0	0	0
154	4	vaccine	1	0	0	0	0
155	4	vaccine	1	0	0	0	0
156	4	vaccine	1	0	0	0	0
157	4	vaccine	1	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
158	4	vaccine	1	0	0	0	0
159	4	vaccine	1	0	0	0	0
160	4	vaccine	1	0	0	0	0
161	4	vaccine	1	0	0	0	0
162	4	vaccine	1	0	0	0	0
163	4	vaccine	1	0	0	0	0
164	4	vaccine	1	0	0	0	0
165	4	vaccine	1	0	0	0	0
166	4	vaccine	1	0	0	0	0
167	4	vaccine	1	-	-	0	0
168	4	vaccine	1	0	0	0	0
169	4	vaccine	1	0	0	0	0
170	4	vaccine	1	0	0	0	0
171	4	vaccine	1	0	0	24	0
119	4	control	2	0	0	0	0
120	4	control	2	0	0	0	0
121	4	control	2	0	0	0	0
122	4	control	2	-	-	-	-
123	4	control	2	-	-	-	-
124	4	control	2	0	0	0	0
125	4	control	2	0	0	0	0
126	4	control	2	0	0	0	0
127	4	control	2	0	0	0	0
128	4	control	2	0	0	0	0
129	4	control	2	0	0	0	0
130	4	control	2	0	0	0	0
131	4	control	2	0	0	0	0
132	4	control	2	0	0	0	0
133	4	control	2	0	0	0	0
134	4	control	2	-	-	0	0
135	4	control	2	0	0	0	0
136	4	control	2	-	-	0	0
137	4	control	2	0	0	0	0
138	4	control	2	0	0	0	0
139	4	control	2	0	0	0	0
140	4	control	2	0	0	0	0
141	4	control	2	0	0	0	0
142	4	control	2	0	0	0	0
143	4	control	2	0	0	0	0
144	4	control	2	0	0	0	0
145	4	vaccine	2	0	0	24	192
146	4	vaccine	2	0	0	48	384
147	4	vaccine	2	0	0	48	192
148	4	vaccine	2	0	0	24	384
149	4	vaccine	2	0	0	0	192
150	4	vaccine	2	0	0	0	384
151	4	vaccine	2	0	0	24	384
152	4	vaccine	2	0	0	96	384
153	4	vaccine	2	0	0	24	192
154	4	vaccine	2	0	0	24	192
155	4	vaccine	2	0	0	-	-
156	4	vaccine	2	0	0	0	0
157	4	vaccine	2	0	0	24	192
158	4	vaccine	2	0	0	48	96
159	4	vaccine	2	0	0	96	384
160	4	vaccine	2	0	0	0	192
161	4	vaccine	2	0	0	96	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
162	4	vaccine	2	0	0	0	0
163	4	vaccine	2	0	0	24	48
164	4	vaccine	2	0	0	24	384
165	4	vaccine	2	0	0	24	384
166	4	vaccine	2	0	0	96	48
167	4	vaccine	2	0	0	24	96
168	4	vaccine	2	0	0	24	192
169	4	vaccine	2	0	0	0	192
170	4	vaccine	2	0	0	24	192
171	4	vaccine	2	0	0	0	24
119	4	control	3	0	0	0	0
120	4	control	3	0	0	0	0
121	4	control	3	0	0	0	0
122	4	control	3	0	0	0	0
123	4	control	3	-	-	-	-
124	4	control	3	0	0	0	0
125	4	control	3	0	0	0	0
126	4	control	3	0	0	0	0
127	4	control	3	0	0	0	0
128	4	control	3	0	0	0	0
129	4	control	3	0	0	0	0
130	4	control	3	0	1	0	0
131	4	control	3	0	0	0	0
132	4	control	3	0	0	0	0
133	4	control	3	0	0	0	0
134	4	control	3	0	0	0	0
135	4	control	3	0	0	0	0
136	4	control	3	0	0	0	0
137	4	control	3	0	0	0	0
138	4	control	3	0	0	0	0
139	4	control	3	0	0	0	0
140	4	control	3	0	0	0	0
141	4	control	3	0	0	0	0
142	4	control	3	0	0	0	0
143	4	control	3	0	0	0	0
144	4	control	3	0	0	0	0
145	4	vaccine	3	-	-	-	-
146	4	vaccine	3	0	0	-	-
147	4	vaccine	3	0	0	-	-
148	4	vaccine	3	0	0	-	-
149	4	vaccine	3	-	-	-	-
150	4	vaccine	3	0	0	-	-
151	4	vaccine	3	0	0	-	-
152	4	vaccine	3	0	0	-	-
153	4	vaccine	3	-	-	-	-
154	4	vaccine	3	0	0	-	-
155	4	vaccine	3	0	0	-	-
156	4	vaccine	3	0	0	-	-
157	4	vaccine	3	0	0	-	-
158	4	vaccine	3	0	0	-	-
159	4	vaccine	3	0	0	-	-
160	4	vaccine	3	0	0	-	-
161	4	vaccine	3	0	0	-	-
162	4	vaccine	3	0	0	-	-
163	4	vaccine	3	0	0	-	-
164	4	vaccine	3	0	0	-	-
165	4	vaccine	3	0	0	-	-

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
166	4	vaccine	3	0	0	-	-
167	4	vaccine	3	0	0	-	-
168	4	vaccine	3	0	0	-	-
169	4	vaccine	3	0	0	-	-
170	4	vaccine	3	0	0	-	-
171	4	vaccine	3	0	0	-	-
119	4	control	4	0	0	0	0
120	4	control	4	0	0	0	0
121	4	control	4	0	0	0	0
122	4	control	4	0	1	192	0
123	4	control	4	0	0	0	0
124	4	control	4	0	0	768	0
125	4	control	4	0	0	0	0
126	4	control	4	0	0	0	0
127	4	control	4	0	0	0	0
128	4	control	4	0	1	0	0
129	4	control	4	0	0	0	0
130	4	control	4	0	1	0	0
131	4	control	4	0	0	0	0
132	4	control	4	-	-	0	0
133	4	control	4	0	0	0	0
134	4	control	4	0	0	0	0
135	4	control	4	0	0	0	0
136	4	control	4	0	0	0	0
137	4	control	4	0	0	0	0
138	4	control	4	0	0	0	0
139	4	control	4	0	0	0	0
140	4	control	4	0	0	0	0
141	4	control	4	0	0	0	0
142	4	control	4	0	0	0	0
143	4	control	4	0	0	0	0
144	4	control	4	0	0	0	0
145	4	vaccine	4	0	0	-	-
146	4	vaccine	4	0	0	-	-
147	4	vaccine	4	0	0	-	-
148	4	vaccine	4	0	0	-	-
149	4	vaccine	4	0	0	-	-
150	4	vaccine	4	0	0	-	-
151	4	vaccine	4	0	0	-	-
152	4	vaccine	4	0	0	-	-
153	4	vaccine	4	0	0	-	-
154	4	vaccine	4	0	0	-	-
155	4	vaccine	4	0	-	-	-
156	4	vaccine	4	0	0	-	-
157	4	vaccine	4	0	0	-	-
158	4	vaccine	4	0	0	-	-
159	4	vaccine	4	0	0	-	-
160	4	vaccine	4	0	0	-	-
161	4	vaccine	4	0	0	-	-
162	4	vaccine	4	-	-	-	-
163	4	vaccine	4	0	0	-	-
164	4	vaccine	4	0	0	-	-
165	4	vaccine	4	0	0	-	-
166	4	vaccine	4	0	0	-	-
167	4	vaccine	4	0	0	-	-
168	4	vaccine	4	0	0	-	-
169	4	vaccine	4	0	0	-	-

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
170	4	vaccine	4	0	0	-	-
171	4	vaccine	4	-	-	-	-
172	5	control	1	0	0	0	0
173	5	control	1	0	0	0	0
174	5	control	1	0	0	0	0
175	5	control	1	0	0	0	0
176	5	control	1	-	-	0	0
177	5	control	1	0	0	0	0
178	5	control	1	0	0	0	0
179	5	control	1	0	0	0	0
180	5	control	1	0	0	0	0
181	5	control	1	0	0	0	0
182	5	control	1	0	0	0	0
183	5	control	1	0	0	0	0
184	5	control	1	0	0	0	0
185	5	control	1	0	0	0	0
186	5	control	1	0	0	0	0
187	5	control	1	0	0	0	0
188	5	control	1	0	0	0	0
189	5	control	1	0	0	0	0
190	5	control	1	0	0	0	0
191	5	control	1	0	0	0	0
192	5	control	1	0	0	0	0
193	5	control	1	0	0	0	0
194	5	control	1	0	0	0	0
195	5	control	1	-	-	0	0
196	5	vaccine	1	0	0	0	0
197	5	vaccine	1	0	0	0	0
198	5	vaccine	1	0	0	0	0
199	5	vaccine	1	0	0	0	0
200	5	vaccine	1	0	0	0	0
201	5	vaccine	1	0	0	0	0
202	5	vaccine	1	0	0	0	0
203	5	vaccine	1	0	0	0	0
204	5	vaccine	1	0	0	0	0
205	5	vaccine	1	0	0	0	0
206	5	vaccine	1	-	-	0	0
207	5	vaccine	1	0	0	0	0
208	5	vaccine	1	0	0	24	0
209	5	vaccine	1	0	0	0	0
210	5	vaccine	1	0	0	0	0
211	5	vaccine	1	0	0	0	0
212	5	vaccine	1	0	0	0	0
213	5	vaccine	1	-	-	0	0
214	5	vaccine	1	0	0	0	0
215	5	vaccine	1	0	0	0	0
216	5	vaccine	1	0	0	0	0
217	5	vaccine	1	0	0	0	0
218	5	vaccine	1	0	0	0	0
219	5	vaccine	1	0	0	0	0
220	5	vaccine	1	0	0	0	0
172	5	control	2	0	0	0	0
173	5	control	2	0	0	0	0
174	5	control	2	0	0	0	0
175	5	control	2	0	0	0	0
176	5	control	2	0	0	0	0
177	5	control	2	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
178	5	control	2	0	0	0	0
179	5	control	2	0	0	0	0
180	5	control	2	0	0	0	0
181	5	control	2	0	0	0	0
182	5	control	2	0	0	0	0
183	5	control	2	0	0	0	0
184	5	control	2	-	-	-	-
185	5	control	2	0	0	0	0
186	5	control	2	0	0	0	0
187	5	control	2	0	0	0	0
188	5	control	2	0	0	0	0
189	5	control	2	0	0	0	0
190	5	control	2	0	0	0	0
191	5	control	2	0	0	0	0
192	5	control	2	0	0	0	0
193	5	control	2	0	0	0	0
194	5	control	2	0	0	0	0
195	5	control	2	-	-	-	-
196	5	vaccine	2	0	0	0	24
197	5	vaccine	2	0	0	0	192
198	5	vaccine	2	0	0	0	96
199	5	vaccine	2	0	0	0	96
200	5	vaccine	2	0	0	0	96
201	5	vaccine	2	0	0	24	96
202	5	vaccine	2	0	0	0	96
203	5	vaccine	2	0	0	0	48
204	5	vaccine	2	0	0	0	96
205	5	vaccine	2	0	0	96	192
206	5	vaccine	2	0	0	24	48
207	5	vaccine	2	0	0	0	96
208	5	vaccine	2	0	0	0	192
209	5	vaccine	2	0	0	24	192
210	5	vaccine	2	0	0	0	192
211	5	vaccine	2	0	0	0	192
212	5	vaccine	2	0	0	24	24
213	5	vaccine	2	0	0	24	24
214	5	vaccine	2	0	0	24	96
215	5	vaccine	2	-	-	-	-
216	5	vaccine	2	0	0	24	96
217	5	vaccine	2	-	-	-	-
218	5	vaccine	2	-	-	-	-
219	5	vaccine	2	0	0	24	384
220	5	vaccine	2	0	0	0	96
172	5	control	3	0	0	0	0
173	5	control	3	0	0	0	0
174	5	control	3	0	0	0	0
175	5	control	3	0	0	0	0
176	5	control	3	0	0	-	-
177	5	control	3	0	0	0	0
178	5	control	3	0	0	0	0
179	5	control	3	0	0	0	0
180	5	control	3	0	0	0	0
181	5	control	3	0	0	0	0
182	5	control	3	0	0	0	0
183	5	control	3	0	0	0	0
184	5	control	3	0	0	0	0
185	5	control	3	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
186	5	control	3	0	0	0	0
187	5	control	3	0	0	0	0
188	5	control	3	0	0	0	0
189	5	control	3	0	0	0	0
190	5	control	3	0	0	0	0
191	5	control	3	0	0	0	0
192	5	control	3	0	0	0	0
193	5	control	3	0	0	0	0
194	5	control	3	0	0	0	0
195	5	control	3	-	-	0	0
196	5	vaccine	3	0	0	-	-
197	5	vaccine	3	0	0	-	-
198	5	vaccine	3	0	0	-	-
199	5	vaccine	3	0	0	-	-
200	5	vaccine	3	0	0	-	-
201	5	vaccine	3	0	0	-	-
202	5	vaccine	3	0	0	-	-
203	5	vaccine	3	0	0	-	-
204	5	vaccine	3	0	0	-	-
205	5	vaccine	3	0	0	-	-
206	5	vaccine	3	0	0	-	-
207	5	vaccine	3	0	0	-	-
208	5	vaccine	3	0	0	-	-
209	5	vaccine	3	0	0	-	-
210	5	vaccine	3	0	0	-	-
211	5	vaccine	3	0	0	-	-
212	5	vaccine	3	0	0	-	-
213	5	vaccine	3	0	0	-	-
214	5	vaccine	3	0	0	-	-
215	5	vaccine	3	0	0	-	-
216	5	vaccine	3	0	0	-	-
217	5	vaccine	3	0	0	-	-
218	5	vaccine	3	-	-	-	-
219	5	vaccine	3	0	0	-	-
220	5	vaccine	3	0	0	-	-
172	5	control	4	0	0	0	0
173	5	control	4	-	-	0	0
174	5	control	4	0	0	0	0
175	5	control	4	0	0	0	0
176	5	control	4	0	0	0	0
177	5	control	4	0	0	0	0
178	5	control	4	-	-	-	-
179	5	control	4	0	0	0	0
180	5	control	4	0	0	0	0
181	5	control	4	0	0	0	0
182	5	control	4	0	0	0	0
183	5	control	4	0	0	0	0
184	5	control	4	0	0	0	0
185	5	control	4	0	0	0	0
186	5	control	4	.	.	0	0
187	5	control	4	0	0	0	0
188	5	control	4	0	0	0	0
189	5	control	4	0	0	0	0
190	5	control	4	0	0	0	0
191	5	control	4	0	0	0	0
192	5	control	4	0	0	0	0
193	5	control	4	0	0	0	0

Deer no.	Farm no.	Status	Period	Culture	PCR	Hardjobovis	Pomona
194	5	control	4	0	0	-	-
195	5	control	4	0	0	0	0
196	5	vaccine	4	0	0	-	-
197	5	vaccine	4	0	0	-	-
198	5	vaccine	4	0	0	-	-
199	5	vaccine	4	0	0	-	-
200	5	vaccine	4	-	-	-	-
201	5	vaccine	4	0	0	-	-
202	5	vaccine	4	0	0	-	-
203	5	vaccine	4	0	0	-	-
204	5	vaccine	4	0	0	-	-
205	5	vaccine	4	0	0	-	-
206	5	vaccine	4	0	0	-	-
207	5	vaccine	4	0	0	-	-
208	5	vaccine	4	0	0	-	-
209	5	vaccine	4	0	0	-	-
210	5	vaccine	4	0	0	-	-
211	5	vaccine	4	-	-	-	-
212	5	vaccine	4	0	0	-	-
213	5	vaccine	4	0	0	-	-
214	5	vaccine	4	0	0	-	-
215	5	vaccine	4	0	0	-	-
216	5	vaccine	4	0	0	-	-
217	5	vaccine	4	0	0	-	-
218	5	vaccine	4	0	0	-	-
219	5	vaccine	4	0	0	-	-
220	5	vaccine	4	-	-	-	-

Appendix 2c. Raw data of urine shedding in no-streptomycin control deer

(Period: 2 = May, 4 = November) (Culture and PCR: 0 = negative, 1 = positive)

Deer no.	Farm no.	Period	Culture	PCR
1	1	2	0	0
2	1	2	0	0
3	1	2	0	0
4	1	2	0	0
5	1	2	0	0
6	1	2	0	0
7	1	2	0	0
8	1	2	0	0
9	1	2	0	0
10	1	2	0	0
11	1	2	0	0
12	1	2	0	0
13	1	2	0	0
14	1	2	0	0
15	1	2	0	0
16	1	2	0	0
17	1	2	0	0
18	1	2	0	0
19	1	2	0	0
20	1	4	1	1
21	1	4	1	1
22	1	4	1	1
23	1	4	0	1
24	1	4	0	1
25	1	4	0	1
26	1	4	0	0
27	1	4	0	1
28	1	4	0	1
29	1	4	1	1
30	1	4	0	1
31	1	4	0	1
32	1	4	0	1
33	1	4	0	1
34	1	4	1	1
35	1	4	0	0
36	1	4	0	0
37	1	4	1	1
38	2	2	0	0
39	2	2	0	0
40	2	2	0	0
41	2	2	0	0
42	2	2	0	0
43	2	2	0	0
44	2	2	0	0
45	2	2	0	0
46	2	2	0	0
47	2	2	0	0
48	2	2	0	0
49	2	2	0	0
50	2	2	0	0
51	2	2	0	0
52	2	2	0	0
53	2	2	0	0
54	2	2	0	0

Deer no.	Farm no.	Period	Culture	PCR
55	2	2	0	0
56	2	2	0	0
57	2	2	0	0
58	2	4	0	0
59	2	4	0	0
60	2	4	0	0
61	2	4	0	0
62	2	4	0	0
63	2	4	0	0
64	2	4	0	0
65	2	4	0	0
66	2	4	0	0
67	2	4	0	0
68	2	4	0	0
69	2	4	0	0
70	2	4	0	0
71	2	4	0	0
72	2	4	0	0
73	2	4	0	0
74	2	4	0	0
75	2	4	0	0
76	2	4	0	0
77	2	4	0	0
78	3	2	0	0
79	3	2	0	0
80	3	2	0	0
81	3	2	0	0
82	3	2	0	0
83	3	2	0	0
84	3	2	0	0
85	3	2	0	0
86	3	2	0	0
87	3	2	0	0
88	3	2	0	0
89	3	2	0	0
90	3	2	0	0
91	3	2	0	0
92	3	2	0	0
93	3	2	0	0
94	3	2	0	0
95	3	2	0	0
96	3	2	0	0
97	3	2	0	0
98	3	4	0	0
99	3	4	0	0
100	3	4	0	0
101	3	4	0	0
102	3	4	0	0
103	3	4	0	0
104	3	4	0	0
105	3	4	0	0
106	3	4	0	0
107	3	4	0	0
108	3	4	0	0
109	3	4	0	0
110	3	4	0	0
111	3	4	0	0

Deer no.	Farm no.	Period	Culture	PCR
112	3	4	0	0
113	3	4	0	0
114	3	4	0	0
115	3	4	0	0
116	3	4	0	0
117	4	2	0	0
118	4	2	0	0
119	4	2	0	0
120	4	2	0	0
121	4	2	0	0
122	4	2	0	0
123	4	2	0	0
124	4	2	0	0
125	4	2	0	0
126	4	2	0	0
127	4	2	0	0
128	4	2	0	0
129	4	2	0	0
130	4	2	0	0
131	4	2	0	0
132	4	2	0	0
133	4	2	0	0
134	4	2	0	0
135	4	2	0	0
136	4	4	0	0
137	4	4	0	0
138	4	4	0	0
139	4	4	0	0
140	4	4	0	0
141	4	4	0	0
142	4	4	0	0
143	4	4	0	0
144	4	4	0	0
145	4	4	0	0
146	4	4	0	0
147	4	4	0	0
148	4	4	0	1
149	4	4	0	0
150	4	4	0	0
151	4	4	0	0
152	4	4	0	1
153	4	4	0	1
154	4	4	0	1
155	4	4	0	1
156	5	2	0	0
157	5	2	0	0
158	5	2	0	0
159	5	2	0	0
160	5	2	0	0
161	5	2	0	0
162	5	2	0	0
163	5	2	0	0
164	5	2	0	0
165	5	2	0	0
166	5	2	0	0
167	5	2	0	0
168	5	2	0	0

Deer no.	Farm no.	Period	Culture	PCR
169	5	2	0	0
170	5	2	0	0
171	5	2	0	0
172	5	2	0	0
173	5	2	0	0
174	5	2	0	0
175	5	2	0	0
176	5	4	0	0
177	5	4	0	0
178	5	4	0	0
179	5	4	0	0
180	5	4	0	0
181	5	4	0	0
182	5	4	0	0
183	5	4	0	0
184	5	4	0	0
185	5	4	0	0
186	5	4	0	0
187	5	4	0	0
188	5	4	0	0
189	5	4	0	0
190	5	4	0	0
191	5	4	0	0
192	5	4	0	0
193	5	4	0	0
194	5	4	0	0
195	5	4	0	0

Appendix 3a. Raw data of maternal status and serology.

(Period: 1 = October'07 (carriage of foetus to term), 2 = March'08 (lactating at weaning)) (Mother status: 0 = No, 1 = yes)

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
1	1	Control	1	1	192	0
2	1	Control	1	1	96	0
3	1	Control	1	1	0	0
4	1	Control	1	1	0	0
5	1	Control	1	1	0	0
6	1	Control	1	1	0	0
7	1	Control	1	0	192	0
8	1	Control	1	1	384	0
9	1	Control	1	1	0	0
10	1	Control	1	1	96	0
11	1	Control	1	1	192	0
12	1	Control	1	1	0	0
13	1	Control	1	1	-	-
14	1	Control	1	1	0	0
15	1	Control	1	1	192	0
16	1	Control	1	1	0	0
17	1	Control	1	1	0	0
18	1	Vaccine	1	1	-	-
19	1	Vaccine	1	1	-	-
20	1	Vaccine	1	1	-	-
21	1	Vaccine	1	1	-	-
22	1	Vaccine	1	1	-	-
23	1	Vaccine	1	1	-	-
24	1	Vaccine	1	1	-	-
25	1	Vaccine	1	1	-	-
26	1	Vaccine	1	1	-	-
27	1	Vaccine	1	1	-	-
28	1	Vaccine	1	1	-	-
29	1	Vaccine	1	1	-	-
30	1	Vaccine	1	1	-	-
31	1	Vaccine	1	1	-	-
32	1	Vaccine	1	1	-	-
33	1	Vaccine	1	1	-	-
34	1	Vaccine	1	1	-	-
35	1	Vaccine	1	1	-	-
1	1	Control	2	1	96	0
2	1	Control	2	0	48	0
3	1	Control	2	1	0	0
4	1	Control	2	1	0	0
5	1	Control	2	0	0	0
6	1	Control	2	1	0	0
7	1	Control	2	0	-	-
8	1	Control	2	1	384	48
9	1	Control	2	0	0	0
10	1	Control	2	1	192	0
11	1	Control	2	1	192	0
12	1	Control	2	1	0	0
13	1	Control	2	1	48	0
14	1	Control	2	1	96	0
15	1	Control	2	1	0	0
16	1	Control	2	1	0	0
17	1	Control	2	1	-	-
18	1	Vaccine	2	1	-	-
19	1	Vaccine	2	1	-	-
20	1	Vaccine	2	0	-	-

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
21	1	Vaccine	2	1	-	-
22	1	Vaccine	2	0	-	-
23	1	Vaccine	2	1	-	-
24	1	Vaccine	2	1	-	-
25	1	Vaccine	2	1	-	-
26	1	Vaccine	2	1	-	-
27	1	Vaccine	2	0	-	-
28	1	Vaccine	2	1	-	-
29	1	Vaccine	2	1	-	-
30	1	Vaccine	2	1	-	-
31	1	Vaccine	2	0	-	-
32	1	Vaccine	2	1	-	-
33	1	Vaccine	2	1	-	-
34	1	Vaccine	2	1	-	-
35	1	Vaccine	2	1	-	-
36	2	Control	1	1	24	0
37	2	Control	1	1	24	0
38	2	Control	1	1	24	0
39	2	Control	1	1	24	0
40	2	Control	1	1	24	0
41	2	Control	1	1	24	0
42	2	Control	1	1	24	0
43	2	Control	1	1	48	0
44	2	Control	1	1	24	0
45	2	Control	1	1	24	0
46	2	Control	1	1	-	-
47	2	Control	1	1	0	0
48	2	Control	1	1	0	0
49	2	Control	1	1	48	0
50	2	Control	1	1	-	-
51	2	Control	1	1	-	-
52	2	Control	1	1	24	0
53	2	Control	1	1	24	0
54	2	Control	1	1	0	0
55	2	Control	1	1	24	0
56	2	Control	1	1	24	0
57	2	Control	1	1	0	0
58	2	Control	1	1	96	0
59	2	Control	1	1	0	0
60	2	Vaccine	1	1	-	-
61	2	Vaccine	1	1	-	-
62	2	Vaccine	1	1	-	-
63	2	Vaccine	1	1	-	-
64	2	Vaccine	1	1	-	-
65	2	Vaccine	1	1	-	-
66	2	Vaccine	1	1	-	-
67	2	Vaccine	1	1	-	-
68	2	Vaccine	1	1	-	-
69	2	Vaccine	1	1	-	-
70	2	Vaccine	1	1	-	-
71	2	Vaccine	1	1	-	-
72	2	Vaccine	1	1	-	-
73	2	Vaccine	1	1	-	-
74	2	Vaccine	1	1	-	-
75	2	Vaccine	1	1	-	-
76	2	Vaccine	1	1	-	-
77	2	Vaccine	1	1	-	-

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
78	2	Vaccine	1	1	-	-
36	2	Control	2	0	-	-
37	2	Control	2	1	-	-
38	2	Control	2	1	24	0
39	2	Control	2	1	-	-
40	2	Control	2	1	-	-
41	2	Control	2	1	24	0
42	2	Control	2	1	24	0
43	2	Control	2	1	-	-
44	2	Control	2	0	-	-
45	2	Control	2	1	-	-
46	2	Control	2	0	0	0
47	2	Control	2	1	24	0
48	2	Control	2	1	-	-
49	2	Control	2	1	-	-
50	2	Control	2	1	-	-
51	2	Control	2	1	48	0
52	2	Control	2	1	-	-
53	2	Control	2	1	-	-
54	2	Control	2	1	-	-
55	2	Control	2	1	-	-
56	2	Control	2	1	-	-
57	2	Control	2	1	-	-
58	2	Control	2	1	48	0
59	2	Control	2	1	-	-
60	2	Vaccine	2	1	-	-
61	2	Vaccine	2	1	-	-
62	2	Vaccine	2	1	-	-
63	2	Vaccine	2	1	-	-
64	2	Vaccine	2	1	-	-
65	2	Vaccine	2	1	-	-
66	2	Vaccine	2	1	-	-
67	2	Vaccine	2	0	-	-
68	2	Vaccine	2	1	-	-
69	2	Vaccine	2	1	-	-
70	2	Vaccine	2	1	-	-
71	2	Vaccine	2	1	-	-
72	2	Vaccine	2	1	-	-
73	2	Vaccine	2	1	-	-
74	2	Vaccine	2	1	-	-
75	2	Vaccine	2	1	-	-
76	2	Vaccine	2	1	-	-
77	2	Vaccine	2	1	-	-
78	2	Vaccine	2	1	-	-
79	3	Control	1	1	24	0
80	3	Control	1	0	0	0
81	3	Control	1	1	48	0
82	3	Control	1	1	24	0
83	3	Control	1	1	48	0
84	3	Control	1	1	24	0
85	3	Control	1	1	48	0
86	3	Control	1	1	24	0
87	3	Control	1	1	-	-
88	3	Control	1	1	0	0
89	3	Control	1	1	48	0
90	3	Control	1	1	96	0
91	3	Control	1	1	0	0

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
92	3	Control	1	1	0	0
93	3	Control	1	1	-	-
94	3	Control	1	1	24	0
95	3	Control	1	1	24	0
96	3	Control	1	1	48	0
97	3	Control	1	1	-	-
98	3	Control	1	1	48	0
99	3	Control	1	1	24	0
100	3	Control	1	1	48	0
101	3	Control	1	1	24	0
102	3	Control	1	1	24	0
103	3	Control	1	1	24	0
104	3	Control	1	1	24	0
105	3	Control	1	1	48	0
106	3	Control	1	1	24	0
107	3	Control	1	1	48	0
108	3	Control	1	1	48	0
109	3	Control	1	1	48	0
110	3	Control	1	1	24	0
111	3	Vaccine	1	1	-	-
112	3	Vaccine	1	1	-	-
113	3	Vaccine	1	1	-	-
114	3	Vaccine	1	1	-	-
115	3	Vaccine	1	1	-	-
116	3	Vaccine	1	1	-	-
117	3	Vaccine	1	1	-	-
118	3	Vaccine	1	1	-	-
119	3	Vaccine	1	1	-	-
120	3	Vaccine	1	1	-	-
121	3	Vaccine	1	1	-	-
122	3	Vaccine	1	1	-	-
123	3	Vaccine	1	1	-	-
124	3	Vaccine	1	1	-	-
125	3	Vaccine	1	1	-	-
126	3	Vaccine	1	1	-	-
127	3	Vaccine	1	1	-	-
128	3	Vaccine	1	1	-	-
129	3	Vaccine	1	1	-	-
130	3	Vaccine	1	1	-	-
131	3	Vaccine	1	1	-	-
132	3	Vaccine	1	1	-	-
133	3	Vaccine	1	1	-	-
134	3	Vaccine	1	1	-	-
135	3	Vaccine	1	1	-	-
136	3	Vaccine	1	1	-	-
137	3	Vaccine	1	1	-	-
138	3	Vaccine	1	1	-	-
139	3	Vaccine	1	1	-	-
140	3	Vaccine	1	1	-	-
141	3	Vaccine	1	1	-	-
79	3	Control	2	1	24	0
80	3	Control	2	0	-	-
81	3	Control	2	1	24	0
82	3	Control	2	1	24	0
83	3	Control	2	1	48	0
84	3	Control	2	1	48	0
85	3	Control	2	1	24	0

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
86	3	Control	2	1	48	0
87	3	Control	2	1	48	0
88	3	Control	2	1	24	0
89	3	Control	2	1	48	0
90	3	Control	2	1	48	0
91	3	Control	2	1	0	0
92	3	Control	2	1	0	0
93	3	Control	2	0	-	-
94	3	Control	2	1	24	0
95	3	Control	2	1	24	0
96	3	Control	2	1	48	0
97	3	Control	2	1	0	0
98	3	Control	2	1	48	0
99	3	Control	2	1	0	0
100	3	Control	2	0	-	-
101	3	Control	2	1	48	0
102	3	Control	2	1	24	0
103	3	Control	2	0	24	0
104	3	Control	2	1	0	0
105	3	Control	2	1	48	0
106	3	Control	2	1	24	0
107	3	Control	2	1	48	0
108	3	Control	2	0	48	0
109	3	Control	2	1	24	0
110	3	Control	2	1	24	0
111	3	Vaccine	2	1	-	-
112	3	Vaccine	2	1	-	-
113	3	Vaccine	2	0	-	-
114	3	Vaccine	2	1	-	-
115	3	Vaccine	2	1	-	-
116	3	Vaccine	2	1	-	-
117	3	Vaccine	2	1	-	-
118	3	Vaccine	2	1	-	-
119	3	Vaccine	2	1	-	-
120	3	Vaccine	2	1	-	-
121	3	Vaccine	2	1	-	-
122	3	Vaccine	2	1	-	-
123	3	Vaccine	2	1	-	-
124	3	Vaccine	2	1	-	-
125	3	Vaccine	2	0	-	-
126	3	Vaccine	2	1	-	-
127	3	Vaccine	2	1	-	-
128	3	Vaccine	2	1	-	-
129	3	Vaccine	2	1	-	-
130	3	Vaccine	2	1	-	-
131	3	Vaccine	2	1	-	-
132	3	Vaccine	2	1	-	-
133	3	Vaccine	2	1	-	-
134	3	Vaccine	2	1	-	-
135	3	Vaccine	2	1	-	-
136	3	Vaccine	2	1	-	-
137	3	Vaccine	2	1	-	-
138	3	Vaccine	2	1	-	-
139	3	Vaccine	2	1	-	-
140	3	Vaccine	2	1	-	-
141	3	Vaccine	2	1	-	-
142	4	Control	1	1	24	0

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
143	4	Control	1	1	24	0
144	4	Control	1	1	0	0
145	4	Control	1	1	48	0
146	4	Control	1	1	0	0
147	4	Control	1	1	0	0
148	4	Control	1	1	0	0
149	4	Control	1	1	0	0
150	4	Control	1	1	0	0
151	4	Control	1	1	0	0
152	4	Control	1	1	0	0
153	4	Control	1	1	0	0
154	4	Control	1	1	0	0
155	4	Control	1	1	24	0
156	4	Control	1	1	0	0
157	4	Control	1	1	24	0
158	4	Control	1	1	24	0
159	4	Control	1	1	192	0
160	4	Control	1	1	0	0
161	4	Control	1	1	48	0
162	4	Control	1	1	0	0
163	4	Control	1	0	48	0
164	4	Control	1	1	-	-
165	4	Control	1	1	0	0
166	4	Control	1	1	24	0
167	4	Control	1	1	-	-
168	4	Control	1	1	24	0
169	4	Control	1	1	24	0
170	4	Control	1	1	0	0
171	4	Control	1	1	0	0
172	4	Control	1	1	0	0
173	4	Control	1	1	0	0
174	4	Control	1	1	24	0
175	4	Vaccine	1	1	-	-
176	4	Vaccine	1	1	-	-
177	4	Vaccine	1	1	-	-
178	4	Vaccine	1	1	-	-
179	4	Vaccine	1	1	-	-
180	4	Vaccine	1	1	-	-
181	4	Vaccine	1	1	-	-
182	4	Vaccine	1	1	-	-
183	4	Vaccine	1	1	-	-
184	4	Vaccine	1	1	-	-
185	4	Vaccine	1	1	-	-
186	4	Vaccine	1	1	-	-
187	4	Vaccine	1	1	-	-
188	4	Vaccine	1	1	-	-
189	4	Vaccine	1	1	-	-
190	4	Vaccine	1	1	-	-
191	4	Vaccine	1	1	-	-
192	4	Vaccine	1	1	-	-
193	4	Vaccine	1	1	-	-
194	4	Vaccine	1	1	-	-
195	4	Vaccine	1	1	-	-
196	4	Vaccine	1	1	-	-
197	4	Vaccine	1	1	-	-
198	4	Vaccine	1	1	-	-
199	4	Vaccine	1	1	-	-

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
200	4	Vaccine	1	1	-	-
201	4	Vaccine	1	1	-	-
202	4	Vaccine	1	1	-	-
203	4	Vaccine	1	1	-	-
142	4	Control	2	0	0	0
143	4	Control	2	1	24	0
144	4	Control	2	1	0	0
145	4	Control	2	1	24	0
146	4	Control	2	0	0	0
147	4	Control	2	1	0	0
148	4	Control	2	1	24	0
149	4	Control	2	1	0	0
150	4	Control	2	1	0	0
151	4	Control	2	1	24	0
152	4	Control	2	1	48	0
153	4	Control	2	1	0	0
154	4	Control	2	1	24	0
155	4	Control	2	1	48	0
156	4	Control	2	1	24	0
157	4	Control	2	1	24	0
158	4	Control	2	1	0	0
159	4	Control	2	1	48	0
160	4	Control	2	1	0	0
161	4	Control	2	1	48	0
162	4	Control	2	1	0	0
163	4	Control	2	0	0	0
164	4	Control	2	0	48	0
165	4	Control	2	1	0	0
166	4	Control	2	1	48	0
167	4	Control	2	0	96	0
168	4	Control	2	0	24	0
169	4	Control	2	1	0	0
170	4	Control	2	1	0	0
171	4	Control	2	1	0	0
172	4	Control	2	1	0	0
173	4	Control	2	1	0	0
174	4	Control	2	1	0	0
175	4	Vaccine	2	1	-	-
176	4	Vaccine	2	1	-	-
177	4	Vaccine	2	1	-	-
178	4	Vaccine	2	0	-	-
179	4	Vaccine	2	1	-	-
180	4	Vaccine	2	1	-	-
181	4	Vaccine	2	1	-	-
182	4	Vaccine	2	1	-	-
183	4	Vaccine	2	1	-	-
184	4	Vaccine	2	1	-	-
185	4	Vaccine	2	1	-	-
186	4	Vaccine	2	1	-	-
187	4	Vaccine	2	1	-	-
188	4	Vaccine	2	1	-	-
189	4	Vaccine	2	1	-	-
190	4	Vaccine	2	1	-	-
191	4	Vaccine	2	0	-	-
192	4	Vaccine	2	1	-	-
193	4	Vaccine	2	1	-	-
194	4	Vaccine	2	1	-	-

Deer no.	Farm no.	Status	Period	Mother status	Hardjobovis	Pomona
195	4	Vaccine	2	1	-	-
196	4	Vaccine	2	0	-	-
197	4	Vaccine	2	1	-	-
198	4	Vaccine	2	0	-	-
199	4	Vaccine	2	1	-	-
200	4	Vaccine	2	1	-	-
201	4	Vaccine	2	1	-	-
202	4	Vaccine	2	1	-	-
203	4	Vaccine	2	1	-	-

Appendix 3b. Raw data of urine shedding in no-streptomycin control deer.

(Period: 1 = June'07, 2 = March'08) (Culture and PCR: 0 = negative, 1 = positive)

Deer no.	Farm no.	Period	Culture	PCR
1	1	1	0	0
2	1	1	0	0
3	1	1	0	0
4	1	1	0	0
5	1	1	0	1
6	1	1	0	0
7	1	1	0	0
8	1	1	0	1
9	1	1	0	0
10	1	1	0	0
11	1	1	0	0
12	1	1	0	1
13	1	1	0	0
14	1	1	0	0
15	1	1	0	0
16	1	1	0	0
17	1	1	0	0
18	1	1	0	1
19	1	1	0	0
20	1	2	0	0
21	1	2	0	0
22	1	2	0	1
23	1	2	0	0
24	1	2	0	0
25	1	2	0	0
26	1	2	0	0
27	1	2	0	0
28	1	2	0	0
29	1	2	0	0
30	1	2	0	0
31	1	2	0	0
32	1	2	0	1
33	1	2	0	0
34	1	2	0	0
35	1	2	0	0
36	1	2	0	0
37	1	2	0	0
38	1	2	0	0
39	1	2	0	0
40	1	2	0	0
41	2	1	0	0
42	2	1	0	0
43	2	1	0	0
44	2	1	0	0
45	2	1	0	0
46	2	1	0	0
47	2	1	0	0
48	2	1	0	0
49	2	1	0	0
50	2	1	0	0
51	2	1	0	0
52	2	1	0	0
53	2	1	0	0
54	2	1	0	0

Deer no.	Farm no.	Period	Culture	PCR
55	2	1	0	0
56	2	1	0	0
57	2	1	0	0
58	2	1	0	0
59	2	1	0	0
60	2	1	0	0
61	2	2	0	0
62	2	2	0	0
63	2	2	0	0
64	2	2	0	0
65	2	2	0	0
66	2	2	0	0
67	2	2	0	0
68	2	2	0	0
69	2	2	0	0
70	2	2	0	0
71	2	2	0	0
72	2	2	0	0
73	2	2	0	0
74	2	2	0	0
75	2	2	0	0
76	2	2	0	0
77	2	2	0	0
78	2	2	0	0
79	2	2	0	0
80	2	2	0	0
81	3	1	0	0
82	3	1	0	0
83	3	1	0	0
84	3	1	0	0
85	3	1	0	0
86	3	1	0	0
87	3	1	0	0
88	3	1	0	0
89	3	1	0	0
90	3	1	0	0
91	3	1	0	0
92	3	1	0	0
93	3	1	0	0
94	3	1	0	0
95	3	1	0	0
96	3	1	0	0
97	3	1	0	0
98	3	1	0	0
99	3	1	0	0
100	3	1	0	0
101	3	2	0	0
102	3	2	0	0
103	3	2	0	0
104	3	2	0	0
105	3	2	0	0
106	3	2	0	0
107	3	2	0	0
108	3	2	0	0
109	3	2	0	0
110	3	2	0	0
111	3	2	0	0

Deer no.	Farm no.	Period	Culture	PCR
112	3	2	0	0
113	3	2	0	0
114	3	2	0	0
115	3	2	0	0
116	3	2	0	0
117	3	2	0	0
118	3	2	0	0
119	3	2	0	0
120	3	2	0	0
121	4	1	0	0
122	4	1	0	0
123	4	1	0	0
124	4	1	0	0
125	4	1	0	0
126	4	1	0	0
127	4	1	0	0
128	4	1	0	0
129	4	1	0	0
130	4	1	0	0
131	4	1	0	0
132	4	1	0	0
133	4	1	0	0
134	4	1	0	0
135	4	1	0	0
136	4	1	0	0
137	4	1	0	0
138	4	1	0	0
139	4	1	0	0
140	4	1	0	0
141	4	2	0	0
142	4	2	0	1
143	4	2	0	0
144	4	2	0	0
145	4	2	0	0
146	4	2	0	0
147	4	2	0	0
148	4	2	0	1
149	4	2	0	0
150	4	2	0	0
151	4	2	0	0
152	4	2	0	1
153	4	2	0	1
154	4	2	0	1
155	4	2	0	1
156	5	2	0	0
157	5	2	0	0
158	5	2	0	0
159	5	2	0	0
160	5	2	0	0

Appendix 4a. Farm questionnaire.

Massey University

Mixed-species farm leptospirosis survey project

Farm owner/manager questionnaire

Section A: General information

Farm name:

……

Contact Person (Owner or Manager):

……

Postal address:

……

Farm location:

……

Tel: ……Mobile: ……Email: ……

Section B: Stock number/Breed

Please fill in your current number and breed of the animals in each species/class in the following table.

Deer	Number		Breed	**Cattle**	Number		Breed	**Sheep**	Number		Breed
	M	F			M	F			M	F	
Weaner (0-12 M)				Calf (0-12 M)				Lamb (0-12 M)			
Yearling (12-24 M)				Yearling (12-24 M)				Hogget (12-24 M)			
Hinds (>24 M)				Cow (>24 M)				Ewe (>24 M)			
Stags (>24 M)				Bull (>24 M)				Ram (>24 M)			

Other species (please describe)…… Total number……

Section C: Farm geography

Total area of farm: …… acre/ha

Effective area of farm: …… acre/ha

Deer fenced area of farm: …… acre/ha

Number of Paddocks.....

Number of paddocks with deer fenced.....

Source of water: Troughs ☐ Dams ☐ Streams ☐ Irrigation ditches ☐

Others ☐ (please describe).....

Please obtain your farm map (print or photo). Describe and estimate (%) the land type (e.g. hill country, downland, flat), areas that would collect drainage (ponds, streams, irrigation ditches, water wells) and identify animal species/class usually grazing in each area.

Section D: Purpose of farms

Please cross (×) your purposes of each livestock on farm as appropriate (can be > 1)

Deer		**Cattle**		**Sheep**	
Breeding		Breeding		Breeding	
Finishing		Finishing		Finishing	
Velvet		Dairy		Wool	
Venison		Beef		Mutton	
Other(describe)		Other(describe)		Other(describe)	

If you are a finishing farm, do you finish your own-bred stock only or buy in animals?

Own stock ☐ Buy in ☐

If you buy in, have you brought in any stock to finish during the past year (Aug 07- Jul 08)? Yes ☐ No ☐

If yes, please fill in the details in the table below as appropriate.

Mob number	**Buy in animals**			**Time period (Months)**	**Animal source**
	Species	**Class**	**No. of animal**		

Section E: Grazing management

Did either of your animal species cross-graze (same paddock, different time) or co-graze (same paddock, same time) during the past year (Aug 07- Jul 08)? Yes ☐ No ☐

If yes, please cross (×) the type of animal grazing in the table below as appropriate (leave it blank if there are no cross/co-graze).

Animal Species	Winter		Spring		Summer		Autumn	
	Cross	Co	Cross	Co	Cross	Co	Cross	Co
Deer + Sheep								
Deer + Cattle								
Sheep + Cattle								
Deer + Sheep + Cattle								

Did any of your animals graze off-farm during the past year (Aug 07- Jul 08)?
Yes ☐ No ☐

If yes, please fill in the detail in the table below.

Your livestock No. / Detail		Species your livestock graze after or with			
		Deer	Cattle	Sheep	None
1	Animal species				
	Number				
	Period (months)				
2	Animal species				
	Number				
	Period (months)				
3	Animal species				
	Number				
	Period (months)				

Do you share-graze any stock not belonging to you on your paddocks? Yes ☐ No ☐
If yes, please fill in the details in **section I**.

Section F: Herd health

Please indicate mortality rate in each animal species/class during the past year (Aug 07- Jul 08) in the following table.

Species	Class	Mortality Rate (%)
Deer	Weaner	
	Yearling	
	Hinds	
	Stags	
Cattle	Calf	
	Yearling	
	Cow	
	Bull	
Sheep	Lamb	
	Hogget	
	Ewe	
	Ram	

Please cross (×) the type of vaccine administered to your animal species/class during the past year (Aug 07- Jul 08).

Vaccination regime	Deer				Cattle				Sheep			
	Weaner	Yearling	Hinds	Stags	Calf	Yearling	Cows	Bulls	Lamb	Hogget	Ewes	Rams
No Vaccine												
5 in 1 (Clostridia)												
7 in 1 (Clostridia,Lepto)												
Yersinia												
Lepto												
Tetanus												
Salmonella												
BVD												
Toxoplasma												
Campylobacter												
Other(describe)												

Have your animals being diagnosed or suspected with any of the following conditions during the past year (Aug 07- Jul 08)? If yes, please cross (×) in the box as appropriate. If no, please leave it blank.

Condition	Deer				Cattle				Sheep			
	Weaner	Yearling	Hinds	Stags	Calf	Yearling	Cows	Bulls	Lamb	Hogget	Ewes	Rams
Yersiniosis												
Leptospirosis												
Salmonellosis												
Malignant Catarrhal Fever												
E. coli diarrhoea												
Bovine Viral Diarrhoea												
Cryptosporidiosis												
Foot lameness												
Pneumonia												
Parapox												
Internal parasitism												
Black leg												
Black disease												
Malignant oedema												
Pulpy kidney												
Tetanus												
Other(describe)												

Section G: Replacement policy

Is your farm open or closed? Open (buy in animals on regular basis) ☐ Closed ☐

Do you quarantine replacement animals? Yes ☐ No ☐

If yes, do you have a quarantine paddock for replacement animals? Yes ☐ No ☐

If no, do you keep replacement animals separated from your other livestock?

Yes ☐ No ☐

Have you brought in any animals for replacement during the past year (Aug 07- Jul 08)? Yes ☐ No ☐

If yes, please fill in the details in the table below as appropriate.

Mob no.	Replacement			Time period (months)	Quarantine Length (days)
	Species	Class	No. of animal		

Section H: Leptospirosis

Has leptospirosis ever been diagnosed by a veterinarian in your animals? Yes ☐ No ☐

If yes, in what kind and class of animals?

.....

If yes, when did it happen?

.....

If yes, what were the signs? (e.g. death, anaemia, red water, abortions, jaundice)

.....

If yes, what tests were done for diagnosis? (can be > 1)

Serological test ☐ Bacteria culture test ☐ No test were done ☐

Have you ever seen animals with signs such as anaemia/ red water/ jaundice on your property? Yes ☐ No ☐

Have you ever discussed leptospirosis issues with your veterinarian? Yes ☐ No ☐

What are the sources of your knowledge of leptospirosis? (can be > 1)

Veterinarian ☐ Other farmers ☐ Magazines ☐ Drug company ☐

ACC/OSH ☐ Internet ☐ Others ☐ (please describe).....

How do you rate leptospirosis as a threat to your animals?

Significant risk ☐ Minor risk ☐ No risk at all ☐

Have you or your workers ever been diagnosed with leptospirosis? Yes ☐ No ☐

If yes, when did it happen?

……

If yes, what were the signs? (e.g. fever, headache, myalgia, photophobia)

……

How do you rate leptospirosis as far as your and your family's health is concerned?

Significant risk ☐ Minor risk ☐ No risk at all ☐

Section I: Neighbour

Please indicate animal species and time period of neighbour livestock share-graze with your deer, cattle and sheep (leave it blank if there is no share-graze between your livestock and neighbour)

Neighbour No. / Detail		Your livestock property		
		Deer	Cattle	Sheep
1	Animal species			
	Period (months)			
2	Animal species			
	Period (months)			
3	Animal species			
	Period (months)			
4	Animal species			
	Period(months)			

Is there any water flow from neighbours onto your property? Yes ☐ No ☐

If yes, please indicate animal species on neighbour's farms (can be > 1).

Deer ☐ Beef Cattle ☐ Dairy Cattle ☐ Sheep ☐ Goat ☐

Other ☐ (please describe) ……

Section J: Veterinarians

Your current veterinarian(s:)

……

Clinic:

……

How frequently do you seek a veterinary service?

Once a month ☐ Once in 3 months ☐ Once in 6 months ☐ Once a year ☐

What is your main purpose of seeking a veterinary service?

Animal health problems ☐ Pregnancy scanning ☐ Management consult ☐

Other ☐ (Please describe).....

Will you agree if we send your farm's leptospirosis test results to your veterinarian?

Yes ☐ No ☐

Section K: Signature of farm owner/manager

I understand that all information contained with in this form is totally confidential to the researchers or where agreed, your veterinary practitioners. No individual farmer or property will be identifiable to other parties when this data is used for any purpose.

…… ……..

Signature of farm owner/manager Date

Appendix 4b. Raw data of serology for deer, cattle and sheep from 2006 to 2008.

*Farm 8 was lost to follow-up in 2007 and 2008

Animal	Farm	Year	Species	Hardjobovis	Pomona
1	1	2006	Deer	0	0
2	1	2006	Deer	0	0
3	1	2006	Deer	0	0
4	1	2006	Deer	0	0
5	1	2006	Deer	0	0
6	1	2006	Deer	0	0
7	1	2006	Deer	0	0
8	1	2006	Deer	0	0
9	1	2006	Deer	0	0
10	1	2006	Deer	0	0
11	1	2006	Deer	0	0
12	1	2006	Deer	0	0
13	1	2006	Deer	48	0
14	1	2006	Deer	0	0
15	1	2006	Deer	0	0
16	1	2006	Deer	0	0
17	1	2006	Deer	0	0
18	1	2006	Deer	0	0
19	1	2006	Deer	24	0
20	2	2006	Deer	0	0
21	2	2006	Deer	0	0
22	2	2006	Deer	0	0
23	2	2006	Deer	0	0
24	2	2006	Deer	0	0
25	2	2006	Deer	0	0
26	2	2006	Deer	0	0
27	2	2006	Deer	0	0
28	2	2006	Deer	0	0
29	2	2006	Deer	0	0
30	2	2006	Deer	0	0
31	2	2006	Deer	0	0
32	2	2006	Deer	0	0
33	2	2006	Deer	0	0
34	2	2006	Deer	24	0
35	2	2006	Deer	0	0
36	2	2006	Deer	0	0
37	2	2006	Deer	0	0
38	2	2006	Deer	0	0
39	2	2006	Deer	0	0
40	3	2006	Deer	48	0
41	3	2006	Deer	0	0
42	3	2006	Deer	24	0
43	3	2006	Deer	0	0
44	3	2006	Deer	0	0
45	3	2006	Deer	0	0
46	3	2006	Deer	0	0
47	3	2006	Deer	0	0
48	3	2006	Deer	0	0
49	3	2006	Deer	0	0
50	3	2006	Deer	0	0
51	3	2006	Deer	0	0
52	3	2006	Deer	0	0
53	3	2006	Deer	0	0
54	3	2006	Deer	24	0
55	3	2006	Deer	0	0
56	3	2006	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
57	3	2006	Deer	0	0
58	3	2006	Deer	0	0
59	3	2006	Deer	0	0
60	4	2006	Deer	0	0
61	4	2006	Deer	24	0
62	4	2006	Deer	24	96
63	4	2006	Deer	0	0
64	4	2006	Deer	0	0
65	4	2006	Deer	0	192
66	4	2006	Deer	0	384
67	4	2006	Deer	0	0
68	4	2006	Deer	96	0
69	4	2006	Deer	0	0
70	4	2006	Deer	0	24
71	4	2006	Deer	0	192
72	4	2006	Deer	0	96
73	4	2006	Deer	0	0
74	4	2006	Deer	0	0
75	4	2006	Deer	0	24
76	4	2006	Deer	0	0
77	4	2006	Deer	0	192
78	4	2006	Deer	0	24
79	4	2006	Deer	0	192
80	4	2006	Deer	0	0
81	4	2006	Cattle	0	96
82	4	2006	Cattle	96	0
83	4	2006	Cattle	192	0
84	4	2006	Cattle	96	48
85	4	2006	Cattle	0	0
86	5	2006	Deer	0	0
87	5	2006	Deer	96	0
88	5	2006	Deer	0	0
89	5	2006	Deer	0	0
90	5	2006	Deer	24	96
91	5	2006	Deer	0	48
92	5	2006	Deer	0	48
93	5	2006	Deer	0	0
94	5	2006	Deer	0	96
95	5	2006	Deer	0	0
96	5	2006	Deer	0	96
97	5	2006	Deer	0	0
98	5	2006	Deer	48	384
99	5	2006	Deer	0	48
100	5	2006	Deer	0	0
101	5	2006	Deer	48	0
102	5	2006	Deer	0	0
103	5	2006	Deer	0	96
104	5	2006	Deer	0	192
105	5	2006	Deer	0	96
106	5	2006	Deer	0	0
107	5	2006	Cattle	48	0
108	5	2006	Cattle	0	0
109	5	2006	Cattle	96	0
110	5	2006	Cattle	48	0
111	5	2006	Cattle	24	0
112	5	2006	Cattle	384	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
113	5	2006	Cattle	96	0
114	5	2006	Cattle	24	0
115	5	2006	Cattle	48	0
116	5	2006	Cattle	192	0
117	5	2006	Cattle	48	0
118	5	2006	Cattle	24	0
119	5	2006	Cattle	24	0
120	5	2006	Cattle	48	0
121	5	2006	Cattle	48	0
122	5	2006	Cattle	48	0
123	5	2006	Cattle	24	0
124	5	2006	Cattle	24	0
125	5	2006	Cattle	48	0
126	5	2006	Cattle	96	0
127	6	2006	Deer	48	0
128	6	2006	Deer	48	0
129	6	2006	Deer	0	0
130	6	2006	Deer	48	0
131	6	2006	Deer	0	0
132	6	2006	Deer	0	0
133	6	2006	Deer	0	0
134	6	2006	Deer	0	0
135	6	2006	Deer	0	0
136	6	2006	Deer	0	0
137	6	2006	Deer	0	0
138	6	2006	Deer	0	0
139	6	2006	Deer	0	0
140	6	2006	Deer	48	192
141	6	2006	Deer	0	0
142	6	2006	Deer	48	0
143	6	2006	Deer	0	0
144	6	2006	Deer	96	0
145	6	2006	Deer	48	0
146	6	2006	Deer	48	0
147	6	2006	Cattle	48	0
148	6	2006	Cattle	96	0
149	6	2006	Cattle	48	0
150	6	2006	Cattle	96	24
151	6	2006	Cattle	192	0
152	6	2006	Cattle	384	0
153	6	2006	Cattle	0	0
154	6	2006	Cattle	192	0
155	6	2006	Cattle	24	0
156	6	2006	Cattle	48	0
157	6	2006	Cattle	96	0
158	6	2006	Cattle	192	0
159	6	2006	Cattle	96	48
160	6	2006	Cattle	0	24
161	6	2006	Cattle	24	0
162	6	2006	Cattle	96	0
163	6	2006	Cattle	384	0
164	6	2006	Cattle	384	0
165	6	2006	Cattle	192	0
166	6	2006	Cattle	384	0
167	7	2006	Deer	0	0
168	7	2006	Deer	48	0
169	7	2006	Deer	24	0
170	7	2006	Deer	48	192
171	7	2006	Deer	48	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
172	7	2006	Deer	0	0
173	7	2006	Deer	96	0
174	7	2006	Deer	0	0
175	7	2006	Deer	96	0
176	7	2006	Deer	0	0
177	7	2006	Deer	24	0
178	7	2006	Deer	0	0
179	7	2006	Deer	24	0
180	7	2006	Deer	48	0
181	7	2006	Deer	0	0
182	7	2006	Deer	0	0
183	7	2006	Deer	0	0
184	7	2006	Deer	0	0
185	7	2006	Deer	24	0
186	7	2006	Deer	0	0
187	7	2006	Sheep	0	0
188	7	2006	Sheep	0	0
189	7	2006	Sheep	0	0
190	7	2006	Sheep	0	0
191	7	2006	Sheep	0	0
192	7	2006	Sheep	0	0
193	7	2006	Sheep	0	0
194	7	2006	Sheep	0	0
195	7	2006	Sheep	0	0
196	7	2006	Sheep	0	0
197	7	2006	Sheep	0	0
198	7	2006	Sheep	0	0
199	7	2006	Sheep	0	0
200	7	2006	Sheep	0	0
201	7	2006	Sheep	0	0
202	7	2006	Sheep	0	0
203	7	2006	Sheep	0	0
204	7	2006	Sheep	0	0
205	7	2006	Sheep	0	0
206	7	2006	Sheep	0	0
207	7	2006	Sheep	0	0
208	7	2006	Sheep	0	0
209	7	2006	Sheep	0	0
210	7	2006	Sheep	0	0
211	7	2006	Sheep	0	0
212	7	2006	Sheep	0	0
213	7	2006	Sheep	0	0
214	7	2006	Sheep	0	0
215	7	2006	Sheep	0	0
216	7	2006	Sheep	0	0
217	8*	2006	Deer	0	0
218	8*	2006	Deer	0	48
219	8*	2006	Deer	0	0
220	8*	2006	Deer	0	0
221	8*	2006	Deer	48	0
222	8*	2006	Deer	0	0
223	8*	2006	Deer	0	0
224	8*	2006	Deer	0	0
225	8*	2006	Deer	0	0
226	8*	2006	Deer	0	0
227	8*	2006	Deer	0	0
228	8*	2006	Deer	0	48
229	8*	2006	Deer	0	0
230	8*	2006	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
231	8*	2006	Deer	0	0
232	8*	2006	Deer	0	0
233	8*	2006	Deer	0	0
234	8*	2006	Deer	0	0
235	8*	2006	Deer	0	0
236	8*	2006	Sheep	192	0
237	8*	2006	Sheep	0	0
238	8*	2006	Sheep	0	0
239	8*	2006	Sheep	96	0
240	8*	2006	Sheep	384	0
241	8*	2006	Sheep	0	0
242	8*	2006	Sheep	0	0
243	8*	2006	Sheep	0	96
244	8*	2006	Sheep	768	0
245	8*	2006	Sheep	0	0
246	8*	2006	Sheep	0	0
247	8*	2006	Sheep	0	0
248	8*	2006	Sheep	192	0
249	8*	2006	Sheep	0	0
250	8*	2006	Sheep	0	0
251	8*	2006	Sheep	0	0
252	8*	2006	Sheep	1536	0
253	8*	2006	Sheep	0	0
254	8*	2006	Sheep	192	0
255	8*	2006	Sheep	384	0
256	8*	2006	Sheep	0	0
257	8*	2006	Sheep	0	0
258	8*	2006	Sheep	96	0
259	8*	2006	Sheep	96	0
260	8*	2006	Sheep	0	0
261	8*	2006	Sheep	0	0
262	8*	2006	Sheep	384	0
263	8*	2006	Sheep	96	0
264	8*	2006	Sheep	0	0
265	8*	2006	Sheep	0	0
266	8*	2006	Sheep	768	0
267	8*	2006	Sheep	0	0
268	8*	2006	Sheep	384	0
269	8*	2006	Sheep	0	0
270	8*	2006	Sheep	0	0
271	8*	2006	Sheep	0	0
272	8*	2006	Sheep	0	0
273	8*	2006	Sheep	0	0
274	8*	2006	Sheep	284	0
275	8*	2006	Sheep	24	0
276	8*	2006	Sheep	192	0
277	8*	2006	Sheep	0	0
278	8*	2006	Sheep	0	0
279	8*	2006	Sheep	0	0
280	8*	2006	Sheep	0	0
281	8*	2006	Sheep	384	0
282	8*	2006	Sheep	0	0
283	8*	2006	Sheep	768	0
284	8*	2006	Sheep	96	0
285	8*	2006	Sheep	24	0
286	8*	2006	Sheep	768	0
287	8*	2006	Sheep	0	0
288	8*	2006	Sheep	0	0
289	8*	2006	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
290	8*	2006	Sheep	48	0
291	8*	2006	Sheep	0	0
292	9	2006	Deer	48	0
293	9	2006	Deer	24	0
294	9	2006	Deer	0	0
295	9	2006	Deer	48	0
296	9	2006	Deer	24	0
297	9	2006	Deer	0	0
298	9	2006	Deer	0	0
299	9	2006	Deer	0	0
300	9	2006	Deer	48	48
301	9	2006	Deer	24	0
302	9	2006	Deer	0	0
303	9	2006	Deer	24	0
304	9	2006	Deer	96	0
305	9	2006	Deer	24	0
306	9	2006	Deer	24	0
307	9	2006	Deer	48	0
308	9	2006	Deer	48	0
309	9	2006	Deer	0	0
310	9	2006	Deer	0	192
311	9	2006	Deer	0	0
312	9	2006	Cattle	48	0
313	9	2006	Cattle	48	48
314	9	2006	Cattle	24	0
315	9	2006	Cattle	0	0
316	9	2006	Cattle	96	0
317	9	2006	Cattle	48	0
318	9	2006	Cattle	24	0
319	9	2006	Cattle	24	192
320	9	2006	Cattle	0	0
321	9	2006	Cattle	48	0
322	9	2006	Cattle	48	0
323	9	2006	Cattle	96	0
324	9	2006	Cattle	48	0
325	9	2006	Cattle	48	0
326	9	2006	Cattle	24	0
327	9	2006	Cattle	96	0
328	9	2006	Cattle	24	0
329	9	2006	Sheep	24	0
330	9	2006	Sheep	96	0
331	9	2006	Sheep	48	0
332	9	2006	Sheep	48	0
333	9	2006	Sheep	24	0
334	9	2006	Sheep	24	0
335	9	2006	Sheep	24	0
336	9	2006	Sheep	48	0
337	9	2006	Sheep	48	0
338	9	2006	Sheep	24	0
339	9	2006	Sheep	48	0
340	9	2006	Sheep	384	0
341	9	2006	Sheep	24	0
342	9	2006	Sheep	0	0
343	9	2006	Sheep	24	0
344	9	2006	Sheep	0	0
345	9	2006	Sheep	24	0
346	9	2006	Sheep	192	0
347	9	2006	Sheep	0	0
348	9	2006	Sheep	24	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
349	9	2006	Sheep	48	0
350	9	2006	Sheep	48	96
351	9	2006	Sheep	48	0
352	9	2006	Sheep	96	0
353	9	2006	Sheep	384	384
354	9	2006	Sheep	48	0
355	9	2006	Sheep	24	0
356	9	2006	Sheep	48	0
357	9	2006	Sheep	24	0
358	9	2006	Sheep	48	0
359	9	2006	Sheep	24	0
360	9	2006	Sheep	24	0
361	9	2006	Sheep	24	0
362	9	2006	Sheep	24	0
363	9	2006	Sheep	48	0
364	9	2006	Sheep	48	0
365	9	2006	Sheep	24	0
366	9	2006	Sheep	24	0
367	9	2006	Sheep	24	0
368	9	2006	Sheep	24	0
369	9	2006	Sheep	24	0
370	9	2006	Sheep	0	0
371	9	2006	Sheep	24	0
372	9	2006	Sheep	24	0
373	9	2006	Sheep	0	0
374	9	2006	Sheep	24	0
375	9	2006	Sheep	24	0
376	9	2006	Sheep	24	0
377	9	2006	Sheep	24	0
378	9	2006	Sheep	24	0
379	9	2006	Sheep	24	0
380	9	2006	Sheep	24	0
381	9	2006	Sheep	0	0
382	9	2006	Sheep	0	0
383	9	2006	Sheep	0	0
384	9	2006	Sheep	24	0
385	9	2006	Sheep	24	0
386	9	2006	Sheep	24	0
387	9	2006	Sheep	0	0
388	9	2006	Sheep	24	0
389	10	2006	Deer	0	0
390	10	2006	Deer	0	0
391	10	2006	Deer	0	0
392	10	2006	Deer	0	0
393	10	2006	Deer	0	0
394	10	2006	Deer	0	0
395	10	2006	Deer	0	0
396	10	2006	Deer	24	0
397	10	2006	Deer	0	0
398	10	2006	Deer	0	0
399	10	2006	Deer	0	0
400	10	2006	Deer	0	0
401	10	2006	Deer	0	0
402	10	2006	Deer	0	0
403	10	2006	Deer	0	0
404	10	2006	Deer	0	0
405	10	2006	Deer	0	0
406	10	2006	Deer	0	192
407	10	2006	Deer	0	0
408	10	2006	Deer	0	0
409	10	2006	Cattle	96	0
410	10	2006	Cattle	24	0
411	10	2006	Cattle	96	0
412	10	2006	Cattle	96	0
413	10	2006	Cattle	48	0
414	10	2006	Cattle	0	0
415	10	2006	Cattle	96	24
416	10	2006	Cattle	192	0
417	10	2006	Cattle	48	192
418	10	2006	Cattle	48	24
419	10	2006	Cattle	0	24
420	10	2006	Cattle	48	48
421	10	2006	Cattle	48	0
422	10	2006	Cattle	48	24
423	10	2006	Cattle	96	0
424	10	2006	Cattle	96	0
425	10	2006	Cattle	96	0
426	10	2006	Cattle	48	0
427	10	2006	Cattle	0	0
428	10	2006	Cattle	96	0
429	10	2006	Sheep	0	0
430	10	2006	Sheep	0	0
431	10	2006	Sheep	0	0
432	10	2006	Sheep	0	0
433	10	2006	Sheep	0	0
434	10	2006	Sheep	0	0
435	10	2006	Sheep	0	0
436	10	2006	Sheep	0	0
437	10	2006	Sheep	0	0
438	10	2006	Sheep	0	0
439	10	2006	Sheep	24	0
440	10	2006	Sheep	0	0
441	10	2006	Sheep	24	0
442	10	2006	Sheep	0	0
443	10	2006	Sheep	0	0
444	10	2006	Sheep	0	0
445	10	2006	Sheep	0	0
446	10	2006	Sheep	0	0
447	10	2006	Sheep	0	0
448	10	2006	Sheep	0	0
449	10	2006	Sheep	0	0
450	10	2006	Sheep	24	0
451	10	2006	Sheep	0	0
452	10	2006	Sheep	0	0
453	10	2006	Sheep	0	0
454	10	2006	Sheep	24	0
455	10	2006	Sheep	0	0
456	10	2006	Sheep	0	0
457	10	2006	Sheep	24	0
458	10	2006	Sheep	48	0
459	10	2006	Sheep	24	0
460	10	2006	Sheep	0	0
461	10	2006	Sheep	24	0
462	10	2006	Sheep	24	0
463	10	2006	Sheep	0	0
464	10	2006	Sheep	0	0
465	10	2006	Sheep	0	0
466	10	2006	Sheep	96	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
467	10	2006	Sheep	192	0
468	10	2006	Sheep	0	0
469	10	2006	Sheep	24	192
470	10	2006	Sheep	0	0
471	10	2006	Sheep	24	0
472	10	2006	Sheep	0	0
473	10	2006	Sheep	24	0
474	10	2006	Sheep	0	0
475	10	2006	Sheep	24	0
476	10	2006	Sheep	0	0
477	10	2006	Sheep	0	0
478	10	2006	Sheep	0	0
479	10	2006	Sheep	0	0
480	10	2006	Sheep	0	0
481	10	2006	Sheep	0	0
482	10	2006	Sheep	0	0
483	10	2006	Sheep	0	0
484	10	2006	Sheep	0	0
485	10	2006	Sheep	0	0
486	10	2006	Sheep	0	0
487	10	2006	Sheep	0	0
488	10	2006	Sheep	0	0
489	11	2006	Deer	0	0
490	11	2006	Deer	0	0
491	11	2006	Deer	0	0
492	11	2006	Deer	0	0
493	11	2006	Deer	0	0
494	11	2006	Deer	24	0
495	11	2006	Deer	0	0
496	11	2006	Deer	24	0
497	11	2006	Deer	48	24
498	11	2006	Deer	0	0
499	11	2006	Deer	0	0
500	11	2006	Deer	48	0
501	11	2006	Deer	0	0
502	11	2006	Deer	0	0
503	11	2006	Deer	0	0
504	11	2006	Deer	0	0
505	11	2006	Deer	0	0
506	11	2006	Deer	96	0
507	11	2006	Deer	0	0
508	11	2006	Cattle	0	0
509	11	2006	Cattle	0	24
510	11	2006	Cattle	24	0
511	11	2006	Cattle	24	0
512	11	2006	Cattle	0	0
513	11	2006	Cattle	0	0
514	11	2006	Cattle	0	0
515	11	2006	Cattle	0	0
516	11	2006	Cattle	24	0
517	11	2006	Cattle	24	0
518	11	2006	Cattle	0	0
519	11	2006	Cattle	0	0
520	11	2006	Cattle	0	0
521	11	2006	Cattle	0	0
522	11	2006	Cattle	0	0
523	11	2006	Cattle	0	0
524	11	2006	Cattle	0	0
525	11	2006	Cattle	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
526	11	2006	Cattle	0	0
527	11	2006	Cattle	0	0
528	11	2006	Sheep	96	0
529	11	2006	Sheep	0	0
530	11	2006	Sheep	48	0
531	11	2006	Sheep	24	0
532	11	2006	Sheep	0	0
533	11	2006	Sheep	0	0
534	11	2006	Sheep	0	0
535	11	2006	Sheep	0	0
536	11	2006	Sheep	96	0
537	11	2006	Sheep	0	0
538	11	2006	Sheep	0	0
539	11	2006	Sheep	0	0
540	11	2006	Sheep	192	0
541	11	2006	Sheep	0	0
542	11	2006	Sheep	0	0
543	11	2006	Sheep	0	0
544	11	2006	Sheep	0	0
545	11	2006	Sheep	48	0
546	11	2006	Sheep	48	0
547	11	2006	Sheep	48	0
548	11	2006	Sheep	96	0
549	11	2006	Sheep	0	0
550	11	2006	Sheep	0	0
551	11	2006	Sheep	0	0
552	11	2006	Sheep	0	0
553	11	2006	Sheep	0	0
554	11	2006	Sheep	0	0
555	11	2006	Sheep	0	0
556	11	2006	Sheep	0	0
557	11	2006	Sheep	0	0
558	11	2006	Sheep	0	0
559	11	2006	Sheep	0	0
560	11	2006	Sheep	0	0
561	11	2006	Sheep	48	0
562	11	2006	Sheep	0	0
563	11	2006	Sheep	0	0
564	11	2006	Sheep	48	0
565	11	2006	Sheep	0	0
566	11	2006	Sheep	0	0
567	11	2006	Sheep	96	0
568	11	2006	Sheep	0	0
569	11	2006	Sheep	0	0
570	11	2006	Sheep	24	0
571	11	2006	Sheep	0	0
572	11	2006	Sheep	96	0
573	11	2006	Sheep	48	0
574	11	2006	Sheep	0	0
575	11	2006	Sheep	384	0
576	11	2006	Sheep	0	0
577	11	2006	Sheep	0	0
578	11	2006	Sheep	48	0
579	11	2006	Sheep	0	0
580	11	2006	Sheep	192	0
581	11	2006	Sheep	96	0
582	11	2006	Sheep	96	0
583	11	2006	Sheep	0	0
584	11	2006	Sheep	96	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
585	11	2006	Sheep	96	0
586	11	2006	Sheep	0	0
587	11	2006	Sheep	0	0
588	12	2006	Deer	48	0
589	12	2006	Deer	0	0
590	12	2006	Deer	0	0
591	12	2006	Deer	0	0
592	12	2006	Deer	0	0
593	12	2006	Deer	24	0
594	12	2006	Deer	0	0
595	12	2006	Deer	0	0
596	12	2006	Deer	0	0
597	12	2006	Deer	0	0
598	12	2006	Deer	24	0
599	12	2006	Deer	48	0
600	12	2006	Deer	0	0
601	12	2006	Deer	0	0
602	12	2006	Deer	0	0
603	12	2006	Deer	0	0
604	12	2006	Deer	24	0
605	12	2006	Deer	0	0
606	12	2006	Deer	24	0
607	12	2006	Deer	48	0
608	12	2006	Cattle	0	0
609	12	2006	Cattle	0	0
610	12	2006	Cattle	384	0
611	12	2006	Cattle	0	0
612	12	2006	Cattle	0	0
613	12	2006	Cattle	24	0
614	12	2006	Cattle	0	0
615	12	2006	Cattle	48	0
616	12	2006	Cattle	0	0
617	12	2006	Cattle	0	0
618	12	2006	Cattle	0	0
619	12	2006	Cattle	0	0
620	12	2006	Cattle	0	0
621	12	2006	Cattle	0	0
622	12	2006	Cattle	0	0
623	12	2006	Cattle	0	0
624	12	2006	Cattle	0	0
625	12	2006	Cattle	0	0
626	12	2006	Cattle	0	0
627	12	2006	Cattle	48	0
628	12	2006	Sheep	0	0
629	12	2006	Sheep	0	0
630	12	2006	Sheep	0	0
631	12	2006	Sheep	96	0
632	12	2006	Sheep	0	0
633	12	2006	Sheep	0	0
634	12	2006	Sheep	0	0
635	12	2006	Sheep	24	0
636	12	2006	Sheep	0	0
637	12	2006	Sheep	24	0
638	12	2006	Sheep	0	0
639	12	2006	Sheep	0	0
640	12	2006	Sheep	0	0
641	12	2006	Sheep	0	0
642	12	2006	Sheep	0	0
643	12	2006	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
644	12	2006	Sheep	0	0
645	12	2006	Sheep	0	0
646	12	2006	Sheep	0	0
647	12	2006	Sheep	0	0
648	12	2006	Sheep	0	0
649	12	2006	Sheep	0	0
650	12	2006	Sheep	0	0
651	12	2006	Sheep	0	0
652	12	2006	Sheep	0	0
653	12	2006	Sheep	0	0
654	12	2006	Sheep	0	0
655	12	2006	Sheep	0	0
656	12	2006	Sheep	0	0
657	12	2006	Sheep	0	0
658	12	2006	Sheep	0	0
659	12	2006	Sheep	0	0
660	12	2006	Sheep	0	0
661	12	2006	Sheep	0	0
662	12	2006	Sheep	0	0
663	12	2006	Sheep	0	0
664	12	2006	Sheep	0	0
665	12	2006	Sheep	0	0
666	12	2006	Sheep	0	0
667	12	2006	Sheep	0	0
668	12	2006	Sheep	0	0
669	12	2006	Sheep	0	0
670	12	2006	Sheep	0	0
671	12	2006	Sheep	0	0
672	12	2006	Sheep	0	0
673	12	2006	Sheep	0	0
674	12	2006	Sheep	0	0
675	12	2006	Sheep	0	0
676	12	2006	Sheep	0	0
677	12	2006	Sheep	0	0
678	12	2006	Sheep	0	0
679	12	2006	Sheep	0	0
680	12	2006	Sheep	0	0
681	12	2006	Sheep	0	0
682	12	2006	Sheep	0	0
683	12	2006	Sheep	0	0
684	12	2006	Sheep	0	0
685	12	2006	Sheep	0	0
686	12	2006	Sheep	0	0
687	12	2006	Sheep	0	0
688	13	2006	Deer	48	0
689	13	2006	Deer	48	0
690	13	2006	Deer	48	0
691	13	2006	Deer	48	0
692	13	2006	Deer	0	0
693	13	2006	Deer	96	0
694	13	2006	Deer	48	0
695	13	2006	Deer	48	0
696	13	2006	Deer	24	0
697	13	2006	Deer	48	0
698	13	2006	Deer	48	0
699	13	2006	Deer	24	0
700	13	2006	Deer	24	0
701	13	2006	Deer	0	0
702	13	2006	Cattle	96	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
703	13	2006	Cattle	48	0
704	13	2006	Cattle	0	0
705	13	2006	Cattle	0	0
706	13	2006	Cattle	48	0
707	13	2006	Cattle	384	0
708	13	2006	Cattle	48	0
709	13	2006	Cattle	48	0
710	13	2006	Cattle	192	0
711	13	2006	Cattle	48	0
712	13	2006	Cattle	192	0
713	13	2006	Cattle	192	0
714	13	2006	Cattle	192	0
715	13	2006	Cattle	48	0
716	13	2006	Cattle	48	0
717	13	2006	Cattle	384	0
718	13	2006	Cattle	96	0
719	13	2006	Cattle	96	0
720	13	2006	Cattle	384	0
721	13	2006	Sheep	0	0
722	13	2006	Sheep	0	0
723	13	2006	Sheep	0	0
724	13	2006	Sheep	0	0
725	13	2006	Sheep	0	0
726	13	2006	Sheep	0	0
727	13	2006	Sheep	0	0
728	13	2006	Sheep	0	0
729	13	2006	Sheep	0	0
730	13	2006	Sheep	0	0
731	13	2006	Sheep	0	0
732	13	2006	Sheep	0	0
733	13	2006	Sheep	0	0
734	13	2006	Sheep	0	0
735	13	2006	Sheep	0	0
736	13	2006	Sheep	0	0
737	13	2006	Sheep	0	0
738	13	2006	Sheep	0	0
739	13	2006	Sheep	0	0
740	13	2006	Sheep	0	0
741	13	2006	Sheep	0	0
742	13	2006	Sheep	0	0
743	13	2006	Sheep	0	0
744	13	2006	Sheep	0	0
745	13	2006	Sheep	0	0
746	13	2006	Sheep	0	0
747	13	2006	Sheep	0	0
748	13	2006	Sheep	0	0
749	13	2006	Sheep	0	0
750	13	2006	Sheep	0	0
751	13	2006	Sheep	0	0
752	13	2006	Sheep	0	0
753	13	2006	Sheep	0	0
754	13	2006	Sheep	0	0
755	13	2006	Sheep	0	0
756	13	2006	Sheep	0	0
757	13	2006	Sheep	0	0
758	13	2006	Sheep	0	0
759	13	2006	Sheep	0	0
760	13	2006	Sheep	0	0
761	13	2006	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
762	13	2006	Sheep	0	0
763	13	2006	Sheep	0	0
764	13	2006	Sheep	0	0
765	13	2006	Sheep	0	0
766	13	2006	Sheep	0	0
767	13	2006	Sheep	0	0
768	13	2006	Sheep	0	0
769	13	2006	Sheep	0	0
770	13	2006	Sheep	0	0
771	13	2006	Sheep	0	0
772	13	2006	Sheep	0	0
773	13	2006	Sheep	0	0
774	13	2006	Sheep	0	0
775	13	2006	Sheep	0	0
776	13	2006	Sheep	0	0
777	13	2006	Sheep	0	0
778	13	2006	Sheep	0	0
779	13	2006	Sheep	0	0
780	13	2006	Sheep	0	0
781	14	2006	Deer	192	0
782	14	2006	Deer	96	96
783	14	2006	Deer	24	24
784	14	2006	Deer	192	0
785	14	2006	Deer	24	0
786	14	2006	Deer	48	0
787	14	2006	Deer	24	48
788	14	2006	Deer	48	96
789	14	2006	Deer	48	192
790	14	2006	Deer	384	0
791	14	2006	Deer	0	0
792	14	2006	Deer	0	24
793	14	2006	Deer	192	384
794	14	2006	Deer	0	24
795	14	2006	Deer	192	192
796	14	2006	Deer	0	0
797	14	2006	Deer	24	0
798	14	2006	Deer	0	0
799	14	2006	Cattle	48	192
800	14	2006	Cattle	384	0
801	14	2006	Cattle	384	192
802	14	2006	Cattle	192	0
803	14	2006	Cattle	768	0
804	14	2006	Cattle	96	0
805	14	2006	Cattle	384	96
806	14	2006	Cattle	192	0
807	14	2006	Cattle	192	384
808	14	2006	Cattle	48	0
809	14	2006	Cattle	96	0
810	14	2006	Cattle	96	0
811	14	2006	Cattle	96	0
812	14	2006	Cattle	384	0
813	14	2006	Cattle	192	0
814	14	2006	Cattle	192	0
815	14	2006	Cattle	96	96
816	14	2006	Cattle	192	0
817	14	2006	Cattle	192	0
818	14	2006	Cattle	48	0
819	14	2006	Sheep	384	0
820	14	2006	Sheep	768	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
821	14	2006	Sheep	384	0
822	14	2006	Sheep	384	0
823	14	2006	Sheep	384	0
824	14	2006	Sheep	192	0
825	14	2006	Sheep	384	0
826	14	2006	Sheep	384	0
827	14	2006	Sheep	0	0
828	14	2006	Sheep	192	0
829	14	2006	Sheep	96	0
830	14	2006	Sheep	0	0
831	14	2006	Sheep	192	0
832	14	2006	Sheep	384	0
833	14	2006	Sheep	24	0
834	14	2006	Sheep	192	0
835	14	2006	Sheep	192	0
836	14	2006	Sheep	0	0
837	14	2006	Sheep	24	0
838	14	2006	Sheep	24	0
839	14	2006	Sheep	192	0
840	14	2006	Sheep	0	0
841	14	2006	Sheep	96	0
842	14	2006	Sheep	24	0
843	14	2006	Sheep	24	0
844	14	2006	Sheep	48	0
845	14	2006	Sheep	384	0
846	14	2006	Sheep	0	0
847	14	2006	Sheep	0	0
848	14	2006	Sheep	0	0
849	14	2006	Sheep	24	0
850	14	2006	Sheep	0	0
851	14	2006	Sheep	24	0
852	14	2006	Sheep	192	96
853	14	2006	Sheep	384	0
854	14	2006	Sheep	192	0
855	14	2006	Sheep	192	0
856	14	2006	Sheep	96	0
857	14	2006	Sheep	0	0
858	14	2006	Sheep	96	0
859	14	2006	Sheep	192	0
860	14	2006	Sheep	96	0
861	14	2006	Sheep	192	0
862	14	2006	Sheep	96	0
863	14	2006	Sheep	384	0
864	14	2006	Sheep	96	0
865	14	2006	Sheep	384	0
866	14	2006	Sheep	96	0
867	14	2006	Sheep	0	0
868	14	2006	Sheep	24	0
869	14	2006	Sheep	384	0
870	14	2006	Sheep	384	0
871	14	2006	Sheep	384	0
872	14	2006	Sheep	192	0
873	14	2006	Sheep	192	0
874	14	2006	Sheep	192	768
875	14	2006	Sheep	96	0
876	14	2006	Sheep	96	0
877	14	2006	Sheep	384	0
878	14	2006	Sheep	0	0
879	15	2006	Deer	0	0
880	15	2006	Deer	24	0
881	15	2006	Deer	48	0
882	15	2006	Deer	0	0
883	15	2006	Deer	24	0
884	15	2006	Deer	24	0
885	15	2006	Deer	0	0
886	15	2006	Deer	96	0
887	15	2006	Deer	0	0
888	15	2006	Deer	24	0
889	15	2006	Deer	48	0
890	15	2006	Deer	0	0
891	15	2006	Deer	24	0
892	15	2006	Deer	24	0
893	15	2006	Deer	0	0
894	15	2006	Deer	0	0
895	15	2006	Deer	24	0
896	15	2006	Deer	48	0
897	15	2006	Deer	48	0
898	15	2006	Cattle	96	0
899	15	2006	Cattle	24	0
900	15	2006	Cattle	0	0
901	15	2006	Cattle	192	0
902	15	2006	Cattle	24	0
903	15	2006	Cattle	96	0
904	15	2006	Cattle	96	0
905	15	2006	Cattle	192	0
906	15	2006	Cattle	96	0
907	15	2006	Cattle	24	0
908	15	2006	Cattle	192	0
909	15	2006	Cattle	192	0
910	15	2006	Cattle	96	0
911	15	2006	Cattle	0	96
912	15	2006	Cattle	24	0
913	15	2006	Cattle	192	0
914	15	2006	Cattle	48	0
915	15	2006	Cattle	96	0
916	15	2006	Cattle	96	0
917	15	2006	Sheep	192	0
918	15	2006	Sheep	384	0
919	15	2006	Sheep	96	0
920	15	2006	Sheep	192	0
921	15	2006	Sheep	96	0
922	15	2006	Sheep	384	0
923	15	2006	Sheep	192	0
924	15	2006	Sheep	384	0
925	15	2006	Sheep	384	0
926	15	2006	Sheep	0	0
927	15	2006	Sheep	0	0
928	15	2006	Sheep	384	0
929	15	2006	Sheep	96	0
930	15	2006	Sheep	96	0
931	15	2006	Sheep	192	0
932	15	2006	Sheep	0	0
933	15	2006	Sheep	96	0
934	15	2006	Sheep	384	0
935	15	2006	Sheep	96	0
936	15	2006	Sheep	96	0
937	15	2006	Sheep	192	0
938	15	2006	Sheep	192	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
939	15	2006	Sheep	384	0
940	15	2006	Sheep	96	0
941	15	2006	Sheep	192	0
942	15	2006	Sheep	48	0
943	15	2006	Sheep	96	0
944	15	2006	Sheep	768	0
945	15	2006	Sheep	96	0
946	15	2006	Sheep	48	0
947	15	2006	Sheep	192	0
948	15	2006	Sheep	96	0
949	15	2006	Sheep	384	0
950	15	2006	Sheep	96	0
951	15	2006	Sheep	48	0
952	15	2006	Sheep	384	0
953	15	2006	Sheep	384	0
954	15	2006	Sheep	192	0
955	15	2006	Sheep	192	0
956	15	2006	Sheep	192	0
957	15	2006	Sheep	96	0
958	15	2006	Sheep	192	0
959	15	2006	Sheep	96	0
960	15	2006	Sheep	96	0
961	15	2006	Sheep	24	0
962	15	2006	Sheep	96	0
963	15	2006	Sheep	96	0
964	15	2006	Sheep	192	0
965	15	2006	Sheep	48	0
966	15	2006	Sheep	384	0
967	15	2006	Sheep	96	0
968	15	2006	Sheep	96	0
969	15	2006	Sheep	48	0
970	15	2006	Sheep	96	0
971	15	2006	Sheep	96	0
972	15	2006	Sheep	96	0
973	15	2006	Sheep	192	0
974	15	2006	Sheep	192	0
975	15	2006	Sheep	192	0
976	16	2006	Deer	96	0
977	16	2006	Deer	24	0
978	16	2006	Deer	48	0
979	16	2006	Deer	0	0
980	16	2006	Deer	96	0
981	16	2006	Deer	24	0
982	16	2006	Deer	24	0
983	16	2006	Deer	48	0
984	16	2006	Deer	48	0
985	16	2006	Deer	0	0
986	16	2006	Deer	0	0
987	16	2006	Deer	24	0
988	16	2006	Deer	24	0
989	16	2006	Deer	24	0
990	16	2006	Deer	0	0
991	16	2006	Deer	24	0
992	16	2006	Deer	48	0
993	16	2006	Deer	24	0
994	16	2006	Deer	48	0
995	16	2006	Deer	192	0
996	16	2006	Cattle	0	0
997	16	2006	Cattle	0	0
998	16	2006	Cattle	192	0
999	16	2006	Cattle	192	0
1000	16	2006	Cattle	48	0
1001	16	2006	Cattle	24	0
1002	16	2006	Cattle	384	0
1003	16	2006	Cattle	96	0
1004	16	2006	Cattle	96	0
1005	16	2006	Cattle	192	0
1006	16	2006	Cattle	24	0
1007	16	2006	Cattle	0	0
1008	16	2006	Cattle	384	0
1009	16	2006	Cattle	0	0
1010	16	2006	Cattle	96	0
1011	16	2006	Cattle	192	0
1012	16	2006	Cattle	192	0
1013	16	2006	Cattle	48	0
1014	16	2006	Cattle	48	0
1015	16	2006	Cattle	24	0
1016	16	2006	Sheep	0	0
1017	16	2006	Sheep	0	0
1018	16	2006	Sheep	0	0
1019	16	2006	Sheep	0	0
1020	16	2006	Sheep	0	0
1021	16	2006	Sheep	0	0
1022	16	2006	Sheep	0	0
1023	16	2006	Sheep	0	0
1024	16	2006	Sheep	0	0
1025	16	2006	Sheep	0	0
1026	16	2006	Sheep	0	0
1027	16	2006	Sheep	0	0
1028	16	2006	Sheep	0	0
1029	16	2006	Sheep	0	0
1030	16	2006	Sheep	0	0
1031	16	2006	Sheep	0	0
1032	16	2006	Sheep	0	0
1033	16	2006	Sheep	0	0
1034	16	2006	Sheep	0	0
1035	16	2006	Sheep	0	0
1036	16	2006	Sheep	0	0
1037	16	2006	Sheep	0	0
1038	16	2006	Sheep	0	0
1039	16	2006	Sheep	0	0
1040	16	2006	Sheep	192	0
1041	16	2006	Sheep	0	0
1042	16	2006	Sheep	0	0
1043	16	2006	Sheep	0	0
1044	16	2006	Sheep	0	0
1045	16	2006	Sheep	0	0
1046	16	2006	Sheep	0	0
1047	16	2006	Sheep	0	0
1048	16	2006	Sheep	0	0
1049	16	2006	Sheep	0	0
1050	16	2006	Sheep	384	0
1051	16	2006	Sheep	0	0
1052	16	2006	Sheep	0	0
1053	16	2006	Sheep	0	0
1054	16	2006	Sheep	0	0
1055	16	2006	Sheep	0	0
1056	16	2006	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1057	16	2006	Sheep	0	0
1058	16	2006	Sheep	0	0
1059	16	2006	Sheep	0	0
1060	16	2006	Sheep	192	0
1061	16	2006	Sheep	384	0
1062	16	2006	Sheep	0	0
1063	16	2006	Sheep	0	0
1064	16	2006	Sheep	0	0
1065	16	2006	Sheep	192	0
1066	16	2006	Sheep	0	0
1067	16	2006	Sheep	0	0
1068	16	2006	Sheep	0	0
1069	16	2006	Sheep	0	0
1070	16	2006	Sheep	0	0
1071	16	2006	Sheep	0	0
1072	16	2006	Sheep	0	0
1073	16	2006	Sheep	0	0
1074	16	2006	Sheep	0	0
1075	16	2006	Sheep	0	0
1076	17	2006	Deer	0	0
1077	17	2006	Deer	0	0
1078	17	2006	Deer	0	0
1079	17	2006	Deer	0	0
1080	17	2006	Deer	24	0
1081	17	2006	Deer	0	0
1082	17	2006	Deer	0	0
1083	17	2006	Deer	0	0
1084	17	2006	Deer	0	0
1085	17	2006	Deer	0	0
1086	17	2006	Deer	0	0
1087	17	2006	Deer	0	0
1088	17	2006	Deer	0	0
1089	17	2006	Deer	0	0
1090	17	2006	Deer	0	0
1091	17	2006	Deer	0	0
1092	17	2006	Deer	0	0
1093	17	2006	Deer	0	0
1094	17	2006	Deer	0	0
1095	17	2006	Deer	0	0
1096	17	2006	Cattle	0	0
1097	17	2006	Cattle	0	0
1098	17	2006	Cattle	48	0
1099	17	2006	Cattle	24	0
1100	17	2006	Cattle	24	0
1101	17	2006	Cattle	0	0
1102	17	2006	Cattle	48	0
1103	17	2006	Cattle	0	0
1104	17	2006	Cattle	0	0
1105	17	2006	Cattle	384	0
1106	17	2006	Cattle	0	0
1107	17	2006	Cattle	0	0
1108	17	2006	Cattle	48	0
1109	17	2006	Cattle	48	0
1110	17	2006	Cattle	48	0
1111	17	2006	Sheep	0	0
1112	17	2006	Sheep	192	0
1113	17	2006	Sheep	0	0
1114	17	2006	Sheep	24	0
1115	17	2006	Sheep	384	0
1116	17	2006	Sheep	192	0
1117	17	2006	Sheep	48	0
1118	17	2006	Sheep	0	0
1119	17	2006	Sheep	96	0
1120	17	2006	Sheep	0	0
1121	17	2006	Sheep	0	0
1122	17	2006	Sheep	192	0
1123	17	2006	Sheep	0	0
1124	17	2006	Sheep	192	0
1125	17	2006	Sheep	96	24
1126	17	2006	Sheep	0	0
1127	17	2006	Sheep	96	0
1128	17	2006	Sheep	0	0
1129	17	2006	Sheep	96	0
1130	17	2006	Sheep	0	0
1131	17	2006	Sheep	96	0
1132	17	2006	Sheep	0	0
1133	17	2006	Sheep	192	0
1134	17	2006	Sheep	96	0
1135	17	2006	Sheep	96	0
1136	17	2006	Sheep	192	0
1137	17	2006	Sheep	0	0
1138	17	2006	Sheep	96	0
1139	17	2006	Sheep	192	0
1140	17	2006	Sheep	0	0
1141	17	2006	Sheep	48	0
1142	17	2006	Sheep	192	0
1143	17	2006	Sheep	96	0
1144	17	2006	Sheep	384	0
1145	17	2006	Sheep	0	0
1146	17	2006	Sheep	768	0
1147	17	2006	Sheep	96	48
1148	17	2006	Sheep	768	0
1149	17	2006	Sheep	96	0
1150	17	2006	Sheep	24	0
1151	17	2006	Sheep	96	0
1152	17	2006	Sheep	384	0
1153	17	2006	Sheep	0	0
1154	17	2006	Sheep	192	0
1155	17	2006	Sheep	0	0
1156	17	2006	Sheep	0	0
1157	17	2006	Sheep	96	0
1158	17	2006	Sheep	0	0
1159	17	2006	Sheep	96	0
1160	17	2006	Sheep	96	0
1161	17	2006	Sheep	96	0
1162	17	2006	Sheep	48	0
1163	17	2006	Sheep	96	0
1164	17	2006	Sheep	24	0
1165	17	2006	Sheep	96	0
1166	17	2006	Sheep	192	0
1167	17	2006	Sheep	48	0
1168	17	2006	Sheep	0	0
1169	17	2006	Sheep	384	0
1170	17	2006	Sheep	0	0
1171	18	2006	Deer	0	0
1172	18	2006	Deer	0	0
1173	18	2006	Deer	0	24
1174	18	2006	Deer	48	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1175	18	2006	Deer	0	768
1176	18	2006	Deer	0	0
1177	18	2006	Deer	0	384
1178	18	2006	Deer	0	192
1179	18	2006	Deer	0	0
1180	18	2006	Deer	0	192
1181	18	2006	Deer	96	0
1182	18	2006	Deer	0	48
1183	18	2006	Deer	0	0
1184	18	2006	Deer	0	0
1185	18	2006	Deer	0	0
1186	18	2006	Deer	0	0
1187	18	2006	Deer	48	192
1188	18	2006	Deer	96	1536
1189	18	2006	Deer	0	0
1190	18	2006	Deer	0	0
1191	18	2006	Cattle	768	0
1192	18	2006	Cattle	384	0
1193	18	2006	Cattle	0	0
1194	18	2006	Cattle	192	0
1195	18	2006	Cattle	0	0
1196	18	2006	Cattle	192	0
1197	18	2006	Cattle	0	0
1198	18	2006	Cattle	0	0
1199	18	2006	Cattle	0	0
1200	18	2006	Cattle	0	0
1201	18	2006	Cattle	0	96
1202	18	2006	Cattle	384	192
1203	18	2006	Cattle	0	0
1204	18	2006	Cattle	0	0
1205	18	2006	Cattle	384	0
1206	18	2006	Cattle	0	0
1207	18	2006	Cattle	0	0
1208	18	2006	Cattle	384	0
1209	18	2006	Cattle	0	0
1210	18	2006	Cattle	0	0
1211	18	2006	Sheep	0	0
1212	18	2006	Sheep	0	0
1213	18	2006	Sheep	0	0
1214	18	2006	Sheep	0	0
1215	18	2006	Sheep	0	0
1216	18	2006	Sheep	768	0
1217	18	2006	Sheep	0	0
1218	18	2006	Sheep	0	0
1219	18	2006	Sheep	0	0
1220	18	2006	Sheep	0	0
1221	18	2006	Sheep	0	0
1222	18	2006	Sheep	0	0
1223	18	2006	Sheep	0	0
1224	18	2006	Sheep	0	0
1225	18	2006	Sheep	0	0
1226	18	2006	Sheep	0	0
1227	18	2006	Sheep	0	0
1228	18	2006	Sheep	0	0
1229	18	2006	Sheep	0	0
1230	18	2006	Sheep	0	0
1231	18	2006	Sheep	0	0
1232	18	2006	Sheep	0	0
1233	18	2006	Sheep	0	0
1234	18	2006	Sheep	0	0
1235	18	2006	Sheep	768	0
1236	18	2006	Sheep	0	0
1237	18	2006	Sheep	0	0
1238	18	2006	Sheep	0	0
1239	18	2006	Sheep	0	0
1240	18	2006	Sheep	0	0
1241	18	2006	Sheep	0	0
1242	18	2006	Sheep	0	0
1243	18	2006	Sheep	0	0
1244	18	2006	Sheep	384	0
1245	18	2006	Sheep	0	0
1246	18	2006	Sheep	0	0
1247	18	2006	Sheep	0	0
1248	18	2006	Sheep	0	0
1249	18	2006	Sheep	768	0
1250	18	2006	Sheep	384	0
1251	18	2006	Sheep	0	0
1252	18	2006	Sheep	0	0
1253	18	2006	Sheep	0	0
1254	18	2006	Sheep	0	0
1255	18	2006	Sheep	768	0
1256	18	2006	Sheep	0	0
1257	18	2006	Sheep	0	0
1258	18	2006	Sheep	0	0
1259	18	2006	Sheep	0	0
1260	18	2006	Sheep	0	0
1261	18	2006	Sheep	0	0
1262	18	2006	Sheep	0	0
1263	18	2006	Sheep	0	0
1264	18	2006	Sheep	0	0
1265	18	2006	Sheep	1536	0
1266	18	2006	Sheep	0	0
1267	18	2006	Sheep	768	0
1268	18	2006	Sheep	24	0
1269	18	2006	Sheep	0	0
1270	18	2006	Sheep	0	0
1271	19	2006	Deer	24	0
1272	19	2006	Deer	0	0
1273	19	2006	Deer	48	0
1274	19	2006	Deer	0	0
1275	19	2006	Deer	0	0
1276	19	2006	Deer	384	0
1277	19	2006	Deer	384	0
1278	19	2006	Deer	48	0
1279	19	2006	Deer	0	0
1280	19	2006	Deer	24	0
1281	19	2006	Deer	0	0
1282	19	2006	Deer	24	0
1283	19	2006	Deer	24	0
1284	19	2006	Deer	24	0
1285	19	2006	Deer	0	0
1286	19	2006	Deer	192	0
1287	19	2006	Deer	0	0
1288	19	2006	Deer	48	0
1289	19	2006	Deer	0	0
1290	19	2006	Deer	0	0
1291	19	2006	Cattle	384	0
1292	19	2006	Cattle	384	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1293	19	2006	Cattle	192	0
1294	19	2006	Cattle	192	0
1295	19	2006	Cattle	768	0
1296	19	2006	Cattle	768	0
1297	19	2006	Cattle	384	0
1298	19	2006	Cattle	384	0
1299	19	2006	Cattle	768	0
1300	19	2006	Cattle	96	0
1301	19	2006	Cattle	96	0
1302	19	2006	Cattle	384	0
1303	19	2006	Cattle	192	0
1304	19	2006	Cattle	384	0
1305	19	2006	Cattle	384	0
1306	19	2006	Cattle	384	0
1307	19	2006	Cattle	384	0
1308	19	2006	Cattle	384	0
1309	19	2006	Cattle	384	0
1310	19	2006	Cattle	192	0
1311	19	2006	Sheep	48	0
1312	19	2006	Sheep	192	0
1313	19	2006	Sheep	384	0
1314	19	2006	Sheep	48	0
1315	19	2006	Sheep	24	0
1316	19	2006	Sheep	24	0
1317	19	2006	Sheep	0	0
1318	19	2006	Sheep	0	0
1319	19	2006	Sheep	96	0
1320	19	2006	Sheep	0	0
1321	19	2006	Sheep	0	0
1322	19	2006	Sheep	48	0
1323	19	2006	Sheep	48	0
1324	19	2006	Sheep	48	0
1325	19	2006	Sheep	24	0
1326	19	2006	Sheep	384	0
1327	19	2006	Sheep	24	0
1328	19	2006	Sheep	48	0
1329	19	2006	Sheep	768	0
1330	19	2006	Sheep	768	0
1331	19	2006	Sheep	0	0
1332	19	2006	Sheep	48	0
1333	19	2006	Sheep	24	0
1334	19	2006	Sheep	768	0
1335	19	2006	Sheep	0	0
1336	19	2006	Sheep	24	0
1337	19	2006	Sheep	192	0
1338	19	2006	Sheep	24	0
1339	19	2006	Sheep	384	0
1340	19	2006	Sheep	192	0
1341	19	2006	Sheep	768	0
1342	19	2006	Sheep	384	0
1343	19	2006	Sheep	0	0
1344	19	2006	Sheep	96	0
1345	19	2006	Sheep	48	0
1346	19	2006	Sheep	0	0
1347	19	2006	Sheep	384	0
1348	19	2006	Sheep	96	0
1349	19	2006	Sheep	0	0
1350	19	2006	Sheep	0	0
1351	19	2006	Sheep	48	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1352	19	2006	Sheep	192	0
1353	19	2006	Sheep	96	0
1354	19	2006	Sheep	24	0
1355	19	2006	Sheep	24	0
1356	19	2006	Sheep	0	0
1357	19	2006	Sheep	24	0
1358	19	2006	Sheep	24	0
1359	19	2006	Sheep	768	0
1360	19	2006	Sheep	48	0
1361	19	2006	Sheep	24	0
1362	19	2006	Sheep	384	0
1363	19	2006	Sheep	96	0
1364	19	2006	Sheep	384	0
1365	19	2006	Sheep	96	0
1366	19	2006	Sheep	192	0
1367	19	2006	Sheep	48	0
1368	19	2006	Sheep	48	0
1369	19	2006	Sheep	192	0
1370	19	2006	Sheep	384	0
1371	20	2006	Deer	24	48
1372	20	2006	Deer	0	0
1373	20	2006	Deer	24	96
1374	20	2006	Deer	0	24
1375	20	2006	Deer	24	0
1376	20	2006	Deer	0	0
1377	20	2006	Deer	0	192
1378	20	2006	Deer	96	0
1379	20	2006	Deer	24	48
1380	20	2006	Deer	24	0
1381	20	2006	Deer	96	0
1382	20	2006	Deer	24	96
1383	20	2006	Deer	0	192
1384	20	2006	Deer	192	48
1385	20	2006	Deer	0	24
1386	20	2006	Deer	192	0
1387	20	2006	Deer	0	0
1388	20	2006	Deer	96	0
1389	20	2006	Deer	48	48
1390	20	2006	Deer	0	0
1391	20	2006	Deer	192	0
1392	20	2006	Cattle	0	0
1393	20	2006	Cattle	0	0
1394	20	2006	Cattle	0	0
1395	20	2006	Cattle	0	0
1396	20	2006	Cattle	48	0
1397	20	2006	Cattle	0	0
1398	20	2006	Cattle	0	0
1399	20	2006	Cattle	24	0
1400	20	2006	Cattle	0	0
1401	20	2006	Cattle	0	0
1402	20	2006	Cattle	0	0
1403	20	2006	Cattle	48	0
1404	20	2006	Cattle	384	0
1405	20	2006	Cattle	0	0
1406	20	2006	Cattle	0	0
1407	20	2006	Cattle	192	0
1408	20	2006	Cattle	768	0
1409	20	2006	Cattle	0	0
1410	20	2006	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1411	20	2006	Sheep	0	0
1412	20	2006	Sheep	384	0
1413	20	2006	Sheep	0	0
1414	20	2006	Sheep	0	0
1415	20	2006	Sheep	0	0
1416	20	2006	Sheep	0	0
1417	20	2006	Sheep	0	0
1418	20	2006	Sheep	48	0
1419	20	2006	Sheep	0	0
1420	20	2006	Sheep	0	0
1421	20	2006	Sheep	0	0
1422	20	2006	Sheep	0	0
1423	20	2006	Sheep	0	0
1424	20	2006	Sheep	0	0
1425	20	2006	Sheep	0	0
1426	20	2006	Sheep	0	0
1427	20	2006	Sheep	0	0
1428	20	2006	Sheep	0	0
1429	20	2006	Sheep	0	0
1430	20	2006	Sheep	0	0
1431	20	2006	Sheep	0	0
1432	20	2006	Sheep	0	0
1433	20	2006	Sheep	0	0
1434	20	2006	Sheep	0	0
1435	20	2006	Sheep	0	0
1436	20	2006	Sheep	24	0
1437	20	2006	Sheep	384	0
1438	20	2006	Sheep	0	0
1439	20	2006	Sheep	0	0
1440	20	2006	Sheep	0	0
1441	20	2006	Sheep	0	0
1442	20	2006	Sheep	0	0
1443	20	2006	Sheep	96	0
1444	20	2006	Sheep	0	0
1445	20	2006	Sheep	0	0
1446	20	2006	Sheep	0	0
1447	20	2006	Sheep	0	0
1448	20	2006	Sheep	0	0
1449	20	2006	Sheep	0	0
1450	20	2006	Sheep	0	0
1451	20	2006	Sheep	0	0
1452	20	2006	Sheep	0	0
1453	20	2006	Sheep	0	0
1454	20	2006	Sheep	0	0
1455	20	2006	Sheep	0	0
1456	1	2007	Deer	0	0
1457	1	2007	Deer	0	0
1458	1	2007	Deer	96	0
1459	1	2007	Deer	0	0
1460	1	2007	Deer	0	0
1461	1	2007	Deer	0	0
1462	1	2007	Deer	0	0
1463	1	2007	Deer	0	0
1464	1	2007	Deer	0	0
1465	1	2007	Deer	0	0
1466	1	2007	Deer	0	0
1467	1	2007	Deer	0	0
1468	1	2007	Deer	0	0
1469	1	2007	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1470	1	2007	Deer	0	0
1471	1	2007	Deer	0	0
1472	1	2007	Deer	0	0
1473	1	2007	Deer	0	0
1474	1	2007	Deer	0	0
1475	2	2007	Deer	48	0
1476	2	2007	Deer	0	0
1477	2	2007	Deer	24	0
1478	2	2007	Deer	0	0
1479	2	2007	Deer	24	0
1480	2	2007	Deer	24	0
1481	2	2007	Deer	24	0
1482	2	2007	Deer	48	0
1483	2	2007	Deer	0	0
1484	2	2007	Deer	48	0
1485	2	2007	Deer	48	0
1486	2	2007	Deer	0	0
1487	2	2007	Deer	24	0
1488	2	2007	Deer	0	0
1489	2	2007	Deer	24	0
1490	2	2007	Deer	24	0
1491	2	2007	Deer	48	0
1492	2	2007	Deer	24	0
1493	2	2007	Deer	0	0
1494	3	2007	Deer	0	0
1495	3	2007	Deer	0	0
1496	3	2007	Deer	0	0
1497	3	2007	Deer	0	0
1498	3	2007	Deer	0	0
1499	3	2007	Deer	0	0
1500	3	2007	Deer	0	0
1501	3	2007	Deer	0	0
1502	3	2007	Deer	0	0
1503	3	2007	Deer	0	0
1504	3	2007	Deer	0	0
1505	3	2007	Deer	0	0
1506	3	2007	Deer	0	0
1507	3	2007	Deer	0	0
1508	3	2007	Deer	0	0
1509	3	2007	Deer	0	0
1510	3	2007	Deer	0	0
1511	3	2007	Deer	0	0
1512	3	2007	Deer	0	0
1513	3	2007	Deer	0	0
1514	4	2007	Deer	48	0
1515	4	2007	Deer	0	48
1516	4	2007	Deer	24	192
1517	4	2007	Deer	0	24
1518	4	2007	Deer	0	24
1519	4	2007	Deer	48	0
1520	4	2007	Deer	48	0
1521	4	2007	Deer	0	0
1522	4	2007	Deer	48	48
1523	4	2007	Deer	0	48
1524	4	2007	Deer	0	0
1525	4	2007	Deer	0	0
1526	4	2007	Deer	0	0
1527	4	2007	Deer	24	0
1528	4	2007	Deer	24	24

Animal	Farm	Year	Species	Hardjobovis	Pomona
1529	4	2007	Deer	0	24
1530	4	2007	Cattle	0	0
1531	4	2007	Cattle	0	0
1532	4	2007	Cattle	0	0
1533	4	2007	Cattle	0	0
1534	4	2007	Cattle	0	0
1535	4	2007	Cattle	0	0
1536	4	2007	Cattle	0	0
1537	4	2007	Cattle	0	0
1538	5	2007	Deer	0	0
1539	5	2007	Deer	0	0
1540	5	2007	Deer	0	0
1541	5	2007	Deer	0	0
1542	5	2007	Deer	0	0
1543	5	2007	Deer	0	0
1544	5	2007	Deer	384	0
1545	5	2007	Deer	0	0
1546	5	2007	Deer	0	0
1547	5	2007	Deer	0	0
1548	5	2007	Deer	0	0
1549	5	2007	Deer	0	0
1550	5	2007	Deer	0	0
1551	5	2007	Deer	0	0
1552	5	2007	Deer	0	0
1553	5	2007	Deer	0	0
1554	5	2007	Deer	0	0
1555	5	2007	Deer	0	0
1556	5	2007	Deer	0	0
1557	5	2007	Deer	0	0
1558	5	2007	Cattle	0	0
1559	5	2007	Cattle	0	0
1560	5	2007	Cattle	0	0
1561	5	2007	Cattle	0	0
1562	5	2007	Cattle	0	0
1563	5	2007	Cattle	0	0
1564	5	2007	Cattle	0	0
1565	5	2007	Cattle	0	0
1566	5	2007	Cattle	0	0
1567	5	2007	Cattle	0	0
1568	5	2007	Cattle	0	24
1569	5	2007	Cattle	0	0
1570	5	2007	Cattle	0	0
1571	5	2007	Cattle	0	0
1572	5	2007	Cattle	0	0
1573	5	2007	Cattle	0	0
1574	5	2007	Cattle	0	0
1575	5	2007	Cattle	0	0
1576	5	2007	Cattle	0	0
1577	6	2007	Deer	24	0
1578	6	2007	Deer	192	0
1579	6	2007	Deer	48	0
1580	6	2007	Deer	192	0
1581	6	2007	Deer	0	0
1582	6	2007	Deer	0	0
1583	6	2007	Deer	192	0
1584	6	2007	Deer	96	0
1585	6	2007	Deer	48	0
1586	6	2007	Deer	48	0
1587	6	2007	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1588	6	2007	Deer	48	0
1589	6	2007	Deer	96	0
1590	6	2007	Deer	96	0
1591	6	2007	Deer	48	0
1592	6	2007	Deer	24	0
1593	6	2007	Deer	0	0
1594	6	2007	Deer	24	0
1595	6	2007	Deer	192	0
1596	6	2007	Deer	24	0
1597	6	2007	Cattle	192	0
1598	6	2007	Cattle	96	0
1599	6	2007	Cattle	192	0
1600	6	2007	Cattle	192	0
1601	6	2007	Cattle	48	0
1602	6	2007	Cattle	48	0
1603	6	2007	Cattle	96	0
1604	6	2007	Cattle	96	0
1605	6	2007	Cattle	96	0
1606	6	2007	Cattle	96	0
1607	6	2007	Cattle	192	0
1608	6	2007	Cattle	384	0
1609	6	2007	Cattle	96	0
1610	6	2007	Cattle	48	0
1611	6	2007	Cattle	96	0
1612	6	2007	Cattle	0	24
1613	6	2007	Cattle	48	0
1614	6	2007	Cattle	96	0
1615	6	2007	Cattle	0	0
1616	6	2007	Cattle	0	0
1617	7	2007	Deer	0	0
1618	7	2007	Deer	0	0
1619	7	2007	Deer	0	0
1620	7	2007	Deer	24	0
1621	7	2007	Deer	0	0
1622	7	2007	Deer	48	0
1623	7	2007	Deer	24	0
1624	7	2007	Deer	48	0
1625	7	2007	Deer	48	0
1626	7	2007	Deer	0	0
1627	7	2007	Deer	0	0
1628	7	2007	Deer	96	96
1629	7	2007	Deer	0	0
1630	7	2007	Deer	0	0
1631	7	2007	Deer	96	0
1632	7	2007	Deer	0	0
1633	7	2007	Deer	0	0
1634	7	2007	Deer	48	0
1635	7	2007	Deer	0	0
1636	7	2007	Deer	0	0
1637	7	2007	Sheep	0	0
1638	7	2007	Sheep	0	0
1639	7	2007	Sheep	0	0
1640	7	2007	Sheep	0	0
1641	7	2007	Sheep	0	0
1642	7	2007	Sheep	0	0
1643	7	2007	Sheep	0	0
1644	7	2007	Sheep	0	0
1645	7	2007	Sheep	0	0
1646	7	2007	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1647	7	2007	Sheep	0	0
1648	7	2007	Sheep	0	0
1649	7	2007	Sheep	0	0
1650	7	2007	Sheep	0	0
1651	7	2007	Sheep	0	0
1652	7	2007	Sheep	0	0
1653	7	2007	Sheep	0	0
1654	7	2007	Sheep	0	0
1655	7	2007	Sheep	0	0
1656	9	2007	Deer	24	192
1657	9	2007	Deer	0	0
1658	9	2007	Deer	0	24
1659	9	2007	Deer	0	0
1660	9	2007	Deer	0	0
1661	9	2007	Deer	0	24
1662	9	2007	Deer	0	0
1663	9	2007	Deer	0	0
1664	9	2007	Deer	0	0
1665	9	2007	Deer	0	24
1666	9	2007	Deer	24	0
1667	9	2007	Deer	0	24
1668	9	2007	Deer	0	0
1669	9	2007	Deer	24	0
1670	9	2007	Deer	0	0
1671	9	2007	Deer	0	0
1672	9	2007	Deer	0	0
1673	9	2007	Deer	24	96
1674	9	2007	Deer	0	48
1675	9	2007	Cattle	96	48
1676	9	2007	Cattle	96	0
1677	9	2007	Cattle	48	0
1678	9	2007	Cattle	24	0
1679	9	2007	Cattle	24	48
1680	9	2007	Cattle	0	96
1681	9	2007	Cattle	24	24
1682	9	2007	Cattle	24	0
1683	9	2007	Cattle	96	48
1684	9	2007	Cattle	0	0
1685	9	2007	Cattle	96	0
1686	9	2007	Cattle	0	0
1687	9	2007	Cattle	0	96
1688	9	2007	Cattle	192	24
1689	9	2007	Cattle	0	48
1690	9	2007	Cattle	24	48
1691	9	2007	Cattle	0	48
1692	9	2007	Cattle	96	0
1693	9	2007	Cattle	0	768
1694	9	2007	Sheep	0	48
1695	9	2007	Sheep	96	0
1696	9	2007	Sheep	0	0
1697	9	2007	Sheep	0	0
1698	9	2007	Sheep	0	0
1699	9	2007	Sheep	0	0
1700	9	2007	Sheep	24	0
1701	9	2007	Sheep	48	0
1702	9	2007	Sheep	96	0
1703	9	2007	Sheep	24	0
1704	9	2007	Sheep	48	0
1705	9	2007	Sheep	48	0
1706	9	2007	Sheep	48	0
1707	9	2007	Sheep	48	0
1708	9	2007	Sheep	96	0
1709	9	2007	Sheep	96	0
1710	9	2007	Sheep	48	0
1711	10	2007	Deer	24	0
1712	10	2007	Deer	0	0
1713	10	2007	Deer	96	24
1714	10	2007	Deer	24	0
1715	10	2007	Deer	96	96
1716	10	2007	Deer	48	0
1717	10	2007	Deer	0	96
1718	10	2007	Deer	0	96
1719	10	2007	Deer	0	96
1720	10	2007	Deer	48	96
1721	10	2007	Deer	48	48
1722	10	2007	Deer	0	192
1723	10	2007	Deer	0	48
1724	10	2007	Deer	0	192
1725	10	2007	Deer	0	48
1726	10	2007	Deer	48	384
1727	10	2007	Deer	0	96
1728	10	2007	Deer	0	0
1729	10	2007	Deer	24	0
1730	10	2007	Cattle	0	0
1731	10	2007	Cattle	24	0
1732	10	2007	Cattle	0	96
1733	10	2007	Cattle	0	0
1734	10	2007	Cattle	24	0
1735	10	2007	Cattle	0	0
1736	10	2007	Cattle	0	0
1737	10	2007	Cattle	48	0
1738	10	2007	Cattle	24	48
1739	10	2007	Cattle	192	0
1740	10	2007	Cattle	24	0
1741	10	2007	Cattle	0	0
1742	10	2007	Cattle	48	24
1743	10	2007	Cattle	0	0
1744	10	2007	Cattle	0	0
1745	10	2007	Cattle	48	0
1746	10	2007	Cattle	24	0
1747	10	2007	Cattle	48	0
1748	10	2007	Sheep	48	0
1749	10	2007	Sheep	96	0
1750	10	2007	Sheep	0	24
1751	10	2007	Sheep	48	96
1752	10	2007	Sheep	0	0
1753	10	2007	Sheep	96	0
1754	10	2007	Sheep	0	0
1755	10	2007	Sheep	0	0
1756	10	2007	Sheep	0	0
1757	10	2007	Sheep	0	0
1758	10	2007	Sheep	48	0
1759	10	2007	Sheep	48	0
1760	10	2007	Sheep	0	48
1761	10	2007	Sheep	0	0
1762	10	2007	Sheep	0	96
1763	10	2007	Sheep	192	0
1764	10	2007	Sheep	24	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1765	10	2007	Sheep	24	0
1766	10	2007	Sheep	0	0
1767	10	2007	Sheep	0	0
1768	11	2007	Deer	48	0
1769	11	2007	Deer	48	0
1770	11	2007	Deer	24	0
1771	11	2007	Deer	24	0
1772	11	2007	Deer	96	0
1773	11	2007	Deer	96	0
1774	11	2007	Deer	48	0
1775	11	2007	Deer	48	0
1776	11	2007	Deer	96	0
1777	11	2007	Deer	48	0
1778	11	2007	Deer	48	0
1779	11	2007	Deer	48	0
1780	11	2007	Deer	96	0
1781	11	2007	Deer	24	0
1782	11	2007	Deer	48	0
1783	11	2007	Deer	96	0
1784	11	2007	Deer	96	0
1785	11	2007	Deer	96	0
1786	11	2007	Deer	96	0
1787	11	2007	Deer	96	0
1788	11	2007	Cattle	48	24
1789	11	2007	Cattle	0	0
1790	11	2007	Cattle	24	24
1791	11	2007	Cattle	0	0
1792	11	2007	Cattle	24	0
1793	11	2007	Cattle	0	0
1794	11	2007	Cattle	0	0
1795	11	2007	Cattle	0	0
1796	11	2007	Cattle	0	0
1797	11	2007	Cattle	192	0
1798	11	2007	Cattle	48	0
1799	11	2007	Cattle	0	0
1800	11	2007	Cattle	0	0
1801	11	2007	Cattle	0	0
1802	11	2007	Cattle	24	0
1803	11	2007	Cattle	0	0
1804	11	2007	Cattle	48	0
1805	11	2007	Cattle	24	0
1806	11	2007	Cattle	192	0
1807	11	2007	Cattle	24	24
1808	11	2007	Sheep	96	0
1809	11	2007	Sheep	0	0
1810	11	2007	Sheep	192	0
1811	11	2007	Sheep	0	0
1812	11	2007	Sheep	192	0
1813	11	2007	Sheep	0	0
1814	11	2007	Sheep	192	0
1815	11	2007	Sheep	0	0
1816	11	2007	Sheep	192	0
1817	11	2007	Sheep	768	0
1818	11	2007	Sheep	96	0
1819	11	2007	Sheep	0	0
1820	11	2007	Sheep	0	0
1821	11	2007	Sheep	0	0
1822	11	2007	Sheep	0	0
1823	11	2007	Sheep	192	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1824	11	2007	Sheep	0	0
1825	11	2007	Sheep	0	0
1826	11	2007	Sheep	0	0
1827	11	2007	Sheep	96	0
1828	12	2007	Deer	0	0
1829	12	2007	Deer	0	0
1830	12	2007	Deer	0	0
1831	12	2007	Deer	0	0
1832	12	2007	Deer	0	0
1833	12	2007	Deer	24	0
1834	12	2007	Deer	96	0
1835	12	2007	Deer	24	0
1836	12	2007	Deer	96	0
1837	12	2007	Deer	0	0
1838	12	2007	Deer	0	0
1839	12	2007	Deer	0	0
1840	12	2007	Deer	0	0
1841	12	2007	Deer	0	0
1842	12	2007	Deer	0	0
1843	12	2007	Deer	0	0
1844	12	2007	Deer	0	0
1845	12	2007	Deer	48	0
1846	12	2007	Deer	0	0
1847	12	2007	Deer	96	0
1848	12	2007	Cattle	0	0
1849	12	2007	Cattle	0	0
1850	12	2007	Cattle	0	0
1851	12	2007	Cattle	48	0
1852	12	2007	Cattle	0	0
1853	12	2007	Cattle	0	0
1854	12	2007	Cattle	96	0
1855	12	2007	Cattle	0	0
1856	12	2007	Cattle	0	0
1857	12	2007	Cattle	0	0
1858	12	2007	Cattle	96	0
1859	12	2007	Cattle	0	0
1860	12	2007	Cattle	48	0
1861	12	2007	Cattle	192	0
1862	12	2007	Cattle	96	0
1863	12	2007	Cattle	96	0
1864	12	2007	Cattle	0	0
1865	12	2007	Cattle	0	0
1866	12	2007	Cattle	0	0
1867	12	2007	Cattle	0	0
1868	12	2007	Sheep	24	96
1869	12	2007	Sheep	96	0
1870	12	2007	Sheep	96	0
1871	12	2007	Sheep	24	0
1872	12	2007	Sheep	0	0
1873	12	2007	Sheep	48	0
1874	12	2007	Sheep	48	0
1875	12	2007	Sheep	0	0
1876	12	2007	Sheep	48	0
1877	12	2007	Sheep	48	0
1878	12	2007	Sheep	24	0
1879	12	2007	Sheep	0	0
1880	12	2007	Sheep	96	0
1881	12	2007	Sheep	48	0
1882	12	2007	Sheep	48	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
1883	12	2007	Sheep	48	0
1884	12	2007	Sheep	48	0
1885	12	2007	Sheep	24	0
1886	12	2007	Sheep	48	0
1887	12	2007	Sheep	96	0
1888	13	2007	Deer	48	0
1889	13	2007	Deer	0	0
1890	13	2007	Deer	24	0
1891	13	2007	Deer	24	0
1892	13	2007	Deer	24	0
1893	13	2007	Deer	48	0
1894	13	2007	Deer	24	0
1895	13	2007	Deer	24	0
1896	13	2007	Deer	48	24
1897	13	2007	Deer	0	0
1898	13	2007	Deer	48	0
1899	13	2007	Deer	96	0
1900	13	2007	Deer	96	0
1901	13	2007	Deer	24	0
1902	13	2007	Deer	0	0
1903	13	2007	Deer	24	0
1904	13	2007	Deer	48	0
1905	13	2007	Deer	96	0
1906	13	2007	Deer	48	0
1907	13	2007	Deer	24	0
1908	13	2007	Sheep	0	0
1909	13	2007	Sheep	0	0
1910	13	2007	Sheep	0	0
1911	13	2007	Sheep	0	0
1912	13	2007	Sheep	0	0
1913	13	2007	Sheep	0	0
1914	13	2007	Sheep	0	0
1915	13	2007	Sheep	0	0
1916	13	2007	Sheep	0	0
1917	13	2007	Sheep	0	0
1918	13	2007	Sheep	0	0
1919	13	2007	Sheep	0	0
1920	13	2007	Sheep	0	0
1921	13	2007	Sheep	0	0
1922	13	2007	Sheep	0	0
1923	13	2007	Sheep	0	0
1924	13	2007	Sheep	0	0
1925	13	2007	Sheep	0	0
1926	13	2007	Sheep	0	0
1927	13	2007	Sheep	0	0
1928	14	2007	Deer	0	96
1929	14	2007	Deer	0	192
1930	14	2007	Deer	0	0
1931	14	2007	Deer	0	96
1932	14	2007	Deer	0	0
1933	14	2007	Deer	24	0
1934	14	2007	Deer	96	48
1935	14	2007	Deer	0	0
1936	14	2007	Deer	96	96
1937	14	2007	Deer	0	96
1938	14	2007	Deer	0	0
1939	14	2007	Deer	0	48
1940	14	2007	Deer	0	0
1941	14	2007	Deer	0	96

Animal	Farm	Year	Species	Hardjobovis	Pomona
1942	14	2007	Deer	0	0
1943	14	2007	Deer	24	0
1944	14	2007	Deer	96	0
1945	14	2007	Deer	48	48
1946	14	2007	Deer	0	384
1947	14	2007	Deer	0	0
1948	14	2007	Cattle	0	0
1949	14	2007	Cattle	0	0
1950	14	2007	Cattle	0	0
1951	14	2007	Cattle	0	0
1952	14	2007	Cattle	0	0
1953	14	2007	Cattle	0	0
1954	14	2007	Cattle	0	0
1955	14	2007	Cattle	0	0
1956	14	2007	Cattle	0	0
1957	14	2007	Cattle	0	0
1958	14	2007	Cattle	0	0
1959	14	2007	Cattle	0	0
1960	14	2007	Cattle	0	96
1961	14	2007	Cattle	0	0
1962	14	2007	Cattle	0	0
1963	14	2007	Cattle	0	0
1964	14	2007	Cattle	0	0
1965	14	2007	Cattle	0	0
1966	14	2007	Cattle	0	0
1967	14	2007	Cattle	0	0
1968	14	2007	Sheep	0	0
1969	14	2007	Sheep	24	0
1970	14	2007	Sheep	48	0
1971	14	2007	Sheep	0	0
1972	14	2007	Sheep	24	0
1973	14	2007	Sheep	24	0
1974	14	2007	Sheep	48	0
1975	14	2007	Sheep	0	0
1976	14	2007	Sheep	0	0
1977	14	2007	Sheep	0	0
1978	14	2007	Sheep	0	0
1979	14	2007	Sheep	48	0
1980	14	2007	Sheep	0	0
1981	14	2007	Sheep	0	0
1982	14	2007	Sheep	192	0
1983	14	2007	Sheep	0	0
1984	14	2007	Sheep	96	0
1985	14	2007	Sheep	96	48
1986	14	2007	Sheep	0	0
1987	14	2007	Sheep	0	0
1988	15	2007	Deer	0	0
1989	15	2007	Deer	0	0
1990	15	2007	Deer	0	0
1991	15	2007	Deer	0	0
1992	15	2007	Deer	0	0
1993	15	2007	Deer	0	0
1994	15	2007	Deer	0	0
1995	15	2007	Deer	0	0
1996	15	2007	Deer	0	0
1997	15	2007	Deer	0	0
1998	15	2007	Deer	0	0
1999	15	2007	Deer	0	0
2000	15	2007	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2001	15	2007	Deer	0	0
2002	15	2007	Deer	0	0
2003	15	2007	Deer	0	0
2004	15	2007	Deer	384	0
2005	15	2007	Deer	48	0
2006	15	2007	Deer	0	0
2007	15	2007	Cattle	192	0
2008	15	2007	Cattle	0	0
2009	15	2007	Cattle	0	0
2010	15	2007	Cattle	24	0
2011	15	2007	Cattle	192	0
2012	15	2007	Cattle	96	0
2013	15	2007	Cattle	48	0
2014	15	2007	Cattle	0	0
2015	15	2007	Cattle	0	0
2016	15	2007	Cattle	384	0
2017	15	2007	Cattle	48	0
2018	15	2007	Cattle	48	0
2019	15	2007	Cattle	48	0
2020	15	2007	Cattle	24	0
2021	15	2007	Cattle	48	0
2022	15	2007	Cattle	192	0
2023	15	2007	Cattle	0	0
2024	15	2007	Cattle	96	0
2025	15	2007	Cattle	0	0
2026	15	2007	Cattle	0	0
2027	15	2007	Sheep	0	0
2028	15	2007	Sheep	48	0
2029	15	2007	Sheep	0	0
2030	15	2007	Sheep	96	0
2031	15	2007	Sheep	48	0
2032	15	2007	Sheep	48	0
2033	15	2007	Sheep	0	0
2034	15	2007	Sheep	96	0
2035	15	2007	Sheep	96	0
2036	15	2007	Sheep	0	0
2037	15	2007	Sheep	96	0
2038	15	2007	Sheep	96	0
2039	15	2007	Sheep	96	0
2040	15	2007	Sheep	48	0
2041	15	2007	Sheep	96	0
2042	15	2007	Sheep	24	0
2043	15	2007	Sheep	48	0
2044	15	2007	Sheep	0	0
2045	15	2007	Sheep	96	0
2046	16	2007	Deer	0	0
2047	16	2007	Deer	0	0
2048	16	2007	Deer	0	0
2049	16	2007	Deer	0	0
2050	16	2007	Deer	0	0
2051	16	2007	Deer	0	0
2052	16	2007	Deer	0	0
2053	16	2007	Deer	0	0
2054	16	2007	Deer	0	0
2055	16	2007	Deer	0	0
2056	16	2007	Deer	0	0
2057	16	2007	Deer	0	0
2058	16	2007	Deer	48	0
2059	16	2007	Deer	0	0
2060	16	2007	Deer	48	0
2061	16	2007	Deer	0	0
2062	16	2007	Deer	0	0
2063	16	2007	Deer	0	0
2064	16	2007	Deer	48	0
2065	16	2007	Deer	24	0
2066	16	2007	Cattle	0	0
2067	16	2007	Cattle	0	0
2068	16	2007	Cattle	0	0
2069	16	2007	Cattle	0	0
2070	16	2007	Cattle	0	0
2071	16	2007	Cattle	0	0
2072	16	2007	Cattle	0	0
2073	16	2007	Cattle	0	0
2074	16	2007	Cattle	0	0
2075	16	2007	Cattle	0	0
2076	16	2007	Cattle	0	0
2077	16	2007	Cattle	0	24
2078	16	2007	Cattle	0	0
2079	16	2007	Cattle	0	0
2080	16	2007	Cattle	0	0
2081	16	2007	Cattle	0	0
2082	16	2007	Cattle	0	0
2083	16	2007	Cattle	0	0
2084	16	2007	Cattle	0	0
2085	16	2007	Cattle	0	0
2086	16	2007	Sheep	0	0
2087	16	2007	Sheep	0	0
2088	16	2007	Sheep	0	0
2089	16	2007	Sheep	0	0
2090	16	2007	Sheep	0	0
2091	16	2007	Sheep	0	0
2092	16	2007	Sheep	0	0
2093	16	2007	Sheep	0	0
2094	16	2007	Sheep	0	0
2095	16	2007	Sheep	0	0
2096	16	2007	Sheep	0	0
2097	16	2007	Sheep	0	0
2098	16	2007	Sheep	0	0
2099	16	2007	Sheep	0	0
2100	16	2007	Sheep	0	0
2101	16	2007	Sheep	0	0
2102	16	2007	Sheep	0	0
2103	16	2007	Sheep	0	0
2104	16	2007	Sheep	0	0
2105	16	2007	Sheep	0	0
2106	17	2007	Deer	48	0
2107	17	2007	Deer	48	0
2108	17	2007	Deer	96	0
2109	17	2007	Deer	0	0
2110	17	2007	Deer	48	0
2111	17	2007	Deer	96	0
2112	17	2007	Deer	96	0
2113	17	2007	Deer	0	0
2114	17	2007	Deer	48	24
2115	17	2007	Deer	48	24
2116	17	2007	Deer	48	0
2117	17	2007	Deer	48	0
2118	17	2007	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2119	17	2007	Deer	0	0
2120	17	2007	Deer	96	0
2121	17	2007	Deer	192	48
2122	17	2007	Deer	0	24
2123	17	2007	Deer	0	0
2124	17	2007	Deer	48	0
2125	17	2007	Cattle	48	0
2126	17	2007	Cattle	96	0
2127	17	2007	Cattle	384	0
2128	17	2007	Cattle	768	0
2129	17	2007	Cattle	768	0
2130	17	2007	Cattle	384	0
2131	17	2007	Cattle	384	0
2132	17	2007	Sheep	0	0
2133	17	2007	Sheep	0	0
2134	17	2007	Sheep	0	0
2135	17	2007	Sheep	0	0
2136	17	2007	Sheep	0	0
2137	17	2007	Sheep	0	0
2138	17	2007	Sheep	48	0
2139	17	2007	Sheep	0	0
2140	17	2007	Sheep	0	0
2141	17	2007	Sheep	0	0
2142	17	2007	Sheep	24	0
2143	17	2007	Sheep	0	0
2144	17	2007	Sheep	0	0
2145	17	2007	Sheep	0	0
2146	17	2007	Sheep	96	0
2147	17	2007	Sheep	48	0
2148	17	2007	Sheep	0	0
2149	17	2007	Sheep	48	0
2150	17	2007	Sheep	0	0
2151	17	2007	Sheep	0	0
2152	18	2007	Deer	24	48
2153	18	2007	Deer	48	192
2154	18	2007	Deer	0	96
2155	18	2007	Deer	24	48
2156	18	2007	Deer	0	0
2157	18	2007	Deer	0	0
2158	18	2007	Deer	0	48
2159	18	2007	Deer	24	48
2160	18	2007	Deer	192	96
2161	18	2007	Deer	96	0
2162	18	2007	Deer	192	0
2163	18	2007	Deer	0	0
2164	18	2007	Deer	0	0
2165	18	2007	Deer	48	96
2166	18	2007	Deer	0	192
2167	18	2007	Deer	0	192
2168	18	2007	Deer	96	48
2169	18	2007	Deer	96	384
2170	18	2007	Deer	48	0
2171	18	2007	Deer	0	0
2172	18	2007	Cattle	192	0
2173	18	2007	Cattle	48	0
2174	18	2007	Cattle	48	0
2175	18	2007	Cattle	192	0
2176	18	2007	Cattle	96	0
2177	18	2007	Cattle	96	0
2178	18	2007	Cattle	192	0
2179	18	2007	Cattle	192	0
2180	18	2007	Cattle	0	0
2181	18	2007	Cattle	96	0
2182	18	2007	Cattle	0	0
2183	18	2007	Cattle	96	0
2184	18	2007	Cattle	48	0
2185	18	2007	Cattle	96	0
2186	18	2007	Cattle	48	0
2187	18	2007	Cattle	48	0
2188	18	2007	Cattle	24	0
2189	18	2007	Cattle	192	0
2190	18	2007	Cattle	48	24
2191	18	2007	Cattle	96	0
2192	18	2007	Sheep	0	0
2193	18	2007	Sheep	0	0
2194	18	2007	Sheep	0	0
2195	18	2007	Sheep	0	0
2196	18	2007	Sheep	0	0
2197	18	2007	Sheep	0	0
2198	18	2007	Sheep	384	0
2199	18	2007	Sheep	0	0
2200	18	2007	Sheep	0	0
2201	18	2007	Sheep	0	0
2202	18	2007	Sheep	0	0
2203	18	2007	Sheep	0	0
2204	18	2007	Sheep	0	0
2205	18	2007	Sheep	0	0
2206	18	2007	Sheep	0	0
2207	18	2007	Sheep	0	0
2208	18	2007	Sheep	0	0
2209	18	2007	Sheep	0	0
2210	18	2007	Sheep	0	0
2211	19	2007	Deer	24	0
2212	19	2007	Deer	24	0
2213	19	2007	Deer	0	0
2214	19	2007	Deer	24	0
2215	19	2007	Deer	24	24
2216	19	2007	Deer	0	0
2217	19	2007	Deer	0	0
2218	19	2007	Deer	0	0
2219	19	2007	Deer	0	0
2220	19	2007	Deer	0	0
2221	19	2007	Deer	48	0
2222	19	2007	Deer	0	0
2223	19	2007	Deer	48	0
2224	19	2007	Deer	0	0
2225	19	2007	Deer	96	0
2226	19	2007	Deer	24	0
2227	19	2007	Deer	24	0
2228	19	2007	Deer	0	0
2229	19	2007	Deer	0	0
2230	19	2007	Deer	0	0
2231	19	2007	Deer	24	48
2232	19	2007	Deer	0	0
2233	19	2007	Deer	0	96
2234	19	2007	Deer	48	48
2235	19	2007	Deer	192	0
2236	19	2007	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2237	19	2007	Deer	96	0
2238	19	2007	Deer	0	0
2239	19	2007	Deer	0	0
2240	19	2007	Deer	0	0
2241	19	2007	Deer	0	48
2242	19	2007	Deer	0	0
2243	19	2007	Deer	192	0
2244	19	2007	Deer	0	0
2245	19	2007	Deer	0	0
2246	19	2007	Deer	0	0
2247	19	2007	Deer	0	0
2248	19	2007	Deer	0	0
2249	19	2007	Deer	48	24
2250	19	2007	Deer	192	0
2251	19	2007	Cattle	0	0
2252	19	2007	Cattle	24	0
2253	19	2007	Cattle	0	0
2254	19	2007	Cattle	24	0
2255	19	2007	Cattle	96	0
2256	19	2007	Cattle	96	0
2257	19	2007	Cattle	24	0
2258	19	2007	Cattle	96	0
2259	19	2007	Cattle	48	0
2260	19	2007	Cattle	48	0
2261	19	2007	Cattle	24	0
2262	19	2007	Cattle	48	0
2263	19	2007	Cattle	48	0
2264	19	2007	Cattle	48	0
2265	19	2007	Cattle	48	0
2266	19	2007	Cattle	0	0
2267	19	2007	Cattle	48	0
2268	19	2007	Cattle	96	0
2269	19	2007	Cattle	96	0
2270	19	2007	Cattle	0	0
2271	19	2007	Cattle	0	0
2272	19	2007	Cattle	0	0
2273	19	2007	Cattle	0	0
2274	19	2007	Cattle	0	0
2275	19	2007	Cattle	0	0
2276	19	2007	Cattle	0	0
2277	19	2007	Cattle	0	0
2278	19	2007	Cattle	0	0
2279	19	2007	Cattle	0	0
2280	19	2007	Cattle	0	0
2281	19	2007	Cattle	0	0
2282	19	2007	Cattle	0	0
2283	19	2007	Cattle	0	0
2284	19	2007	Cattle	0	0
2285	19	2007	Cattle	0	0
2286	19	2007	Cattle	0	0
2287	19	2007	Cattle	0	0
2288	19	2007	Cattle	0	0
2289	19	2007	Cattle	0	0
2290	19	2007	Cattle	0	0
2291	19	2007	Sheep	0	384
2292	19	2007	Sheep	0	192
2293	19	2007	Sheep	0	0
2294	19	2007	Sheep	768	0
2295	19	2007	Sheep	192	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2296	19	2007	Sheep	768	0
2297	19	2007	Sheep	0	96
2298	19	2007	Sheep	0	192
2299	19	2007	Sheep	0	192
2300	19	2007	Sheep	0	384
2301	19	2007	Sheep	0	0
2302	19	2007	Sheep	48	192
2303	19	2007	Sheep	0	192
2304	19	2007	Sheep	0	192
2305	19	2007	Sheep	0	192
2306	19	2007	Sheep	96	192
2307	19	2007	Sheep	48	96
2308	19	2007	Sheep	96	384
2309	19	2007	Sheep	48	48
2310	19	2007	Sheep	384	192
2311	20	2007	Deer	48	48
2312	20	2007	Deer	48	192
2313	20	2007	Deer	0	0
2314	20	2007	Deer	0	48
2315	20	2007	Deer	24	0
2316	20	2007	Deer	48	96
2317	20	2007	Deer	0	96
2318	20	2007	Deer	0	48
2319	20	2007	Deer	0	0
2320	20	2007	Deer	48	96
2321	20	2007	Deer	96	48
2322	20	2007	Deer	24	24
2323	20	2007	Deer	0	192
2324	20	2007	Deer	96	0
2325	20	2007	Deer	48	0
2326	20	2007	Deer	48	96
2327	20	2007	Deer	96	96
2328	20	2007	Deer	0	0
2329	20	2007	Deer	96	0
2330	20	2007	Deer	0	0
2331	20	2007	Cattle	0	0
2332	20	2007	Cattle	0	0
2333	20	2007	Cattle	96	96
2334	20	2007	Cattle	24	192
2335	20	2007	Cattle	0	0
2336	20	2007	Cattle	0	0
2337	20	2007	Cattle	0	0
2338	20	2007	Cattle	0	0
2339	20	2007	Cattle	0	0
2340	20	2007	Cattle	96	0
2341	20	2007	Cattle	0	0
2342	20	2007	Cattle	96	0
2343	20	2007	Cattle	0	0
2344	20	2007	Cattle	48	0
2345	20	2007	Cattle	0	0
2346	20	2007	Cattle	0	0
2347	20	2007	Cattle	0	96
2348	20	2007	Cattle	0	0
2349	20	2007	Cattle	0	0
2350	20	2007	Cattle	0	0
2351	20	2007	Sheep	0	0
2352	20	2007	Sheep	0	96
2353	20	2007	Sheep	24	0
2354	20	2007	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2355	20	2007	Sheep	0	0
2356	20	2007	Sheep	0	0
2357	20	2007	Sheep	0	0
2358	20	2007	Sheep	0	0
2359	20	2007	Sheep	0	0
2360	20	2007	Sheep	0	0
2361	20	2007	Sheep	0	0
2362	20	2007	Sheep	0	24
2363	20	2007	Sheep	0	0
2364	20	2007	Sheep	0	0
2365	20	2007	Sheep	0	0
2366	20	2007	Sheep	0	0
2367	20	2007	Sheep	24	0
2368	20	2007	Sheep	0	0
2369	20	2007	Sheep	0	0
2370	1	2008	Deer	0	0
2371	1	2008	Deer	0	0
2372	1	2008	Deer	0	0
2373	1	2008	Deer	0	0
2374	1	2008	Deer	0	0
2375	1	2008	Deer	0	0
2376	1	2008	Deer	96	0
2377	1	2008	Deer	24	0
2378	1	2008	Deer	0	0
2379	1	2008	Deer	0	0
2380	1	2008	Deer	0	0
2370	1	2008	Deer	0	0
2371	1	2008	Deer	0	0
2372	1	2008	Deer	0	0
2373	1	2008	Deer	0	0
2374	1	2008	Deer	0	0
2375	1	2008	Deer	0	0
2376	1	2008	Deer	96	0
2377	1	2008	Deer	24	0
2378	1	2008	Deer	0	0
2379	1	2008	Deer	0	0
2380	1	2008	Deer	0	0
2381	1	2008	Deer	0	0
2382	1	2008	Deer	0	0
2383	1	2008	Deer	0	0
2384	1	2008	Deer	0	0
2385	1	2008	Deer	0	0
2386	1	2008	Deer	0	0
2387	1	2008	Deer	0	0
2388	1	2008	Deer	0	0
2389	1	2008	Deer	0	0
2390	2	2008	Deer	0	0
2391	2	2008	Deer	24	0
2392	2	2008	Deer	0	0
2393	2	2008	Deer	24	0
2394	2	2008	Deer	0	0
2395	2	2008	Deer	0	0
2396	2	2008	Deer	0	0
2397	2	2008	Deer	48	0
2398	2	2008	Deer	0	0
2399	2	2008	Deer	0	0
2400	2	2008	Deer	0	0
2401	2	2008	Deer	0	0
2402	2	2008	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2403	2	2008	Deer	0	0
2404	2	2008	Deer	0	0
2405	2	2008	Deer	48	0
2406	2	2008	Deer	0	0
2407	2	2008	Deer	0	0
2408	2	2008	Deer	0	0
2409	3	2008	Deer	0	0
2410	3	2008	Deer	0	0
2411	3	2008	Deer	0	0
2412	3	2008	Deer	0	0
2413	3	2008	Deer	0	0
2414	3	2008	Deer	0	0
2415	3	2008	Deer	0	0
2416	3	2008	Deer	0	0
2417	3	2008	Deer	0	0
2418	3	2008	Deer	0	0
2419	3	2008	Deer	24	0
2420	3	2008	Deer	0	0
2421	3	2008	Deer	48	0
2422	3	2008	Deer	0	0
2423	3	2008	Deer	0	0
2424	3	2008	Deer	0	0
2425	3	2008	Deer	0	0
2426	3	2008	Deer	0	0
2427	3	2008	Deer	0	0
2428	3	2008	Deer	0	0
2429	4	2008	Deer	0	0
2430	4	2008	Deer	0	384
2431	4	2008	Deer	0	0
2432	4	2008	Deer	0	0
2433	4	2008	Deer	0	96
2434	4	2008	Deer	0	0
2435	4	2008	Deer	0	1536
2436	4	2008	Deer	0	0
2437	4	2008	Deer	0	0
2438	4	2008	Deer	0	768
2439	4	2008	Deer	0	0
2440	4	2008	Deer	0	0
2441	4	2008	Deer	0	96
2442	4	2008	Deer	0	0
2443	4	2008	Deer	0	384
2444	4	2008	Deer	0	0
2445	4	2008	Deer	0	96
2446	4	2008	Deer	0	0
2447	4	2008	Deer	0	48
2448	4	2008	Deer	0	768
2449	4	2008	Cattle	0	0
2450	4	2008	Cattle	0	0
2451	4	2008	Cattle	0	0
2452	4	2008	Cattle	0	0
2453	4	2008	Cattle	0	0
2454	4	2008	Cattle	0	0
2455	4	2008	Cattle	0	0
2456	5	2008	Deer	0	0
2457	5	2008	Deer	0	0
2458	5	2008	Deer	0	0
2459	5	2008	Deer	0	0
2460	5	2008	Deer	0	0
2461	5	2008	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2462	5	2008	Deer	0	0
2463	5	2008	Deer	0	0
2464	5	2008	Deer	0	0
2465	5	2008	Deer	0	0
2466	5	2008	Deer	0	0
2467	5	2008	Deer	0	0
2468	5	2008	Deer	0	0
2469	5	2008	Deer	24	0
2470	5	2008	Deer	0	0
2471	5	2008	Deer	0	0
2472	5	2008	Deer	0	0
2473	5	2008	Deer	0	0
2474	5	2008	Deer	0	24
2475	5	2008	Deer	0	24
2476	5	2008	Cattle	48	1536
2477	5	2008	Cattle	0	384
2478	5	2008	Cattle	0	768
2479	5	2008	Cattle	0	768
2480	5	2008	Cattle	0	768
2481	5	2008	Cattle	0	96
2482	5	2008	Cattle	0	768
2483	5	2008	Cattle	192	768
2484	5	2008	Cattle	0	768
2485	5	2008	Cattle	0	768
2486	5	2008	Cattle	0	384
2487	5	2008	Cattle	24	768
2488	5	2008	Cattle	0	384
2489	5	2008	Cattle	0	96
2490	5	2008	Cattle	0	3072
2491	5	2008	Cattle	0	768
2492	5	2008	Cattle	0	1536
2493	5	2008	Cattle	0	384
2494	5	2008	Cattle	0	24
2495	5	2008	Cattle	0	768
2496	6	2008	Deer	0	0
2497	6	2008	Deer	48	0
2498	6	2008	Deer	192	0
2499	6	2008	Deer	0	0
2500	6	2008	Deer	48	0
2501	6	2008	Deer	96	0
2502	6	2008	Deer	0	0
2503	6	2008	Deer	48	0
2504	6	2008	Deer	192	0
2505	6	2008	Deer	0	0
2506	6	2008	Deer	48	0
2507	6	2008	Deer	48	0
2508	6	2008	Deer	96	0
2509	6	2008	Deer	48	0
2510	6	2008	Deer	192	0
2511	6	2008	Deer	0	0
2512	6	2008	Deer	24	0
2513	6	2008	Deer	768	0
2514	6	2008	Deer	96	0
2515	6	2008	Deer	24	0
2516	6	2008	Cattle	0	0
2517	6	2008	Cattle	24	0
2518	6	2008	Cattle	0	0
2519	6	2008	Cattle	0	0
2520	6	2008	Cattle	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2521	6	2008	Cattle	0	0
2522	6	2008	Cattle	0	0
2523	6	2008	Cattle	24	0
2524	6	2008	Cattle	0	0
2525	6	2008	Cattle	0	0
2526	6	2008	Cattle	0	0
2527	6	2008	Cattle	192	0
2528	6	2008	Cattle	0	0
2529	6	2008	Cattle	0	0
2530	6	2008	Cattle	0	0
2531	6	2008	Cattle	24	0
2532	6	2008	Cattle	0	0
2533	6	2008	Cattle	0	0
2534	6	2008	Cattle	24	0
2535	6	2008	Cattle	0	0
2536	7	2008	Deer	384	0
2537	7	2008	Deer	24	0
2538	7	2008	Deer	24	0
2539	7	2008	Deer	24	0
2540	7	2008	Deer	24	0
2541	7	2008	Deer	24	0
2542	7	2008	Deer	192	0
2543	7	2008	Deer	24	0
2544	7	2008	Deer	48	0
2545	7	2008	Deer	24	0
2546	7	2008	Deer	48	0
2547	7	2008	Deer	48	48
2548	7	2008	Deer	24	0
2549	7	2008	Deer	24	0
2550	7	2008	Deer	48	0
2551	7	2008	Sheep	0	0
2552	7	2008	Sheep	0	0
2553	7	2008	Sheep	0	0
2554	7	2008	Sheep	0	0
2555	7	2008	Sheep	0	0
2556	7	2008	Sheep	0	0
2557	7	2008	Sheep	0	0
2558	7	2008	Sheep	0	0
2559	7	2008	Sheep	0	0
2560	7	2008	Sheep	0	0
2561	7	2008	Sheep	0	24
2562	7	2008	Sheep	0	0
2563	7	2008	Sheep	0	0
2564	7	2008	Sheep	0	0
2565	7	2008	Sheep	0	0
2566	7	2008	Sheep	0	0
2567	7	2008	Sheep	0	0
2568	7	2008	Sheep	0	0
2569	7	2008	Sheep	0	0
2570	9	2008	Deer	0	0
2571	9	2008	Deer	48	96
2572	9	2008	Deer	0	48
2573	9	2008	Deer	192	48
2574	9	2008	Deer	0	96
2575	9	2008	Deer	0	192
2576	9	2008	Deer	0	0
2577	9	2008	Deer	48	0
2578	9	2008	Deer	48	96
2579	9	2008	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2580	9	2008	Deer	0	96
2581	9	2008	Deer	0	0
2582	9	2008	Deer	0	192
2583	9	2008	Deer	0	0
2584	9	2008	Deer	0	0
2585	9	2008	Deer	24	384
2586	9	2008	Deer	0	48
2587	9	2008	Deer	24	96
2588	9	2008	Cattle	0	24
2589	9	2008	Cattle	0	192
2590	9	2008	Cattle	0	48
2591	9	2008	Cattle	24	192
2592	9	2008	Cattle	0	0
2593	9	2008	Cattle	0	96
2594	9	2008	Cattle	0	0
2595	9	2008	Cattle	0	0
2596	9	2008	Cattle	0	0
2597	9	2008	Cattle	48	0
2598	9	2008	Cattle	96	0
2599	9	2008	Cattle	96	24
2600	9	2008	Cattle	96	0
2601	9	2008	Cattle	0	0
2602	9	2008	Cattle	0	0
2603	9	2008	Cattle	0	192
2604	9	2008	Cattle	48	0
2605	9	2008	Cattle	0	384
2606	9	2008	Cattle	0	1536
2607	9	2008	Cattle	0	0
2608	9	2008	Sheep	24	0
2609	9	2008	Sheep	24	24
2610	9	2008	Sheep	48	48
2611	9	2008	Sheep	96	24
2612	9	2008	Sheep	48	0
2613	9	2008	Sheep	96	384
2614	9	2008	Sheep	24	0
2615	9	2008	Sheep	96	24
2616	9	2008	Sheep	24	0
2617	9	2008	Sheep	96	384
2618	9	2008	Sheep	0	24
2619	9	2008	Sheep	24	192
2620	9	2008	Sheep	192	0
2621	9	2008	Sheep	96	0
2622	9	2008	Sheep	48	0
2623	9	2008	Sheep	24	0
2624	9	2008	Sheep	96	384
2625	9	2008	Sheep	48	96
2626	9	2008	Sheep	24	0
2627	9	2008	Sheep	96	0
2628	10	2008	Deer	0	0
2629	10	2008	Deer	0	96
2630	10	2008	Deer	0	0
2631	10	2008	Deer	0	48
2632	10	2008	Deer	96	0
2633	10	2008	Deer	48	48
2634	10	2008	Deer	0	0
2635	10	2008	Deer	48	96
2636	10	2008	Deer	96	0
2637	10	2008	Deer	96	0
2638	10	2008	Deer	24	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2639	10	2008	Deer	96	0
2640	10	2008	Deer	48	0
2641	10	2008	Deer	24	0
2642	10	2008	Deer	96	24
2643	10	2008	Deer	48	0
2644	10	2008	Deer	0	0
2645	10	2008	Deer	24	24
2646	10	2008	Deer	96	0
2647	10	2008	Deer	24	0
2648	10	2008	Cattle	0	0
2649	10	2008	Cattle	192	0
2650	10	2008	Cattle	0	0
2651	10	2008	Cattle	192	0
2652	10	2008	Cattle	96	0
2653	10	2008	Cattle	0	0
2654	10	2008	Cattle	0	0
2655	10	2008	Cattle	192	0
2656	10	2008	Cattle	0	0
2657	10	2008	Cattle	0	0
2658	10	2008	Cattle	0	96
2659	10	2008	Cattle	192	0
2660	10	2008	Cattle	96	0
2661	10	2008	Cattle	192	0
2662	10	2008	Cattle	768	0
2663	10	2008	Cattle	48	24
2664	10	2008	Cattle	192	0
2665	10	2008	Cattle	24	96
2666	10	2008	Cattle	192	0
2667	10	2008	Cattle	0	0
2668	10	2008	Sheep	192	0
2669	10	2008	Sheep	192	0
2670	10	2008	Sheep	768	0
2671	10	2008	Sheep	96	0
2672	10	2008	Sheep	384	0
2673	10	2008	Sheep	96	0
2674	10	2008	Sheep	0	0
2675	10	2008	Sheep	0	0
2676	10	2008	Sheep	192	24
2677	10	2008	Sheep	192	0
2678	10	2008	Sheep	0	0
2679	10	2008	Sheep	0	0
2680	10	2008	Sheep	0	0
2681	10	2008	Sheep	0	0
2682	10	2008	Sheep	192	0
2683	10	2008	Sheep	192	0
2684	10	2008	Sheep	192	0
2685	10	2008	Sheep	192	0
2686	10	2008	Sheep	0	0
2687	10	2008	Sheep	0	0
2688	11	2008	Deer	48	0
2689	11	2008	Deer	96	0
2690	11	2008	Deer	0	0
2691	11	2008	Deer	24	0
2692	11	2008	Deer	0	0
2693	11	2008	Deer	0	0
2694	11	2008	Deer	48	0
2695	11	2008	Deer	0	0
2696	11	2008	Deer	48	0
2697	11	2008	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2698	11	2008	Deer	0	0
2699	11	2008	Deer	0	0
2700	11	2008	Deer	48	0
2701	11	2008	Deer	48	0
2702	11	2008	Deer	48	0
2703	11	2008	Deer	0	0
2704	11	2008	Deer	48	0
2705	11	2008	Deer	48	0
2706	11	2008	Deer	0	0
2707	11	2008	Deer	24	0
2708	11	2008	Cattle	0	0
2709	11	2008	Cattle	0	0
2710	11	2008	Cattle	0	0
2711	11	2008	Cattle	48	0
2712	11	2008	Cattle	0	0
2713	11	2008	Cattle	0	0
2714	11	2008	Cattle	0	0
2715	11	2008	Cattle	0	0
2716	11	2008	Cattle	0	0
2717	11	2008	Cattle	0	0
2718	11	2008	Cattle	0	0
2719	11	2008	Cattle	0	0
2720	11	2008	Cattle	0	24
2721	11	2008	Cattle	24	192
2722	11	2008	Cattle	0	0
2723	11	2008	Cattle	0	0
2724	11	2008	Cattle	0	24
2725	11	2008	Cattle	0	0
2726	11	2008	Cattle	0	0
2727	11	2008	Cattle	0	384
2728	11	2008	Sheep	0	0
2729	11	2008	Sheep	0	24
2730	11	2008	Sheep	48	24
2731	11	2008	Sheep	96	0
2732	11	2008	Sheep	0	0
2733	11	2008	Sheep	0	0
2734	11	2008	Sheep	0	0
2735	11	2008	Sheep	0	0
2736	11	2008	Sheep	0	0
2737	11	2008	Sheep	0	24
2738	11	2008	Sheep	48	0
2739	11	2008	Sheep	0	24
2740	11	2008	Sheep	96	0
2741	11	2008	Sheep	192	0
2742	11	2008	Sheep	0	0
2743	11	2008	Sheep	96	0
2744	11	2008	Sheep	96	0
2745	11	2008	Sheep	0	24
2746	11	2008	Sheep	0	0
2747	11	2008	Sheep	0	0
2748	12	2008	Deer	48	0
2749	12	2008	Deer	96	0
2750	12	2008	Deer	96	0
2751	12	2008	Deer	48	0
2752	12	2008	Deer	0	0
2753	12	2008	Deer	48	0
2754	12	2008	Deer	0	0
2755	12	2008	Deer	48	0
2756	12	2008	Deer	96	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
2757	12	2008	Deer	48	0
2758	12	2008	Deer	24	0
2759	12	2008	Deer	48	0
2760	12	2008	Deer	48	0
2761	12	2008	Deer	24	0
2762	12	2008	Deer	96	0
2763	12	2008	Cattle	0	0
2764	12	2008	Cattle	384	0
2765	12	2008	Cattle	192	0
2766	12	2008	Cattle	192	0
2767	12	2008	Cattle	768	0
2768	12	2008	Cattle	192	0
2769	12	2008	Cattle	0	0
2770	12	2008	Cattle	384	0
2771	12	2008	Cattle	0	0
2772	12	2008	Cattle	0	0
2773	12	2008	Cattle	0	0
2774	12	2008	Cattle	1536	0
2775	12	2008	Cattle	384	0
2776	12	2008	Cattle	192	0
2777	12	2008	Cattle	192	0
2778	12	2008	Cattle	192	0
2779	12	2008	Cattle	0	0
2780	12	2008	Cattle	0	0
2781	12	2008	Cattle	48	0
2782	12	2008	Cattle	384	0
2783	12	2008	Sheep	384	0
2784	12	2008	Sheep	768	0
2785	12	2008	Sheep	192	0
2786	12	2008	Sheep	192	0
2787	12	2008	Sheep	0	0
2788	12	2008	Sheep	384	0
2789	12	2008	Sheep	384	0
2790	12	2008	Sheep	192	0
2791	12	2008	Sheep	192	0
2792	12	2008	Sheep	384	0
2793	12	2008	Sheep	384	0
2794	12	2008	Sheep	192	0
2795	12	2008	Sheep	96	0
2796	12	2008	Sheep	192	0
2797	12	2008	Sheep	192	0
2798	12	2008	Sheep	384	0
2799	12	2008	Sheep	192	0
2800	12	2008	Sheep	0	0
2801	12	2008	Sheep	192	0
2802	13	2008	Deer	0	48
2803	13	2008	Deer	0	0
2804	13	2008	Deer	0	0
2805	13	2008	Deer	0	0
2806	13	2008	Deer	0	0
2807	13	2008	Deer	0	0
2808	13	2008	Deer	0	0
2809	13	2008	Deer	0	0
2810	13	2008	Deer	0	0
2811	13	2008	Deer	0	0
2812	13	2008	Deer	0	0
2813	13	2008	Deer	0	0
2814	13	2008	Deer	0	0
2815	13	2008	Deer	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
3111	18	2008	Cattle	0	48
3112	18	2008	Cattle	48	0
3113	18	2008	Cattle	0	0
3114	18	2008	Cattle	0	0
3115	18	2008	Sheep	0	0
3116	18	2008	Sheep	0	0
3117	18	2008	Sheep	0	0
3118	18	2008	Sheep	0	0
3119	18	2008	Sheep	0	0
3120	18	2008	Sheep	0	0
3121	18	2008	Sheep	0	0
3122	18	2008	Sheep	0	0
3123	18	2008	Sheep	0	0
3124	18	2008	Sheep	0	0
3125	18	2008	Sheep	0	0
3126	18	2008	Sheep	0	0
3127	18	2008	Sheep	0	0
3128	18	2008	Sheep	0	0
3129	18	2008	Sheep	0	0
3130	18	2008	Sheep	0	0
3131	18	2008	Sheep	0	0
3132	18	2008	Sheep	0	0
3133	18	2008	Sheep	0	0
3134	18	2008	Sheep	0	0
3135	19	2008	Deer	0	0
3136	19	2008	Deer	0	0
3137	19	2008	Deer	0	0
3138	19	2008	Deer	0	0
3139	19	2008	Deer	0	0
3140	19	2008	Deer	0	0
3141	19	2008	Deer	0	0
3142	19	2008	Deer	0	0
3143	19	2008	Deer	0	0
3144	19	2008	Deer	0	0
3145	19	2008	Deer	0	0
3146	19	2008	Deer	0	0
3147	19	2008	Deer	0	0
3148	19	2008	Deer	0	0
3149	19	2008	Deer	0	0
3150	19	2008	Deer	0	0
3151	19	2008	Deer	0	0
3152	19	2008	Deer	0	0
3153	19	2008	Deer	0	0
3154	19	2008	Deer	0	0
3155	19	2008	Deer	48	0
3156	19	2008	Deer	24	0
3157	19	2008	Deer	48	0
3158	19	2008	Deer	48	0
3159	19	2008	Deer	24	0
3160	19	2008	Deer	24	0
3161	19	2008	Deer	0	0
3162	19	2008	Deer	0	0
3163	19	2008	Deer	24	0
3164	19	2008	Deer	0	0
3165	19	2008	Deer	48	0
3166	19	2008	Deer	24	0
3167	19	2008	Deer	24	0
3168	19	2008	Deer	0	0
3169	19	2008	Deer	24	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
3170	19	2008	Deer	0	0
3171	19	2008	Deer	0	0
3172	19	2008	Deer	0	0
3173	19	2008	Deer	48	0
3174	19	2008	Cattle	96	0
3175	19	2008	Cattle	96	0
3176	19	2008	Cattle	96	0
3177	19	2008	Cattle	24	0
3178	19	2008	Cattle	96	0
3179	19	2008	Cattle	48	0
3180	19	2008	Cattle	48	0
3181	19	2008	Cattle	192	0
3182	19	2008	Cattle	0	0
3183	19	2008	Cattle	48	0
3184	19	2008	Cattle	96	0
3185	19	2008	Cattle	768	0
3186	19	2008	Cattle	96	0
3187	19	2008	Cattle	0	0
3188	19	2008	Cattle	192	0
3189	19	2008	Cattle	96	0
3190	19	2008	Cattle	0	0
3191	19	2008	Cattle	384	0
3192	19	2008	Cattle	24	0
3193	19	2008	Cattle	96	192
3194	19	2008	Cattle	96	48
3195	19	2008	Cattle	96	0
3196	19	2008	Cattle	384	24
3197	19	2008	Cattle	96	96
3198	19	2008	Cattle	96	192
3199	19	2008	Cattle	192	192
3200	19	2008	Cattle	96	0
3201	19	2008	Cattle	192	384
3202	19	2008	Cattle	192	48
3203	19	2008	Cattle	192	0
3204	19	2008	Cattle	192	0
3205	19	2008	Cattle	96	48
3206	19	2008	Cattle	48	48
3207	19	2008	Cattle	96	0
3208	19	2008	Cattle	192	0
3209	19	2008	Cattle	192	0
3210	19	2008	Cattle	96	384
3211	19	2008	Cattle	192	0
3212	19	2008	Cattle	48	24
3213	19	2008	Sheep	96	0
3214	19	2008	Sheep	192	0
3215	19	2008	Sheep	192	0
3216	19	2008	Sheep	0	96
3217	19	2008	Sheep	0	192
3218	19	2008	Sheep	0	0
3219	19	2008	Sheep	0	0
3220	19	2008	Sheep	0	192
3221	19	2008	Sheep	192	0
3222	19	2008	Sheep	24	48
3223	19	2008	Sheep	96	0
3224	19	2008	Sheep	96	0
3225	19	2008	Sheep	0	0
3226	19	2008	Sheep	96	0
3227	19	2008	Sheep	48	0
3228	19	2008	Sheep	0	0

Animal	Farm	Year	Species	Hardjobovis	Pomona
3229	19	2008	Sheep	0	48
3230	19	2008	Sheep	0	0
3231	19	2008	Sheep	0	0
3232	19	2008	Sheep	96	0
3233	20	2008	Deer	0	0
3234	20	2008	Deer	48	24
3235	20	2008	Deer	0	384
3236	20	2008	Deer	96	96
3237	20	2008	Deer	0	192
3238	20	2008	Deer	48	24
3239	20	2008	Deer	96	0
3240	20	2008	Deer	384	24
3241	20	2008	Deer	48	96
3242	20	2008	Deer	0	0
3243	20	2008	Deer	0	24
3244	20	2008	Deer	0	0
3245	20	2008	Deer	192	96
3246	20	2008	Deer	24	384
3247	20	2008	Deer	48	48
3248	20	2008	Deer	48	96
3249	20	2008	Deer	24	0
3250	20	2008	Deer	24	0
3251	20	2008	Deer	24	24
3252	20	2008	Deer	48	96
3253	20	2008	Cattle	0	0
3254	20	2008	Cattle	0	0
3255	20	2008	Cattle	0	0
3256	20	2008	Cattle	0	0
3257	20	2008	Cattle	0	0
3258	20	2008	Cattle	0	0
3259	20	2008	Cattle	0	0
3260	20	2008	Cattle	0	0
3261	20	2008	Cattle	0	0
3262	20	2008	Cattle	0	0
3263	20	2008	Sheep	192	0
3264	20	2008	Sheep	0	0
3265	20	2008	Sheep	0	384
3266	20	2008	Sheep	0	0
3267	20	2008	Sheep	0	0
3268	20	2008	Sheep	0	0
3269	20	2008	Sheep	0	0
3270	20	2008	Sheep	0	0
3271	20	2008	Sheep	0	0
3272	20	2008	Sheep	96	0
3273	20	2008	Sheep	0	0
3274	20	2008	Sheep	0	0
3275	20	2008	Sheep	0	0
3276	20	2008	Sheep	0	0
3277	20	2008	Sheep	0	0
3278	20	2008	Sheep	0	0
3279	20	2008	Sheep	0	0
3280	20	2008	Sheep	0	0
3281	20	2008	Sheep	24	48
3282	20	2008	Sheep	0	0

Appendix 6a. Raw data of serology against 23 reference panel serovars.

Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
1	1	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
2	1	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
3	1	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
4	1	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
5	1	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
6	2	West Coast	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
7	2	West Coast	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
8	2	West Coast	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
9	2	West Coast	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
10	2	West Coast	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
11	3	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
12	3	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
13	3	Otago	<50	**200**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
14	3	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
15	3	Otago	<50	**400**	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
16	4	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
17	4	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
18	4	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
19	4	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
20	4	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
21	5	Northland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
22	5	Northland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
23	5	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
24	5	Northland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
25	5	Northland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
26	6	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
27	6	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
28	6	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
29	6	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
30	6	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
31	7	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
32	7	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
33	7	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
34	7	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
35	7	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
36	8	Otago	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
37	8	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
38	8	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
39	8	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
40	8	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
41	9	Otago	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
42	9	Otago	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
43	9	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
44	9	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
45	9	Otago	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
46	10	Northland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
47	10	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
48	10	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
49	10	Northland	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
50	10	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
51	11	Northland	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
52	11	Northland	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
53	11	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
54	11	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
55	11	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
56	12	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
57	12	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
58	12	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
59	12	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
60	12	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
61	13	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
62	13	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
63	13	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
64	13	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
65	13	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
66	14	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
67	14	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
68	14	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**800**	<50	<50	<50	**100**	<50	<50	<50
69	14	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
70	14	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
71	15	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
72	15	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
73	15	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
74	15	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
75	15	Northland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
76	16	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
77	16	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
78	16	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
79	16	Bay of Plenty	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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91	19	West Coast	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
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93	19	West Coast	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
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273	55	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
274	55	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
275	55	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
276	56	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	**100**	<50	**50**
277	56	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
278	56	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
279	56	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
280	56	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50
281	57	Canterbury	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
282	57	Canterbury	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	**100**	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50
283	57	Canterbury	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
284	57	Canterbury	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
285	57	Canterbury	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
286	58	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
287	58	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
288	58	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**100**	**50**	<50	<50	<50	**50**	<50	<50
289	58	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
290	58	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
291	59	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
292	59	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
293	59	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
294	59	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
295	59	Otago	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
296	60	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
297	60	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
298	60	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
299	60	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
300	60	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50
301	61	Southland	<50	**200**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
302	61	Southland	<50	**200**	<50	<50	<50	<50	<50	<50	<50	<50	**50**	**100**	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
303	61	Southland	<50	**400**	<50	<50	<50	<50	<50	<50	<50	<50	**50**	**200**	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
304	61	Southland	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
305	61	Southland	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
306	62	Southland	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
307	62	Southland	**400**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
308	62	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
309	62	Southland	**400**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
310	62	Southland	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
311	63	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
312	63	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
313	63	Hawkes Bay	**200**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
314	63	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
315	63	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
316	64	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
317	64	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
318	64	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
319	64	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
320	64	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
321	65	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
322	65	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
323	65	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
324	65	Hawkes Bay	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
325	65	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
326	66	Hawkes Bay	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
327	66	Hawkes Bay	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
328	66	Hawkes Bay	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

Serum number	Farm number	Region	Pomona	Hardjo	Tarassovi	Grippotyphosa	Celledoni	Copenhageni	Australis	Zanoni	Robinsoni	Canicola	Kremastos	Szwajizak	Medanensis	Bulgarica	Cynopteri	Arborea	Ballum	Bataviae	Djasiman	Javanica	Panama	Shermani	Topaz
329	66	Hawkes Bay	**200**	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50
330	66	Hawkes Bay	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
331	67	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
332	67	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
333	67	Manawatu	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
334	67	Manawatu	**200**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**100**	<50	<50	<50	<50
335	67	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
336	68	Manawatu	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
337	68	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
338	68	Manawatu	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	<50	<50	<50	<50	<50	<50	<50
339	68	Manawatu	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
340	68	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
341	69	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
342	69	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
343	69	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
344	69	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
345	69	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
346	70	Manawatu	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
347	70	Manawatu	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
348	70	Manawatu	<50	**100**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
349	70	Manawatu	<50	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
350	70	Manawatu	<50	**400**	<50	<50	<50	<50	<50	<50	<50	<50	<50	**50**	**50**	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50

Appendix 6b. Raw data of follow-up serology (additional serum bank samples) for Arborea and Ballum and farm region.

Sample no.	Farm no.	Region	Arborea	Ballum
1	14	Canterbury	<50	<50
2	14	Canterbury	<50	<50
3	14	Canterbury	<50	<50
4	14	Canterbury	<50	<50
5	14	Canterbury	<50	<50
6	14	Canterbury	<50	<50
7	14	Canterbury	<50	<50
8	14	Canterbury	<50	<50
9	14	Canterbury	<50	<50
10	14	Canterbury	<50	<50
11	14	Canterbury	<50	<50
12	14	Canterbury	<50	<50
13	14	Canterbury	<50	<50
14	14	Canterbury	<50	<50
15	14	Canterbury	<50	<50
16	16	Bay of Plenty	<50	<50
17	16	Bay of Plenty	<50	<50
18	16	Bay of Plenty	<50	<50
19	16	Bay of Plenty	<50	<50
20	16	Bay of Plenty	<50	<50
21	16	Bay of Plenty	<50	<50
22	16	Bay of Plenty	<50	<50
23	16	Bay of Plenty	<50	<50
24	16	Bay of Plenty	<50	<50
25	31	Southland	<50	<50
26	31	Southland	<50	<50
27	31	Southland	<50	<50
28	31	Southland	<50	<50
29	31	Southland	<50	<50
30	31	Southland	<50	<50
31	31	Southland	<50	<50
32	31	Southland	<50	<50
33	31	Southland	<50	<50
34	31	Southland	<50	<50
35	31	Southland	<50	<50
36	31	Southland	<50	<50
37	31	Southland	<50	<50
38	31	Southland	<50	<50
39	31	Southland	<50	<50
40	50	Southland	<50	<50
41	50	Southland	<50	<50
42	50	Southland	<50	<50
43	50	Southland	<50	<50
44	50	Southland	<50	<50
45	50	Southland	**50**	<50
46	50	Southland	<50	<50
47	50	Southland	<50	<50
48	51	Southland	**50**	<50
49	51	Southland	<50	<50
50	51	Southland	<50	<50
51	51	Southland	<50	<50
52	51	Southland	<50	<50
53	51	Southland	**50**	<50
54	51	Southland	<50	<50

Sample no.	Farm no.	Region	Arborea	Ballum
55	51	Southland	<50	<50
56	51	Southland	<50	<50
57	51	Southland	<50	<50
58	51	Southland	<50	<50
59	51	Southland	<50	<50
60	51	Southland	**50**	<50
61	51	Southland	<50	<50
62	51	Southland	<50	<50
63	52	Manawatu	<50	<50
64	52	Manawatu	<50	<50
65	52	Manawatu	<50	<50
66	52	Manawatu	<50	<50
67	52	Manawatu	<50	<50
68	52	Manawatu	<50	<50
69	52	Manawatu	<50	<50
70	52	Manawatu	<50	<50
71	52	Manawatu	<50	<50
72	52	Manawatu	**50**	**50**
73	52	Manawatu	<50	<50
74	52	Manawatu	<50	<50
75	52	Manawatu	<50	<50
76	52	Manawatu	<50	<50
77	52	Manawatu	<50	<50
78	52	Manawatu	<50	<50
79	52	Manawatu	<50	<50
80	52	Manawatu	<50	<50
81	56	Southland	<50	<50
82	56	Southland	<50	<50
83	56	Southland	<50	<50
84	56	Southland	<50	<50
85	56	Southland	<50	<50
86	56	Southland	<50	<50
87	56	Southland	<50	<50
88	56	Southland	<50	<50
89	56	Southland	<50	<50
90	56	Southland	<50	<50
91	56	Southland	<50	<50
92	57	Canterbury	<50	<50
93	57	Canterbury	<50	<50
94	57	Canterbury	<50	<50
95	57	Canterbury	<50	<50
96	57	Canterbury	<50	<50
97	57	Canterbury	<50	<50
98	57	Canterbury	<50	<50
99	57	Canterbury	<50	<50
100	57	Canterbury	<50	<50
101	57	Canterbury	<50	<50
102	57	Canterbury	<50	<50
103	57	Canterbury	<50	<50
104	57	Canterbury	<50	<50
105	57	Canterbury	<50	<50
106	57	Canterbury	<50	<50
107	57	Canterbury	<50	<50
108	57	Canterbury	<50	<50
109	57	Canterbury	<50	<50
110	57	Canterbury	<50	<50
111	57	Canterbury	<50	<50

Sample no.	Farm no.	Region	Arborea	Ballum
112	60	Southland	<50	<50
113	60	Southland	<50	<50
114	60	Southland	<50	<50
115	60	Southland	<50	<50
116	60	Southland	<50	<50
117	60	Southland	<50	<50
118	60	Southland	<50	<50
119	60	Southland	<50	<50
120	60	Southland	<50	<50
121	60	Southland	<50	<50
122	60	Southland	<50	<50
123	60	Southland	<50	<50
124	60	Southland	<50	<50
125	60	Southland	<50	<50
126	60	Southland	<50	<50

Appendix 6c. Raw data of serology for additional blood samples against Arborea and Ballum and Farm region.

Sample no.	Farm no.	Region	Arborea	Ballum
1	50	Southland	<50	<50
2	50	Southland	<50	<50
3	50	Southland	<50	<50
4	50	Southland	<50	<50
5	50	Southland	<50	<50
6	50	Southland	<50	<50
7	50	Southland	<50	<50
8	50	Southland	<50	<50
9	51	Southland	<50	<50
10	51	Southland	<50	<50
11	51	Southland	<50	<50
12	51	Southland	<50	<50
13	51	Southland	<50	<50
14	51	Southland	<50	<50
15	51	Southland	<50	<50
16	51	Southland	<50	<50
17	51	Southland	<50	<50
18	51	Southland	<50	<50
19	51	Southland	<50	<50
20	51	Southland	<50	<50
21	51	Southland	<50	<50
22	51	Southland	<50	<50
23	51	Southland	<50	<50
24	51	Southland	<50	<50
25	51	Southland	<50	<50
26	51	Southland	<50	<50
27	51	Southland	<50	<50
28	51	Southland	<50	<50
29	51	Southland	<50	<50
30	51	Southland	<50	<50
31	51	Southland	<50	<50
32	51	Southland	<50	<50
33	51	Southland	<50	<50
34	51	Southland	<50	<50
35	51	Southland	<50	<50
36	51	Southland	<50	<50
37	51	Southland	<50	<50
38	51	Southland	<50	<50
39	51	Southland	<50	<50
40	51	Southland	<50	<50
41	51	Southland	**50**	**50**
42	51	Southland	<50	<50
43	51	Southland	<50	<50

Appendix 7. Raw data of culture and PCR for kidney, uterus and foetus (if available) and serology.

(PCR and culture: 0 = negative, 1 = positive, n/a = not available)

Sample no.	Line no.	Kidney culture	Kidney PCR	Uterus culture	Uterus PCR	Hardjobovis	Pomona	Foetus culture	Foetus PCR
1	1	0	0	0	0	0	0	n/a	n/a
2	1	1	0	0	0	96	192	n/a	n/a
3	1	0	0	0	0	96	0	n/a	n/a
4	1	0	0	0	0	48	0	n/a	n/a
5	1	1	0	0	0	48	192	n/a	n/a
6	1	0	0	0	0	48	0	n/a	n/a
7	1	0	0	0	0	0	96	n/a	n/a
8	1	0	0	0	0	0	96	n/a	n/a
9	1	0	0	0	0	0	0	n/a	n/a
10	1	0	0	0	0	192	0	n/a	n/a
11	1	0	0	0	0	48	0	n/a	n/a
12	1	1	1	0	0	96	0	n/a	n/a
13	1	1	1	0	0	96	0	n/a	n/a
14	2	0	0	0	0	0	0	n/a	n/a
15	2	0	0	0	0	0	0	n/a	n/a
16	2	0	0	0	0	0	0	n/a	n/a
17	2	0	0	0	0	0	0	n/a	n/a
18	2	0	0	0	0	0	48	n/a	n/a
19	2	0	0	0	0	0	768	n/a	n/a
20	2	0	0	0	0	0	0	n/a	n/a
21	2	0	0	0	0	0	0	n/a	n/a
22	2	0	0	0	0	0	0	n/a	n/a
23	2	0	0	0	0	0	0	n/a	n/a
24	2	0	0	0	0	0	0	n/a	n/a
25	2	0	0	0	0	0	0	n/a	n/a
26	2	0	0	0	0	0	0	n/a	n/a
27	2	0	0	0	0	0	0	n/a	n/a
28	3	0	0	0	0	0	0	n/a	n/a
29	3	0	0	0	0	0	0	n/a	n/a
30	3	0	0	0	0	0	0	n/a	n/a
31	3	0	0	0	0	0	0	n/a	n/a
32	3	0	0	0	0	0	0	n/a	n/a
33	3	0	0	0	0	0	0	n/a	n/a
34	3	0	0	0	0	48	0	n/a	n/a
35	3	0	0	0	0	48	0	n/a	n/a
36	3	0	0	0	0	0	0	n/a	n/a
37	3	0	0	0	0	0	0	n/a	n/a
38	3	0	0	0	0	0	0	n/a	n/a
39	3	0	0	0	0	0	0	n/a	n/a
40	3	0	0	0	0	0	0	n/a	n/a
41	3	0	0	0	0	0	0	n/a	n/a
42	4	0	0	0	0	96	0	n/a	n/a
43	4	0	0	0	0	48	96	n/a	n/a
44	4	0	0	0	0	48	0	n/a	n/a
45	4	0	0	0	0	0	0	n/a	n/a
46	4	0	0	0	0	0	0	n/a	n/a
47	4	0	0	0	0	0	0	n/a	n/a
48	4	0	0	0	0	0	0	n/a	n/a
49	4	0	0	0	0	48	0	n/a	n/a
50	4	0	0	0	0	48	0	n/a	n/a
51	4	0	0	0	0	48	0	n/a	n/a
52	4	0	0	0	0	48	48	n/a	n/a
53	4	1	1	0	0	24	0	n/a	n/a
54	4	0	0	0	0	0	24	n/a	n/a

Sample no.	Line no.	Kidney culture	Kidney PCR	Uterus culture	Uterus PCR	Hardjobovis	Pomona	Foetus culture	Foetus PCR
55	4	0	0	0	0	0	0	n/a	n/a
56	4	1	1	0	0	0	0	n/a	n/a
57	5	0	0	0	0	0	0	n/a	n/a
58	5	0	0	0	0	0	0	n/a	n/a
59	5	0	0	0	0	0	0	n/a	n/a
60	5	0	0	0	0	0	0	n/a	n/a
61	5	0	0	0	0	0	0	n/a	n/a
62	5	0	0	0	0	0	0	n/a	n/a
63	5	0	0	0	0	0	0	n/a	n/a
64	5	0	0	0	0	0	0	n/a	n/a
65	5	0	0	0	0	0	0	n/a	n/a
66	5	0	0	0	0	0	0	n/a	n/a
67	5	0	0	0	0	0	0	n/a	n/a
68	5	0	0	0	0	0	0	n/a	n/a
69	5	0	0	0	0	0	0	n/a	n/a
70	5	0	0	0	0	0	0	n/a	n/a
71	5	0	0	0	0	0	0	n/a	n/a
72	6	0	0	0	0	n/a	n/a	n/a	n/a
73	6	0	0	0	0	n/a	n/a	0	0
74	6	0	0	0	0	n/a	n/a	0	0
75	6	0	0	0	0	n/a	n/a	n/a	n/a
76	6	0	0	0	0	48	48	0	0
77	6	0	0	0	0	192	0	0	0
78	6	0	0	0	0	24	0	0	0
79	6	0	0	0	0	24	0	0	0
80	6	0	0	0	0	0	0	0	0
81	6	0	0	0	0	48	0	n/a	n/a
82	6	0	0	0	0	0	0	n/a	n/a
83	6	0	0	0	0	0	0	0	0
84	7	0	0	0	0	0	0	0	0
85	7	0	0	0	0	192	0	0	1
86	7	0	0	0	0	48	0	0	0
87	7	0	0	0	0	24	0	0	0
88	7	0	0	0	0	0	0	0	0
89	7	0	0	0	0	0	0	0	0
90	7	0	0	0	0	24	0	0	0
91	7	0	0	0	0	0	0	n/a	n/a
92	7	0	0	0	0	0	0	n/a	n/a
93	7	0	0	0	0	0	384	0	0
94	7	0	0	0	0	96	0	0	0
95	7	1	1	0	0	192	384	0	0
96	7	0	0	0	0	0	0	n/a	n/a
97	7	0	0	0	0	24	48	0	0
98	8	0	0	0	0	24	0	n/a	n/a
99	8	0	0	0	0	24	0	n/a	n/a
100	8	0	0	0	0	24	0	n/a	n/a
101	8	0	0	0	0	192	0	n/a	n/a
102	8	0	0	0	0	24	0	0	0
103	8	0	0	0	0	48	0	n/a	n/a
104	8	0	0	0	0	24	0	n/a	n/a
105	8	0	0	0	0	48	0	n/a	n/a
106	8	0	0	0	0	48	48	n/a	n/a
107	8	0	0	0	0	48	0	n/a	n/a
108	9	0	0	0	0	48	0	0	0
109	9	0	0	0	0	48	0	n/a	n/a
110	9	0	0	0	0	48	0	0	0
111	9	0	0	0	0	0	0	n/a	n/a

Sample no.	Line no.	Kidney culture	Kidney PCR	Uterus culture	Uterus PCR	Hardjobovis	Pomona	Foetus culture	Foetus PCR
112	9	0	0	0	0	0	0	n/a	n/a
113	9	0	0	0	0	48	0	0	0
114	9	0	0	0	0	0	0	n/a	n/a
115	9	0	0	0	0	24	0	0	0
116	9	0	0	0	0	24	0	n/a	n/a
117	9	0	0	0	0	24	0	0	0
118	9	0	0	0	0	0	0	0	0
119	9	0	0	0	0	0	0	0	0
120	9	0	0	0	0	0	0	n/a	n/a