INVESTIGATIONS INTO THE CONTROL OF NEOSPOROSIS IN CATTLE

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“It is a riddle,

wrapped in a mystery,

inside an enigma;

but perhaps there is a key.”

Winston Churchill

October, 1939

General Abstract

The research presented in this thesis was undertaken to further understanding of the control of neosporosis in cattle. A prospective cohort study of primiparous heifers on a farm with a history of *Neospora*-associated abortion found a 0.65 risk of abortion among seropositive heifers, suggesting that identification and culling of seropositive heifer replacements may be cost-effective.

A clinical trial of a registered *Neospora caninum* vaccine utilising 2,246 cattle from five farms with endemic *N. caninum* infection was assessed for efficacy in preventing abortion and vertical transmission. Overall vaccine efficacy was 0.25 (p=0.12) and vaccination increased the risk of vertical transmission. Histopathological and serological results from 148 cases of abortion from this trial were compiled to establish aetiological diagnoses. Nine of 34 cases where the fetus was examined had histopathological evidence of *N. caninum* infection. Histopathology revealed dual infectious aetiologies in 2 cases and serology suggested that, in another 17 cases, there had been recent exposure to a second infectious agent capable of causing abortion in conjunction with *N. caninum* lesions in the fetus or fetal bacteraemia.

As a prelude to cattle challenge trials, a challenge study conducted on pregnant sheep revealed a strong dose-response for abortion and that indirect fluorescent antibody test results did not correlate well with infection status or pregnancy outcome. A novel challenge method of applying tachyzoites to an abraded oral mucosa was undertaken in pregnant heifers to establish whether oral lesions could facilitate the direct horizontal transmission of *N. caninum* between cattle. One of eight heifers seroconverted, her calf and one other were seropositive when sampled within 12 hours of birth, and three other heifer-calf pairs had at least one positive polymerase chain reaction result at parturition. This method of transmission between cattle may be responsible for only a small proportion of infections but is a major new finding in the epidemiology of *N. caninum* infection and warrants further investigation.

Finally, inoculation with mouse-passaged *N. caninum* tachyzoites prior to mating did not prevent abortion when heifers were challenged again on Day 70 of gestation, suggesting that live inoculation may not be a suitable control option.

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Abbreviations

AI	Artificial insemination
BLAST	Basic local alignment search tool
BVDV	Bovine viral diarrhoea virus
CD	Cluster of differentiation system
CI	Confidence interval
CMI	Cell-mediated immunity
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAT	Direct agglutination test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphosphate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
IB	Immunoblot
IBR	Infectious bovine rhinotracheitis
ICT	Immunochromatographic test
IFAT	Indirect fluorescent antibody test
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ISCOM	Immunostimulating complex
ITS	Internal transcribed spacer
IVABS	Institute of Veterinary, Animal and Biomedical Sciences (Massey University)
kDa	Kilodalton
k/o	*knock out*
MAT	Microscopic agglutination test
MEM	Minimum essential media

MLST	Multilocus sequence typing
MUAEC	Massey University Animal Ethics Committee
N. caninum	*Neospora caninum*
Nc-NZ	*N. caninum* New Zealand
NcSAG1	*N. caninum* surface antigen 1
NcSRS2	*N. caninum* surface antigen-1 related sequence 2
NK	Natural Killer
NPV	Negative predictive value
OD	Optical density
PAG	Pregnancy-associated glycoprotein
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Persistently infected
PP	Percent positivity
PPV	Positive predictive value
PSM	Planned start of mating
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RR	Relative risk
RT-PCR	Reverse transcription polymerase chain reaction
SAG	Surface antigen
SAS	Statistical analysis system
SD	Standard deviation
SDS	Sodium dodecyl sulphate
S/P ratio	Sample to positive ratio
TEM	Transmission electron microscopy
T. gondii	*Toxoplasma gondii*
Th	T cell helper
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VMRD	Veterinary Medical Research and Development
VNT	Virus neutralisation test

Preface

This thesis is presented as a series of papers which have been published (or submitted for publication). Consequently, there is some repetition, particularly in the Material & Methods sections. Different formats between journals mean that the format of chapters within this thesis will also vary. The references for each chapter have been collated at the end of this thesis in the Bibliography.

The literature review (Chapter Two) provides basic details of the life cycle, epidemiology, clinical consequences and immune response to *Neospora caninum* infection in cattle with a detailed review of the diagnosis of neosporosis in cattle and current recommendations for its control.

Chapter Three details the risk of abortion in primiparous Friesian-cross dairy heifers according to their serostatus for *N. caninum*. The cattle on the study farm had been sampled quarterly for the previous six years to measure antibody to *N. caninum* following an abortion outbreak in 1997. The relative risk for abortion among ELISA-positive heifers was 23.6 compared to the seronegative heifers and eleven of seventeen seropositive heifers aborted. These findings suggested that identification and culling of infected heifer replacements may be a cost-effective control option on farms with a history of abortion due to *N. caninum* infection.

In the same year as the prospective cohort study was being carried out, a killed vaccine containing *N. caninum* tachyzoites was licensed for use in New Zealand and several other countries. A large-scale clinical trial of this product was conducted over five commercial dairy farms in four regions of New Zealand that had a history of abortion associated with *N. caninum* infection (Chapter Four). A total of 2,246 cattle were enrolled in this trial of the first, commercially available vaccine with a claim to reduce the risk of abortion in cattle. The vaccine was found to have a significant effect on reducing abortion in one of five farms in this study suggesting that farm-specific effects need to be elucidated to identify farms which may benefit from such a product. Vaccination increased the risk of vertical transmission.

A thorough investigation was conducted on all cases of abortion from cows and heifers from the farms that were enrolled in the clinical trial. This consisted of histopathological examination of fetus and placenta as well as serological testing of the dam for antibody to *N. caninum* and bovine viral diarrhoea virus and compared to antibody status the time of at enrolment in the trial. A diagnosis of abortion due to *N. caninum* infection was made in nine of 34 cases of abortion where fetal histopathology was undertaken and on one of the five farms, there was no evidence that any of the 33 abortions were due to *N. caninum* infection. Although *N. caninum* was the most common cause of abortion on these farms, there were at least as many abortions that were associated with other infectious causes, or in which there was evidence of co-infection with other organisms. It is likely that the importance of *N. caninum* as a cause of abortion is over-estimated on some farms following the confirmation of its involvement in cases of abortion. Ongoing diagnostic efforts should be made to identify all causes of abortion on an affected farm as some may be able to be controlled and the removal of other immunological stressors may decrease the incidence of abortion in cows that are infected with *N. caninum*.

Pregnant sheep were used as a model for challenge prior to undertaking such studies in cattle due to their earlier onset of puberty, shorter gestation length and the lower cost. New Zealand isolates of *Neospora caninum* that had been kept in Vero cell culture at Massey University were used to produce the tachyzoite challenge in all subsequent studies. A strong dose-response relationship was observed with five of ten sheep aborting when challenged with 5 x 10^3 tachyzoites intravenously at 70-90 days gestation and all sheep aborting at higher doses. Additionally, it was found that there was little agreement between two different serological methods used to detect antibody to *N. caninum* in these animals which called into question the use of the indirect fluorescent antibody test on sheep sera.

A challenge study involving the inoculation of pregnant cattle with *Nesopora caninum* tachyzoites on Day 70 of gestation was then conducted. Intravenous inoculation with 2 x 10^8 tachyzoites resulted in seroconversion in all eight heifers but only one heifer subsequently aborted, suggesting that the New Zealand isolates were of low virulence. A novel challenge model involving the application of tachyzoites to an abraded oral mucosa resulted in seroconversion in one of eight heifers, two calves from these heifers were seropositive and *N. caninum* DNA was found in samples from three other heifer-calf pairs in this group. These results were consistent with the hypothesis that aborting and

parturient cattle may be a source of infection for other cattle that become infected by direct horizontal transmission if tachyzoites gain entry to the bloodstream via lesions in the oral mucosa.

The final study involved a two-by-two trial design in which cattle were inoculated with either *N. caninum* tachyzoites that had been passaged through mice or with serum-free tissue culture media prior to mating. Pregnant heifers within each group were then randomly allocated to inoculation with either *N. caninum* tachyzoites or serum-free tissue culture media on Day 70 of gestation. Other authors had reported that pre-mating inoculation with *N. caninum* tachyzoites was protective against abortion when cattle were subsequently challenged. A challenge dose of 2×10^9 tachyzoites intravenously was chosen to increase the risk of abortion. Two of six heifers aborted that had previously been inoculated with *N. caninum* tachyzoites aborted when subsequently challenged as opposed to five of six heifers aborting that received a *N. caninum* tachyzoite challenge on Day 70 of gestation and had previously been inoculated with serum-free tissue culture media. This study casts doubt on the efficacy of pre-mating inoculation with live tachyzoites to prevent abortion due to *N. caninum*.

For each chapter my input was greatest. The preliminary ideas for some of the studies were a collaboration of several of the authors but I devised the details of the research, undertook the fieldwork, analysed the data and wrote the manuscripts. I was, however, assisted by my co-authors and a declaration of authors' contribution to each paper is included at the conclusion of each chapter that reports a published paper. Further assistance such as technical work and editorial input is reported in the Acknowledgements section of each chapter.

All experiments documented in this body of work were approved by the Massey University Animal Ethics Committee as documented within each chapter.

Chapter One

Introduction

1.0 Introduction

Neospora caninum is an obligate, intracellular, protozoan parasite of the family Sarcocystidae in the phylum Apicomplexa (Ellis et al. 1994) capable of infecting a wide range of animals but most commonly associated with neuromuscular disease in dogs and abortion in cattle (Dubey et al. 2007). *Neospora caninum* is closely related to, but antigenically different from, *Toxoplasma gondii* (Bjerkås and Presthus 1988) and there are important differences between the immunological response to the two parasites (Innes et al. 2007). The closely related *Neospora hughesi* has been described only in horses (Marsh et al. 1998).

Neospora caninum was first reported in 1984 as an un-named, cyst-forming sporozoon parasite associated with encephalitis and myositis in dogs (Bjerkås et al. 1984). In 1988 the parasite was named *Neospora caninum* when tissues from dogs that were presumed to have died from toxoplasmosis were re-examined, and morphological differences between the two parasites were described (Dubey et al. 1988a).

Abortion in cattle due to neosporosis was first recognised in 1989 (Thilsted and Dubey 1989) and *N. caninum* is now the most commonly diagnosed cause of bovine abortion in many countries (Thornton et al. 1991; Hemphill and Gottstein 2000; Innes et al. 2005). *Neospora caninum* is often associated with abortion epidemics in dairy and beef herds, with up to 57% of pregnant cows in a herd aborting; however endemic and sporadic losses also occur (Thornton et al. 1994; Jenkins et al. 2000; Dubey et al. 2007). Herds that have experienced an abortion epidemic often subsequently experience endemic abortion in at least the following year (Moen et al. 1998; Pfeiffer et al. 2002; Björkman et al. 2003). Estimates of the economic impact of neosporosis on cattle industries are US$35 million annually in the state of California (Barr et al. 1998), AUS$100 million annually in Australia and New Zealand (Reichel 2000) and €9.7 million annually in Switzerland (Hasler et al. 2006a).

Some herds experiencing epidemic abortion have serological evidence of recent point-source exposure to *N. caninum* and horizontal transmission (McAllister et al. 2000; Schares et al. 2002). However, one herd was known to have a high rate of seroconversion with no increase in abortion incidence (Dijkstra et al. 2002a).

Evidence from herds with endemic neosporosis suggests that the risk of abortion decreases with parity (Thurmond and Hietala 1997a; López-Gatius et al. 2005a) as does the incidence of vertical transmission (Anderson et al. 1995; Romero and Frankena 2003) suggesting that immunity develops – at least in some cows. However, repeated abortion due to neosporosis has been reported in a small proportion of cows (Anderson et al. 1995; Obendorf et al. 1995; Moen et al. 1998).

In the past 20 years there has been a vast expansion of the literature on *N. caninum*. Despite this, many aspects of its biology have yet to be elucidated. The aetiological diagnosis of *Neospora*-associated abortion can be complicated and there are limited options for its control. Current control options for neosporosis in cattle consist of:

1) Identification of infected breeding and replacement females and removing them from the herd (test and cull; Hall et al. 2005) or not keeping replacement heifers from them (Reichel and Ellis 2002; Hasler et al. 2006b).

2) Biosecurity measures to prevent the introduction of infected cattle.

3) Embryo transfer from infected females to prevent vertical transmission (Baillargeon et al. 2001; Landmann et al. 2002).

4) Chemotherapy to treat the parasite within the host. A number of substances have been assessed, with toltrazuril showing the most promise (Kritzner et al. 2002; Haerdi et al. 2006) although there is no product currently licensed for use in cattle for the treatment of neosporosis.

5) Vaccination: a killed tachyzoite vaccine (Bovilis NeoGuard; Intervet B.V. Ltd, Boxmeer, The Netherlands; Choromanski et al. 2001) was commercially available in New Zealand and other markets at the time much of this research was undertaken but has since been withdrawn from all markets.

6) On-farm management strategies to limit contact between cattle and dogs (Reichel and Ellis 2002).

Options 1 - 4 above rely on serological identification of infected animals which is limited by the fluctuation of antibody levels that may drop below the cut-off value for the serological test used (Conrad et al. 1993; Stenlund et al. 1999), even following abortion (Jenkins et al. 2002). Proving that *N. caninum* infection causes abortion relies on assessing the severity of histopathological lesions (Barr et al. 1991a) and demonstration of the

presence of the parasite in aborted material (Lindsay and Dubey 1989). Aborted material is commonly not available and when it is, an aetiological diagnosis is made in less than 50% of cases (Anderson 2007). Statistically significant associations between serostatus and abortion aid diagnosis (Thurmond and Hietala 1995). The costs and difficulties associated with abortion diagnosis mean that once a small number of abortions have been diagnosed as being due to *N. caninum* infection, further abortions are often attributed to neosporosis. Current literature shows that the risk of abortion in *N. caninum* infected cattle can be increased by other stressors (López-Gatius et al. 2005a) or immunosuppressive factors (Hässig and Gottstein 2002) and these may contribute to abortion incidence.

Control measures should be tailored to each farm according to herd seroprevalence, risk factors for infection and abortion, and the farmer's goals and expectations. Consideration of control options should incorporate a cost-benefit analysis to compare the costs of the control method with the expected benefits from reducing losses from *N. caninum* infection (Larson et al. 2004; Hasler et al. 2006a; Reichel and Ellis 2006).

The research reported in this thesis was undertaken to evaluate current recommendations for the control of neosporosis and to perform preliminary investigations into the development of a novel vaccine against *N. caninum* in cattle.

Chapter Two

Literature Review

2.0 Literature Review

This review focuses on the immune response to *N. caninum* infection, methods of diagnosis and options for the control of neosporosis in cattle. More detail on other aspects of *N. caninum* infection in cattle are found in the following excellent reviews by Dubey and others: biology (Dubey and Lindsay 1996; Dubey et al. 2002; Hemphill and Gottstein 2006; Dubey and Schares 2011), epidemiology (Dubey et al. 2007) and pathogenesis (Dubey et al. 2006).

2.1 Life cycle and basic biology of *Neospora caninum*

Neospora caninum has a heteroxenous life cycle as illustrated in Figure 2.1. There are three infectious stages of *N. caninum*; sporozoites, tachyzoites and bradyzoites. All three are important in transmission of the parasite. Tachyzoites and bradyzoites are asexually proliferating stages that occur in tissues of infected hosts, whereas sporozoites are present in sexually produced oocysts that are excreted in the faeces of definitive hosts. Dogs, coyotes (*Canis latrans*) and dingoes (*Canis lupus dingo*) are confirmed definitive hosts (McAllister et al. 1998; Gondim et al. 2004a; King et al. 2010), but other wild canids such as wolves (*Canis lupus*), foxes (both *Vulpes vulpes* and *Urocyon cinereoargenteus*) and others from Brazil (*Lycalopex gymnocercus* and *Cerdocyon thous*) show serological evidence of infection (Rosypal and Lindsay 2005) and may be involved in horizontal transmission to intermediate grazing hosts. Intermediate hosts for *N. caninum* are commonly considered to be cattle, dogs, sheep (Dubey et al. 1990a) and goats (Dubey et al. 1996a). However Eld's deer (*Cervus eldi siamensis*; Dubey et al. 1996b), lesser kudu (*Tragelaphus imberbis*; Peters et al. 2001a), black-tailed deer (*Odocoileus hemionus columbianus*; Woods et al. 1994) white-tailed deer (*Odocoileus virginianus*; Dubey et al. 1999), water buffalo (*Bubalus bubalis*; Rodrigues et al. 2004) and chickens (*Gallus domesticus*; Costa et al. 2008) are also thought to be intermediate hosts. Serological evidence suggests that many other mammalian and avian species may also fulfil this role (Dubey et al. 2007). Domestic and sylvatic life cycles occur, making control difficult in situations where there is interaction between domesticated livestock and wildlife (Barling et al. 2000a; Gondim et al. 2004b; Rosypal and Lindsay 2005).

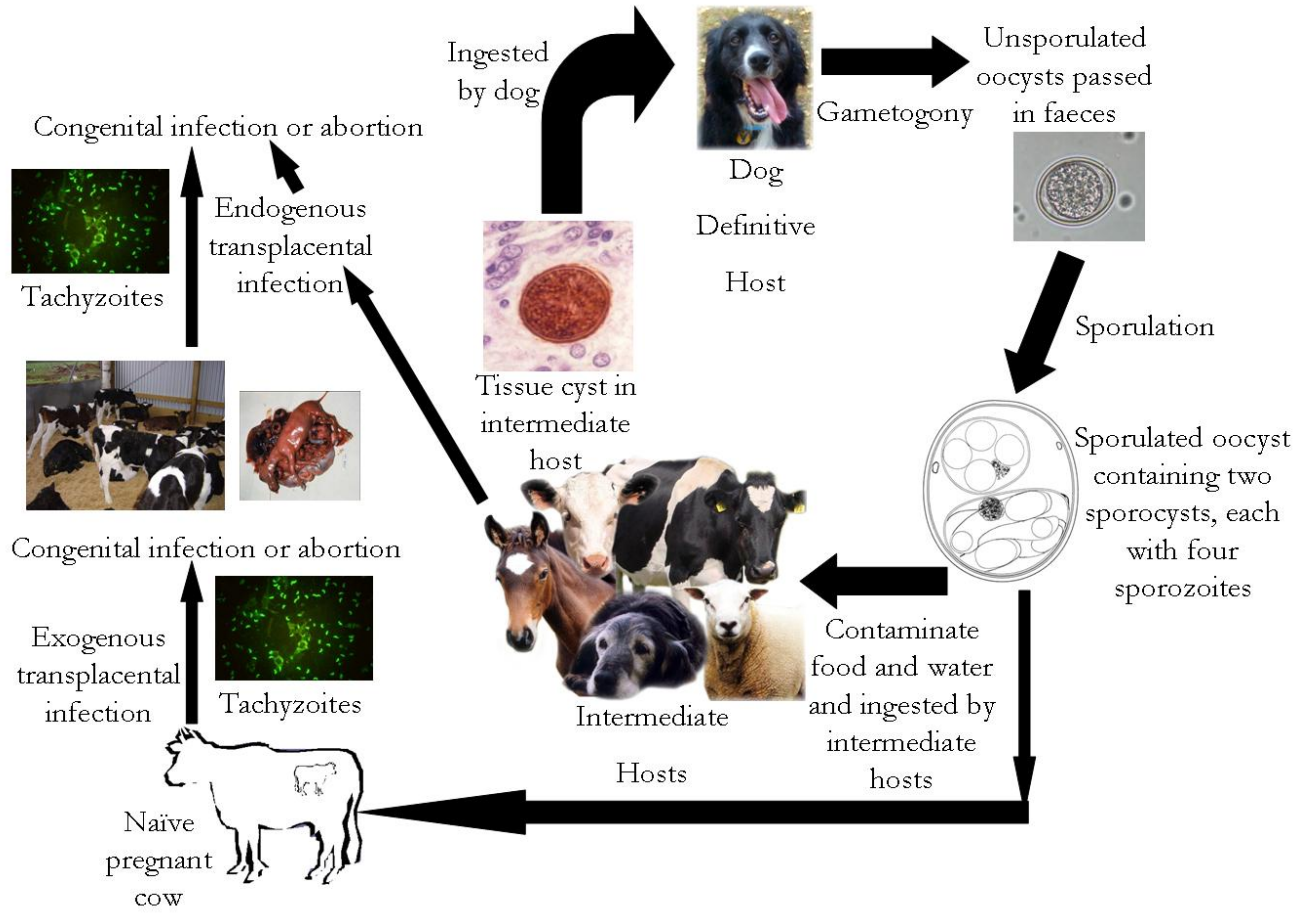

Figure 2.1 The life cycle of *Neospora caninum*; images from Śmielewska-Łoś et al. (2003), Doležalová et al. (2004), www.testapet.com/test/Neospora.html and http://people.upei.ca/sgreenwood/html/protozoa.html.

A number of serological studies of human populations (including those focused on agricultural workers and women who have suffered multiple spontaneous abortions) have found no evidence that *N. caninum* is zoonotic (Graham et al. 1999; Petersen et al. 1999; McCann et al. 2008). However, a paper by Lobato et al. (2006) reported the presence of IgG antibodies to *N. caninum* in HIV patients and people with neurological disorders. Similarly, challenge studies of Rhesus macaque monkeys (*Macaca mulatta*) have shown that maternal infection could be established and that this resulted in transplacental transmission (Barr et al. 1994a; Ho et al. 1997a). Further investigation into the zoonotic potential of *N. caninum* is warranted.

Many different strains of *N. caninum* have been identified (Regidor-Cerrillo et al. 2006) which vary in their virulence in challenge studies with mice (Lindsay and Dubey 1990; Atkinson et al. 1999). Evidence for different levels of virulence in strains that affect cattle was implied by Dijkstra et al. (2002a) who found one dairy herd with a high level of seroconversion without any increase in abortion incidence.

2.1.1 Sporozoites

Unsporulated oocysts (measuring 10 x 12 μm) are excreted transiently in the faeces of definitive hosts (Basso et al. 2001). Little is known about the frequency and intensity of shedding of oocysts by definitive hosts, although it has been shown that infected dogs shed oocysts on more than one occasion (McGarry et al. 2003). Sporulation occurs within a few days of excretion. After sporulation, the oocyst contains two sporocysts, with four sporozoites within each sporocyst (McAllister et al. 1998). Sporozoites measure 6.5 x 2 μm (Lindsay et al. 1999). The ability of oocysts to survive in the environment is unknown but their contamination of pasture, fodder for grazing animals and water sources is regarded as the most likely source of horizontal transmission to cattle and other intermediate hosts (McAllister et al. 2000; Dijkstra et al. 2002b).

2.1.2 Tachyzoites

Tachyzoites are found in the tissues of intermediate and definitive hosts. They have a central nucleus and can be ovoid, lunate or globular depending on the stage of division (Dubey and Lindsay 1996) and measure approximately 2 x 6 μm (Dubey et al. 2006). Tachyzoites are enclosed within a parasitophorous vacuole that is usually located adjacent to the host cell nucleus (Speer et al. 1999). They divide rapidly by endodyogeny within a

wide range of host cells, including neural cells, vascular endothelial cells, myocytes, hepatocytes, renal cells, alveolar macrophages and placental trophoblasts (Barr et al. 1991b; Dubey et al. 2002). Proliferating tachyzoites lyse host cells, and released tachyzoites invade neighbouring cells. Tachyzoites can damage the placenta and be transmitted haematogenously to the fetus. At some point, tachyzoites differentiate to the more slowly dividing bradyzoite stage, probably triggered by the host's immune response (Williams and Trees 2006).

2.1.3 Bradyzoites and tissue cysts

Bradyzoites are the encysted stage of the parasite. They multiply slowly by endodyogeny (Buxton et al. 2002), allowing infection to persist protected from the host's immune response in a latent form in infected cattle. It is assumed that ingestion of tissue cysts containing bradyzoites transmits *N. caninum* infection to a definitive host (Dubey et al. 2007). Bradyzoites can be morphologically distinguished from tachyzoites by their terminally located nucleus, the fact that they contain amylopectin granules and are somewhat more slender (6.5 x 1.5 μm; Dubey et al. 2004; 2006). Immunohistochemistry can also be used to distinguish between tachyzoite and bradyzoite stages of the parasite due to differences in antigenic structure (McAllister et al. 1996a).

Tissue cysts are round to elliptical and are found predominantly in the central and peripheral nervous system. They vary considerably in size, typically containing 20 – 100 bradyzoites (Speer et al. 1999). They are enclosed in a cyst wall, whose thickness depends on how long infection has persisted (Jardine 1996). The cyst wall is resistant to acid pepsin digestion in the stomach which allows the bradyzoites to be released in the gut (Lindsay and Dubey 1990). Tissue cysts up to 107 μm in diameter and with a cyst wall up to 4 μm thick have been reported in dogs (Dubey and Lindsay 1996), although tissue cysts in the brain and spinal cord of bovine fetuses and congenitally infected calves, are rarely more than 50 μm in diameter with a cyst wall that is usually < 2.5 μm thick (Dubey et al. 1989; Barr et al. 1991b). Tissue cysts containing *N. caninum* bradyzoites have also been reported in the skeletal muscle of naturally infected dogs and cattle (Peters et al. 2001b). Tissue cysts are also found in placental tissue from cows that deliver congenitally infected calves (Dijkstra et al. 2001a; Fioretti et al. 2003) and such material has been shown to be infective for dogs.

Bradyzoites differentiate back to tachyzoites but the control of this is not fully understood. Of particular importance to bovine neosporosis, it is thought that this reverse differentiation can occur in latently infected cattle during pregnancy due to down-regulation of specific cell proliferation and IFN-γ responses in the T helper (Th) 2-type environment that predominates during pregnancy (Innes et al. 2001a). This can result in endogenous transplacental infection of the fetus.

2.2 Transmission of *Neospora caninum* to cattle

Cattle can be infected by horizontal (post-natal; De Marez et al. 1999; Trees et al. 2002) or vertical (congenital) transmission (Björkman et al. 1996). Vertical transmission has been further categorised as (i) exogenous transplacental infection following a *de novo* infection of the pregnant cow or (ii) endogenous transplacental infection when the parasitaemia occurs as a result of recrudescence of a previously established *N. caninum* infection (Trees and Williams 2005). Such recrudescence is probably assisted by a down-regulation of cell-mediated immunity during pregnancy in the persistently infected cow (Entrican 2002). Epidemiological studies suggest that *Neospora*-associated abortion epidemics in cattle herds most commonly occur due to postnatal exposure to an oocyst challenge (Jenkins et al. 2000; McAllister et al. 2000; Schares et al. 2002). By contrast, in herds undergoing endemic abortion, the main mode of transmission appears to be vertical with a positive association between the serostatus of mothers and daughters (Thurmond et al. 1997; Schares et al. 2002).

2.2.1 Horizontal transmission

Horizontal transmission in cattle appears to occur only by ingestion of food or water contaminated by faeces from a definitive host which contain sporulated oocysts (De Marez et al. 1999; Trees et al. 2002). An increasing proportion of seropositive cows with age (Jensen et al. 1999; Rinaldi et al. 2005) and a lack of correlation between serostatus of dams and daughters (Thurmond and Hietala 1997a; Schares et al. 1998; Dijkstra et al. 2002a; Dijkstra et al. 2002b) provide evidence for horizontal transmission. In herds where there is epidemiological evidence for point-source exposure, presumably from an infected definitive host, mean annual rates of horizontal transmission in the range of 20-30% are commonly reported (Pfeiffer et al. 2002; Björkman et al. 2003; Romero and Frankena

2003) but 47% of cattle have been reported to seroconvert in a 6 month period (Dijkstra et al. 2002a). Once *N. caninum* infection has become established within a herd however, vertical transmission becomes the most important means of transmission and an annual rate of horizontal transmission of 2-4% is more likely (Davison et al. 1999a).

To date there is no direct evidence in the literature that cow-to-cow transmission of *N. caninum* can occur. However, *N. caninum* may be excreted in milk or uterine discharges from infected cattle, so there is the potential for these to be sources of infection for other cattle (Davison et al. 2001). Bradyzoites within tissue cysts can also be found within placentae from infected cattle: placentophagia has been postulated as a means of horizontal transmission (Modrý et al. 2001) but this idea was refuted by Schares and Conraths (2001). The recognition of *N. caninum* DNA in semen from naturally infected bulls suggests that venereal transmission of *N. caninum* is possible but it appears that viable organisms are only shed in small quantities and infrequently (Ortega-Mora et al. 2003; Caetano-da-Silva et al. 2004a; Ferre et al. 2005) so this is probably not an important natural route of transmission.

2.2.2 Vertical transmission

Transplacental transmission is thought to be the most important means of infection of *N. caninum* in cattle (Antony and Williamson 2001) as, once infection has been established within a herd, very high rates of vertical transmission maintain a population of infected female replacements. *Neospora caninum* can be transmitted in consecutive pregnancies (Fioretti et al. 2003) and through successive generations (Schares et al. 1998). Herd-level serological testing of cattle suggests that vertical transmission is responsible for almost all infections in some herds (Paré et al. 1996; Anderson et al. 1997). Vertical transmission rates of up to 95% have been reported (Davison et al. 1999b), which makes *N. caninum* a highly efficient congenitally-transmitted parasite. Importantly however, modelling has shown that despite the high rates of vertical transmission, the increased risks of abortion and culling in infected animals means that *N. caninum* infection within a herd cannot be maintained in the long term without ongoing horizontal transmission (French et al. 1999).

Experimental studies have shown that the stage of gestation at which the fetus is exposed to *N. caninum* is an important determinant of the outcome of infection. Infection

on Day 70 of gestation in naïve cows results in fetopathy in most animals, whereas infection in Week 30 of gestation results in the birth of clinically normal but congenitally-infected calves (Williams et al. 2000; Innes et al. 2001a).

2.2.3 Experimental methods of transmission

Further methods of transmission have been demonstrated experimentally but have not been reported to occur naturally. Firstly, oral transmission occurred in neonatal calves fed colostrum or milk spiked with tachyzoites (Uggla et al. 1998; Davison et al. 2001). Secondly, artificial insemination using semen that had been inoculated with tachyzoites resulted in seroconversion of some cows and lower pregnancy rates at higher doses (Serrano-Martínez et al. 2007), although such a high parasite load is unlikely to occur under natural conditions. Calves produced from such trials were, however, seronegative (Serrano et al. 2006).

2.3 The clinical outcomes of *Neospora caninum* infection in cattle

The main clinical sign of *N. caninum* infection in cattle is abortion, although stillborn calves and the birth of calves with congenital neurological defects have also been reported (Buxton et al. 1997a; Uggla et al. 1998). A brief period of pyrexia has been reported from challenge studies (Innes et al. 2001a; Macaldowie et al. 2004).

2.3.1 Abortion

Abortion occurs most commonly at 5-6 months of gestation, but *Neospora*-associated abortion has been reported to occur from 3 months gestation to full term (Dubey 2003a). Earlier loss of pregnancy may occur but is less likely to be recognised or fetal material be available to confirm the diagnosis (Innes et al. 2005). Fetal death before 5 months gestation is likely to lead to mummification and retention in the uterus, whilst fetuses that die in the earliest stages of gestation may be reabsorbed. The evidence that *N. caninum* infection causes early embryonic loss or infertility is contradictory, with Latham (2003) reporting an increased number of services per conception in infected cattle, while López-Gatius et al. (2005b) found no effect on conception rate. Moreover, Santolaria et al. (2009) found that cows that aborted due to *N. caninum* infection rapidly returned to normal fertility.

Cattle infected with *N. caninum* have a 3 to 7-fold greater risk of abortion than non-infected cattle (Thurmond and Hietala 1997a; Wouda et al. 1998a). Likewise, there is evidence that the risk of abortion in congenitally-infected heifers is much higher in their first pregnancy than in their non-infected herd-mates (Weston et al. 2005). Together, such results suggest that the immunocompetence of the host when it first encounters the parasite affects the immune response. This notion is supported by studies in which experimental challenge of cattle with tachyzoites prior to pregnancy prevented abortion and/or vertical transmission to their calves (Williams et al. 2000; Innes et al. 2001a).

The pathogenesis of *Neospora*-associated abortion is not completely understood as although characteristic histopathologic lesions can be identified in aborted fetuses, similar lesions are found in some congenitally-infected but clinically-normal calves (Gondim et al. 2004c). The severity and outcome of fetal lesions probably depend primarily upon fetal age and immunocompetence (Innes 2007a). There is also evidence that abortion may result from the maternal immune response to *N. caninum* within the placenta (Dubey et al. 2006) or from a maternal systemic change from a predominantly T helper (Th) 2-type regulatory response to a predominantly Th1-type pro-inflammatory response, which will assist in the control of parasitaemia but is detrimental to the maintenance of pregnancy (Raghupathy 1997).

Experimental infections in cattle have shown that infection in the first trimester of pregnancy may have more severe consequences for the fetus than infection later in gestation (Barr et al. 1994b; Williams et al. 2000; Maley et al. 2003; Macaldowie et al. 2004). Fortunately, transmission to the fetus during early pregnancy is not inevitable, as several studies have reported only 50-83% vertical transmission when a tachyzoite challenge was given on Day 70 of gestation compared with 100% vertical transmission when challenged on Day 140 or 210 of gestation (Williams et al. 2000; Innes et al. 2001a; Macaldowie et al. 2004).

Challenge trials of pregnant sheep have shown that *N. caninum* infection can cause abortion and vertical transmission (Buxton et al. 1998) but it is not thought to be an important abortifacient in sheep or goats under natural conditions (Owen and Trees 1999; Hässig et al. 2003; Eleni et al. 2004).

2.3.2 Stillbirths and congenital abnormalities

Infection of cows with *N. caninum* commonly results in an increased incidence of stillborn calves (Waldner et al. 1998; Stenlund et al. 1999) and more rarely, in a range of congenital neurological abnormalities (Dubey et al. 2006). The latter include low birthweight and recumbency (O'Toole and Jeffrey 1987), flexural deformities of the limbs (Dubey and de Lahunta 1993), ataxia, encephalomyelitis and proprioceptive deficits (Barr et al. 1993), exophthalmia or asymmetric appearance of the eyes (O'Toole and Jeffrey 1987), scoliosis (Bryan et al. 1994), hydrocephalus (Dubey et al. 1998) and narrowing of the spinal cord (Dubey et al. 1990b).

A recent study (Brickell et al. 2010) has reported an increased risk of perinatal mortality (defined as stillborn calves or dying within 24 hours of parturition) amongst heifers infected with *N. caninum* in their first ($p < 0.01$) and second ($p < 0.1$) parity. It was not, however, clear from the study whether any of these exhibited clinical signs consistent with congenital neosporosis.

2.3.3 Other production effects

No other health consequences have been reported in cattle infected with *N. caninum*. There is, however, a consistently increased risk of culling that has been reported in several studies from different countries and production systems (Thurmond and Hietala 1996; Tiwari et al. 2005; Bartels et al. 2006a). In most studies, the increased risk of culling can be attributed to abortion. Interestingly, a study of one Californian dairy herd showed that the risk of culling was increased in seropositive cattle independent of abortion (Thurmond and Hietala 1996), whereas another study carried out among dairy herds in Ontario, Canada, found that seropositivity was not associated with culling (Cramer et al. 2002).

Whether infection with *N. caninum* (once the effects of abortion are excluded) affects milk production is less certain, as some studies have reported that yield is decreased by 3 - 4% (Thurmond and Hietala 1997b; Hernandez et al. 2001; Romero et al. 2005). In another study this effect only occurred in the first 100 days of lactation (Bartels et al. 2006a). Two other studies found no effect of seropositivity on milk production (Hobson et al. 2002; van Leeuwen et al. 2002) and one New Zealand study reported increased milk production in seropositive cows (Pfeiffer et al. 2000). It might be that lesions similar to

those in aborted fetuses occur in congenitally infected animals and may compromise organ function and thereby limit a cow's ability to produce large quantities of milk, or even that the energy cost of the immune response might affect milk production; but it is difficult to imagine how infection with *N. caninum* could have a positive effect on milk production.

Studies on *N. caninum* infection in beef cattle have found that seropositive calves have lower weaning weights and an increased risk of culling (Kasari et al. 1999), as well as lower growth rates after weaning resulting in a lower carcass weight and profit (Barling et al. 2000b).

In addition to the aforementioned losses there may be rebreeding costs, expenses associated with establishing a diagnosis and replacement costs if aborting cows are culled. Not all of these costs will be relevant to all cattle farming enterprises but the net result will be a decrease in farm profitability.

2.4 The immune response to *Neospora caninum* infection in cattle

Humoral and cell-mediated immune responses are elicited against *N. caninum* infection. These responses are not entirely protective, since repeated vertical transmission can occur in subsequent pregnancies (Björkman et al. 1996). The immune response affords at least some degree of protection as (i) previously infected cattle are less likely to abort than naïve cattle when there is point source exposure to the parasite (McAllister et al. 2000) and (ii) the risks of abortion (Thurmond and Hietala 1997a) and vertical transmission decrease in subsequent pregnancies (Anderson et al. 1995; Romero et al. 2002; Dijkstra et al. 2003).

One challenge study reported that inoculation with live *N. caninum* tachyzoites before mating protected against a subsequent tachyzoite challenge during pregnancy (Williams et al. 2007). Other challenge studies have shown that the timing of parasite challenge (relative to stage of gestation) affects the outcome, illustrating that there is a complex interaction between the different stages of the parasite's life cycle, and the maternal and fetal immune systems (Williams et al. 2000; Innes et al. 2001a; Bartley et al. 2004). There can be marked differences in pregnancy outcome in cattle infected with *N. caninum*

depending on whether the tachyzoite challenge is the result of an exogenous or endogenous challenge (Guy et al. 2001; Williams et al. 2003).

A thorough understanding of the immune response to *N. caninum* infection and how this is affected by the immunological changes that occur during pregnancy is essential to the development of control strategies (and particularly vaccines). In general, antibodies control the numbers of parasites in blood and tissue fluids, whereas cell-mediated responses target intracellular parasites (Hemphill 1999; Tizard and Schubot 2004). Vaccines tend to stimulate humoral immunity and have little effect on cell-mediated immunity (Williams and Trees 2006).

2.4.1 Humoral immunity

Antibodies to *N. caninum* help prevent invasion of host cells (Haldorson et al. 2006) and this is borne out by challenge trials in immunodeficient mice (Eperon et al. 1999). However, the control of *N. caninum* infection relies mainly on the cytokines of the cell-mediated immune system that trigger effector cells to respond to infection.

Antibody responses are commonly detected in cattle within 14 days of experimental challenge by intravenous inoculation with tachyzoites or ingestion of oocysts (Maley et al. 2001; Bartley et al. 2004). Specific IgM concentrations peak approximately 2 weeks after infection and then decline rapidly (De Marez et al. 1999) while IgG concentrations increase during the first weeks of infection and can continue to rise for 3-6 months after primary infection (Dubey et al. 1996c; Uggla et al. 1998) with an initial rise in concentration of specific IgG_1 and a slightly delayed surge of IgG_2 (Williams et al. 2000; Andrianarivo et al. 2001). After primary infection, the avidity of specific IgG antibodies increases over time and this can be used to assess whether infection occurred recently (Björkman et al. 1999; Björkman et al. 2003; Björkman et al. 2005).

2.4.2 Cell-mediated immunity

Experimental studies in cattle and mice have shown the importance of cell-mediated immunity in controlling *N. caninum* infection. Natural killer (NK) cells are probably involved in the initial innate response, as these have been shown to lyse fibroblasts that were infected with *N. caninum*. They also produce interferon-gamma (IFN-γ; Boysen et al. 2006) which controls intracellular parasite multiplication and may prime the cytokine

environment towards a Th1-type response (Gazzinelli et al. 1996). T cells recognise infected host cells (Innes et al. 2000; Innes et al. 2002), leading to antigen-specific cell proliferation and the production of IFN-γ (Lundén et al. 1998; Marks et al. 1998; De Marez et al. 1999; Williams et al. 2000; Andrianarivo et al. 2001; Trees et al. 2002). *In vitro* studies have shown that IFN-γ and tumour necrosis factor alpha (TNF-α) inhibit parasite multiplication in a variety of cell cultures (Innes et al. 1995; Yamane et al. 2000). In addition, some cell surface glycoproteins on T-cells (e.g. CD4+; as classified by the cluster of differentiation system [CD]) are cytotoxic for cells infected with *N. caninum* (Staska et al. 2003; Staska et al. 2005).

Cattle do not have such a clear-cut differentiation between Th1 and Th2 response in T cell clones and the same cells can express both IFN-γ and interleukin four (IL-4) (Brown et al. 1998; Rosbottom et al. 2007). However, it appears that the Th1-type cell mediated immune response and particularly the activity of IFN-γ, tumour necrosis factor alpha (TNF-α) and IL-12 are important in controlling infection (Innes et al. 1995; Baszler et al. 1999a; Yamane et al. 2000). An important component of the cell-mediated immune response to *N. caninum* is the production of large amounts of IFN-γ by mononuclear cells in the peripheral blood, lymph nodes and spleen (Marks et al. 1998; Williams et al. 2000; Andrianarivo et al. 2001) CD4+ T cells are the major producers of IFN-γ, although other cells such as NK cells are likely to be important additional sources of IFN-γ early in infection. Moreover, NK cells appear to be of particular importance in maternal caruncular tissue of the placentome (Williams and Trees 2006).

2.4.3 The effect of pregnancy on the immune response to *Neospora caninum*

Any pregnant mammal must modify its immune system to allow it to carry the essentially foreign fetus to term. During pregnancy, a Th2-type environment predominates at the materno-fetal interface with regulatory cytokines IL-10, IL-4 and transforming growth factor beta (TGF-β) counteracting the more inflammatory cytokines such as IFN-γ (Tangri and Raghupathy 1993). A histopathological comparison of placentae from aborting and non-aborting cattle found increased numbers of CD4+ and CD8+ T cells, NK cells and IFN-γ when fetal death had occurred and no such response in non-aborting cattle (Maley et al. 2006).

It has been postulated that the immunomodulation that must occur to maintain pregnancy may trigger recrudescence of *N. caninum* bradyzoites to the rapidly-dividing tachyzoite stage and a subsequent parasitaemia (Innes et al. 2001a) and that the consequent (normal; Th1-type) inflammatory immune response to parasites at the materno-fetal interface may cause fetal death whilst the same response in the rest of the dam controls the infection.

Similarly, infection with *T. gondii* in sheep and humans has occasionally been reported to reactivate with a parasitaemia of the tachyzoite stage. Although this usually occurs in conjunction with severe immunosuppression of the host, for example chemotherapy following transplant surgery or infection with human immunodeficiency virus (HIV) (Williams and Trees 2006), it may also rarely occur during pregnancy (Duncanson et al. 2001; Silveira et al. 2003; Kodjikian et al. 2004).

2.4.4 The fetal response to *Neospora caninum* infection

The immunocompetence of the fetus develops gradually during gestation (Osburn et al. 1982) with mitogenic cellular responses being detected in bovine fetal spleen and thymic cells at about Week 14-15 of gestation (Innes et al. 2005). This means that the time during gestation when the fetus first encounters the parasite determines whether the fetus is killed or survives to term (Williams et al. 2000). This may be further complicated by immune responses at the materno-fetal interface which may compromise placental function leading to fetal death.

From about Day 120 of gestation the fetus is able to produce specific antibodies (Almería et al. 2003; Bartley et al. 2004) and thus may be able to resolve active *N. caninum* infection and be born seropositive. This same response is recognised in fetal infection with bovine viral diarrhoea virus (BVDV), such that fetuses which encounter the virus earlier in pregnancy are at risk of fetal death or remaining immunologically tolerant and persistently infected (PI) with the virus, whereas infection after about Day 120 of gestation results in an uninfected calf that is serologically positive (Brownlie and Clarke 1993). There may be similarities with *N. caninum* infection in that congenitally (and persistently) infected cattle appear to have higher risks for abortion and vertical transmission compared to post-natally infected cattle (Weston et al. 2005). One important difference between this

scenario and that of the BVD PI animal is that cattle congenitally infected with *N. caninum* are seropositive while cattle persistently infected with BVD remain antibody negative.

Further evidence for a difference in the efficacy of the immune response between congenitally and post-natally infected cattle has been demonstrated by challenge studies. Cattle challenged with *N. caninum* tachyzoites prior to mating and then again at a stage of gestation that would normally result in abortion, did not abort (Williams et al. 2007) and cattle receiving a primary infection as primiparous heifers did not produce congenitally infected calves in a subsequent pregnancy (McCann et al. 2007).

2.5 Diagnosis of neosporosis in cattle

The lack of clinical signs other than abortion in *N. caninum*-infected adult cattle means that serological diagnosis is an important diagnostic tool for *in vivo* use and in testing fluids from aborted fetuses. Molecular techniques (such as PCR) have high sensitivity and specificity and can be used on aborted material or on post-mortem tissue samples (Baszler et al. 1999b). PCR can also be used to detect tachyzoites in whole blood (Okeoma et al. 2004a) and serum (McInnes et al. 2006a) although parasitaemia is short-lived so this technique is only really applicable to research settings. Histopathology is useful to demonstrate lesions consistent with protozoal infection in cases of abortion (Barr et al. 1991a) but such lesions are not specific for *N. caninum* and need to be confirmed by the use of immunohistochemistry (Lindsay and Dubey 1989) or PCR and the severity of lesions also needs to be assessed as congenitally infected but clinically normal calves have been found to have similar lesions (Thurmond et al. 1999).

Antibody concentrations fluctuate during an animal's lifetime (Maley et al. 2001; Okeoma et al. 2004b; Kyaw et al. 2005) and although useful for detecting animals that have previously been exposed to *N. caninum* (and are probably still infected) serological diagnostic techniques are limited by the fact that antibody concentrations may fall below normal cut-points resulting in false-negative results (Dannatt 1997; Wouda et al. 1998b; Dijkstra et al. 2003). Subsequent increases in antibody concentrations appear to reflect recrudescence of the parasite (Guy et al. 2001) and can be a predictor for abortion or

vertical transmission (Paré et al. 1997). False-positive results can occur following vaccination against *N. caninum* (Choromanski and Block 2000; Schetters et al. 2004).

In a clinical setting it is important to differentiate between a cow being infected with *N. caninum* and diagnosing that an abortion is due to *N. caninum* infection. The former is relatively easy (although fluctuating antibody levels mean that serological tests lack sensitivity; Dannatt 1997; Wouda et al. 1998b; Dijkstra et al. 2003) whilst the latter is more difficult and relies on assessing the severity of the lesions in the fetus and/or placenta (with the presence of tachyzoites) as well as excluding other causes of abortion (Anderson et al. 1991; Barr et al. 1991a; Wouda et al. 1997a). Sometimes a diagnosis can only be made at the herd level, particularly where there is little aborted material available for examination. Herd-level diagnosis depends upon comparing the *N. caninum* serostatus of aborting and non-aborting cows to determine if there is a statistically significant association (Thurmond and Hietala 1995).

2.5.1 Serological techniques – indirect detection

The development of any serological test requires that an organism has been isolated and can be kept in culture so that specific antigens can be identified (Björkman and Uggla 1999). Following the isolation of *Neospora caninum* in 1988 (Dubey et al. 1988b), serology has been the main diagnostic tool for neosporosis and has been used by many researchers in clinical situations and challenge studies (Thornton et al. 1994; Williams et al. 1997; Maley et al. 2001; Dijkstra et al. 2002b; Trees et al. 2002). Current serological techniques are aimed at the tachyzoite stage of *N. caninum* (Jenkins et al. 2002) and positive results can be detected within 14 days of initial exposure to the parasite (Maley et al. 2001; Bartley et al. 2004). A variety of enzyme-linked immunosorbent assays (ELISA), an agglutination test kit and reagents for *N. caninum* indirect fluorescent antibody tests (IFAT) are commercially available.

Some serological techniques cross-react with closely related apicomplexan parasites (Packham et al. 1998; Schares et al. 1999a; Wapenaar et al. 2007) and the sensitivity of most techniques is compromised by a drop in antibody concentrations below detectable limits in some individual animals (Bartels et al. 2005). Another potential problem with serological techniques relates to the *in vitro* propagation of *N. caninum* tachyzoites and the production of specific antibodies for the development of diagnostic assays using fetal bovine serum

(FBS), as many commercial batches of FBS already contain antibodies to *N. caninum* and may produce false positive results (Torres and Ortega 2006).

A consideration for all serological techniques is the selection of the cut-point at which samples are considered positive. This depends on the host species being tested, age and sex of the animal, stage of gestation and whether the test is being employed to detect abortion due to *N. caninum* or to identify infected animals (Hemphill 1999; Reichel and Pfeiffer 2002; Álvarez-García et al. 2003; Wapenaar et al. 2007). Most serological tests were developed to detect *Neospora*-associated abortion and most aborting cattle will have high concentrations of antibodies soon after abortion (Quintanilla-Gozalo et al. 2000). The cut-point may need to be lowered if the aim is to find infected animals, many of which will have a lower titre (Schares et al. 1999a). The validation of serological tests for *N. caninum* is, however, made more difficult as there is no appropriate "gold-standard" by which to define a truly seropositive or truly seronegative reference group. Various methods based on Bayesian statistics can be used to validate diagnostic tests in the absence of a gold-standard (Enøe et al. 2000; Greiner and Gardner 2000) and have been used to assess serological methods for the diagnosis of *N. caninum* infection in cattle (Frössling et al. 2003).

Serological techniques can also be used to identify specific antibodies in aborted fetuses using serum or fluid from the fetal abdominal or thoracic cavities or from abomasal contents (Barr et al. 1995; Reichel and Drake 1996; De Meerschman et al. 2002). Such tests tend to have a high positive predictive value but a low negative predictive value, as fetuses aborted before 5 months of gestation are unlikely to have mounted an antibody response to the parasite (Barr et al. 1995; Wouda et al. 1997b) and there is commonly a short interval between infection and fetal death (Söndgen et al. 2001). Pre-colostral sampling should be undertaken to assess if calves have been congenitally infected, as colostral IgG_1 antibodies will be absorbed by the calf and may persist for several months (Hietala and Thurmond 1999). Occasionally, an infected animal may not develop antibodies to *N. caninum* as was reported by De Marez et al. (1999) in a calf experimentally infected with oocysts.

In most situations only a low proportion of cattle infected with *N. caninum* abort and most of their offspring are born infected but healthy (Paré et al. 1997; Thurmond and Hietala 1997a; Guy et al. 2001). Thus, the presence of antibodies to *N. caninum* in a cow that has aborted does not confirm that abortion was due to *N. caninum*. However, if a cow is sampled close to the time of abortion and is found to be seronegative then it is considered unlikely that the abortion was due to neosporosis, although occasionally, seropositive fetuses have been aborted by seronegative cows (Davison et al. 1999a; Sager et al. 2001). The finding of seronegative cattle that have *N. caninum* DNA in their serum detected by polymerase chain reaction (PCR; McInnes et al. 2006a) suggests that the cutpoint used in serological tests might not be low enough to identify all infected cattle or that there are some infected cattle that are immunotolerant.

2.5.1.1 Modified Agglutination Test (MAT)

This test relies on the fact that formalin-treated tachyzoites agglutinate in the presence of specific (IgG) antibodies. Tachyzoites are placed on the bottom of a microtitre well, test serum is added and the agglutination reaction is read after an incubation period (usually 24 hours). This can be done by direct visualisation or by using a microplate spectrophotometer coupled to a computer with agglutination-reading software.

The benefit of this technique is that it does not require species-specific secondary antibodies or special equipment and results are easily reproducible between laboratory personnel (Ortega-Mora et al. 2006). *Neospora caninum* MATs have been developed for use in cattle (Packham et al. 1998) and dogs (Romand et al. 1998) and have been used to test sera from a variety of species with reasonable sensitivity and specificity. False positive reactions may occur when testing fetal fluids due to the presence of particulate matter or the haemolysis of fetal serum. A disadvantage of the MAT is that large numbers of tachyzoites are required in their production (Björkman and Uggla 1999).

2.5.1.2 Indirect Fluorescent Antibody Test (IFAT)

An IFAT was initially developed to demonstrate antibodies to *N. caninum* in dogs (Dubey et al. 1988b) and became the first serological assay used for *N. caninum* infection in cattle (Conrad et al. 1993). IFAT is considered the gold standard for comparison against other serological techniques as it shows little or no cross-reactivity with other

apicomplexan parasites (Trees et al. 1994; Dubey et al. 1996c). Air-dried or fixed cell culture-derived tachyzoites are attached to microscope slides which then have diluted test sera added and are incubated with fluoroscein-labelled antibodies directed against immunoglobulins of the animal species under investigation (Björkman and Uggla 1999). A positive result occurs when fluorescence appears over the entire surface of the tachyzoite. Non-specific apical fluorescence may be a cross-reaction due to other apicomplexan parasites (Paré et al. 1995a). Reagents are commercially available but there can be poor correlation between laboratories using this technique due to differences in equipment (the fluorescence microscope) and subjective interpretation when deciding the highest dilution at which complete fluorescence occurs. Consequently, comparison of IFAT titres between laboratories is difficult.

The cut-point in IFAT titre for identifying cattle infected with *N. caninum* differs between laboratories. For adult cattle, cut-offs from 1:100 to over 1:640 have been reported although a 1:200 dilution is usually recommended (von Blumröder et al. 2004). Lower antibody concentrations are found in bovine fetuses and a cut-point in the range of 1:16 to 1:25 is considered positive (Álvarez-García et al. 2003). It must always be borne in mind that the quality of the equipment and the experience of the reader of the slides should be taken into account when comparing IFAT results between laboratories.

2.5.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

A variety of ELISA types have been developed to detect antibodies to *N. caninum*. These include whole tachyzoite lysate antigen indirect ELISA (Paré et al. 1995b), fixed whole tachyzoite indirect ELISA (Williams et al. 1997), ISCOM antigen indirect ELISA (Björkman et al. 1997), single native antigen indirect ELISA (Schares et al. 2000), recombinant antigen indirect ELISA (Lally et al. 1996a), antigen-capture indirect ELISA (Schares et al. 1999b), competitive inhibition ELISA (Baszler et al. 1996), antigen-capture competitive inhibition ELISA (Dubey et al. 1997) and an avidity ELISA which distinguishes between recent and chronic infections (Björkman et al. 1999; Schares et al. 2002). These assays have been comprehensively characterised by Björkman & Uggla (1999), Bartels et al. (2005), and Dubey & Schares (2006) and vary in sensitivity and specificity.

Indirect ELISA uses multi-well microtitre plates coated with an antigen preparation. Plates are incubated with diluted sera and then an enzyme-labelled, species-specific anti-immunoglobulin antibody (conjugate) is applied. Finally, a substrate is added which, in the presence of the conjugate, is transformed to a coloured product that can be measured by spectrophotometer as absorbance or optical density (OD; Venkatesan and Wakelin 1993). This provides a quantitative indication of antibody concentrations which is compared to a positive control sample and the result is reported as the percent positivity (PP) value or sample to positive (S/P) ratio. However, ELISA results are commonly reported back to clinicians as a dichotomous result (positive or negative), so much of the quantitative information is lost. An advantage of ELISA techniques is that automated processing allows many samples to be processed quickly and relatively cheaply.

The basis of an avidity ELISA is that the first antibodies synthesised after exposure to an antigen have a lower affinity for the antigen than those produced later (Jenkins et al. 2000). With this technique, antibodies are allowed to bind, but low affinity antibodies are then eluted by incubation with urea whilst the high-avidity antibodies remain bound. The titres obtained with and without incubation with urea are then calculated to assess whether infection is recent or chronic. However, the avidity ELISA does not discriminate between a *de novo* infection with *N. caninum* and recrudescence of infection (Aguado-Martínez et al. 2005).

Some ELISA can be modified to measure antibody concentrations in milk (Björkman et al. 1997; Chanlun et al. 2002; Schares et al. 2004a). Such assays can be used to determine herd serostatus on bulk milk samples. A positive result is usually obtained when the seroprevalence in the herd is > 10 - 15% (Chanlun et al. 2002; Schares et al. 2003). Antibody concentrations in milk tend to be lower than in sera (Álvarez-García et al. 2007) but are concentrated in colostrum (Jenkins et al. 2002).

ELISA can be modified to increase sensitivity or specificity, with some ELISA reported to have greater sensitivity and specificity than IFAT (Paré et al. 1995b; Schares et al. 1998); however, they may cross-react with other apicomplexan parasites (Schares et al. 1999a; Wapenaar et al. 2007). A two-graph receiver operating characteristic (ROC) analysis can be

used to select an appropriate cut-point when interpreting results obtained using different tests between seroepidemiological studies (Greiner et al. 1995; Reichel and Pfeiffer 2002).

2.5.1.4 Immunochromatographic Test (ICT)

Immunochromatographic tests (also known as lateral flow tests) are simpler than ELISA techniques with no need for special materials or equipment and can be carried out in clinical or field applications. An ICT is a nitrocellulose membrane-based test that can detect antigen, antibodies, hormones or proteins. A qualitative measurement can be made of any ligand that binds to a visually detectable solid support, such as dyed microspheres. These tests are relatively inexpensive to produce, require little in the way of sample processing, have long-term stability in a wide range of climates and results are rapidly reported. Common examples include test kits to detect ovulation or pregnancy in women, streptococcal throat infection in humans and HIV antigen. ICT have been developed for the diagnosis of other apicomplexan parasites including malaria (Mills et al. 1999; Moody 2002), toxoplasmosis (Huang et al. 2004a) and babesiosis (Huang et al. 2004b). A rapid ICT for *N. caninum* using the tachyzoite recombinant surface antigen NcSAG1 has been developed (Liao et al. 2005). These tests require more antigen for the production of kits than an ELISA but this is feasible when using recombinant antigens as they can be produced relatively easily in large quantities and in a standardised format.

2.5.1.5 Immunoblot (IB)

In an immunoblot (IB), tachyzoite proteins are separated by electrophoresis in a sodium dodecyl sulphate (SDS) polyacrylamide gel and then transferred onto a nitrocellulose or polyvinylidene fluoride (PVDF) membrane where they bind in the same pattern as formed in the gel. Antigen-coated membrane strips are incubated with the test sera and then with enzyme-labelled anti-species immunoglobulin. A substrate is added and the antigen pattern that is recognised by the antigen-specific antibodies present in the tested serum recorded (Björkman et al. 2007).

Barta and Dubey (1992) then Bjerkås et al. (1994) described immunoblot techniques to identify *N. caninum* infections using either reduced or non-reduced antigens. Stronger reactions have been observed using non-reduced antigens and they show less cross-reactivity to *Toxoplasma* and *Sarcocystis* species (Stenlund et al. 1997). An avidity IB has been

developed to detect the pattern of IgG avidity maturation against different specific antigens of *N. caninum* tachyzoites (Aguado-Martínez et al. 2005).

Immunoblot techniques can be used to detect antibodies in sera, milk or cerebrospinal fluid (CSF) and western blot is the most specific of these tests. Western blot analysis has been used to study the IgG response of cattle to infection with *N. caninum* (Harkins et al. 1998) and has greater sensitivity compared to IFAT and ELISA when used on fetal serum (Söndgen et al. 2001). However, immunoblot techniques are time consuming and are usually reserved for diagnosis after the event to confirm equivocal results from other serological techniques by recognition of certain defined immunodominant antigens (Bartels et al. 2006b).

2.5.1.6 T-cell proliferation and IFNγ detection

Intracellular parasites such as *N. caninum* trigger a strong cell-mediated immune response. Measuring this response may prove more useful than the detection of specific antibodies when the infection has become sequestered within tissue cysts and antibody concentrations decline (Innes 2007b). Lymphocytes from peripheral blood will proliferate and synthesise IFN-γ *in vitro* when stimulated with specific antigen (Lundén et al. 1998; Marks et al. 1998). The determination of specific IFN-γ allows an indirect quantification of cell-mediated responses although to date, this method has only been used in research. Several methods of measuring this lymphoproliferative response and IFN-γ responses have been reported but in general they require the collection of whole blood and removal of the buffy coat to collect peripheral blood mononuclear cells (Innes 2007b). There is the potential to refine these assays by using a panel of different antigens (from tachyzoites and/or bradyzoites) which may increase the sensitivity of the test and provide information on the duration of infection.

2.5.1.7 Herd level diagnosis using serology

The expense associated with histopathological examination of aborted material and the facts that such material is often not available and that an aetiological diagnosis is only made in < 50% of cases of bovine abortion (Cabell 2007) means that it is often necessary to rely on serological results in a clinical setting. Serology has been valuable in elucidating the epidemiology of *Neospora*-associated abortion in cattle and statistical analysis of

seroprevalence among aborting and non-aborting cows can be used to assess whether there is a significant relationship between infection and reproductive outcome (Thurmond and Hietala 1995). Careful consideration needs to be given to define which animals in a herd are included in any such calculation, as cows need to be "at risk" of aborting during the time period under consideration.

Serological screening is also useful to attempt to establish the main means of transmission within a herd by sampling the herd and establishing (i) if seropositive animals are distributed across the different age groups and (ii) whether seropositive dams have seropositive daughters (Dijkstra et al. 2001b). An avidity ELISA could also be employed to assess whether the *N. caninum* antibody response is indicative of a recent introduction to the herd (Björkman et al. 2003).

2.5.2 Direct detection

Neospora caninum infection can be widespread in some herds and appears to be more common where climatic conditions such as warmer temperatures and adequate rainfall enhance sporulation of oocysts (Bartels et al. 2006b). This, along with the fact that many infected cattle do not abort, means that the demonstration of lesions considered detrimental to the fetus and the presence of tachyzoites or tissue cysts in aborted tissue is valuable in diagnosing *Neospora*-associated abortion.

Methods for the detection of *N. caninum* within the infected animal rather than specific antibodies indicating prior exposure include immunohistochemistry (IHC) and polymerase chain reaction (PCR). Such techniques usually have lower sensitivity and higher specificity, but are also more expensive and time-consuming, so are usually reserved for research projects rather than clinical diagnosis.

Gross lesions in the aborted fetus are rare in cases of *Neospora* abortion but white linear foci have been reported in skeletal muscle and myocardium, minute pale to dark foci may be seen in the brain (Dubey and Lindsay 1996; Anderson et al. 2000) and focal areas of discolouration may be seen in placental cotyledons (Fioretti et al. 2003).

2.5.2.1 Histopathologic lesions

Histopathology using haematoxylin and eosin (H&E) staining is commonly used to examine aborted material for evidence of infection with *N. caninum*. Fetal brain is considered the most useful sample, although the probability of diagnosing the infection increases when other tissues such as the heart, liver and lung are also examined (Wouda et al. 1997a). Many aborted fetuses (and particularly the brain) are likely to be autolysed (Dillman 1976; Barr et al. 1990) and although this reduces diagnostic sensitivity it does not preclude a diagnosis being made.

Lesions that range from degenerative to inflammatory may be found throughout fetal tissues, most commonly in the central nervous system (CNS), heart, liver and lung (Barr et al. 1990; Wouda et al. 1997a). Focal areas of necrosis may also be seen in placental cotyledons (Fioretti et al. 2003). Neural lesions are present in the spinal cord and the brain (cerebrum, brainstem and cerebellum) and consist of nonsuppurative encephalomyelitis characterised by multifocal nonsuppurative infiltration, with or without multifocal necrosis, and multifocal to diffuse nonsuppurative leukocytic infiltration of the meninges (Dubey and Lindsay 1996). The characteristic lesion of the CNS is a focus of mononuclear cell infiltration around an area of central necrosis. Protozoa may be observed in this central area (Barr et al. 1990) but have usually been destroyed by the immune response (Dubey and Lindsay 1996). Glial proliferation is seen in some fetuses aborted in the third trimester (Peters et al. 2001b) and occasionally, the foci of necrosis may be mineralised (Boulton et al. 1995). The lesions in the brain may be accompanied by vascular endothelial hyperplasia with infiltration of mononuclear cells and leukomalacia in the cerebral white matter; although such lesions are not specific to neosporosis (Ortega-Mora et al. 2006).

Nonsuppurative myocarditis and nonsuppurative pneumonia are also commonly observed (Pescador et al. 2007). Lesions in the liver consist of periportal hepatitis with infiltration of mononuclear cells and multifocal hepatocellular necrosis (Barr et al. 1990; Wouda et al. 1997a). A non-suppurative placentitis, sometimes with mineralisation, has been reported (Maley et al. 2003) and experimental challenge in early gestation produces acute necrosis of fetal placental villi (Macaldowie et al. 2004).

Examination of aborted fetuses of differing gestational ages has shown that lesions are widespread throughout the fetus, and that the parasite load and severity of lesions is highest in the younger fetuses (especially those from the first trimester). Fetuses aborted in the third trimester contained fewer parasites, and the lesions were less severe and restricted predominantly to the fetal brain (Collantes-Fernández et al. 2006a).

Neospora caninum organisms are not easily identified in H&E stained sections but tachyzoites may occasionally be seen within the lumen of blood vessels or lying in clusters within the brain parenchyma. Small tissue cysts may occasionally be observed (Barr et al. 1990; Wouda et al. 1997a). Tachyzoites are easily confused with degenerate host cells in H&E sections, although they can be distinguished by their vesicular nucleus. Bradyzoites within tissue cysts are more easily identified with periodic acid Schiff (PAS) staining which stains their terminal nucleus red (Dubey et al. 2002).

Histological diagnosis is complicated by the fact that encephalomyelitis has occasionally been reported in congenitally infected calves and in calves born with neurological deficits, meaning that such lesions may be compatible with fetal survival (De Meerschman et al. 2005). It has been suggested that lesions should be assessed for the degree of severity to support a diagnosis of *N. caninum*-associated abortion (Wouda et al. 1997a) although standardised guidelines have not been developed. In addition, other protozoa such as *Sarcocystis* spp. may cause similar lesions in aborted fetuses (Jenkins et al. 2002) so confirmation using immunohistochemistry or PCR should be undertaken to prove that *N. caninum* is the agent responsible.

2.5.2.2 Immunohistochemistry (IHC)

Immunohistochemistry can provide an aetiological diagnosis of *Neospora*-associated abortion by demonstrating *N. caninum* parasites in areas of fetal tissue containing lesions due to protozoal infection. The technique relies on the use of specific antibody which binds to *N. caninum* antigen in tissue sections which is then visualised by applying reagents that locate the bound antibody and produce a colour reaction (Wouda and Buxton 2007). An avidin-biotin-peroxidase conjugate is commonly used, but peroxidase-antiperoxidase and other techniques can be used. Fetal brain is the tissue of choice for examination, but fetal liver and heart are also suitable for examination (Wouda et al. 1997a). Tissues

containing a high concentration of peroxidase, especially placenta, should be treated with trypsin or pepsin prior to processing and the diagnosis should not be made unless parasite outlines are visible, as diffuse staining may be non-specific (Dubey and Schares 2006).

Polyclonal antibodies specific to *N. caninum* are commercially available (Cole et al. 1994), but cross-reactivity between a *T. gondii* bradyzoite antigen and *N. caninum* has been reported (McAllister et al. 1996a; van Maanen et al. 2004) so serological testing should also be undertaken. Immunohistochemistry has also been used to show clusters of zoites in the brain and spinal cord of dogs with clinical neosporosis (Patitucci 1995).

Although IHC can confirm that fetal lesions are due to *N. caninum* rather than other apicomplexan parasites, positive immunoperoxidase stained tissues have also been found in experimentally infected fetuses that did not abort (Barr et al. 1994b) and in congenitally infected calves (Barr et al. 1993; Bryan et al. 1994) meaning that such fetal lesions are not incompatible with life. An advantage that IHC has over diagnosis by PCR is it confirms that the parasites are associated with the histological lesions. On the other hand, IHC has lower sensitivity than PCR (Gottstein et al. 1998). Moreover, differences in methodology and operator experience mean that the sensitivity of the procedure may vary considerably between laboratories (van Maanen et al. 2004).

2.5.2.3 Polymerase Chain Reaction (PCR)

Polymerase chain reaction methods can be used to amplify extracted DNA from fresh, frozen, formalin-fixed or paraffin-embedded tissues (Baszler et al. 1999b) and have also been used to demonstrate *N. caninum* in whole blood (Okeoma et al. 2005), serum (McInnes et al. 2006a), milk (Moskwa et al. 2003), semen (Caetano-da-Silva et al. 2004a; Ferre et al. 2005) and amniotic fluid (Ho et al. 1997b) from infected cows. Fetal brain appears to be the best sample for PCR detection of *N. caninum* when investigating cases of abortion but fetal heart, lung, kidneys and placenta have also yielded positive results (Gottstein et al. 1998; Baszler et al. 1999b). PCR techniques work well on autolysed tissues and have been used in epidemiological studies to test environmental samples and cattle fodder and water for the presence of *N. caninum* (Dubey and Schares 2006). As with serology however, the presence of parasite DNA in maternal or fetal tissue following abortion does not mean that *N. caninum* was the cause of the abortion. The finding that

cattle and fetuses may contain parasite DNA yet are seronegative (McInnes et al. 2006a) further confuses the interpretation of diagnostic methods for *N. caninum* infection.

Standard PCR techniques were initially used (Kaufmann et al. 1996; Payne and Ellis 1996; Yamage et al. 1996) then modifications such as nested or semi-nested PCR (Ellis et al. 1999) and small-subunit rRNA sequence probe hybridisation (Ho et al. 1996) were developed to increase sensitivity and specificity. Semi-quantitative (Liddell et al. 1999) and quantitative PCR methods (Collantes-Fernández et al. 2002) have also been developed and are useful to estimate parasite load when assessing putative vaccines or chemotherapeutics (Collantes-Fernández et al. 2004; Collantes-Fernández et al. 2006b). Recently a PCR method has been reported for detecting *N. caninum* oocysts in dog faeces (Hill et al. 2001).

PCR-based methods for the diagnosis of neosporosis were initially developed based around the internal transcribed spacer 1 (ITS1) region of the rRNA gene (Holmdahl and Mattsson 1996; Payne and Ellis 1996) and the repeated *Neospora*-specific Nc5 sequence (Kaufmann et al. 1996; Müller et al. 1996). Genes coding for rRNA and the pNc5 gene have become important targets for PCR technology due to their repetitive character which increases the sensitivity of the technique (Ho et al. 1996). Real-time PCR techniques have the advantage that amplification, detection and quantification all take place within the same tube and there is no need for post-PCR manipulation. Hence, there is less risk of sample contamination. PCR techniques are fast and efficient but assays need to be standardised and quality control measures implemented in order to achieve uniformity in the molecular diagnosis of neosporosis (Conraths and Schares 2006). As lesions are not distributed throughout fetal tissues it is important that fetal tissues are carefully homogenised prior to DNA extraction (van Maanen et al. 2004).

Any PCR method is susceptible to false-negative and false-positive results. PCR techniques are highly sensitive and false-positive results are usually a result of cross contamination of samples (Mattsson and Müller 2007). Positive PCR amplicon samples should be purified and sequenced for confirmation.

Several approaches to molecular characterisation of *N. caninum* have been attempted. One method using comparison of ribosomal DNA sequences from the 18S subunit and

the ITS1 regions suggested that there were no differences between geographically distinct isolates from cattle and dogs (Barber et al. 1995; Stenlund et al. 1997; Sreekumar et al. 2004). In addition, comparative analyses of the *NcSAG1* and *NcSRS2* genes from six *N. caninum* isolates from dogs and cattle from geographically distinct regions revealed complete sequence conservation (Marsh et al. 1999). The use of random amplification of polymorphic DNA PCR detected variation among 6 isolates of *N. caninum* (Schock et al. 2001) and most recently, a nested PCR procedure has been developed for the application of *N. caninum* microsatellite markers which has revealed extensive genetic diversity (Regidor-Cerrillo et al. 2006). This procedure may assist with determining differences instrain virulence (Pedraza-Díaz et al. 2009) and prove useful for tracking isolates in live vaccine development. To date, most microsatellite array analysis has focused on laboratory isolates but this is starting to be applied to clinical samples (Regidor-Cerrillo et al. 2008; Basso et al. 2010) which should increase our understanding of the natural transmission routes of the parasite.

2.5.2.4 Isolation by bioassay in rodents or cell culture

Isolation of viable *N. caninum* by bioassay in mice, gerbils (*Meriones* spp.), sand rats (*Psammomys obesus*) or cell culture has proven difficult as most parasite stages within the fetus die when the fetus succumbs to infection or when the host cell is destroyed by the parasite (Dubey and Schares 2006). Isolation of viable *N. caninum* from tissue cysts has generally been more successful as they are more resistant to autolysis. A method for isolation of *N. caninum* from bovine tissues was reported by Dubey and Schares (2006). Such techniques and the maintenance of *N. caninum* in cell cultures, are undertaken in research laboratories but are not available for commercial diagnostics.

2.6 Methods of control of neosporosis in cattle

Control of neosporosis may be aimed at preventing abortion in infected cattle, but, in the long-term, attempts should be made to reduce the prevalence in a herd by preventing vertical and horizontal transmission. Control measures are likely to incur financial costs, which may be significant, including the loss of genetic potential from premature culling of high-producing cattle. Implementing control measures also incurs significant time costs. A cost-benefit analysis specific to the particular farm should be carried out to assess whether control measures should be instigated. This can be particularly difficult due to the variation in ongoing abortion losses in subsequent pregnancies and the fact that other factors may interact with *N. caninum* infection to influence the outcome of pregnancy.

2.6.1 General hygiene

Measures should be implemented to prevent the transmission of *N. caninum* between intermediate and definitive hosts on a property. These include preventing stray or feral definitive hosts (particularly pregnant females and juveniles of the *Canidae* family) from contaminating food and water sources. The number of dogs kept on farms has been identified as a risk factor for bovine neosporosis in many epidemiological studies (Paré et al. 1998; Mainar-Jaime et al. 1999; Schares et al. 2003), so limiting the number of dogs on farms would be prudent. It should, however, be noted that resident dogs on a farm are likely to already be infected (Antony and Williamson 2003) and may be less likely to transmit infection to cattle than introduced dogs, as the latter may suffer a primary infection and shed oocysts (Dijkstra et al. 2002b). It may be possible to train dogs to defaecate before they work or are exercised over the rest of the farm in areas not used for grazing. Aborted material, placentae and dead livestock should be promptly and securely disposed of to prevent scavenging (which could continue the life cycle of *N. caninum*; McAllister et al. 1998; Basso et al. 2001; Gondim et al. 2002) or spread other infectious causes of abortion.

Seropositive rodents, chickens and pigeons have been reported (McGuire et al. 1999; Huang et al. 2004c; Hughes et al. 2006; Costa et al. 2008) and although they are probably intermediate hosts and not infective for cattle, they may be infective for dogs on the farm so should be controlled if possible.

2.6.2 Test and cull

A 'test and cull' strategy has been recommended by many authors to reduce herd seroprevalence on farms where endogenous transplacental transmission is an important route of infection (Reichel and Ellis 2002; Larson et al. 2004; Haddad et al. 2005; Hall et al. 2005; Conraths et al. 2007; Dubey et al. 2007). However, all control strategies that rely on the identification of animals infected with *N. caninum* are limited by the sensitivity and cut-points used to interpret serological tests. Test sensitivity can be improved by sampling at a time when cows are more likely to have higher antibody titres such as in mid-late gestation or early lactation (Innes et al. 2005). This is particularly applicable in seasonal farming systems such as in the New Zealand dairy industry where all cows on the farm are at approximately the same stage of gestation.

A test and cull strategy may not be possible on farms where herd seroprevalence is > 50% (Pfeiffer et al. 2002; Bartels et al. 2006b). In such circumstances, as an interim measure, it may be possible to identify seropositive cattle and cull them when it is feasible to do so, but not keep their offspring as replacements. Alternatively, calves could be serologically tested to determine whether or not they are congenitally infected. This is difficult however, as it necessitates either pre-colostral sampling (which is not feasible in most situations) or testing when maternal antibody has waned. Such testing would need to occur when calves are > 4 months old (Hietala and Thurmond 1999) but considerable expense will have been incurred to raise them to this age.

In most instances when a disease has been eradicated, a vaccination and/or biosecurity programme needs to be implemented on the farm as the herd is now immunologically naïve. To some extent this is also true for *N. caninum* infection, as there is evidence that previously infected cattle have some level of protection in the face of a new challenge (McAllister et al. 2000). High levels of ongoing vertical transmission and the high risk of abortion in congenitally infected heifers means that living with *N. caninum* infection in the herd can be costly. However, risk of cattle becoming infected due to oocyst contamination of pasture is low (at least in Germany; Conraths et al. 2007) so a test and cull strategy is worth considering.

Economic analysis should consider the possible costs of epidemic neosporosis if there was subsequent re-introduction of *N. caninum* in addition to the ongoing costs of living with neosporosis in the herd and the costs of testing and removing infected animals. The observation that climatic and immunosuppressive factors in conjunction with *N. caninum* infection affect the risk of *Neospora*-associated abortion (Björkman et al. 2000; López-Gatius et al. 2005a) makes the estimation of these costs difficult.

2.6.3 Biosecurity

Preventing the introduction of animals that are seropositive to *N. caninum* into a herd is difficult due to the lack of sensitivity of serological testing. However, purchasing cattle from farms that are known to have had *Neospora*-associated abortions should be avoided. Bulls present a particular problem in terms of biosecurity, as their serology is difficult to assess. Bulls have been reported to have lower antibody titres than cows (Caetano-da-Silva et al. 2004b), presumably because parasitaemia is a one-off event (i.e. they do not encounter the recrudescence of parasitaemia associated with pregnancy). Hence, their exposure to tachyzoite antigen may have been less recent and antibody concentrations will have declined. On the other hand, venereal transmission of *N. caninum* from bulls is probably of little significance in the epidemiology of the disease so the only consequence of them on the farm may be as a source of infection if fed to dogs.

2.6.4 Minimising other stressors

Factors that allow or enable the conversion of *N. caninum* bradyzoites to tachyzoites are not fully understood and it has been hypothesised that changes in host immunity during pregnancy may be implicated (Innes et al. 2000; Quinn et al. 2002). Other stressors and management factors that may have a negative impact on the host's immune system have been reported to increase the risk of abortion in cattle infected with *N. caninum*. These include fungal contamination of feed and feeding remnant fodder which may be of inferior quality (Thurmond et al. 1995; Bartels et al. 1999; Wouda et al. 1999a). However, as lower quality feed may also be contaminated with mycotoxins which can cause abortion in their own right, this effect could be due to confounding. Similarly, other infectious causes of abortion such as BVDV, *Chlamydophila psittaci*, *Coxiella burnetii*, *Salmonella* spp. and *Leptospira* spp. may have a direct effect on the cow or could cause stress or immune suppression, making the recrudescence of a chronic *N. caninum* infection more likely (Thurmond and Hietala 1995; Björkman et al. 2000; Williamson et al. 2000; Hässig and Gottstein 2002).

However, other studies have found no relationship between the presence of antibodies to other infectious causes of abortion in herds with *Neospora*-associated abortion (Bartels et al. 1999) but this could be due to adequate herd immunity providing protection against active infection. Vaccination against other infectious causes of abortion will reduce their impact and could also reduce the level of stress in the herd and may reduce the likelihood of *Neospora*-associated abortions (Hobson et al. 2005).

Various climatic factors have been positively associated with herd seroprevalence to *N. caninum* (Bartels et al. 2006b) and the incidence of *Neospora*-associated abortion (López-Gatius et al. 2005a). It has been hypothesised that higher minimum temperatures in winter and adequate rainfall or humidity allows more rapid sporulation of oocysts and enhances oocyst survival in the environment, thus facilitating horizontal transmission to cows. Warm and humid conditions also increase the likelihood of fungal contamination of stored feed which could have direct or indirect effects on increasing the risk of abortion in cattle infected with *N. caninum*. High rainfall may also increase stress and decrease immune competence in cattle by a direct physical effect, by decreasing food quality and decreasing levels of hygiene (López-Gatius et al. 2005a).

Other management factors such as stocking rate, housing of cattle and the design of housing systems may lower immunity in cattle if they are stressed and may facilitate the horizontal transfer of *N. caninum* to cattle (Hässig and Gottstein 2002; Schares et al. 2004b). Such effects are impossible to quantify and will depend on the presence and management of definitive hosts. While the effect of many of these stressors on *Neospora*-associated abortion remains unproven, it makes sense from animal production and animal welfare viewpoints to minimise physical and social stress among cattle and to vaccinate against other pathogens. Adequate shelter from harsh climatic conditions and adequate, quality feed should be provided if it is economically feasible to do so.

2.6.5 Reproductive management

Embryo transfer can be used to prevent vertical transmission and produce non-infected offspring from seropositive cows (Baillargeon et al. 2001; Landmann et al. 2002). Cows used in any programme must be free of *N. caninum* infection.

The use of beef breed bulls as sires over dairy cows has been shown to reduce abortion (López-Gatius et al. 2005c; Almería et al. 2009; Yániz et al. 2010). The immune response at the materno-fetal interface may be enhanced in crossbreed pregnancies via an increased concentration of pregnancy-associated glycoproteins (PAG; Serrano et al. 2009). Higher levels of *N. caninum* IgG antibodies have been demonstrated throughout pregnancy in beef cattle than in dairy cattle while dairy cattle mated to beef bulls, hence carrying a crossbred pregnancy, had intermediate levels of *N. caninum* IgG antibodies (Santolaria et al. 2011).

Although crossbreeding appears to be a practical method of reducing the incidence of abortion, it would require dairy farmers to purchase heifer replacements rather than breeding their own. Bought-in heifers should be tested for antibody to *N. caninum* to ensure herd seroprevalence decreases and the introduction of other pathogens to the herd needs to be considered.

2.6.6 Vaccination

Attempts to develop a vaccine to control bovine neosporosis have met with limited success. A POLYGEN-adjuvanted killed *N. caninum* tachyzoite preparation failed to prevent fetal infection in pregnant cattle following experimental challenge (Andrianarivo et al. 2000). A killed tachyzoite preparation (Bovilis Neoguard; Intervet B.V., Boxmeer, The Netherlands) was licensed for use in countries, including New Zealand and the USA to aid in the control of abortion due to *N. caninum*. Its efficacy was assessed by field trials carried out in Costa Rica (Romero et al. 2004) and New Zealand (Schetters et al. 2004; Weston et al. 2012 as reported in Chapter Four in this thesis). These trials showed that the response to the vaccine varied substantially between farms, but on some farms, it reduced abortion by approximately 50%. This product has since been withdrawn from the market internationally so there is no vaccine currently available.

Control of intracellular parasites relies primarily upon cell-mediated immunity (Marks et al. 1998). Antibody responses provide some protection as demonstrated by trials in immunosuppressed mice (Eperon et al. 1999), but most protozoal vaccines that have been registered for veterinary use are based on live, attenuated formulations (Williams and Trees 2006) as these are more effective than killed vaccines at stimulating cell-mediated immunity. Examples of such vaccines include the S48 strain of *T. gondii* tachyzoites

(Toxovax; Intervet Schering Plough Animal Health, Upper Hutt, New Zealand) and the Paracox (Pitman-Moore Europe Ltd, Harefield, Uxbridge, UK) vaccine used to control coccidiosis in poultry. Vaccines against intracellular parasites have proven difficult to develop, so there is still no vaccine against the important human pathogen *Plasmodium* spp. (the apicomplexan which causes malaria) despite the obvious potential value of such a product. Problems with attenuated, live vaccines include short shelf-life, risk of reversion to virulence, danger of contamination with other pathogens and risk to the operator in case of accidental self-injection. In future, it is likely that the use of live-attenuated organisms will be superseded by recombinant or nucleic acid vaccines that include gene products or genes for desirable cytokines as well as the appropriate antigen (Cox 1997).

2.6.7 Chemotherapy

A wide range of antimicrobial substances have been tested *in vitro* against *N. caninum* tachyzoites (Lindsay et al. 1994). However, there are no substances currently licensed for use in cattle to treat neosporosis, although experimental trials using toltrazuril, and its derivative ponazuril, have shown some promise *in vitro* (Darius et al. 2004), in mice (Gottstein et al. 2001; Ammann et al. 2004) and in calves (Kritzner et al. 2002; Haerdi et al. 2006). Calves inoculated with *N. caninum* tachyzoites and then treated with ponazuril did not return PCR positive results in brain or muscle tissue in a small scale pilot study (Kritzner et al. 2002). Pregnant mice treated with toltrazuril on the same day as they were infected with *N. caninum* tachyzoites had reduced pre-natal and peri-natal losses, did not transmit *Neospora* in their next pregnancy and had decreased PCR detection in the brains of the mothers (Gottstein et al. 2005). Further trials to demonstrate ongoing efficacy of chemotherapeutic substances for prevention of vertical transmission and to elucidate the most appropriate and cost-effective treatment protocols are required.

2.7 Conclusions

Neospora caninum is an exceptionally well adapted parasite, with a life stage that can be harboured as a latent infection within the host and high rates of vertical transmission. Apart from the increased risk of culling (which is usually related to abortion) *N. caninum* has few deleterious effects on its hosts, meaning that it is easily maintained within cattle herds. The host's immune response is poor (e.g. in comparison to infection with *T. gondii*) and is

complicated by the immunomodulation that occurs during pregnancy. This is thought to allow recrudescence of parasitaemia. Immunological methods of control are likely to be hampered by the delicate balance between Th1- and Th2-type cell-mediated immunity and the difficulties associated with stimulating this response. Fluctuation of specific antibody titres in infected animals makes it difficult to identify all infected animals in a herd with a single serological screening test, making "test and cull" an inexact and expensive method of control, particularly when there is the chance that *N. caninum* may be reintroduced to the herd (e.g. from a definitive host).

The research presented in this thesis was conducted to assess the recognised approaches for the control of bovine neosporosis by focusing on the importance of congenitally infected heifers in a herd, assessing the efficacy of a commercially available killed tachyzoite vaccine and undertaking preliminary work on the development of a live-attenuated tachyzoite vaccine. As an adjunct to this, challenge trials were carried out in pregnant heifers and young, pregnant sheep to validate isolation and vaccination procedures.

Chapter Three

Associations between pregnancy outcome and serological response to *Neospora caninum* among a group of dairy heifers

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3.0 Preface

This trial was carried out in a herd of dairy cattle that experienced an abortion epidemic associated with *Neospora caninum* infection from February to July 1997 with 67 abortions among 745 animals at risk (9.0% abortion risk; 95% CI: 7.1 – 11.3%) but the abortion risk among heifers was significantly higher ($p < 0.001$) at 19%. In subsequent years, the herd experienced ongoing abortion losses of ~ 3% (Pfeiffer et al. 2002). From May 1997, the herd was monitored by quarterly serological testing by veterinarians from IVABS until October 2005. Multiple serological results for individual animals and their dams made this an ideal herd for further study.

This trial's aim was to compare the reproductive outcomes between primiparous heifers according to serostatus to *N. caninum* and to examine serological patterns in seropositive heifers that did or did not abort.

3.1 Abstract

AIM: To monitor pregnancy in a group of dairy heifers on a farm on which abortion due to *Neospora caninum* was known to occur in previous years.

METHODS: A prospective cohort study group of 164 rising 2-year-old heifers was pregnancy tested and blood-sampled at 4-5 week intervals throughout gestation. When loss of pregnancy was detected, a *N. caninum* indirect fluorescent antibody test (IFAT) was conducted retrospectively on stored sera collected the month before abortion, the month abortion was detected, and for the following 2 months, from heifers that aborted. All fetal and placental material detected following abortion was subjected to gross post-mortem and histopathological examination.

RESULTS: Eleven of 17 (65%) heifers that were seropositive and 4/146 (3%) heifers that were seronegative to *N. caninum* by ELISA, aborted. The relative risk for abortion among ELISA-positive heifers was 23.6. Abortion occurred predominantly between Days 120 and 152 gestation among the ELISA-positive heifers and throughout gestation among the ELISA-negative heifers. IFAT titres rose around the time of abortion in most of the heifers that were previously seropositive by ELISA, but dropped rapidly again in post-abortion samples. IFAT titres among 4/6 ELISA-positive heifers that did not abort

increased, but later in gestation than the time other heifers aborted. IFAT titres remained negative in heifers that aborted that were ELISA-negative.

CONCLUSIONS: Heifers that were seropositive to *N. caninum* by ELISA had a much greater risk of abortion than seronegative heifers. Most seropositive heifers showed evidence of a reactivation of infection during pregnancy. High (≥1:2,000) *N. caninum* IFAT titres also occurred in non-aborting heifers.

CLINICAL RELEVANCE: Culling of replacement heifers seropositive to *N. caninum* may be a cost-effective strategy for minimising risk of abortion. Pregnancy testing heifers before 5 months gestation may overestimate the number that calve in *N. caninum*-infected herds, but would assist in documenting the occurrence of abortion. Reliance on a high (>1:2,000) IFAT titre to rule-in *N. caninum* as a cause of abortion is likely to produce false-positive results.

3.2 Introduction

Neospora caninum is a protozoal parasite that was first discovered as a cause of disease in dogs (Bjerkås et al. 1984) and has since been found to be associated with abortion in dairy (Thilsted and Dubey 1989) and beef (Dubey et al. 1990c) cattle. *Neospora caninum* is now the most commonly diagnosed cause of abortion in cattle in many countries including New Zealand (Thornton et al. 1991). The life cycle has been reviewed by several authors (Dubey 1999; Reichel 2000; Antony and Williamson 2001). Once cows within a herd become infected, a high incidence of vertical transmission, at rates of up to 95%, maintains a reservoir of infected young animals entering the herd as replacements (Paré et al. 1996), although modelling studies have demonstrated that this is insufficient to maintain infection indefinitely within a herd (French et al. 1999).

Abortion due to neosporosis may occur as a result of a lethal infection of the fetus following a maternal parasitaemia, or damage to the placenta resulting in fetal compromise (Innes et al. 2002). This may occur when a pregnant cow is first exposed to *N. caninum* or when recrudescence of infection occurs. Fetal death may occur at any time during gestation and stillbirth of calves has been attributed to *N. caninum* infection (Dubey and Lindsay 1996). The main risk period for abortion is between the fifth and seventh months gestation (Anderson et al. 1991). It has been suggested that loss of pregnancy may occur

earlier than this and not be recognised as abortion but simply as a failure to conceive (Dubey and Lindsay 1996). A recent study in Spain found no association between *N. caninum* infection and loss of pregnancy before 90 days of gestation (López-Gatius et al. 2004).

The timing of exposure of the fetus to *N. caninum* infection has a major effect on the outcome of pregnancy (Innes et al. 2002). Infection can result in abortion, birth of a congenitally-infected calf or birth of a normal calf. Cattle naturally or experimentally infected with *N. caninum* produce a specific antibody and predominantly T helper (Th) 1-type cell-mediated immune response, and the production of interferon gamma (IFN-γ) and other pro-inflammatory cytokines (Lundén et al. 1998). Normal pregnancy is characterised by a lack of strong maternal cell-mediated anti-fetal immunity and a dominant humoral and Th 2-predominant immune response (Wegmann et al. 1993).

Gestational age and immunocompetence of the fetus at the time of infection also affects pregnancy outcome (Williams et al. 2000); infection of the fetus later in gestation being less likely to result in abortion than early infection, presumably due to the relative maturity of the fetal immune system. Following experimental infection of pregnant cattle at 23 weeks gestation, it was found that all fetuses had specific antibodies to the parasite but that cell-proliferation and IFN-γ responses were highly variable between individuals (Andrianarivo et al. 2001).

Definitive diagnosis of abortion due to *N. caninum* infection remains difficult and relies on characteristic histopathological changes in fetal tissues (Barr et al. 1990) or demonstration of *N. caninum* DNA using a polymerase chain reaction assay (PCR; Lally et al. 1996b). Immunohistochemistry using antibodies to *N. caninum* can also be used to identify tissue cysts and tachyzoites within fetal tissue (Barr et al. 1991a). A high *N. caninum* antibody titre in the dam is suggestive of the parasite being involved in the loss of pregnancy, and comparison of titres among aborting and non-aborting cattle can assist diagnosis of neosporosis as a cause of abortion when a number of cattle are affected (Paré et al. 1995a). However, diagnosis of *N. caninum* as the cause of abortion when there is no fetal tissue available for examination remains speculative.

Various serological tests that detect *N. caninum*-specific antibodies in cattle are commercially available (von Blumröder et al. 2004). Cattle that produce positive results to these tests have circulating antibody and this provides evidence for previous infection, but the significance of infection is difficult to establish and many pregnant, seropositive cattle go on to deliver full-term, healthy calves (Dubey and Lindsay 1996). A PCR test using whole blood has recently been developed (Okeoma et al. 2004a) but as the parasite may no longer be circulating by the time abortion is detected, its use may be limited to experimental studies. Diagnostic tests that detect *N. caninum* antibodies have been calibrated for different purposes; the detection of infected animals and detecting among infected animals those cattle aborting due to *N. caninum* infection (von Blumröder et al. 2004). For this reason, different cut-off points are used to determine positivity and the significance of test results, and there is not always agreement between tests.

It has been shown that the *N. caninum* IFAT titre can rise sharply around the time of abortion and then decrease markedly over the next 2 months (Cox et al. 1998). However, other studies have shown fluctuations in antibody titre among pregnant cattle that do not abort, suggesting that recrudescence of infection does not always lead to fetal death (Stenlund et al. 1999). Stressors such as parturition and negative energy balance in early lactation can suppress immunity (Lloyd 1983) and may allow recrudescence to occur. Changes occurring as a result of abortion from any cause may alter the balance of cell-mediated immunity (CMI) and result in recrudescence of a latent *N. caninum* infection, as has been recently reported to occur during pregnancy in infected animals (Okeoma et al. 2005).

Previous infection with *N. caninum* or the presence of antibodies to it do not confer protective immunity and abortion may still occur (Dubey and Lindsay 1996). Repeat abortion in the same animals has been reported (Thornton et al. 1994) but the prevalence of this can be difficult to quantify as many cows that abort are culled.

The current study is the first prospective cohort study of a group of naturally infected first-calving dairy heifers, managed in an extensive grazing environment. The aim was to compare the rates and timing of abortion between heifers that were seropositive *vs* seronegative for *N. caninum* antibodies detected using an ELISA, and to measure *N. caninum*

IFAT titres in the month before and 2 months after abortion, to determine their usefulness as a diagnostic tool on a dairy farm with a confirmed history of abortion due to *N. caninum* over the preceding 7 years.

3.3 Material and Methods

3.3.1 History

The cows in the herd under study (n~600) and all replacement stock had previously been tested for antibodies to *N. caninum* quarterly since 1997. An abortion storm in which 9% of the herd and 19% of the rising 2-year-old replacement heifers had aborted occurred in that year (Pfeiffer et al. 2002). Other findings from this herd have been reported by Williamson et al. (2000). Since the initial outbreak, the incidence of abortion on the farm had dropped to about 3% of pregnant animals in the milking herd but was higher among the rising 2-year-old replacement heifers.

3.3.2 Animals

The current prospective cohort study was conducted during the 2003-2004 season, to monitor the pregnancies and seroprevalence of *N. caninum* antibodies amongst the rising 2-year-old heifers (n=166) in that season. The heifers were Friesian-Jersey crossbreds and were run with Jersey bulls from 20 October 2003 until 12 January 2004. The seroprevalence of *N. caninum* antibodies was determined in July 2003, November 2003, January 2004 and May 2004 using an ELISA (HerdChek, IDEXX Laboratories Inc, Maine, USA). A cut-off value of 0.50 in the sample to positive (S/P) ratio was used to distinguish positive from negative results. The group was scanned transrectally using ultrasound (Sector Scanner; BCF Technologies, North Carolina, USA) to diagnose the occurrence and stage of pregnancy, from 8 weeks after the planned start of mating (PSM) and then at 4-5-week intervals until calving. All procedures involving the experimental use of animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand.

Dates of conception and abortion were estimated from the results of pregnancy tests. Dates of abortion were estimated by selecting the mid-point in time between the last positive and first negative pregnancy test that detected the abortion. In some cases, abortion was judged to be recent and the date of occurrence was estimated from the size

and characteristics of the uterus determined by manual palpation per rectum. Stage of gestation at the date of abortion was then calculated from the difference between this and the estimated date of conception.

3.3.3 Sampling

Serum and whole blood samples were collected by coccygeal venepuncture at the time of each pregnancy-testing visit, and sera were stored at -20°C. When abortion was detected, serum samples from the aborting heifer for the month before abortion, the month the abortion was detected, and for the following 2 months were analysed for *N. caninum* IFAT titres using a commercial kit (VMRD Inc, Pullman, USA) at an initial dilution of 1:200. As a control, stored sera from heifers that were seropositive to the ELISA but that did not abort, from the months of February, March, April and May, were also tested using the IFAT; these samples were chosen because these were the months when abortion and a fluctuating titre were most prevalent.

Any fetal or placental material that was detected following an abortion was examined post-mortem and fixed samples of fetal heart, liver and brain were examined histopathologically. The seroprevalence of antibodies to bovine viral diarrhoea (BVD) in all heifers was tested using an ELISA (Institut Pourquier, Montpellier, France) from the blood samples collected in May 2004, and compared with those samples collected in July 2003.

3.3.4 Statistical analysis

Chi-squared analysis was performed to test the null hypothesis that there was no difference in the rate of abortion between heifers seropositive *vs* seronegative for *N. caninum* antibodies measured using the ELISA. A two-tailed student's *t*-test was used to compare the stage of gestation at which IFAT titres peaked in *N. caninum* ELISA-positive heifers that aborted compared with those that did not abort. Relative risk, confidence intervals (CI), Chi-squared tests and survival curves for pregnancies were calculated using Microsoft Excel (Microsoft Corporation, Washington, USA).

3.4 Results

Two heifers failed to conceive, one of which was positive to the *N. caninum* antibody ELISA. Thus, the study group consisted of 164 remaining pregnant heifers, of which 18 were initially classified as seropositive using the *N.* caninum antibody ELISA; this was reduced to 17 when one seropositive heifer was euthanased at approximately Day 104 of gestation after fracturing a limb. The ELISA results for the 18 seropositive heifers were consistently positive from the time they were first sampled at approximately 4 months of age. These 18 heifers were born to 17 cows in the herd (one cow contributed twin heifers to the 2002 cohort) that were consistently seropositive for *N. caninum* antibody, nine of which were born in 1995 and were rising 2-year-old heifers at the time of the initial abortion outbreak in 1997, indicating that they had most likely been congenitally infected. Comparison of the serology of the heifers included in this study with that of their dams indicated that the vertical transmission rate of *N. caninum* was at least 73%.

Abortion was estimated to have occurred at 85, 104, 120, 120, 122, 122, 124, 128, 150, 152 and 152 (mummified fetus detected) days gestation (mean=125.4 days, (SD 20.5); median = 122 days) 11/17 seropositive heifers. No fetal or placental material was recovered from any of these animals. It was estimated that abortion occurred at 72, 99, 141 and 210 days of gestation (mean=130.5 days, (SD 60.1); median = 120 days) in 4/146 heifers classified as seronegative to the *N. caninum* antibody ELISA. Only 1/15 abortion events was observed and twin fetuses were collected but post mortem and histopathological examination detected no significant lesions and no cause was attributed to the event. Figure 3.1 illustrates the frequency and timing of abortion in seropositive and seronegative heifers.

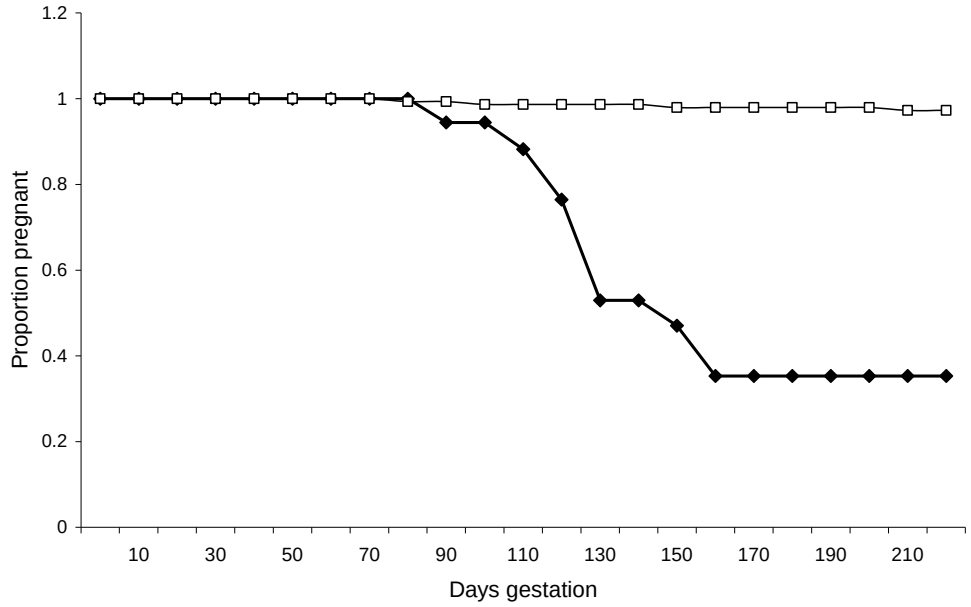

Figure 3. 1 Survival curve for pregnancy in 164 rising 2-year-old heifers which were (◆) *Neospora*-positive or (□) *Neospora*-negative.

The risk of abortion among heifers that were positive to the *N. caninum* antibody ELISA was 0.65 (95% CI=0.42-0.87), compared with 0.03 (95% CI=0.001-0.05) among the seronegative heifers. The relative risk of abortion was 23.6 times greater (95% CI=8.5-66.0) among the seropositive heifers, and the attributable risk of abortion among the seropositive heifers was 0.62 (95% CI=0.39-0.84). There was a significant difference ($p<0.001$) between observed and expected rates of abortion between seropositive and seronegative heifers.

The *N. caninum* IFAT titres among the 11 infected heifers that aborted ranged from 1:200 to >1:2,000 in the month of abortion (Figure 3.2). On average, the IFAT titre 1 month after abortion dropped by 55% compared with the titre at the time of abortion and dropped by a further 50% by the second month. IFAT titres were consistently negative in all of the heifers classified as seronegative by ELISA that aborted.

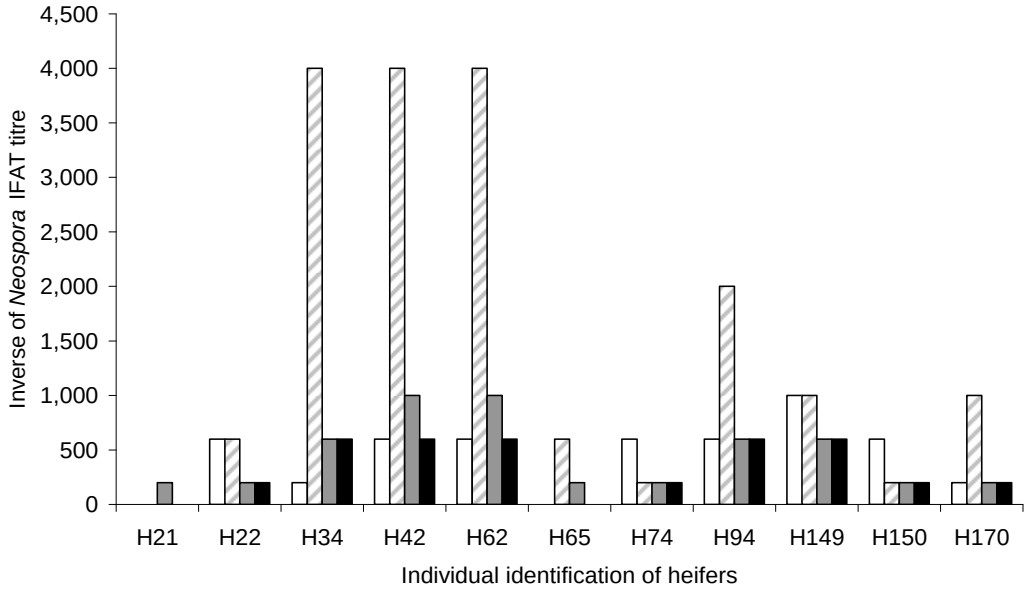

Figure 3.2 *Neospora caninum* indirect fluorescent antibody test (IFAT) titre around the time of abortion among 11 heifers seropositive by enzyme-linked immunosorbent assay (☐) 1 month before abortion was detected, (▨) in the month that abortion was detected, (▩ **)** 1 month after abortion was detected, and (■) 2 months after the abortion was detected. NB. Heifer 21 was diagnosed as not pregnant after removal of the bulls and was transported to a run-off property where she was later observed to abort; other blood samples were not available to be tested.

The IFAT results for the six heifers classified as seropositive by ELISA that did not abort showed detectable titres in all the months sampled (February, March, April, May) ranging from 1:200 (n = 2 throughout this period) to a peak of ≥1:1,000 (n = 3; Figure 3.3). Among the heifers seropositive by ELISA, the average peak IFAT titre occurred at 134.5 (SD 22.0) days gestation in those that aborted compared with 159.8 (SD 41.5) days gestation in those that did not abort ($p = 0.14$).

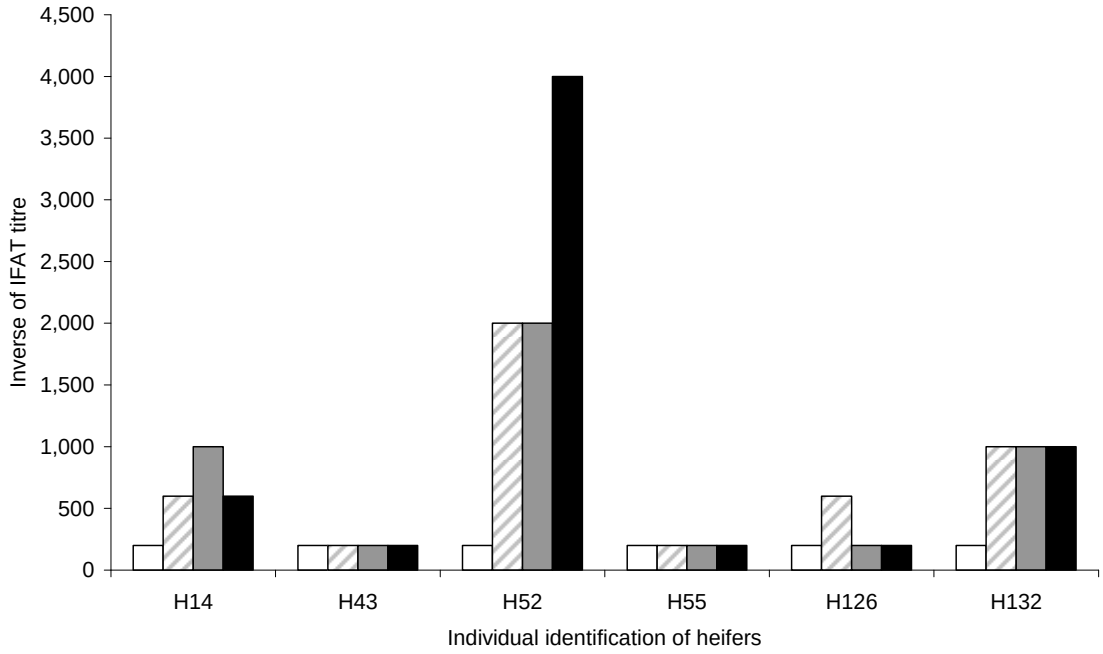

Figure 3.3 *Neospora caninum* indirect fluorescent antibody test (IFAT) titre in (☐) February, (▨) March, **(▩)** April, and (■ **)** May 2004, in six heifers previously classified as seropositive by enzyme-linked immunosorbent assay that did not abort.

Using an S/P ratio cut-off of 0.50 in the ELISA test, 19/166 heifers were seropositive for *N. caninum* antibodies in July 2003, 22/166 in November 2003, 22/166 in January 2004 and 21/165 in May 2004. Only one previously seronegative heifer became consistently seropositive. All heifers that were initially classified as seropositive remained so throughout the study, and heifers that were seronegative and aborted remained seronegative. Consecutive ELISA S/P ratios for several heifers in the study are presented in Table 3.1.

Table 3.1 Enzyme-linked immunosorbent assay (ELISA) sample-to-positive (S/P) ratios, used to determine the serological status for *Neospora caninum*, of all heifers that had at least one positive ELISA result from a cohort of 164 pregnant Friesian-cross heifers; standard cut-off in the S/P ratio for the ELISA is 0.50.

			ELISA S/P ratio						
ID	Status[a]	Abort	Dec 02	Mar 03	Jul 03	Nov 03	Jan 04	May 04	Oct 04
H150	Pos	Yes	2.73	1.84	0.73	0.76	0.94	1.90[b]	na
H022	Pos	Yes	0.80	0.44	0.86	0.97	1.09	1.74[b]	na
H074	Pos	Yes	2.20	1.88	1.39	1.14	2.11	2.41[b]	na
H065	Pos	Yes	1.98	1.16	1.50	0.81	1.11	1.50[b]	na
H170	Pos	Yes	3.04	2.38	1.68	1.21	2.14	2.39[b]	na
H034	Pos	Yes	2.59	2.65	1.72	1.53	2.13	2.71[b]	na
H021	Pos	Yes	0.73	0.66	2.11	1.88	1.67	3.16[b]	na
H094	Pos	Yes	2.02	2.93	2.27	2.00	2.49	3.32[b]	na
H062	Pos	Yes	1.83	4.16	2.91	2.67	3.69[b]	3.47	na
H149	Pos	Yes	2.74	4.35	3.32	2.75	3.43[b]	3.22	na
H042	Pos	Yes	1.65	4.73	3.44	2.39	4.73	3.47[b]	na
H149	Pos	-	2.82	2.72	1.77	1.36	N/A	4.01	3.53
H132	Pos	-	2.35	4.38	2.28	2.71	2.32	3.58	2.74
H052	Pos	-	2.54	2.37	2.99	2.25	3.10	4.11	3.34
H055	Pos	-	1.09	1.45	3.02	1.57	1.47	2.82	1.8
H126	Pos	-	2.82	3.49	4.40	1.75	2.13	2.59	3.28
H043	Pos	-	1.80	1.78	na	1.34	1.81	1.43	1.96
H102	Neg	Yes	-0.05	0.03	0.03	0.06	0.04	0.01[b]	na
H097	Neg	Yes	-0.06	0.20	0.05	0.03	0.22[b]	0.02	na
H169	Neg	Yes	-0.05	-0.02	0.06	0.05	0.13[b]	0.08	na
H098	Neg	Yes	-0.04	0.17	na	0.24	0.21	0.36[b]	na
H136	Neg	-	-0.04	0.07	0.01	0.24	0.21	0.55	0.53
H134	Neg	-	-0.03	0.32	0.03	0.25	0.19	0.80	1.47
H002	Neg	-	0.38	1.53	0.05	0.11	0.10	0.15	0.74
H044	Neg	-	-0.05	0.22	0.06	0.51	0.60	0.43	0.19
H031	Neg	-	-0.01	0.40	0.07	0.60	0.02	0.12	0.08
H106	Neg	-	-0.05	0.26	0.08	0.62	0.28	1.47	0.38
H061	Neg	-	0.02	0.15	0.10	0.41	0.89	0.27	0.26
H058	Neg	-	0.00	0.50	0.18	0.39	0.83	0.28	0.14
H036	Neg	-	0.00	-0.02	0.21	0.15	0.96	0.25	0.48
H154	Neg	-	-0.04	0.15	0.25	0.14	0.16	0.12	0.64
H049	Neg	-	0.74	0.43	0.27	0.37	0.32	0.33	0.98
H029	Neg	-	1.60	1.26	0.49	0.51	1.07	3.09	1.89

[a] Status (serological status for *N. caninum*) was allocated for the purposes of this trial from the July 2003 result
[b] This is the ELISA result closest to the time of abortion
na = heifer was not available for sampling (heifers that aborted were culled before October 2004); Pos = positive; Neg = negative

3.5 Discussion

Abortion among the heifers that were seropositive using the *N. caninum* antibody ELISA were clustered in the fourth and fifth months gestation while those among seronegative heifers occurred between months 3 and 7 (Figure 3.1). Abortion among the seropositive heifers in this study occurred earlier than is typically reported in studies of abortion due to *N. caninum* (Dubey and Lindsay 1996). In a similar study that followed the pregnancies of 18 naturally-infected heifers for two consecutive gestations, abortions occurred in the fifth (twice), seventh (twice) and eighth months (Stenlund et al. 1999). Paré et al. (1997) followed 254 adult cows in a herd with an abortion rate of 17.3% and found that the median survival time of fetuses was 147 days for the 30/146 *N. caninum*- seropositive cows that aborted, and 117.5 days in the 13/108 seronegative cows that aborted; in our study the median survival time of fetuses was 122 and 120 days of gestation for seropositive and seronegative heifers, respectively.

The risk of abortion among seropositive heifers was 0.65 (95% CI=0.42-0.87), considerably greater than in the study reported by Stenlund et al. (1999) in which the corresponding risk was 0.17 (95% CI=0-0.38). The relative risk of abortion among *N. caninum*-seropositive heifers was 23.6 times greater than that of the seronegative heifers, demonstrating a very strong association between serological status for *N. caninum* and risk of abortion. In a study in California, congenitally-infected heifers had a 7.4-fold higher risk of abortion in their initial pregnancy than non-infected heifers (Thurmond and Hietala 1997a). Paré et al. (1996) reported a 2-fold greater risk of abortion among seropositive cows compared with seronegative cows, and another study following four Dutch dairy herds found a 2-3-fold increased risk of abortion in seropositive cows (Moen et al. 1998). Both of these studies were of multiparous cattle and it was not reported whether they were congenitally or post-natally infected. In our study, results from regular testing of the heifers for *N. caninum* antibodies from approximately 4 months of age suggested that those classified as seropositive for the purposes of this study were congenitally infected. We propose that such animals may have a less effective immune response, dependant on the level of fetal immunocompetence at the time of initial infection, than cattle that are infected post-natally, and the consequent risk of abortion may be significantly higher.

The results presented here provided strong evidence of an association between serological status for *N. caninum* and risk of abortion. However, it was not possible to ascertain the cause of abortion or to definitively conclude that *N. caninum* infection was responsible for any of the abortions that occurred. BVD was ruled out as a cause of abortion and there was little evidence of active BVD infection within the study group, as only three heifers seroconverted over a 12-month period. The heifers were managed as a group and pasture-fed. Immunosuppression due to mycotoxins, which has been hypothesised to be associated with abortion due to *N. caninum* in some herds (Bartels et al. 1999), was considered unlikely in this situation.

Changes in antibody titre in the seropositive heifers suggests either recrudescence of infection or new exposure to the parasite during pregnancy occurred. New exposure would have been a risk for all heifers, resulting in more abortions in seronegative heifers or at least an increase in the number of seropositive heifers, so this is unlikely to have occurred. None of the seronegative heifers that aborted had a detectable IFAT titre (the lowest dilution tested was 1:200) during the study nor was seroconversion evident from ELISA test results (Table 3.1), suggesting that abortions that occurred amongst these heifers were not due to *N. caninum*.

One non-aborting heifer may have been misclassified as seronegative in this study, as all previous and subsequent ELISA results for that animal were positive. Inclusion of this heifer in the seropositive group would reduce the relative risk of abortion amongst seropositive *vs* seronegative heifers to 22.2 (95% CI=7.9-62.3). A further 16 heifers that were classified as seronegative had sporadic positive ELISA results in their first 29 months of life, evident from samples collected before, during and after this study, and none of those heifers aborted. Of these occasionally seropositive animals, 7/16 had two positive ELISA results and for four of these animals the positive results occurred in consecutive samples, suggesting that some horizontal transmission or recrudescence of latent infection occurred.

Cox et al. (1998) and Dubey et al. (1996c) reported that *N. caninum* IFAT titre did not always rise to high levels during abortion outbreaks. It has also been observed that IFAT titres can fall from as high as 1:4,000 to 1:200 within 2 months (Reichel and Drake 1996).

In most abortion outbreaks, there has not been the opportunity for serial blood collection prior to the event so the temporal pattern of change in titres has not been elucidated as it has been in the current study.

The wide variability in the peak IFAT titres of affected animals and the consistently rapid decline in circulating antibody levels (Figure 3.2) illustrates the difficulty of diagnosing *N. caninum* as a cause of abortion, even when detected close to the time of occurrence. If a loss of pregnancy is detected some months after it has occurred, *N. caninum* serology will be of little diagnostic value, as a positive titre only indicates previous infection and a negative result does not rule-out *N. caninum* as the cause. Attributing cause is not possible in an individual animal but consideration of serological results among groups of aborting and non-aborting cattle can be useful when investigating herd outbreaks (Thurmond and Hietala 1999). The detection of high titres in non-aborting cattle (Figure 3.3) further confuses the diagnostic picture and supports the use of herd serology, not individual animal samples, where this is possible.

IFAT is widely regarded as the 'gold standard' when comparing serological tests for the diagnosis of *N. caninum* infection in cattle (Reichel and Pfeiffer 2002), although this was recently challenged by Frössling et al. (2003), as the IFAT had a lower sensitivity than an ELISA in that study. The IFAT is often conducted at an initial dilution of 1:200. The fact that 2/4 samples from a seropositive heifer (H65; Figure 3.2) taken around the time of abortion in our study were negative on IFAT, while all ELISA results for this heifer had an S/P ratio >0.8, suggests that the standard IFAT testing conducted for commercial purposes may lack sensitivity, particularly in cases where the immunological challenge may have occurred several months earlier. However, it is possible that this heifer may have aborted from another cause. An alternative testing regime using an ELISA, and reporting quantitative results in the form of an S/P ratio accompanied with a guide to interpretation, should be considered (Álvarez-García et al. 2003).

The increase in IFAT titres among most of the 11 aborting and seropositive heifers suggests a recrudescence of infection may have occurred in these animals prior to abortion. The increase in titre in 4 / 6 seropositive but non-aborting heifers (Figure 3.3) indicates that these heifers were also immunologically re-exposed to *N. caninum* antigen. Stenlund et

al. (1999) followed 18 naturally-infected heifers through their first two pregnancies and found a consistent increase in antibody titre at 5-6 months gestation among both aborting and non-aborting cattle, that then decreased prior to parturition. In our study, the peak in IFAT titre tended to occur later in gestation than that. The time of the increase in IFAT titre evident in our study did not vary significantly between aborting and non-aborting heifers, but this comparison was only made between group sizes of 10 and 5, respectively, which provides little power for comparison. These results support the finding of Williams et al. (2000) that the time of recrudescence during pregnancy determines fetal survival. This may be a result of fetal immunocompetence or the differing effects of Th1- *vs* Th2-type immunological responses in pregnant animals at different stages of gestation.

In another study, nine cows that were naturally and persistently infected with *N. caninum* were housed and sampled intensively during pregnancy to monitor abortion and serological responses (Guy et al. 2001). A marked increase in maternal antibody occurred in one cow at 17 weeks gestation and a fetus was aborted. Five cows produced congenitally-infected calves and had an increase in maternal antibody between Weeks 22 and 36 gestation. The remaining three cows showed no changes in maternal antibody and did not produce congenitally infected calves.

Infection of a pregnant cow with *N. caninum* will activate the Th1-type response and sway the balance of CMI away from the Th2-type response which protects the pregnancy. The Th1-type cytokines can have a direct embryotoxic effect and damage the placental trophoblast (Raghupathy 1997). In cattle that have a latent *N. caninum* infection, the significant down-regulation of specific cell proliferation and IFN-γ responses that occur around mid-gestation may be a factor contributing to recrudescence in persistently-infected animals (Innes et al. 2002).

Progesterone is known to bias a T-cell response towards a Th2 phenotype (Piccinni et al. 1995) and the presence of prostaglandin E_2 will bias the priming of naïve T-cells towards a Th2 phenotype (Kalinski et al. 1997), so fluctuations in maternal hormone levels may also interact to regulate immune response during pregnancy. Oestrogen concentrations in plasma increase in pregnant cows at approximately the fourth month of gestation (Hoffman et al. 1997), and some studies suggest that this suppresses CMI and

enhances the formation of systemic antibodies (Styrt and Sugarman 1991). Thus, the increased maternal antibody titre could possibly reflect either an increased release of parasites from tissue cysts due to suppressed immunity, a hormone-triggered antibody production, or both. Alternatively, the rise in antibody titre may be due to antigenic stimulation from an *N. caninum*-infected fetus.

Under-reporting of abortions during the first trimester is likely, regardless of cause, as it is less likely that an expelled fetus would be noticed, particularly among heifers under conditions of pastoral management. Regular pregnancy testing of animals confirmed as being pregnant is most useful for diagnosing abortion and has been used in other studies (López-Gatius et al. 2004). Pregnancy diagnosis before about 160 days gestation (Figure 3.1) in our group of heifers would not have correlated well with calving results and so early pregnancy diagnosis may not be accurate in predicting the number of heifers to calve in herds where animals are seropositive. Early pregnancy diagnosis does, however, provide a basis for diagnosing early abortion and allows the economic benefit of removal of non-pregnant animals.

It is likely that the pattern of abortion due to *N. caninum* differs according to whether the infection is endemic or epidemic. In congenitally-infected heifers, previous exposure (probably *in utero*) primes the immune system to respond and a heifer's immune response to a re-exposure or recrudescence of infection causes the abortion (Quinn et al. 2002). In naïve animals, abortions occur over a wider range of gestational ages according to time of exposure. It is unknown whether the parasite is lethal to the fetus during the phase of tachyzoite proliferation, through damage to the fetus or the placenta, or whether the maternal cell-mediated immune response is detrimental to the maintenance of pregnancy. Categorising cows simply as seropositive or seronegative may be misleading, as variation in antibody levels may provide useful information; some animals have high antibody levels for their entire life-time, some have detectable levels for only a short time period, and some alternate between seropositive and seronegative states repeatedly over their life-time. These differences in serological state may be associated with different reproductive outcomes, the highest rates of abortion and vertical transmission occurring among cows that are consistently seropositive. Cattle challenged with *N. caninum* 6 weeks prior to mating were protected against vertical transmission when a second challenge occurred on

Day 140 of gestation (Innes et al. 2001a). This suggests that there is a difference in immune response and ability to transmit the infection *in utero* between cattle that are infected as adults and those that are congenitally infected.

The very high (0.61 or 0.65 depending on the inclusion of one heifer) risk of abortion during a first pregnancy in heifers that were seropositive for *N. caninum* in this study suggests that testing and culling of seropositive heifers may be indicated on some farms. However, to be effective, a test with high sensitivity and specificity is required and, as has been discussed, interpretations of serological test results vary. If the serological status of the dam is also known then this is a useful preditor of the serological status for *N. caninum* of the offspring (Anderson et al. 1997) and may be considered in the decision-making. The apparent reliability of pre-colostral testing to determine the serological status of vertically-infected potential replacement heifers before significant investment is made raising them commend this as an approach to reducing the number of heifers within a herd with a propensity to abort.

3.6 Acknowledgements

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3.7 Authors' contributions to this study

NB Williamson helped with sample collection and provided advice on trial design as well as providing editorial advice.

WE Pomroy provided advice on trial design and editorial advice.

JF Weston designed and conducted the experiment, was responsible for pregnancy testing and sample collection, and wrote the manuscript.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

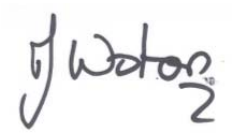
Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Associations between pregnancy outcome and serological response to *Neospora caninum* among a group of dairy heifers

In which Chapter is the Published Work: Three

What percentage of the Published Work was contributed by the candidate: 80%.



29th August 2011

Candidate's Signature

2nd September 2011

Principal Supervisor's signature

Chapter Four

Efficacy of a *Neospora caninum* killed tachyzoite vaccine in preventing abortion and vertical transmission in dairy cattle

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4.0 Preface

The observation of a 0.65 risk of abortion among heifers that were seropositive to *Neospora caninum* was higher than is commonly reported in primiparous heifers. A high economic cost due to *Neospora*-associated abortion means that costs associated with controlling *N. caninum* infection within a herd are likely to be worthwhile. Vaccination or pharmacological treatment to control or eliminate the parasite in already-infected cattle, or to prevent new infections has been suggested to be more practical than eliminating natural transmission routes to cattle. Research into the development of such vaccines or treatments has been ongoing over the past 15 years. This study was funded by Intervet B.V. Ltd (Boxmeer, The Netherlands) to carry out a clinical trial of Bovilis Neoguard, a killed tachyzoite vaccine comprising inactivated *N. caninum* tachyzoites (3×10^6 mL^{-1} at harvest), 10% of Havlogen adjuvant, 5% of stabilisers and 5% of phosphate buffered saline. At the time of this trial, this product had recently been licensed for use in several countries including New Zealand and the United States of America.

4.1 Abstract

A clinical trial was undertaken to assess the efficacy of Bovilis Neoguard, a killed *Neospora caninum* tachyzoite vaccine on 5 commercial dairy farms in New Zealand with a history of *Neospora*-associated abortion. Cattle were enrolled in the trial at 30-60 days of gestation and randomly allocated to treatment or control groups. Treatment consisted of 5 mL doses of Bovilis Neoguard administered subcutaneously at enrolment then 4 weeks later. Isotonic saline was administered to the control group. Of 2,246 cattle enrolled in the trial, 10.7% of cows and 12.6% of heifers were seropositive to *N. caninum*. Sampling of a randomly selected proportion of enrolled animals 6 weeks after the second treatment showed that 188/232 (81.0%) vaccinated with Bovilis Neoguard had seroconverted, while 11/130 (8.5%) cows and 10/36 (27.8%) heifers in the control group had seroconverted. Forty-eight vaccinated and 63 control animals aborted. On one farm 12.5% of control animals and 6.1% of vaccinated animals aborted (vaccine efficacy 0.61; $p = 0.03$). On another farm with a high level of abortion 8.4% of control animals and 8.7% of vaccinates aborted. On the remaining 3 farms fewer abortions occurred than expected. A modified Poisson regression approach was used to calculate relative risks for abortion and vertical

transmission. Overall vaccine efficacy was 0.25 (p = 0.12). Heifer replacement calves from the animals enrolled in the trial were sampled for antibodies to *N. caninum* at 6-9 months of age. Fourteen of 17 calves from vaccinated, seropositive cows were seropositive as were 13/23 calves from seropositive cows in the control group. The interaction between dam serostatus and treatment group was significant (p = 0.05) with vaccination increasing the risk of vertical transmission. It was concluded that vaccination after conception prevented 61% abortions in one of five herds and that vaccination may have increased the risk of early embryonic death.

Keywords: Neospora caninum; Dairy cattle; Vaccination; Abortion; Vertical transmission

4.2 Introduction

Neospora caninum is an important cause of bovine abortion in many countries (Dubey et al. 2007). Losses occur in dairy (Anderson et al. 1995) and beef cattle (Waldner et al. 1999) but losses are more likely to be noticed, investigated and reported on dairy farms due to daily observation of cows at milking. Epidemic and endemic abortion patterns have been associated with *N. caninum* infection (Wouda et al. 1999a). Epidemic abortion, with up to 33% of animals aborting, has been reported (Thornton et al. 1994) and is thought to occur when a naïve herd is exposed to *N. caninum* oocysts excreted by a definitive host (Jenkins et al. 2000) or in a chronically infected herd with recrudescence of latent infection (Wouda et al. 1999a). Endemic abortion may occur in herds following epidemic abortion (Moen et al. 1998; Pfeiffer et al. 2002). Heifer replacements infected by vertical transmission maintain a proportion of seropositive cows in a herd despite infected and aborting cows having an increased risk of being culled (Thurmond and Hietala 1996). Economic losses attributable to *N. caninum* include an increase in calving interval, a decrease in milk production, a decrease in stock value and increased culling (Trees et al. 1999; Bartels et al. 2006a).

The immune response to *N. caninum* infection includes specific cell-mediated and antibody responses, and the Th1-type response important in controlling intracellular parasitic infections (Innes et al. 2002). An antibody response is commonly detected within 14 days of experimental challenge and may help prevent parasite invasion of other host cells (Innes et al. 2002). The bradyzoite stage of the parasite is thought to evade the host's

immune response and it has been postulated that immunomodulation during pregnancy alters the balance of Th1 and Th2-type immune responses, allowing recrudescence of the parasite and subsequent parasitaemia (Entrican 2002). Parasitaemia in a pregnant cow leading to transplacental transmission and/or abortion may occur in cattle experiencing a primary infection (exogenous transplacental transmission) or where a chronic infection has recrudesced (endogenous transplacental transmission; Trees and Williams, 2005). Abortion may be due to tissue destruction in the fetus, placental insufficiency due to inflammation, the maternal immune response or a combination of these (Dubey et al. 2006).

Recommendations for the control of bovine neosporosis include undertaking serological testing and culling of infected cattle (Hall et al. 2005), quarantine and testing of replacement and purchased cattle (Haddad et al. 2005), preventing transmission from dogs and other potential definitive hosts (Reichel and Ellis 2002) and minimising exposure to stressors such as mycotoxins (Bartels et al. 1999) and bovine viral diarrhoea virus (Quinn et al. 2004) that may promote recrudescence of infection. Transfer of embryos from infected cows to uninfected recipients can prevent endogenous transplacental transmission (Baillargeon et al. 2001). Treatment of infected calves and mice with toltrazuril (or its derivative ponazuril) has shown some efficacy in preventing vertical transmission in small-scale trials (Gottstein et al. 2005; Haerdi et al. 2006) but is not currently recommended as a treatment.

A killed tachyzoite vaccine (Bovilis Neoguard; Intervet International B.V., Boxmeer, The Netherlands) was registered for use in cattle in New Zealand in 2001 as an aid in the prevention of abortion due to *N. caninum*. The product had been assessed in experimental challenges for safety (Choromanski and Block 2000; Barling et al. 2003) and had been trialled under controlled and field conditions. A dairy herd in Minnesota reported 25% abortions annually for 2 years (with some confirmed as being associated with *N. caninum* infection) prior to introducing Neoguard vaccination, after which the abortion incidence dropped to less than 4% (Choromanski and Shawnee 2002). A challenge trial of seronegative heifers showed a significant reduction in abortion among the vaccinated animals (Choromanski and Shawnee 2002). It was also suggested that the vaccine may protect against vertical transmission since 6 heifer calves from the Minnesota herd were seronegative at age 6 months despite their dams being seropositive prior to vaccination

(Choromanski and Shawnee 2002). A field trial in Costa Rica in 25 dairy herds involving 876 cows reported 11.2% abortions in vaccinated animals and 20.8% abortions in the control group, a vaccine efficacy of 0.46 (95% confidence interval [CI]: 0.26, 0.61; Romero et al. 2004).

The objectives of this clinical trial were to assess the efficacy of Bovilis Neoguard in reducing abortion on commercial dairy farms in New Zealand with a history of *Neospora*-associated abortion and its effect on the vertical transmission of *N. caninum*.

4.3 Material and methods

4.3.1 Animals and serological testing

The owners of five commercial dairy farms from three regions in New Zealand (three from Taranaki, one from Canterbury and one from Southland) were recruited to participate in this clinical trial due to the documented history of high abortion rates (>8%) on their farm for at least two previous years that had been diagnosed as due to *Neospora caninum* infection. Cows were predominantly Friesian or Friesian/Jersey crossbreds and calved annually from late July to early October. Feed consisted predominantly of grazed pasture, supplemented with grass or maize silage at times during lactation, or with hay during the winter (dry period). Multiparous cows and home-bred, replacement heifers that would calve for the first time at approximately 24 months old were included in the trial. All cattle had been vaccinated against leptospirosis [serovars *Hardjobovis* and *Pomona* (Leptavoid 2, Schering Plough Animal Health, Upper Hutt, NZ) or serovars *Hardjobovis, Pomona* and *Copenhageni* (Lepto-3-way, Virbac, Auckland, NZ)] with annual boosters administered in mid-gestation (March-May). A bovine viral diarrhoea virus vaccination (BVDV) programme (Bovilis BVD, Intervet International B.V., Boxmeer, The Netherlands) had been instigated on Farm A.

Cows and primiparous heifers were enrolled once pregnancy was confirmed by transrectal ultrasonography conducted 8 weeks after the planned start of mating for each herd. Allocation to the treatment or control group was by systematic random allocation using a coin toss for the first, then every alternate animal. A second pregnancy test was conducted 4 weeks later to confirm pregnancy in the enrolled animals and to allow

enrolment of further animals. Animals were 30-60 days pregnant at the time of enrolment. Antibodies to *N. caninum* were measured by indirect fluorescent antibody test (IFAT; VMRD Inc., Pullman, Washington, USA) in serum collected from enrolled animals. Initial dilution was 1:100 then positive samples were serially diluted two-fold to determine a fluorescence end-point. An IFAT titre of ≥ 1:200 was considered positive. Sera were also tested for antibodies to BVDV by enzyme-linked immunosorbent assay (ELISA; Institut Pourquier, Montpellier, France). Pregnant cattle were enrolled in the trial in December 2001 and January 2002.

4.3.2 Treatment

Vaccinated animals received 5 mL of Bovilis Neoguard (batch 244016; Intervet International B.V., Boxmeer, The Netherlands) administered twice, at an interval of 4 weeks, by subcutaneous injection in either the anterior neck or the fossa lateral to the tail base. The second dose of vaccine was administered at no later than 3 months gestation. The vaccine comprised inactivated *N. caninum* tachyzoites (3×10^6 mL^{-1} at harvest), 10% of Havlogen adjuvant, 5% of stabilisers and 5% of phosphate buffered saline. Control animals similarly received a placebo (5 mL of 0.9% sodium chloride; Baxter Healthcare Pty. Ltd., NSW, Australia), subcutaneously. Farmers, their veterinarians and laboratory staff were unaware of the vaccination status of the animals enrolled in the trial. A sample of vaccinated and control adult cows from all farms (n = 125 after the first treatment, n = 228 after the second treatment) were assessed for injection site reactions by their local veterinarian 72 hours after each vaccination. This was achieved by palpating the injection site of those cows that were accessible while the cows were yarded for milking. Reactions were scored as 0 = no response, 1 = mild response (< 20 mm diameter), 2 = marked response (> 20 mm diameter).

Approximately 6 weeks after the second treatment, serum was collected from 326 randomly selected cows and 103 heifers across the 5 farms from both treatment groups to measure *N. caninum* antibody by IFAT. This represented 21.2% of vaccinated cows, 24.5% of vaccinated heifers, and 15.8% of cows and 17.8% of heifers in the control group.

4.3.3 Abortion investigation

When abortion was detected, the fetus and placenta were collected if available and submitted to a veterinary diagnostic laboratory (Gribbles Veterinary Pathology, Palmerston North, New Zealand) for examination. Brain, myocardium, liver, lung and placenta were fixed in 10% formalin then trimmed, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for histopathological examination. Serum collected from aborting cows when abortion was detected, and approximately four weeks later where possible, was tested for antibodies to *N. caninum* by IFAT, BVDV by ELISA and leptospirosis (serovars *Pomona and Hardjobovis*) by microscopic agglutination test (MAT; original cultures supplied by Environmental Science and Research, Wallaceville, Upper Hutt, New Zealand).

4.3.4 Vertical transmission

Logistically, it was not possible to collect pre-colostral serum from calves born to animals enrolled in this trial. Instead, serum was collected from heifer replacement calves (Farms A, B, C and E) at 6-9 months of age when maternal antibody would have waned (Hietala and Thurmond 1999) and was tested by IFAT for antibodies to *N. caninum*. Calves with titres of ≥ 1:200 were classified as infected.

4.3.5 Statistical analysis

The sample size was calculated to achieve a power of 80% to test the hypothesis of a reduction in abortion incidence from 10% to 5% with 95% confidence (i.e. 50% vaccine efficacy). This resulted in 435 animals per group, a total of 870 animals. A design effect of 2 was chosen to double the sample size as it was assumed that abortion incidence and vaccine effect would differ between herds. Additional animals to compensate for loss to follow up resulted in approximately 1,900 animals being required for the clinical trial.

All information was recorded in Microsoft Access 2000 (Microsoft Corporation, WA, USA). Statistical analysis of proportional data was carried out using chi-square or Fisher's exact test as appropriate. Binary outcome variables (e.g. seroconversion to *N. caninum*, failure to deliver a live calf) at the animal level were analysed by logistic regression, using SAS System for Windows version 9 (SAS Institute Inc., Cary, NC, USA). The model, with abortion as the outcome, included vaccination as the main effect with age (cow/heifer),

herd (A-E) and pre-vaccination *N. caninum* serostatus (positive when IFAT titre ≥1:200; else negative) as potential confounders. Interaction terms were initially evaluated using the likelihood ratio test and non-significant ($p > 0.05$) effects were removed manually. The model included interaction terms for vaccination by herd, vaccination by age (cow / heifer) and vaccination by pre-vaccination *N. caninum* serostatus (positive / negative) along with all main effects.

An odds ratio is a biased estimate of relative risk (RR) and as vaccine efficacy (1-RR) was the outcome of interest, further analysis was required to calculate relative risks from the data. The analysis of a vaccination effect was based on the Wald chi-square statistic testing the null hypothesis of relative risks being different from unity. Relative risks (RR) and 95% confidence intervals for RR were calculated as described by Zou (2004) for binary data, using a Poisson model with a log-link function and the same covariates as for the logistic model, but including a repeated statement in a GEE marginal model with animal-ID as subject and an unstructured correlation pattern. Vaccine efficacy was defined as 1 – RR for both means and 95% confidence intervals. Cumulative frequency of abortion, by month after enrolment in the trial, was evaluated by relative risk and tested for significance.

The relative risk for a calf to be seropositive according to dam serostatus was calculated for the vaccinated and control groups using 2 x 2 tables. The proportion of vertical transmission was calculated as the attributable fraction (AF), to correct for the low level of apparent horizontal transmission i.e. the fraction of vertical transmission that was attributable to having a seropositive dam. $AF = (RR - 1) / RR$, where the relative risk (RR) is p_1/p_0 and p_1 is the proportion of seropositive calves from seropositive dams and p_0 is the proportion of seropositive calves from seronegative dams (Rothman and Greenland, 1998; pg 295). Additionally, a logistic regression model using GEE, where calf identification was the subject for repeated measures and the farm effect was fixed with robust standard error estimates (Zou, 2004) was run to compare the proportion of vertical transmission in vaccinated and control animals. The RR was obtained using the modified Poisson regression approach (Zou, 2004) as described for vaccine efficacy. The model included the interaction between the IFAT-status of the dam and vaccination status of the dam as a single interaction effect. Least square means were extracted from the model to

obtain p_1 and p_0 and contrasts were used to estimate the RR of IFAT-positive calves from vaccinated and control dams, respectively.

All procedures involving the experimental use of animals were approved by the Massey University Animal Ethics Committee (MUAEC 01/5).

4.4 Results

4.4.1 Animals

A total of 2,246 cows and heifers were enrolled, 1,697 (75.6%) of them at the first pregnancy test, with 886 cows and 245 heifers allocated to the vaccine group and 874 cows and 241 heifers allocated to the control group. Since entire herds were enrolled, the final sample size was greater than the 1,900 animals required. On serological testing, 10.7% of cows and 12.6% of heifers were seropositive to *N. caninum* by IFAT and 49.2% of cows and 17.3% of heifers were positive or suspicious for BVD antibody by ELISA at the commencement of the trial. The proportion seropositive to *N. caninum* and BVD varied between farms (Table 4.1). There was no significant difference in seroprevalence to *N. caninum* between the vaccinated and control groups on any farm.

4.4.2 Injection site reaction

A mild reaction (< 20 mm diameter) was seen at the injection site in 9/66 (13.6%) vaccinated animals and none of the control animals after the first inoculation. After the second inoculation, 16/118 (13.6%) vaccinated animals and 1/110 (0.9%) control animals had a mild injection site reaction and 4/118 (3.4%) vaccinated but no control animals had a marked injection site reaction (> 20 mm diameter). The proportion of mild reactions was similar on all farms but all strong reactions occurred on Farm E.

4.4.3 Antibody response

Sera collected 6 weeks after the last treatment showed that, among the animals that were initially seronegative to *N. caninum*, 140/166 (84.3%) vaccinated cows and 48/66 (72.7%) vaccinated heifers had become seropositive to *N. caninum* whilst 11/130 (8.5%) cows and 10/36 (27.8%) heifers in the control group seroconverted in the same time period. The

incidence of seroconversion was significantly higher in vaccinated animals than in the controls. The results stratified by farm are presented in Table 4.2. On farm D the proportion of vaccinated cows seroconverting was significantly lower ($p < 0.01$) than on the other farms. In vaccinated animals, the *N. caninum* IFAT titre increased by at least 2 dilutions in 75.5% of vaccinated cows and 83.3% of vaccinated heifers, whilst in placebo-treated animals, 9.4% of cows and 18.6% of heifers showed a similar increase in titre.

The average increase in titre was 2.71 dilutions for vaccinated cows (median = 3), 2.68 dilutions for vaccinated heifers (median = 3), 0.48 dilutions for control cows (median = 0) and 0.72 dilutions for control heifers (median = 1). The magnitude of change in titre dilution by age and treatment group is shown in Figure 4.1.

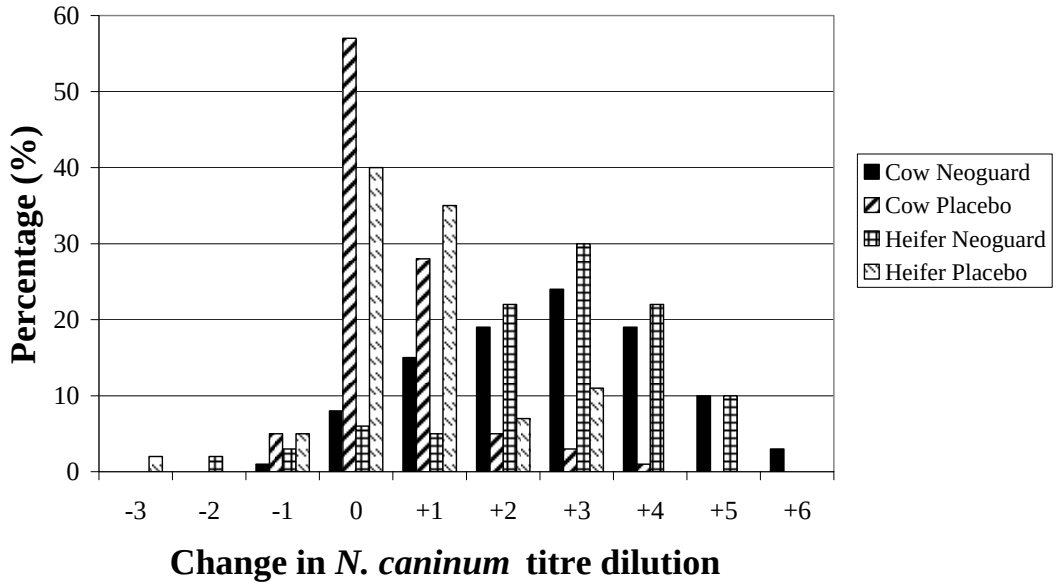

Figure 4.1 Change in *Neospora caninum* IFAT titre (number of dilutions) 6 weeks after the second treatment with Bovilis Neoguard or a 0.9% sodium chloride placebo.

Table 4.1 Pre-trial serological status of animals enrolled in a clinical trial of Bovilis Neoguard to *Neospora caninum* (IFAT ≥ 1:200) and bovine viral diarrhoea virus (BVDV; ELISA).

Farm	Age	# seropositive to *N. caninum* (Neoguard)	# seropositive to *N. caninum* (control)	# seropositive to BVDV* (Neoguard)	# seropositive to BVDV* (control)
A	Cows	9/111 (8.1%)	17/111 (15.3%)	75/111 (67.6%)	68/111 (61.3%)
	Heifers	5/31 (16.1%)	6/29 (20.7%)	0/3	0/2
B	Cows	12/182 (6.6%)	16/180 (8.9%)	46/182 (25.3%)	47/180 (26.1%)
	Heifers	0/29	2/29 (6.9%)	0/29	0/29
C	Cows	34/134(25.4%)	20/129 (15.5%)	95/134 (70.9%)	85/129 (65.9%)
	Heifers	6/31 (19.4%)	4/31 (12.9%)	7/31 (22.6%)	2/31 (6.5%)
D	Cows	17/256 (6.6%)	8/253 (3.2%)	196/256 (76.6%)	182/253 (71.9%)
	Heifers	10/104 (9.6%)	9/102 (8.8%)	25/104 (24.0%)	32/102 (31.4)
E	Cows	25/203 (12.3%)	31/201 (15.4%)	36/203 (17.7%)	40/201 (19.9%)
	Heifers	10/50 (20.0%)	9/50 (18.0%)	4/50 (8.0%)	5/50 (10.0%)
All	Cows	97/886 (10.9%)	92/874 (10.5%)	448/886 (50.6%)	422/874 (48.3%)
	Heifers	31/245 (12.7%)	30/241 (12.4%)	36/217 (16.6%)	39/214 (18.2%)

* Includes animals that were positive or suspicious by ELISA

4.4.4 Abortion and vaccine efficacy

Forty-eight vaccinated and 63 control animals aborted, including 39 animals (20 vaccinated) that failed to calve and were found to be non-pregnant. Regardless of treatment, the relative risk of abortion (using the modified Poisson regression approach) tended to be greater in cows than heifers (RR = 1.52, 95% CI: 0.94 - 2.45, $p = 0.051$) and was significantly higher in animals that were initially seropositive compared to seronegative, being 2.92 in cows (95% CI: 1.93 – 4.43, $p < 0.01$), 10.10 in heifers (95% CI: 3.97 – 25.70, $p < 0.01$) and 3.57 in cows and heifers combined (95% CI: 2.50 – 5.15, $p < 0.01$).

Abortion occurred in 5.7% of control animals (2.9%, 3.8%, 12.5%, 2.8% and 8.4% on Farms A-E respectively). The proportion aborting among control animals was significantly ($p < 0.05$) higher on Farms C and E than on Farms A, B and D. Overall 4.2% of vaccinated animals aborted (2.7%, 2.4%, 6.1%, 1.9% and 8.7% on Farms A-E respectively). A summary of abortion by farm, age and treatment is presented in Table 4.3.

The results of the logistic regression and the modified Poisson regression analysis are presented in Table 4.4. The efficacy of Neoguard in reducing abortion on farm C was 0.61 ($p = 0.03$) and on farms B and D was 0.41 and 0.42 respectively though the results were non-significant. There was no apparent vaccine effect on Farm E where 8.5% of enrolled cows aborted. Across the entire trial, the efficacy of Neoguard in preventing abortion was 0.25 ($p = 0.12$). There was no effect of the GEE on the vaccine model with coefficients remaining the same and standard errors decreasing by 6-9%.

Table 4.2 Proportion of animals that seroconverted to *Neospora caninum* (IFAT ≥ 1/200) when sampled 6 weeks after the second treatment in a clinical trial of Bovilis Neoguard.

Farm		A	B	C	D	E	Total
Vaccinated	Cows	28/28 (100%)	26/27 (96.3%)	15/15 (100%)	36/57 (63.2%)	35/39 (89.7%)	140/166 (84.3%)
	Heifers	7/9 (77.8%)	7/8 (87.5%)	4/5 (80.0%)	19/22 (86.4%)	11/12 (91.7%)	48/66 (72.7%)
Control	Cows	0/21	0/28	2/21 (9.5%)	0/18	9/42 (21.4%)	11/130 (8.5%)
	Heifers	3/4 (75.0%)	2/9 (22.2%)	2/4 (50.0%)	2/13 (15.4%)	1/6 (16.7%)	10/36 (27.8%)

Time to abortion was calculated as months since enrolment in the trial at which point the animals were 30-60 days pregnant. Among the 72 animals for which the date of abortion was known, the average time to abortion was 3.52 months (standard deviation, SD = 1.83) for the vaccinated animals and 3.79 months for the control animals (SD = 1.63). When all 111 cases of abortion were included, the average time to recognition of abortion was 5.65 months (SD = 2.35) in the vaccinated animals and 5.17 months (SD = 2.22) in the control animals. Figure 4.2 shows the relative risk of abortion between vaccinated and control animals. Relative risk stabilised at 0.60 and became significant in Months 6 and 7 (p = 0.04 and 0.02 respectively). However, some abortions were detected only when cows failed to calve and were found to be non-pregnant. When these cases were added to the data (according to months between enrolment and detection of abortion), the overall vaccine effect became non-significant. The proportion of cattle in which the timing of abortion was unknown was higher in vaccinated animals (20/48, 41.7%) than control animals (19/63, 30.2%) with a relative risk of 1.38 (95% CI: 0.84 – 2.29, $p = 0.21$).

Table 4.3 Abortion among animals from 5 farms according to age and initial serostatus to *Neospora caninum* in a clinical trial evaluating Bovilis Neoguard.

Treatment		Vaccine		Control	
Initial serological status		Positive	Negative	Positive	Negative
Farm	Age				
A	Cows	1/9 (11.1%)	3/102 (2.9%)	2/17 (11.8%)	1/94 (1.1%)
	Heifers	0/5	0/26	0/6	1/23 (4.3%)
B	Cows	2/12 (16.7%)	3/170 (1.8%)	2/16 (12.5%)	6/164 (3.7%)
	Heifers	0/0	0/29	0/2	0/27
C	Cows	4/34 (11.8%)	4/100 (4.0%)	6/20 (30.0%)	10/109 (9.2%)
	Heifers	2/6 (33.3%)	0/25	4/10 (40.0%)	0/21
D	Cows	0/17	6/239 (2.5%)	0/8	8/245 (3.3%)
	Heifers	0/10	1/94 (1.1%)	0/9	2/93 (2.2%)
E	Cows	4/25 (16.0%)	13/178 (7.3%)	7/31 (22.6%)	12/170 (7.1%)
	Heifers	4/10 (40.0%)	1/40 (2.5%)	1/9 (11.1%)	1/41 (2.4%)
Total	Cows	11/97 (11.3%)	29/789 (3.7%)	17/92 (18.5%)	37/782 (4.7%)
	Heifers	6/31 (19.4%)	2/214 (1.0%)	5/36 (13.9%)	4/205 (2.0%)

Table 4.4 Farm-specific efficacy of Bovilis Neoguard in reducing abortion in dairy herds with endemic *Neospora caninum* infection compared to a control group (1131 vaccinated animals and 1115 controls) adjusted for age and pre-vaccination status for *N. caninum* infection.

Farm	Odds Ratio of abortion (95% CI)	Relative Risk of abortion (95% CI)	p-value	Vaccine efficacy (1-RR)
A	1.04 (0.22 – 4.89)	1.03 (0.24 – 4.43)	0.96	-0.03
B	0.58 (0.16 – 2.12)	0.59 (0.17 – 2.01)	0.41	0.41
C	0.34 (0.12 – 0.96)	0.39 (0.16 – 0.96)	0.04	0.61
D	0.56 (0.17 – 1.83)	0.58 (0.19 – 1.77)	0.34	0.42
E	1.13 (2.88 – 6.68)	1.11 (0.64 – 1.95)	0.71	-0.11
All animals	0.73 (0.49 – 1.09)	0.75 (0.52 – 1.08)	0.12	0.25

A definitive diagnosis of the cause of abortion was made in 22 of 37 cases in which fetal and/or placental tissue was available for examination. Nine were attributed to *N. caninum* infection following histological examination. These occurred in a vaccinated cow on Farm A, a control cow on Farm B, three cows (one vaccinated and two controls) on Farm C and four cows on Farm E (one vaccinated and three controls). Fifteen cases had fetal bacteraemia and/or bacterial placentitis (two of these also had histological evidence of neosporosis). Another 24 animals from the control group (two from Farm A, three from Farm B, nine from farm C and ten from Farm E) and 25 vaccinates (one from Farm A, three from Farm B, four from Farm C and 17 from Farm E) had high *N. caninum* IFAT titres (≥1:1,000) at the time of abortion but no fetal tissue was available to confirm the diagnosis. A tentative diagnosis of BVDV abortion was made in a further nine cases where the dam had seroconverted to BVDV during the trial (one case from Farms A and B, two cases from Farms C and E and three cases from Farm D), four of these had a high IFAT titre (≥ 1:1,000) but in the other five cases this was the only finding. In addition, eight cases of abortion had maternal MAT titres to *Leptospira* serovars *Hardjobovis* or *Pomona* of ≥ 1:200, these were in 2 cows from Farm C and 6 cows from Farm E, seven of which also had a high IFAT titre (≥1:1,000) although four of them had been vaccinated with Neoguard.

4.4.5 Vertical transmission

Heifer replacement calves from Farms A, B, C and E (n = 305) were sampled at 6-9 months of age to test for the presence of *N. caninum* antibodies. Forty of these were from cows that were seropositive for *N. caninum* at the commencement of the trial; 155 calves were from cows that had received Neoguard while 150 calves were progeny of the control group. Forty-three calves were seropositive with 27 being from cows that were initially seropositive. Complete results by farm, treatment group and dam's initial serostatus are presented in Table 4.5. It appeared that some horizontal transmission had been occurring on the farms, either during pregnancy in the enrolled cows or in their offspring, with 16/265 (6.0%) calves from seronegative cows being seropositive.

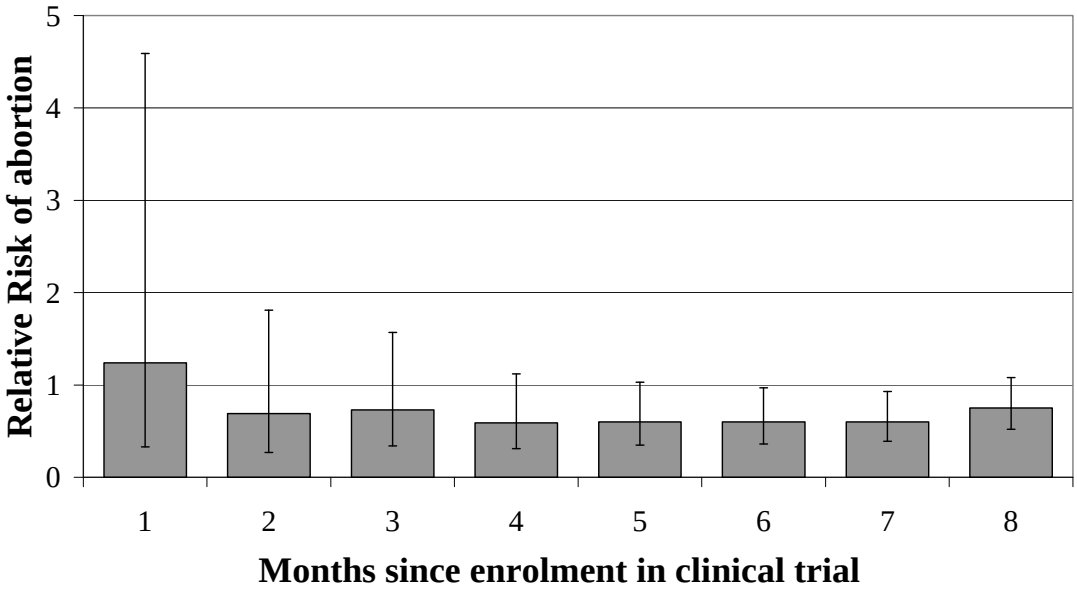

Figure 4.2 Relative risk of abortion with 95% confidence intervals for cows vaccinated with Bovilis Neoguard (n = 1131) compared to control animals (n = 1115) calculated by cumulative frequency of detected abortion since enrolment at 30 – 60 days gestation.

Table 4.5 Proportion of calves seropositive for *Neospora caninum* when sampled at 6-9 months of age whose dams were enrolled in a clinical trial of Bovilis Neoguard.

	Farm	A	B	C	E	Total
	Dam Serostatus					
Vaccinated	Seropositive	2 / 3 (66.7%)	3 / 3 (100%)	6 / 8 (75.0%)	3 / 3 (100%)	14 / 17 (82.4%)
	Seronegative	1 / 31 (3.2%)	3 / 37 (8.1%)	0 / 20	2 / 50 (4.0%)	6 / 138 (4.3%)
Control	Seropositive	5 / 7 (71.4%)	2 / 4 (50.0%)	1 / 2 (50.0%)	5 / 10 (50.0%)	13 / 23 (56.5%)
	Seronegative	2 / 21 (9.5%)	3 / 37 (8.1%)	1 / 24 (4.2%)	4 / 45 (8.9%)	10 / 127 (7.9%)

Crude relative risks from two-by-two tables and results of the modified Poisson regression analysis comparing the relative risks of a calf being seropositive due to having a seropositive *vs* seronegative dam from the vaccinated and control groups, as well as the attributable fractions of vertical transmission are presented in Table 4.6. Vaccination with Neoguard increased the risk of vertical transmission (p = 0.04) as evidenced by the differences in relative risk for calves being seropositive. In addition, the interaction term between treatment group and dam serostatus was significant (p = 0.05) with vaccination increasing the risk of vertical transmission. The seroprevalence in calves from seropositive dams increased from 53.3% in the control to 89.5% in vaccinated dams (p = 0.03), whereas the seroprevalence in calves from seronegative dams did not differ between vaccinated (4.2%) and control groups (7.3%; p = 0.26).

Table 4.6 Relative risk (RR) for a calf to be seropositive at 6-9 months of age according to dam serostatus and the attributable fraction (AF) of vertical transmission that was due to the dam being seropositive, with 95% confidence intervals, in a clinical trial of a *Neospora caninum* killed tachyzoite vaccine.

Parameter	Two-by-two table	Modified Poisson regression
RR vaccine	18.94 (8.40 – 42.70)	21.56 (10.19 – 45.61)
RR placebo	7.18 (3.58 – 14.38)	7.30 (3.46 – 15.40)
AF vaccine	0.94 (0.88 – 0.98)	0.95 (0.90 – 0.98)
AF placebo	0.86 (0.72 – 0.93)	0.86 (0.71 – 0.94)

4.5 Discussion

The prevention of abortion in vaccinated animals was not statistically significant (25% efficacy; $p = 0.12$) however vaccine effect varied between the farms. A significant vaccine effect (61% efficacy, $p = 0.03$) was detected on Farm C but on Farm E there were 22 abortions among 253 vaccinated animals and 21 abortions among 251 animals in the control group. The incidence of abortion on Farms A, B and D was lower than expected and this reduced the statistical power to detect a significant effect of vaccination.

Initial seroprevalence to *N. caninum* on the farms was also lower than expected and ranged from 4.9% to 20.5% of cows and 3.4% to 19.0% of heifers. Reichel (1998) reported that 7.6% of 800 cows from 40 dairy farms in New Zealand were seropositive to *N. caninum* but a seroprevalence of 10.9% to 53.0% has been reported among herds that have a history of *Neospora* abortion (Schares et al. 1999a). Herds that have had a longstanding problem with *Neospora* abortion are more likely to have culled seropositive and/or aborting cattle, either due to their failure to contribute to milk production or as part of a 'test and cull' programme in an attempt to control the problem. In addition, herd seroprevalence tends to decline due to the introduction of naïve animals and a decline in measurable antibody in previously infected cattle (Wouda et al. 1998b; Dijkstra et al. 2003). The herds used in this trial may previously have had a higher seroprevalence to *N. caninum* with previously aborting cows having been culled. Farms A, D and E had a higher proportion of heifers seropositive than cows, suggesting that vertical transmission was an important mode of transmission in these herds, that *N. caninum* infection had been present for some time and that culling of older, seropositive animals may have occurred.

Analysis of bulk-milk samples by ELISA has been used to estimate herd seroprevalence (Chanlun et al. 2002) and although the sensitivity of these tests is limited to detecting herds with at least 10-20% seroprevalence (Dubey and Schares 2006) this may have been useful for selecting herds for this trial. Alternatively, serological testing of a proportion of cows from a larger number of herds could have been used to identify those herds that were most likely to have an ongoing abortion problem.

Most injection site reactions were mild and all classified as marked were on Farm E. This may have been due to observer bias as each farm was examined by a local veterinarian. The only other large-scale trial of the Bovilis Neoguard vaccine reported no

injection site reactions or other adverse effects such as decreased milk yield or behavioural changes in the cows (Romero et al. 2004).

Most, but not all, seronegative animals became seropositive following vaccination. In previous trials, Choromanski and Block (2000) reported that all 15 vaccinated heifers developed IFAT titres ≥ 1:320 following vaccination while Barling et al. (2003) found that 23 of 30 steers seroconverted following vaccination. It is not known whether a failure to seroconvert is a sign of vaccine failure but the vaccine was administered by veterinarians according to manufacturer's directions. Occasionally, an infected animal fails to develop antibodies to *N. caninum* (De Marez et al. 1999) so we can not conclude that the animals that remained seronegative following vaccination were not antigenically challenged. Seroconversion in control animals suggested that ongoing horizontal transmission was occurring on the farms, that there had been a recrudescence of infection in these cows that increased antibody levels to a point at which they were classified as seropositive or that the IFAT cut-point used to determine infection was too high. Different serological cutpoints have been used in IFAT measurement of *N. caninum* antibodies according to the reason for testing and the species involved. A comparison of IFAT serology is further complicated by differences in equipment and individual interpretation between laboratories (Björkman and Uggla 1999). Dubey and Lindsay (1996) recommend that for adult cattle a dilution of 1:200 be used and this was the cut-point used to determine infection status in this trial. Fluctuations in *N. caninum* antibody levels, particularly during pregnancy, have been reported (Paré et al. 1997) so it is not possible to determine an animal's true infection status from a single serum sample. It is possible that some cows in the control group that were initially classified as seronegative and were subsequently found to be seropositive were misclassified due to fluctuating antibody levels and hence initial seroprevalence to *N. caninum* on the farms was underestimated. This is supported by the magnitude of titre change in control animals which was lower than in the vaccinated animals (Figure 4.1) suggesting that, in most cases, IFAT titres were just below the cut-off of 1:200.

The risk of abortion among seropositive animals in this trial was 3.6 times greater than in seronegative animals and this is comparable to other studies (Thurmond and Hietala 1997a; Wouda et al. 1998a). The relative risk of abortion in seropositive animals was higher in heifers than cows and others have reported increasing parity to be protective for

both abortion and vertical transmission in herds with endemic abortion associated with *N. caninum* (Anderson et al. 1995; López-Gatius et al. 2005a).

Vaccination appeared to be significantly protective across all farms in the 6th and 7th months after first vaccination (Figure 4.2). At expected calving however, the proportion of animals that were found to be non-pregnant was greater in the vaccinated group than in the control group. This finding negated the apparent protection in the earlier months. It is expected that an abortion would be detected in the later stages of gestation as the more advanced and larger fetus is more likely to be expelled and noticed by farm staff. *N. caninum* mainly causes abortion between 3 and 8 months with a mean occurrence at 5.5 months of gestation (Dubey et al. 2006). Fetuses dying before 5 months gestation are more likely to be mummified and remain in the uterus for some months (McAllister et al. 1996b) and early losses may simply be resorbed (Williams et al. 2000). Therefore it is likely that the abortions that were not immediately detected occurred early in gestation. Abortion was detected in some cases when cows displayed oestrous behaviour but, in a seasonal calving system, oestrous detection is not ongoing throughout the year and some cases of abortion were only detected when cows failed to calve. Antigenic stimulation from the vaccine may upset the balance of the host-parasite interaction by stimulating a Th-1 type response with the pro-inflammatory cytokines interleukin (IL)-2, IL-12 and gamma interferon (IFN-γ) potentially damaging the fetus and the maintenance of pregnancy (Innes et al. 2002).

Neospora caninum has exceedingly efficient transplacental transmission of up to 95% (Davison et al. 1999a) which may occur over several generations and in successive pregnancies (Björkman et al. 1996). Attempts to control *N. caninum* infection in a herd must therefore target this route of infection. Vaccine developed against bovine neosporosis should protect against abortion as well as preventing vertical transmission. Neoguard did not have a claim to prevent vertical transmission although one report suggested that it may do so (Choromanski and Shawnee 2002). The present study has shown that vaccination with Neoguard at 2-3 months of gestation did not prevent vertical transmission, in fact there was a trend for seropositive cows that were vaccinated to be more likely to have calves that were seropositive ($p = 0.05$). It has been reported that maternal antibody from ingesting pooled colostrum wanes by 128 days of age (Hietala and Thurmond 1999) so the antibody detected in the calves in this study is not from passive

transfer but is from antibody produced by the calf itself. Most of the seropositive calves born to seropositive cows thus represent vertical transmission, although the observation that some calves that were born from seronegative cows had become seropositive by 6-9 months of age indicates that horizontal transmission may have been occurring on the farms or that some cows were initially misclassified as seronegative. Romero and Frankena (2003) calculated an AF of 0.64 for vertical transmission (i.e. the probability of a calf being seropositive due to vertical transmission) from serological results for 747 dam-daughter pairs in a multi-farm study where the probability of horizontal infection was 0.22. A higher AF was calculated in the calves from cows involved in the present study with vaccination increasing the risk of vertical transmission.

A clinical trial involving a 250 cow Holstein-Friesian herd in North America reported preliminary results that Neoguard reduced the incidence of *Neospora*-associated abortion but the level of efficacy and significance was not reported (Estill 2004). A large scale trial of the same vaccine in Costa Rica reported a protective effect of vaccination between days 150 and 200 of gestation. Given the very high level of abortion in the herds (20.8% in the control group) this was sufficient to achieve an overall vaccine efficacy of 0.49 (Romero et al. 2004). However, as is expected in clinical trials with abortion as an outcome, the effect was farm-specific with a positive effect in 15 herds, no effect in 4 herds and a small negative effect in 6 herds. The farm-specific effect observed in the current New Zealand trial is therefore not unexpected and further research should be undertaken to identify factors which indicate that vaccination may be cost-effective on certain farms. Further clinical trials with *Neospora* vaccines are required in settings where aborted material is more likely to be collected and where calves are sampled pre-colostrally for antibodies to *N. caninum* to assess efficacy in preventing vertical transmission.

4.6 Conclusion

This large-scale clinical trial of Bovilis Neoguard on 5 commercial dairy farms in New Zealand showed a significant effect of vaccination on reducing the occurrence of abortion on one of five farms. While the vaccine provided 61% efficacy on this farm, there were insufficient cases of abortion on 3 other farms to demonstrate a significant effect and the remaining farm (E) had more than 8% abortions with no difference between the vaccinated and the control group. The low incidence of abortion on 3 farms may relate to the lower

than expected seroprevalence to *N. caninum*. Despite extensive sampling, it was not possible to elucidate the cause of abortion in most cases. Assessment of the serostatus of the calves born to the cows enrolled in this clinical trial showed that the vaccine did not prevent vertical transmission.

4.7 Acknowledgements

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Conflict of Interest Statement

None of the authors have any financial or personal relationship with other people or organisations that could have inappropriately influenced this work.

4.8 Authors' contributions to this study

C Heuer secured the contract for the trial and contributed to the trial design and analysis, helped with sample collection and provided editorial advice.

NB Williamson provided editorial advice.

JF Weston assisted with trial design and sample collection, maintained the database and assisted with statistical analysis and wrote the manuscript.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

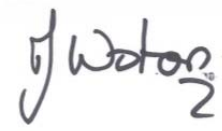
Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Efficacy of a *Neospora caninum* killed tachyzoite vaccine in preventing abortion and vertical transmission in cattle

In which Chapter is the Published Work: Four

What percentage of the Published Work was contributed by the candidate: 50%.



29th August 2011

Candidate's Signature

2nd September 2011

Principal Supervisor's signature

Chapter Five

Causes of abortion on New Zealand dairy farms with a history of abortion associated with *Neospora caninum*

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5.0 Preface

Thorough diagnostic investigations were carried out on cases of abortion among cows enrolled in a clinical trial of Bovilis Neoguard. This involved testing of maternal serology for antibodies to *Neospora caninum* and bovine viral diarrhoea virus and comparing this to antibody status at enrolment as well as histopathological examination of the fetus and placenta when these were available. In addition, cattle that conceived later in the mating period in these seasonally calving herds and which were excluded from enrolment in the clinical trial were monitored and those that aborted were investigated. This study was undertaken to assess whether abortions on these farms with a history of *Neospora*-associated abortion were due to *N. caninum* infection or whether other causes of abortion were occurring; in which case stimulating immunity against *N. caninum* was likely to be of little use.

5.1 Abstract

AIM: To investigate the cause of abortion in cows from farms with a history of abortion associated with *Neospora caninum*.

METHODS: Cows and primiparous heifers (n = 2,246) that were enrolled in a clinical trial of a vaccine for *N. caninum,* and a further 403 cows and heifers that conceived > 6 weeks after the planned start of mating (PSM), were monitored for abortion on five dairy farms with a history of abortion associated with *N. caninum*. When abortion was detected, the fetus and/or placenta were submitted for histopathological examination and maternal paired sera were collected for the detection of antibodies to *N. caninum* using the indirect fluorescent antibody test (IFAT), bovine viral diarrhoea virus (BVDV) using ELISA, and *Leptospira* spp. using the microscopic agglutination test (MAT).

RESULTS: At the start of the clinical trial, 189/1,760 (10.7%) of cows and 61/486 (12.6%) of heifers had IFAT titres ≥1:200 for *N. caninum*. Abortions were detected in 111 (4.9%) enrolled and 37 (9.2%) non-enrolled cows and heifers. The relative risk of abortion was 4.21 (95% CI 2.92-6.08) times greater in enrolled animals that were seropositive to *N. caninum* than seronegative animals (p<0.01). Among all animals, histopathological lesions suggestive of protozoal abortion were seen in nine, and of bacterial infection in 15, of the 40 cases where the fetsus and/or placenta were recovered. IFAT titres for *N. caninum* ≥1:1,000: were detected in 66 cows at abortion; including 7/9 cases where there were fetal lesions indicative of *N. caninum* infection. In nine cows that aborted, there was evidence of

seroconversion to BVDV and eight cows had MAT titres for *Leptospira* spp. ≥1:200. Histopathology revealed dual infectious aetiologies in two cases and, in another 17 cases, there was serological evidence of recent exposure to a second infectious agent capable of causing abortion in conjunction with *N. caninum* lesions in the fetus or fetal bacteraemia.

CONCLUSIONS: Multiple infectious aetiologies occur at herd-level and in individual cows on farms with a history of abortion associated with *N. caninum*. A diagnosis of abortion due to *N. caninum* infection was made in 9/34 (26.5%) cases where fetal histopathology was undertaken.

CLINICAL RELEVANCE: Thorough and ongoing diagnostic investigations should be undertaken in herds with higher than expected abortion losses to identify all causative agents as control measures could be implemented that may also reduce the risk of abortion in cows infected with *N. caninum*.

5.2 Introduction

Pregnancy losses may be due to genetic factors, hormonal dysfunction, an abnormal uterine environment, nutritional factors, toxic substances, trauma, heat stress, infectious agents and other conditions that trigger a systemic inflammatory response (Bulman 1979; López-Gatius et al. 1996; Santos et al. 2004a; Santos et al. 2004b). The expected abortion rate, determines the point at which abortions are investigated. It depends on the stage of pregnancy at which confirmation takes place (McDougall et al. 2005), what is considered "normal loss" by the farmer and veterinarian, and their previous experience of achieving a diagnosis (Kinsel 2002). Attempts should be made to achieve an aetiological diagnosis of bovine abortion, as it may be possible to prevent further losses. However, an aetiological diagnosis is made in only approximately 50% of cases even when the entire fetus is submitted to a veterinary diagnostic laboratory (Thornton 1996; Anderson 2007; Cabell 2007). The ability to reach a diagnosis is compromised when a full range of samples is not submitted.

The relative frequencies of the pathogens diagnosed as causing bovine abortion have altered over the past 40 years (e.g. United States: Anderson 2007; New Zealand: Anonymous 1974; Thornton 1996). Some have been eradicated (e.g. *Brucella abortus*) or can be controlled by vaccination (e.g. *Leptospira* spp. and bovine viral diarrhoea virus [BVDV]), whilst others such as *Neospora caninum* have become recognised. The change in reported

frequency reflects true changes in prevalence of causal agents, refinements in diagnostic techniques and submissions to veterinary diagnostic laboratories.

Neospora caninum has become one of the most commonly diagnosed infectious causes of abortion in many countries (Dubey et al. 2007) since its recognition as a cause of abortion in cattle (Thilsted and Dubey 1989) and has been associated with epidemic and endemic abortion patterns (Moen et al. 1998). Maternal serology provides evidence for infection with *N. caninum*, although infected cows commonly give birth to healthy, congenitally-infected calves (Anderson et al. 1997). Consequently, the presence of antibody is not sufficient to establish that *N. caninum* was the cause of abortion, and a combination of appropriate gestational age, the presence of compatible disseminated inflammatory lesions in the fetus, the presence of parasites in the fetus and the absence of other abortifacient agents are required to confirm the diagnosis (Anderson et al. 2000). Alternatively, a seroepidemiological approach, comparing serological status of aborting *vs* non-aborting cows can be applied (Thurmond and Hietala 1995).

Once a diagnosis of *N. caninum*-associated abortion is made on a farm experiencing an abortion epidemic, further abortions in that and subsequent seasons are frequently attributed to neosporosis (Dubey and Lindsay 1996), as abortion outbreaks are well documented and there is ongoing vertical transmission with an increased risk of abortion in congenitally-infected animals (Weston et al. 2005). However, multiple infectious or non-infectious factors may be involved at both the farm- and cow-level (Yildiz et al. 2009). Immunosuppressive factors such as mycotoxins and BVDV may increase the risk of abortion in cows infected with *N. caninum* (Bartels et al. 1999; Björkman et al. 2000), as well as causing abortion in their own right. Identification and mitigation of other causes should reduce the incidence of abortion. Currently, there are few practical options to prevent *N. caninum* infection or abortions (Dubey et al. 2007) and long-term strategies to reduce the proportion of seropositive animals in a herd may not be economically viable (Reichel and Ellis 2006). Other causative and exacerbating factors may be controlled more easily.

The present study was undertaken to establish the causes of abortion among cows from 5 seasonally-calving dairy herds in New Zealand that had a history of abortion associated with *N. caninum* over at least 2 years. Most of the cattle in this study were enrolled in a clinical trial of a *N. caninum* killed tachyzoite vaccine. Vaccine efficacy has already been

reported (Weston et al. 2012, Chapter Four in this thesis), this paper focuses on the aetiological diagnosis of the abortions.

5.3 Material and Methods

5.3.1 Animals

Five commercial dairy farms in three regions of New Zealand (Taranaki, South Canterbury and Southland) were selected for the study due to their annual abortion incidence being >8% for the previous two years and a previous confirmation of the involvement of *N. caninum* infection in a substantial proportion of those abortions. Cows were predominantly Holstein-Friesian or Holstein-Friesian / Jersey crossbreds. All animals calved annually from late July to early October (late winter to early spring) and were bred between late October and the end of December. Cows grazed at pasture and were supplemented with grass silage or maize silage at times during lactation, and with hay during the winter (dry period). Home-bred, rising 2-year-old replacement heifers that were grazed on the farm or at a nearby property were included in the study. All cattle had been vaccinated against leptospirosis [serovars Hardjo-bovis and Pomona (Leptavoid 2, Schering Plough Animal Health, Upper Hutt, NZ) or serovars Hardjo-bovis, Pomona and Copenhageni (Lepto-3-way, Virbac, Auckland, NZ)] with annual boosters administered mid-gestation (March - May). Cows on one farm (Farm A) had also been vaccinated against BVDV (Bovilis BVD, Intervet International B.V., Boxmeer, The Netherlands).

5.3.2 Enrolment in the clinical trial

Cows and primiparous heifers that conceived during the first 6 weeks of the mating period were identified by transrectal ultrasonography at 8 and 12 weeks after the start of mating for each herd. These animals were enrolled in a clinical trial of a *N. caninum* vaccine (Bovilis Neoguard; Intervet International B.V., Boxmeer, The Netherlands); consisting of inactivated *N. caninum* tachyzoites and Havlogen adjuvant. The animals were 30 - 60 days pregnant at the time of enrolment and were allocated to vaccinated (n = 888 cows and 245 heifers) and control (n = 874 cows and 241 heifers) groups by systematic random allocation. The vaccination protocol followed the manufacturer's instructions; namely a 5 mL dose by subcutaneous injection, repeated after 4 weeks, with both vaccinations given within the first trimester of pregnancy. The control group received two doses of 5 mL of

0.9% sodium chloride solution (Baxter Healthcare Pty. Ltd., NSW, Australia) subcutaneously, at the same stages of gestation.

Serum was collected by caudal venepuncture into plain glass tubes (Vacutainer; Becton Dickinson and Company, Plymouth, UK) at the time of enrolment, to measure antibodies to *N. caninum* by indirect fluorescent antibody test (IFAT; VMRD Inc., Pullman, Washington, USA). Initial dilution was at 1:100 and positive samples were then serially diluted two-fold to determine a fluorescence end-point. An IFAT titre of 1:200 or higher was considered positive. Sera were also tested for antibodies to BVDV by ELISA (Institut Pourquier, Montpellier, France) with results interpreted according to the manufacturer's instructions, namely positive results had ≤40% inhibition and suspicious results had 40 - 50% inhibition.

Approximately 6 weeks after the second vaccination, serum was collected from 317 cows and 100 heifers that were randomly selected across the five farms and both treatment groups to measure IFAT titres for *N. caninum*.

A further whole herd pregnancy test was carried out 6 weeks after the end of the mating period to confirm pregnancy in the enrolled animals and to ascertain pregnancy status in those cows which conceived more than six weeks after the planned start of mating (PSM) which were not enrolled in the clinical trial. These cows and heifers (n = 403) were also observed for abortion. All procedures involving the experimental use of animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand (MUAEC 01/5).

5.3.3 Abortion investigation

For the purposes of this trial, abortion was defined as (a) the observed expulsion of a fetus and/or placenta at least 10 days prior to expected calving date, (b) failure of an animal that had been confirmed pregnant to calve, or (c) loss of a pregnancy between the first and second ultrasonographic examinations.

When an abortion was detected the fetus and placenta were collected, if available, and submitted to a veterinary diagnostic laboratory (Gribbles Veterinary Pathology, Palmerston North, New Zealand) for examination. Samples of brain, myocardium, liver, lung and

placenta were collected and fixed in 10% formol saline. These tissues were then processed by routine paraffin wax embedding, sectioned and stained with H&E for histopathological examination. Serum was collected from aborting cows at the time abortion was detected and, where possible, approximately four weeks later. Sera were tested for antibodies to *N. caninum* by IFAT, BVDV by ELISA and *Leptospira* spp. (serovars Pomona and Hardjo-bovis) by microscopic agglutination test (MAT; original cultures supplied by Environmental Science and Research, Wallaceville, Upper Hutt, New Zealand).

5.3.4 Statistical analysis

All animal information and diagnostic results from the cases of abortion was recorded and analysed using pivot tables in Microsoft Excel 2000 (Microsoft Corporation, Seattle WA, USA). Differences between the groups were tested for significance using Fisher's exact test (www.graphpad.com/quickcalcs/contingency1.cfm) or the Chi-square test, as appropriate, and the relative risk of abortion, with 95% CI, calculated.

5.4 Results

5.4.1 Animals

The clinical trial included 1,760 cows and 486 heifers, the details of which are reported in Table 5.1. Serological testing revealed that 10.7% of cows and 12.6% of heifers were seropositive to *N. caninum* at the start of the trial. Farms A, C and E had a higher proportion of seropositive animals (11.7, 20.5 and 13.9% of cows and 18.3, 16.1 and 19.0% of heifers, respectively) than Farms B and D (7.7% and 4.9% of cows, and 3.4% and 9.2% of heifers, respectively). There was variable incidence of exposure to BVDV across the farms, with 18.8 – 74.3% of cows and 0 - 53.8% of heifers being positive or suspicious for BVDV antibody.

Table 5.1 Number of animals enrolled in a clinical trial of Bovilis Neoguard (Intervet International B.V., Boxmeer, The Netherlands) from 5 farms in New Zealand with a history of *Neospora caninum*-associated abortion where cause of abortion was investigated and the percentage that were initially seropositive to *N. caninum* (IFAT[a] titre ≥ 1:200).

Farm	A	B	C	D	E	All farms
Cows seropositive to *N. caninum*	26 / 222 (11.7%)	28 / 362 (7.7%)	54 / 263 (20.5%)	25 / 509 (4.9%)	56 / 404 (13.9%)	189 / 1760 (10.7%)
Heifers seropositive to *N. caninum*	11 / 60 (18.3%)	2 / 58 (3.4%)	10 / 62 (16.1%)	19 / 206 (9.2%)	19 / 100 (19.0%)	61 / 486 (12.6%)
Seropositive animals that aborted	3 / 37 (8.1%)	4 / 30 (13.3%)	16 / 70 (22.9%)	0 / 44	16 / 75 (21.3%)	39 / 256 (15.2%)
Seronegative animals that aborted	5 / 245 (2.0%)	9 / 390 (2.3%)	14 / 255 (5.5%)	17 / 671 (2.5%)	27 / 429 (6.3%)	72 / 1990 (3.6%)
Relative risk of abortion among seropositive animals	3.97 (0.99 – 15.94)	5.78 (1.89 – 17.67)	4.16 (2.14 – 8.11)	0	3.39 (1.92 – 5.98)	4.21 (2.92 – 6.08)

[a] Indirect fluorescent antibody test (IFAT; VMRD Inc., Pullman, WA, USA)

After vaccination with Neoguard, 140 / 166 (84.3%) cows that were initially seronegative became seropositive to *N. caninum*, the median increase in titre being 3 dilutions (min -1, max 6). Amongst heifers, 48 / 66 (72.7%) seroconverted following vaccination with the median increase in titre being 3 dilutions (min -2, max 5). In the control group, 11 / 130 (8.5%) cows and 10 / 36 (27.8%) heifers that were seronegative at the time of enrolment became seropositive in the 10 weeks between blood sample collections, the seroconversion rate in heifers being higher than in cows ($p = 0.004$). The median increase in titre in control cows was 0 dilutions (min -1, max 4) and for heifers was 1 dilution (min -3, max 3).

5.4.2 Abortion Investigation

The number of abortions that were detected at occurrence and the number of fetuses and placentas collected are summarized in Table 5.2. Abortion occurred in 48/1,131 (4.2%) animals in the vaccinated group and 63 / 1,115 (5.7%) in the control group. More enrolled animals aborted on Farms C and E (9.2% and 8.5% respectively) than on Farms A, B and D (2.8%, 3.1% and 2.4% respectively). Animals that were seropositive to *N. caninum* at the time of enrolment had a relative risk of abortion 4.21 (95% CI 2.92–6.08) times greater than seronegative animals ($p < 0.01$), but this risk varied between farms (Table 5.1). When further categorised by age, the relative risk of abortion was 3.53 (95% CI 2.33 – 5.34; $p < 0.01$) for seropositive *vs* seronegative cows and 12.77 (95% CI 4.90 – 33.29; $p < 0.01$) for heifers.

Serological and histopathological results from the enrolled animals that aborted are summarised in Table 5.3. There were 31 cases of abortion from which the fetus and maternal serology were collected; however eight fetsuses were mummified and/or severely autolysed which limited the ability to make a diagnosis. Histopathological lesions characteristic of *N. caninum* infection were seen in the fetus and, in some cases, the placenta in three vaccinated cows (one each from Farms A, C and E) and six cows in the control group (one from Farm B, two from Farm C and three from Farm E). Lesions included multifocal leucomalacia, granulomatous myocarditis, chronic multifocal encephalitis, neuropil degeneration with dense areas of microglial cells, multifocal regions of necrosis in the cotyledon and aggregations of macrophages and lymphocytes in portal areas of the liver.

Table 5.2 Details of the cases of abortion in cows and heifers enrolled in a clinical trial of Bovilis Neoguard (Intervet International B.V., Boxmeer, The Netherlands) from 5 farms in New Zealand with a history of *Neospora caninum*-associated abortion from which the cause of abortion was investigated.

	Group		
	Vaccinated	Control	Non-enrolled
Enrolled animals	1131	1115	403
Total cases of abortion	48	63	37
Abortions detected at the time of occurrence	28	44	14
Fetus only collected	8	13	2
Placenta only collected	3	3	0
Fetus and placenta collected	4	6	1

Table 5.3 Diagnostic findings from 111 cases of abortion among 2246 cows and primiparous heifers enrolled in a clinical trial of Bovilis Neoguard (Intervet International B.V., Boxmeer, The Netherlands) from 5 commercial dairy farms with a history of *Neospora caninum*-associated abortion.

	N. caninum status	*n*	Antibodies to *Leptospira* spp. (MAT)[a]	Other bacterial infection	Seroconversion to BVDV[b]	No other relevant findings
Vaccinated	Lesions[c]	3	1	0	0	2
	IFAT titre ≥1:1000	25	2	3	3	17
	IFAT titre <1:1000	20	1	1	3	15
Control	Lesions[c]	6	0	2	0	4
	IFAT ≥1:1000	24	4	6	1	13
	IFAT <1:1000	33	0	3	2	28
Total		111	8	15	9	79

[a] *Leptospira interrogans Pomona and Leptospira borgpetersenii Hardjo-bovis* by microscopic agglutination test (MAT; original cultures supplied by Environmental Science and Research, Wallaceville, Upper Hutt, New Zealand), titre ≥ 1:200

[b] ELISA (Institut Pourquier, Montpellier, France)

[c] Histopathological lesions in the fetus consistent with *Neospora caninum* infection, all except 2 of these cases also had an IFAT titre ≥1:1000

A diagnosis of abortion due to *Neospora caninum* infection was made in 9 / 31 cases (0.29; 95% CI 0.13 – 0.45) in which the entire fetus had been submitted to the veterinary diagnostic laboratory.

Serological results and estimated gestational age of the nine abortions with histopathological lesions suggestive of *N. caninum* are shown in Table 5.4. Of these nine cases, all except two had IFAT titres for *N. caninum* ≥1:1,000 at, or just after, abortion. Additionally, one vaccinated cow in which fetal granulomatous myocarditis was attributed to *N. caninum* infection, had serial IFAT titres of 1:100 and a MAT titre ≥1:1,600 against *Leptospira* serovar Pomona when sampled after aborting (>4 months after the cows had received their leptospirosis annual vaccination). One cow from the control group in which the fetus had a multifocal granulomatous pericarditis had IFAT titres of 0 and then 1:100 within a month of aborting. Bradyzoite tissue cysts were not seen in any of the samples. In addition to histopathological lesions consistent with protozoal infection, bacterial placentitis was diagnosed in two cases. One of these cases had areas of malacia of the neuropil, paired maternal sera with an IFAT of 1:2,000 and then 1:4,000 and a placentitis with moderate numbers of Gram positive bacteria present. The other case had multifocal areas of cotyledonary necrosis and a chronic granulomatous myocarditis, as well as a heavy mixed growth from fetal stomach contents.

A further 49 cases of abortion had maternal IFAT titres for *N. caninum* ≥1:1,000 at, or just after, aborting with 19 of these cases also having serological or histopathological evidence of the presence of other infectious agents capable of causing abortion (Table 5.3). In the 44 cases of abortion with a positive IFAT titre (≥1:200) after abortion where paired maternal serology was tested, 15 cases had an increase in titre (median = 2 dilutions, range 1 – 4), 18 cases had a decrease in titre (median = 2 dilutions, range 1 – 3) and there was no change in the remaining 11 cases. There were 43 cases of abortion in cows where IFAT titres were <1:1,000 and no other aetiological diagnosis was able to be made. Among the vaccinated cows in this category 5 had an IFAT titre of 1:600 when sampled, 6 had a titre of 1:200 and 4 had negative titres (0 or 1:100). Among the 28 control cows 3 had a titre of 1:600, 5 had a titre of 1:200 and the remaining 20 were consistently seronegative.

Table 5.4 Maternal IFAT[a] titre to *Neospora caninum* and estimated stage of gestation at which abortion occurred in 9 cases of abortion with histopathological evidence of *N. caninum* infection from cows and primiparous heifers enrolled in a clinical trial of Bovilis Neoguard (Intervet International B.V., Boxmeer, The Netherlands). Cows had serum collected to measure antibodies to *N. caninum* at enrolment in the clinical trial (Day 30 - 60 gestation; Pre), at the time of abortion (Post1) and 4 weeks later (Post2).

Group	Farm	Pre	Estimated stage of gestation	Post1	Post2
Vaccinated	A	1:2,000	118 d	1:2,000	1:2,000
Control	B	0	176 d	0	1:100
Control	C	1:200	210 d	1:1,000	1:1,000
Control	C	1:200	187 d	1:4,000	1:2,000
Vaccinated	C	0	180 d	1:2,000	1:4,000
Vaccinated	E	0	165 d	1:100	1:100
Control	E	0	170 d	1:600	1:4,000
Control	E	1:200	170 d	1:4,000	1:2,000
Control	E	1:200	180 d	1:8,000	na

Among the aborting cows with no evidence of *N. caninum* infection (IFAT titre <1:200) the sole finding was histological evidence of bacterial infection of the fetus and/or placenta (3 animals), seroconversion of the dam to BVDV between enrolment and the detection of abortion (3 animals), and one case with histological evidence of bacterial placentitis and a MAT titre to *Leptospira* serovar Pomona in the dam of ≥1:1,600 at the time of abortion, at least 4 months since leptospirosis vaccination.

In total, there were 15 cases in which bacterial infection of the fetus and/or placenta was recognised. Bacterial species present were: *Arcanobacter pyogenes* (1 animal), coliforms and alpha haemolytic *Streptococci* (1 animal), Gram positive rods (2 animals), Gram positive cocci (4 animals) and seven cases in which the bacteria were not identified.

[a] Indirect fluorescent antibody test (IFAT; VMRD Inc., Pullman, WA, USA)
NA Not available

5.4.3 Non-enrolled cows

Thirty-seven abortions (9.2%) occurred among the 403 cows and heifers that conceived >6 weeks after the PSM (one, five, 11, 16 and four cases on Farms A – E, respectively), which was more than among the enrolled cows ($p < 0.001$). At farm-level, the difference was significant only on Farm D on which 1.9% of vaccinated cows, 2.8% of cows in the control group and 10.9% of non-enrolled cows aborted ($p < 0.001$).

Of the 37 abortions, 14 (38%) were detected at the time of abortion. Blood samples were collected from these 14 cases whilst the fetus was collected from two cows and both fetus and placenta from another cow. Ten aborting cows had IFAT titres ≥1:1,000 for *N. caninum* (one, one, six, zero and two from Farms A – E, respectively), one of which also had mycotic placentitis. A further six cows were seropositive for *N. caninum* (IFAT titre of 1:200 or 1:600), comprising four cows from Farm B and one each from Farms C and D. No diagnosis was made in the remaining 21 cases.

5.4.4 Cause of abortion by farm

A summary of abortion aetiology by farm is presented in Table 5.5. When the results from the enrolled and non-enrolled cows were combined, abortion associated with *N. caninum* was suspected on all farms except Farm D, diagnosed by either histopathological lesions consistent with protozoal infection or IFAT titres ≥1:1,000. At least one (and no more than three) aborting cows on each farm had seroconverted to BVDV during the course of the trial. Fetal bacteraemia and/or placentitis was observed on all farms except Farm B. Positive titres to *Leptospira* spp. were detected on two farms. No diagnosis was able to be inferred in a total of 65 cases. Some cases of abortion had evidence of more than one aetiology, so the sum of the numbers in each category exceeds the total number of abortions on each farm.

Table 5.5 Diagnostic findings from cases of abortion among cows (*n* = 2,125) and primiparous heifers (*n* = 524) from five farms with a history of *Neospora caninum*-associated abortion where 1,760 cows and 486 heifers were enrolled in a clinical trial of Bovilis Neoguard (Intervet International B.V., Boxmeer, The Netherlands).

Farm	A	B	C	D	E	All farms
Histopathological lesions consistent with *N. caninum* infection	1	1	3	0	4	9
IFAT titre to *N. caninum* of at least 1:1,000	4	6	18	0	29	57
Fetal bacteraemia and/or placentitis	1	1	5	1	7	15
BVDV[a]	1	2	2	3	2	10
Positive MAT titre to *Leptospira* spp.[b]	0	0	2	0	6	8
Mycotic abortion	0	0	0	0	1	1
No significant findings	3	10	18	29	10	70
Total abortions[c]	9	18	41	33	47	148

Despite the fact that few abortions could be confirmed histopathologically as being due to *N. caninum* infection, seroprevalence to *N. caninum* among the aborting cows (range 0 – 0.53, average 0.35) was significantly higher ($p < 0.05$) than among the non-aborting cows (range 0.04 – 0.14, average 0.09) on all farms except Farm D.

[a] Cow seroconverted between 6-8 weeks gestation and abortion
[b] MAT titre to *Leptospira interrogans* serovar *Pomona* or *Leptospira borgpetersenii* serovar *Hardjo-bovis* of at least 1:200
[c] The sum of different aetiologies for each farm does not equal the total number of abortions as some cases had evidence of more than one possible aetiology

5.5 Discussion

The main finding of this study is that, in herds with a history of abortion associated with *N. caninum* infection, that organism was the most commonly diagnosed cause of abortion; yet in all of those herds, there were at least as many abortions that were associated with other infectious causes, or in which there was evidence of co-infection with other organisms as well as *N. caninum*. In addition, only 66 / 148 (0.45; 95% CI 0.37 – 0.53) cases of abortion had either histopathological or serological (IFAT titre ≥1:1,000) evidence of recent *N. caninum* exposure.

A diagnosis of abortion due to *Neospora caninum* infection was made in 9 / 34 cases, including non-enrolled cows, in which the fetus had been submitted to the veterinary diagnostic laboratory, and a definitive diagnosis in a further 14 of these cases. However, no fetus was available in 114 of the 148 cases of abortion, which limited the ability to reach a definitive diagnosis. Hence, a diagnosis could only be inferred from maternal serology in the remaining cases. Abortion was not detected at occurrence in 62 cases, so sampling was delayed and maternal serology was less likely to provide useful information. Despite this, histopathological or serological evidence for the cause of abortion was elucidated in 78 cases of abortion; 23 of which had evidence of more than one infectious aetiology.

Serological testing of the dam to determine the cause of abortion due to infectious agents is commonly undertaken, as this may be the only sample available. Problems with this approach include determining whether the infection is recent or of significance and, in some cases, whether an increase in titre may be due to vaccination. Testing of paired sera may be useful in some circumstances but, following infection with *N. caninum*, maternal seroconversion usually precedes abortion and the titre may be declining rather than rising after abortion (Anderson 2007). IFAT titres in aborting cows in the present study were increasing in 12 / 44 cases but decreasing or staying the same in the majority of cases.

To help address this problem, an avidity ELISA can be used to determine whether infection is recent, as the first antibodies synthesised after exposure to an antigen have a lower affinity for the antigen than those produced later (Jenkins et al. 2000). With this technique, antibodies are allowed to bind, but low affinity antibodies are then eluted by incubation with urea whilst the high-avidity antibodies remain bound. The titres obtained with and without incubation with urea are then calculated to assess whether infection is

recent or chronic (Björkman et al. 2003). However, the avidity ELISA does not discriminate between a *de novo* infection with *N. caninum* and recrudescence of infection (Aguado-Martínez et al. 2005).

In the present study, serum had been collected from the animals at the time of enrolment in the clinical trial and antibody status to *N. caninum* and BVDV was known and could be compared to results at the time of abortion. Vaccination with killed *N. caninum* tachyzoites provoked an antibody response in most animals, which further complicated the interpretation of *N. caninum* serology. The development of marker vaccines with serological tests that distinguish the antibody response between vaccinated and naturally infected cattle would be useful. In the present study, a cut-off in the IFAT titre of 1:1,000 was used for a serological diagnosis of *N. caninum*-associated abortion, although this may have overestimated the effect of *N. caninum* among vaccinated animals. When reliance is placed on serology alone to establish a likely cause in cases of bovine abortion it is essential to compare the results of aborting cows *vs* pregnant non-aborting cows in the herd (Thurmond and Hietala 1995) as many cows with evidence of previous infection with these pathogens do not abort.

The IFAT is regarded as providing a definitive serological diagnosis of neosporosis in cattle and dogs (Dubey and Lindsay 1996). However, the interpretation and comparison of serological results with IFAT techniques varies, as the result reported is largely dependent on both the quality of the equipment used for fluorescence microscopy and different individuals' visual interpretations (Dubey and Schares 2006). This makes comparison of IFAT results between different laboratories difficult and cut-offs that are reported in the literature may not be applicable to each laboratory. For example, an IFAT titre of 1:640 has been reported for the diagnosis of abortion due to *N. caninum* infection (Conrad et al. 1993; Otter et al. 1997a). Conversely, Schares et al. (1999b) calculated that an IFAT titre of ≥1:1,000 had a negative predictive value (NPV) of 0.97 and a positive predictive value (PPV) of 0.46 for diagnosing abortion due to *N. caninum*, whilst an IFAT titre of ≥1:2,000 had a NPV of 0.93 and a PPV of 0.50. Different cut-offs in serological tests are also recommended for detecting infection with *N. caninum* according to age, species, gender and stage of gestation (Hemphill 1999; Álvarez-García et al. 2003; Wapenaar et al. 2007). One case of abortion in the present study illustrates the problems with setting a serological cut-off to diagnose abortion due to *N. caninum*: inasmuch as it had fetal lesions that were

consistent with *N. caninum* infection and a maternal IFAT titre of 1:600 at abortion which then rose to 1:4,000 (Table 5.4).

Farms A, B and D had a relatively low seroprevalence to *N. caninum* and reported fewer abortions than did Farms C and E. On Farm D, none of the seropositive cows aborted and *N. caninum* did not appear to be involved in any of the 33 cases of abortion. One case of bacterial abortion was diagnosed and a presumptive diagnosis of abortion due to BVDV infection was made in a further 3 cases based on maternal seroconversion. It is likely that given the previous history of this farm, samples from these aborting cows would not have been tested if they were not participating in this clinical trial, and these cases would have been incorrectly assumed to be due to *N. caninum* infection.

Farms C and E had the highest herd seroprevalences to *N. caninum* and the highest abortion incidences, 9.2% and 8.5% respectively; a relationship that has been reported by many other authors (e.g. Paré et al. 1998; Bartels et al. 1999; Schares et al. 2004b). *Neospora caninum* was implicated in 21 / 41 cases on Farm C and 33 / 47 cases on Farm E, but there was also evidence of recent bacterial infections (including leptospirosis; Table 5.5). Farms C and E also had the highest incidence of abortion among animals that were initially seronegative to *N. caninum* and 11 / 41 non-vaccinated animals that aborted were found to have seroconverted. Together, these findings suggested that ongoing horizontal transmission was occurring. Alternatively, it could be merely that the cut-off in the IFAT (1:200) was too high to detect all infected animals, as titres are known to fluctuate (Conrad et al. 1993; Stenlund et al. 1999).

Despite the history of abortion associated with *N. caninum* on the properties, only nine cases were confirmed as having histopathological lesions consistent with *N. caninum* infection. There were a further five cases (two among non-vaccinated animals) in which no significant lesions were seen in fetal brain yet maternal IFAT at abortion was ≥1:1,000. Furthermore, two cases (one from a vaccinated cow) with fetal lesions consistent with *N. caninum* infection had maternal IFAT titres at abortion and 4 weeks later of 0 or 1:100, suggesting that *N. caninum* was not the cause of abortion. A small-scale trial where seropositive pregnant cows were slaughtered at different stages of pregnancy showed that 4 / 8 fetuses had histopathologic lesions consistent with *N. caninum* infection and presence of positive immunoperoxidase staining (Thurmond et al. 1999). It was then calculated that the

positive predictive value for these combined tests ranged between 0.08 – 0.18 depending on estimates of sensitivity of the technique ranging between 0.46 and 1.0 i.e. a false positive rate of 82 – 92%. The fact that assessment of fetal histopathology is subjective, in conjunction with parasites being rarely seen in histological sections and immunohistochemistry or PCR not routinely carried out in commercial diagnostic laboratories to confirm the presence of *N. caninum* suggests that abortion due to neosporosis may be over-diagnosed (Jenkins et al. 2002).

The cases of bacterial abortion reported in this study were likely to be sporadic infections associated with a maternal bacteraemia, since the presence of bacteria was associated with histopathological lesions ruling out contamination of samples. *Bacillus licheniformis*, *Arcanobacterium pyogenes* and *Salmonella* species are the most commonly isolated bacteria in cases of bovine abortion in the UK, where abortion due to *Brucella abortus* and *Leptospira* spp. have largely been controlled (Cabell 2007) and similar findings might be expected in New Zealand. Hill (2011) recently reported in a non-refereed publication that bacterial abortion was commonly diagnosed in New Zealand with *Listeria monocytogenes*, *Bacillus cereus*, *Streptococcus uberis*, *Streptococcus bovis* and *Leptospira* spp. being most commonly reported, although *Bacillus licheniformis*, *Salmonella* spp., *Campylobacter jejuni*, *Arcanobacterium pyogenes*, *Escherichia coli* and *Fusobcaterium necrophorum* were also cultured.

The abortion incidence of 9.2% in the later-conceiving cows was significantly higher than in the vaccinated animals ($p < 0.001$) and in the control animals ($p = 0.014$). These cows were excluded from the clinical trial of the Neoguard vaccine due to the logistical difficulties involved in confirming their date of conception and ensuring that they received the vaccination at the correct stage of gestation and, hence, their IFAT status to *N. caninum* in early gestation was unknown. Cows that conceive later in the mating period under New Zealand dairy farming systems are commonly those that have had a shorter period between calving and the PSM, those that calved at lower body condition score and those that have lost more condition between calving and the start of mating (Macmillan 2002). It is also possible that these cows were still in a state of negative energy balance when they conceived: it has been shown that animals in negative energy balance have lower progesterone secretion which, in turn, reduces fetal survival (Spicer et al. 1990).

Pregnancy outcome in a *N. caninum* infected cow is affected by maternal and fetal immune responses as well as the complex interaction between host and parasite that, together, result in the death (or survival) of the fetus (Innes et al. 2005). Other environmental and infectious conditions that adversely affect the host's immune system have been identified as risk factors for *Neospora*-associated abortion. These include mycotoxins in feed (Bartels et al. 1999), climatic stress (López-Gatius et al. 2005a) and other infectious conditions (Thurmond and Hietala 1995) - particularly BVDV (Björkman et al. 2000; Hässig and Gottstein 2002). Serological evidence for active infection with other agents capable of causing abortion was seen on all five farms and may have influenced the maternal immune response in cows that were infected with *N. caninum*. It is well established, and supported by this trial, that cows that are seropositive to *N. caninum* have a significantly greater risk of aborting than their seronegative herd-mates. Whether *N. caninum* infection is solely responsible for this increased risk of abortion is unknown, since it may be that such animals are more likely to abort in the presence of another infectious or immunosuppressive agent, and that animals exposed to both *N. caninum* and another stressor experience worse reproductive outcomes than those that are exposed to a single risk factor.

The involvement of other infectious aetiologies on the farms was not unexpected, as abortion in cattle has multiple infectious and non-infectious causes. However, evidence for more than one aetiology occurring within an individual animal raises the question of the relative importance of the pathogens. Thurmond et al. (1997) reported multiple infectious aetiologies occurring on 20 farms that had previously experienced an abortion epidemic which was presumed to have been caused by *N. caninum*. They found serological evidence of recent infection with BVDV on three farms and leptospirosis on one other and concluded that the importance of *N. caninum* may be over-estimated when diagnosis during an abortion epidemic is based solely on the results of fetal histopathology. The results from the present study support this finding, with evidence of more than one infectious aetiology being present at both the individual-cow and at farm levels.

In conclusion, the role of *N. caninum* on farms with higher than expected abortion incidence may be over-estimated when the diagnosis is made solely on fetal histopathology from a limited number of cases. We recommend that thorough and ongoing diagnostic investigations be undertaken to identify all factors that may be implicated, as some may be

controlled more easily than *N. caninum* and the removal of other immunological stressors may decrease the incidence of abortion in cows that are infected with *N. caninum*.

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5.7 Authors' contributions to this study

NB Williamson provided editorial and analytical advice.

TJ Parkinson provided editorial advice as well as suggestions on how to present the results from the animals that were not enrolled in the clinical trial.

C Heuer obtained the initial contract to carry out the clinical trial, assisted with trial design and analysis and provided editorial advice.

JF Weston assisted with trial design, maintained the database, compiled and then analysed all diagnostic results, and wrote the manuscript.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

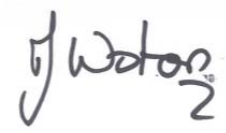
Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Cause of abortion on New Zealand dairy farms with a history of *Neospora caninum*-associated abortion

In which Chapter is the Published Work: Five

What percentage of the Published Work was contributed by the candidate: 80%.



Candidate's Signature

29th August 2011

Principal Supervisor's signature

2nd September 2011

Chapter Six

Dose-titration challenge of young pregnant sheep with *Neospora caninum* tachyzoites

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6.0 Preface

The variable efficacy of Bovilis Neoguard vaccine and ongoing interest in the development of an alternative vaccine led us to contract to work with AgVax Developments Ltd. (Upper Hutt, New Zealand) to develop a live tachyzoite vaccine that would be administered to cattle prior to mating. In preparation for carrying out challenge trials in cattle, young, pregnant sheep were inoculated with *Neospora caninum* tachyzoites to test a challenge model.

6.1 Abstract

Pregnant ewe lambs (7-8 months old at breeding) were inoculated intravenously at 90 days after joining with the ram with 50, 5 x 10^3, 10^6, or 10^8 *Neospora caninum* tachyzoites and outcomes were compared to a control group. Seroconversion was measured by indirect fluorescent antibody test (IFAT) at fortnightly intervals and by enzyme-linked immunosorbent assay (ELISA) at 28 days post-challenge. Seroconversion (by IFAT) occurred in all animals except for 2 sheep in the lowest dose group, including the 9 sheep in the control group. IFAT titres rose and fell rapidly, peaking at 1:800 and in 8 animals were negative within 7 days of abortion. ELISA results more closely reflected infection dose and outcome of pregnancy than IFAT serology did. All 10 ewes in each of the 2 highest dose groups aborted, 5 of 10 ewes in the mid-dose group aborted and no ewes in the lowest dose or control groups aborted. Histological lesions consistent with *N. caninum* infection were seen in the brains of all 25 aborted lambs, as well as in 2 live-born premature lambs from the group receiving 5 x 10^3 tachyzoites and 2 clinically normal lambs (one from the control group). Polymerase chain reaction (PCR) detected *N. caninum* DNA in a variety of tissues from lambs, aborted fetuses and dams including 3 of 9 ewe-lamb pairs from the control group. The results from this study showed a strong relationship between challenge dose of *N. caninum* tachyzoites, the sample to positive (S/P) percentage in an ELISA test 28 days after challenge and pregnancy outcome. IFAT results did not correlate well with the level of challenge or the outcome of pregnancy and their relevance in studies of this kind should be questioned.

6.2 Introduction

Neospora caninum, an apicomplexan parasite, was first described in 1984 associated with encephalitis and myositis in dogs in Norway (Bjerkås et al. 1984). It has since been recognised as the leading cause of bovine abortion in many countries including New Zealand (Thornton et al. 1991). The report of a naturally infected dog shedding *N. caninum* oocysts (McAllister et al. 1998) identified that dogs are definitive hosts but much current understanding of the life cycle is based on knowledge of similar organisms.

Natural congenital infection in a lamb was first reported in England (Dubey et al. 1990a) and subsequent experimental infections have shown that *N. caninum* infection of sheep has similar pathogenesis and outcomes to cattle, including repeated abortion and congenital infection (McAllister et al. 1996c; Buxton et al. 1997b; Buxton et al. 1998; Jolley et al. 1999). Thus, sheep may be a useful model for studying infection. Serological surveys report varying evidence for exposure among sheep populations but abortion associated with infection is not commonly reported (Otter et al. 1997b; Hässig et al. 2003; Masala et al. 2007).

Histopathological lesions due to *N. caninum* infection of the fetus and placenta are indistinguishable from those of *Toxoplasma gondii* (Buxton et al. 1997b). It was not until the advent of immunohistochemical and molecular methods for diagnosis that the two were distinguished (Lindsay and Dubey 1989; Müller et al. 1996; Harkins et al. 1998; Baszler et al. 1999b). Serology has also been used to distinguish between these two protozoal infections (Hässig et al. 2003) but a serological test for *N. caninum* infection in sheep has not been commercially available in New Zealand. Indirect fluorescent antibody testing (IFAT) is considered sensitive and specific for serological diagnosis in cattle (Slotved et al. 1999) and has been used in the absence of a gold standard to validate a variety of enzyme-linked immunosorbent assays (Schares et al. 1999a; von Blumröder et al. 2004; Wapenaar et al. 2007). IFAT has been used as a serological test in experimentally infected sheep (Dubey et al. 1996c; Buxton et al. 1998). *N. caninum* antibody levels fluctuate in cattle and at times may be below the usual cut-off values used in serological testing so false negative results occur (Conrad et al. 1993). False positives are also possible if fetal bovine serum in the cell culture medium has antibodies to *N. caninum*, a common occurrence (Torres and Ortega 2006). The sensitivity and specificity of serological tests of naturally-infected sheep is unknown.

Recently, West et al. (2006) suggested that *N. caninum* could be responsible for abortions in some flocks in New Zealand with aborting ewes being more likely to be seropositive than non-aborting ewes and fetuses being seropositive to *N. caninum*. Further examination of samples from these farms in subsequent years used PCR to distinguish between *N. caninum* and *T. gondii*. This confirmed that *N. caninum* DNA was present in whole blood and fetal tissues from some aborting animals (Howe et al. 2008) and found that PCR had greater diagnostic sensitivity than IFAT serology. Similar testing of samples from ovine and caprine abortion in Italy found 2% of ovine placentae were positive on PCR to *N. caninum* with 8.6% of caprine samples being positive (Masala et al. 2007).

New Zealand's extensive pasture-based farming systems mean that there is opportunity for farm or stray dogs to maintain the life cycle of *N. caninum* with aborted fetuses, placentae and dead stock being scavenged and with cull animals being slaughtered and fed to dogs. One study (Antony and Williamson 2003) found high *N. caninum* seroprevalence in New Zealand dogs from urban and rural properties with 30.7% of urban dogs, 74.5% of dogs from dairy farms and 96.8% of dogs from sheep/beef farms being seropositive by IFAT. This seroprevalence is high when compared to reports from other countries which are usually less than 50% (Dubey et al. 2007). An earlier study in New Zealand found 22% of randomly selected, clinically normal dogs positive by IFAT (Reichel 1998).

Previous experimental challenges of sheep have used Nc-1 (Buxton et al. 1998; Buxton et al. 2001; Innes et al. 2001b; Rettigner et al. 2004), Nc-2 (McAllister et al. 1996c; Jolley et al. 1999), Nc-Liverpool (McAllister et al. 1996c; Buxton et al. 1997b; Jolley et al. 1999) and Nc-Illinois (Jenkins et al. 2004) isolates. The present study aimed to investigate the level of challenge of local *N. caninum* isolates necessary to cause abortion in pregnant ewe lambs and whether the lambs born following challenge would be congenitally infected. These isolates have not been characterised and their pathogenicity in sheep is unknown so a dose-titration challenge was carried out. An assessment of the sensitivity of a variety of diagnostic procedures was also made.

6.3 Materials and Methods

6.3.1 Animals

Pregnant ewe lambs (n=49, 7-8 months at breeding, Romney-cross) from a commercial sheep and beef farm were used in the trial. They were previously vaccinated with Toxovax (Intervet Ltd, New Zealand) and Campyvax 4 (Intervet Ltd, New Zealand) and were pregnant with a single fetus conceived during the first 17 days of breeding, confirmed using transabdominal ultrasound*. Ram mating-harnesses with different colour crayons were used to establish mating dates. The ewes' rectal temperature was measured before challenge and daily for 7 days. Weekly transabdominal ultrasound scanning was conducted to monitor fetal viability/abortion. Following challenge, the sheep were run as one group on a commercial farm; dogs were unable to be excluded from the property but were not used in the management of this flock. Ewes were checked twice daily to monitor the general health of the animals and paddocks examined to collect aborted material or lambs born. Any lambs born were euthanased with pentobarbitone (Pentobarb 500 mg/mL, National Veterinary Supplies, Auckland, New Zealand) within 24 h of birth and the dams were similarly euthanased within 7 days of parturition.

6.3.2 Inoculum

N. caninum tachyzoites from three New Zealand isolates (Nc-NZ1, Nc-NZ2 and Nc-NZ3; Okeoma et al. 2004c) were maintained by periodic passage in Vero cell cultures. Heavily infected cells were left to lyse then tachyzoites were pelleted and washed twice by centrifugation at 1000 x g for 10 min. The tachyzoites were resuspended in sterile PBS (pH 7.4) and counted to produce each infective dose. Equal proportions of the three isolates were prepared in 2 mL doses. The 49 ewe lambs were randomly allocated to 5 groups and were intravenously inoculated with *N. caninum* tachyzoites via the jugular vein at 90 days after joining with the ram (therefore 73-90 days gestation). The challenge inoculates were group A (n=9) Vero cell culture control group; group B (n=10) 50 tachyzoites; group C (n=10) $5x10^3$ tachyzoites; group D (n=10) $1x10^6$ tachyzoites; and group E (n=10) $1x10^8$ tachyzoites.

* Ovi-scan 6, BCF Ultrasound Australasia Ltd, Christchurch, New Zealand.

6.3.3 Serology

Blood samples were taken from 100 ewe lambs at the farm of origin 8 weeks before the start of the experiment to select non-infected animals to enter the trial. These were tested using IFAT for *N. caninum* as described by Paré et al. (1995a) but was modified using fluoroscein-labelled donkey anti-sheep IgG as conjugate (Jackson ImmunoResearch). Positive samples were then serially two-fold diluted to determine a fluorescence end-point. A positive control serum sample from an experimentally-infected ewe (1:6,400) and a negative control serum sample from an uninfected ewe were included in every test run. A second serum sample was taken on the day of challenge to confirm their negative *N. caninum* antibody status. Following challenge, serum was collected fortnightly and tested by IFAT and a final sample was collected for testing just prior to the euthanasia of the ewe. Sera collected 28 days after inoculation were additionally tested using an ELISA as per manufacturer's instructions (Chekit* Neospora, IDEXX Laboratories Inc, Maine, USA) and sample to positive results (S/P%) ≥ 11.8% were regarded as seropositive (Reichel et al. 2008). This additional testing was carried out to assess agreement between two serological tests. Serum was also collected within 12 h of birth from lambs (which may have drunk colostrum) and tested by IFAT.

6.3.4 PCR

Heparinised whole blood was collected from lambs within 24 h of birth and from ewes just prior to euthanasia for PCR detection of *N. caninum* DNA. The brain was collected from each ewe and lamb with 10 μg of tissue containing white and grey matter from one hemisphere being tested by PCR. Any placenta that could be identified to a particular ewe was also tested by PCR. Fluid from the thoracic or abdominal cavity of the fetus was also collected for PCR, as a proxy for the whole blood which was collected from live-born lambs.

All samples tested by PCR for *Neospora* DNA were processed using the DNeasy Tissue Kit (Qiagen, Victoria, Australia) following the manufacturer's instructions. Water blanks were included as sample processing controls to confirm a lack of contamination during testing. Purified DNA from *N. caninum* Nc-NZ1 tachyzoites (Massey University) was used as a positive control. Single-tube nested PCR reactions were performed as described by Ellis et al. (1999) to amplify the internal transcribed spacer 1 (ITS1) region of *N. caninum* using the internal (NR1 and NS2) and external (NF1 and SR1) primer pairs. The 50 μL

PCR reaction mixture contained: up to 5 μL of test sample (approximately 200 ng total genomic DNA), 1 x PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM NS2 and NR1, 0.015 μM NF1 and SR1, and 1 unit of Platinum Taq DNA polymerase (Invitrogen, CA, USA). Thermal cycle conditions were similar to those described by Ellis et al. (1999) with an increase to 20 cycles from 15 in the second round of amplification. PCR products were electrophoresed through a 1.5% (w/v) ultra-pure agarose gel (Invitrogen, CA, USA) containing ethidium bromide and visualised under UV light on a transilluminator.

All positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, CA, USA) to confirm their genomic sequence.

6.3.5 Histopathology

The brain was collected from each ewe and lamb with one hemisphere fixed in 10% formalin. Other aborted materials (fetus and placenta) were collected and samples of fetal brain and placenta (where available) were submitted for histopathological examination. The formalin-fixed samples were trimmed into tissue blocks, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Three tissue blocks were examined from each brain.

6.3.6 Statistics

Data were recorded and analysed using Microsoft Excel (Microsoft Corporation, Washington, USA). SPSS (SPSS Inc., Chicago, IL, USA) was used to generate figures that compared serological methods. Differences between the groups were tested for significance using Fisher's exact test (www.exactoid.com). All procedures involving the experimental use of animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand (MUAEC 06/35).

6.4 Results

Eight of 100 ewe lambs tested in pre-trial sampling were serologically positive for *N. caninum* by IFAT (6 at 1/100 and 2 at 1/200) so were not eligible for selection. The first 50 IFAT-negative ewe lambs were selected to enter the study however only 49 animals were delivered and 3 of them had not previously been sampled. Six ewes that entered the

study (none that had been allocated to the control group) were seropositive based on samples collected on the day of challenge, having titres of up to 1/800. Five of these had been seronegative 8 weeks earlier and one had not been sampled.

6.4.1 Clinical observations

Eight of 10 ewes in group E and 3 of 10 in group D had a fever (>40.0°C) 24 h after inoculation with *N. caninum* tachyzoites that generally persisted for 1-2 days but 5 animals in group E were still febrile 6 days after inoculation. Also, 2 of 9 animals in group A, 1 of 10 in group B and 1 of 10 in group C had a fever on Day 6 or 7 after inoculation.

Transabsdominal ultrasound identified 2 group C, 4 group D and 3 group E sheep that were about to abort. No animals in groups A or B aborted and all in groups D and E aborted. None of the aborted lambs were mummified but most had undergone some autolysis. In group C, 5 abortions, 2 premature but live and 3 normal births occurred. The differences in pregnancy outcome between groups A and C, B and C, C and D, and C and E were significant ($p < 0.05$). Two group A ewes with dystocia were assisted to deliver dead lambs. The times to abortion or parturition after inoculation are shown in Table 6.1.

Table 6.1 Days to abortion or parturition of ewes following intravenous inoculation with *Neospora caninum* at mid-gestation.

Group (dose)	Abortion	Days to abortion or parturition	Average
A (control)	0 / 9	55 - 65	59.2
B (50)	0 / 10	56 - 79	59.8
C (5×10^3)	5 / 10	36 - 63	49.2
D (10^6)	10 / 10	37 – 54	44.7
E (10^8)	10 / 10	26 - 56	37.6

6.4.2 Serology

All control group ewes had at least one positive result by IFAT serology during the trial. Only one animal (from group B) had no detectable IFAT titre throughout the trial. The highest titre measured was 1/800. The titre peaked and declined rapidly in most animals

and in 8 of 25 ewes that aborted it was negative within 7 days of abortion. Full serological results are presented in Table 6.2.

There were no differences in the proportion of inoculated sheep that were seropositive by IFAT between dose groups at any sampling occasion. However, none of 8 lambs born from group A or 10 lambs from group B were seropositive by IFAT while 3 of 5 lambs born from group C were seropositive (1 at 1/200 and 2 at 1/400). These differences between groups A and B to group C were significant ($p < 0.05$).

ELISA S/P ratio at 28 days post-challenge increased with inoculation dose and showed little agreement with the IFAT result from the same sample, [kappa agreement between tests κ=0.025 (95% CI 0-0.28); Figure 6.1]. The proportion of inoculated animals that were seropositive by ELISA at 28 days after inoculation differed between groups A and C, B and C, and group C compared to groups D and E combined ($p < 0.05$).

A) B)

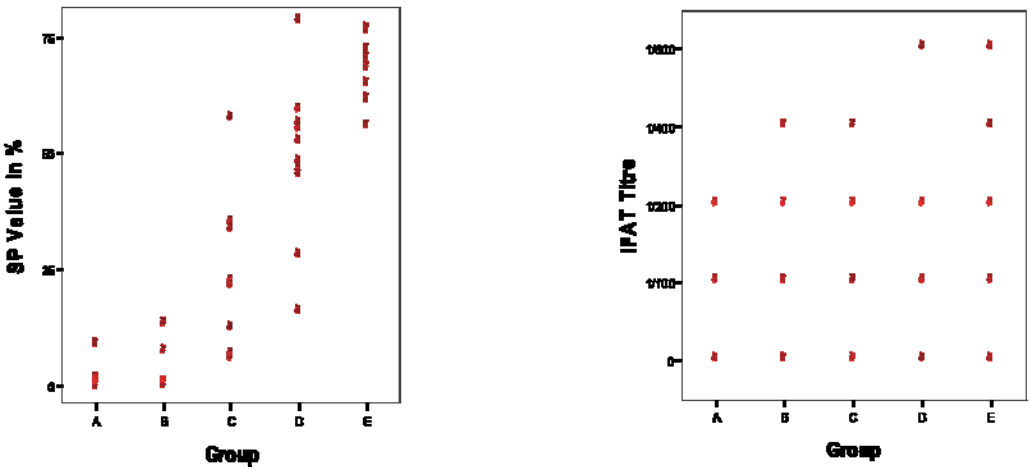

Figure 6.1 Comparison of serological results by A) IDEXX ELISA and B) IFAT at 28 days after intravenous inoculation with *N. caninum* tachyzoites. Group A = control, group B = 50 tachyzoites, group C = 5,000 tachyzoites, group D = 10^6 tachyzoites, group E = 10^8 tachyzoites.

Table 6.2 Serological results for ewes related to the time of intravenous inoculation with *N. caninum* tachyzoites at mid-gestation (A=control, B=50 tachyzoites, C=5x10^3 tachyzoites, D=10^6 tachyzoites, E=10^8 tachyzoites).

ID	Day -56 IFAT	Day 0 IFAT	Day 14 IFAT	Day 28 IFAT	Day 28 ELISA S/P* (%)	Day 42 IFAT	Lamb at Birth IFAT	Ewe at parturition IFAT	Pregnancy outcome
A1	N	N	N	1/200	1.19	1/200	na	N	Stillborn
A2	N	N	N	1/100	-0.24	1/200	na	1/100	Stillborn
A3	N	N	N	1/200	-0.24	N	N	1/200	Live lamb
A4	N	N	1/400	N	0.47	1/100	N	N	Live lamb
A5	N	N	1/400	N	0.00	N	N	N	Live lamb
A6	N	N	1/200	1/200	-0.59	1/200	N	1/200	Live lamb
A7	N	N	N	1/200	0.83	N	N	1/800	Live lamb
A8	N	N	1/400	1/200	-0.36	1/200	N	N	Live lamb
A9	N	N	1/200	1/100	8.42	N	N	N	Live lamb
B1	N	N	N	1/200	13.00	1/200	N	N	Live lamb
B2	N	N	N	1/400	-0.13	1/200	N	N	Live lamb
B3	N	N	1/800	1/200	0.38	1/200	N	N	Live lamb
B4	N	N	N	1/100	-0.36	N	N	1/100	Live lamb
B5	N	N	N	N	0.25	N	N	1/200	Live lamb
B6	N	N	N	1/200	0.00	N	N	1/200	Live lamb
B7	na	N	N	1/100	0.25	N	N	1/100	Live lamb
B8	N	N	N	1/400	0.13	N	N	1/200	Live lamb
B9	N	N	N	N	0.25	N	N	N	Live lamb
B10	N	N	N	1/200	7.13	N	N	N	Live lamb
C1	N	N	1/200	1/200	22.22	1/800	na	1/400	Abortion
C2	N	N	N	N	33.18	1/800	na	1/400	Abortion
C3	na	N	N	N	12.01	1/200	1/200	1/200	Premature
C4	N	N	N	1/200	5.86	N	N	1/400	Live lamb
C5	N	N	N	1/200	57.21	na	na	1/200	Abortion
C6	na	1/800	1/200	1/100	21.17	N	na	N	Abortion
C7	N	1/200	N	1/400	34.83	1/200	1/400	N	Premature
C8	N	N	1/200	1/200	21.13	N	na	N	Abortion
C9	N	N	N	N	6.46	1/200	1/400	N	Live lamb
C10	N	1/200	N	1/200	5.56	1/100	N	N	Live lamb
D1	N	N	N	1/200	47.90	1/200	na	1/200	Abortion
D2	N	N	1/200	1/100	45.00	NA	na	1/100	Abortion
D3	N	N	N	1/800	59.13	N	na	N	Abortion
D4	N	1/800	1/100	1/200	55.89	N	na	N	Abortion
D5	N	N	N	1/100	54.82	1/400	na	1/400	Abortion
D6	N	N	N	1/100	15.54	1/200	na	1/200	Abortion
D7	N	N	1/200	N	46.10	1/800	na	1/400	Abortion
D8	N	N	1/200	1/200	27.78	1/200	na	1/100	Abortion
D9	N	N	1/200	N	78.13	NA	na	1/200	Abortion
D10	N	N	N	1/200	52.10	1/200	na	1/200	Abortion
E1	N	N	1/800	N	61.07	na	na	N	Abortion
E2	N	N	N	1/200	76.43	na	na	1/200	Abortion
E3	N	1/400	1/100	1/800	72.00	N	na	N	Abortion
E4	N	N	1/800	1/100	70.18	na	na	1/100	Abortion
E5	N	N	N	1/200	75.89	N	na	N	Abortion
E6	N	N	N	1/400	55.36	na	na	1/400	Abortion
E7	N	N	1/200	1/800	61.43	N	na	1/400	Abortion
E8	N	N	N	1/200	68.04	na	na	1/200	Abortion
E9	N	1/200	N	N	67.86	1/200	na	1/200	Abortion
E10	N	N	1/400	1/100	64.64	N	na	N	Abortion

* Chekit* Neospora ELISA, IDEXX Laboratories Inc, Maine, USA
na = not available

6.4.3 PCR

Cotyledons from 8/9 group E animals were positive on PCR for *N. caninum*, in group D 3/6 were positive, 4/4 from group C were positive and one placenta associated with a ewe-lamb pair from group A (control) was negative (Table 6.3). PCR testing of cotyledon when compared to lamb/fetal brain histopathology had a diagnostic sensitivity of 0.79 (95% confidence interval (CI) 0.61-0.97).

Twenty-one of 29 lamb/fetal brains with histopathological evidence of protozoal infection were positive for *N. caninum* DNA by PCR testing (Table 6.3), a sensitivity of 0.72 (95% CI 0.56-0.89) compared with histopathology. Another 3 lamb brains that had no histopathological lesions were positive by PCR testing; 2 from group A and one from a lamb born in group C. The proportion of lamb/fetal brain with positive PCR results differed ($p < 0.05$) between groups A and C, and B and C.

Table 6.3 PCR and histopathology results from ewe and lamb/fetus samples following intravenous inoculation with *N. caninum* tachyzoites at mid-gestation. (A=control, B=50 tachyzoites, C=5x10^3 tachyzoites, D=10^6 tachyzoites, E=10^8 tachyzoites).

ID	Pregnancy Outcome	Lamb Brain PCR	Lamb Blood PCR	Lamb Brain Histopath	Placenta PCR	Ewe Brain PCR	Ewe Blood PCR
A1	Stillborn	-	-	Normal	na	-	-
A2	Stillborn	-	-	Normal	na	-	-
A3	Live lamb	Pos	-	Normal	na	-	-
A4	Live lamb	-	-	Normal	na	-	-
A5	Live lamb	-	-	Normal	na	-	-
A6	Live lamb	-	-	Normal	na	-	Pos
A7	Live lamb	-	-	Normal	na	-	-
A8	Live lamb	Pos	-	Normal	-	-	-
A9	Live lamb	-	-	Lesions	na	-	-
B1	Live lamb	-	-	Normal	na	-	-
B2	Live lamb	-	-	Normal	na	-	-
B3	Live lamb	-	-	Normal	na	-	-
B4	Live lamb	-	-	Normal	na	-	-
B5	Live lamb	-	-	Normal	na	-	-
B6	Live lamb	-	-	Normal	na	-	-
B7	Live lamb	-	-	Normal	na	-	-
B8	Live lamb	-	-	Normal	na	-	-
B9	Live lamb	-	-	Normal	na	Pos*	-
B10	Live lamb	-	-	Lesions	na	-	-
C1	Abortion	Pos	-	Lesions	Pos	-	-
C2	Abortion	Pos	Pos	Lesions	Pos	-	Pos

* None of the brains from ewes showed any histopathological evidence of *N. caninum* infection

C3	Premature	Pos	-	Lesions	Pos	-	-
C4	Live lamb	Pos	-	Normal	na	-	-
C5	Abortion	Pos	Pos	Lesions	na	-	na
C6	Abortion	Pos	na	Lesions	Pos	Pos	-
C7	Premature	Pos	-	Lesions	na	-	-
C8	Abortion	Pos	Pos	Lesions	na	-	-
C9	Live lamb	-	-	Normal	na	-	-
C10	Live lamb	-	-	Normal	na	-	-
D1	Abortion	-	-	Lesions	na	-	-
D2	Abortion	-	-	Lesions	na	-	na
D3	Abortion	Pos	Pos	Lesions	Pos	-	-
D4	Abortion	Pos	na	Lesions	na	Pos	Pos
D5	Abortion	Pos	Pos	Lesions	-	-	-
D6	Abortion	Pos	-	Lesions	-	-	-
D7	Abortion	Pos	na	Lesions	Pos	-	-
D8	Abortion	-	na	Lesions	na	-	-
D9	Abortion	Pos	-	Lesions	Pos	-	na
D10	Abortion	Pos	-	Lesions	-	-	-
E1	Abortion	-	-	Lesions	Pos	-	-
E2	Abortion	Pos	-	Lesions	-	-	-
E3	Abortion	Pos	Pos	Lesions	Pos	-	-
E4	Abortion	Pos	Pos	Lesions	Pos	Pos	-
E5	Abortion	-	-	Lesions	Pos	-	-
E6	Abortion	Pos	-	Lesions	Pos	-	na
E7	Abortion	-	-	Lesions	na	-	-
E8	Abortion	Pos	Pos	Lesions	Pos	-	na
E9	Abortion	Pos	-	Lesions	Pos	-	-
E10	Abortion	Pos	na	Lesions	Pos	-	Pos

Whole blood from live-born lambs or thoracic fluid from aborted fetuses was positive by PCR in 9/44 samples (none from group A or B, 3/9 from group C, 3/7 from group D and 3/9 from group E). The proportion of lambs/fetuses with blood or thoracic fluid that was positive by PCR differed ($p < 0.05$) when lambs from groups A and B were combined and compared to all other groups. Whole blood collected from 4/44 ewes at the time of parturition was positive by PCR (1 ewe each from groups A, C-E) and 4/49 adult brains were positive by PCR (1 ewe each from groups B-E) when ewes were euthanased and examined within 3 days of aborting or lambing. Complete PCR and histopathology results are shown in Table 6.3.

Six of 9 lambs born to group A control ewes were negative for *N. caninum* in all samples, 2 had positive brain PCR results and 1 had histopathological lesions in the brain suggestive of protozoal infection. Nine of 10 lambs born to group B ewes were negative in all samples while 1 had lesions in the brain detected by histopathology. Five lambs were born alive to the 10 group C ewes; 1 had no evidence of *N. caninum* infection, 3 were positive on

brain PCR, 2 were positive on brain histopathology and 3 were positive on IFAT serology. All aborted fetuses from group D and E animals were classified as infected by histopathology and these results were largely supported by positive PCR results, particularly from fetal brain. However, the brains of 7/25 aborted fetuses were negative by PCR (1/5 from group C, and 3/10 from each of groups D and E).

6.4.4 Histopathology

Twenty samples of placenta were collected that could be traced to the ewe. These were predominantly from aborting ewes as they remained close to the abortus. The placentae from ewes B10, D7, D10 and E10 had inflammatory foci, some of which were calcified, together with fibrin exudation, prominent trophoblastic cells and some epithelial cells containing yellow pigment granules. Signs of protozoal infection were seen in the brains from all aborted fetuses as well as 2 live-born premature lambs from group C, one lamb from group B and one lamb from group A (the control group). Observed lesions were congestion, foci of leukomalacia, isolated granulomas or necrogranulomas (some with mineralised centres), occasional tissue cysts, as well as small foci of gliosis and multifocal periventricular haemorrhage associated with malacia. The proportion of lambs/fetuses showing histopathological evidence of protozoal infection in the brain differed ($p < 0.05$) between groups A and C, B and C, and group C compared to groups D and E combined. No abnormalities were observed in the brains from any of the ewes.

6.5 Discussion

This study demonstrated a strong dose-response relationship for the occurrence of abortion and time to abortion in pregnant ewe lambs intravenously inoculated with *N. caninum* tachyzoites at 90 days after joining with the ram. A dose of 5,000 tachyzoites was required to cause abortion in 50% of the young ewes and at higher doses all ewes aborted. No abortions occurred in the ewes inoculated with only 50 tachyzoites. There was also a dose-response relationship with congenital infection of offspring although determination of this effect is confused by seroconversion among ewes in the control group including some positive results among their offspring.

IFAT has been used for serological diagnosis in cattle and is considered to have high sensitivity and specificity, for this reason it had been used in earlier work in New Zealand

to indicate previous exposure to *N. caninum* among sheep (West et al. 2006; Howe et al. 2008). Also, IFAT was available and relatively inexpensive to perform in the absence of a commercially available test. An initial dilution of 1/100 was used as at lower dilutions the background reactivity was too high. Serological results using IFAT did not relate to infection dose in terms of proportion of each group that were seropositive at each sampling occasion, the strength of the titre response or the outcome of pregnancy. Repeated testing of the samples collected 28 days after inoculation using an ELISA gave more consistent results with a strong correlation between dose and S/P% as shown in Figure 6.1. The ELISA results 28 days after challenge were also a strong indicator of pregnancy outcome with 25 of 28 ELISA-positive animals aborting and none of 21 ELISA-negative animals aborting. These results support the findings of Reichel et al. (2008) that a cut-off in the S/P % of 11.8 be regarded as a positive result. Animals used to calculate that cut-off were experimentally infected, as were the ewes in this trial, so the performance of the ELISA in naturally infected animals remains unknown and comparisons between fetal brain histopathology and maternal serology could be useful in determining this.

Ewes in this experiment had serum collected 8 weeks before challenge and on the day of challenge in an attempt to include only non-exposed animals. Errors in identifying animals at the source farm led to some animals entering the trial with one serological result on the day of challenge and other ewes had seroconverted in the 8 weeks before the trial commenced. The validity of the IFAT results have however been called into question with ELISA results from 28 days post-challenge suggesting that all animals in groups A and B were seronegative. Nevertheless, 3 of 9 lambs born to control group ewes had at least one positive result indicating transplacental transmission with 2 lambs having parasite DNA detected in their brains and 1 lamb having histopathological lesions consistent with protozoal infection. Another ewe in this group was positive by PCR on a whole blood sample collected at the time of parturition. These results are consistent with *N. caninum* infection occurring within control group ewes. It is possible that the control group ewes were infected at the start of the experiment given the questionable value of IFAT serology and that only one or two samples were taken before starting the experiment. Ewes may have been infected by horizontal transmission just before or during the experiment, or sample contamination during collection or processing may have occurred though attempts were made to minimise this. Standard laboratory handling procedures were used for the

removal of brains of adults and lambs and equipment was cleaned between animals. Dogs had not been excluded from the farm of origin or the property on which the sheep were pastured during the experiment so horizontal transmission from *N. caninum* oocysts in the environment is possible. Conducting the experiment under housed conditions, with groups of animals kept separately, would reduce the chance of infection but animals could still be exposed to *N. caninum* oocysts through the introduction of pasture and other feed supplements. Re-testing of all sera from this experiment by ELISA and western blot to assess humoral response and infection status would be valuable and is the focus of a subsequent investigation.

Intravenous inoculation of tachyzoites is an artificial means of infection. How such dosing equates to the ingestion of sporulated oocysts from definitive hosts and what level of environmental contamination is required to produce a similar outcome through natural exposure is not known. A low level of exposure earlier in pregnancy may have severe consequences for the fetus, as demonstrated by experimental inoculation of cattle (Barr et al. 1994b; Williams et al. 2000). In sheep (McAllister et al. 1996c), all ewes inoculated on Day 65 of gestation aborted, those inoculated on Day 90 aborted or gave birth to weak or clinically normal lambs, and all ewes inoculated on Day 120 gave birth to clinically normal lambs. In this trial, 10-month-old ewe lambs considered to have conceived during the first 17 days of mating were challenged at 90 days since joining with the ram, resulting in challenge between 73 and 90 days gestation. Other challenge trials have used predominantly the Nc-1 and Liverpool strains and most commonly with a subcutaneous challenge over the prefemoral lymph node. Jolley et al. (1999) used a mixture of Nc-1 and Liverpool strains and intravenous inoculation with 1.7×10^5 tachyzoites on Day 67 of gestation with a result that both ewes aborted. With 3 New Zealand isolates 5 of 10 ewes inoculated with 5×10^3 tachyzoites aborted and all 10 ewes aborted with a dose of 10^6 tachyzoites (both given intravenously).

If determining the cause of abortion in sheep is the object of an investigation, then histopathological examination of fetal brain appears to be the most sensitive diagnostic test in *N. caninum* abortion. Collection of 3 tissue blocks from one hemisphere of each fetal brain was sufficient to detect lesions in each aborted lamb and in some lambs that were born alive. Inflammation in fetal brains may be diffuse or multifocal and severity and distribution of lesions may be affected by gestational age (Dubey and Lindsay 1996). In the

present trial, the diagnostic sensitivity of fetal histopathology was high. Whether similar lesions would be found in all cases of natural infection is not known but they have been reported in some cases (Dubey et al. 1990a; Hässig et al. 2003) and lesions may also be seen in fetal heart and lung (Barr et al. 1990; Wouda et al. 1997a). Other reports suggest that histopathology of mummified lambs including those with severely autolysed tissues is worthwhile to reveal evidence of protozoal infection (Dubey and Lindsay 1996). However, lesions are indistinguishable from those associated with *T. gondii* infection and require immunohistochemistry or serology to differentiate.

PCR testing of aborted material (fetal brain and cotyledon) may be more appropriate in severely autolysed tissue. Maternal serology using IFAT is apparently of little use following abortion as many animals in this experiment were seronegative at the time of abortion or shortly afterwards and this IFAT methodology has been called into question. Once further serological methods are validated and become commercially available these could be of use in seroepidemiological studies of ovine abortion. As with *N. caninum*-associated abortion in cattle, positive serology only provides evidence of exposure and not a definitive diagnosis of the cause of abortion, so non-aborting animals should also be sampled to allow statistical hypothesis testing.

Further studies are required to ascertain the prevalence of natural infection among New Zealand sheep but the low sensitivity of antibody detection methods requires serial sampling of many animals. One study using the IDEXX ELISA test found 4 of 640 rams to be seropositive (Reichel et al. 2008) while initial sampling for the current experiment found 8 of 100 8-month-old ewe lambs to be positive by a now discredited IFAT. If *N. caninum* infection is common in the sheep population but reproductive loss only occurs sporadically, factors that increase the pathogenesis of *N. caninum* infection need to be elucidated. Factors that cause immunosuppression may impact on pregnancy outcome as has been shown to occur in cattle with bovine viral diarrhoea virus infection and the feeding of mouldy hay (Bartels et al. 1999).

Congenital infection of lambs was shown to occur in this experiment at varying levels depending on the challenge dose of organism. At a dose when 5 of 10 ewe lambs aborted, 4 of the 5 lambs born to the remaining sheep showed evidence of infection. Ewes inoculated with a low challenge dose and control group ewes produced 19 lambs of which

4 showed evidence of *N. caninum* infection in the brain. The relative importance of horizontal transmission compared to vertical transmission (endogenous or exogenous; Trees and Williams 2005) for maintaining *N. caninum* infection in sheep is unknown and warrants further investigation.

The response to the dose-titration challenge in this experiment could be used in future challenge experiments in sheep to assess vaccination and treatment protocols. It would be valuable to monitor the offspring of pregnant, challenged sheep to assess their reproductive performance as this is likely to be similar to outcomes in naturally infected animals. Ongoing abortion losses from congenitally infected cattle are an important feature of endemic *N. caninum* and this may also occur in sheep. The present study has expanded our understanding of the potential for *Neospora* to cause abortion in sheep but many questions remain to be answered.

6.6 Acknowledgements

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6.7 Authors' contributions to this study

L Howe assisted with sample processing, carried out the PCR analysis and provided editorial advice.

MG Collett assisted with sample processing and carried out the histopathological examination.

RS Pattison performed the indirect fluorescent antibody testing.

NB Williamson provided advice for the trial design and editorial advice.

DM West provided advice for the trial design.

WE Pomroy provided advice for the trial design and editorial advice.

SS Syed-Hussain performed the enzyme-linked immunosorbent assays.

ST Morris secured the funding for this study and provided advice for the trial design.

PR Kenyon provided advice for the trial design.

JF Weston designed the study, was responsible for the inoculation and sampling of animals, analysed the results and wrote the manuscript.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Dose-titration challenge of young pregnant sheet with *Neospora caninum* tachyzoites

In which Chapter is the Published Work: Six

What percentage of the Published Work was contributed by the candidate: 80%.

_____ 29th August 2011

Candidate's Signature

_____ 2nd September 2011

Principal Supervisor's signature

Chapter Seven

Experimental evidence for direct horizontal transmission of *Neospora caninum* in cattle

7.0 Preface

Following the dose-response trial using *Neospora caninum* tachyzoites to inoculate pregnant sheep by intravenous injection, a challenge trial was conducted in pregnant heifers to establish a challenge model for future experiments where control methods including putative vaccines could be assessed. This heifer challenge trial consisted of intravenous inoculation to establish whether the New Zealand strains of *N. caninum* tachyzoites that had been maintained in Vero cell culture could reliably induce abortion. Additionally, an oral challenge whereby pregnant heifers had tachyzoites applied to an abraded oral mucosa was conducted to test the hypothesis that horizontal transmission could occur in cattle if oral lesions are present and they ingest tachyzoites following contamination of food or water by aborting or parturient cattle.

7.1 Abstract

Pregnant heifers that were seronegative for *Neospora caninum* were inoculated with 1 x 10^8 *N. caninum* tachyzoites intravenously (n = 8) or via abraded oral mucosa (n = 8), or received uninfected culture medium intravenously (negative controls; n = 7) on Day 70 of gestation. Pregnancy status was assessed 2x then 1x weekly (transrectal ultrasonography/palpation) until animals had calved or aborted. Blood samples were collected throughout pregnancy and after parturition for serology and PCR analysis. Placenta or vaginal discharge was collected for PCR after abortion or parturition. Blood samples were collected from new-born calves for serology and PCR analysis; brains were collected from these calves after euthanasia for histopathology and PCR analysis. All intravenously inoculated heifers became, and remained, seropositive to *N. caninum* within 28 days of inoculation. One heifer from this group aborted and one calf was seropositive when sampled within 12 hours of birth. Of 8 heifers inoculated orally, one became consistently seropositive 28 days after inoculation, her calf and one other were seropositive, and PCR positive results were obtained from the vaginal discharge of 3 heifers and the blood or brain of two calves. None of the negative control heifers seroconverted to *N. caninum,* but 3 of 7 heifer-calf pairs of the control group had at least one positive PCR result. These results are consistent with the hypothesis that cattle with oral lesions may become infected with *N. caninum* by ingesting tachyzoites.

Keywords: Neospora caninum; Horizontal transmission; Abortion; Cattle

7.2 Introduction

Serological evidence for infection with the apicomplexan parasite *Neospora caninum* has been detected in many mammals (Dubey and Lindsay 1996). Dogs (Basso et al. 2001), coyotes (*Canis latrans*; Gondim et al. 2004b) and dingoes (*Canis lupus dingo*; King et al. 2010) are definitive hosts, and domestic and sylvatic life cycles are known to occur naturally (Rosypal and Lindsay 2005). Infection with *N. caninum* is associated with neurological disease in dogs (Barber and Trees 1996) and abortion in cattle, being one of the most commonly diagnosed causes of bovine abortion worldwide (Anderson et al. 1994). Abortion associated with *N. caninum* infection has been reported in dairy and beef cattle (Barr et al. 1990; Dubey et al. 1990c), sheep (Dubey et al. 1990a), goats (Barr et al. 1992) and deer (Woods et al. 1994).

Recognised methods of transmission to cattle are the ingestion of sporulated oocysts from definitive hosts (Björkman et al. 2005) and vertical transmission from infected cow to fetus during either a *de novo* infection or during recrudescence of a previously latent infection (Anderson et al. 1997). *Neospora caninum* tachyzoites have been used in experimental challenge studies to establish infection by intravenous, intramuscular and subcutaneous routes in pregnant and non-pregnant cattle of varying ages (Barr et al. 1994b; Andrianarivo et al. 2000; Macaldowie et al. 2004). Young calves seroconverted following oral inoculation with colostrum or milk spiked with tachyzoites (Uggla et al. 1998; Davison et al. 2001) and pregnant heifers seroconverted following conjunctival inoculation with tahcyzoites (de Yaniz et al. 2007). Attempts to produce venereal transmission via semen infected with tachyzoites have produced conflicting results (Canada et al. 2004; Serrano-Martínez et al. 2007) but this does not appear to be an important method of transmission in natural infections, despite *N. caninum* DNA being reported in the semen of infected bulls (Caetano-da-Silva et al. 2004a).

Dogs infected with *N. caninum* shed oocysts sporadically, although difficulties in oocyst identification mean that estimates of levels of shedding are highly variable from as few as 19 to 114,000 oocysts per gram of faeces (Schares et al. 2005a). Young dogs and those that are immunosuppressed have the highest levels of oocyst shedding (Dubey et al. 2007). Oral doses of 600 oocysts can prompt *Neospora*-specific antibody responses in cattle (Trees

et al. 2002), but dose and timing of challenge affect the occurrence of vertical transmission or abortion (Gondim et al. 2004c).

Neospora caninum infection within a herd can be widespread. During an abortion epidemic in a New Zealand dairy herd, up to 50% of cows became seropositive and up to 33% aborted (Thornton et al. 1994), whilst a 600-cow dairy herd that suffered an abortion epidemic, and was blood sampled quarterly for the next 2 years to detect *N. caninum* antibodies, had only 27% of cows seronegative in all samples (Pfeiffer et al. 2002). Cows that are infected with *N. caninum* have an increased risk of being culled (Bartels et al. 2006a) and French et al. (1999) concluded that without reintroduction of infection by a definitive host, infection within a dairy herd would not be maintained in the long term.

The incidence of *N. caninum* abortion has been associated with the number of dogs on a farm (Wouda et al. 1999b) and feeding practices that could aid the spread of feed contaminated with dog faeces (Dijkstra et al. 2002b). Other associations identified from epidemiological studies include positive correlations with bovine viral diarrhoea virus (BVDV) infection (Björkman et al. 2000), the presence of poultry on the farm and exposure to mycotoxins (Bartels et al. 1999). On farms where *N. caninum* infection is endemic, the highest incidence of abortion occurs in primiparous heifers but declines with subsequent pregnancies (López-Gatius et al. 2005a). We hypothesised that oral lesions (such as during tooth eruption) could facilitate infection by allowing tachyzoites to pass into the bloodstream, providing a means of horizontal transmission between cattle. The present study was undertaken to assess whether cattle could become infected by tachyzoites applied to an abraded oral mucosa, via a novel oral challenge model.

7.3 Material and methods

7.3.1 Experimental Design

Twenty three heifers that were seronegative to *N. caninum* (as defined in 7.3.2 Animals) were randomly allocated to one of three groups which received inoculation with *N. caninum* tachyzoites on Day 70 of gestation, either (i) intravenously (n=8), or (ii) by applying the tachyzoites directly to an abraded oral mucosa (n=8), or (iii) a negative control group which received intravenous injection of Vero cell culture medium (n=7). All heifers were subsequently monitored clinically and via blood samples collected for serology and for whole blood polymerase chain reaction (PCR) analysis. Pregnancy diagnosis was performed

manually and by transrectal ultrasonography to (a) confirm that conception had occurred and (b) to detect fetal death or abortion.

All heifers were grazed in a single group on a commercial farm (with no history of *N. caninum* infection) throughout the trial until four weeks before the planned start of calving, when the heifers in the negative control group and one heifer from the oral challenge group had to be returned to their farm of origin (Farm A). Blood samples were collected from calves as soon as possible after birth, generally within 12 h. The calves were then euthanased and their brains removed for PCR testing and histopathology.

7.3.2 Animals

Twenty six 14-month-old Friesian and Friesian/Jersey cross heifers were obtained from two commercial dairy farms. All were serologically negative for *N. caninum* at the start of the trial, and all were the progeny of seronegative dams. Seronegativity was based on testing of the heifers and their dams which had been undertaken quarterly for the past 7 years on Farm A (n =18) or by a single test on Farm B (n = 8). All heifers had been vaccinated against clostridial disease and leptospirosis (7 in 1, CSL, Melbourne, Australia) and bovine viral diarrhoea virus (Bovilis BVD, Intervet International B.V., Boxmeer, The Netherlands) before the start of the trial.

Oestrous cycles of heifers were synchronised using a standard protocol of CIDR-B devices (Pfizer Animal Health, Auckland, New Zealand) inserted for 10 days, prostaglandin $F_{2\alpha}$ injection (Estroplan, Parnell Laboratories Ltd., Alexandria, NSW, Australia) 6 days after CIDR insertion and oestradiol benzoate injections (1 mg; Cidirol, Bomac Laboratories Ltd., Auckland, New Zealand) on the day of CIDR insertion and 24 h after CIDR removal. Fixed-time artificial insemination (FTAI) was performed 24 h after the final oestradiol benzoate injection, when the heifers were expected to be in oestrus. Two 2-year-old Jersey bulls (which were seronegative on a single test for *N. caninum*) were also run with the heifers for 10 weeks from the time of CIDR-B removal. Pregnancy diagnosis was performed weekly by transrectal ultrasonography from 5 weeks after AI to allow estimation of conception dates and to confirm pregnancy until inoculation on Day 70 of gestation.

7.3.3 Inoculum

The inoculated tachyzoites were a mixture of Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates (Okeoma et al. 2004c) that had been maintained by periodic passage in Vero cell culture. Heavily infected cells were left to lyse, after which tachyzoites were pelleted and washed twice by centrifugation at 1,000 x *g* for 10 min. The tachyzoites were counted and re-suspended in sterile PBS (pH 7.4) to produce each infective dose. Equal proportions of the three isolates were prepared in 2 mL aliquots, each containing a total of 1 x 10^8 tachyzoites. Intravenous inoculation was administered via the jugular vein. For oral inoculation, the site of inoculation was prepared by applying 5 mL 10% lignocaine (Lopaine, Phoenix Pharm Distributors Ltd., Auckland, New Zealand) topically to the buccal mucosa adjacent to the left second and third incisors. After 5 minutes the area was rinsed with distilled water then a scalpel blade was used to superficially abrade the oral mucosa. The tachyzoites were then applied directly to the wound by syringe. The negative control group was inoculated with 2 mL of tissue culture medium containing Vero cells via the jugular vein. To confirm viability of the tachyzoites, an extra dose of inoculum was returned to the laboratory and placed into Vero cell culture.

7.3.4 Clinical observations

Temperature, heart rate and respiratory rate were recorded, and heifers were observed for signs of ill health or behavioural change before, then daily for 10 days after, inoculation. Thereafter, heifers were checked twice-daily in their paddock throughout the remainder of the trial. Pregnancy testing occurred twice-weekly by trans-rectal ultrasonography or manual palpation for 10 weeks after inoculation and then weekly until abortion or parturition.

7.3.5 Blood and tissue sampling

Blood samples (10 mL) were collected from heifers on Days 35 and 70 of gestation, then every 2 weeks until 10 weeks after inoculation and then every 4 weeks for the remainder of the trial, by caudal venepuncture into evacuated collection tubes (BD vacutainer Ref 367895, no anticoagulant; Becton, Dickinson and Co., Plymouth, UK). Heparinised whole blood (10 mL; BD vacutainer Ref 367880, lithium heparin 144 USP units) was also collected from the heifers at the time of challenge and then 7, 14, 21, 63, 70 and 84 days later. Within 12 h of parturition heifers, and the calves born to them, had serum and whole blood collected (intravenous and oral inoculation groups) or by a single

visit to Farm A (at which point the calves were 2-11 days old) for the negative control group. Serum samples were immediately submitted to a commercial veterinary diagnostic laboratory (New Zealand Veterinary Pathology, Palmerston North, New Zealand) for assay and thereafter, serum was stored at -20°C. Whole blood samples were transferred to 15 mL high density polypropylene conical tubes (Greiner Bio-One Ltd., article 188261; Stonehouse, Great Britain) then stored at -20°C until used for PCR.

Immediately after blood sampling, the calves were euthanased by intravenous injection of pentobarbitone (Pentobarb 500, Provet NZ Pty Ltd., Auckland, New Zealand), for collection of brain tissue. One hemisphere of the brain was fixed in 10% buffered formol saline for histopathological examination and the other hemisphere was frozen at -20°C for PCR. Equipment used in this collection was cleaned and disinfected between samples. In addition, samples of placenta (where available) or vaginal discharge were collected for PCR into single-use containers from heifers after calving or abortion.

7.3.6 Serology

Sera were tested for antibody to *N. caninum* using the HerdChek* Neospora ELISA (IDEXX Laboratories Inc, Maine, USA) for the pre-trial selection process and throughout the trial. A cut-off in the sample-to-positive (S/P) ratio of 0.21, rather than the manufacturer's recommended cutpoint of 0.50 was used when selecting heifers for the trial to improve test sensitivity (Reichel and Pfeiffer 2002). Thereafter, the standard cutpoint in the S/P ratio was used to determine serostatus consistent with the manufacturer's recommendations for the test.

7.3.7 Polymerase chain reaction (PCR)

The whole blood samples that were collected just prior to inoculation and on Days 7, 14, 21, 63, 70 and 84 days post-inoculation, as well as those blood, placental and calf brain samples that were collected after parturition or abortion, were subjected to PCR to detect *N. caninum* DNA.

DNA was extracted from whole blood and samples of placenta or vaginal discharge using the DNeasy Tissue Kit (Qiagen, Victoria, Australia) according to manufacturer's instructions. Five grams of each fresh calf brain sample was placed into a sterile, single use, 50 mL conical tissue grinder (VWR, PA, USA) containing 10 mL of sterile PBS (pH 7.4;

137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO). After homogenisation, 100 μL aliquots of the suspension of brain tissue were used for DNA extraction.

Single-tube nested *N. caninum* PCR reactions to amplify the ITS1 region were performed as described by Ellis et al. (1999), using the internal (NR1 and NS2) and external (NF1 and SR1) primer pairs with the following modifications. The 50 μL PCR reaction mixture contained: 5 μL of test sample DNA, 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM NS2 and NR1, 0.015 μL NF1 and SR1, 1 unit of Platinum Taq DNA polymerase with 1 PCR buffer [10X buffer consisting of 200 mM Tris HCl (pH 8.4), 500 mM KCl; Invitrogen, CA, USA]. The thermal cycle conditions were as follows: 95°C for 5 minutes; five cycles of 94°C for 30 seconds (s), 60°C for 150 s, 72°C for 30 s; 20 cycles of 88°C for 30 s, 60°C for 30 s, 72°C for 30 s, ten cycles of 88°C for 30 s, 54°C for 30 s, 72°C for 30 s; 25 cycles of 86°C for 30 s, 54°C for 30 s, 72°C for 30 s; and 72°C for 10 min. PCR products were eluted on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen, CA, USA) containing ethidium bromide (5 μg/mL) at 100V for 45 min and visualized under UV light on a transilluminator (Bio-Rad, New South Wales, Australia). Water blanks were included as sample processing controls to confirm lack of contamination during sample manipulation and purified DNA from *N. caninum* -NZ1 tachyzoites (Massey University) was used as a positive control for each PCR run.

Positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Inc, CA, USA) and the ABI3730 Genetic Analyzer (Applied Biosystems Inc.). Sequencing results were submitted to the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation of ITS1 gene amplification.

7.3.8 Histopathology

The formalin-fixed samples of one hemisphere from the brain of each calf were trimmed and a tissue block from each of the brain stem, cerebellum, midbrain and cerebrum were embedded in paraffin wax, cut into 3 μm sections and stained with haematoxylin and eosin (H&E).

7.3.9 Statistics

Data were recorded and analysed using Microsoft Excel (Microsoft Corporation, WA, USA). Fisher's exact test (www.exactoid.com) was used to test differences in outcome between the groups for significance. All procedures involving the use of animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand (MUAEC 04/114).

7.4 Results

7.4.1 Clinical observations

Eighteen heifers conceived to the first AI and a further 5 conceived at the next oestrus, providing a total of 23 pregnant heifers. Eight heifers were randomly allocated to each of the intravenous and oral inoculation groups but the heifers that comprised the negative control group were pre-selected as a condition of their use and were leased from Farm A. The dose of inoculum returned to the laboratory after the heifers had been challenged continued to multiply in Vero cell culture confirming tachyzoite viability.

One heifer from the intravenous inoculation group had an increased temperature (39.5 - 40.0°C) at 24 and 48 h after inoculation. All other heifers had normal temperatures (<39.5°C) following inoculation. There were no localised reactions at the site of intravenous or oral inoculation. No other abnormal clinical parameters or ill effects were observed.

7.4.2 Abortion and Parturition

The heifer that was pyrexic following intravenous inoculation (Heifer 2) aborted between Days 42 and 45 after the challenge. The fetus was not recovered despite extensive searching, but autolysed placenta was passed 18 days after abortion. Samples of mucopurulent vaginal discharge were collected 6 and 19 days after the abortion was detected.

Two heifers from the intravenously challenged group experienced dystocia at calving (full term) that required veterinary intervention. These two calves and one calf from the negative control group were stillborn. A failure by farm staff to accurately identify cow-calf pairs meant that samples were collected from only 4/7 calves in the negative control group.

7.4.3 Serology

Serological response following challenge is shown in Figure 7.1. Fourteen days after inoculation, 2/8 of the intravenously inoculated heifers had seroconverted (as defined by a S/P ratio of ≥ 0.50); the other 6 heifers had an increase in the S/P ratio by Day 14 and were seropositive 28 days after inoculation. All 8 heifers remained seropositive throughout the trial (with the exception of one heifer which was seronegative only on Day 70). Of the 8 heifers that were inoculated orally, one was seropositive 28 days post-inoculation and remained seropositive throughout the trial. None of the other heifers in this group became seropositive. None of the 7 negative control heifers became seropositive. The proportion of animals developing an antibody response in the intravenously inoculated animals was significantly greater ($p < 0.01$) than in either of the other groups.

One of the 7 calves born from the intravenously inoculated heifers was seropositive when sampled within 12 hours of birth. Two of 8 calves from the heifers that had received oral inoculation were also seropositive; one of these being the offspring of the heifer that had seroconverted. A third calf from this group had a mild increase in S/P ratio (0.39). None of the calves from the negative control group from which blood samples were collected ($n = 4$) were seropositive, although all exhibited non-significant increases in S/P ratio (0.15 - 0.24).

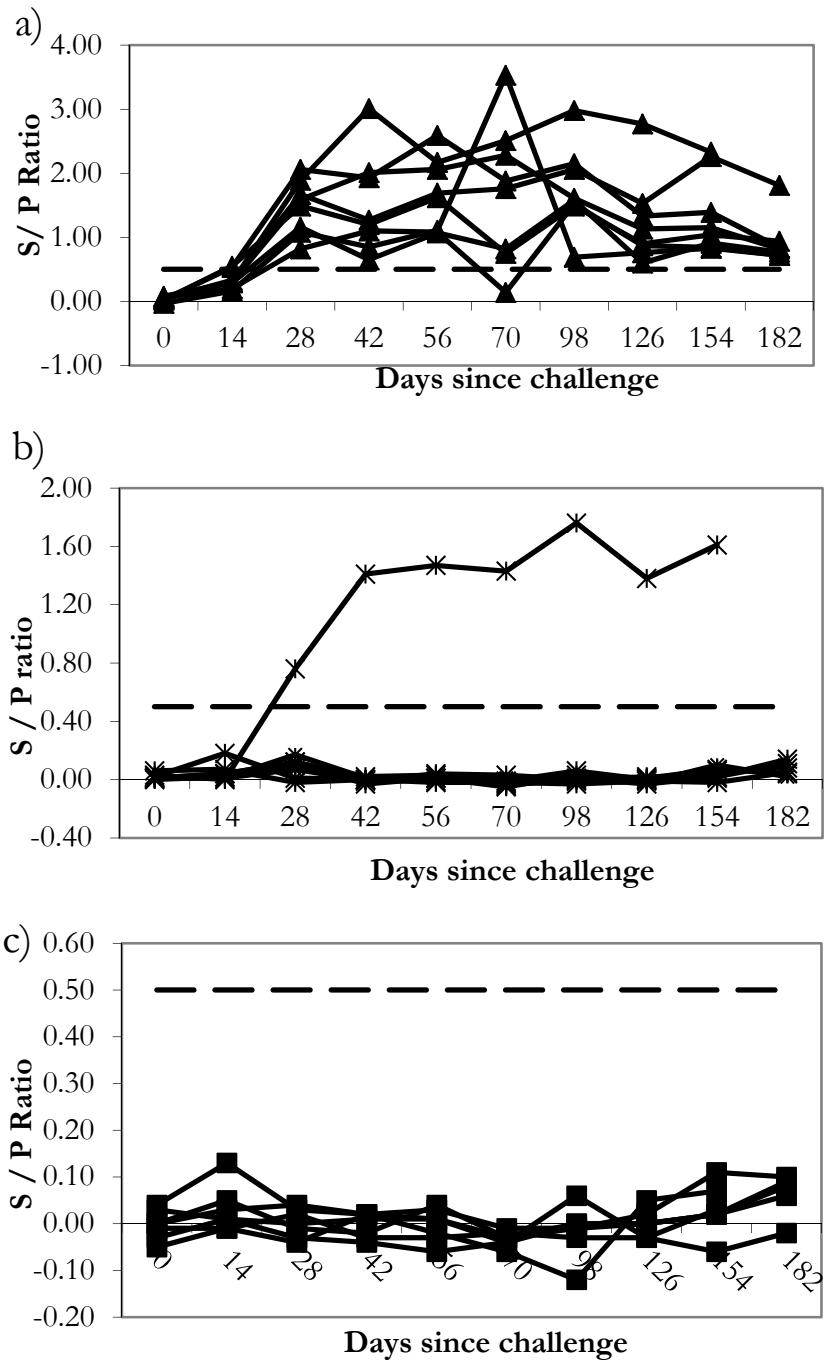

Figure 7.1 Serological response following challenge of pregnant heifers on Day 70 of gestation with *Neospora caninum* tachyzoites expressed as Sample / Positive (S/P) ratio using the HerdChek Neospora ELISA (IDEXX Laboratories, Maine, USA) where S/P ≥ 0.50 is positive among a) heifers challenged with 10^8 tachyzoites inoculated intravenously b) heifers challenged with 10^8 tachyzoites applied to abraded oral mucosa c) negative control heifers challenged intravenously with tissue culture medium containing Vero cells. Note: the scale of the y-axis differs between the graphs.

7.4.4 PCR

A full summary of PCR and serological results at parturition (abortion in Heifer 2) is presented in Table 7.1. Whole blood samples collected 14 days after inoculation yielded positive PCR results in 2/8 heifers receiving intravenous inoculation, and from 2 other heifers in this group 70 days after inoculation i.e. Day 140 of gestation. One heifer in the oral inoculation group had *N. caninum* DNA detected in whole blood collected 70 days after inoculation. In the negative control group, whole blood samples collected from two heifers 63 days after inoculation were positive. All other samples of whole blood from the heifers (including those collected after parturition or abortion) were negative, other than one heifer that had received intravenous inoculation, whose post-parturient whole blood sample was positive.

Two samples of vaginal discharge (collected 6 and 19 days after abortion was detected) from the heifer that aborted, as well as the sample of fetal membranes collected 18 days after abortion, were positive by PCR. Four of the remaining seven heifers in the intravenous inoculation group had *N. caninum* DNA detected by PCR in their placenta or vaginal discharge within 12 hours of calving. Two heifers from the oral inoculation group had vaginal discharge that was positive by PCR, whilst a third (whose calf also had a positive PCR result for brain tissue) had PCR positive placental tissue. Two of the 7 heifers in the negative control group had vaginal discharge that was positive by PCR.

Whole blood samples collected from 5 of the 7 calves from the intravenous inoculation group were PCR positive. The brains from 3 of these calves were also PCR positive. A sample of whole blood from one of the calves from the oral inoculation group was also positive by PCR. The dam of this calf had been seronegative throughout the trial. Another calf from the oral inoculation group had *N. caninum* DNA detected in its brain. The brain from one calf in the negative control group was positive by PCR. There were no statistically significant differences in the proportion of PCR positive samples between the three experimental groups ($p < 0.05$). All positive PCR amplicons were sequenced as *Neospora caninum* using the NCBI BLAST database.

7.4.5 Histopathology

Histopathology of the sections of brainstem, cerebellum, midbrain and cerebrum from the calves revealed no abnormalities or evidence of infection with *N. caninum*.

Table 7.1 Serological[#] and PCR results from heifers and their progeny inoculated on Day 70 of gestation by either intravenous (I/V) or abraded oral mucosa (oral) with 1 x 10^8 *Neospora caninum* tachyzoites or intravenously with tissue culture medium containing Vero cells (negative control) when sampled at abortion or parturition.

Treatment group	Heifer ID	Heifer serostatus[*]	Abortion	Calf serostatus[*]	Calf whole blood PCR	Calf brain PCR	Placenta/vaginal discharge PCR	Heifer whole blood PCR
I / V	1	0.72	-	-0.15	Pos	-	-	Pos
	2	3.01	Y	na	na	na	Pos	-
	3	0.70	-	-0.10	-	-	-	-
	4	0.83	-	-0.09	-	Pos	Pos	-
	5	1.56	-	-0.14	Pos	Pos	Pos	-
	6	0.94	-	1.49	Pos	Pos	-	-
	7	1.89	-	-0.10	Pos	-	-	-
	8	0.59	-	-0.10	Pos	-	Pos	-
Oral	9	0	-	0.10	Pos	-	Pos	-
	10	-0.02	-	-0.12	-	Pos	Pos	-
	11	0.13	-	0.39	-	-	-	-
	12	-0.07	-	0.12	-	-	-	-
	13	0.11	-	-0.15	-	-	-	-
	14	-0.03	-	0.13	-	-	Pos	-
	15	0	-	0.69	-	-	-	-
	16	1.37	-	1.07	-	-	-	-
Control	17	-0.05	-	0.16	-	Pos	Pos	-
	18	-0.07	-	na	na	na	na	Pos
	19	0.03	-	0.24	-	-	-	-
	20	-0.03	-	0.22	-	-	-	-
	21	-0.07	-	na	na	na	-	-
	22	0.03	-	0.15	-	-	-	-
	23	0.10	-	na	na	na	Pos	-

[#] HerdChek* Neospora ELISA; IDEXX Laboratories Inc, Maine, USA

[*] Sample to positive (S/P) ratio in the ELISA; ≥ 0.50 considered positive; sampling was not pre-colostral.

na = not available

7.5 Discussion

To the authors' knowledge, this is the first report of challenge with *N. caninum* tachyzoites applied directly to an abraded oral mucosa. This method of challenge was used to mimic risk factors for *N. caninum* infection associated with oral lesions such as BVDV infection (Björkman et al. 2000) or tooth eruption (Hietala and Thurmond 1999). Serology showed that 1 of 8 heifers in the oral inoculation group seroconverted within the same time-frame as heifers that were challenged by intravenous inoculation and remained seropositive throughout gestation. The calf from this heifer was also seropositive, further indicating that infection with *N. caninum* had been successfully established. Two other calves from the oral inoculation group exhibited elevated S/P ratios (albeit one of these was < 0.50). Inclusion of PCR results (two calves with *N. caninum* DNA in either brain or blood and three heifers with *N. caninum* DNA in placenta or vaginal discharge) showed evidence of infection with *N. caninum* in 5/8 heifer-calf pairs in the oral inoculation group.

As expected, intravenous inoculation with *N. caninum* tachyzoites on Day 70 of gestation caused seroconversion in all heifers within 28 days. This is in agreement with previous similar trials using different strains of *N. caninum* (Andrianarivo et al. 2001; Innes et al. 2001a). The incidence of abortion was lower than expected, with only 1 of 8 animals in the intravenous inoculation group aborting, compared with the results of Williams et al. (2007), in which 5 of 6 fetuses died *in utero* following an intravenous tachyzoite challenge (10^7 Nc-Liverpool) on Day 70 of gestation. Although it was not possible to incontrovertibly prove that the abortion in the one heifer in the present study was due to *N. caninum* infection, the timing of abortion, serological response and positive PCR results from the placenta and vaginal discharge following abortion all strongly indicate that this was the case. Moreover, other results from heifers and calves in the intravenous inoculation group confirm that this was a successful *N. caninum* challenge model; as 6 of 7 calves from heifers that were inoculated intravenously had at least one positive result in PCR or serology suggesting that transplacental transmission had occurred even if chronic infection had not been established (as evidenced by only 1 of 7 calves being seropositive at, or shortly after, birth).

The low prevalence of abortion (1/8) and congenital infection in the intravenously inoculated group suggests that the New Zealand isolates used in this trial (Nc-NZ1, Nc-NZ2 and Nc-NZ3) may have had low virulence or that their virulence had been reduced by collection and storage procedures. Other studies provide support for the suggestion that

N. caninum isolates may vary in virulence (Atkinson et al. 1999; Rojo-Montejo et al. 2009) and a high level of seroconversion in a dairy herd that did not experience an increase in abortion incidence has been reported (Dijkstra et al. 2002a). A strain of the closely related Apicomplexan parasite, *Toxoplasma gondii*, that was maintained by twice-weekly passage through mice (about 3,000 times) lost the ability to develop bradyzoites in tissue cysts and was incapable of oocyst production following infection of the definitive host (Buxton and Innes 1995). This attenuated strain was used to produce the live vaccine that prevents ovine abortion (Toxovax; MSD Animal Health, Upper Hutt, New Zealand, Wilkins et al.1998).

All 4 calves that were sampled from the control group and 4/8 calves from the oral challenge group had a S/P ratio between 0.10 and 0.39 in the *Neospora* ELISA and 2 other calves in the oral challenge group were seropositive. The S/P ratios for all other calves were negative. It is not possible to differentiate whether the antibody detected in these samples was derived from the fetus or was present as a result of ingesting maternal antibody. The calves in the control group would have received pooled colostrum from a herd known to have long-standing *N. caninum* infection for up to 11 days and the other calves may have suckled their dam or other heifers in the paddock between birth and sampling. Low levels of antibody (S/P < 0.50) in calves therefore may reflect passive transfer of maternal antibody. It is interesting however, that the 6 seronegative calves from heifers that had been inoculated intravenously all had a S/P ratio <0 when their dams were in fact seropositive. Another trial that was able to obtain pre-colostral sample from calves and then re-sampled them several days later found some overlap in antibody concentrations between calves that were congenitally infected and those that were naïve; although the level of antibody in the congenitally infected calves was significantly higher (Hietala and Thurmond 1999). Further sampling of neonatal calves on farms with endemic *N. caninum* infection may allow the development of reference ranges that indicate congenital infection or maternal antibody relative to the age of the calf at sampling.

Positive PCR results from samples collected from the heifers and calves of the negative control group could be explained if i) the heifers in this group were not free of *N. caninum* infection at the commencement of the trial although testing seronegative, ii) there was contamination of samples during collection or processing, or iii) infection became

established during the study period. Heifers in this group were leased from Farm A (which had endemic *N. caninum* infection) because there was a seven year history of quarterly serological testing of all cattle on the farm. This combination of long-standing negative serological results for the heifers and their dams and the use of a lower cut-off in the S/P ratio (of 0.21) for the ELISA (Reichel and Pfeiffer 2002) allowed confidence that the heifers from that farm were not infected with *N. caninum* if being consistently seronegative to *N. caninum* indicates freedom from infection. The pre-entry serological status of the heifers that were enrolled from Farm B was also evaluated using the lower S/P ratio cut-off. This farm had no history of *Neospora*-associated abortion, but even so, these animals were not considered for allocation to the negative control group. Reports of *N. caninum* infection in fetuses from dams without detectable antibodies (Sager et al. 2001; Kyaw et al. 2005) suggests that some cattle may be immunotolerant to infection, possibly due to early *in utero* infection (McInnes et al. 2006a) and it is possible that the heifers used in the present study could be infected with *N. caninum* as evidenced by parasite DNA in their blood at times during the trial. Analysis of serum and fetal tissues to detect antibodies to *N. caninum* or DNA by PCR in 79 pregnant cows at slaughter in Western Australia found *N. caninum* DNA in 12 cases that were serologically negative (McInnes et al. 2006a). Therefore, it is possible that some of the heifers in the present study may have already been infected with *N. caninum* despite our best efforts to prevent this.

To address issues of sample contamination, single-use consumables were used for sample collection when appropriate, all other equipment was cleaned between sampling and water blanks were included as sample processing controls to confirm lack of contamination during sample manipulation.

Alternative explanations for the observations are that the negative control group animals acquired *de novo* infections from ingesting oocysts, as dogs were not able to be excluded from the property, or these positive PCR results support an hypothesis of horizontal transmission between cattle. Four of the 7 control heifers had mild transitory increases in the S/P ratio following the abortion that occurred in the heifer from the intravenous inoculation group; moreover, all of the positive PCR results came from these heifers or their calves. The fact that all positive PCR results were sequenced as *N. caninum* further ensures the specificity of the result. Approximately 5 years after the trial was carried out, an attempt was made to use microsatellite array analysis to confirm that the positive PCR

results were genetically identical to the challenge strains. Unfortunately, the samples had degraded over the intervening time and could not be typed.

Other authors have previously reported that seronegative cows which were housed with seropositive and aborting cows did not seroconvert (Guy et al. 2001; Fioretti et al. 2003); and, similarly, the in-contact heifers in the control group of the present study did not seroconvert. However, infection with *N. caninum* may be more widespread than currently recognised due to the limitations of currently available tests. The use of PCR and immunohistochemistry (IHC) has been shown to increase the sensitivity of testing for the presence of *N. caninum* infection (Baszler et al. 1999b). Moreover, serology may not be the best indicator of *N. caninum* infection status in cattle, since there are reports of cattle (De Marez et al. 1999) and dogs (Dijkstra et al. 2001a) that do not seroconvert despite other evidence of infection. Hence, it is possible that infection was successfully established in more heifers and calves in the oral challenge group, as suggested by PCR results, but at a level that did not result in the production of detectable concentrations of specific antibodies. These findings, call into question our current methods for detecting infected cattle which, to date, have relied on repeated serological screening. The development of nested PCR techniques that are able to detect *N. caninum* DNA appear to have very high sensitivity (Ellis 1998) and the application of these techniques to serum or whole blood makes the repeated sampling of animals convenient and opens up new avenues of research to elucidate the epidemiology of this parasite. The significance of infections that do not provoke an antibody response is unknown. It is widely recognised that seropositive cattle have a greater risk of abortion and it has previously been suggested that it is in fact, the maternal immune response to active infection that causes abortion (Innes et al. 2002). The true prevalence of *N. caninum* infection in cattle may be much greater than serological surveys suggest, although it is possible that infections which do not provoke an antibody response are of little consequence. PCR testing of whole blood samples collected weekly, for 12 weeks, from heifers in this trial revealed that the 7/23 animals that had a positive sample were positive on only one occasion so parasitaemia is short-lived.

Neospora caninum DNA has been demonstrated in bovine placenta and amniotic fluid by PCR (Ho et al. 1997b; Gottstein et al. 1998) and immunohistochemistry (IHC; McAllister et al. 1996a), and it has been suggested that the ingestion of fetal membranes or amniotic fluid containing tachyzoites could be a means of postnatal infection in cattle (Schares et al.

1998; Davison et al. 1999a) There has also been direct identification of *N. caninum* tachyzoites in bovine placenta (Shivaprasad et al. 1989) which can be infective when fed to dogs (Dijkstra et al. 2001a). A study that monitored serologic responses among cows from two drylot dairies with endemic *N. caninum* infection found that most cases of horizontal transmission occurred in cattle between 20 and 35 months of age, and noted that this coincided with the animals' first exposure to pregnant cows (and, possibly, to aborted fetuses; Hietala and Thurmond, 1999). It is also frequently the time of permanent tooth eruption in cattle.

Cattle are inquisitive and explore novel objects in their environment by smell, taste and the flehmen response (Wood-Gush et al. 1985; Nielsen and Luescher 1988; Paterson and Morris 1995). In ungulates, parturition frequently attracts the attention of herd-mates, even males (Lent 1974). Multiparous cows show maternal responsiveness prior to their own parturition and may adopt calves leading to mis-mothering (Edwards 1983; Owens et al. 1985). Social interactions between cows around parturition are proportional to cattle density and, within the first few hours of parturition, other cows frequently lick the dam and, to a lesser extent, the calf. Pinheiro Machado et al. (1997) offered placenta or amniotic fluid mixed with silage to peri-parturient cows and found that cows were attracted to placenta from other cows after they had given birth and, moreover, in the case of donor amniotic fluid, from 12 h before to at least 24 h after they themselves had given birth, they preferred to eat silage contaminated with donor amniotic fluid rather than silage on its own.

Placentophagia has been suggested as a means of transmission of *N. caninum* among cattle by several authors (Schares et al. 1998; Davison et al. 1999a; Wouda et al. 2000; Davison et al. 2001; Modrý et al. 2001), although others have been cautious in their support for this idea (Schares and Conraths 2001). Infected placenta or amniotic fluid is a potential means of transmission of *N. caninum* (Ho et al. 1997b) and tachyzoites have proven infective for mice after oral inoculation (Lindsay and Dubey 1990), either by unexpectedly surviving passage through the stomach or by penetrating the mucosa of the oral cavity or oesophagus and thereby bypassing the stomach. The latter route of infection might be aided by trauma to the mucosa or by the presence of pre-existing lesions. Tachyzoites in amniotic fluid or placenta may not survive for long in the environment but have been shown to cause infection when added to milk and colostrum fed to calves (Uggla et al.

1998; Davison et al. 2001) so it is clear that they are not immediately inactivated in such media. While such transmission may be responsible for only a small proportion of infections in cattle, there may be considerable contamination of the environment by tachyzoites during a *Neospora*-associated abortion epidemic. Hence, even though the tachyzoite stage of the parasite may not survive for long in the environment, high levels of challenge, maternal responsiveness of other peri-parturient cows and a potential oral route of infection provide a route by which horizontal infection could occur.

7.6 Conclusions

This study demonstrated that cattle can become infected with *N. caninum* after direct inoculation of tachyzoites onto abraded oral mucosa. The key observations to support this conclusion are that in the group of eight animals that received oral inoculation, firstly, one previously uninfected heifer became consistently seropositive, secondly, that her calf was also seropositive and, thirdly, that there were positive PCR results for *N. caninum* DNA in another three heifers or their calves. These results are consistent with the hypothesis that aborting and calving cows contaminating pasture with infective stages of *N. caninum* are a potential source of infection for other cattle on the farm and that horizontal transmission among cattle by placentophagia or contamination of feed or water with tachyzoites in amniotic fluid or vaginal discharge from infected cows may occur. Although this route may be much less frequent than horizontal transmission from definitive hosts and vertical transmission, models of herd infection rates should take this into account. Recommendations to collect and safely dispose of aborted material and isolating aborting and seropositive cows at parturition to limit the spread of infection within a herd would be prudent. These findings suggest that further studies to investigate possible mechanisms of horizontal transmission between cattle are desirable.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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7.8 Authors' contritubtions to this study

N.B. Williamson assisted with trial design, monitoring and sampling of animals and provided editorial advice.

L. Howe assisted with sample collection from aborted fetuses, was responsible for PCR and microsatellite array analysis and provided editorial advice.

W.E. Pomroy assisted with trial design and provided editorial advice.

M.G. Collett carried out the histopathological examination of tissues and provided editorial advice.

T.J. Parkinson provided editorial advice.

J.F. Weston assisted with trial design, was responsible for running the trial and carried out the majority of the treatment, monitoring and sampling of animals and wrote the manuscript.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

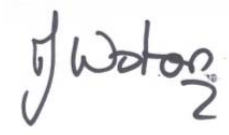
Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Experimental evidence for direct horizontal transmission of *Neospora caninum* in cattle

In which Chapter is the Published Work: Seven

What percentage of the Published Work was contributed by the candidate: 75%.



_____ 29th August 2011

Candidate's Signature

_____ 2nd September 2011

Principal Supervisor's signature

Chapter Eight

Inoculation of cattle before breeding with a strain of *Neospora caninum* tachyzoites previously passaged through mice does not prevent fetal deaths

8.0 Preface

The variable efficacy of Bovilis Neoguard in preventing abortion on farms with a history of *Neospora caninum*-associated abortion and its failure to prevent vertical transmission showed a need for an alternative vaccine to be developed. The findings that prior infection with *N.caninum* prevented fetal death (Williams et al. 2003) when pregnant cattle were subsequently challenged with *N. caninum* tachyzoites and that vertical transmission did not occur in infected cattle that were challenged with tachyzoites at mid-gestation (Innes et al. 2001a), suggested that a modified live vaccine may be useful in controlling bovine neosporosis. The live, attenuated vaccine that prevents abortion due to *Toxoplasma gondii* (Toxovax; MSD Animal Health, Upper Hutt, New Zealand) is a model for such an approach. Dairy heifers were again sourced from the farm reported in Chapters Three and Seven and were randomly allocated to receive inoculation prior to mating with either *N. caninum* tachyzoites that had previously been passaged through mice or with serum-free tissue culture media. The heifers were then mated and half were challenged with a mixture of the New Zealand strains of *N. caninum* on Day 70 of gestation, the other half were challenged with serum-free tissue culture media at the same stage of gestation. A greater challenge dose was given compared to the trial reported in Chapter Seven (2×10^9 tachyzoites intravenously and 2×10^8 subcutaneously *vs* 1×10^8 tachyzoites intravenously) in an attempt to achieve 100% abortions in the cattle which received serum-free tissue culture media prior to mating and then a tachyzoite challenge on Day 70 of gestation.

8.1 Abstract

Neospora caninum is an important cause of infectious bovine abortion. Attempts to develop a vaccine to prevent abortion and vertical transmission have so far been unsuccessful. However, several experimental challenge studies found that inoculation with *N. caninum* tachyzoites prior to mating does not result in endogenous vertical transmission in the subsequent pregnancy, but prevented abortion following challenge with tachyzoites on Day 70 of gestation. A trial was conducted that used a New Zealand isolate of *N. caninum* that had been passaged through mice to inoculate heifers prior to mating. Two of six heifers inoculated with mouse-passaged *Neospora caninum* tachyzoites 42 days before mating aborted following a *N. caninum* tachyzoite challenge on Day 70 of gestation compared with five of six heifers only challenged with tachyzoites on Day 70 ($p = 0.12$). One of five heifers inoculated with the mouse-passaged *N. caninum* tachyzoites aborted in

the absence of any further *N. caninum* challenge. These findings show that the use of inoculation of cattle with *N. caninum* tachyzoites prior to mating with field strains as a control option may not be suitable. Molecular comparison using nested polymerase chain reaction to amplify microsatellite markers revealed genetic differences between the New Zealand isolates of *N. caninum* and that the New Zealand isolates differed from other isolates that have previously been reported.

Keywords: Neospora caninum; Cattle; Abortion; Vaccination; Mouse

8.2 Introduction

Neospora caninum is an Apicomplexan parasite, that has dogs, coyotes (*Canis latrans*) and dingoes (*Canis lupus dingo*) as its definitive hosts (McAllister et al. 1998; Gondim et al. 2004a; King et al. 2010) and cattle, sheep, goats and dogs as intermediate hosts (Dubey and Lindsay 1996). Many other mammals and birds have serological evidence of infection and may be natural intermediate hosts (Dubey et al. 2007). Neurological disease in dogs (Bjerkås et al. 1984) and abortion in cattle (Thilsted and Dubey 1989) are the main clinical syndromes associated with infection. The risk of abortion is commonly reported to be increased four-fold in cattle that are seropositive to *N. caninum* (Thurmond and Hietala 1997a; Wouda et al. 1998a) although the risk varies according to the serological assay and cut-point used to determine infection status (Schares et al. 1999a).

The proportion of pregnant cattle aborting in a herd diagnosed with *Neospora*-associated epidemic abortion is often greater than 10% and levels as high as 56% are reported (Wouda et al. 1999a). The risk of abortion in infected animals tends to decrease with increasing parity (Thurmond and Hietala 1997a), suggesting that some protective immunity develops in congenitally-infected cattle – even though repeat abortions may occur (Obendorf et al. 1995; Moen et al. 1998).

Control of *N. caninum* infection in cattle has proven difficult and until now has focussed on limiting contact between cattle and dogs, identifying and culling infected cattle or culling replacement heifers from infected cows (Reichel and Ellis 2006). Embryo transfer utilising known non-infected recipient cows can be used to produce non-infected offspring from infected cattle (Baillargeon et al. 2001). Pharmacological treatments have been tested in an

attempt to kill all stages of the parasite in infected cattle (Kritzner et al. 2002; Gottstein et al. 2005), but no effective treatment protocol has yet been established. Economic modelling suggests that vaccination would probably be the most cost-effective means of control of bovine neosporosis in dairy cattle (Reichel and Ellis 2006; 2008) if an effective vaccine were available.

Over the past 30 years, efforts have been directed to develop vaccines against a variety of Apicomplexan parasites of veterinary and human importance (particularly malaria). Few effective vaccines have been released onto the market. Exceptions include toxoplasmosis in sheep (an attenuated S48 strain of *T. gondii*; Toxovax; MSD Animal Health, Upper Hutt, New Zealand; Wilkins et al. 1988) and coccidiosis in poultry (Paracox, MSD Animal Health, Upper Hutt, New Zealand; Williams et al. 1999). Problems associated with the development of vaccines against parasitic diseases include the complexity of immunological changes in an infected host, antigenic differences in different stages of the parasite, the chronic nature of many parasitic infections and the fact that cell-mediated immunity as opposed to specific antibody appears to be responsible for the control of parasitic infections (Cox 1997). Issues that need to be addressed in the case of live vaccines include: 1) production, shelf-life and handling to ensure that the vaccine is still viable when administered, 2) contamination of the vaccine with other pathogens, the most important of these being the transmissible spongiform encephalopathies, and 3) the differentiation between vaccinated and naturally-infected cattle if other control strategies such as serological testing are employed or when livestock are traded. The first two issues have been successfully addressed with the use of Toxovax to protect sheep against toxoplasmosis and millions of doses of this product are used annually in New Zealand (Reichel and Ellis 2009) although the seasonal nature of the farming system facilitates planning for production of the vaccine.

Vaccination of cattle against neosporosis may aim to prevent infection in naïve cattle, prevent the recrudescence of infection in infected cattle (which should prevent both abortion and vertical transmission), prevent or reduce abortion or ideally, a combination of any of these (Reichel and Ellis 2009). Attempts to develop a vaccine involving live, inactivated, subunit or vector vaccines have been undertaken using mouse models and some cattle trials. These have been reviewed by Reichel and Ellis (2009). A *Neospora caninum* killed tachyzoite vaccine (Bovilis Neoguard; Intervet International B.V., Boxmeer,

The Netherlands) that stimulated both humoral and cell-mediated immunity in cattle (Andrianarivo et al. 1999) was licensed in 2001 for use in several countries including New Zealand and the United States. Large-scale clinical trials, with reduction in abortion being the outcome of interest, were carried out in Costa Rica and New Zealand. Vaccine effect varied markedly across farms in both trials. The trial in Costa Rica reported a significant vaccine effect with abortions being reduced in the vaccinated group by 46% (Romero et al. 2004). In the New Zealand trial, a significant vaccine effect occurred on only one of five farms, so the overall vaccine effect was non-significant (Chapter Four). Due to low market demand, the New Zealand product registration was allowed to lapse in 2009 and the product has not been sold internationally since.

A report that inoculation prior to pregnancy with *N. caninum* tachyzoites prevented vertical transmission when subsequently challenged with *N. caninum* tachyzoites on Day 140 of gestation (Innes et al. 2001a) suggests that a modified live vaccine may provide acceptable protection. Both humoral and cell-mediated immune responses were elicited following live immunisation and although there was marked down-regulation of the cell-mediated immune response around mid-gestation, vertical transmission was prevented. Another challenge trial, this time conducted on Day 70 of gestation, found that chronically infected cows did not abort (although 3 of 5 calves were born seropositive) while naïve cattle challenged at the same stage of gestation aborted (Williams et al. 2003). Although chronically infected cows have an increased risk of abortion there appears to be an interesting distinction between immunity to exogenous and endogenous transplacental infection (as defined by Trees and Williams 2005).

Continuous passage of *Toxoplasma gondii* in mice has been shown to lead to an inability to sexually reproduce and failure to shed oocysts following infection of a definitive host (Frenkel et al. 1976). A similar methodology was undertaken to attenuate the S48 strain of *T. gondii* which led to the development of Toxovax – a live attenuated vaccine that protects against abortion in sheep (O'Connell et al. 1988). The present trial was undertaken to assess the efficacy of a mouse-passaged strain of *N. caninum* in preventing abortion and vertical transmission in cattle.

8.3 Material and Methods

8.3.1 Experimental Design

Heifers that were consistently seronegative to *N. caninum* were randomly allocated to receive a subcutaneous injection in the anterior neck of either mouse-passaged *N. caninum* tachyzoites (n=13) or serum-free tissue culture media (Invitrogen, Carlsbad, CA, USA; n=13) as a control 42 days before the Planned Start of Mating (PSM). Following inoculation, heifers were monitored clinically and samples were collected for serology and for whole blood polymerase chain reaction (PCR) testing. Pregnancy diagnosis was performed manually and by transrectal ultrasonography to confirm conception. Pregnant heifers were then randomly allocated to receive either a *N. caninum* tachyzoite challenge (intravenous and subcutaneous inoculation; n=12) or an intravenous injection of serum-free tissue culture media (n=11) on Day 70 of gestation, resulting in a 2 x 2 trial design with heifers vaccinated with either mouse-passaged tachyzoites or a placebo and then challenged with either *N. caninum* tachyzoites or a control substance. Clinical monitoring and blood sampling continued along with pregnancy diagnosis to assess fetal viability and to detect abortion. The heifers were grazed as one group on a commercial farm throughout the trial. Dogs were not used in the direct management of the animals but had been present on the farm for many years.

Each heifer was euthanased following fetal mummification, abortion or parturition to allow collection of tissue samples. Blood samples were collected from calves within 12 h of birth; these samples were not necessarily pre-colostral. Thereafter, the calves were euthanased and their brains removed for PCR testing and histopathology. All procedures involving the use of animals were approved by the Massey University Animal Ethics Committee, Palmerston North, New Zealand (MUAEC 05/102).

8.3.2 Passage of *N. caninum* in mice

A strain of *Neospora caninum* (Nc-NZ1; Okeoma et al. 2004c) was propagated in interferon-gamma knockout (IFN-γ *k/o*) mice. Mice were injected intraperitoneally with tachyzoites and euthanased 5 days later by cervical dislocation, after which parasites were recovered by peritoneal lavage. At the conclusion of the 26th passage, the lavage fluid was washed twice in PBS at 1,400 *g* for 10 min, the pellet was re-suspended in 25 mL of Advanced MEM with 2% fetal bovine serum, 2 mM L-glutamine and 30 ug/mL

gentamycin added (all from Invitrogen) and incubated for 1 h at 37°C with 5% CO_2. The media was removed from a flask of Vero cells at approximately 50% confluence and the 25 mL of incubated parasites and media added to the flask. Parasite infected Vero cells were incubated at 37°C with 5% CO_2 for one week until 98% of Vero cells had lysed. *N. caninum* cultures were expanded for use as the cattle inoculum, hereafter referred to as P26NZ1. Heavily infected cells were left to lyse and tachyzoites were pelleted and washed twice by centrifugation at 1,000 *g* for 10 min. The tachyzoites were counted and re-suspended in serum-free tissue culture media to produce each infective dose of mouse-passaged tachyzoites.

8.3.3 Animals

Twenty-six 14-month-old Friesian / Jersey crossbred heifers were obtained from a commercial dairy farm where quarterly serological testing for *N. caninum* antibodies had been undertaken for the past 7 years. From 1997 to 1999 an ELISA developed at Wallaceville Animal Health Laboratory (Upper Hutt, New Zealand) was used (Reichel and Drake 1996) and samples with an optical density of ≥ 0.15 at 450 nm were classified as positive. From 2000 this test was no longer available and it was replaced by the HerdChek* Neospora ELISA (IDEXX Laboratories Inc., Maine, USA). All heifers were seronegative to *N. caninum* when tested quarterly prior to the trial and were the progeny of consistently seronegative dams. All had been vaccinated against clostridial disease (5 in 1, CSL, Melbourne, Australia), leptospirosis (Leptavoid3, Schering Plough Animal Health, Upper Hutt, New Zealand) and bovine viral diarrhoea virus (Bovilis BVD, Intervet, Boxmeer, The Netherlands) before the trial commenced.

Thirteen randomly-allocated heifers received subcutaneous inoculation in the right anterior neck with 2 mL (containing 10^7 parasites/mL) of mouse-passaged Nc-NZ1 42 days before the PSM. The remaining 13 heifers were similarly injected with 2 mL serum-free tissue culture media only.

Oestrous cycles of heifers were synchronised using a standard protocol of an intravaginal progesterone device (CIDR-B; Pfizer Animal Health, Auckland, New Zealand) inserted for 10 days, prostaglandin $F_{2\alpha}$ injection (Estroplan, Parnell Laboratories Ltd., Alexandria, NSW, Australia; 2 mL) 6 days after CIDR-B insertion and oestradiol benzoate injections (Cidirol, Bomac Laboratories Ltd., Auckland, New Zealand; 1 mL) on the day of

CIDR-B insertion and 24 h after CIDR removal. Heifers were run with two 2-year-old Jersey bulls (seronegative for *N. caninum* on a single screening test) for 9 weeks from the time of CIDR-B removal. Fixed-time artificial insemination (AI) was performed 24 h after the final oestradiol benzoate injection when the heifers were expected to be in oestrus. Pregnancy diagnosis was performed weekly by transrectal ultrasonography from 5 weeks after AI to allow estimation of conception dates and to confirm pregnancy until challenge with *N. caninum* tachyzoites on Day 70 of gestation.

8.3.4 Challenge strains

The inoculated tachyzoites were an equal mixture of Nc-NZ1, Nc-NZ2 and Nc-NZ3 isolates (Okeoma et al. 2004c) that had been maintained by periodic passage in Vero cell culture. Heavily infected cells were left to lyse, after which tachyzoites were pelleted and washed twice by centrifugation at 1,000 *g* for 10 min. The tachyzoites were counted and re-suspended in sterile PBS (pH 7.4) to produce each dose. Two mL aliquots were prepared containing equal proportions of the three isolates.

The heifers were challenged with *N. caninum* tachyzoites on Day 70 of gestation via both intravenous and subcutaneous routes. Intravenous inoculation consisted of 2 mL of 1 x 10^9 tachyzoites/mL administered via the jugular vein. The 2 mL subcutaneous inoculation was administered over the left pre-scapular lymph node and consisted of 1 x 10^8 tachyzoites/mL. The skin in this area was clipped to facilitate ongoing assessment for site reactions and methylated spirits was used to clean the skin prior to inoculation. The control group was given 2 mL of serum-free tissue culture media in the jugular vein. To confirm viability of the tachyzoites, an extra dose of inoculum was returned to the laboratory and placed into Vero cell culture.

8.3.5 Clinical Observations

Temperature, heart rate and respiratory rate were recorded and heifers were observed for signs of ill health or behavioural change prior to challenge inoculation, then daily for 7 days after inoculation. Thereafter, heifers were checked twice-daily in their paddock throughout the remainder of the trial. Pregnancy testing occurred twice weekly by trans-rectal ultrasonography for 10 weeks after inoculation and then weekly by manual palpation until 8 months gestation.

8.3.6 Blood and Tissue Sampling

Blood samples for serum were collected from heifers on the day of inoculation with P26NZ1 tachyzoites (42 days before PSM) then fortnightly on 4 occasions and heparinised whole blood was collected on the day of inoculation as well as 7, 14 and 28 days later. In addition, whole blood for serum was collected 14 and 28 days after challenge inoculation with *N. caninum* tachyzoites (i.e. Days 84 and 98 of gestation). Serum and heparinised whole blood were collected from the heifers and the calves born to them within 12 h of parturition. Immediately after blood sampling, the calves were euthanased by intravenous injection of pentobarbitone (Pentobarb 500, Provet NZ Pty Ltd., Auckland, New Zealand), for collection of brain tissue. The brain from each calf was removed, with one hemisphere fixed in 10% formol saline for histopathological examination and the other hemisphere collected and frozen at -20°C for PCR. Equipment used in this collection was cleaned and disinfected between samples. In addition, samples of placental tissue (where available) or vaginal discharge were collected into single-use containers after parturition or abortion, for PCR to detect *N. caninum* DNA.

8.3.7 Serology

Serum was tested for antibody to *N. caninum* using the HerdChek* Neospora ELISA (IDEXX Laboratories Inc, Maine, USA; testing conducted by New Zealand Veterinary Pathology Ltd, Palmerston North) in the pre-trial selection process and throughout the trial. A cut-off in the sample-to-positive (S/P) ratio of 0.21 rather than the standard cut-off of 0.50 was used when selecting heifers for the trial to improve test sensitivity (Reichel and Pfeiffer 2002). Subsequent serological results were considered positive at a S/P ratio of ≥ 0.50.

8.3.8 Polymerase Chain Reaction (PCR)

Whole blood, vaginal discharge and placental samples destined for PCR detection of *N. caninum* DNA were processed using the DNeasy Tissue Kit (Qiagen, Victoria, Australia) as per the manufacturer's instructions for DNA extraction from tissue. Five grams of each fresh fetal, calf or cow brain sample was placed into a sterile, single use, 50 mL conical tissue grinder (VWR, PA, USA) containing 10 mL sterile PBS. Brain tissues were homogenized and 100 µL used for DNA extraction using the DNeasy Tissue Kit.

Single-tube nested *N. caninum* PCR reactions to amplify the ITS1 region were performed as described by Ellis et al. (1999), using the internal (NR1 and NS2) and external (NF1 and SR1) primer pairs, with the following modifications. The 50 μL PCR reaction mixture contained: 5 μL of test sample DNA, 1 x PCR buffer, 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM NS2 and NR1, 0.015 μL NF1 and SR1, and 1 unit of Platinum Taq DNA polymerase (Invitrogen, CA, USA). The thermal cycle conditions were as follows: 95°C for 5 min; five cycles of 94°C for 30 seconds (s), 60°C for 150 s, 72°C for 30 s; 20 cycles of 88°C for 30 s, 60°C for 30 s, 72°C for 30 s, ten cycles of 88°C for 30 s, 54°C for 30 s, 72°C for 30 s; 25 cycles of 86°C for 30 s, 54°C for 30 s, 72°C for 30 s; and 72°C for 10 min. PCR products were run on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen, CA, USA) containing ethidium bromide at 100V for 45 min and visualized under UV light on a transilluminator. DNA extracted from cell-cultured *N. caninum* was used as a positive control and was included with water blanks as a negative control for each PCR run to confirm lack of contamination during sample manipulation.

Positive PCR amplicons were purified (PureLink PCR purification kit, Invitrogen, CA, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, CA, USA). Sequencing results were submitted to the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation of ITS1 gene amplification.

8.3.9 PCR amplification of microsatellite (MLST)-containing sequences of *Neospora caninum* from bovine tissues

Positive DNA samples by *N. caninum* nested-PCR from tissues were analysed for the microsatellites MS1B, MS2, MS3, MS4 and MS5 as described by Basso et al. (2009). DNA extracted from cell-cultured *N. caninum* Nc-NZ1 was used as a positive control and nuclease free water was included as a negative control for each PCR run. PCR products were visualised, purified and subjected to automatic dye-terminator cycle sequencing as described above. MLST sequencing results were compared to previously published sequences (Regidor-Cerrillo et al. 2006; Basso et al. 2009; Pedraza-Díaz et al. 2009).

8.3.10 Histopathology

The formalin-fixed samples of one hemisphere from the brain of each calf and heifer were trimmed and tissue blocks from each of the brain stem, cerebellum, midbrain and cerebrum were embedded in paraffin wax, cut into 3 μm sections and stained with H&E for histopathological examination.

8.3.11 Statistics

All data were recorded using Microsoft Excel 2007 (Microsoft Corporation, WA, USA). Fisher's exact test was used to compare binomial outcomes between the groups for significance.

8.4 Results

8.4.1 Clinical observations

None of the heifers had an elevated rectal temperature (> 39.5°C) following vaccination with either the mouse-passaged strain of *N. caninum* tachyzoites or the serum-free tissue culture media. Two of 13 heifers injected with the control substance had a mild site reaction (subcutaneous swelling of < 5 mm height) that was noted only on a single day (one 3 days and the other 5 days) after treatment. Twelve of 13 heifers that were inoculated with the passaged tachyzoites exhibited a site reaction within 3 – 7 days of treatment (average = 3.9 days). Six of these were mild and persisted for 1-11 days (average = 4.3 days) the other 6 had a mild site reaction that became moderate (height of subcutaneous swelling 5 – 10 mm) for 1 – 3 days. The duration of the site reaction was at least 10 days in five of six heifers and at least 25 days in two of six heifers.

Ten of 13 heifers inoculated with mouse-passaged *N. caninum* tachyzoites conceived at the start of the mating period, as did 12 / 13 animals that were inoculated with serum-free tissue culture media. Two of 13 heifers inoculated with mouse-passaged *N. caninum* tachyzoites did not conceive after being run with bulls for 9 weeks. When pregnancy was confirmed, heifers were randomly allocated to receive either a *N. caninum* tachyzoite challenge or serum-free tissue culture media on Day 70 of gestation. The treatments for each group of animals are detailed in Table 8.1.

Table 8.1 Treatment protocol for dairy heifers that were seronegative to *Neospora caninum* and that were mated at 16 months old.

Group	Inoculation 42 days before mating	Challenge on Day 70 of gestation	*n*
A (N / N)	Mouse-passaged *N. caninum* tachyzoites (subcutaneous)	*N. caninum* tachyzoites, intravenous and subcutaneous	6
B (N / -)	Mouse-passaged *N. caninum* tachyzoites (subcutaneous)	Serum-free tissue culture media, intravenous and subcutaneous	5
C (- / N)	Serum-free tissue culture media	*N. caninum* tachyzoites, intravenous and subcutaneous	6
D (- / -)	Serum-free tissue culture media	Serum-free tissue culture media, intravenous and subcutaneous	6

After inoculation with *N. caninum* tachyzoites or serum-free tissue culture media on Day 70 of gestation, 4 heifers from Group A had an elevated rectal temperature (> 39.5°C). In 3 animals this occurred after 24 hours and one heifer also had an elevated temperature on Day 4 after challenge. The fourth heifer had an elevated temperature only on the second day after challenge. One group C heifer had an elevated temperature on the third day after challenge.

Fewer and smaller injection site reactions were seen after challenge on Day 70 of gestation. Mild reactions (< 5 mm swelling) were seen in two of six Group A animals, two of five Group B animals, four of six Group C animals and one of six Group D animals and these were observed on only one day in six of nine animals. A moderate reaction was observed in a Group C heifer that had a mild reaction on Days 1 and 4 after challenge and then a moderate reaction on Day 5.

8.4.2 Serology

Serological results are summarised in Table 8.2. Two weeks after inoculation with mouse-passaged *N. caninum* tachyzoites, 10/13 heifers were seropositive and, after a further 2 weeks, all inoculated heifers were seropositive. No heifer that received cell-free tissue culture media seroconverted. Following challenge with *N. caninum* tachyzoites on Day 70 of gestation, all six heifers in Group A showed a further increase in antibody titre (as measured by the sample to positive [S/P] ratio in the ELISA). Among the Group B

heifers, four of five had declining antibody titres and the remaining heifer had no change. All six Group C heifers seroconverted within 28 days of challenge while all six heifers in Group D remained seronegative. All heifers in Groups A, B and C remained seropositive until abortion or parturition while all group D heifers were seronegative at parturition.

8.4.3 Abortion and parturition

Pregnancy outcome and calves' serological status to *N. caninum* are shown in Table 8.2. Eight abortions occurred among the 23 heifers that comprised the study group; being two of six heifers in Group A (at 49 and 62 days after the tachyzoite challenge), one of five heifers in Group B and five of six heifers in Group C (no statistically significant difference occurred between Groups A and C; p=0.24). The heifer in Group B was found to have aborted 92 days after challenge and, despite concerted efforts, the fetus was not found. The fetuses in the Group C heifers were identified as undergoing mummification between 38 and 69 days after challenge (average = 55.8).

The four calves born to the Group A heifers at full-term were all seropositive for *N. caninum*. The four remaining heifers in Group B calved at full term with three of four calves being seropositive. The fourth calf had a S/P ratio of 0.47 (the cut-off for the ELISA being 0.50). The remaining heifer in Group C calved at full-term and delivered a seronegative calf. All six Group D heifers delivered live calves, two of which were seropositive.

8.4.4 Polymerase chain reaction and microsatellite sequencing

Samples of whole blood collected from the 13 heifers allocated to inoculation prior to breeding with *N. caninum* tachyzoites previously passaged through mice were positive for *N. caninum* DNA in five instances 7 days after inoculation and one heifer was still positive a further 7 days later. Of the 13 heifers that were inoculated with serum-free tissue culture media on the same day, five were positive by PCR 7 days later and two of these were still positive 14 days after inoculation. No whole blood samples collected 28 days after inoculation were positive.

PCR results from tissue samples collected within 12 h of parturition are shown in Table 8.2. Samples of placenta or vaginal discharge from 6 / 8 aborting heifers gave positive PCR results for *N. caninum* DNA, as did the fetal brain samples from the 3/5 samples that were able to be tested. The samples of brain from one aborting heifer (C2) and one (B1) that delivered a full-term, seronegative calf also gave positive PCR results. In addition, vaginal discharge from heifer D5 (calf was seropositive when tested despite the dam being consistently seronegative) was positive by PCR.

Microsatellite typing was subsequently performed on positive PCR-amplicon samples once the technique became available for use (these samples had been stored for 4 years). Results are shown in Table 8.3. Only the reverse primer could be used in the MS3 marker region. A sample from each case of abortion that yielded a positive PCR result was analysed; this included placenta from each of the aborting heifers in Group C, fetal brain from heifer A5 and the brain from heifer B1 which did not abort. The *Neospora* amplicon in the brain from heifer B1 could not be typed as there were multiple overlying peaks on the electropherogram, suggesting that either multiple strains or contamination was present. Similarly, the placenta from heifer C1 could only be typed using MS2. All other samples had an allele pattern that was identical to that of one of the challenge strains, except for the placenta from heifer C6. This differed from the challenge strains at MS2, having a repetitive sequence of 6/10/2 rather than 6/11/2 or 6/9/2.

Table 8.2 Serological and PCR results at parturition or abortion from heifers that were inoculated with either a mouse-passaged strain of *Neospora caninum* (A & B) or serum-free tissue culture media (C & D) before mating and then were challenged with *N. caninum* tachyzoites (A & C) or serum-free tissue culture media (B & D) on Day 70 of gestation.

Animal ID	Heifer serostatus (S/P ratio)*	Days to abortion	Calf serostatus (S/P ratio)*	Calf brain PCR	Heifer brain PCR	Placenta / vaginal discharge PCR
A1	Pos (3.97)	-	Pos (4.17)	Neg	Neg	Neg
A2	Pos (1.92)	-	Pos (1.71)	Neg	Neg	Neg
A3	Pos (1.95)	-	Pos (2.66)	Neg	Neg	Neg
A4	Pos (2.87)	62	NA	NA	Neg	Neg
A5	Pos (2.53)	49	NA	Pos	Neg	Pos
A6	Pos (2.22)	-	Pos (2.47)	Neg	Neg	Neg
B1	Pos (0.67)	-	Neg (0.47)	Neg	Pos	Neg
B2	Pos (1.42)	-	Pos (2.04)	Neg	Neg	Neg
B3	Pos (1.56)	92	NA	NA	Neg	Neg
B4	Pos (0.78)	-	Pos (1.32)	Neg	Neg	Neg
B5	Pos (2.03)	-	Pos (2.21)	Neg	Neg	Neg
C1	Pos (2.40)	38	NA	Neg	Neg	Pos
C2	Pos (2.43)	55	NA	Pos	Pos	Pos
C3	Pos (0.97)	-	Neg (-0.11)#	Neg	Neg	Neg
C4	Pos (2.09)	55	NA	Neg	Neg	Pos
C5	Pos (3.22)	62	NA	Pos	Neg	Pos
C6	Pos (2.45)	69	NA	NA	Neg	Pos
D1	Neg (-0.11)	-	Pos (0.76)	Neg	Neg	Neg
D2	Neg (0.03)	-	Neg (-0.23)#	Neg	Neg	Neg
D3	Neg (-0.01)	-	Neg (0.26)	Neg	Neg	Neg
D4	Neg (-0.16)	-	Neg (-0.07)#	Neg	Neg	Neg
D5	Neg (0.03)	-	Pos (0.62)	Neg	Neg	Pos
D6	Neg (-0.23)	-	Neg (-0.24)#	Neg	Neg	Neg

* Samples are classified as positive at a Sample to Positive (S/P) ratio of ≥ 0.50
# Sample was pre-colostral
NA = not available

Table 8.3: Summary of results of microsatellite alleles obtained from bovine tissue.

DNA Sample	Tissue source	Microsatellite length (bp) (MS3, MS4 and MS5) or sequence (MS1B and MS2)				
		MS1B	**MS2**	**MS3**	**MS4**	**MS5**
		$(AT)_xAC(AT)_y$	$(AT)_xTTGTATC(AT)_yGT(AT)_z$	$(AT)_n$	$(AT)_n$	$(TA)_n$
Nc-NZ1	Cow brain	8/3	6/9/2	12	15	9
Nc-NZ2	Calf brain	8/3	6/11/2	14	15	9
Nc-NZ3	Calf brain	8/3	6/11/2	14	15	9
P26NZ1 Mouse attenuated	Tissue culture	8/3	6/11/2	14	15	9
A5	Calf brain	8/3	6/11/2	14	15	9
B1	Cow brain	*	*	*	*	*
C1	Placenta	*	6/11/2	*	*	*
C2	Placenta	8/3	6/11/2	14	15	9
C4	Placenta	8/3	6/11/2	14	15	9
C5	Placenta	8/3	6/11/2	14	15	9
C6	Placenta	8/3	6/10/2	14	15	9
Nc-Liv[a]		8/3	5/11/2	14	20	11
Nc-1[b]		9/3	7/10/2	16	12	12

*Not able to be typed
a As described in Regidor Cerrillo et al. 2006; GenBank AY935166-170
b As described in Basso et al. 2009; GenBank EU

8.4.5 Histopathology

Between one and three perivascular cuffs were seen in the brains of three of the eight heifers that aborted (A5, C5 and C6). Another heifer that aborted (C2) had mild, locally extensive gliosis, with a few small, perivascular cuffs in the region of the hypothalamus near Rathke's pouch (pituitary stalk). Also, in a section from the cerebellum, there was a focus of three adjacent, large, protozoal cysts: these were not associated with an inflammatory reaction in the granular layer (grey matter). The largest of these was 160 µ long x 70 µ wide and the zoites within were 3 – 5 µ wide and 10-12 µ long and resembled those of *Sarcocystis* spp. No abnormalities were seen in the brain from the other 4 heifers that aborted or in any of the remaining heifers or calves.

A cotyledon was collected for examination from each heifer that aborted. Two showed mild lesions with infiltration of lymphoctyes and plasma cells into the caruncular endometrium (Heifers A4 and C1). The placental tissue from the other aborting heifers had more obvious signs of inflammation, with extensive necrosis of cotyledonary tissue, clumps of neutrophils interspersed with mucus and fibrin, and mineralisation of aggregates of trophoblastic cells. The placentome from Heifer B3 had numerous thrombi within larger blood vessels, together with necrosis and haemorrhage of the cotyledon which had led to infarction of the placentome.

No protozoa were seen in any of the sections examined apart from in the cerebellum of Heifer C2. All fetuses submitted for histopathological examination were markedly autolysed and although sections of their brain were examined, no histological structure was apparent.

8.5 Discussion

An ideal vaccine against bovine neosporosis would prevent both *de novo* infection and recrudescence of existing infection in animals that may have been infected congenitally or after birth. This in turn would prevent both abortion due to neosporosis and vertical transmission of the parasite. The prevention of abortion alone would result in a need for ongoing control due to the high levels of vertical transmission (up to 95%; Davison et al. 1999a). Previous challenge studies in cattle that were infected with *N. caninum* prior to mating appeared to achieve at least one of these objectives (Williams et al. 2000; Innes et

al. 2001a Williams et al. 2003; McCann et al. 2007; Williams et al. 2007), indicating that live immunisation may be the most promising prospect to date in vaccine development. By contrast, in the present study, two of six heifers that had been inoculated before conception with *N. caninum* tachyzoites that had been passaged through mice, aborted after challenge on Day 70 of gestation. There appeared to be no effect on transplacental transmission since three of four of the calves born to heifers that received only inoculation before mating were seropositive, suggesting that endogenous transplacental transmission had occurred. Differences between the present study and those previously reported in the literature include (i) the strain of *Neospora caninum* used to stimulate immunity before conception and (ii) the challenge dose, which was both larger and by both intravenous and subcutaneous routes.

The fact that one of five heifers that were given only mouse-passaged tachyzoites before conception aborted (i.e. which were not subsequently given an experimental *Neospora* challenge; Group B) shows that inoculation with tachyzoites prior to mating may not be a suitable control strategy. Whilst the proportion of heifers that aborted in Group A (two of six) was less than that in Group C (challenged on Day 70 of gestation without administration of mouse-passaged tachyzoites before conception; five of six heifers), there was no evidence that the administration of mouse-passaged tachyzoites had conferred a sufficient level of protection against *Neospora* abortion to support this as a useful method of vaccinating cattle against the disease.

In the present study, a challenge dose of 2×10^9 tachyzoites was injected intravenously in addition to a subcutaneous dose over the left prescapular lymph node of 2×10^8 on Day 70 of gestation. Other authors have used up to 1×10^7 tachyzoites intravenously as a challenge dose at this stage of gestation, to induce abortion in almost all animals (Williams et al. 2000; Innes et al. 2001a; Williams et al. 2003; Williams et al. 2007; Gibney et al. 2008). A higher challenge dose was selected for the present study as an earlier trial that was conducted to test the challenge model with the New Zealand *N. caninum* isolates resulted in only one of eight heifers aborting following an intravenous challenge of 1×10^8 tachyzoites (as reported in Chapter Seven in this thesis). It is therefore possible that the challenge dose used in the present study overwhelmed any protective effect of the pre-mating immunisation.

Difficulties associated with challenge studies of putative vaccines include the monetary and ethical cost in running such trials in cattle, the generation-interval for cattle and the fact that most challenges involve intravenous inoculation of tachyzoites and this does not mimic the accepted natural routes of infection, being via ingestion of oocysts or transplacental infection. The natural dose that is infectious to cattle is also unknown, so it is impossible to accurately judge if an artificial challenge mimics what happens under field conditions.

A major limitation of the present study was the inability to collect pre-colostral serum from the calves. This was a logistical difficulty because cattle were left to calve naturally while grazing at pasture and insufficient staff were available to continuously observe the animals through this period. Pre-colostral serum would have enabled us to document whether antibodies detected when the calf was sampled were due to transplacental infection or were maternally-derived. The inability to sample the fetus from one cow in Group B that aborted (immunised pre-mating with tachyzoites then challenged with a placebo on Day 70 of gestation) meant that it was not possible to determine the cause of that abortion.

The complex interactions between *Neospora caninum*, the bovine fetus and the maternal immune system have previously been documented (Innes et al. 2005; Williams and Trees 2006), but are still not fully understood. The outcome of parasitaemia in a pregnant host is affected by the downregulation of immunity in the dam with a bias towards the regulatory Th2-type response (Entrican 2002), which impairs the ability of the host to mount an effective immune response. In addition, the development of fetal immunocompetence means that infection later in gestation is less likely to prove fatal to the fetus although it commonly results in the birth of a calf that is congenitally infected (Williams et al. 2000). Hence, the timing, duration, infective dose and strain of parasitaemia also influence the outcome in a pregnant animal.

There is evidence that some degree of protective immunity occurs against neosporosis inasmuch as previously infected cattle are less likely to abort than are their naïve herdmates following a point-source exposure to the parasite (McAllister et al. 2000). Moreover, the risk of abortion in infected animals is highest in their first pregnancy and decreases with subsequent pregnancies (Thurmond and Hietala 1997a). However, repeat abortion can

occur (Moen et al. 1998) and repeated vertical transmission is common (Barr et al. 1993). Experimental challenge studies have shown that infection with *N. caninum* tachyzoites prior to conception prevented transplacental infection when cattle were subsequently challenged during pregnancy (Innes et al. 2001a; Williams et al. 2003) i.e. exogenous transplacental infection was prevented. However, there is little evidence that maternal immunity can prevent endogenous transplacental infection (Trees and Williams 2005), so successive generations of congenitally-infected heifers maintain *N. caninum* infection within the herd. These observations have led to attempts to identify non-pathogenic strains of *N. caninum* which could be used to immunise cattle prior to mating with no ill-effects in their offspring.

In the present study, seven of eight calves born to heifers inoculated with passaged tachyzoites before mating were seropositive when sampled shortly after birth (< 12 hours). As pre-colostral sampling could not be conducted, it is possible that maternal antibody was detected, although the level of antibody in most of these calves was sufficiently high (Table 8.2) to suggest a fetal source. Hietala and Thurmond (1999) used a kinetic ELISA (cutoff in the S/P ratio of 0.45) to show that S/P ratios were higher in congenitally infected calves (0.56 – 1.88, mean 0.99 +/- 0.35); than in calves sampled at 2-5 days old which were not infected but had ingested pooled colostrum (0.04 – 1.53, mean 0.49 +/- 0.41).

Another similar trial reported that immunisation of six heifers 9 weeks before mating with live Nc-Nowra prevented fetal death when the heifers were subsequently challenged on Day 70 of gestation with Nc-Liverpool (Williams et al. 2007). The calves born to these heifers showed no evidence of infection with *N. caninum*, being serologically and PCR negative. These tachyzoite challenges did not appear to result in persistent infection of the heifers as they were negative on all samples tested by PCR. The same group of authors have also reported that infection of cattle before pregnancy with either tachyzoites or occysts did not result in persistent infection or subsequent endogenous transplacental transmission (Williams et al. 2000; McCann et al. 2007).

Neospora caninum DNA was found in whole blood from heifers in this trial seven days after inoculation with *N. caninum* (five of 13 heifers) and in vaginal discharge at parturition from Heifer D5 that was inoculated with serum-free tissue culture media at both treatment points. Additionally, the calves from Heifers D1 and D5 were seropositive when sampled

shortly after birth despite their dams being seronegative at that time and throughout the trial. *Neospora caninum* DNA has been reported in the blood of seronegative cattle (McInnes et al. 2006a) as well as in blood and tissue from cattle in the control group of the study reported in Chapter Seven of this thesis. Single-use sampling materials and standard laboratory protocols were used to prevent the contamination of samples and all positive PCR amplicons were sequenced and analysed by microsatellite array analysis for comparison against the strains of *N. caninum* that were used in the study.

There is considerable evidence of strain variation in *Neospora* isolates. In the present study, Nc-NZ1 differed from Nc-NZ2 and Nc-NZ3 at the MS2 and MS3 marker regions. Moreover, after passage through mice, the strain of Nc-NZ1 differed from the native type and more closely resembled Nc-NZ2 and Nc-NZ3 at the MS2 and MS3 regions. Similarly, the MS2 region of the strain of *Neospora* that was isolated from the placenta of Heifer C6 differed from any of those that were administered to the heifers or which were isolated from other tissues. The presence of this strain in Heifer C6 suggests that either this strain was also present in the tachyzoite challenge that was administered on Day 70 of gestation or that this was a result of natural infection during the trial. The differences between the Nc-NZ1 and Nc-NZ2 isolates are the more interesting in that they were recovered from an individual cow-calf pair (Okeoma et al. 2004c). In other words, differences in microsatellite length between the strains used in, and recovered during the present study suggest that different strains of *N. caninum* exist between farms, between and within individual animals and, probably, within isolates. Different strains of *N. caninum*, identified by microsatellite analysis, have been reported within and between regions in both Germany and Spain, isolated from bovine fetuses and dog faeces (Basso et al. 2009; Pedraza-Díaz et al. 2009). It may be that some strains are more virulent in certain species or tissues (for example mice and bovine fetuses) and replicate more successfully, thereby being more likely to be isolated from PCR amplicons. Repeated microsatellite typing of the New Zealand isolates should be carried out to confirm the presence of multiple strains in these field-derived samples and other naturally-occurring New Zealand isolates should also be characterised to assess the level of genetic diversity in bovine, ovine and canine populations. A final observation of note from this study relates to the microsatellite analysis of the New Zealand strains of *Neospora caninum*. The New Zealand strains had previously been reported as having the same repetitive sequences at Tand-3 (equates to MS10), Tand-12 and Tand-13 as Nc-Liverpool (Al-Qassab et al. 2009). The present results

show that the New Zealand isolates are genetically distinct from Nc-Liverpool, on the basis of sequence differences at the MS2, MS4 and MS5 regions.

The development of the vaccine against ovine toxoplasmosis centred around production of an attenuated strain (S48) of *T. gondii* from an aborted ovine fetus (Wilkins et al. 1987). This strain was maintained as tachyzoites passaged about 3,000 times through mice (twice-weekly) until the strain was shown to be unable to develop bradyzoites in tissue cysts and incapable of oocyst production following infection of the definitive host (Buxton and Innes 1995). A similar approach may prove fruitful in the development of a live, attenuated *Neospora* vaccine but would be time-consuming. A live vaccine developed from a strain isolated from New Zealand cattle would have the benefit of being guaranteed free from contamination with prions of the transmissible spongiform encephalopathies. The present study does not support the current literature suggesting that pre-mating infection of naïve cattle prevents abortion when cattle receive an exogenous *N. caninum* tachyzoite challenge. Further studies need to be performed to determine whether an attenuated strain of *N. caninum* tachyzoites, or one of low virulence, could be developed as a live vaccine against bovine neosporosis.

8.6 Conclusion

Pre-mating inoculation of heifers that were previously seronegative to *Neospora caninum* with live tachyzoites did not prevent fetal death or transplacental transmission following tachyzoite challenge on Day 70 of gestation. In addition, infection of heifers prior to mating resulted in endogenous transplacental infection in three of four calves born to the heifers in Group B. These results are in contrast to similar trials that have been reported (Williams et al. 2000; Innes et al. 2001a; Williams et al. 2007). It is feasible that the New Zealand strains of *N. caninum* are of lower virulence, given the high challenge dose that was required to induce abortion in heifers in Group C. In addition, the New Zealand strains are genetically distinct to other strains that have been identified by microsatellite typing.

8.7 Acknowledgements

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8.8 Authors' contributions to this study

J.F. Weston initiated the trial design, was responsible for running the trial and carried out most of the animal treatments and sampling and wrote the manuscript.

L. Howe conducted the mouse passage, supervised the PCR and microsatellite array analysis and provided editorial advice.

N.B. Williamson assisted with trial design and animal sampling and provided editorial advice.

W.E. Pomroy assisted with trial design and animal sampling and provided editorial advice.

M.G. Collett examined histopathological sections and provided editorial advice.

T.J. Parkinson provided editorial advice.

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

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

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

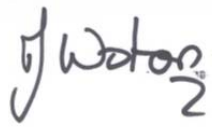
Name of Candidate: Jennifer F. Weston

Name/Title of Principal Supervisor: Professor Norman B. Williamson

Name of Published Paper: Inoculation of cattle with a strain of *Neospora caninum* tachyzoites after passage through mice does not prevent fetal deaths

In which Chapter is the Published Work: Eight

What percentage of the Published Work was contributed by the candidate: 75%.



29th August 2011

Candidate's Signature

2nd September 2011

Principal Supervisor's signature

Chapter Nine

General Discussion

9.0 General Discussion

The work presented in this thesis assessed control options for *Neospora caninum* that could be of practical use on commercial dairy farms. Current recommendations to control *N. caninum* revolve around limiting contact between dogs and cattle, identification and culling of infected animals to reduce herd seroprevalence or, if this is not possible, identification of infected animals and not keeping calves from them, and embryo transfer to prevent vertical transmission. Research has been ongoing to develop a vaccine to prevent abortion and/or vertical transmission or to develop chemotherapeutics that will inactivate all stages of the parasite in a chronically infected host. However, no products are currently licensed to control neosporosis in cattle.

The experiments described in this thesis aimed to:

(i) Calculate the risk of abortion in primiparous heifers that were seropositive to, and probably congenitally-infected with, *N. caninum*, as one of the main control methods that has been proposed is the removal of seropositive animals from infected herds.

(ii) Determine the efficacy of a commercial, killed tachyzoite vaccine in reducing abortion and vertical transmission in five herds with a history of abortion associated with *N. caninum*.

(iii) Document causes of abortion on farms with endemic *N. caninum* infection to determine the relative contributions of *N. caninum* and other causes to the incidence of abortion.

(iv) Develop a challenge model using the New Zealand strains of *N. caninum* and assess its virulence in sheep as a prelude to challenge studies in cattle.

(v) Test the hypothesis that cattle with oral lesions may acquire infection by tachyzoites, supporting hypothesised transmission from parturient or aborting cattle which are infected with *N. caninum*.

(vi) Conduct a pilot study to assess whether a mouse-passaged strain of *N. caninum* tachyzoites would protect against fetopathy and vertical transmission when pregnant heifers were subsequently challenged with *N. caninum* tachyzoites on Day 70 of gestation.

9.1 Specific contributions to the field of knowledge from research presented in this thesis

The most important finding from this work was evidence that direct horizontal transmission of *N. caninum* between cattle appears possible. Even if this mechanism of transmission is responsible for only a small proportion of infections in cattle, it should be considered when modelling *N. caninum* infection within a herd. The management of cattle at calving could be altered to minimise the risk of horizontal transmission (e.g. by reducing stocking rate or limiting contact between cattle). The possibility of direct horizontal transmission between cattle also increases the benefit of removing infected animals from the herd as not only are they likely to produce congenitally-infected calves and be a source of infection for dogs on the farm, but they may be infective for other cattle.

Another important finding was the particularly high relative risk of abortion among primiparous heifers that were seropositive to *N. caninum* compared to their seronegative herdmates. If this finding is applicable to other infected herds, then identification and removal of replacement heifers that are congenitally infected with *N. caninum* is likely to be a cost-effective control option. Alternatively, identifying infected cattle within the herd and not breeding replacement heifers from them would avoid the difficulties of assessing serostatus in young calves when the interpretation of serological results can be confused by the presence of colostral antibody.

The efficacy of a killed tachyzoite vaccine to prevent abortion in herds with endemic *N. caninum* infection was variable. Many of the abortions that occurred in these herds showed no evidence of being due to neosporosis. Thus, ongoing diagnostic efforts should be made to achieve an aetiological diagnosis in as many cases of abortion as are possible on farms suffering higher than expected abortion losses. Some causes of abortion may be more easily prevented than is currently feasible for neosporosis. Evidence of more than one infectious agent being present in some cases of abortion makes it difficult to interpret the importance of *N. caninum* infection. A greater understanding of how other infectious agents and stressors impact the outcome of pregnancy in cattle infected with *N. caninum* could lead to an overall reduction in abortion incidence.

Positive PCR results were obtained from a number of consistently seronegative cattle (and their offspring) from the control groups of the two challenge trials (reported in Chapters Seven and Eight). This highlights the difficulties associated with determining *N. caninum* infection status in cattle, as it appears that some cattle may produce very little or no antibody when infected (McInnes et al. 2006a). It is recognised that antibody concentrations in cattle can fluctuate and, at times, may drop below detectable limits (Bartels et al. 2005), but it is possible that some infected cattle may never seroconvert. It appeared that parasitaemia was transient, with most cattle having parasite DNA detected in their blood on a single occasion when sampled weekly (Sections 7.3.4 and 8.3.4. in this thesis). This means that identifying truly non-infected cattle for research or clinical purposes may not be possible. The significance of infections that do not provoke an antibody response is unknown, as it is well-established that animals that are seropositive to *N. caninum* have an increased risk of abortion. This finding provides support for the hypothesis that it is the maternal immune response to *Neospora* infection that is deleterious to the maintenance of pregnancy, rather than the infection itself. On the other hand, positive PCR results in previously seronegative animals may be explicable in terms of evidence for the direct horizontal transmission of *Neospora caninum* between cattle. Such results may indicate the early stages of infection (parasitaemia) which may or may not result in persistent infection.

Inoculation with a mouse-passaged strain of *N. caninum* tachyzoites prior to mating provided limited protection against fetopathy when heifers were subsequently challenged on Day 70 of gestation. This finding was at variance with one earlier challenge study, in which inoculation with *N. caninum* tachyzoites before mating prevented abortion (although different strains of *N. caninum* and lower dose rates were used for that challenge; Williams et al. 2007). A further complication of challenge studies is that it is not known what level and route of challenge mimics natural infection.

9.1.1 Control of *Neospora caninum* infection by culling infected cattle

The relative risk (RR) of abortion among primiparous heifers that were seropositive to *N. caninum* compared to seronegative heifers of 23.6 (95% CI: 8.5 – 66.0; Weston et al. 2005) was higher than has previously been reported. A study in California found that congenitally-infected heifers had a 7.4-fold higher risk of abortion in their first pregnancy compared to non-infected heifers (Thurmond and Hietala 1997a; López-Gatius et al. 2005a). Other studies have reported that seropositive cows are approximately 2-3 times more likely to abort than seronegative cows (Paré et al. 1996; Moen et al. 1998); and that the risk of abortion is highest in primiparous heifers, with the risk of abortion decreasing with increasing parity (Thurmond and Hietala 1997a; Hernandez et al. 2002; López-Gatius et al. 2005a). The relative risk will vary according to the serological assay used and the cut-point that is applied (Waldner et al. 1998; Schares et al. 1999a). The heifers that were seropositive to *N. caninum* in the study reported in Chapter Three (using a commercial ELISA and a standard S/P ratio cut-point) had a risk of abortion of 0.65 (95% CI: 0.42 – 0.87) compared to similar studies reported by Stenlund et al. (1999), in which the risk of abortion was 0.17 (95% CI: 0 – 0.38) and Thurmond and Hietala (1997a) in which the risk of abortion was 0.13 (95% CI: 0.09 – 0.20; RR = 7.4).

A higher risk of abortion among heifers that are seropositive to *N. caninum* makes the losses associated with such infection more costly. A partial budget that considers two alternative approaches to *N. caninum* control in the heifers reported in Chapter Three of this thesis is shown in Table 9.1. The financial return from two control options was calculated: i) test calves for antibody to *N. caninum* at 4 months of age (when colostral antibody will have waned; Hietala and Thurmond 1999) and cull infected calves, ii) as for the first scenario but additionally, purchase pregnant replacement heifers at 20 months of age to maintain herd size. The second scenario maintains the ability to cull mixed-age cows for poor performance and should maintain expected herd milk production levels in the coming season. These control options are compared to what happened on the farm in the season that the study reported in Chapter Three was conducted, since although the serostatus of the heifers was determined, no management changes were made.

Assumptions for the partial budget are as follows:

- Cost of serology per animal $25
- Value of culled calves at 4 months old $400
- Purchase of replacement heifers at 20 months old $1200
- Slaughter value of heifers at 28 months old $700
- Feed costs from 4 – 28 months old $750
- Primiparous heifer milk production 280 kgMS
- Milk payout $6 /kgMS
- Cost of milk production $3 /kgMS

Table 9.1 Example of a partial budget that considers two alternative approaches to *N. caninum* control in a group of heifers in which 18 of 164 are seropositive to *N. caninum*. Eleven seropositive heifers aborted as did 4 seronegative heifers.

Identify and cull seropositive calves with no replacement		Identify and cull seropositive calves then replace with pregnant heifers at 20 months of age	
Extra costs		**Extra costs**	
Serological testing	4,100	Serological testing	4,100
		Purchasing pregnant heifers at 20 months old	13,200
		Feeding costs 20-24 months old	1,650
Returns no longer obtained		**Returns no longer obtained**	
Slaughter value at 28 months old	7,700	Slaughter value at 28 months old	7,700
TOTAL COSTS	**11,800**	**TOTAL COSTS**	**26,650**
Extra returns		**Extra returns**	
Sale of calves at 4 months old	4,400	Sale of calves at 4 months old	4,400
		Extra milk produced (minus costs of production)	9,240
Costs no longer incurred		**Costs no longer incurred**	
Feeding costs 4-28 months old	8,250	Feeding costs 4-28 months old	8,250
TOTAL BENEFITS	**12,650**	**TOTAL BENEFITS**	**21,890**
BENEFITS - COSTS	**850**	**BENEFITS - COSTS**	**-4,760**

The first scenario in the partial budget presented in Table 9.1 considers only the rearing cost of the heifers that abort in association with being seropositive to *N. caninum* and shows an economic benefit in identifying and selling infected calves ($850). Dairy farming systems are complex entities and modelling ongoing costs and benefits from changes in management is difficult, particularly in pasture-based systems where feed supply is primarily weather-dependent as opposed to a system where feed is purchased according to demand. A reduced stocking rate on the farm due to non-replacement of aborting heifers may result in increases in individual animal production such that total milk production is not affected but this would only occur if the farm was over-stocked. Another possible benefit of a reduced stocking rate would be an improvement in cow body condition which may improve herd fertility and future milk production in a seasonally-calving system.

An immediate benefit from the strategy is the income from the sale of seropositive calves. An economic benefit that is difficult to quantify is the reduction in proportion of calves that are congenitally-infected (from the seropositive heifers that do not abort) although, on most dairy farms in New Zealand, replacement calves are not kept from heifers. Probably the greatest economic benefit from the identification and removal of cattle infected with *N. caninum* would be the more rapid reduction in herd seroprevalence with a resultant decrease in abortions. A policy of annual culling of infected cattle has been shown to be the most effective method of control in the short term (French et al. 1999; Hasler et al. 2006b) although the economic feasibility of this will vary depending on the value of livestock and their products (Reichel and Ellis 2006; 2008). A policy of not keeping replacements from infected cattle will also reduce herd seroprevalence in the medium term and avoids the high cost of replacing culled cattle (French et al. 1999). The cost of sampling cattle to identify those that are infected with *N. caninum* are relatively high and the sensitivity of the available tests means that not all infected cattle will be identified if a herd is sampled on one occasion (Bartels et al. 2005). Modification of the interpretation of serological tests to improve sensitivity will compromise specificity and result in higher costs due to the unnecessary culling of some cattle.

The second scenario presented in Table 9.1 shows that the benefits of replacing the calves that were seropositive and maintaining the number of heifers entering the herd are outweighed by the costs. This partial budget does not account for the ongoing costs and benefits from the ability to cull mixed-age cows, maintain herd size and thus milk production as well as reducing herd seroporevalence to *N. caninum* infection. The direct

production losses and treatment costs in herds infected with *N. caninum* have been calculated to be similar to those in herds infected with Johne's disease (*Mycobacterium avium* subspecies *paratuberculosis* infection) and bovine viral diarrhoea virus infection (Chi et al. 2002). Deterministic simulation modelling in 1998 calculated a median annual loss of $5,400 on New Zealand dairy farms and that on 70% of infected farms, the annual loss would be at least $3,000 (Pfeiffer et al. 1998). Since that time, average herd size increased from 229 to 322 in 2006 (Livestock Improvement Corporation Ltd., New Zealand National Dairy Statistics annual reports) when analysis of the economic benefits of controlling *N. caninum* infection suggested that at 5% seroprevalence, bovine neosporosis costs the average New Zealand dairy farm NZ$2,897.50 annually which rises to NZ$25,375 annually at 50% seroprevalence (Reichel and Ellis 2006). A model that considered data from dairy farms in the Maritime Provinces of Canada calculated that total annual costs from neosporosis in an average, infected 50 cow herd was $2,304 (Chi et al. 2002). These ongoing costs mean that purchasing replacement heifers to maintain replacement rate would probably be cost effective at a seroprevalence of 11% and a risk of abortion of 0.65 among seropositive heifers.

The financial analysis of strategies for the control of bovine neosporosis is further complicated by the variation in rates for vertical and horizontal transmission that have been reported. Vertical transmission rates from as low as 30% (Dijkstra et al. 2001b) through to > 95% (Baillargeon et al. 2001; Campero et al. 2003) have been reported. Rates of horizontal transmission are generally thought to be low (Björkman et al. 1996; Waldner et al. 1998; Davison et al. 1999a) once the source of infection has been removed but can be as high as 71% in herds with evidence of point source exposure to *N. caninum* (Waldner et al. 1999). Although abortion epidemics suggesting recent point-source exposure are relatively rare, farms that have already experienced this probably still have many of the risk factors for introduction of *N. caninum* infection from a definitive host and the risk of re-introduction to a naïve herd should be considered if attempts are made to cull all infected cattle.

Examination of serological results from calves born during the oral challenge and mouse-passaged inoculation studies in this thesis provided 30 dam-calf pairs in which the calves were sampled within 12 h of birth. Individual animal results are presented in Appendix 8.4 and are summarised in Table 9.2. It appears that using a cut-off in the S/P ratio of 1.0 in the HerdChek Neospora ELISA would correctly identify most congenitally-

infected calves although repeated sampling of calves from other herds with endemic *N. caninum* infection is needed to validate this approach.

Table 9.2 Serological results of calves sampled within 12 hours of birth using the HerdChek Neospora ELISA (IDEXX Laboratories Inc., Maine, USA) with results considered positive at sample to positive (S/P) ratio ≥ 0.50.

Dam and calf serostatus	*n*	Minimum S/P ratio	Maximum S/P ratio	Mean S/P ratio	Std. Dev. S/P ratio
Dam seropos Calf seropos	9	1.07	4.17	2.13	0.93
Dam seroneg Calf seropos	3	0.62	0.76	0.69	0.07
Dam seropos Calf seroneg	7	-0.15	0.47	-0.03	0.22
Dam seroneg Calf seroneg	15[a]	-0.23	0.39	0.05	0.20

Another approach to identifying congenitally-infected calves that would avoid the complication of colostral antibody effects would be to ascertain whether or not the dam is infected with *N. caninum*. This technique has several limitations, including (i) assuming that all calves from seropositive dams are congenitally-infected, (ii) mis-mothering of calves or (iii) incorrect identification of dam-calf pairs by farm staff may lead to misclassification. Several ELISA kits have been modified to detect antibodies to *N. caninum* in milk (Moskwa et al. 2003; Schares et al. 2004a; Bartels et al. 2005; Schares et al. 2005b). Using an ELISA to test colostrum rather than serum from the dams is less invasive and may be cheaper, as sampling can be carried out by farm staff at the first post-partum milking for each cow. This may improve the sensitivity of maternal testing, as antibodies to *N. caninum* are concentrated in colostrum (Jenkins et al. 2002). Despite the aforementioned limitations, testing of colostrum deserves further investigation as a means of early identification and culling of replacement heifer calves from seropositive cows, as it would be a practical and relatively inexpensive method of reducing abortions in primiparous heifers and overall herd seroprevalence.

[a] Four calves in this group were older when sampled; 2, 4, 10 and 11 days old

9.1.2 Reducing abortion in herds infected with *Neospora caninum* by identifying and controlling other causes of abortion

After excluding the cows that received the killed tachyzoite vaccine, abortion incidence varied between 2.7% - 13.4% (average = 6.6%) on five farms with a history of abortion associated with *N. caninum*. The histopathological and serological evidence for *N. caninum* involvement in these abortions ranged from 0 to 60% between the farms (average 40%) and an aetiological diagnosis could not be made in 47.3% of abortions. Not surprisingly, other causes of abortion were identified, including BVD, leptospirosis, bacteraemia and fungal abortion. What was unexpected was that 23 of 58 (39.7%) cases that had evidence of *N. caninum* involvement, also had histopathological or serological evidence of other pathogens, making it difficult to determine the contribution of *N. caninum* infection to the abortion.

Previous studies have reported the involvement of other infectious causes of abortion in herds experiencing epidemic or endemic abortion that had been attributed to *N. caninum*. Thurmond et al. (1997) found a significant association between seropositivity to *N. caninum* and abortion in only 7 / 20 American herds in which previous abortions had been attributed to *N. caninum*. They also found evidence of infection with *Leptospira* spp. or BVDV in a further 4 herds and, in those herds, the seroprevalence of *N. caninum* in non-aborting cows ranged from 7 – 86%. Davison et al. (1999b) reported herd-specific and age-specific *N. caninum* seroprevalence in 14 English dairy herds, but many of these herds also had a history of abortions due to BVDV, *Leptospira* spp. and/or infectious bovine rhinotracheitis (IBR) in the previous 5 years.

Establishing *N. caninum* as a cause of abortion is complicated by the fact that many seropositive cows do not abort and histopathological lesions suggestive of *N. caninum* infection could be a result of infection with other apicomplexan parasites. Moreover, even if lesions are due to *N. caninum* infection, identical lesions have been reported in congenitally infected calves, suggesting that fetal neosporosis is not incompatible with survival (Gondim et al. 2004c).

The following criteria have been suggested (Anderson et al. 2000) as necessary to confirm that *N. caninum* infection is responsible for a case of bovine abortion:

1) Compatible gestational age
2) The fetus has some degree of autolysis
3) There are disseminated inflammatory lesions in the fetus
4) *N. caninum* can be detected in the fetus by immunohistochemistry (IHC) and/or there is serological evidence of infection
5) No other abortifacients are detected.

Fulfilling these criteria is difficult for a number of reasons. *Neospora caninum* infection is commonly associated with abortion between the fifth and sixth month of gestation (Dubey 2003b), but fetal death and mummification at approximately 3 months of gestation has been reported (McAllister et al. 1996b) as has the birth of stillborn calves (Waldner et al. 1998). Experimental challenge by intravenous inoculation with tachyzoites on Day 70 of gestation commonly results in fetal death (Williams et al. 2000; Williams et al. 2003; Macaldowie et al. 2004; Maley et al. 2006; Williams et al. 2007; Gibney et al. 2008), although whether this mimics a situation that occurs naturally is unknown. The impact of *N. caninum* infection on early embryonic loss is yet to be determined, since there are studies reporting conflicting evidence on this aspect (Latham 2003; López-Gatius et al. 2005b). Nonetheless, the consensus of evidence appears to be that fetal death could be caused by *N. caninum* infection at almost any stage of gestation.

Aborted material may not be available for examination either due to resorption, mummification or being scavenged if expelled. The detection of characteristic inflammatory lesions in the fetus is not guaranteed and the severity of lesions should be assessed, a subjective judgment based on the experience of the pathologist, to determine whether they are sufficient to cause fetal death (Dubey and Schares 2006). The concentration and timing of antibody response relative to the occurrence of abortion may vary (Schares et al. 1999b) although in most instances, a cow that has aborted due to *N. caninum* infection will be seropositive and have at least a moderate concentration of specific antibodies against *N. caninum* at the time of abortion. This would equate to a positive result in the HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA) or an IFAT titre of ≥1:800. A cow that is sampled at the time of abortion and is seronegative to *N. caninum* is unlikely to have aborted due to neosporosis, although such cases are occasionally reported (Davison et al. 1999a; Sager et al. 2001). Fetal serology can

also be assessed and has a high positive predictive value but a low negative predictive value (Barr et al. 1995; Wouda et al. 1997b). IHC or PCR are not routinely carried out in abortion investigations and are unlikely to be available through most veterinary diagnostic laboratories.

The cost of diagnostic testing often precludes a complete diagnostic effort on every case of abortion that occurs on a farm. When a herd experiences an abortion epidemic, however, it is prudent to establish a diagnosis with the goal that preventive or control initiatives be implemented. In herds where *Neospora*-associated abortion has been diagnosed in the past, maternal serology should be undertaken at the least, to identify that aborting cows are seropositive to *N. caninum*. If a higher than acceptable level of abortion is occurring in seronegative animals (and that would be > 2 - 3% in New Zealand herds; Thobokwe and Heuer 2004), further investigation should be conducted to determine the presence of other causes of abortion.

The interaction between host, pathogen and immune response for *N. caninum* infection in cattle is not completely understood. However, there is evidence that co-infection with other infectious causes of abortion is likely to increase the risk of abortion in *N. caninum*-infected animals (Thurmond and Hietala 1995; Björkman et al. 2000), so attempts should be made to diagnose and control such pathogens. The development of herd-level testing of milk for antibodies to BVDV and PCR testing to detect BVDV DNA now makes it possible to monitor dairy herd status for BVDV (Houe et al. 2006). Such testing allows rapid recognition of the introduction of BVDV and is recommended in herds where there is introduction of animals or contact with other cattle. In beef herds, annual serological testing of a sample of young stock (> 9 months old to allow for the decline of maternal antibody) should be undertaken to establish the presence of active BVDV infection (Houe et al. 1995).

Vaccination against leptospirosis has proven effective in reducing abortion in cattle due to the commonly encountered serovars (Marshall et al. 1976; Mackintosh et al. 1980). Apparent vaccine breakdown could be due to infection with serovars not included in the vaccine, a failure to adequately vaccinate stock (frequency and timing of doses), incorrect handling or storage of vaccine or the introduction of cattle that are already infected and shedding leptospires. Adequate biosecurity and vaccination protocols would mitigate most of these risks but histopathological examination and culture of aborted fetuses and

serological testing of aborting cows is necessary to identify the presence of *Leptospira* spp. (Anderson 2007). Care in the production and storage of supplementary feed will limit the risk of abortion due to listeriosis and mycotoxins (Austwick 1976; Low and Donachie 1997). In addition, attention to the provision of adequate nutrition and shelter will minimise stress and protect animal welfare and production.

The final step in ascertaining the importance of *N. caninum* infection in a herd with a high incidence of abortion is to compare *N. caninum* seroprevalence in aborting and non-aborting cattle (Thurmond and Hietala 1995). The strength and significance of the association between *N. caninum* infection and risk of abortion can be quantified to allow conclusions to be drawn. All aborting and sufficient non-aborting cattle should be sampled to achieve power in this comparison. Seropositive cattle are commonly reported to have a two to four-fold greater risk of abortion and this figure should be used when calculating how many non-aborting cattle to sample.

In summary, even when *Neospora*-associated abortion has been diagnosed in a herd, ongoing assessment of the significance of *N. caninum* infection should be carried out at the herd and individual cow level. Cows that are seronegative to *N. caninum* at the time of abortion are unlikely to have aborted as a result of *N. caninum* infection. In addition, a BVDV monitoring and control programme, a risk management and thorough vaccination programme against leptospirosis, and careful production and storage of supplementary feed should be undertaken. Such steps will not only minimise the risks from the relevant infectious agents but are also likely to reduce the risk of abortion in cattle infected with *N. caninum*.

9.1.3 Control of *Neospora caninum* infection by minimising the risk of direct horizontal transmission between cattle

The number of oocysts shed and the duration of shedding by naturally-infected dogs is variable, and factors that affect oocyst shedding are largely unknown (Basso et al. 2001; Šlapeta et al. 2002; McGarry et al. 2003; Schares et al. 2005a; McInnes et al. 2006b). However, relatively few oocysts are shed by most dogs (Dubey et al. 2007). In some instances, a herd-level seroprevalence to *N. caninum* of > 50% has been reported (Jensen et al. 1999; Pfeiffer et al. 2002; Frössling et al. 2005). This finding, combined with the relatively low level of oocyst shedding by dogs has led others to postulate that horizontal transmission between cattle may occur (Davison et al. 1999a; Modrý et al. 2001).

Oral inoculation of pregnant heifers was carried out to mimic cattle with oral lesions that may ingest tachyzoites from feed contaminated by amniotic fluid or vaginal discharge following abortion or parturition, or when cattle lick newborn calves or placenta. Infection was established in at least one of eight heifers following the application of *N. caninum* tachyzoites to abraded oral mucosa, suggesting that direct horizontal transmission between cattle is possible in some circumstances. Indeed, the strongest evidence that the method of oral challenge reported in this thesis (Chapter Seven) was successful in establishing infection, was that that the timing of the development of the serological response in Heifer 16 matched those of the heifers that were intravenously inoculated and that her calf was seropositive for *N. caninum* when sampled shortly after birth.

While it is clear that the majority of cattle acquire *N. caninum* infection *in utero* (Davison et al. 1999a), there is evidence that horizontal (post-natal) transmission occurs; this is attributed to a point-source exposure from a definitive host with oocyst contamination of feed and/or water (De Marez et al. 1999; Trees et al. 2002; Gondim et al. 2004c). Direct horizontal transmission between cattle could be responsible for some cases of post-natal infection. The presence of *N. caninum* has been demonstrated in placental tissue (Shivaprasad et al. 1989; Fioretti et al. 2000; Bergeron et al. 2001) and amniotic fluid (Ho et al. 1997b). Several authors have suggested that access to fetal fluids could be a risk factor for horizontal infection in dogs and cattle (Thurmond and Hietala 1995; Davison et al. 1999a; Dijkstra et al. 2002c).

Placentophagia has been suggested by several authors as a possible means of horizontal transmission of *N. caninum* between cattle (Davison et al. 1999a; Wouda et al. 2000; Davison et al. 2001; Modrý et al. 2001). Cattle are known to undertake placentophagia, although this usually refers to the parturient cow eating her own placenta rather than that of another cow (Kristal 1980). This behaviour may assist with deterring predators but has been found to enhance opioid-mediated analgesia in some mammalian species and enhances maternal acceptance of the neonate (Kristal 1991). Ingestion of another cow's placenta is probably not a common occurrence in cattle, but peri-parturient cattle are attracted to feed contaminated with bovine amniotic fluid from 12 h before until at least 24 h after parturition (Pinheiro Machado et al. 1997). In ungulates, parturition frequently attracts the attention of herd-mates, even males (Lent 1974) and cattle will lick calves other than their own (Illmann and Spinka 1993; Lidfors et al. 1994). Cattle are known to explore

new objects in the environment predominantly by sniffing and licking, and the flehmen response may also be involved (Wood-Gush et al. 1985; Nielsen and Luescher 1988). Additionally, following parturition, other cattle will spend time licking the recently calved cow (Lidfors et al. 1994) meaning that there is ample opportunity for cows to ingest amniotic fluid. The frequency of such interactions between cattle increases with stocking rate (Lidfors et al. 1994).

While it is difficult to envision placentophagia being responsible for more than occasional post-natal transmission of *N. caninum* infection in cattle, the ingestion of potentially infective amniotic fluid is more likely, given that (i) it will be more widely distributed in the environment, (ii) feed contaminated with such fluid is preferred by peri-parturient cattle to uncontaminated feed and (iii) licking of calves other than their own (and possibly aborted fetuses) is a common behaviour of cattle. Schares and Conraths (2001) have commented that placentophagia is unlikely to play an important role in transmission of *N. caninum* in endemically infected herds. Notwithstanding this assertion, in order to accurately model infection within a herd it is important to consider all possible routes of infection, so this potential means of transmission should be investigated further.

The ability of *N. caninum* tachyzoites to consistently produce infection in cattle following oral challenge is uncertain. *In vitro* experiments have shown that tachyzoites are inactivated by acidic pepsin but not by trypsin (Lindsay and Dubey 1990) so theoretically, an oral tachyzoite challenge should not be infective for cattle. However, oral tachyzoite challenges have successfully infected mice (Lindsay and Dubey 1990) and calves (Uggla et al. 1998; Davison et al. 2001) even after intestinal closure for the absorption of colostral antibody. Lactogenic transmission has been reported in mice (Cole et al. 1995), but fostering seronegative calves onto seropositive cows did not establish infection (Davison et al. 2001). Tachyzoites appear to establish infection prior to reaching the stomach as delivery by stomach tube did not establish infection, whilst calves which were bottle-fed colostrum or milk became seropositive (Uggla et al. 1998), suggesting that damage in the oropharynx facilitates haematogenous uptake of tachyzoites. If damage to the oral or oesophageal mucosa is required to allow haematogenous spread of tachyzoites, cattle with teeth erupting, and herds with active BVDV infection (or any other diseases that produce lesions in the oropharynx) or which are being fed rough, stalky material that could cause lesions may be at risk of horizontal transmission of *Neospora caninum* from other cattle in the herd.

The estimation of rate of horizontal transmission under normal farming conditions, when faecal contamination by definitive hosts can not be excluded, is complicated by the limitations of serological testing (i.e. antibody concentrations can drop below detectable levels resulting in false-negative results) and the fact that serum samples collected prior to a *Neospora*-associated abortion epidemic are not usually available. Rates of horizontal transmission that have been calculated are highly variable, ranging from approximately 1% annually (Hietala and Thurmond 1999) to 45 of 95 seronegative cows seroconverting in a period of 6 months (Dijkstra et al. 2002a). Higher rates of horizontal transmission have been associated with the presence of dogs on the farm, as well as in herds with an initial seroprevalence to *N. caninum* of > 10% (Bartels et al. 2007), although the mechanism of post-natal infection in such herds has never been confirmed. Farming systems in which cattle are housed and fed total mixed rations may facilitate the spread of oocysts compared to herds in which cattle are grazed and only discrete areas of feed are contaminated. However, high rates of seroconversion have also been reported in extensive grazing systems (Pfeiffer et al. 2002; Romero and Frankena 2003) and in association with a high incidence of abortion. Proving that horizontal transmission is occurring between cattle remains difficult as a higher incidence of abortion increases the amount of potentially infective material available to both cattle and definitive hosts. Studies that have kept infected and non-infected cattle in close contact, including throughout pregnancy and parturition, have reported that horizontal transmission between cattle did not occur (Guy et al. 2001; Fioretti et al. 2003). This suggests that such transmission is not common if it does occur. More studies of seronegative cattle kept with seropositive cattle throughout pregnancy and parturition are required, under circumstances which prevent horizontal transmission from definitive hosts, to determine whether horizontal transmission between cattle is possible. Microsatellite typing of positive PCR amplicons could then be used to confirm whether the strains of *N. caninum* are identical among in-contact cattle. The likelihood of such transmission may be increased when cattle are aborting due to *N. caninum* infection and are kept at high stocking rates.

If horizontal transmission between cattle is possible as a result of ingestion of tachyzoites from amniotic fluid contaminating the feed or by cattle licking neonatal calves or placenta, measures should be taken to limit such contact. Cattle at lower stocking rates have fewer interactions around parturition (Lidfors et al. 1994), so reducing stocking rates or housing peri-parturient cattle individually would limit the risk of horizontal transmission. The collection and safe disposal of aborted fetuses and placentas would reduce the risk of

infection to cattle and other hosts. Minimising the prevalence of oral lesions in cattle by implementing a BVDV control programme, which may include vaccination, could be undertaken and the feeding of rough or stalky material should be avoided.

9.1.4 Control of *Neospora caninum* infection by vaccination

The clinical trial of a whole, killed *N. caninum* tachyzoite vaccine did not demonstrate a significant reduction in the risk of abortion. For two farms with an incidence of abortion >8%, a significant vaccine effect occurred on one while *N. caninum* appeared to be involved in at least 60% of the cases of abortion on the other. The vaccine did not prevent vertical transmission. In fact, there was evidence that vaccination increased the risk of vertical transmission. A clinical trial of the same vaccine in Costa Rica also found that vaccine efficacy varied by farm, with a positive effect in 15 herds, no effect in four herds and a small negative effect in six herds (Romero et al. 2004). This previously-registered product has since been removed from all markets and there is currently no licensed vaccine against bovine neosporosis.

Experience with the development of other vaccines against parasitic diseases suggests that live, attenuated vaccines or those based on recombinant proteins, carried by other vectors or iscom antigens are more likely to prove successful. A killed or inactivated vaccine may be capable of protecting naïve cattle from post-natal infection but is unlikely to be able to prevent recrudescence and vertical transmission in previously, and mostly congenitally, infected animals (Innes et al. 2002).

The success of a live, attenuated vaccine against ovine toxoplasmosis (O'Connell et al. 1988) and the finding that inoculation of cattle with *N. caninum* tachyzoites before mating prevented fetal death when subsequently challenged during pregnancy (Williams et al. 2007) suggested that a live, attenuated vaccine may be successful in preventing abortion due to *N. caninum* in cattle. Additionally, transplacental transmission did not occur in chronically infected cattle that were challenged with *N. caninum* tachyzoites at mid-gestation (Innes et al. 2001a) suggesting that such an approach would also eventually lead to a decline in the proportion of cattle seropositive to *N. caninum*.

Challenge studies have revealed that the timing, dose, strain and life stage of *N. caninum* used to inoculate cattle affects pregnancy outcome; a summary of these studies is presented in Table 9.3. Several challenge studies in which pregnant cattle were inoculated with Nc-

Liverpool tachyzoites on Day 70 of gestation achieved 100% fetopathy with a dose of 1 x 10^7 tachyzoites (Williams et al. 2000; Williams et al. 2003; Williams et al. 2007; Gibney et al. 2008). Conversely, one of the studies presented in this thesis (Chapter Seven) showed that only one of eight heifers aborted following intravenous inoculation with 1 x 10^8 tachyzoites (a mixture of the three New Zealand strains). These findings, in conjunction with challenge studies in mice (Okeoma et al. 2004c), suggests that the New Zealand strains are less pathogenic that the Nc-Liverpool strain. Concerns that live vaccines may be contaminated with prions from transmissible spongiform encephalopathies restricts the geographical pool from which a live *N. caninum* vaccine could be developed. The *N. caninum* isolates from New Zealand and Australia (Nc-NZ1, Nc-NZ2, Nc-NZ3 and Nc-Nowra) appear to be suitable candidates for further study even though the study presented in Chapter Eight did not show a protective effect of live inoculation with *N. caninum* tachyzoites pre-mating in preventing abortion subsequent to a further tachyzoite challenge.

The delicate balance of immune response in the pregnant mammal means that there may be difficulties associated with the development of a vaccine for bovine neosporosis. Current understanding of the immune response to intracellular parasites such as *N. caninum* and the downregulation of immunity during pregnancy suggests that it may be the maternal immune response to *N. caninum* infection that is deleterious to pregnancy (Innes 2007b). Infection with *N. caninum* appears to be chronic and may be lifelong (Hietala and Thurmond 1999). The bradyzoite stage of the parasite appears to be protected against the host's immune response and may also be protected against chemotherapeutic agents that may eliminate other stages of the infection. Vaccination of cattle against neosporosis may invoke humoral and cell-mediated immune responses, but whether cell-mediated immunity can be maintained during pregnancy such that *N. caninum* infection is controlled but not at the cost of the pregnancy itself remains unknown.

Table 9.3 A summary of findings from challenge studies involving the experimental inoculation of *Neospora caninum* in cattle

Strain of *N.caninum*	Dose and route	Stage of gestation	Outcome	Reference
Nc-Illinois	1 x 10^7 tachyzoites IV	Day 110	1/8 fetal death by 6 weeks	Almeria et al. 2010
Nc-BPA1	2 x 10^7 tachyzoites IV and IM	Day 91	14/19 fetal death or resorption by 25-33 d	Andrianarivo et al. 2000
Nc-BPA1	1 x 10^6 tachyzoites fetal IM	Day 118	2/2 fetal death within 17 d	Barr et al. 1994b
Nc-BPA1	3 x 10^6 tachyzoites IV and 5 x 10^6 IM	Day 85	1/3* fetal death after 63 d	Barr et al. 1994b
Nc-1, Nc-2, Nc-3	13 x 10^6 tachyzoites SC and 13 x 10^6 IM	Day 81	1/1 aborted after 74 d	Dubey et al. 1992
Nc-1, Nc-2, Nc-3	13 x 10^6 tachyzoites SC and 13 x 10^6 IM	Day 126	1/1 aborted after 101 d	Dubey et al. 1992
Nc-Liverpool	1 x 10^7 tachyzoites IV	Day 70	6/6 fetal death at 22.7 +/- 1.2 d	Gibney et al. 2008
Nc-Liverpool	1 x 10^7 tachyzoites IV	Day 210	6/6 fetuses infected but alive after 22 d	Gibney et al. 2008
Nc-beef	41,000 oocysts oral	Day 120	1/4 fetal death after 44 d	Gondim et al. 2004c
Nc-1	5 x 10^8 tachyzoites IV	Day 70	6/6 fetal death by 28 d	Macaldowie et al. 2004
Nc-1	5 x 10^8 tachyzoites SQ	Day 70	3/6 fetal death by 28-56 d	Macaldowie et al. 2004
Nc-1	5 x 10^8 tachyzoites SQ	Day 70	Fetal death after 22-28 d, aborted 42-56 d	Maley et al. 2006
Nc-1	5 x 10^8 tachyzoites SQ	Day 70	50% fetal death by 28-56 d	Maley et al. 2006
Nc-1	Up to 5 x 10^8 tachyzoites SQ	Day 140	28/28 fetal infection but lesions resolve	Maley et al. 2003
Nc-Liverpool	40,000 oocysts oral	Day 120	1/6 fetal death after 33 d	McCann et al. 2007
Nc-Liverpool	1 x 10^7 tachyzoites IV	Day 70	Fetal death within 21 – 30 d	Williams et al. 2007
Nc-Liverpool	1 x 10^7 tachyzoites IV	Day 70	Fetal death estimated to have occurred ~ 21 d	Williams et al. 2000
Nc-Liverpool	1 x 10^7 tachyzoites IV	Day 70	Fetal death within 3-5 weeks	Williams et al. 2003

* Other fetuses had been surgically removed at an earlier stage

During pregnancy, a Th2-type cell-mediated immune response predominates at the materno-fetal interface (Tangri and Raghupathy 1993) to allow the maintenance of pregnancy. The Th1-type environment and particularly the activity of IFN-γ, TNF-α and IL-12 are important in controlling *N. caninum* infection. The downregulation of the Th1-type response during pregnancy may be what allows recrudescence in animals infected with *N. caninum*. The antigenic stimulation of a tachyzoite parasitaemia may then provoke a Th1-type response to control the parasite and this may cause abortion (Quinn et al. 2002). Fetal immunocompetence also plays a role in determining the outcome of infection in the pregnant cow that becomes infected later in gestation (typically after about Day 120) that does not abort but results in the birth of a congenitally-infected calf (Williams et al. 2000).

9.1.5 Serological diagnosis of *Neospora caninum* infection in sheep

The indirect fluorescent antibody test is commonly regarded as the gold standard test for the serological diagnosis of *Neospora caninum* infection in cattle and dogs (Trees et al. 1994; Dubey et al. 1996c) and has been used to detect antibodies to *N. caninum* in sheep (West et al. 2006; Howe et al. 2008). IFAT results from the challenge of pregnant sheep (Table 6.2) did not correlate with infection dose or pregnancy outcome and there was little agreement between IFAT titres and the sample to positive result in a commercial ELISA from the serum samples that were collected 28 days after inoculation (kappa agreement between the tests was 0.025). By comparison, the commercial ELISA (Chekit Neospora, IDEXX Laboratories Inc., Maine, USA) gave results that were consistent with the dose of challenge that the sheep were given. A cut-point in the S/P ratio of ≥ 11.8%, as suggested by Reichel et al. (2008), appeared to correctly differentiate between infected and non-infected sheep.

The apparently poor performance of the IFAT in experimentally challenged sheep brings into question its use in this species and possibly in cattle. Both IFAT and a commercial ELISA were used in the prospective cohort study of primiparous heifers reported in Chapter Three where the S/P ratio in the ELISA was consistently high in most infected animals while IFAT titre fluctuated between 1:200 and 1:600 over the same time period. For example, the S/P ratios in heifers H43, H55 and H126 (none of whom went on to abort) in January 2004 were 1.81, 1.47 and 2.13 respectively and then 1.43, 2.82 and 2.59 respectively in May 2004 (the recommended cut-point in the S/P ratio was 0.50). The IFAT titres for these animals over the same time period weres 1:200 when tested monthly

throughout February to May 2004 except on one occasion in March when H126 had an IFAT titre of 1:600. Whilst all of these serological results would be classified as positive, it is widely regarded that the cut-point in the S/P ratio for the ELISA equates to an IFAT titre of approximately 1:800 and that does not hold true for the heifers in this study. Further comparison of IFAT and the HerdChek* Neospora ELISA (IDEXX Laboratories Inc., Maine, USA) should be carried out to validate their use in both aborting and persistently infected cattle as the ELISA may have greater sensitivity when there has not been recent exposure to *N. caninum* antigen.

9.2 Limitations of the research presented in this thesis

All studies reported in this thesis were carried out under normal grazing conditions on commercial farms and it was not possible to guarantee that the environment was not contaminated with *N. caninum* oocysts in dog faeces. It would have been necessary to house and feed cattle in a controlled environment to eliminate the possibility that any positive results in control animals were due to horizontal transmission from dogs and not the result of horizontal transmission from other heifers which were experimentally challenged. Microsatellite typing confirmed that the *N. caninum* DNA in positive PCR samples from one trial was the same (except for one sample) as that used to challenge the animals, meaning that it was less likely that the positive PCR results in control animals were a result of horizontal transmission from dogs. Microsatellite results also suggested that multiple strains of *N. caninum* may have been present in some of the New Zealand isolates.

The numbers of cattle in each group for the challenge trials was limited by both cost and availability of suitable animals as only animals that were consistently seronegative and which had a low chance of pre-existing infection with *N. caninum* were used. Researchers at Massey University had been sampling cattle from one farm for the previous seven years to monitor serological status, so this farm was the ideal source of animals for the trials. It was possible to request they keep more heifer replacement calves than needed for their own requirements to supply animals for the studies reported in this thesis. This enabled selection of calves whose dams had a lifelong history of being seronegative. The finding that cattle can be infected with *N. caninum* yet remain seronegative (McInnes et al. 2006a; also supported by studies reported in this thesis) means it is difficult to be certain that cattle selected for trials are not infected. However, repeated serological testing remains the best

screening tool currently available. Numbers of animals were limited to 6-8 in each group which meant that differences between the groups were less likely to be statistically significant. However, *a priori* power analysis for the trial reported in Chapter Eight showed that the allocation of six heifers to each Group had a power of 0.80 to detect a significant effect of pre-mating inoculation with *N. caninum* tachyzoites to prevent abortion if all heifers that were not inoculated prior to mating but were challenged on Day 70 of gestation (Group C) aborted.

Positive PCR results were unexpectedly found in animals and offspring in the control group in all challenge studies reported in this thesis. It was not possible to ensure that such positive results were not due to horizontal transmission from dogs. PCR positive results from dam-neonate pairs that did not seroconvert in the challenge studies brings into question how widespread infection with *N. caninum* really is and why some animals are infected but do not seroconvert. It may be that the parasite challenge is too low to establish infection or there may be differences in the host-parasite relationship and immune balance.

Logistics prevented pre-colostral sampling of calves and lambs born during the studies reported in this thesis. Although it would have been valuable to collect pre-colostral samples from calves born during the studies reported in Chapters Seven and Eight, the cost to employ research assistants to monitor parturition and collect samples from calves at birth was beyond the available budget.

It would also have been valuable to ascertain whether calves born to the heifers in the study reported in Chapter 3 were congenitally-infected with *N. caninum*, particularly those calves born to the seropositive heifers that did not abort, to establish whether vertical transmission had occurred. The cost of collecting such samples and the fact that the herd was approximately 300 km distant from Massey University precluded such sampling. Serum was collected monthly from all 164 heifers that were monitored throughout pregnancy during this study. Antibody testing was only carried out on samples from the month before abortion, when abortion was detected and the following 2 months in heifers that aborted and for 4 consecutive months when abortion had most commonly occurred in the remaining 6 seropositive heifers that did not abort. Further funding would have

allowed more samples to be tested, but it was decided that the information that might be gained from more testing could not be justified with the funds available.

The lower than expected abortion incidence on 3 of the 5 farms selected for the clinical trial of a whole, killed *N. caninum* tachyzoite vaccine limited the power of the study to detect a significant vaccine effect in reducing abortion. Herd seroprevalence to *N. caninum* at enrolment varied between 6.2 – 19.7% and, in general, the herds with the highest seroprevalence had the highest abortion incidence. Pre-trial screening using a bulk milk antibody test or stratified serological screening may have identified other herds with higher seroprevalence that may have been more likely to demonstrate an effect of vaccination on the incidence of abortion.

9.3 Avenues for further research

9.3.1 Methods to diagnose *Neospora caninum* infection in young calves

The identification and culling of heifer replacements that are infected with *N. caninum* seems to be the most practical and cost-effective control option to derive from this research. Serological testing is complicated by the presence of colostral antibody, which may be present at variable levels until 4 months of age (Hietala and Thurmond 1999), whilst pre-colostral sampling is not practical. Congenitally-infected calves are likely to have higher antibody concentrations than calves that have received colostral antibody. However, in one study that collected pre-colostral and subsequent samples from 181 calves, an overlap in antibody concentration between infected and non-infected calves occurred following the ingestion of pooled colostrum (Hietala and Thurmond 1999). Using a kinetic ELISA with a cut-off of S/P $V_{max} \geq 0.45$, initially seronegative calves ranged from 0.04 – 1.53 (mean = 0.49 +/- 0.41) at 2-5 days of age while the congenitally-infected calves ranged from 0.73 – 1.87 (mean = 1.2 +/- 0.32) at 2-5 days of age. The calves sampled in that study came from 2 dairy farms with a high level of seroprevalence to *N. caninum* with 30% of calves on one farm and 49% on the other being classified as infected.

Further serological studies in herds endemically infected with *N. caninum* are required to validate serological tests for the identification of congenitally-infected calves at 1-2 weeks of age where antibody may be due to feeding of pooled colostrum. Receiver-operating characteristic (ROC) analysis could be used to select a cut-off that distinguishes between

colostral and fetal antibody in congenitally-infected calves. Sampling and identification of these animals before much expense had been incurred in their rearing would probably be cost-effective and would allow other, non-infected calves to be selected as replacements. Serial testing of calves from several herds with endemic infection would need to be carried out to compare pre-colostral antibody concentrations with serology results when the calves are 1-2 weeks old. Alternative serological or other diagnostic tests that detect cattle infected with *N. caninum* and are not confounded by maternal antibody should be investigated. Such tests may rely on the detection of specific immunoglobulins which are derived from the fetus (as opposed to IgG_1 which is the predominant antibody in bovine colostrum). Cow-side tests such as an immunochromatographic test would be useful to determine the infection status of the dam and thus, the likely infection status of the calf. The methodology behind such testing assumes a high rate of vertical transmission and that accurate identification of cow-calf pairs is possible. If infected calves are selected on the basis of their dam's serostatus then non-infected animals will be needlessly culled due to the fact that vertical transmission rates are not 100% and are thought to decline with parity.

Currently, PCR and IHC have low sensitivity but high specificity in detecting infected animals. It may be possible to develop a protocol using samples collected *ante mortem* from calves that provides higher sensitivity such as the antigen-capture ELISA that can be used to detect calves persistently infected with BVDV from an ear notch sample (Cornish et al. 2005). Such techniques would not be affected by the presence of maternal antibody.

There will always be limitations on the ability of tests to detect infected animals. When the aim is to reduce herd seroprevalence and not to guarantee that all infected animals are detected, as in a disease eradication programme, it is reasonable to accept such tests subject to a cost-benefit analysis. Removal of most congenitally-infected heifer replacements will reduce the economic losses associated with abortion in primiparous heifers as well as reducing herd seroprevalence which will have ongoing economic benefits.

9.3.2 Direct horizontal transmission of *Neospora caninum* between cattle

The success in infecting at least one heifer by the application of *Neospora caninum* tachyzoites to an abraded oral mucosa suggests that direct horizontal transmission of *N. caninum* between cattle is possible. There is strong sero-epidemiological evidence that vertical transmission is responsible for most *N. caninum* infections in cattle (Paré et al. 1996;

Anderson et al. 1997). Horizontal transmission from dogs and other definitive hosts that excrete oocysts to contaminate food or water that is then ingested by cattle is proposed as an important means of transmission for introducing infection into naïve herds (Jenkins et al. 2000; McAllister et al. 2000; Schares et al. 2002) although this route has never been proven. Direct horizontal transmission between cattle would require contamination of the environment from an infected cow. Tachyzoites are present in placenta (Shivaprasad et al. 1989) and *N. caninum* DNA has been demonstrated in amniotic fluid by PCR (Ho et al. 1997b). Factors that affect the survival of tachyzoites in the environment are unknown, but their survival would probably be reduced compared to oocysts. Results from calves fed colostrum that was spiked with tachyzoites suggest that infection can only be established when the tachyzoites contact the oropharynx (Uggla et al. 1998). The presence of lesions in the oropharynx may facilitate haematogenous spread of tachyzoites if cattle ingest food or water contaminated with tachyzoites such as amniotic fluid on pasture or by licking calves or placenta from infected cows.

Authors have previously reported that seronegative cows which were housed with seropositive and aborting cows did not seroconvert (Guy et al. 2001; Fioretti et al. 2003). It would not be expected that direct horizontal transmission occurs commonly, so further studies to monitor in-contact cattle should be conducted to investigate this question. The finding that it is not uncommon for cattle to have *N. caninum* DNA in their blood yet be seronegative (McInnes et al. 2006a), suggests that there is more to understand about the dynamics of infection in cattle. Some low levels of infection may fail to become established, whilst other cattle seroconvert and appear to be susceptible to repeated recrudescence of infection throughout their life. A challenge trial similar to the one reported in Chapter Seven could be undertaken in housed cattle to exclude the risk of horizontal transmission from a definitive host, although bought-in feed may need to be irradiated or otherwise treated to ensure that it is not contaminated with oocysts.

9.3.3 Isolation and characterisation of other New Zealand isolates of *Neospora caninum*

Relatively few isolates of *Neospora caninum* have been reported internationally and there is evidence for differences in virulence in challenge studies with mice (Lindsay and Dubey 1990; Atkinson et al. 1999). Natural infections in cattle have been reported to result in

many cattle seroconverting without any increase in the incidence of abortion (Dijkstra et al. 2002a).

Two of the three New Zealand isolates were from a dam-calf pair in the Bay of Plenty (Nc-NZ1 and Nc-NZ2 respectively) while the third was from a calf from a farm in the Waikato region (Nc-NZ3). Microsatellite array analysis showed some differences between the isolates from the dam and her calf. Differences in the microsatellite alleles between the dam-calf pair suggests that the dam was infected with more than one isolate, and that at least one of these was transmitted vertically to her calf. Some isolates may have a predilection for certain tissues and be more likely to cross the placenta and establish infection in the fetus. Analysis of multiple positive PCR-amplicon samples from individual cows may confirm the presence of mixed infections. Microsatellite typing may also assist with understanding the epidemiology of *N. caninum* at the herd level by identifying isolates that differ between individual animals and host species.

The challenge trials reported in this thesis suggest that the strains used had low virulence, as a relatively high dose (2×10^9 tachyzoites) was administered by intravenous inoculation and produced abortion in five of six heifers. Under natural conditions however, at least 33% of pregnant cows aborted associated with *N. caninum* infection in one season on a New Zealand dairy farm (Thornton et al. 1994) suggesting that either some New Zealand isolates are more virulent or that large infective doses can be acquired naturally. It is possible that the strains used in the challenge trials had reduced virulence from being kept in Vero cell culture over 28 months, as continuous passage of *T. gondii* in cell culture reduced its ability to develop tissue cysts when inoculated into mice (Frenkel et al. 1976). The characterisation of other isolates may lead to the identification of strains that may be useful in the development of a live, attenuated vaccine.

9.3.4 Vaccination to prevent infection with, or abortion due to, *Neospora caninum*

The successful control of abortion in sheep due to toxoplasmosis has led to the proposition that a live, attenuated vaccine may be useful in preventing abortion in cattle due to *Neospora caninum* infection. However, there are important differences in the immunological response to these closely related parasites that suggest that such an

approach may not be successful. Innes and colleagues (2007a) have summarised these differences as follows:

i. There is a higher rate of endogenous transplacental transmission in *N. caninum* infection and this occurs over several generations and in successive pregnancies.
ii. Bovine neosporosis may occur following either a primary exogenous infection (as with *T. gondii*) or following recrudescence of a persistent endogenous infection.
iii. The immune response that is provoked following primary infection with *T. gondii* in sheep generally protects against further disease. By comparison, cattle infected with *N. caninum* may develop some degree of immunity but this is not fully protective.
iv. Fetal infection with *T. gondii* is usually fatal even when this occurs later in gestation, so few lambs are born congenitally-infected. This is not the case with bovine neosporosis, in which vertical transmission is thought to be responsible for the majority of cases. It may be that infection of the fetus with *N. caninum* when its immune system is not fully mature leads to some degree of immunotolerance compared to cattle that are infected after birth.

It has previously been reported that the inoculation of cattle with live tachyzoites prior to mating protects against fetal death (Williams et al. 2007) and vertical transmission (Innes et al. 2001a) when the cattle are challenged with *N. caninum* tachyzoites during pregnancy. Although these trials were carried out using relatively small numbers of cattle in each group, there was 100% protection. These findings suggested that a live, vaccine approach may be useful although following inoculated cattle through subsequent pregnancies to test ongoing efficacy needs to be carried out. The study reported in this thesis (Chapter Eight) in which cattle were inoculated with *N. caninum* tachyzoites prior to mating and then challenged on Day 70 of gestation did not provide such conclusive results suggesting that this approach requires further trials to be validated.

9.3.5 The significance of cattle infected with, but seronegative to, *Neospora caninum*

Neospora caninum DNA was detected in samples from sheep or cattle that were in the control group in all of the challenge studies reported in this thesis. Efforts had been made to ensure that all animals selected for the trial were serologically negative and thus were not

previously infected with *N. caninum*. Additionally, standard laboratory practices to prevent cross-contamination of samples were adhered to and positive and negative control samples were included in each PCR run. Parasitaemia in these animals appeared to be transient with most animals being PCR-positive on one occasion during weekly testing. The development of PCR techniques capable of detecting *N. caninum* DNA in serum and whole blood allows *ante mortem* testing of cattle to determine the incidence of infection in seronegative animals. To ensure that cross reaction with *N. caninum* DNA is not occurring, positive PCR amplicon samples should be sequenced.

The clinical significance of *N. caninum* infection in seronegative cattle is yet to be determined. It may be that these animals are not at increased risk of abortion, do not develop chronic infections and do not vertically transmit the parasite. Investigations to determine why some cattle do not become seropositive may elucidate factors that can be used to protect naïve cattle from developing a chronic infection when first exposed to *N. caninum*.

Microsatellite array analysis of these positive PCR-amplicon samples may reveal genetic differences that are linked to virulence and thus identify suitable isolates for further studies on vaccine development.

9.3.6 Diagnostic methods for bovine neosporosis – what does it all mean?

It is widely recognised that many cows that are infected with *Neospora caninum* do not abort and thus, that infection with *N. caninum* in an aborting cow is not proof of causation. Even the presence of histopathological lesions consistent with *N. caninum* infection in an aborted fetus does not guarantee that *N. caninum* caused the abortion, as such lesions may not be sufficient to cause fetal death. Serology, histopathology, PCR and immunohistochemistry can be used to demonstrate infection with *N. caninum* in cattle. However, none of these methods can positively conclude that *N. caninum* was the cause of an abortion. The finding that 23 of 58 cases of abortion that had evidence of infection with *N. caninum* also had evidence of other infectious aetiologies shows that the interpretation of results from an abortion investigation can be complicated. A thorough diagnostic investigation using serology, histopathology and microbial culture will provide the most complete information. Such investigations can be expensive but will provide the most meaningful results to instigate appropriate control options.

While it has been recognised that antibody concentrations in cattle infected with *N. caninum* can fluctuate and, at times, drop below detectable levels, it was expected that most infected cattle would be seropositive at some stage if serial sampling was conducted and that sampling in mid-late gestation or early lactation improves test sensitivity. The detection of *N. caninum* DNA in blood or tissue from consistently seronegative animals supports the findings of McInnes et al. (2006a) who reported *N. caninum* DNA in the serum of cattle that were seronegative at that point in time. These findings reinforce that seronegative as well as seropositive results are difficult to interpret. Further epidemiological investigations should be conducted using PCR to establish how widespread infection with *Neospora caninum* is among individual cows and herds.

Appendices

List of Appendices

Relating to Chapter Eight: Inoculation of cattle with a strain of *Neospora caninum* tachyzoites after passage through mice does not prevent fetal deaths.

Appendix 3.1 Serological results using the HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA); a cut-off in the sample-to-positive (S/P) ratio of 0.50 is considered positive.

Animal ID	Dec-02	Mar-03	Jul-03	Nov-03	Jan-04	May-04	Abort
02/001	-0.01	0.06	0.13	0.11	0.29	0.04	0
02/002	0.38	**1.53**	0.05	0.11	0.1	0.15	0
02/003	-0.02	0.05	0.05	0.01	0.38	0.13	0
02/004	-0.02	0.13	0.06	0.11	0.37	0.14	0
02/005	0.00	0.23	0.15	0.18	0.31	0.24	0
02/006	-0.04	-0.02	0.03	-0.03	0	0.1	0
02/007	-	0.03	-0.01	0.01	0.29	0.03	0
02/008	-0.02	0.07	0.16	0.03	0.08	0.28	0
02/009	-0.03	0.09	0.09	0.02	0.15	0.07	0
02/010	-0.01	0.32		0.2	0.38	0.21	0
02/011	-0.01	0.28	0.32	0.37	0.14	0.15	0
02/012	-0.06	0.07	0.02	0.34	0.43	0.3	0
02/013	-0.03	0.02	0.05	0.23	0.38	0.33	0
02/014	**2.82**	**2.72**	**1.77**	**1.36**	-	**4.01**	0
02/015	-0.05	0.05	-	0.1	0.1	0.12	0
02/016	-0.02	0.09	0.27	0.1	0.29	0.16	0
02/017	-0.04	0.03	0.07	0.12	0.16	0.22	0
02/018	-0.03	0.7	0.06	0.06	0.14	0.13	0
02/019	-0.02	0.02	0.01	0.17	0.24	0.14	0
02/020	-0.04	0	0.02	-0.03	0.12	0.06	0
02/021*	**0.73**	**0.66**	**2.11**	**1.88**	**1.67**	Culled	-
02/022	**0.80**	0.44	**0.86**	**0.97**	**1.09**	Culled	1
02/023	0.12	0.26	0.13	0.12	0.48	0.12	0
02/024	-0.03	0.08	-0.02	0.24	0.43	0.4	0
02/025	-0.04	0.22	0.03	0.14	0.35	0.26	0
02/026	-0.04	-0.01	0.03	-0.02	0.1	0.09	0
02/027	-0.03	0.03	0	-0.01	0.1	0.04	0
02/028	-0.04	0.09	0.05	0.12	0.21	0.16	0
02/029	**1.60**	**1.26**	0.49	**0.51**	**1.07**	**3.09**	0
02/030	-0.04	0.01	0.05	0.01	0.1	0.12	0
02/031	-0.01	0.4	0.07	0.6	0.02	0.12	0
02/032	-0.04	0.05	0.02	-0.01	0.02	0.04	0
02/033	-0.01	0.06	0.05	0.04	0.11	0.03	0
02/034	**2.59**	**2.65**	**1.72**	**1.53**	**2.13**	Culled	1
02/035	-0.01	0.11	0.09	0.02	0.12	0.04	0
02/036	0.00	-0.02	0.21	0.15	**0.96**	0.25	0
02/037	-0.02	0.15	0.05	0.05	0.13	0.05	0
02/038	0.00	0.03	0.07	0.09	0.18	0.12	0
02/039	-0.03	-0.01	0.09	0.08	0.15	0.09	0
02/040	-0.02	0.09	0.13	0.27	0.32	0.03	0

* Did not conceive

Animal ID	Dec-02	Mar-03	Jul-03	Nov-03	Jan-04	May-04	Abort
02/041	-0.03	0.13	0.03	0.1	0.2	0.33	0
02/042	**1.65**	**4.73**	**3.44**	**2.39**	**4.73**	Culled	1
02/043	**1.80**	**1.78**	-	**1.34**	**1.81**	**1.43**	0
02/044	-0.05	0.22	0.06	0.51	0.6	0.43	0
02/045	-0.04	0.03	0.07	0.04	0.13	0.18	0
02/046	-0.03	0.15	-	0.08	0.05	0.12	0
02/047	-0.04	-0.01	0.08	-0.01	0.09	0.08	0
02/048	-0.04	0.18	0.03	0.06	0.2	0.16	0
02/049	0.74	0.43	0.27	0.37	0.32	0.33	0
02/050	-0.05	0.04	0.04	0.4	0.49	0.05	0
02/051	-0.02	0.13	0.07	0.2	0.14	0.35	0
02/052	**2.54**	**2.37**	**2.99**	**2.25**	**3.1**	**4.11**	0
02/053	-0.04	0.22	0.14	0.08	0.21	Culled	1
02/054	-0.03	0.2	0.11	0.25	0.26	0.35	0
02/055	**1.09**	**1.45**	**3.02**	**1.57**	**1.47**	**2.82**	0
02/056	-0.05	0	0.01	0.11	0.2	0.06	0
02/057	-0.04	0.01	0.04	0.03	0.3	-	0
02/058	0.00	0.5	0.18	0.39	**0.83**	0.28	0
02/059	-0.01	-0.03	-0.07	0.02	0.08	0.08	0
02/060	-0.05	-0.05	-0.02	0.02	0.15	0.22	0
02/061	0.02	0.15	0.1	0.41	0.89	0.27	0
02/062	**1.83**	**4.16**	**2.91**	**2.67**	**3.69**	Culled	1
02/063	-0.01	0.05	0.29	0.16	0.23	0.31	0
02/065	**1.98**	**1.16**	**1.5**	**0.81**	**1.11**	Culled	1
02/066	0.02	0.15	0.02	0	0.11	0.28	0
02/067	-0.04	0.07	0.03	0.11	0.32	0.14	0
02/068	-0.05	-0.01	0.05	0.02	0.13	0.21	0
02/069	-0.04	0.05	0.08	0.01	0.17	0.1	0
02/070	-0.04	0.38	0.22	0.2	0.47	0.1	0
02/071	-0.05	-0.04	0.14	0.08	0.19	0.17	0
02/072	-0.05	0.09	0.01	0.13	0.09	0.18	0
02/073	0.00	0.06	0.14	-0.03	0.03	0.18	0
02/074	**2.20**	**1.88**	**1.39**	**1.14**	**2.11**	Culled	1
02/075	-0.02	0.25	0.12	0.38	0.45	0.22	0
02/076	-0.03	0.04	0.07	0	0.05	0.09	0
02/077	-0.03	0.11	-	0.04	0.25	-	0
02/078	-0.04	0.17	0.02	0.08	0.25	0.16	0
02/079	-0.02	-0.04	0.09	0.02	0.3	0.04	0
02/080	0.00	0.13	0.01	0.23	0.18	0.17	0
02/081	-0.05	0.02	0.06	0.09	0.27	0.09	0
02/082	-0.05	0	0.03	-0.01	0.05	0.08	0
02/083	-0.05	0.06	0.09	0.18	0.12	0.13	0
02/084	-0.01	0.18	-0.01	0.1	0.22	0.05	0
02/085	-0.03	-	0.01	0.02	0.28	0.06	0
02/086	-0.03	-	0.27	0.27	0.45	0.09	0
02/087	-0.05	0.03	0	0.12	0.21	0.15	0

Animal ID	Dec-02	Mar-03	Jul-03	Nov-03	Jan-04	May-04	Abort
02/088⊕	-0.04	0.19	0.26	0.24	Culled	Culled	0
02/089	-0.04	-0.07	0.05	-0.01	-0.05	0.04	0
02/090	-0.04	0.06	0.22	0.04	0.19	0.42	0
02/091	-0.02	0.21	0.05	0.23	0.31	0	0
02/092	-0.04	0.11	0.15	0.23	0.32	0.2	0
02/093	-0.02	0.02	-	0.09	0.18	0.12	0
02/094	**2.02**	**2.93**	**2.27**	**2.0**	**2.49**	Culled	1
02/095	-0.04	0.13	0.11	0.07	0.2	0.12	0
02/096	-0.04	-0.02	0.05	0.09	0.12	0.05	0
02/097	-0.06	0.2	0.05	0.03	0.22	Culled	1
02/098	-0.04	0.17	-	0.24	0.21	Culled	1
02/099	-0.05	0.1	0.05	0.12	0.17	0.22	0
02/101	-0.01	-0.02	0.03	0.03	0.07	0.07	0
02/102	-0.05	0.03	0.03	0.06	0.04	0.01	1
02/103	-0.02	0.01	-0.02	0	0.11	0.31	0
02/104	-0.03	0.04	-0.04	0.01	0.09	0.1	0
02/105	-0.02	0.31	0.12	0.28	0.41	0.1	0
02/106	-0.05	0.26	0.08	**0.62**	0.28	**1.47**	0
02/107	-0.04	-0.02	0.01	0.01	0.08	0.19	0
02/108	-0.05	0.17	0.13	0.08	0.22	0.32	0
02/110	-0.04	-0.02	0.12	0.04	0.24	0.28	0
02/111	-0.05	-0.01	0.18	0.17	0.29	0.39	0
02/112	-0.01	0.17	0.08	0.42	0.38	0.07	0
02/113	-0.03	0.15	0.12	0.08	0.19	0.23	0
02/114*	**1.78**	**1.77**	**1.93**	**2.0**	**1.96**	Culled	0
02/115	-0.04	0.01	0.1	0.18	0.14	0.29	0
02/116	-0.04	0	0	0	0.04	0.02	0
02/117	-0.05	-0.01	0.24	0.11	0.11	0.18	0
02/118	-0.05	0.01	0.03	-0.02	0.02	0.04	0
02/119	-0.06	0.28	0.19	0.19	0.14	0.03	0
02/120	-0.05	0.04	0.02	0.04	0.09	0.02	0
02/121	-0.02	-0.04	0	0.05	0.06	0.11	0
02/122	-0.05	0.09	0	0.01	0.04	0.16	0
02/123	-0.04	0.03	0.05	-0.01	0.05	0.07	0
02/124	-0.02	0.23	0.26	0.28	0.23	0.36	0
02/126	**2.82**	**3.49**	**4.4**	**1.75**	**2.13**	**2.59**	0
02/127	-0.04	-0.04	0.01	0.04	0.05	0.03	0
02/128	-0.02	0.2	0.02	0.1	0.08	0.31	0
02/129	-0.02	0.1	0.08	0.07	0.05	0.22	0
02/130	0.00	0.13	0.07	0.01	0.11	0.11	0
02/131	-0.05	0.02	-0.06	0.03	0.07	0.09	0
02/132	**2.35**	**4.38**	**2.28**	**2.71**	**2.32**	**3.58**	0
02/133	-0.04	-0.03	0.19	0.09	0.04	0.08	0
02/134	-0.03	0.32	0.03	0.25	0.19	0.8	0
02/135	-0.04	0.02	0.03	0	0.05	0.32	0

⊕ Uterus unicornis

* Did not conceive

Animal ID	Dec-02	Mar-03	Jul-03	Nov-03	Jan-04	May-04	Abort
02/136	-0.04	0.07	0.01	0.24	0.21	**0.55**	0
02/137	-0.06	0.08	0.26	0.29	0.22	0.07	0
02/138	-0.04	-0.05	0.05	0.06	0.12	0.08	0
02/139	-0.04	0.06	0.09	-0.02	0.29	0.16	0
02/140	-0.03	0.04	0.15	0.36	0.27	0.08	0
02/141	-0.04	0.03	0.13	0.1	0.1	0.19	0
02/143	-0.01	0.13	0.04	0.19	0.14	0.18	0
02/144	-0.04	0.03	0.01	0.01	0.08	0.09	0
02/145	-0.04	-0.01	0.13	0.05	0.16	0.25	0
02/146	-0.02	0.12	0.23	0.17	0.16	0.11	0
02/147[#]	-0.03	0.05	0.15	0.14	0.13	Culled	0
02/148	-0.03	-0.03	0	0.1	0.18	0.3	0
02/149	**2.74**	**4.35**	**3.32**	**2.75**	**3.43**	Culled	1
02/150	**2.73**	**1.84**	**0.73**	**0.76**	**0.94**	Culled	1
02/151	-0.05	-0.05	-0.02	-0.05	0.01	0.03	0
02/152	-0.03	0.44	0.15	0.03	0.16	0.17	0
02/153	-0.05	-	-0.02	0.05	0.1	0.05	0
02/154	-0.04	0.15	0.25	0.14	0.16	0.12	0
02/155	-0.02	0.24	0.15	0.06	0.12	0.09	0
02/156	-0.04	0.06	0.05	0.02	-	0.09	0
02/157	-0.01	-0.01	0.03	0.12	0.12	0.08	0
02/158	-0.04	-0.1	0.01	0.01	0.04	0.1	0
02/159	-0.04	0.07	0.13	0.15	0.12	0.12	0
02/160	-0.02	0.33	-	0.4	0.22	0.38	0
02/161	-0.06	-0.04	0.09	0	0.16	0.15	0
02/162	0.08	-0.02	0.12	0.25	0.11	0.09	0
02/163	-0.02	-0.01	0.07	0.13	0.13	0.46	0
02/164	-0.04	-0.04	0.03	0.27	0.09	-0.03	0
02/165	-0.02	-0.05	-0.02	0.15	0.05	0.22	0
02/166	-0.04	0.03	0.22	0.13	0.06	0.12	0
02/167	-0.06	-0.03	-0.04	0.08	0.08	0.11	0
02/168	-0.04	0.09	0.05	0.05	0.34	-	0
02/169	-0.05	-0.02	0.06	0.05	0.13	Culled	1
02/170	**3.04**	**2.38**	**1.68**	**1.21**	**2.14**	Culled	1

[#] Euthanased due to fractured leg

Appendix 3.2: Details of abortion and *Neospora caninum* serological status for all heifers that aborted and *N. caninum* IFAT[a] results for the ELISA[b] positive heifers that did not abort.

Animal ID	*N. caninum* serological status by ELISA[b]	Estimated date of conception	Date abortion detected	Estimated stage of gestation at abortion (days)	Jan IFAT[a]	Feb IFAT[a]	Mar IFAT[a]	Apr IFAT[a]	May IFAT[a]
02/014	POS	14/11/03	-	-	-	1/200	1/600	1000	1/600
02/022	POS	3/11/03	18/03/04	122	-	1/600	1/600	1/200	1/200
02/029	POS	30/10/03	-	-	-	0	0	1/200	1/600
02/034	POS	5/11/03	18/03/04	120	-	1/200	1/4000	1/600	1/600
02/042	POS	5/11/03	18/03/04	120	-	1/600	1/4000	1/1000	1/600
02/043	POS	5/11/03	-	-	-	1/200	1/200	1/200	1/200
02/052	POS	1/12/03	-	-	-	1/200	1/2000	1/2000	1/4000
02/053	NEG	24/10/03	17/12/03	58	0	0	-	-	-
02/055	POS	1/11/03	-	-	-	1/200	1/200	1/200	1/200
02/062	POS	12/11/03	19/02/04	85	1/600	1/4000	1/1000	1/600	1/200
02/065	POS	1/11/03	18/03/04	124	-	0	1/600	1/200	0
02/074	POS	3/11/03	15/04/04	150	-	0	1/600	1/200	1/200
02/094	POS	1/11/03	15/04/04	152	-	1/200	1/600	1/2000	1/600
02/097	NEG	28/10/03	19/02/04	100	0	0	0	0	-
02/098	NEG	16/11/03	15/04/04	137	-	-	0	0	0
02/126	POS	10/12/03	-	-	-	1/200	1/600	1/200	1/200
02/132	POS	5/11/03	-	-	-	1/200	1/1000	1/1000	1/1000
02/149	POS	24/10/03	19/02/04	104	1/1000	1/1000	1/600	1/600	1/600
02/150	POS	1/11/03	15/04/04	152	-	0	1/600	1/200	1/200
02/169	NEG	5/11/03	25/02/04	98	0	0	0	0	-
02/170	POS	3/11/03	18/03/04	122	-	1/200	1/1000	1/200	1/200

[a] VMRD Inc., Pullman, WA, USA
[b] HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA)

Appendix 3.3 Protocol for Herd Chek Neospora ELISA (IDEXX Laboratories Inc., Maine, USA)

General principles

This enzyme immunoassay is used to detect antibody to *Neospora caninum* in bovine serum. *Neospora caninum* antigens are coated on the microtitre well plates (96 wells) and test samples are added then incubated. Antibody within the sample forms a complex with the coated antigen. Unbound material is then washed from the wells and an anti-bovine:horseradish peroxidise conjugate is added which binds to any bovine antibody attached in the wells. The final step consists of washing away any unbound conjugate then an enzyme substrate (hydrogen peroxide) and a chromogen 3,3', 5,5' tetramethlybenzidine are added to the wells. Any colour detected is proportional to the amount of antibody present in the test sample.

All reagents must be stored at 2-7^{o}C and be brought to room temperature (15-30^{o}C) before use. Care must be taken to prevent contamination of kit components and to observe expiry dates on the components. Reagents should be mixed by gentle swirling or vortexing.

Test procedure

1. Dilute test samples 1:100 using sample diluent and the wash concentrate is also diluted 1:10 using distilled / deionised water.
2. Dispense 100 μL of undiluted negative control into wells A1 and A2.
3. Dispense 100 μL of undiluted positive control into wells A3 and A4.
4. Dispense 100 μL of diluted sample into other wells and record their position (all samples should be run in duplicates).
5. Incubate for 30 minutes at room temperature.
6. Aspirate all liquid contents of all wells into an appropriate waste reservoir.
7. Wash each well with approximately 300 μL of phosphate buffered wash solution, four times. Aspirate liquid contents of all wells after each wash. Avoid the plate drying between washes and prior to the addition of conjugate. Following the final wash fluid aspiration, gently but firmly tap residual wash fluid from each plate onto absorbent material.
8. Dispense 100 μL of anti-bovine:horseradish peroxidise conjugate into each well.

9. Incubate for 30 minutes at room temperature.
10. Repeat steps 6 and 7.
11. Dispense 100 μL of tetramethlybenzidine substrate solution into each test plate well.
12. Incubate for 15 minutes at room temperature.
13. Dispense 100 μL of stop solution into each well of the test plate to stop the reaction.
14. Blank on air.
15. Measure and record the absorbance at 620, 630 or 650 nm.
16. Calculate the results (sample to positive ratio)

Results

For the assay to be valid, the difference between the positive control (PC) mean and the negative control (NC) mean must be ≥ 0.150. In addition, the negative control mean must be ≤ 0.20.

The presence or absence of antibody to *Neospora caninum* is determined by the sample to positive (S/P) ratio for each sample, this should have been carried out in duplicate to calculate the mean absorbance for that sample. The positive control has been standardised and represents a significant level of antibody to *N. caninum* in bovine serum.

$$S/P = \frac{Sample\ \ A650 - NCmean}{PCmean - NCmean}$$

Example: Sample mean = 0.450, PCmean = 0.505, NCmean = 0.085

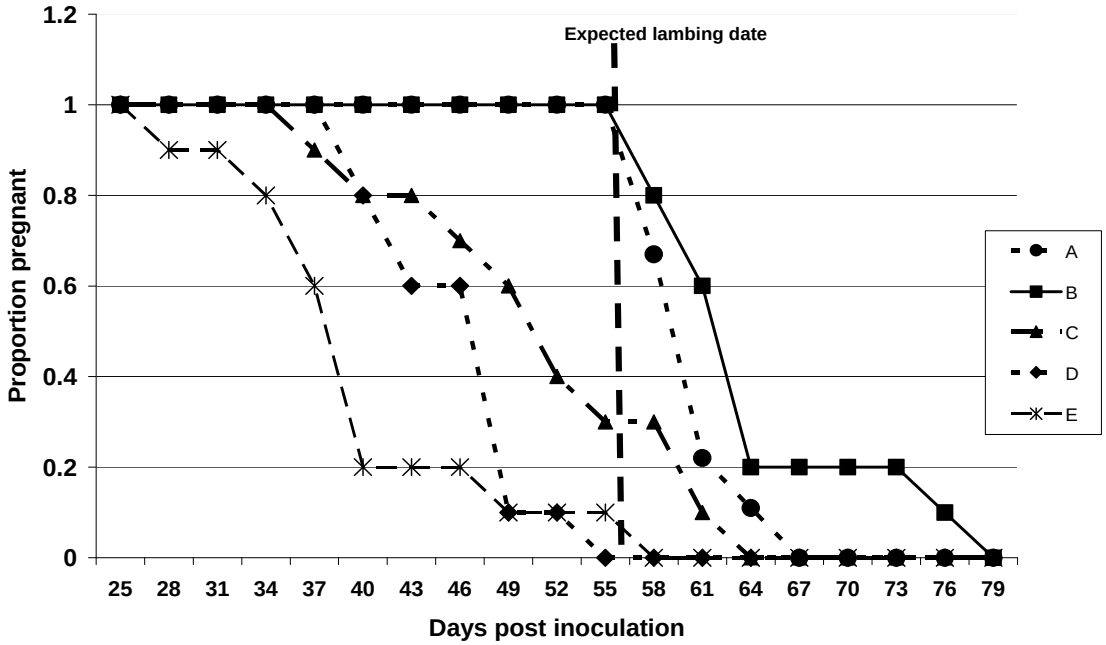
$$S/P = \frac{0.450 - 0.085}{0.505 - 0.085} = \frac{0.365}{0.420} = 0.87$$

Interpretation: serum samples with S/P ratios of < 0.50 are classified as negative for *N. caninum* antibodies. If the S/P ratio is ≥ 0.50 the sample is classified positive for *N. caninum* antibodies. When we wanted to be sure that the cattle we were selecting for trials were not infected with *N. caninum*, a cut-off in the sample-to-positive (S/P) ratio of 0.21, was used to improve test sensitivity (Reichel and Pfeiffer 2002).

Appendix 5.1: Summary of histopathological and serological findings for cases of abortion among cattle from farms with endemic *Neospora caninum* infection.

		Farm					
	GROUP	A	B	C	D	E	ALL
N. caninum lesions	Vaccinated	1	0	1	0	1	3
	Control	0	1	2	0	3	6
	Non-enrolled	0	0	0	0	0	0
High *N. caninum* IFAT	Vaccinated	1	2	4	0	17	24
	Control	2	3	9	0	10	24
	Non-enrolled	1	1	5	0	2	9
Bacteraemia	Vaccinated	0	0	1	0	3	4
	Control	1	0	4	1	4	10
	Non-enrolled	0	0	0	0	0	0
BVDV seroconversion	Vaccinated	1	1	0	2	2	6
	Control	0	1	2	1	0	4
	Non-enrolled	0	0	0	0	0	0
High MAT to *Leptospira spp.*	Vaccinated	0	0	0	0	4	4
	Control	0	0	2	0	2	4
	Non-enrolled	0	0	0	0	0	0
Mycotic abortion	Vaccinated	0	0	0	0	0	0
	Control	0	0	0	0	0	0
	Non-enrolled	0	0	0	0	1	1
No significant findings	Vaccinated	2	2	5	5	1	15
	Control	1	4	8	8	7	28
	Non-enrolled	0	4	5	16	2	27
TOTAL	Vaccinated	4	5	10	7	22	48
	Control	4	8	20	10	21	63
	Non-enrolled	1	5	11	16	4	37

Appendix 6.1: Survival curve for pregnancy in ewe lambs inoculated intravenously with *Neospora caninum* tachyzoites 90 days after joining with the ram; the legend denotes the amount of the tachyzoite challenge.



Group A = Vero cell culture (control group)

Group B = 50 *N. caninum* tachyzoites

Group C = 5 x 10^3 *N. caninum* tachyzoites

Group D = 1 x 10^6 *N. caninum* tachyzoites

Group E = 1 x 10^8 *N. caninum* tachyzoites

Appendix 6.2: Protocol for immunofluorescent antibody test to detect antibodies against *Neospora caninum* in sheep sera

Preparation of antigen slides: Tachyzoites of the New Zealand strains of *Neospora caninum* (Nc-NZ1, Nc-NZ2 and Nc-NZ3; Okeoma et al. 2004c) had been maintained by periodic passage in Vero cell culture. The monolayer was removed into 4 x 50 mL conical tubes (Greiner Bio One, Frickenhausen, Germany) then centrifuged at 1,300 *g* for 10 minutes and the pellets were combined into one tube. This tube was then centrifuged and the supernatant removed to leave 15 mL remaining. The supernatant was then passed twice, through a 25g needle, then through a 5 μm filter (Membrane Solutions, TX, USA). The resulting product was then centrifuged (again at 1,300 *g* for 10 minutes) to produce pelleted tachyzoites and the supernatant removed to leave 1 mL remaining. 5 μL aliquots were then pipetted into each well on commercial teflon-coated glass slides (ProSciTech, Queensland, Australia). Slides were dried and then stored at -70°C until used. Each batch was checked by Giemsa stain and haemocytometer counts.

Preparation of test sera: Serum samples were collected into 10 mL (adult sheep) or 3 mL (neonatal lambs) plain vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) and kept refrigerated at 4°C until they were centrifuged at 500 *g* for 10 minutes (within 24 h of collection). The supernatant (sera) was then removed.

IFAT test:

1. Antigen slides were thawed just prior to use.
2. Serum samples were diluted to 1:50 and then two-fold from that dilution. Samples were screened at 1:100.
3. Serum from a sheep experimentally challenged with Nc-NZ1 was used as a positive control (IFAT titre 1:6,400) and sterile PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO) as a negative control and these were included on each slide.
4. 5 μL of diluted sera was placed into each well of the antigen slide and incubated for 1 h at 37°C.
5. Slides were washed in PBS for 5 minutes and tapped to remove any excess.
6. 5 μL of fluorescein isothiocyanate-labelled donkey anti-sheep IgG (Jackson ImmunoResearch, PA, USA), diluted to 1:200 in PBS, was added as the conjugate

to each well, along with 985uL PBS and 10 μL Evans Blue (Sigma-Aldrich, St Louis, MO, USA) as a counterstain.

7. Slides were incubated at 37 °C for 30 minutes then washed in PBS for 5 minutes and tapped to remove any excess.
8. A cover slip was placed on the slide which was examined microscopically using oil at 400x.
9. A positive result occurs when fluorescence appears over the entire surface of the tachyzoite. Non-specific apical fluorescence may be a cross-reaction due to other apicomplexan parasites such as *Eimeria* spp. *Cryptosporidia* spp. *Sarcocystis* spp. and *Toxoplasma gondii*.
10. Positive samples were serially two-fold diluted to determine a fluorescence end-point which was the last serum dilution with complete tachyzoite peripheral fluorescence.

Note: An initial dilution of 1:100 was used as at lower dilutions the background reactivity was too high. This method was adapted from those reported by Conrad et al. (1993) and Paré et al. (1995a).

Appendix 6.3: Protocol for Chekit Neospora ELISA (IDEXX Laboratories Inc., Maine, USA)

General principles

This enzyme immunoassay is used to detect antibodies against *Neospora caninum* in serum and plasma samples from ruminants. Dilutions of the samples to be tested are incubated in microtitre wells that are coated with *N. caninum* antigen. Any antibody specific for *N. caninum* binds to the antigen in the wells and forms an antigen/antibody complex on the plate well surface. Unbound material is removed from the wells by washing. A peroxidise-labelled anti-ruminant IgG conjugate is added that binds to the ruminant antibodies complexed with the *N. caninum* antigen. Unbound conjugate is removed by washing and the substrate is added to the wells. The degree of colour that develops (optical density measured at 450 nm following the addition of the stop solution) is directly proportional to the amount of antibody specific for *N. caninum* present in the sample. The diagnostic relevance of the result is obtained by comparing the optical density (OD) that develops in wells containing the samples with the OD from the wells containing the positive control.

All reagents must be stored at 2-8°C and be brought to room temperature (15-30°C) before use. Care must be taken to prevent contamination of kit components and to observe expiry dates on the components. Reagents should be mixed by gentle swirling or vortexing.

Test procedure

1. Determine the amount of CHEKIT-wash solution needed for washing the microtitre plates and diluting the samples and controls. Dilute the CHEKIT-10x wash concentrate 1:10 with distilled water.
2. Dispense 90 µL CHEKIT-Neospora sample diluent into each well of the microtitre plate.
3. Add 10 µL of the undiluted samples and controls into the appropriate wells of the microtitre plate (final dilution 1:10).
4. Mix the contents within each well by gently shaking the microtitre plate briefly.
5. Cover the microtitre plate with a lid and incubate for 60 minutes at 37 °C.
6. Wash well with approximately 300 µL CHEKIT wash solution three times. Aspirate liquid contents of all wells after each wash. Following the final aspiration,

firmly tap residual wash fluid from each plate onto absorbent material. Avoid the plate drying between washes and prior to the addition of the next reagent.

7. Dispense 100 µL of CHEKIT-Neospora-anti-ruminant IgG peroxidise conjugate into each well.
8. Cover and incubate the microtitre plate for 60 minutes at 37 °C in a humid chamber.
9. Repeat step 6.
10. Dispense 100 µL tetramethlybenzidine substrate into each well.
11. Incubate the substrate at room temperature (18-25°C) for 15 minutes.
12. Stop the colour reaction by adding 100 µL CHEKIT-stop solution. The stop solution should be dispensed in the same order and at the same speed as the substrate.
13. Read the results using a photometer at a wavelength of 450 nm (must be within 2 hours of adding the stop solution).

Results

To validate the assay the optical density (OD) of the positive control should not exceed 2.0 and the OD of the negative control should not exceed 0.5. The difference between the positive and the negative control must be ≥ 0.3. Analyse the samples in relation to the negative and the positive controls with the formula:

$$Value\ (\%) = \frac{ODsample - ODneg}{ODpos - ODneg} \times 100\%$$

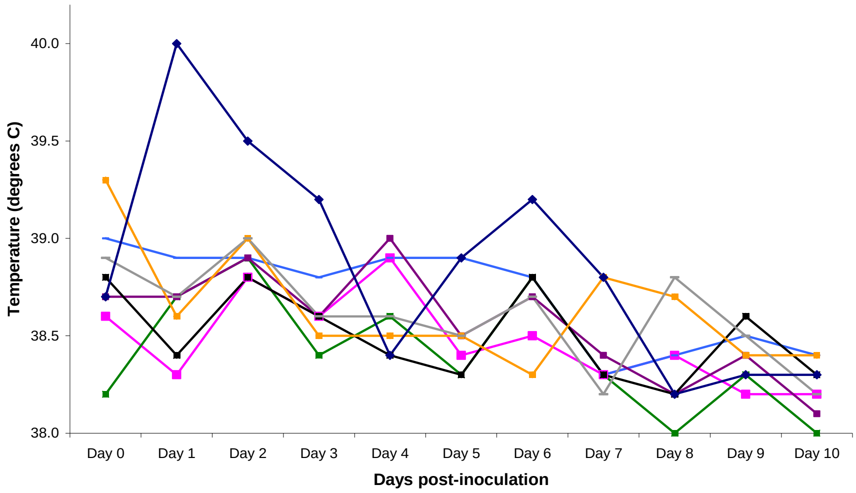
The manufacturer's instructions for interpretation of the optical density are: < 30% the sample is negative, ≥ 30% to < 40% the sample is suspicious, and ≥ 40% the sample is positive. For the purposes of our research and to improve test sensitivity, samples ≥ 11.8% were regarded as seropositive (Reichel et al. 2008).

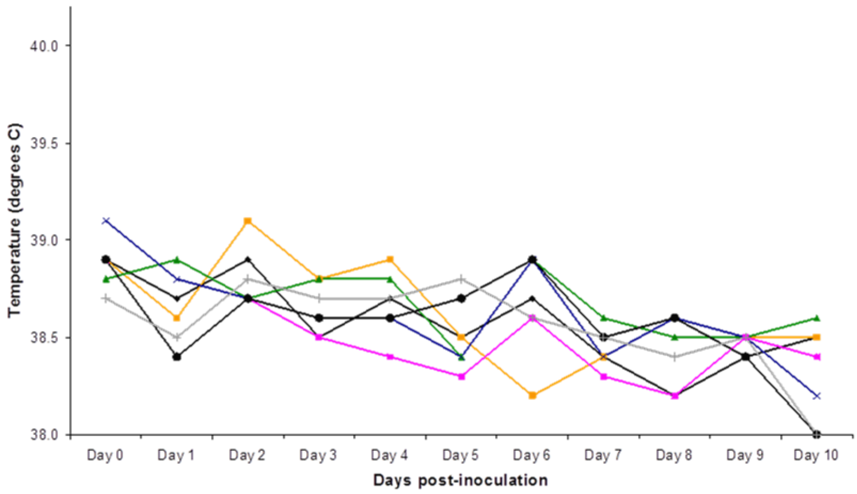
Appendix 7.1: Sample to positive (S/P) ratios using the HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA; a cut-off in the S/P ratio of 0.50 is considered positive) in heifers prior to challenge with *N. caninum* and on days following challenge.

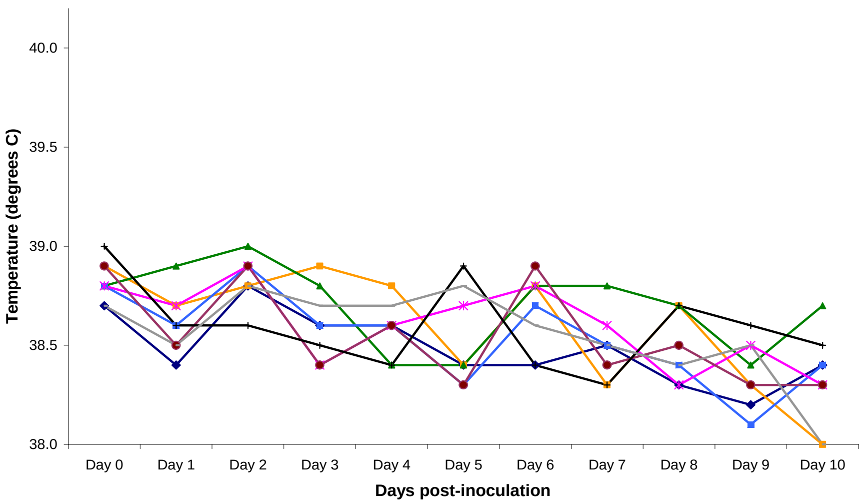
Heifer ID	Challenge method	26/01 2004	12/06 2004	30/09 2004	6/12 2004	Day 0	Day 14	Day 28	Day 42	Day 56	Day 70	Day 98	Day 126	Day 154	Day 182
1	IV	-	-	0.00	0.04	-0.03	0.16	**1.08**	**0.85**	**1.11**	**3.53**	**0.69**	**0.76**	**0.83**	**0.72**
2	IV	-	-	0.16	0.35	0.05	0.30	**1.89**	**3.01**	**2.17**	**2.51**	**2.98**	**2.77**	**2.33**	na[#]
3	IV	-	-	0.05	0.14	0.07	0.30	**1.59**	**2.01**	**2.06**	**2.28**	**1.61**	**1.13**	**1.15**	**0.82**
4	IV	-	-	0.05	0.16	0.06	0.20	**0.82**	**1.10**	**1.08**	**0.14**	**1.52**	**0.87**	**0.84**	**0.72**
5	IV	-0.04	0.05	0.06	0.10	0.02	**0.55**	**1.49**	**1.20**	**1.62**	**0.75**	**1.49**	**0.90**	**1.04**	**0.94**
6	IV	-0.07	0.15	0.10	0.16	0.08	0.29	**1.16**	**0.65**	**1.07**	**0.82**	**1.58**	**0.60**	**0.91**	**0.77**
7	IV	-0.03	0.05	0.08	0.36	0.04	0.33	**1.67**	**1.27**	**1.69**	**1.76**	**2.06**	**1.53**	**2.26**	**1.81**
8	IV	0.00	0.04	0.10	0.09	0.03	0.53	**2.06**	**1.93**	**2.59**	**1.88**	**2.15**	**1.33**	**1.39**	**0.84**
9	Oral	-	-	0.11	0.18	0.01	0.01	0.17	0.01	0.01	0.01	-0.01	-0.02	0.10	0.02
10	Oral	-	-	0.19	0.07	0.01	0.01	0.07	-0.01	0.04	0.03	0.00	0.00	0.06	0.08
11	Oral	-	-	0.11	0.13	0.02	0.18	0.01	0.00	0.01	-0.04	0.01	-0.03	0.02	0.11
12	Oral	-0.04	0.03	0.12	0.07	0.06	0.07	-0.02	0.00	-0.01	-0.01	0.06	0.00	0.01	-0.07
13	Oral	0.14	0.06	0.04	0.11	0.01	0.04	0.12	0.02	0.03	-0.03	-0.01	0.02	0.02	0.14
14	Oral	0.02	0.09	0.06	0.04	0.00	0.02	0.10	0.00	-0.02	-0.02	-0.03	-0.01	-0.02	0.05
15	Oral	0.06	0.03	0.14	0.16	0.01	0.04	0.12	-0.03	0.01	-0.05	0.03	0.01	0.07	0.04
16	Oral	-0.03	0.03	0.02	0.07	0.03	0.00	**0.76**	**1.41**	**1.47**	**1.43**	**1.76**	**1.38**	**1.61**	**1.37**
17	Control	-0.04	0.00	0.00	0.04	-0.03	0.01	-0.03	-0.04	-0.06	-0.04	0.06	-0.03	0.03	-0.05
18	Control	0.04	0.03	0.09	0.09	0.04	0.13	0.03	0.02	-0.02	-0.06	-0.12	0.05	0.07	-0.07
19	Control	-0.07	0.00	0.09	0.07	0.00	0.05	-0.01	-0.02	0.04	-0.03	0.00	0.00	0.02	0.06
20	Control	0.05	-0.07	-0.02	0.02	-0.05	-0.01	0.02	-0.03	-0.03	-0.02	-0.03	-0.03	-0.06	-0.02
21	Control	0.06	0.10	0.05	0.11	0.00	0.03	0.04	0.02	0.03	-0.01	-0.01	0.02	0.11	0.10
22	Control	-0.07	0.07	0.06	0.14	0.03	0.01	0.00	0.01	0.01	-0.03	-0.01	0.00	0.02	0.08
23	Control	-0.02	0.04	0.13	0.06	-0.01	-0.01	-0.04	0.02	0.01	-0.04	0.00	0.00	0.02	0.09

[#] Culled after aborting

Appendix 7.2: Rectal temperature (degrees Celsius) of heifers following inoculation with *Neospora caninum* tachyzoites by intravenous (top) and oral routes (centre) and control group inoculated intravenously with Vero cells (bottom).







Appendix 7.3: PCR protocol to detect *Neospora caninum* DNA used in heifer challenge studies (modified from Ellis et al. 1999).

DNA was extracted from whole blood and samples of placenta or vaginal discharge using the DNeasy Tissue Kit (Qiagen, Victoria, Australia), used according to manufacturer's instructions[a]. Samples are first lysed using Proteinase K. The buffer system is optimised to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. Simple centrifugation processing completely removes contaminants and enzyme inhibitors, such as proteins and divalent cations. Purified DNA is then eluted in water or low-salt buffer. DNeasy purified DNA typically has an A_{260}/A_{280} ratio between 1.7 and 1.9, and is up to 50 kb in size, with fragments of 30 kb predominating. However, the DNeasy procedure also efficiently recovers DNA fragments as small as 100 bp.

Purification of total DNA from whole blood or vaginal discharge:

1. Pipette 20 µL proteinase K into the microcentrifuge tube
2. Add 100 µL anticoagulated blood or vaginal discharge and adjust the total volume to 220 µL with PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO).
3. Add 200 µL Buffer AL, mix thoroughly by vortexing and incubate at 56°C for 10 min
4. Add 200 µL ethanol (96-100%) to the sample and mix thoroughly by vortexing
5. Pipette the mixture into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuge at ≥ 6,000 *g*. Discard flow-through and collection tube
6. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL Buffer AW1 and centrifuge for 1 min at ≥ 6,000 *g*. Discard flow-through and collection tube
7. Place the DNeasy Mini spin column in a new 2 mL collection tube, add 500 µL Buffer AW2 and centrifuge for 3 min at 20,000 *g* to dry the DNeasy membrane. Discard flow-through and collection tube
8. Place the DNeasy Mini spin column in a clean 1.5 mL or 2 mL microcentrifuge tube and pipette 200 µL Buffer AE directly onto the DNeasy membrane. Incubate

[a] (Qiagen DNeasy blood & Tissue handbook 07/2006 http://www.qiagen.com/product/genomicdnastabilizationpurification/dneasytissuesystem/dneasybloodtissuekit.aspx#Tabs=t2 downloaded 17 July 2011).

at room temperature for 1 min and then centrifuge for 1 min at ≥ 6,000 *g* to elute. Repeat this step again to maximise DNA yield.

Purification of total DNA from tissues:

Samples of brain: Five grams of each fresh calf brain sample was placed into a sterile, single use, 50 mL conical tissue grinder (VWR, PA, USA) containing 10 mL of sterile PBS (pH 7.4; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO). After homogenisation, 100 µL aliquots of the suspension of brain tissue were used for DNA extraction from Step 2 of the protocol described above.

Samples of placenta: Twenty-five milligrams of tissue was cut into small pieces and placed in a 1.5 mL microcentrifuge tube and 180 µL of buffer ATL was added. The protocol for purification of total DNA as listed above was then followed from Step 2.

PCR positive and negative controls: purified DNA from *N. caninum* NcNZ1, NcNZ2 or NcNZ3 (Okeoma et al. 2004) tachyzoites (kept in Vero cell culture at Massey University) was used as a positive control for each PCR run. Water blanks were included for negative controls.

Reaction set-up: all manipulations for PCR were performed in a PCR clean room free of extraneous DNA. Standard quality control procedures to prevent contamination as recommended by Dragon et al. (1994) were followed. Amplifications were carried out in 0.2 µL thin-wall PCR tubes (Axygen, Union City, CA, USA).

Reactions were prepared as a master mix containing all reagents except for the template DNA in order to minimise labour, operator error and artefactual variation. Reagents were kept chilled on ice at all times.

Single tube nested PCR: Primers used were:
NF1 (5′-GCGTGATATACTACTCCCTGT), NS2 (5′-CATGTGGATATTTTGCA),
NR1 (5′-AAACTCCTGGAAGTTAAAG) and SR1 (5′-AAATAACGGTGTGGGAAAA).

The 50 µL PCR reaction mixture contained: 5 µL of test sample DNA, 1.75 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 µM NS2 and NR1, 0.015 µL NF1 and SR1, 1 unit of Platinum Taq DNA polymerase (Invitrogen, CA, USA) with 1X PCR buffer [10X buffer consisting of

200 mM Tris HCl (pH 8.4), 500 mM KCl; Invitrogen, CA, USA]. Reaction mixtures were placed in a Px2 Thermocycler (Thermo Scientific, Rockford, IL, USA).

The thermal cycle conditions were as follows: 95°C for 5 minutes; five cycles of 94°C for 30 seconds (s), 60°C for 150 s, 72°C for 30 s; 20 cycles of 88°C for 30 s, 60°C for 30 s, 72°C for 30 s, ten cycles of 88°C for 30 s, 54°C for 30 s, 72°C for 30 s; 25 cycles of 86°C for 30 s, 54°C for 30 s, 72°C for 30 s; and 72°C for 10 min. PCR products were eluted on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen, CA, USA) containing ethidium bromide (5 μg/mL) at 100V for 45 min and visualized under UV light on a transilluminator (Bio-Rad, New South Wales, Australia).

Positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Inc, CA, USA) and the ABI3730 Genetic Analyzer (Applied Biosystems Inc.). Sequencing results were submitted to the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for confirmation of ITS1 gene amplification.

Appendix 8.1: Sample to positive (S/P) ratios using the HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA; a cut-off in the S/P ratio of 0.50 is considered positive) in heifers prior to inoculation and then after challenge with *N. caninum* tachyzoites or Vero cell culture.

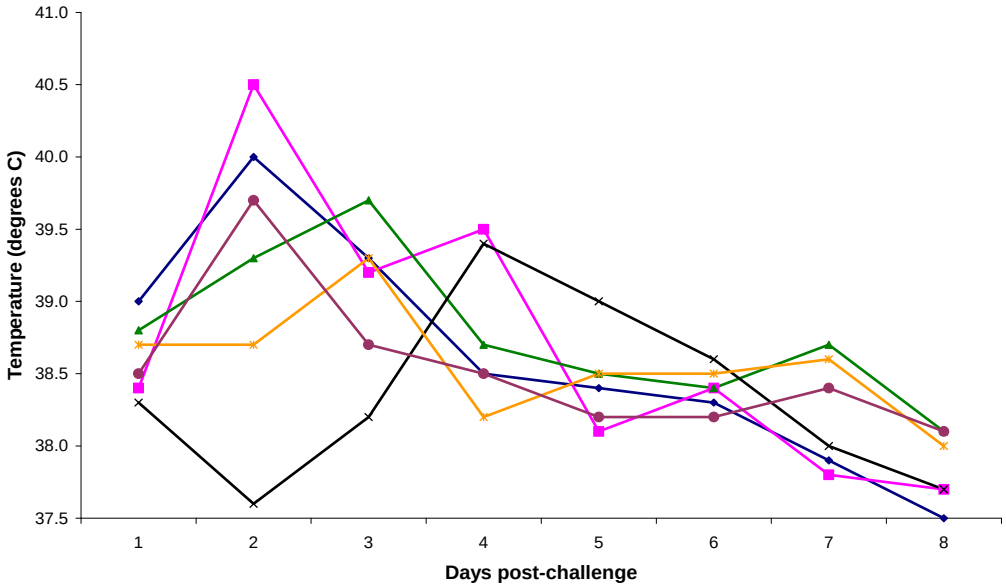
Heifer ID	9 Dec 2004	12 May 2005	28 Jul 2005	4 Oct 2005[#]	11 Nov 2005[a]	25 Nov 2005	9 Dec 2005	23 Dec 2005	6 Jan 2006	31 Mar 2006[b]	Heifer at calving	Calf
A1	-0.02	-0.03	0.00	0.26	0.05	**0.54**	**0.87**	**1.88**	**1.29**	**3.39**	**3.97**	**4.17**
A2	-0.06	-0.03	-0.02	0.12	0.04	**0.64**	**0.97**	**1.21**	**0.93**	**2.99**	**1.93**	**1.72**
A3	-0.09	-0.03	0.02	0.06	0.00	0.36	**1.10**	**1.41**	**0.80**	**3.30**	**1.95**	**2.67**
A4	-0.09	-0.03	-0.02	0.18	-0.02	0.35	**0.69**	**1.25**	**1.00**	**3.11**	**2.87**	na
A5	-0.10	-0.04	-0.01	0.22	0.05	**0.99**	**1.49**	**2.03**	**1.77**	**2.93**	**2.53**	na
A6	-0.01	-0.02	0.00	0.08	0.01	0.35	**0.66**	**0.71**	**0.74**	**3.43**	**2.23**	**2.48**
B1	-0.07	0.00	0.02	0.21	0.04	**0.87**	**1.50**	**1.94**	**1.45**	0.29	**0.67**	**0.47**
B2	-0.08	-0.01	-0.02	0.09	0.11	**0.99**	**1.28**	**1.69**	**0.81**	**0.68**	**1.42**	**2.04**
B3	-0.08	-0.03	-0.03	-0.02	0.00	**1.18**	**2.23**	**2.48**	**2.48**	**1.29**	**1.56**	na
B4	-0.05	0.01	0.03	0.17	0.28	**0.78**	**0.82**	**1.25**	**0.97**	**0.79**	**0.78**	**1.32**
B5	-0.10	-0.02	0.10	0.23	0.13	**0.93**	**1.31**	**1.76**	**1.33**	**1.43**	**2.03**	**2.21**
C1	-0.08	-0.03	-0.02	0.37	0.04	0.03	0.00	-0.01	0.03	**2.40**	**2.40**	na
C2	-0.06	-0.03	-0.02	0.10	0.29	0.38	0.19	0.19	0.11	**2.16**	**2.43**	na
C3	-0.07	-0.04	-0.03	-0.03	-0.02	-0.03	0.00	0.00	0.02	**2.04**	**0.97**	-0.11
C4	-0.08	-0.03	0.04	0.24	0.10	0.15	0.05	0.04	0.07	**2.53**	**2.09**	na
C5	-0.06	-0.03	0.00	0.06	0.01	0.12	0.02	0.09	0.09	**2.73**	**3.22**	na
C6	-0.11	0.00	0.08	0.21	0.11	0.27	0.08	0.15	0.13	**2.36**	**2.45**	na
D1	-0.07	-0.01	-0.04	0.09	0.03	0.04	0.02	0.14	0.10	0.04	-0.11	**0.76**
D2	-0.10	-0.04	-0.04	-0.03	0.01	-0.01	0.01	0.01	0.01	-0.03	0.03	-0.23
D3	-0.05	-0.02	0.00	0.12	-0.01	0.02	0.00	0.00	0.01	0.02	-0.01	0.26
D4	-0.10	-0.04	-0.04	-0.01	-0.03	-0.06	-0.02	-0.05	-0.01	-0.01	-0.16	-0.07
D5	-0.09	-0.01	0.03	0.20	0.06	0.04	0.05	0.07	0.06	0.04	0.03	**0.62**
D6	-0.10	-0.02	0.03	0.11	0.05	0.12	0.06	0.06	0.10	0.03	-0.23	-0.24

[#] The negative control sample in this batch had an S/P ratio of 0.12

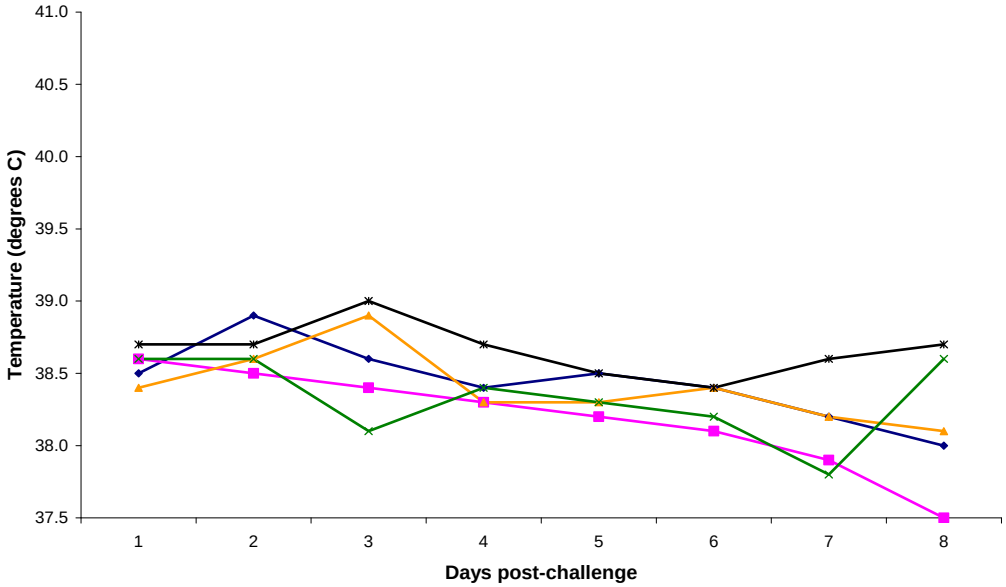
[a] Date of inoculation with either mouse-passaged *N. caninum* tachyzoites or Vero cell culture; pre-mating

[b] 28 days following challenge with either intravenous and subcutaneous *N. caninum* tachyzoites or Vero cell culture on Day 70 of gestation

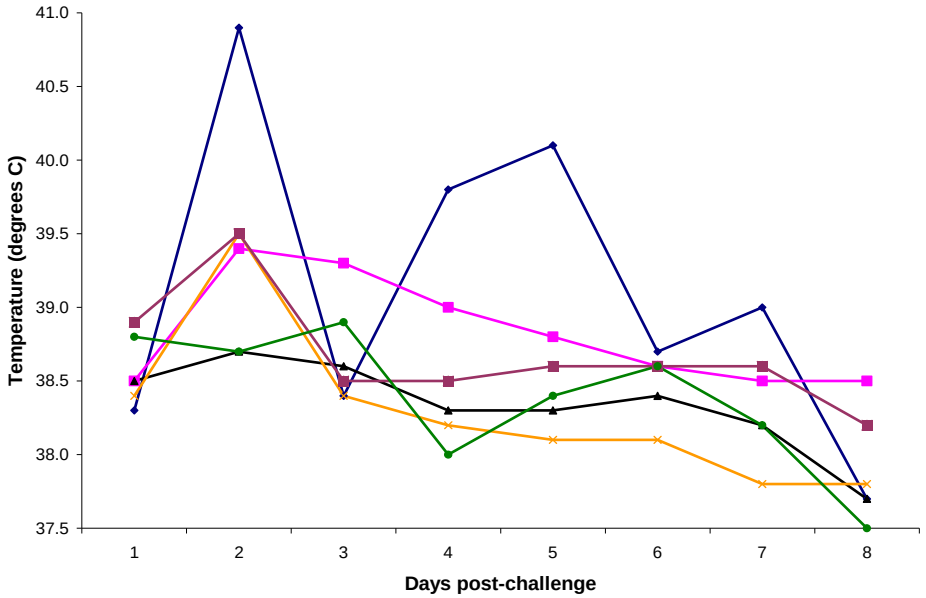
Appendix 8.2: Rectal temperature (degrees Celsius) of heifers following intravenous and subcutaneous challenge with *Neospora caninum* tachyzoites or Vero cells on Day 70 of gestation.



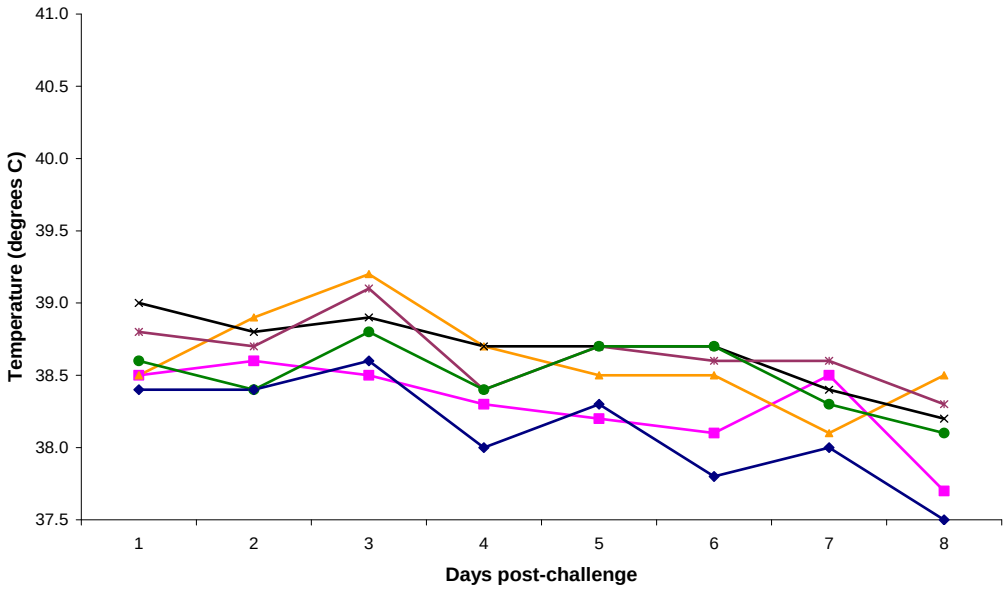
a) Group A heifers: inoculated with mouse-passaged *N. caninum* tachyzoites pre-mating and then challenged with *N. caninum* tachyzoites on Day 70 of gestation.



b) Group B heifers: inoculated with mouse-passaged *N. caninum* tachyzoites pre-mating and then challenged with Vero cell culture on Day 70 of gestation.



c) Group C heifers: inoculated with Vero cell culture pre-mating and then challenged with *N. caninum* tachyzoites on Day 70 of gestation.



d) Group D heifers: inoculated with Vero cell culture pre-mating and then challenged with Vero cell culture on Day 70 of gestation.

Appendix 8.3: Protocol for microsatellite array analysis used to characterise positive PCR amplicon samples from heifers that were inoculated with mouse-passaged *Neospora caninum* tachyzoites and then inoculated on Day 70 of gestation.

Tissue samples that were positive for *N. caninum* DNA using the nested-PCR technique (described in Appendix 7.3) were amplified for microsatellite array analysis. *N. caninum* DNA that had previously been extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Victoria, Australia; also detailed in Appendix 7.3) was then tested using the following nested-PCR technique:

1. The following external primers [described by Basso et al. (2009) and derived from Nc-Liv tachyzoite sequence data] were used for the external amplification step:

Primers	Name	Sequence (5'-3')	Size	Position	Repeat Sequence
Forward	MS1B	TTGGACCTTCGGCTAAA	400	Contig762	$(AT)_n$ AC $(AT)_3$
Reverse	MS1B	CCTCGCTTCCCCCACAAA			
Forward	MS2	ACGCGACGGTGGAGAGGAA	488	Contig1160	$(AT)_n$ TTGTATC$(AT)_n$ GT $(AT)_2$
Reverse	MS2	TAAAACCGAATACCCGAAAAACT			
Forward	MS3	CAGACGATTGCCAGGGAGACT	371	Contig1157	$(AT)_n$
Reverse	MS3	CGCCATAGCATCGCATTTG			
Forward	MS4	CTGGGAGTGAATCGGAAGG	414	Contig801	$(AT)_n$ ACATTT $(AT)_2$
Reverse	MS4	CGTACACCCCATAGACAT			
Forward	MS5	GGTGCACCAAGCCGAGAAC	414	Contig1030	$(TA)_n$ TGTA
Reverse	MS5	CCGCCCGTGTCCTGAACT			

2. Internal primers were as described by Regidor-Cerillo et al. (2006) with the MS5 reverse (R) primer as modified by Basso et al. (2009) and consisted of:

Primers	Name	Sequence (5'-3')	Size	Position	Repeat Sequence
Forward	MS1B	GGAGAACGGACGACGTTAAG	301	Contig762	$(AT)_n$ AC $(AT)_3$
Reverse	MS1B	CCCACAAACTCCCTTTCCTC			
Forward	MS2	CTCTTCTCACCAGCCCAGTC	307	Contig1160	$(AT)_n$ TTGTATC$(AT)_n$ GT $(AT)_2$
Reverse	MS2	GCTGTTACAACCCACGGAAC			
Forward	MS3	GACTTTGGTTTGCCACTGTCG	249	Contig1157	$(AT)_n$
Reverse	MS3	TCCGACATCTACGGACATCG			
Forward	MS4	AGAAGAAGAAACGCGGAATG	299	Contig801	$(AT)_n$ ACATTT $(AT)_2$
Reverse	MS4	TCTGAAACGAATTCCCTTGG			
Forward	MS5	AATCAGGACTCCGTCACACC	310	Contig1030	$(TA)_n$ TGTA
Reverse	MS5	CAGAGAGCTCCCACATCTC			

3. Purified DNA from *N. caninum* Nc-NZ1 tachyzoites (kept in Vero cell culture at Massey University; Okeoma et al. 2004c) was used as a positive control for each PCR run. Nuclease free water was included as a negative control in each run.
4. All manipulations for PCR were performed in a PCR clean room free of extraneous DNA. Standard quality control procedures to prevent contamination as recommended by Dragon et al. (1994) were followed. Amplifications were carried out in 0.2 µL thin-wall PCR tubes (Axygen, Union City, CA, USA). Reagents were prepared as a master mix containing all reagents except for the template DNA in order to minimise labour, operator error and artefactual variation. Reagents were kept chilled on ice at all times.
5. The first round reagent mix (volume 25 µL): A Platinum Taq DNA polymerase kit (Invitrogen, CA, USA) consisting of: 11.35 µL sterile distilled water, 2.5 µL 10x PCR buffer, 0.75 µL $MgCl_2$ (50 mM), 0.7 µL dNTPs (10 mM), 1.25 µL external forward primer (10µM), 1.25 µL external reverse primer (10µM), 5 µL bovine serum albumin (20 µg/ml), 0.2 µL Platinum Taq DNA polymerase (Invitrogen, CA, USA) and 2.0 µL sample DNA.
6. The second round reagent mix (volume 50 µL) consisted of: 38.8 µL sterile distilled water, 5 µL 10x PCR buffer, 2.0 µL $MgCl_2$ (50 mM), 1.0 µL dNTPs (10 mM), 1.0 µL external forward primer (10µM), 1.0 µL external reverse primer (10µM), 0.2 µL Platinum Taq DNA polymerase (Invitrogen, CA, USA) and 1.0 µL of first round DNA product.
7. The thermal cycle conditions were as follows:

First Round	Temp (°C)	Time	No. cycles
Hold	95	5 min	1
Denature Anneal Extension	95 50 72	1 min 1 min 1 min	35
Hold	72 4	10 min ∞	1

Second Round	Temp (°C)	Time	No. cycles
Hold	95	5 min	1
Denature Anneal Extension	95 60 72	1 min 1 min 1 min	40
Hold	72 4	10 min ∞	1

8. The amplification products were visualised after electrophoresis in 1.5% agarose gels stained with ethidium bromide. A 100 bp ladder (Invitrogen, CA, USA) was used as a

size standard. Nc-NZ1 was expected to have amplicons of a similar size to Nc-1 so the following size amplicons were expected after the second round: MS1B=301, MS2=307, MS3=249, MS4=299, MS5=310 although some variation is possible so any slight differences were noted.

9. All amplicons were then purified (PureLink PCR purification kit, Invitrogen, California, USA) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc, California, USA). Sequencing results were submitted to NCDI BLAST to confirm *N. caninum* DNA amplification.

Appendix 8.4: Sample to positive (S/P) ratios using the HerdChek Neospora ELISA (IDEXX Laboratories Inc, Maine, USA; a cut-off in the S/P ratio of 0.50 is considered positive) in dam-calf pairs from the heifer challenge trials reported in Chapters Seven and Eight.

Animal ID	Trial and group	Dam at parturition	Calf within 12 h of birth
1	Challenge - IV	0.72	-0.15
3	Challenge - IV	0.70	-0.10
4	Challenge - IV	0.83	-0.09
5	Challenge - IV	1.56	-0.14
6	Challenge - IV	0.94	1.49
7	Challenge - IV	1.89	-0.10
8	Challenge - IV	0.59	-0.10
9	Challenge - oral	0.00	0.10
10	Challenge - oral	-0.02	-0.12
11	Challenge - oral	0.13	0.39
12	Challenge - oral	-0.07	0.12
13	Challenge - oral	0.11	-0.15
14	Challenge - oral	-0.03	0.13
15	Challenge - oral	0.00	0.69
16	Challenge - oral	1.37	1.07
17	Challenge - control	-0.05	0.16[§]
19	Challenge - control	0.03	0.24[§]
20	Challenge - control	-0.03	0.22[§]
22	Challenge - control	0.03	0.15[§]
A1	Mouse-passaged Nc then Nc mix	3.97	4.17
A2	Mouse-passaged Nc then Nc mix	1.93	1.72
A3	Mouse-passaged Nc then Nc mix	1.95	2.67
A6	Mouse-passaged Nc then Nc mix	2.23	2.48
B1	Mouse-passaged Nc then control	0.67	0.47
B2	Mouse-passaged Nc then control	1.42	2.04
B4	Mouse-passaged Nc then control	0.78	1.32
B5	Mouse-passaged Nc then control	2.03	2.21
C3	Control then Nc mix	0.97	-0.11
D1	Control then control	-0.11	0.76
D2	Control then control	0.03	-0.23
D3	Control then control	-0.01	0.26
D4	Control then control	-0.16	-0.07
D5	Control then control	0.03	0.62
D6	Control then control	-0.23	-0.24

[§] Sampled at 2-11 days old at the farm of origin

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