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Some aspects of the epidemiology of neosporosis in sheep in New Zealand

A thesis presented in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy in Veterinary Science

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2014

khas buat mak dan abah.

"Never say quit till u die....."

Abstract

Recent reports from New Zealand indicate *Neospora caninum* may have a role in causing reproductive problems in sheep. However, knowledge about the epidemiology of neosporosis in sheep in New Zealand is limited. Thus, the research presented in this thesis was undertaken to further understand the mode of transmission, seroprevalence, diagnosis and treatment of *N. caninum* in sheep in New Zealand.

The initial study investigated venereal and vertical transmission. The results suggested that although *N. caninum* DNA can be found in the semen of experimentally infected rams (n=16), the transmission of *N. caninum* to ewes (n=16) via natural mating is unlikely. In a two year study, ewes inoculated prior to mating (n=25 in Year 1; n=7 in Year 2) did not have congenitally infected lambs that year (n=0/44) but did in Year 2 (n=7/11). Ewes re-inoculated on Day 120 of gestation in Year 2 (n=9) had congenitally infected lambs (n=12/12) with more severe lesions than those not re-inoculated (n=2/11) indicating that the initial inoculation did not induce protection. Ewes inoculated for the first time on Day 120 of gestation (n=12) gave birth to lambs (n=10) that were all congenitally infected. Treatment of these congenitally infected newborn lambs (n=11) with toltrazuril (20mg/kg) on Day 1, 7, 14 and 21 was not effective as determined by serology, histopathology and qPCR.

An avidity ELISA assay was able to differentiate between recently and chronically infected sheep. A longitudinal study with serology on 3 farms where *N. caninum* infected sheep were previously identified, found an overall seroprevalence of 0.8% (n=7/880) for *N.*

caninum antibodies. The low seroprevalence observed across selected farms did not allow a meaningful interpretation to be made about the role of neosporosis on these farms.

A consistent observation was the value of using multiple diagnostic tests to detect the presence of *Neospora* rather than relying exclusively on any one of them. Observation of typical lesions was generally more rewarding then the detection of *Neospora* DNA. Overall, further work is required to fully determine if *N. caninum* is causing reproductive problems in sheep in New Zealand.

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List of Abbreviations

AI	artificial insemination
ANOVA	analysis of variance
AV	artificial vagina
BLAST	basic local alignment search tool
cELISA	competitive ELISA
CI	confidence interval
CNS	central nervous system
DAT	direct agglutination test
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphospate
ds	double strand
ELISA	enzyme-linked immunosorbent assay
FBS	foetal bovine serum
G	gravity
H&E	haematoxylin and eosin
IDA	immunodominant antigen
IFAT	indirect fluorescent antibody test
iELISA	indirect ELISA
IFN	interferon
IFN-γ	gamma interferon
Ig	immunoglobulin
IgG	immunoglobulin G
IgM	immunoglobulin M
IH	in-house
IHC	immunohistochemistry
IL	interleukin
ISCOM	immunostimulating complex
IVABS	Institute of Veterinary, Animal and Biomedical Sciences, Massey University
i/v	intravenously
kDa	kilodalton
LAT	latex agglutination test
rDNA	ribosomal DNA

M	molar
MEM	minimum essential medium
NcNZ	*N. caninum* New Zealand
NcSAG1	*N. caninum* surface antigen 1
NcSRS2	*N. caninum* surface antigen-1 related sequence 2
NK	natural killer
O	oocysts
OD	optical density
PBS	phosphate buffer saline
PCR	polymerase chain reaction
qPCR	quantitative or real time PCR
r	ribosomal
RNA	ribonucleic acid
S	Svedberg units
s	seconds
SAG	surface antigen
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S/P	sample to positive ratio
SC	sub cutaneous
ss	single strand
t	tachyzoites
TPI	transplacental infection

Thesis structure and format

This thesis is presented as a series of chapters that are inter-related, thus there are some repetitive elements especially in the Materials and Methods section. Four of the chapters have been published (Chapters 2, 3, 4 and 5) in peer reviewed journals. They are included in the thesis in the form they are presented for publication except that sections have been renumbered and references to appendices have been included. All references for each chapter have been collated at the end of the thesis in the Bibliography section.

Chapter One: ***Neospora caninum* – General introduction and literature review** introduces a brief summary of the objectives of the thesis. It includes a review on various aspects of neosporosis especially in cattle and sheep.

Chapter Two: **Detection of *Neospora caninum* DNA in the semen of experimentally infected rams with no evidence of horizontal transmission in ewes** has been published in the journal Veterinary Parasitology (Syed-Hussain, S.S., Howe, L., Pomroy, W.E., West, D.M., Smith, S.L., Williamson, N.B., 2013, 197, 534-542) which suggested that venereal transmission of *N. caninum* is not a possible route of transmission in sheep although the parasite DNA was detected in the semen of these experimentally infected rams.

Chapter Three: **Adaptation of a commercial ELISA to determine the IgG avidity in sheep experimentally and naturally infected with *Neospora caninum*** has been published in the journal Veterinary Parasitology (Syed-Hussain, S.S., Howe, L., Pomroy, W.E., West, D.M., Smith, S.L., Williamson, N.B., 2014, 203, 21-28) and describes the use of a commercially available ELISA test kit that has been adapted as an IgG avidity assay to differentiate between recently and chronically infected sheep.

Chapter Four: **Vertical transmission of *Neopsora caninum* in experimentally infected sheep** has been published in the journal Veterinary Parasitology (S.S. Syed-Hussain, L. Howe, W.E. Pomroy, D.M. West, M. Hardcastle, and N.B. Williamson., 2015) and describes the importance of vertical transmission as a mode of transmission of neosporosis in sheep. However how this reflects in a natural setting is yet to be known. This study was run concurrently as a part of another study which is described in Chapter Five.

Chapter Five: **A study on the use of toltrazuril to eliminate *Neospora caninum* in congenitally infected lambs born from experimentally infected ewes** has been published in the journal Veterinary Parasitology (S.S. Syed-Hussain, L. Howe, W.E. Pomroy, D.M. West, M. Hardcastle, and N.B. Williamson., 2015) and describes the results of the use of toltrazuril as a mode of treatment for neosporosis in congenitally infected newborn lambs.

Chapter Six: **A longitudinal investigation of *Neospora caninum* serodynamics and seroprevalence in naturally-infected pregnant ewes** describes the serological status of sheep on farms with a previous history of *N. caninum* infection over pregnancy.

Chapter Seven: **General discussion** summarises all the information observed from the studies above which also includes the implications of the findings as well as suggestions for future research work.

Bibliography: To minimise repetition, all references are listed at the end of the thesis.

All experiments conducted in this thesis were approved by the Massey University Animal Ethics Committee.

CHAPTER ONE

Neospora caninum - Introduction and Literature Review

1.1 Introduction

Neospora caninum was first identified in Norway in 1984 as causing a neuromuscular disorder in dogs and has subsequently been recognised as the leading cause of bovine abortion worldwide (Bjerkas et al., 1984; Dubey et al., 1988b; Thornton et al., 1991; Dubey and Schares, 2011). It has also been detected in a wide range of animals including goats, sheep, deer and horses (Dubey, 2003; Dubey and Schares, 2011). In 1990, the first natural infection of *Neospora* in sheep was reported in a congenitally infected lamb with signs of ataxia and weakness after birth (Dubey et al., 1990). Subsequently, naturally occurring ovine neosporosis and abortions have been reported worldwide, including Japan, South America, Australia, Switzerland, Italy, and Spain (Kobayashi et al., 2001; Helmick et al., 2002; Hassig et al., 2003; Masala et al., 2007; Bishop et al., 2010; Moraes et al., 2011; Howe et al., 2012; Moreno et al., 2012).

Until 1988 *N. caninum* was confused with a closely related coccidian, *Toxoplasma gondii,* which has a similar structure and life cycle (Dubey et al., 1988b). There are however, major differences. *N. caninum* has a wide host range but neosporosis is primarily a disease of cattle where dogs and related canids act as definitive hosts. It differs from *T. gondii* which also has a wide intermediate host range but is also recognised as a cause of disease in a wide range of animals especially humans, sheep and goats. It also differs as felids are the definitive hosts. Toxoplasmosis has not been recognised as an important cause of reproductive problems in cattle (Dubey et al., 2007). Unlike *T. gondii*, there is no solid evidence for any zoonotic potential with *N. caninum* as there have been no reports of *N. caninum* DNA or the parasite in human tissues to date (Dubey et al., 2007; McCann et al., 2008).

The overall objectives of this body of work were to better understand the epidemiology of *N. caninum* in sheep and to determine if neosporosis plays a role as an abortifacient agent in sheep on New Zealand farms. This interest followed the recent increase in the number of reports implicating *N. caninum* as a potential cause of abortions and reproductive problems in sheep (West et al., 2006; Howe et al., 2008; Howe et al., 2012).

There were five main aims in this study. The first was to determine if *N. caninum* could be transmitted to ewes via venereal transmission using experimentally infected rams. The second aim was to adapt a commercially available ELISA assay to enable the differentiation between recently and chronically infected animals. The third aim was to determine the rate of vertical transmission from experimentally infected ewes. The fourth aim was to determine the effectiveness of toltrazuril in treating congenitally infected newborn lambs as a model for the use of this drug in cattle. Finally, the fifth aim was to determine the dynamics of neosporosis in sheep from farms with a previous history of *N. caninum*-related reproductive failures by monitoring the serological status of ewes from pre-mating through lambing to weaning.

1.2 Literature Review

1.2.1 Life cycle of *N. caninum*

N. caninum has a heteroxenous life cycle, as shown in Figure 1.1, which involves three infectious stages i.e. the sporozoites (within oocysts), bradyzoites (within tissue cysts) and tachyzoites. Oocysts are only excreted by the definitive host which are dogs (*Canis lapus familiaris*) (McAllister et al., 1998; Lindsay et al., 1999a; Dijkstra et al., 2001) and other canids such as coyotes (*Canis latrans*) (Gondim et al., 2004), Australian dingoes (*Canis*

lapus dingo) (King et al., 2010) and gray wolves (*Canis lapus*) (Dubey et al., 2011). Dogs have been confirmed to act as both an intermediate and definitive host for *N. caninum*. Both bradyzoites and tachyzoites, which occur intracellularly, are the stages found in the intermediate hosts. The range of animals which have been reported to act as intermediate hosts comprises most warm blooded animals including most domestic ruminants, horses (Dubey and Schares, 2011; Dubey et al., 2013; Mesquita et al., 2013; Gharekhani et al., 2013b; Hao et al., 2014), chickens (Martins et al., 2011), sparrows (Gondim et al., 2010), rodents (Ferroglio et al., 2007; Valadas et al., 2010), rabbits (Hughes et al., 2008), hares (Bartova et al., 2010) and these have been reviewed in detail elsewhere (Dubey et al., 2007; Dubey and Schares, 2011). Such a wide range of potential intermediate hosts is a factor as to why neosporosis is so difficult to control. Once the intermediate host ingests oocysts of *N. caninum,* usually in contaminated food or water, sporozoites will be released into the intestinal tract and penetrate the cell walls thus initiating infection. Here, an asexual phase will occur where it will differentiate into tachyzoites and these will rapidly replicate via endodyogeny resulting in rupture of the host cell, liberation of tachyzoites which will infect other cells nearby. This invading process occurs rapidly and leads to development of lesions due to its destruction of cells and associated inflammation (Buxton et al., 2002a).

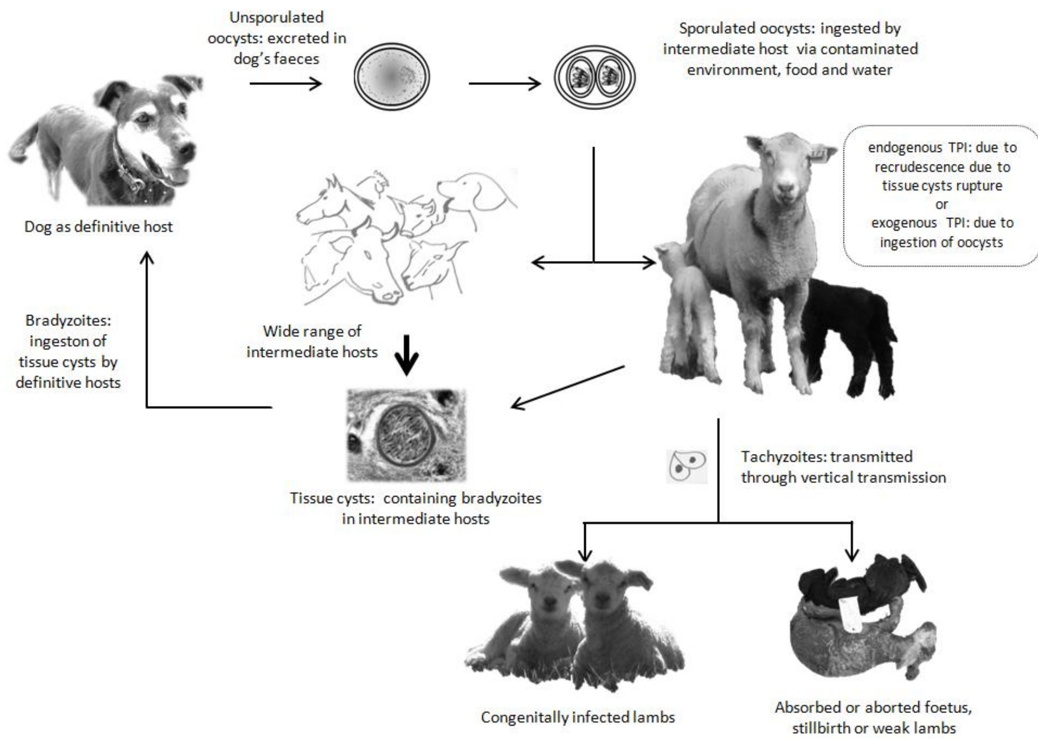

Figure 1.1: The life cycle of *N. caninum* in sheep

1.2.1.1 Oocysts

Upon ingesting infected tissues containing bradyzoites, oocysts were produced during the sexual reproductive stage of the parasite in the intestines of the definitive host (McAllister et al., 1998). These unsporulated *N. caninum* oocysts are about 10 X 12 µm (Lindsay et al., 1999b). They are excreted in faeces and undergo sporulation within 24 to 72 hours once outside the host, depending on the temperature and humidity (Lindsay et al., 1999a). At the end of this process each oocyst will then contain two sporocysts and each of these will contain four sporozoites at the size of 6.5 X 2 µm each (Lindsay et al., 1999b). The *N. caninum* oocysts are morphologically similar to those of *Hammondia heydorni* which are found in dogs and *T. gondii* and *Hammondia hammondi* which are found in cats (Dubey, 1999).

Oocysts are considered to play an important role in maintaining the disease but surprisingly have only been observed a small number of times in dog faeces (Schares et al., 2005; Palavicini et al., 2007; Razmi, 2009). Similar to other coccidia, *N. caninum* oocysts are environmentally resistant (Dubey et al., 2011). Though not many studies have been conducted on the viability and resistance of *N. caninum* oocysts, it has been shown that oocysts were inactivated when treated with high temperatures at 100°C (Uzeda et al., 2007; Alves-Neto et al., 2011).

There is a lot of variation in reports about the total number of oocysts excreted and the period of shedding. Experimental studies have shown that dogs shed oocysts over a period ranging from 5 to 30 days with the total number of oocysts varying from very few up to 1 – 4 million (Dubey et al., 2007). It was also indicated that more oocysts were excreted by puppies than mature dogs. Dogs that were fed infected bovine tissues also excreted more oocysts when compared to those fed with infected murine tissues (Gondim et al.,

2002). Immunosuppressed dogs were also found to shed more oocysts than immunocompetent dogs (Lindsay et al., 1999a; Lindsay et al., 2001). The minimum prepatent period is poorly defined so far (Dubey et al., 2007).

1.2.1.2 Tachyzoites

Tachyzoites are approximately 6 µm X 2 µm and are lunate-shaped. Unlike bradyzoites, they lack amylopectin granules and have a central nucleus (Dubey et al., 2006). Enclosed within a parasitophorous vacuole in the host cell, *N. caninum* tachyzoites multiply rapidly via endodyogeny in a variety of cells including neural cells, vascular endothelial cells, myocytes, hepatocytes, renal cells, alveolar macrophages and placental trophoblasts (Barr et al., 1991; Dubey et al., 2002). This is a stage where the rapidly multiplying tachyzoites cause cells to rupture and can result in severe lesions. In cases where the host's immune response fails to prevent parasite multiplication, death is usually due to the proliferation of tachyzoites that continue to penetrate and multiply progressively causing extensive cell death (Buxton et al., 2002a).

1.2.1.3 Bradyzoites

Bradyzoites are a stage in which the parasite multiplies slowly by endodyogeny within a tissue cyst, thus protected from the host's immune response, leading to persistent infection (Buxton et al., 2002a; Innes et al., 2002). Extrapolating from the life cycle of *T. gondii* (Buxton et al., 2002a), it was suggested that due to the onset of the host protective immune response and other factors, tachyzoites transform into bradyzoites, leading to a persistent dormant infection (Lyons et al., 2002). Measuring about 6.5 X 1.5 µm, the bradyzoites are slender (Dubey et al., 2004). The tissue cysts containing them have been

reported to vary in size and are often round or oval in shape. Typical cysts have been reported to range from 50 – 107 µm in diameter with the cyst wall varying from 2.5 – 4 µm in thickness (Dubey et al., 1989; Barr et al., 1991). Bradyzoites generally stay dormant in the host but can recrudesce if the immune response weakens. This may lead to foetal infection in subsequent pregnancies (Innes, 2007). Infection of the definitive host is by ingestion of tissues cysts containing bradyzoites. Initially tissue cysts were reported in nervous tissue including the central nervous system, retina (Dubey et al., 1988b; Dubey and Lindsay, 1996), , peripheral nerves of a horse (Daft et al., 1997) the brains of experimentally infected mice (McGuire et al., 1997b; McGuire et al., 1997a) but have now also been reported in other tissues including the ocular muscle in a foal (Lindsay et al., 1996a), the skeletal muscle of naturally infected puppies and calves (Peters et al., 2001).

1.2.2 Transmission of *N. caninum*

There is the potential for the term infection to be misinterpreted with regards to apicomplexan protozoa. In this thesis it is defined as the presence of live disease-causing microorganisms in the body of the host. It may or may not result in a clinical presentation of disease (Boden et al., 2005). With neosporosis it is usually expected that once infected an animal will then remain infected for the duration of its life.

1.2.2.1 Horizontal transmission via the venereal route/semen

There are a small number of studies which have investigated the possibility of infection occurring via semen. It is as likely for bulls to be infected as cows but few studies have been conducted with male animals (Dubey et al., 2011). A study conducted on 305

beef bulls in Argentina revealed a seroprevalence of 4.9% indicating a low level of infection (Moore et al., 2003). If these infected animals still have active tachyzoite proliferation, it was queried by these authors if venereal transmission could play a role in transmitting neosporosis. At about this time, Ortega-Mora et al (2003) reported that semen samples collected from an artificial insemination centre had *N. caninum* DNA in the cell fraction of the semen of these naturally infected bulls but did not confirm if this represented viable tachyzoites. *N. caninum* DNA was also detected in semen of bulls 46 days after they were experimentally infected with 10^8 tachyzoites (Serrano-Martinez et al., 2007a; Ferre et al., 2008) which again suggests tachyzoites may be present in the semen but this was not proven. Although these early studies only detected *Neospora* DNA they do support the possibility of venereal transmission. Evidence of infection was found in heifers inseminated with semen artificially contaminated with 10^7 N. caninum tachyzoites. *N. caninum* DNA was found in the blood, brain, liver and uterine horn tissues of these heifers confirming that tachyzoites both survived in the contaminated semen and could infect the heifers (Serrano et al., 2006). Similarly, intrauterine inoculation of heifers with 5×10^4 and 5×10^5 *N. caninum* tachyzoites in experimentally contaminated semen resulted in all of the animals becoming seropositive. Interestingly, one animal of 7 given a low dose of only 10^2 became parasitaemic (Serrano-Martinez et al., 2007b). Collectively these studies with contaminated semen also support the possibility of venereal transmission in cattle and potentially other intermediate hosts. That semen was able to act as mode of transmission for *N. caninum* was further supported when Masuda et al (2007) showed that CB-17 SCID and BALB/c male mice inoculated with 2×10^5 *N. caninum* tachyzoites were able to transmit the disease through mating to immunodeficient female SCID mice and their neonates.

In order to prove the presence of live infectious *N. caninum* in *N. caninum* PCR-positive semen and blood samples, some studies have included a bioassay using inoculation of these test samples into BALB/c mice (Ferre et al., 2005; Serrano-Martinez et al., 2007a; Ferre et al., 2008). However, in none of these studies, were they able to prove the presence of live infectious *N. caninum* organisms in the semen samples which have been shown to have *N. caninum* DNA. This could probably be attributable to the low number of parasites in the semen ranging from 0.1 to 15.6 parasites per ml of semen. As a result, it was suggested that the parasite numbers may be too low in the semen of bulls to initiate infection in the cow as it has been shown that at least 5000 tachyzoites are required to initiate an infection and for serum specific antibodies to be detected (Serrano-Martinez et al., 2007b).

There are two consistent features that can be identified in most of the reports concerning *Neospora* in bull semen. One of them is that *N. caninum* DNA was detected in the cellular fraction and not in the seminal fluid of the semen (Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005; Serrano et al., 2006; Serrano-Martinez et al., 2007b). It was proposed that *N. caninum* are within mononuclear cells that may act as a vehicle for the *N. caninum* tachyzoites and provide protection from the host. Another feature shown in these studies is the low number of parasites found in semen which is depicted by the intermittent detection of *N. caninum* DNA. The reason for this could be due the non-uniformity of distribution combined with the limited number of *N. caninum* and its DNA existing in fluids and tissues (Ferre et al., 2005).

Although the presence of *N. caninum* DNA in semen is suggestive for a role of semen as a mode of horizontal transmission, the low parasite load suggests it may be insufficient to produce an infection in heifers/cows. The reported dose of tachyzoites in some studies

is only about 200 per ejaculation (Serrano-Martinez et al., 2007a; Ferre et al., 2008) which is considerably less than the threshold level of 5000-50,000 tachyzoites suggested to be needed for horizontal transmission via contaminated semen to occur (Serrano-Martinez et al. 2007b). This was also supported with the findings of a study involving natural breeding with bulls experimentally infected with 10^8 *N. caninum* which failed to induce seroconversion in dams (Osoro et al., 2007).

With the current findings, it is likely that venereal transmission in cattle is not a significant route of infection due to an insufficient number of parasites shed in semen to produce an infection in heifers/cows. However, the same cannot be said about the dynamics of this disease in sheep. At present, knowledge about the epidemiology of neosporosis in sheep is limited especially for the mode of transmission. Due to the increasing evidence for the possible role of *N. caninum* in poor reproductive performance in New Zealand (Howe et al., 2012) and lack of transmission studies in sheep under natural farming conditions, further investigation is warranted.

1.2.2.2 Horizontal transmission via ingestion of oocysts

Although the life cycle is not fully understood, the canine family such as dogs, dingoes and coyotes are known to be the definitive hosts and dogs have been shown to shed unsporulated oocysts after being fed infective tissues from intermediate hosts (McAllister et al., 1998; Lindsay et al., 1999a; Schares et al., 2001; Dijkstra et al., 2001; Gondim et al., 2004; King et al., 2010). However, very few studies have shown naturally infected dogs to be shedding *N. caninum* oocysts (Basso et al., 2001; McGarry et al., 2003; Schares et al., 2005; Basso et al., 2009). Serological studies indicate that there has been a high level of infection in dogs and at least some studies indicate that dogs on farms have a

higher seroprevalence than those kept in urban areas. There is also no evidence that dogs which have shed oocysts will necessarily seroconvert (Sawada et al., 1998; Wouda et al., 1999; Antony and Williamson, 2003). Thus the understanding of the dynamics of oocyst shedding in the environment is poor with several unanswered questions.

Access to oocysts has limited the number of studies investigating the infectivity of oocysts for intermediate hosts. In cattle, immune responses were detected 2 to 4 weeks after calves were fed with 10^4-10^5 *N. caninum* oocysts (Marez et al., 1999). Cows fed with 600 sporulated oocysts on Week 10 of gestation did not indicate any sign of vertical transmission (Trees et al., 2002) while only 1 of 6 cows fed with 40000 oocysts on Day 120 of gestation subsequently had an abortion (McCann et al 2007). This latter study also indicated that only cows that were infected orally on Day 210 of gestation and not earlier, subsequently demonstrated vertical transmission in calves born in that year. In sheep, only one study has attempted to orally infect sheep with oocysts (O'Handley et al., 2002). In this case the dose was 10^4 oocysts and after 30 days, *N. caninum* DNA was detected in the brain tissues, although no parasites were actually seen in these tissues. These studies indicated that though ingestion of oocysts is able to lead to an infection and vertical transmission, the rate of infection would appear to be low. To date the majority of studies have used high dose rates of oocysts which is unlikely to be representative of what happens in nature. Future studies will need to investigate the dose response to better understand the epidemiology of infections following ingestion of oocysts.

1.2.2.3 Vertical transmission

Vertical or transplacental infection (TPI) is a major mode of transmission for *N. caninum* in cattle, resulting in foetal mortality or congenitally infected calves. In *N. caninum* infection a number of studies have shown that congenital transmission is very efficient with 60-95% of seropositive cows giving birth to congenitally infected calves (Barr et al., 1993; Conrad et al., 1993a; Pare et al., 1996; Antony and Williamson, 2001; Mazuz et al., 2014).

Trees and Williams (2005) defined TPI into two categories, endogenous and exogenous TPI in an effort to explain the different patterns of infection that were being reported. Endogenous TPI occurs when recrudescence of a pre-existing persistent maternal infection leads to foetal infection, while exogenous TPI occurs when a pregnant dam becomes infected during pregnancy. In sheep, experimental evidence to date indicates that exogenous TPI will still result in congenital infections in subsequent pregnancies (Jolley et al., 1999; Buxton et al., 2001). The occurrence of infection in these subsequent pregnancies indicates that endogenous TPI has also occurred. Interestingly, studies in cattle have suggested that when dams were experimentally infected with *N. caninum* tachyzoites before pregnancy, there was no infection of the foetuses in the following pregnancy, i.e. there was no endogenous TPI observed (Williams et al., 2000; Innes et al., 2001b). This contrasts with the situation observed in naturally infected cattle where endogenous TPI is very successful. Clearly further studies are required to resolve some of these observed differences.

Experimental studies have shown that foetal age at the time of exposure to *N. caninum* plays a role in determining the outcome of the pregnancy. This was observed when infection on Day 70 of gestation in naive cows led mostly to fetopathy, with those

infected at Day 120 of gestation resulting in congenitally infected but clinically healthy calves (Williams et al., 2000; Innes et al., 2001b).

In sheep similar observations were made where ewes that were experimentally inoculated on Day 65 of gestation aborted, those inoculated on Day 90 either aborted, gave birth to weak lambs or birth to clinically normal lambs and those inoculated on Day 120 produced clinically normal lambs (McAllister et al., 1996a).

1.2.3 *N. caninum* in sheep

The first reported case of neosporosis in a sheep was a congenitally infected lamb that was reported in England in 1990 (Dubey et al., 1990). It died one week after birth and at necropsy a unilateral reduction of grey matter in the ventral horn of the spinal cord with local cavitation was observed. *N. caninum* was later detected in Japan in an adult ewe which died of metritis after its twin foetuses were delivered surgically (Kobayashi et al., 2001). Perivascular cuffing with mononuclear cells and the presence of glial nodules were noted in the brain of the foetuses. Tissue cysts and focal gliosis with mononuclear cell perivascular cuffing were seen in the brain of the ewe and *N. caninum* DNA was detected by PCR. Since these early reports and using a variety of diagnostic techniques, a large number of studies have been reported on the occurrence of neosporosis in sheep from around the world as shown in Table 1.1. The seroprevalence has ranged from as low as 0.6% in New Zealand (Reichel et al., 2008) to 47% in Brazil (Rossi et al., 2011) with more than half of these studies reporting less than 10% seroprevalence from either a random selection of a population or flocks with reproductive problems or reported abortions.

Many of these studies used either in-house (IH) or commercially available serological assays that were originally developed for use in cattle but then modified for use in sheep.

Many studies have used the Indirect Fluorescent Antibody Test (IFAT) which should be able to be adapted for any animal. Most of the commercially available ELISA assays, have only been validated for use in cattle (von Blumroder et al., 2004; Wapenaar et al., 2007a; Alvarez-García et al., 2013) and not in sheep. Some indicate that they are for use with all ruminants such as the Chekit ELISA (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories, Australia) and ID-VET ELISA (ID-VET, Innovative Diagnostic, France). The competitive ELISA (VMRD) (*N. caninum* Antibody Test kit-cELISA, VMRD, USA) is theoretically useful for all animal species (Baszler et al., 2001). However, there are no peer reviewed publications supporting the use of any of these commercial ELISA assays with sheep serum except for Reichel et al (2008) who recommended a different cut-off value than that recommended by the manufacturer - see Section 1.2.7.5.1. In addition, the agglutination test should be useful with serum from a variety of animal species (Romand et al., 1998) but has also not been validated for use with sheep serum. Cut-off values for similar assays have also differed between laboratories, especially for IFATs, where these cut-off values have ranged from 1:25 to 1:160. This compares with most cattle studies which have commonly used a higher cut-off value of 1:200 (Dubey and Schares, 2011). The use of lower cut-off values and non-validated assays with sheep serum does allow the possibility of false positive reactions confusing the results obtained. Nevertheless, this collection of seroprevalence studies does indicate that neosporosis in sheep is common.

N. caninum DNA has been detected in the tissues and blood of infected ewes, aborted materials and foetuses (Kobayashi et al., 2001; Hassig et al., 2003; Masala et al., 2007; Howe et al., 2008; Abo-Shehada and Abu-Halaweh, 2010; Howe et al., 2012; Moreno et al., 2012; Castañeda-Hernández et al., 2014). There is also one report of neurological disease in an adult sheep which subseqently was found to have mild non-suppurative

encephalitis (Bishop et al., 2010). Until now, *N. caninum* has only been isolated into cell culture from one pregnant sheep (Koyama et al., 2001) and from two 4 months old lambs (Pena et al., 2007).

Due to the low seroprevalence and general lack of evidence of abortions attributed to *Neospora*, this organism is not considered a major cause of disease affecting sheep production and is not regarded as a significant abortifacient agent in ewes (Otter et al., 1997; Helmick et al., 2002). Nevertheless, not much is known about the epidemiology of this disease in sheep.

To date there have only been 13 studies where sheep have been experimentally infected with *N. caninum* as summarized in Table 1.2. Although the number of studies in sheep is limited they have been used for a variety of purposes, ranging from: studying the immune response; investigating the dose required to induce abortion; investigating the rate of vertical transmission; the pathogenesis of disease in sheep; and vaccination studies. Overall these reports have shown that responses to experimental infection in sheep are generally similar to those in cattle (McAllister et al., 1996a; Buxton et al., 1997; Buxton et al., 1998; Jolley et al., 1999; Innes et al., 2001a; Weston et al., 2009). Consequently, sheep are considered a useful model for studying the disease in cattle (Dubey and Schares, 2011; Benavides et al., 2014). However, more studies are required to confirm this.

Table 1.1: Serologic prevalence and detection of *N. caninum* DNA in sheep worldwide

Country	Number of animals	% Animal pos	Assay	Cut value	Type	Population	Author
Argentina	704	3	IFAT	≥1:50	IH	random	Hecker et al (2013a)
Australia	232	0 (0/184)	iELISA	≥30%	CHEKIT	random + selected	Bishop et al (2010)
		2.2 (5/232)	cELISA	30%	VMRD		
			IFAT	1:100	IH		
		1/6	PCR				
Brazil	597	9	IFAT	≥1:50	IH	random	Figliuolo et al (2004)
	141	29	IFAT	1:50	IH	random	Aguiar et al (2004)
	305	9.5	IFAT	1:50	IH	random	Romanelli et al (2007)
	441	31	IFAT	1:50	IH	random	Andreotti et al (2009)
		32	iELISA	NS	IH		
	409	1.8	IFAT	1:50	IH	random	Soares et al (2009)
	1028	9	IFAT	1:50	IH	random	Ueno et al (2009)
	343	10	IFAT	1:50	IH	random	Faria et al (2010)
	334	8.1	IFAT	1:50	IH	random	Salaberry et al (2010)
	382	13	IFAT	1:25	IH	random	Langoni et al (2011)
	1497	8	IFAT	1:25	IH	random	Machado et al (2011)
	64	4.7	IFAT	1:25	IH	random	Moraes et al(2011)
	155	47	IFAT	≥1:50	IH	random	Rossi et al (2011)
		26	iELISA	≥ 1.3	IH		
	488	13	IFAT	≥1:50	IH	random	da Silva Andrade et al (2012)
	17	23.5	IFAT	1:50	IH	zoo animals	Morikawa et al (2014)

iELISA, indirect ELISA; cELISA, competitive ELISA; IH, in-house; NS, not stated; random=from a non-selected population; selected=animals with reproductive problems

Country	Number of animals	% Animal pos	Assay	Cut value	Type	Population	Author
Czech Rep	547	12	cELISA	≥30%	VMRD	Random	Bartova et al (2009)
Greece	458	16.8	ELISA	NS	IH	Random	Anastasia et al (2013)
Jordan–North	339	19	iELISA	NS	BIOX	Random	Abo-Shehada and Abu-Halaweh (2010)
		1/7	PCR				
Jordan-South	320	4.3	iELISA	NS	IDEXX	Random	Al-Majali et al (2008)
Iran	70 dams	5.7	iELISA	>50%	IDEXX	Selected	Asadpour et al (2013)
	70 foetuses	8.5	PCR				
	586	1.5	iELISA	>50%	ID Screen	random + selected	Ezatpour et al (2013)
	358	2.2	iELISA	≥40%	HerdCheck	Selected	Gharekhani et al (2013a)
Iraq	288	12.2	iELISA	>50%	ID Screen	random + selected	Al-Farwachi et al (2012)
Italy	1010	2	iELISA	≥30%	CHEKIT	Random	Gaffuri et al (2006)
	368	2	PCR			Selected	Masala et al (2007)
Mexico	324	5.5	iELISA	≥50%	IDEXX	Random	Castañeda-Hernández et al (2014)
		25	PCR				

iELISA, indirect ELISA; cELISA, competitive ELISA; IH, in-house; NS, not stated; random=from a non-selected population; selected=animals with reproductive problems

Country	Number of animals	% animal pos	Assay	Cut value	Type	Population	Author
New Zealand	419	26.2	IFAT	≥1:100	IH	selected	West et al (2006)
	67	19	IFAT	≥1:100	IH	selected	Howe et al (2008)
		23/64	PCR				
	640	0.6	iELISA	11.8%	CHEKIT	random–-rams	Reichel et al (2008)
	504	37	IFAT	≥1:100	IH	selected	Howe et al (2012)
		1.4	iELISA	11.8%	CHEKIT		
		23/427 blood	PCR				
		18/138 brain	PCR				
Pakistan	128	27.7	cELISA	≥30%	VMRD	random	Nasir et al (2012)
Philippines	38	26.3%	ELISA, IB	NS	IH	random + selected	Konnai et al (2008)
Slovakia	382	33.7	ELISA	≥50%	ID Screen	random	Špilovská and Reiterová (2008)
	382	3.7	iELISA	≥50%	ID Screen	selected	Špilovská et al (2009)
Spain	178	10.1	cELISA	<30%	VMRD	random	Panadero et al (2010)
	74	6.8	PCR				Moreno et al (2012)
	209	1.9	iELISA	≥50%	ID Screen	random	Astorga et al (2014)
	2400	5.5	iELISA	≥50%	ID Screen	random	Díaz et al (2014)
Switzerland	117	10.3	IFAT	≥1:160	IH	selected	Hassig et al (2003)
		4/20	PCR				
UK	320	0	IFAT	>1:50	IH	selected	Otter et al (1997)
	660	0.45	IFAT	>1:50	IH	selected	Helmick et al (2002)
		4.2	ELISA	>0.38	IH		

iELISA, indirect ELISA; cELISA, competitive ELISA; IH, in-house; NS, not stated; random=from a non-selected population; selected=animals with reproductive problems

Table 1.2: Summary of studies where sheep have been experimentally infected with *N. caninum*

Country	No of animals	Route	Inoculation Dose	Isolates	Purpose	Authors
Belgium	9	T/SC	$2.5X10^6$	NC-1	Immune response	Rettigner et al (2004)
New Zealand	49	T/IV	50, 5 $x10^3$, 10^6, 10^8	NcNZ1, NcNZ2, NcNZ3	Dose titration/VT	Weston et al (2009)
UK	36	T/IV	10^5, 10^6	NC-2, NC-Liv	Infection/VT	McAllister et al (1996a)
	21	T/SC	10^4, 10^6, 10^7	NC- Liv	Infection/VT	Buxton et al (1997)
	4	T/SC	10^6	NC-1	Immune response	Harkins et al (1998)
	30	T/SC	$2.5x10^5$	NC-1	Infection/VT	Buxton et al (1998)
	11	T/SC	10^5, 10^6	NC-2, NC-Liv	Infection/VT	Jolley et al (1999)
	62	T/SC	10^5, 10^7, 10^8	NC-1	Infection/VT	Buxton et al (2001)
	53	T/SC	10^7	NC-1	Infection	Innes et al (2001a)
USA	2	T/IV/IM	$1.5x10^7$	NC-1	Infection	Dubey and Lindsay (1990)
	NS	NS	NS	NS	Immune response	Bjerkas et al (1994)
	6	O/PO	10^4	NC-2	Infection	O'Handley et al (2002)
	30	T/SC	$5x10^7$	NC-1	Vaccine	Jenkins et al (2004)

T, tachyzoites; O, oocysts, SC, subcutaneous; IV, intravascular; IM, intramuscular; PO, per os, NS, not stated; VT, vertical transmission

1.2.4 *N. caninum* in sheep in New Zealand

The principal cause of ovine abortion in New Zealand accounting for 80% of cases are campylobacteriosis, toxoplasmosis and salmonellosis (*Salmonella enterica* serovar Brandenburg) (West, 2002). Less frequent causes of abortion include border disease, listeriosis, infections due to *Fusobacterium* spp., *Bacillus* spp., various fungi, yersiniosis, brucellosis and leptospirosis (West et al., 2006). Major infectious agents causing abortion in sheep such as *Chlamydophila abortus, Coxiella burnetti, Salmonella enterica abortusovis, Brucella melitensis*, Bluetongue virus, Wesselsbron disease virus and Akabane disease virus are not present in this country (West et al., 2006). Progress with the development and extensive use of vaccines in New Zealand against the three most common agents of ovine abortion has greatly reduced the number of abortion cases, especially in maiden ewes (West, 2002). Yet in recent years, unexplained abortions in maiden ewes have been occurring in some sheep flocks. In 2004 a study investigating foetal losses in maiden ewes raised the possibility of the involvement of a previously unrecognised abortion agent (West et al., 2006). This study was conducted when two flocks had late gestation abortions occurring. Serum samples from affected, non-affected ewes and from aborted lambs together with those from 5 flocks of ewes which had abortions the previous year were all tested for the presence of agents such as *Campylobacter* spp., *T. gondii* and also *N. caninum*. *N. caninum* was tested using IFAT with a cut-off titre of 1:100. Significant findings showed that 26/57 ewes in the two flocks that aborted had antibodies to *N. caninum* compared to 4/69 of non-aborted ewes. In one flock, four out of five aborted foetuses were seropositive to *N. caninum* (West et al., 2006). As a consequence *N. caninum* which had previously not been thought as an important abortifacient in sheep in New Zealand has now been included in the list of potential agents. Another investigation (Howe et al., 2008) followed as there were more reports of abortions occurring in ewe hoggets which had been vaccinated for both *Toxoplasma*

and *Campylobacter.* In this study on 3 farms seroprevalence in aborting ewes was 36% (17/47) with titres ranging from 1:100 to 1:800 and 7 of 13 foetal brains were also PCR positive. In addition, *N. caninum* DNA was detected in the blood of 19 of the 44 aborting ewes by PCR. Histological examination of tissues from 13 ewes and some foetuses failed to identify any protozoal organisms but pathological changes were generally consistent with a protozoal cause of abortion. The findings in this study implied that there was an association between neosporosis and the occurrence of abortions, and further supported the possibility that *N. caninum* could be an abortifacient agent in sheep. A study was subsequently conducted looking into the effect on pregnant hoggets challenged with *N. caninum* tachyzoites at different doses of infection. Hoggets were inoculated with vero cells (control group) and 50, 5×10^3, 1×10^5 or 1×10^8 *N. caninum* tachyzoites at around 90 days of gestation period. Abortion and time to abortion was found to be more consistent among those hoggets that were challenged at a higher dose although there was no association found between the antibody response and the challenge dose rates (Weston et al., 2009). The lowest dose rate to induce abortion in this study was 1×10^3 suggesting that a reasonably high dose rate would be required in sheep to result in abortion.

At about the same time in 2008, a prevalence study using a commercial ELISA test kit to test 640 serum samples from rams collected around New Zealand showed a seroprevalence of only 0.6% (Reichel et al, 2008) which did not indicate a widespread level of infection. However, in a more recent study (Howe et al., 2012), *N. caninum* DNA was detected in 7%, 4% and 2% of blood samples from aborting/non pregnant, pregnant and high fertility pregnant ewes respectively whereas the overall ELISA prevalence was only 1.4% (7/504 animals). The seroprevalence is broadly similar between the two studies but the higher value with the PCR

suggests that the use of multiple diagnostic tests for *N. caninum* is important to avoid under-estimation of the true status of neosporosis in sheep in that situation.

1.2.5 Pathogenesis of *N. caninum*

A major factor in the pathogenesis of neosporosis is the rapid multiplication of tachyzoites and their invasion of cells using surface receptors and proteins released from the micronemes, rhoptries and dense granules of *N. caninum*. These multiply within cells by a form of binary fission to the point where cell death occurs and tachyzoites are liberated which then invade more cells. The amount of cell destruction is a balance between the penetration abilities of the tachyzoites and the immune status of the host (Buxton et al., 2002a). The differentiation of tachyzoites to bradyzoites is thought to be initiated by the host immune response and other physiological factors which are considered to be similar to that as described in *T. gondii* (Lyons et al., 2002).

Within the bovine uterus, *N. caninum* invades cells and multiplies, leading to focal destruction at the materno-foetal interface involving both maternal and foetal tissues. Such pathology has been reported in cases of both naturally and experimentally induced neosporosis (Barr et al., 1994; Otter et al., 1995).

Besides initiating inflammatory reactions in both maternal and foetal membranes, *N. caninum* also attacks other foetal tissues, especially the central nervous system (CNS), by invading the foetal bloodstream and dispersing through tissues (Buxton et al., 1998). It was suggested that in the young foetus, the CNS cells were destroyed due to the uncontrollable multiplication of *N. caninum* tachyzoites causing multifocal necrosis but with minimal inflammatory cell reactions. However, in an older foetus with an active immune system,

numerous IgG-containing cells and tissue cysts were the prominent features of the infection (Ogino et al., 1992). Consequently numbers of tachyzoites found are more limited and smaller foci of necrosis are seen. Such damage is generally encircled by microglia, reactive astrocytes and cells of the monocyte and lymphoid series. Mild meningitis has also been reported. Lesions reported in other organs such as the heart, skeletal muscle, liver and lung include destruction of foetal cells with associated lymphoid inflammation (Barr et al., 1994; Otter et al., 1995). A progressive development of the immune system influences the degree of damage as there is an obvious relationship between gestational period and the degree of tissue inflammation (Ogino et al., 1992). During congenital transmission, the outcome of the pregnancy depends on the age of the foetus, dose of inoculation or level of parasitaemia and strain of *N. caninum* (Innes et al., 2002; Weston et al., 2009).

1.2.6 Immunity towards *N. caninum* during pregnancy

In cattle, the foetal immune system develops progressively over the last trimester, with the neonate being born immunologically capable. The thymus, spleen and peripheral lymph nodes are formed during the first third of the gestation period, making this the most susceptible period for the foetus. A host immune response to microorganisms in foetuses only occurs when these organs start to function and recognize parasites, commencing in the middle trimester of pregnancy (Dubey et al., 2006). The first trimester is considered the most critical period as foetuses are very defenceless to infection and survival is very unlikely. During the middle trimester of pregnancy, immune responses are still at the elementary level and therefore are not able to protect the foetus completely (Buxton et al., 2002a), this being the period when the majority of abortion occurs in cattle (Gonzalez et al., 1999). Only in the third trimester, where

there is an increase in the defence mechanisms of the foetus, is immunity to apicomplexan pathogens sufficient to increase the survivability rate (Buxton et al., 2002a).

The ruminant materno-foetal interface comprises approximately 100 placentomes. Each of these placentomes consists of a foetal placental cotyledon which is closely interlocked with a maternal caruncle. Nutrients and oxygen are transferred from the mother to the foetus between the maternal caruncular septa and the foetal placental villi (Buxton et al., 2002a). In the placenta, a unique situation develops where the host can restrict cytokines such as IL-2, IL-12, interferon (IFN) -γ and tumour necrosis factor (TNF) -α whilst allowing (IL) -10 and transforming growth factor (TGF) - β to predominate. This situation allows the mother to nurture an allograft but this also means it would allow some microorganisms to survive in this location such as *N. caninum*.

Abortion could be the outcome of an inappropriate immune reaction due to the invasion and multiplication in the maternal and foetal placental cells by the tachyzoites (Entrican, 2002). It has been proposed that TNF-α could play an important function in the resulting failure of pregnancy in sheep. This is because the foetal production of TNF-α, a cytokine not usually present in the ovine placenta, is produced in the placenta as a reaction to the infection (Buxton et al., 2002b).

As indicated above, the outcome of the pregnancy is greatly influenced by the stage of gestation and maturity of the foetus (McAllister et al., 1996a; Buxton et al., 1998). In one study, groups of pregnant ewes were separately inoculated with *N. caninum* tachyzoites at day 45, 65 and 90 of gestation. All 6 ewes that were inoculated at the earlier time (45 days) had dead foetuses that were either resorbed or aborted. In the group that were infected at 65 days of gestation, six ewes had an abortion while one ewe resorbed her foetus and one ewe had a live

lamb. In the last group infected at 90 days, six ewes had an abortion, one ewe had a stillborn lamb and the other two ewes produced live lambs (Buxton et al., 1998). Similar results were also obtained in studies by McAllister et al (1996a) where the pathogenicity was more significant in cattle inoculated with *N. caninum* at Day 65 of gestation compared to those inoculated at Day 120 of gestation. This clearly indicates that understanding the extent to which animals develop protective immunity following natural infection with the parasite is essential in order to explore the feasibility of vaccination as a control strategy against neosporosis.

1.2.7 Diagnosis of *N. caninum*

Definitive identification of non-infected animals with *N. caninum* is very difficult as infected animals can be harbouring a latent infection and not show any clinical signs (Dubey and Schares, 2006). Infected animals may experience fluctuations in antibody titres during pregnancy which may move below as well as above the cut-off value being used (Okeoma et al., 2004b; Lopez-Gatius et al., 2007; Nogareda et al., 2007). However, showing an animal is infected does not prove that *Neospora* is the cause of the abortion. Similarly, although parasite DNA can be detected this does not necessarily mean that the parasite is the agent causing the abortion (Dubey and Schares, 2011). In order to establish a cause-effect of *N. caninum* as an abortifacient, the use of a comprehensive diagnostic approach is crucial. Diagnosis can be achieved by detecting typical lesions caused by *N. caninum* in the placenta, foetuses, stillborn offspring and neonates. More confidence is achieved if typical organisms are seen associated with these lesions. Immunohistochemistry can be used to confirm these are *Neospora* organisms although the only commercially available antibodies are polyclonal, so some cross-reactions are still possible with other related apicomplexan protozoa such as *T. gondii*. The use of PCR and qPCR are some of the methods to confirm the identity of these organisms.

1.2.7.1 Necropsy examination

Foetuses may be found dead in-utero, resorbed, mummified, autolyzed or stillborn. Gross lesions may be present and despite the fact that lesions can be found in several organs such as the heart, liver, placenta, body fluids or serum, the foetal brain is the most consistently affected organ, making it a preferred sample for diagnosis (Dubey and Schares, 2006). Usually foetuses are autolyzed or mummified and although seldom observed, lesions consisting of pale white foci, minute pale to black foci of necrosis in the brain, hydrocephalus or local areas of discolouration of the placental cotyledons may be present (Dubey and Schares, 2006; Fioretti et al., 2003).

1.2.7.2 Histopathology

At post mortem, congenitally infected neonates that have been necropsied soon after birth usually have encephalomyelitis although clinical signs are rarely seen (Dubey et al., 1989; Barr et al., 1993; Dubey et al., 2006; Zhang et al., 2007). In the placenta, tachyzoites are more likely to be observed in the foetal chorionic epithelium and foetal placental blood vessels with resultant thrombosis in some maternal caruncular blood vessels. Focal degeneration and inflammation of the choriollantois, severe focal necrosis in placentomes and areas of focal discoloration in placental cotyledons may also be found (Fioretti et al., 2003). In the samples of lung and liver obtained from aborted foetuses, mild lymphoid inflammation has been consistently present and focal hepatic necrosis has been observed. In the myocardium, a focus of necrosis with mineralization associated mild lymphoid inflammation is a common finding (Buxton et al., 2001).

Although lesions due to *N. caninum* infection are not pathognomonic, they are suggestive of the parasite infection (Dubey and Schares, 2006). In bovine foetal brain tissues the use of

histopathology in detecting *N. caninum*-related lesions has been shown to be more sensitive than relying on immunohistochemistry (IHC) to identify *N. caninum* (Pescador et al., 2007). Many previous studies have reported that *N. caninum* DNA was consistently detected in the brain of aborted foetuses due to neosporosis (Collantes-Fernandez et al., 2006b; Dubey et al., 2006) and this is tolerant of a certain degree of autolysis but again this does not prove it caused an abortion, only that the organism was present.

N. caninum tachyzoites in haematoxylin and eosin (H&E) sections are usually round in shape to slightly elongated with a vesicular nucleus which allow it to be differentiated from degenerated host cells (Dubey and Schares, 2006). *N. caninum* infection in bovines can be assumed if apicomplexan-like protozoa are found in the brain as other abortifacient protozoans such as *Sarcocystis cruzi* and *T. gondii* have different entities (Dubey and Schares, 2006) as the former has asexual multiplication only as schizonts in endothelial cells and the latter is rare in cattle. However in sheep, it is feasible to confuse *T. gondii* with *N. caninum* (Dubey and Schares, 2006).

1.2.7.3 Immunohistochemistry staining (IHC)

In order to demonstrate the presence of *N. caninum* parasites in histological section, the IHC method is commonly employed (Ortega-Mora et al., 2006). IHC has been utilised in all organs where lesions are commonly detected including the foetal brain, muscle, liver, heart and the lung tissues (Dubey and Schares, 2006; Collantes-Fernandez et al., 2006a; Pescador et al., 2007). However, it has not proven to be very sensitive (Van Maanen, 2004) especially when applied in autolysed tissues (Lindsay and Dubey, 1989); a limitation with many aborted foetuses. For example, typical histological lesions were observed in 92% and 82% of skeletal muscle and

heart of aborted bovine foetuses that were negative on IHC and a similar low association between IHC and brain lesions was also observed (Pescador et al., 2007).

The IHC uses either polyclonal (pAb) or monoclonal (mAb) antibodies as the primary antibody in the reaction (van Maanen et al., 2004; Uzêda et al., 2013). A recent study has shown that using a combination of different mAbs greatly improved the sensitivity of the IHC as compared to the use of a single mAb (Uzêda et al., 2013). The use of pAb has been shown to be prone to cross-reactions with *T. gondii*, *Sarcocystis* spp., and *Besnoitia besnoiti* (McAllister et al., 1996b; Sundermann et al., 1997; van Maanen et al., 2004; Pescador et al., 2007; Uzêda et al., 2013) which is particularly frustrating for the use of IHC in sheep. To date, access to commercially available monoclonal antibodies against *N. caninum* has not been possible.

1.2.7.4 Demonstration of viable parasites by bioassay and cell culture

Demonstration of viable *N. caninum* tachyzoites by bioassay, usually through mice, or by cell culture has been shown to be a less successful diagnostic approach compared to the others mentioned elsewhere (Dubey and Schares, 2006). Since the first report of *N. caninum* only a small number of isolates have been established in cell culture demonstrating the difficulties involved in this approach (Dubey and Schares, 2011, 2006). Isolating the parasite from foetal tissues has been considered difficult as presumably the parasite dies soon after the host tissue is unlikely to be still present in autolyzed or mummified foetal tissue. If the tissue is fresh, there are reports that it is more likely to isolate *N. caninum* from neural tissues than elsewhere in the foetus, presumably due to the presence of more tissue cysts in the CNS (Conrad et al., 1993b; Dubey and Schares, 2006). In New Zealand, *N. caninum* isolates were successfully isolated from brain tissues of a cow, her calf and another stillborn calf (Okeoma et al., 2004a) but to date, there are no isolates from sheep. Bioassay through mice has also been used in some

experimental studies but not for routine diagnostic purposes. For example, viable tachyzoites could not be detected in bull semen using mice bioassay (Ferre et al., 2005; Ferre et al., 2008).

1.2.7.5 Serology

In neosporosis, serology has been one of the most commonly used diagnostic tools although interpreting the serological status of an animal can be difficult (Dubey and Schares, 2011). Various methods have been developed, including the indirect fluorescent antibody test (IFAT), direct agglutination test (DAT), latex agglutination test (LAT), western blot and a range of enzyme-linked immunosorbent assays (ELISAs). These have been reviewed in detail elsewhere (Dubey and Schares, 2006; Björkman and Uggla, 1999; Ortega-Mora et al., 2006; Wapenaar et al., 2007a).

1.2.7.5.1 ELISA

There has been rapid progress since the first ELISA was described in 1994 (Björkman et al.). In determining the efficiency of a serological test, its sensitivity and specificity need consideration. Sensitivity is defined as the proportion of infected animals correctly identified by the test. Specificity is defined as the proportion of non-infected animals which are correctly identified**.** It is crucial to accurately identify a non-infected animal considering that an animal infected with *N. caninum* can be in a latent state of infection and not show any clinical signs (Björkman and Uggla, 1999).

Some research groups still use in-house ELISAs (Osawa et al., 1998; Wouda et al., 1998) but most recent studies have utilised validated commercial ELISAs in their various epidemiological studies of neosporosis (Dubey and Schares, 2006). The Chekit ELISA (IDEXX Laboratories, Australia) has been used in several studies throughout this thesis. It is an indirect

ELISA which detects antibodies against *N. caninum* in ruminants and has been used in studies in sheep and goats (Reichel et al., 2008; Weston et al., 2009; Bishop et al., 2010; Czopowicz et al., 2011). Using sera from experimentally infected sheep, Reichel et al. (2008) recommended the use of a sample to positive ratio (S/P) value of 11.8% as a cut-off value in sheep instead of those recommended by the manufacturer which was for an S/P ratio of 30-39% to be considered suspicious and >40% being positive. The use of 11.8% gave a sensitivity of 100% and specificity of 98% which is similar to that reported if using the cut values suggested by the manufacturer which were 96% and 100% respectively.

Most of the serological assays that have been developed are generally based on *N. caninum* tachyzoites antigens that have been prepared in different ways including: whole; fixed; ISCOM incorporated tachyzoite antigens; native affinity-purified NcSR2; aqueous or detergent based lysate; and recombinant proteins (Dubey and Schares, 2006; Dong et al., 2012; Ghalmi et al., 2014). Several studies have compared the specificity and sensitivity of the different approaches and shown there are differences (von Blumroder et al., 2004; Wapenaar et al., 2007a; Alvarez-García et al., 2013; Ghalmi et al., 2014). For example, ELISAs based on recombinant proteins of NcGRA7 (tachyzoite and bradyzoite-based) and the NcSAG4 (bradyzoite-based) antigens have been shown able to differentiate between acute, chronic and recrudescent infection in individual animals (Aguado-Martinez et al., 2008) whereas earlier ELISAs were not able to achieve this differentiation (Björkman et al., 1999). Using NcSAG4 antigens has shown promise in being able to indicate reactivation of *N. caninum* infection as well as being a good marker for neosporosis in aborting cows (Huang et al., 2007; Hiasa et al., 2012). Recently a latex bead agglutination test using the dense granule protein NcGRA6 has been shown to be an easy and rapid serological test that can be used in the field (Ghalmi et al., 2014) but is not yet commercialised for this purpose. Other ELISAs have used a competitive-inhibition

(CI-ELISAs) approach (Baszler et al., 1996; Baszler et al., 2001) which has the advantage of not being host-species specific but still maintains a high sensitivity (Wapenaar et al., 2007a; Alvarez-García et al., 2013). Besides the use of serum in ELISAs, individual and bulk milk samples have also been tested using some of these ELISAs. The limitation of these bulk milk assays is that they are not able to give a true indication of the true seroprevalence of a herd and can only give a positive value when the seroprevalence is over 10-15%. (Bartels et al., 2005; Frossling et al., 2006; Milne et al., 2006; Wapenaar et al., 2007b; Sotiraki et al., 2008). The sensitivity of the bulk milk assays varied from 47% to 95% where the detection of antibodies greatly depends on the proportion of infected animals as well as their antibody levels, lactation stage and milk production. Consequently care is needed to thoroughly interpret the results (Sekiya et al., 2013).

1.2.7.5.2 IgG avidity ELISA

The ability to determine when infection occurs is a useful factor especially in epidemiological studies. Generally detecting antibodies towards *N. caninum* during diagnosis only implies that the animal has been infected with *Neospora* and does not indicate when the animal was infected (Björkman and Uggla, 1999; Dubey and Schares, 2006). Use of techniques to determine the avidity of the antibody binding to the *N. caninum* antigen will provide information on the duration of infection.

The IgG avidity ELISA assay was first introduced by Hedman et al., (1989) to differentiate between chronic and recent *T. gondii* infections in humans. An IgG avidity ELISA assay was later adapted by Björkman et al., (1999) to distinguish between acute and chronic *Neospora* infections in cattle. Avidity IgG assays work on the basis that antibodies to a recent primary infection have a low affinity or binding strength towards the antigen as compared to those produced later

(Jenum et al., 1997). The general approach is to utilise a hydrogen-bond disrupting agent such as urea that would elute immunoglobulins (Ig) that were not that tightly bound to the immobilized antigen on the ELISA plate. These IgG antibodies with low avidity will then be dissociated while those with high avidity IgG should remain bound to the antigen. By comparing results with and without this urea step, it is possible to calculate the avidity of the IgG and thus able to discriminate between acute and chronic infection.

There have been several studies with cattle utilising various approaches to prepare the *N. caninum* antigen for an IgG avidity assay. These studies used both in-house preparations as well as a range of commercially available ELISA test kits (McAllister et al., 2000; Guy et al., 2001; Dijkstra et al., 2002; Björkman et al., 2003; Björkman et al., 2005; Gottstein et al., 1998; Maley et al., 2001;Sager et al., 2003; Aguado-Martinez et al., 2005; Schares et al., 2002). To date no studies have been reported demonstrating IgG avidity values against *N. caninum* antibodies in sheep.

1.2.7.5.3 Indirect Fluorescent Antibody Test (IFAT)

A gold standard test is often referred to as an established reference method against which newly introduced tests can be compared. In the case of *N. caninum*, the IFAT has been regarded as a gold standard (Björkman et al, 1999). The IFAT has been used to detect antibodies of *N. caninum* in a variety of animals from dogs, cats, foxes, cattle, sheep, goat, water buffaloes, horses, rodents and primates (Björkman and Uggla, 1999).

Antibodies to *N. caninum* were first demonstrated using an IFAT in 1988 (Dubey et al., 1988). Using a fluorescence microscope, evaluation was made by looking at the reaction between intact *N. caninum* tachyzoites affixed onto a microscope slide which was incubated with diluted test serum. This was then probed with a conjugate of a second fluorescein-labelled

antibody directed against the Igs of the test animal. Bright and unbroken peripheral fluorescence of the organisms was the standard to indicate a positive result whereas fluorescence of only part of the organism, in particular only the apical part of the tachyzoites, was considered a non-specific reaction. This "cap" or "polar" staining is considered to be due to cross-reaction with other apicomplexan species (Pare et al., 1995b). In the IFAT, intact tachyzoites are used as antigens and therefore it detects antibodies which are directed against the antigens present on the cell surface.

Various aspects of an IFAT influence its cut-off titre, including conjugate characteristics, dilution factors and the properties of the microscope (Pare et al., 1995b). A range of cut-off values have been reported for sheep (Table 1.1) with the most commonly used being 1:50. Early reports indicated that an IgG IFAT titre of ≤ 1:64 in adult sheep was not indicative of exposure to *N. caninum* (Buxton et al., 1997) although most surveys, most occurring after this comment, have used a lower cut-off value than this. The use of 1:50 is similar to the cut-off value commonly used with dogs (Figliuolo et al., 2004; Romanelli et al., 2007). In cattle, as in sheep, a variety of cut-off values have been used varying from as low as 1:25 to as high as 1:640. However, 1:200 has been the most commonly chosen (Dubey et al., 2007). In a comparison of IFAT with a range of 11 ELISA tests, the k value with a cut off value of 1:200 was consistently high (von Blumroder et al., 2004). It is interesting there is such variation between different studies and also differences in cut-off values used between different host animals. Using an IFAT cut-off threshold at 1:200, Reichel et al (2008) reported that IFAT titres from experimentally-infected sheep were positive at about 14 days post- infection, reaching their highest value at about 3 months post infection after which time they then declined. A similar rise or fall has been observed in studies in cattle (Cox et al., 1998). Hence it has been suggested that IFAT is only suitable to be used in immediate cases of suspected abortion and if used later than 3 months

after the outbreak, the possibilities of getting false-negative results are high. It has also been suggested that the IFAT is the preferred choice of serological assay in individual abortion cases while ELISAs would be more for flock-based investigations (Reichel et al., 2008).

Although the IFAT has been used in earlier investigations of neosporosis, it has some disadvantages. The use of foetal bovine serum (FBS) in cell culture medium in growing *N. caninum* may contain antibodies towards *N. caninum*, hence could give false-positive results in an IFAT or other serological assay using these tachyzoites (Dubey and Schares, 2006). In addition, it also requires trained personnel, is somewhat more cumbersome to undertake during large scale investigations and involves subjectivity in interpretation of the assay. Collectively this leads to a lot of variation in the results obtained (Pare et al., 1995b; Miller et al., 2002; Weston et al., 2009). In a study involving 49 experimentally-infected pregnant hoggets with *N. caninum,* no correlation was found between the values and timing of IFAT titres with the timing of abortion or reproduction outcome (Weston et al., 2009). It has been suggested that the use of IFAT in diagnosing abortion in sheep due to *N. caninum* infection lacks sensitivity (Weston et al., 2009). It has also been noted that IFAT titres in sheep that aborted due to N. caninum were generally lower compared to those in cattle that aborted due to *N. caninum* (West et al., 2006). More recently Howe et al (2012) observed similar inconsistencies between IFAT results and another indirect ELISA (Chekit, IDEXX) especially when involving low-titre serum samples.

1.2.7.5.4 Western blot

Western blot or immunoblot is a commonly used analytical technique and has been widely used for the diagnosis of *N. caninum* (as shown in Table 1.3) especially for experimental purposes. In the western blot, proteins or antigens are separated via gel electrophoresis. These proteins migrate based on molecular weight and smaller sized proteins will migrate faster than

those larger sized ones. Proteins are then transferred to a membrane (commonly PVDF or nitrocellulose) and later blocked to reduce non-specific binding of antibodies to sites that do not contain proteins to prevent false positive results. The membrane is then incubated with the primary antibodies of interest. Unbound antibodies are removed during the washing process and a secondary antibody or conjugate is then added to the membrane to detect the primary antibodies. After a final washing step, a substrate is added which will allow the detection of the bound primary antibodies which will be visualised as a band on the blotting membrane, x-ray film or by the imaging system.

Antibodies may either recognise an epitope of non-continuous amino acids as it exists on the surface of the folded three-dimensional structure of the protein, or those that have been denatured. The use of reduced antigens (use of reducing agents to disrupt disulfide bonds/bridges in proteins) in an immunoblot to diagnose *Neospora* was first described by Barta et al (1992). This was followed by the use of non-reduced antigens (Bjerkas et al., 1994). As shown in Table 1.3, there is a different range of antigens that are subsequently recognised with each approach. Using non-reduced antigens led to stronger reactions being recognized which was probably due to the involvement of conformational epitopes in the antibody response towards *N. caninum* infection. These conformational epitopes were also believed to be species-specific as fewer cross-reactions were seen from animals infected with various other species of the apicomplexan family such as *T. gondii, Sarcocystis* and *N. caninum* (Dubey and Schares, 2006). Nevertheless, most of the recent reports have used reduced antigens (Table 1.3)

Western blot is thought to be a complementary tool for diagnosing *N. caninum* and when combined with other methods the diagnosis of *N. caninum* has been shown to improve. It may have a particularly useful role with foetal serology in *N. caninum* infection. In one study on aborted foetuses, it was observed to have higher relative sensitivity and specificity in contrast to

IFAT, immunohistochemistry of tissues, ELISA and PCR, although it is of limited value when used in bovine foetuses younger than 6 months old without an effective immune system (Sondgen et al., 2001). In another study, it was noted that a western blot had a higher sensitivity of 98% compared to only 87% with an ELISA. In addition, seropositive experimentally infected calves were detected 1 to 3 days earlier using immunoblotting when compared to an ELISA (Staubli et al., 2006a). The phenomenon of transient seronegative results (Pare et al., 1997; Hasler et al., 2006a) where an ELISA titre fluctuates from being seropositive to seronegative in chronically infected animals, may be overcome by using immunoblotting where previously ELISA seronegative sera were found positive by immunoblotting (Staubli et al., 2006a)**.**

A wide range of immunodominant antigens (IDAs) has been reported and it appears that similar IDAs are recognised in different species of animals (Table 1.3). However, different ranges of *N. caninum* IDAs have been reported depending on the electrophoresis conditions as well as the antigen solubilisation and extraction methods used (Barta et al., 1992; Zintl et al., 2006). Consequently, comparison between different studies is difficult. Nevertheless, various studies have shown that IDAs of 17, 29, 30, 37 and 40 kDa proteins were those commonly recognised regardless if they were reduced or non-reduced antigens and therefore are useful for the diagnosis of *N. caninum* (Schares et al., 1999; Sondgen et al., 2001; Bjerkas et al., 1994).

Working with New Zealand *N. caninum* isolates obtained from naturally infected cattle, Okeoma et al (2004b) reported several IDAs ranging from 18 to 116 kDa. In this study, there was a pattern seen between the IDAs recognized by the bovine IgG at different IFAT titres. With higher titres more IDAs were recognised. In a different study, ewes experimentally infected with live *N. caninum* tachyzoites revealed immunodominant antigens of 44, 42, 40, 39 and 28 kDa with 28kDa being detected as early as 14 days post inoculation. Due to its frequency and

intensity of recognition, these IDAs were considered as important antigens for diagnosing *N. caninum* infections in sheep (Rettigner et al., 2004).

The interpretation of immunoblots also varies between authors. Alvarez-Garcia et al (2002; 2003) defined sera that recognized at least one IDA with molecular weight (MW) of 17, 25-29, 30 or 37 kDa as being seropositive for *N. caninum* while Staubli et al (2006a) only considered an animal to be seropositive with the detection of at least 2 or more relevant IDAs whilst Schares et al (1998) suggested it required the presence of at least 3 IDAs to be considered as seropositive to *N. caninum.* To date, there has been no consensus as to how many IDAs need to be detected to indicate an animal is seropositive. It also varies depending on how sensitive the assay is required to be.

Table 1.3: Summary of IDAs detected using western blot in experimentally and naturally infected animals with *N. caninum*

Species	Group	Condition	IDAs (kDA)	Authors
Rabbit	E	NR	16/17, 29, 31, 32, 37, 46, 51, 56, 79	Barta and Dubey (1992)
		R	17, 31, 34, 37, 40.5, 47, 50, 55, 65	
Cattle and dog	E + N	NR	17, 29, 30, 37	Bjerkas et al (1994)
Sheep and goat	N	NR	17, 26, 29, 30, 37	
Rabbit	N	NR	17, 18, 30 - 45, >51	Björkman et al (1994)
Dog	N	NR	17-19, 30-42, 43,60	
Cattle	N	NR+R	17, 29, 30, 37	Pare et al (1995a)
Cattle	E + N	R	14, 20, 25, 31, 35, 42, 54, 65, 76, 116	Baszler et al (1996)
Rabbit	E	NR	16, 28, 31, 40, 83, 91	Stenlund et al (1997)
Cattle	E + N	NR	24, 40, 60, 65, 75	
Cattle, sheep, goat	E	R	17, 25, 26, 27, 31.5, 36.5-38, 45.5-48.5, 52-53.5, 58, 58.5, 59.5, 60.5, 62, 63.5, 64, 66.5, 67.5, 68.5, 69.5,	Harkins et al (1998)

N=naturally, E=experimentally infected animals; NR=non-reducing, R=reducing condition of antigen preparation

Species	Group	Condition	IDAs (kDa)	Authors
Cattle	N	NR	17, 29, 30,33, 37	Schares et al (1998; 1999)
Cattle- foetus	N	NR	17, 29, 30, 33, 37, 40	Sondgen et al (2001)
Cattle	N	R	17-18, 24, 28, 30, 41, 47, 51, 53, 55-57, 60-62, 67, 77	Alvarez-Garcia et al (2002)
Cattle-foetuses	N	R	17-18, 28,34-35, 37, 47, 53, 55-57, 60-62, 67, 77	Alvarez-Garcia et al (2002)
Cattle	N	R	14.4 to > 97.4 but most intense is 18	Osawa et al (1998)
Sheep	E	R	14.4 to > 97.4 but most intense 18, 29, 33, 65	Osawa et al (1998)
Goat	E	R	14.4 to > 97.4 but most intense 29, 65	Osawa et al (1998)
Sheep	E	R	28, 41, 56, 73, 100	O' Handley et al (2002)
Cattle	N	R	18, 25, 33, 35-36, 45, 46, 47, 51, 60-62, 64, 77, 116	Okeoma et al (2004b)
Sheep	E	R	21, 26, 27, 28, 33, 36, 37-38, 39, 40, 42, 44, 49, 54, 59, 93, 94,103	Rettigner et al (2004)
Cattle	E + N	NR	17, 21, 26, 29, 33, 36, 43, 55, 70-80,	Staubli et al (2006a)
Cattle	N	R	9, 16, 21, 27, 31, 34, 36, 38, 40, 43, 47, 48, 53, 58	Goździk and Cabaj (2007)
Cattle	E +N	R	17-18, 25-26, 40, 45, 77, 116	De Yaniz et al (2007)
Cattle	E	R	17-18, 23-24, 26, 28-30, 34, 41-43, 50, 60-62, 74-75, 77-78, 82	Moore et al (2011)

1.2.7.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction or PCR is a powerful tool developed in the 1980s to amplify a specific area of a DNA target producing millions of copies of that particular segment of DNA. It consists of repeated cycles which includes the denaturing step where the temperature is increased to 94-98°C leading to the disruption of the hydrogen bonds of the double strand (ds) DNA into single strand (ss) DNA; the annealing process where the temperature is reduced to 50-65°C to allow the primers to anneal to the ssDNA template; the extension step where the temperature is raised again to 75-80°C where the DNA polymerase will synthesize new DNA strands complementary to the ssDNA present. This cycle is then repeated where the newly formed dsDNA will act as a new template. The final products or amplicons are then subjected to separations using agarose gel electrophoresis and visualised.

Early development of PCR in phylogenetic and diagnostic studies of *N. caninum* (Ellis et al., 1994; Holmdahl et al., 1994; Holmdahl and Mattsson, 1996; Kaufmann et al., 1996; Payne and Ellis, 1996; Yamage et al., 1996) has led to the vast improvement and usage of PCR as an important diagnostic tool. *N. caninum* DNA has been detected in various body tissues of aborted foetuses and infected animals which include samples from fresh unpreserved samples, frozen, formaldehyde-preserved tissues and paraffin-embedded preserved samples (Baszler et al., 1999; Ellis et al., 1999; Collantes-Fernandez et al., 2002). Generally, *N. caninum* DNA is more concentrated in the brain tissues (Baszler et al., 1999; Okeoma et al., 2005) but has been also found in a variety of other tissues including muscle, kidney, liver, lungs, uterus, skin and placenta (Ho et al., 1997; Gottstein et al., 1998; Baszler et al., 1999; Kritzner et al., 2002; Collantes-Fernandez et al., 2002). *N. caninum* DNA has also been detected in the amniotic fluid (Ho et al., 1997), cerebrospinal fluid (Buxton et al.,

2001), faecal samples from dogs or coyotes (Basso et al., 2001; Gondim et al., 2004), blood (Okeoma et al., 2004c; Okeoma et al., 2005), serum (McInnes et al., 2006), milk (Moskwa et al., 2003; Moskwa et al., 2007) and bull semen (Ortega-Mora et al., 2003; Staubli et al., 2006b). This indicates that PCR is an extremely useful technique and can detect *N. caninum* in a wide range of tissues.

The development of PCR to detect *N. caninum* using various targets in ribosomal (r) DNA and genes such as the NC5 has been reviewed, in particular by Dubey and Schares (2006). As shown in Figure 1.2, the rDNA provides the genetic coding for the construction of the rRNA components. In eukaryotes, the rDNA consists of tandem repeats which includes the nonstranscribed spacer (NTS), the external transcribed spacer (ETS), 18S rDNA, internal transcribed spacers (ITS) 1, 5.8S rDNA, ITS2 and 28S rDNA.

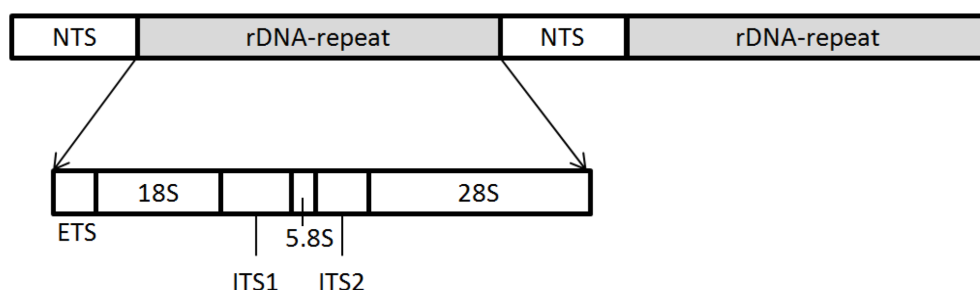

Figure 1.2: An example of eukaryotic rDNA gene segment.

The 18S rDNA (also known as small subunit-rDNA) gene of *N. caninum* has evolved slowly and has sufficient size to be examined. Consequently, it has been used extensively in phylogenetic studies (Al-Qassab et al., 2010). However, it has been shown to have little dissimilarity with *T. gondii* (Ho et al., 1996; Ellis et al., 1998) and thus it is not useful for

diagnostic purposes. The ITS1 region of the rRNA gene has been used widely in the development of species specific PCRs such as the single-step PCR (Holmdahl and Mattsson, 1996), the two-step nested PCR (Uggla et al., 1998; Buxton et al., 1998) and the single-step (single tube) PCR (Ellis et al., 1999). Among these PCRs, the single-step method has been shown to be highly sensitive with the detection range equivalent to 0.1 to 0.01 tachyzoites (Ellis et al., 1999).

A common gene used to detect *N. caninum* is the pNc5 gene which is a repetitive DNA sequence (Kaufmann et al., 1996) and has the advantage of not being present in *T. gondii* or *H. hammondi* (Yamage et al., 1996). Consequently, it is highly sensitive and thus has been used in various *N. caninum* studies (Baszler et al., 1999; Okeoma et al., 2004a; Okeoma et al., 2004c; Abo-Shehada and Abu-Halaweh, 2010). Together these rDNAs sequences and the Nc5 gene sequences have been the prime targets for the detection of *N. caninum* DNA in PCRs.

Using standard PCR methods, the target DNA can only be visualised post-PCR which is time consuming, laborious and the repeated handling increases the chances of sample contamination. Results obtained are based only on size discrimination although bands obtained can be sequenced to improve certainty about the interpretation. One of the earlier methods to estimate parasite load was the quantitative-competitive PCR (QC-PCR) which was based on the inclusion of a known competitor to the Nc5 target. However this method was found to be laborious (Liddell et al., 1999) as compared to the quantitative PCR (qPCR) methods that were developed later (Collantes-Fernandez et al., 2002; Muller et al., 2002; van Maanen et al., 2004).

1.2.7.7 Real time or quantitative PCR (qPCR)

The qPCR method is based on the detection of a fluorescent or other signals that are generated during each cycle and where the increase of signals emitted will be directly proportional to the number of amplicons produced. The assay is monitored in real time and the reactions are measured during the early stages of the cycle which is at the exponential phase of the PCR. This method can then both identify and quantify the number of starting templates in the sample tested. Other advantages include: not requiring post-PCR analysis; the assay is fast and efficient; there is a reduction in carry-over contaminations; it is highly sensitive; and results are reproducible (Collantes-Fernandez et al., 2002; Arya et al., 2005).

As shown in Table 1.4, qPCR is a commonly used analytical technique and has been widely used in studies and for diagnosis of *N. caninum* infections in tissues. It has been used to: determine the pathogenicity and parasite load in tissues (Collantes-Fernandez et al., 2002; Okeoma et al., 2005; Pinitkiatisakul et al., 2008; Nishimura et al., 2013); study the use and efficacy of vaccines and chemotherapeutic drugs (Cannas et al., 2003; Esposito et al., 2005; Debache et al., 2011; Debache and Hemphill, 2013); quantify parasite DNA in semen studies (Collantes-Fernandez et al., 2002; Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005; Serrano-Martinez et al., 2007a; Ferre et al., 2008); and quantify *N. caninum* DNA in blood samples (Okeoma et al., 2005). For the latter, this assay has shown a high degree of sensitivity where it was able to detect the presence of *N. caninum* DNA from <1 *N. caninum* tachyzoites/ml. This particular approach has the advantage of being able to test a large number of blood samples without the need for slaughtering any animals.

Most of the qPCR used in studies (Table 1.4) employed the SYBR Green method developed by Collantes-Fernandez et al (2002). This SBYR Green dye emits fluorescent light when intercalated into dsDNA. The SYBR Green technique is commonly used due to its simplicity, reliability and cost. Nevertheless, accurate primer design as well as optimised reaction conditions are required since the dye will bind to all dsDNA molecules leading to non-specific amplicons and primer dimers. A more specific technique than the SYBR Green is the Taqman probe which requires specific hybridisation between probes and the target DNA sequence. It has also been shown to be sensitive in a recent experimental study in cows (Pereira et al., 2014). The Taqman probe however, is more expensive and time consuming than SYBR Green. In general, the use of qPCR has proven to be a highly sensitive assay and appears to be a practical analytical tool when investigating neosporosis, especially when dealing with larger number of samples.

1 Table 1.4: Summary of some *N. caninum* studies using qPCR

Species	Samples	Target	Size (bp)	Primers	Detection method	Authors
cattle/mice	aborted foetus/brain	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Collantes-Fernandez et al (2002)
cell culture	cell culture	NC5	337	Np21plus (59CCCAGTGCGTCCAATCCTGTAAC39) Np6plus (59CTCGCCAGTCAACCTACGTCTTCT39) Nc5-Np 5LC(5"-TCCCTCGGTTCACCCGTTCACACAC-3) detection probe and anchor probe Np 3FL(5- CACGTATCCCACCTCTCACCGCTACCA-3	dual fluorescent hybridization probe system	Muller et al (2002)
mice/ cell culture	tissues/cell culture	NC5	337	Np21plus (59CCCAGTGCGTCCAATCCTGTAAC39) Np6plus (59CTCGCCAGTCAACCTACGTCTTCT39) Nc5-Np 5LC(5"-TCCCTCGGTTCACCCGTTCACACAC-3) detection probe and anchor probe Np 3FL(5- CACGTATCCCACCTCTCACCGCTACCA-3	dual fluorescent hybridization probe system	Cannas et al (2003)
cattle	semen	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Ortega-Mora et al (2003)
cattle	semen	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Caetano-da-Silva et al (2004)
cattle	tissues	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Van Maanen et al (2004)

Species	samples	Target	Size (bp)	Primers	Detection method	Authors
cattle	blood	NC5	188	NeoF=5'-GTGAGAGGTGGGATACG-3' NeoR=5'-GTCCGCTTGCTCCCTA-3'	SYBR Green 1	Okeoma et al (2005)
cattle	semen	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Ferre et al (2005)
cell culture	cell culture	NCR	337	Np21plus (59CCCAGTGCGTCCAATCCTGTAAC39) Np6plus (59CTCGCCAGTCAACCTACGTCTTCT39) Np 5LC(5"-TCCCTCGGTTCACCCGTTCACACAC-3) detection probe and anchor probe Np 3FL(5- CACGTATCCCACCTCTCACCGCTACCA-3	dual fluorescent hybridization probe system	Esposito et al (2005)
cattle	aborted foetus	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Collantes-Fernandez et al (2006a)
mouse/cattle	tissues	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Collantes-Fernandez et al (2006; 2006b)
cattle	semen	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Serrano-Martinez et al (2007a)
cattle	semen/blood	NC5	76	F=5'-ACTGGAGGCACGCTGAACAC-3' R=5'-AACAATGCTTCGCAAGAGGAA-3'	SYBR Green 1	Ferre et al (2008)
dog	tissues	NC5	265	F=5'-GGGAGTTGGTAGCGGTGAGA-3' R=5'-GCCTCCCAATGCGAACGAAA-3'	SYBR Green	Ghalmi et al (2008)
		NC5	142	NC5-550: GGGTGAACCGAGGGAGTTG NC5-596: ACGTGAGGAATGACTAACCACAA NC5 probe:AGCGGTGAGAGGTGGGATACGTGG.	Taqman	
		NC5	101	NC5PLEX: ACAGAACACTGAACTCTGGATAAGTATCA NC5FRIEND: GGATACGTGGTTTGTGGTTAGTCATTC.	Plexor	

Species	Samples	Target	Size (bp)	Primers	Detection method	Authors
mice	Blood	NC5	337	Np21+ (5’-GGG TGT GCG TCC AAT CCT GTA AC-3’) Np6+ (5’-CTC GCC AGT CAA CCT ACG TCT TCT-3’)	SYBR Green	Pinitkiatisakul et al (2008)
mice	Tissues	NC5	76	F=5’-ACTGGAGGCACGCTGAACAC-3’ R=5’-AACAATGCTTCGCAAGAGGAA-3’	SYBR Green 1	López-Pérez et al (2010)
cattle	Blood	NC5	188	NeoF=5’-GTGAGAGGTGGGATACG-3’ NeoR=5’-GTCCGCTTGCTCCCTA-3’	SYBR Green 1	VanLeeuwen et al (2011)
mice	Tissues	NC5	76	F=5’-ACTGGAGGCACGCTGAACAC-3’ R=5’-AACAATGCTTCGCAAGAGGAA-3’	SYBR Green 1	Rojo-Montejo et al (2011)
mice/cell culture	mice/cell culture	NC5	337	Np21plus (59CCCAGTGCGTCCAATCCTGTAAC39) Np6plus (59CTCGCCAGTCAACCTACGTCTTCT39) Np 5LC(5”-TCCCTCGGTTCACCCGTTCACACAC-3) detection probe and anchor probe Np 3FL(5- CACGTATCCCACCTCTCACCGCTACCA-3	dual fluorescent hybridization probe system	Debache et al (2011); Debache and Hemphill (2012)
cattle	Tissues	NC5	76	F=5’-ACTGGAGGCACGCTGAACAC-3’ R=5’-AACAATGCTTCGCAAGAGGAA-3’	SYBR Green 1	Nishimura et al (2013)
mice	Brain	NC5	103	NcA 5’-GCTACCAACTCCCTCGGTT-3’ NcS 5-GTTGCTCTGCTGACGTGTCG-3’	SYBR Green	Ferreirinha et al (2014)
cattle	foetal tissues	NC5	76	NC5 -F: TCCAATCCTGTAACGTGTTGCT, NC-R: CACAAACAAAAAGGAGCCTTGCT Probe: CTGCGCCCAACAAC	Taqman	Pereira et al (2014)

1.2.8 Treatment for neosporosis

At present no vaccine or chemotherapy has been shown to be safe or effective in cattle for the treatment of neosporosis. Infection causes high economic impact on the bovine dairy industry worldwide, leading to many studies attempting to address the need for the development of effective treatment and prevention measures against bovine neosporosis.

A large variety of chemotherapeutically active components have been tested for their potential use against *N. caninum,* either by *in vitro* or *in vivo* testing. Using *in vitro* cultivated *N. caninum* tachyzoites, Lindsay et al (1994) examined the activities of 43 chemotherapeutic agents in cell culture-based assays. Some of these compounds exhibited some antiparasitic properties including sulfathiazole, piritrexim, lasalocid, monensin, erythromycin, doxycycline and clindamycin, with clindamycin showing the highest activity. Other recent studies have demonstrated some antiparasitic activity for a range of other compounds such as decoquinate, artemisinin, depudecin, new generation diaminidines, ruthenium, nitazoxanide and other thiazolides derivatives using the same general *in vitro* approach (Lindsay et al., 1996b; Lindsay et al., 1997; Kim et al., 2002; Kwon et al., 2003; Darius et al., 2004b; Esposito et al., 2005; Muller et al., 2008; Leepin et al., 2008; Barna et al., 2013). Although some of the results were very promising the *in vitro* approach only targets cultivated *N. caninum* tachyzoites and provides no information on efficacy against the bradyzoite stage. There is also no assurance it will also be effective against the tachyzoite stage in the animal.

There have been fewer *in vivo* studies. Using a mouse model, treatment with sulfadiazine and amprolium against the tachyzoite stage was found to be unsuccessful (Lindsay and Dubey, 1990). Miltefosine has shown promising results in reducing parasite

burden in the CNS as well as clinical signs of neosporosis in infected Balb/c mice (Debache and Hemphill, 2012) but again, this was against the tachyzoite stage. Dicationic arylimidamides have also shown promise but the early report utilised a long term treatment to reduce clinical disease in mice starting treatment 14 days after infection (Schorer et al., 2012). The treatment reduced, but did not totally eliminate mouse death. Artemisone has also shown promising results in treating neosporosis in gerbils which had been recently infected (Mazuz et al., 2012). Neosporosis in dogs with acute neurological signs such as hind limb paresis or ataxia with muscle atrophy has been treated with clindamycin, potentiated sulphonamides and pyrimethamine (Hay et al., 1990; Mayhew et al., 1991; Dubey et al., 1995; Barber and Trees, 1996). However, these are only effective if given early in the course of the infection. In summary, most of these studies were studying the efficacy of the drug against the tachyzoite stage.

One of the more promising chemotherapeutic drugs that seemed to be showing some positive effect is toltrazuril and its derivative ponazuril. Toltrazuril was found to be effective in eliminating *N. caninum* tachyzoites in cell culture (Strohbusch et al., 2008) and to reduce the severity of disease and vertical transmission in murine studies (Gottstein et al., 2001; Ammann et al., 2004; Darius et al., 2004a; Gottstein et al., 2005; Strohbusch et al., 2009). Ammann et al. (2004) suggested that a supportive T cell-immune response was necessary in order for toltrazuril to reach its full effectiveness in clearing the *N. caninum* infection. In experimentally and naturally infected calves toltrazuril or ponazuril reduced the number of parasites that could be detected (Kritzner et al., 2002). In another study it was suggested there was an enhanced antibody response detectable after several months although the mechanism involved was not explained (Haerdi et al., 2006).

Toltrazuril, a symmetrical triazinone, is an anti protozoal agent and can be used against a broad spectrum of cyst-forming and non cyst-forming coccidian parasites (Mehlhorn et al., 1988; Haberkorn, 1996). It has the advantage when used to treat coccidiosis of being effective after a single treatment. During systemic dissemination, toltrazuril is metabolized into toltrazuril sulfone or ponazuril. Both of these drugs are able to pass through the host cell membrane and the cytoplasm causing swelling of the mitochondria and enlargement of the endoplasmic reticulum of the parasite thus affecting the intracellular developmental stages (schizonts, gamonts) (Mehlhorn et al., 1984). Though the specific mode of action of these two drugs is not really known, it was proposed that the respiratory chain of the chloroplast-like organelle and two enzymes involved in the pyrimidine synthesis of the parasite was affected (Harder and Haberkorn, 1989).

1.2.9 Vaccination

To date, no vaccine is available to prevent neosporosis in animals. In 2001 a killed tachyzoite vaccine (Neoguard™) was registered in New Zealand to reduce and prevent abortion in cattle due to *N. caninum*. However this vaccine was later removed from the market as it did not prove to be sufficiently effective, providing only 50% protection (Schetters, 2004) in vaccinated animals and possibly increased the risk of early embryonic death (Weston et al., 2012). In comparison, the Toxovax® vaccine is a modified live vaccine that has been successful in preventing ovine toxoplasmosis worldwide since its introduction in 1988 (Innes et al., 2007). This vaccine is the S48 strain of *T. gondii* which originally was isolated from a ewe in New Zealand and is now an incomplete strain that has lost its ability to form tissue cysts or oocysts (Wilkins et al., 1987; Buxton, 1993). The immunity induced by this vaccine will last up to 18 months post inoculation and has been shown to reduce the formation of tissue cysts in experimentally challenged sheep with *T. gondii* (Buxton et al.,

1993; Katzer et al., 2014). A similar approach has been taken with Neospora to attenuate a live isolate or to use a low virulence strain. To date, studies have shown promising results in live vaccinations with low virulence strains such as Nc-Nowra, NC-6 Argentina and Nc-Spain (Rojo-Montejo et al., 2011; Weber et al., 2013; Hecker et al., 2013b).

CHAPTER TWO

Detection of *Neospora caninum* DNA in the semen of experimentally infected rams with no evidence of horizontal transmission in ewes

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2.1 Abstract

Recent reports from New Zealand indicate *N. caninum* has a possible role in causing abortions in sheep. Transmission of *N. caninum* via semen has been documented in cattle. This study aimed to investigate if horizontal transmission through semen was also possible in sheep. Initially, six month old crossbred ram lambs (n=32), seronegative to *N. caninum,* were divided into 4 equal groups. Group 1 remained uninoculated whilst the remainder were inoculated with *N. caninum* tachyzoites intravenously as follows: Group 2 - 50 tachyzoites; Group 3 - 10^3 tachyzoites; Group 4 - 10^7 tachyzoites. Semen samples were collected weekly for 8 weeks for the detection of *N. caninum* DNA and quantified using quantitative PCR (qPCR). Plasma collected 1 month post-inoculation was subjected to ELISA (IDEXX Chekit) and western blot. At two weeks post-infection, three rams from Group 1 (uninoculated) and three rams from Group 4 (10^7 tachyzoites/ml) were mated with two groups of 16 ewes over two oestrus cycles. Ewe sera collected one and two months post-mating were tested for seroconversion by ELISA and western blot. All experimentally infected rams seroconverted by one month with ELISA S/P % values ranging from 11-36.5% in Group 2, 12-39.5% in Group 3 and 40-81% in Group 4. However, none of the ewes mated with the experimentally infected rams seroconverted. For the western blot, responses towards immunodominant antigens (IDAs) were observed in ram sera directed against proteins at 10, 17, 21, 25-29, 30, 31, 33 and 37 kDa. Rams in Group 2, 3 and 4 were noted to have at least 3 IDAs present. None of the ewes showed any of the 8 prominent IDAs except for the one at 21 kDa which was seen in 30 out of 32 ewes in both groups. *N. caninum* DNA was detected intermittently in the ram's semen up to 5 weeks post-inoculation with the concentrations ranging from that equivalent to 1-889 tachyzoites per ml of semen. Low concentrations of *N. caninum* DNA were also detected in the brain tissue of two rams (Group 2 and 4). These results suggest that although *N. caninum* DNA can be found in the

semen of experimentally infected rams, the transmission of *N. caninum* via natural mating is an unlikely event.

Keywords: *N. caninum*, semen, mating, serology, qPCR, rams, sheep

2.2 Introduction

N. caninum is an obligate intracellular parasite which is recognised as the leading cause of bovine abortion (Thornton et al., 1991). However, *N. caninum* infections have also been reported in horses, goats, deer, and sheep (Dubey, 2003). Natural infection of *N. caninum* in sheep was first identified in 1990 in a congenitally infected lamb with signs of ataxia and weakness after birth (Dubey et al., 1990). Subsequently, naturally occurring ovine neosporosis has been reported worldwide, including Japan, South America, Australia, Switzerland, Italy, Spain and New Zealand (Koyama et al., 2001; Kobayashi et al., 2001; Hassig et al., 2003; Moore, 2005; Masala et al., 2007; Moreno et al., 2012; Reichel et al., 2008; Howe et al., 2008; Bishop et al., 2010). Repeat abortions in subsequent years have also been reported (Jolley et al., 1999). In addition, studies have shown that experimentally infected sheep will seroconvert and abort in a dose dependent manner (Buxton et al., 1998; Buxton et al., 2001; Weston et al., 2009). These reports suggest that *N. caninum* infections can and do occur and cause disease, although it has generally not been regarded as a significant cause of abortion in sheep (Dubey et al., 1990; Dubey and Lindsay, 1990; Otter et al., 1997; Buxton et al., 1998; Helmick et al., 2002).

Seroprevalence studies using ELISA-based assays in sheep flocks from around the world show the prevalence ranges from 2-14% worldwide (Figliuolo et al., 2004; Gaffuri et al., 2006; Panadero et al., 2010; Langoni et al., 2011). Seroprevalence has been reported at 2.2% in New South Wales, Australia, and slightly lower at 0.6% and 1.4% in New Zealand (Reichel et al., 2008; Howe et al., 2012) as compared to the seroprevalence in dairy and beef cattle in New Zealand which is about 30 - 35% and 2.8% respectively (Reichel, 2000; Tennent-Brown et al., 2000). However, the role of *N. caninum* as a significant pathogen and abortifacient in sheep is still unclear although there have been sporadic reports of *N.*

caninum being involved in ovine abortions (Otter et al., 1997; Helmick et al., 2002; Hassig et al., 2003; Moreno et al., 2012). At present, knowledge about the epidemiology of neosporosis in sheep is limited and particularly the mode of transmission.

Transmission of *N. caninum* via semen was thought to be possible when bulls were found to be seropositive towards *N. caninum* and *N. caninum* DNA was detected in the semen of naturally (Moore et al., 2003; Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005) and experimentally infected bulls (Serrano-Martinez et al., 2007a; Ferre et al., 2008). Dose dependent seroconversion and parasitaemia were also seen in heifers inseminated with semen spiked with *N. caninum* tachyzoites, although no seroconversion or detection of *N. caninum* DNA in the embryo or calves was observed (Serrano et al., 2006; Serrano-Martinez et al., 2007b). The possibility that semen was a potential mode of transmission for *N. caninum* was further supported when Masuda et al., (2007) showed that CB-17 SCID and BALB/c male mice inoculated with 2 x 10^5 *N. caninum* tachyzoites were able to transmit the infection through mating to immunodeficient female SCID mice and their neonates.

Due to the increasing evidence for the possible role of *N. caninum* in poor reproductive performance in New Zealand (Howe et al., 2012) and lack of transmission studies in sheep under natural farming conditions, this study was designed to examine the potential for transmission of *N. caninum* through semen in sheep. The specific objectives were to determine if *N. caninum* DNA could be detected in the semen of rams experimentally inoculated with *N. caninum* with doses ranging from 50 to 10^7 tachyzoites (controlled challenge study) and to determine if *N. caninum* could be horizontally transmitted in sheep through semen via natural mating (controlled exposure study). The

findings from this research will help to better understand the epidemiology of *N. caninum* among sheep flocks in New Zealand and worldwide.

2.3 Materials and methods

2.3.1 Experimental design

This trial consisted of two parts. The first part was a controlled challenge study to determine if *N. caninum* DNA would be found in the semen of rams experimentally infected with *N. caninum* tachyzoites. The second part was a controlled exposure study to determine if *N. caninum* could be transmitted to ewes via natural mating with rams experimentally infected with *N. caninum.*

2.3.2 Animals

For the first part of the trial, 32 young rams aged approximately 6 months were selected from a farm with no known history of reproductive problems. The rams were a cross between Borderdale, Romney and Suffolk breeds and were free from *N. caninum* as indicated by negative serology using ELISA when sampled prior to removing them from their home farm and again prior to the experiment commencing. The rams were randomly divided into 4 groups with 8 rams per group. Group 1 was left uninoculated (Control); Group 2 rams were inoculated with 50 *N. caninum* tachyzoites intravenously (i/v); Group 3 rams were inoculated with 10^3 *N. caninum* tachyzoites i/v and Group 4 rams were inoculated with 10^7 *N. caninum* tachyzoites i/v. Inoculated rams grazed in one mob in a paddock separated from the uninoculated control rams in an adjacent paddock.

Semen was collected by electroejaculation using a battery powered intra-rectal ejaculator probe (Ruakura probe, Shoof Int, NZ). Repeated five second stimulations separated by a similar length break were applied and semen was collected in a sterile

container. Semen collection was done once pre-inoculation and then weekly up to 8 weeks post-inoculation. Blood samples were also collected one month post-inoculation to determine serum antibody levels and to look for the presence of *N. caninum* DNA circulating in whole blood. At the end of the study (Week 12) all rams were euthanized and brain tissue was collected and kept at -20° C.

For the second part of the trial 32 adult ewes aged approximately 4 years were purchased from the same farm. These ewes had successfully reared a lamb the previous year. All ewes had been vaccinated for *Toxoplasma gondii* and *Campylobacter fetus fetus* and were determined by a blood sample taken prior to leaving their farm of origin to be seronegative by ELISA for *N. caninum*. In addition, prior to mating, blood samples were collected 3 times via venipuncture to confirm that the ewes were serologically negative by ELISA to *N. caninum*.

The 32 ewes were randomly divided into 2 groups of 16. One group was mated with 3 rams from the control group (Group 1) while the other group was mated with 3 rams inoculated with 10^7 *N. caninum* tachyzoites (Group 4 - ram IDs 40, 43 and 46). Rams were introduced to the ewes one week post-inoculation and these ewes were then mated over 40 days (> 2 oestrous cycles). Mating harnesses were used on rams with crayons changed every week to allow determination of when ewes were mated. Blood samples were collected from the ewes at Weeks 4 and 8 from the commencement of mating to determine the presence of *N. caninum* antibodies and DNA. All ewes were kept till lambing had ended but no further tests were conducted on any of the ewes or lambs born. All animal usage was approved by the Massey University Animal Ethics Committee (MUAEC 08/93).

2.3.3 Inoculums

A New Zealand *N. caninum* isolate (NcNZ1) first discovered by Okeoma et al (2004a) was used as inoculums for the study. The isolate was maintained by periodic passage in Vero cell cultures with minimum essential medium (MEM) (Invitrogen, Grand Island, NY, USA) with each ml of media supplemented with 2% heat inactivated foetal bovine serum (FBS) (Invitrogen), 500 Units of penicillin (Invitrogen), 500 µg of streptomycin (Invitrogen), 200mM Glutamax™-1 (Invitrogen) and 0.1 µg of amphotericin B (Invitrogen). For inoculation trials, flasks with more than 70% of cell lysis were chosen and the flask was gently scraped using a sterile flask scraper to maximize tachyzoite collection. Tachyzoites were washed twice by centrifuging at 1000 times gravity (xg) for 10 minutes with phosphate buffer saline pH 7.4 (PBS) (Invitrogen) and then passed through a 5µm sterile syringe filter (Membrane Solutions, TX, USA) to ensure no whole Vero cells were present. The tachyzoites were then re-suspended using MEM without serum or antibiotic/antimycotics and counted to produce the required inoculation dose. Each inoculum was prepared as a 2 ml dose. Vials were transported in individual inoculum doses to the farm and kept in a polystyrene container away from light until used. Vials were kept at ambient temperature (~18°C) at the farm and were used for inoculation as soon as possible. The total time to inoculate rams was less than 3 hours. An additional 3 vials taken to the farm were returned to the laboratory where two were inoculated onto Vero cells to confirm live tachyzoites were present. Flasks were inspected visually within 48 hours and monitored for one week for cell bed disruption, consistent with actively replicating parasites. In addition, a third vial of inoculums was checked for the viability of the tachyzoites after return from the field using the trypan blue exclusion method (Strober, 2001).

2.3.4 ELISA

All sera collected during this trial were tested using a commercial ELISA test kit (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories, Australia). This indirect assay utilises *N. caninum* antigen with anti-ruminant IgG conjugates and detects antibodies against *N. caninum* antibodies in samples from cattle, goats and sheep. Results were calculated as a corrected sample to positive ratio (S/P) and the value was expressed as a percentage. According to the manufacturer's instructions a value greater than 30% is considered as suspect and above 40% as positive for *N. caninum.* However in this study the findings of Reichel et al (2008) were adopted where an S/P ratio of ≥11.8% was regarded as positive.

2.3.5 Western Blot

All sera from the 32 rams and ewes were analysed using western blot. Sera from rams were tested prior to and 1 month post-inoculation while sera from ewes were tested 1 month post-mating.

2.3.5.1 Preparation of water-soluble *N. caninum* antigen

Tachyzoites of *N. caninum* were harvested when the Vero cells displayed greater than 80% lysis. The tachyzoites were then washed 3 times by suspending them in PBS (pH 7.4) by centrifuging the solution at 1200xg for 10 minutes and removing the supernatant leaving the pellet. The pellet was re-suspended with PBS and the content was then passed through a 5µm filter to remove any cell debris. The tachyzoites were centrifuged again and supernatant was removed leaving a pellet which was stored at -20°C until used. The pelleted tachyzoites were re-suspended in 5ml of distilled water and disrupted by three

cycles of freezing and thawing by cycling them from room temperature to -80°C. This was followed by seven 15 second cycles of sonication on ice (Sonics Vibracell™, Sonics & Materials Inc., CT, USA). The sonicated tachyzoites were centrifuged at 10,000xg for 30 min at 4°C to remove debris and the supernatant containing the water soluble proteins were collected. Protein content was determined using a Nano-spectrophotometer (NanoDrop ND-1000, Thermo Scientific, DE, USA) and stored in aliquots.

2.3.5.2 SDS PAGE and Western blot analysis

SDS PAGE and Western blot were performed as described by Okeoma et al (2004b) with the following modifications. Membrane strips were then blocked overnight at 4°C using 5% Blotto with 1% Tween-20 (Sigma). Strips were incubated with ram or ewe sera (1:100 dilution) 20°C on a slow rocker for 2 hours then washed three times for 10 minutes in PBS with 1% Tween (PBS-T). Secondary antibody (rabbit anti-sheep IgG: HRP, Santa Cruz Biotechnology, CA, USA) at 1:10,000 dilution in 5% Blotto with 1% Tween-20 was added for 1 hour at 20°C with gentle shaking. After final washing with PBS-T the strips were then incubated for 1 minute in chemiluminescent solution (Pierce® ECL-Western Blotting Substrate, Thermo Scientific, IL. USA) and arranged accordingly on an x-ray film. The film was then exposed for 2 minutes and processed, digitalized and bands measured against the standard markers using a log graph. Presence of bands indicating immunodominant antigens (IDAs) of *N. caninum* were recorded. Each western blot was run with a negative and positive control. The positive control serum was from a ewe naturally infected with *N. caninum*. Serum positive to *Toxoplasma gondii* was also tested to determine if there was any cross reaction with the antigen used for the western blot. For this study, sera that recognized at least one IDA with molecular weight (MW) of 17, 25-29, 30-33 or 37 kDa were considered positive for *N. caninum* as defined by Alvarez-Garcia et al (2003). Our positive

control sera showed consistent banding patterns and thus acted as an internal control for protein loading and antibody probing.

2.3.6 Detection and quantification of *N. caninum* DNA in samples

2.3.6.1 DNA extraction

Five grams of each ram's brain sample were placed into a sterile, single use, 50 ml conical tissue grinder (VWR, PA, USA) containing 10 ml of sterile PBS. Homogenized brain tissues and semen (100 µl) 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 or cells respectively. Water blanks were included as sample processing controls to confirm lack of contamination during sample manipulation. All extracted samples were kept at -20°C until further analysis was performed.

2.3.6.2 Real-time Polymerase Chain Reaction (qPCR)

All samples were subjected to qPCR for the detection of *N. caninum* DNA and only those that were positive were later quantified. The qPCR was designed to amplify the Nc-5 gene (Kaufmann et al., 1996) of *N. caninum* using the primers NeoF (5'-GTGAGAGGTGGGATACG-3') and NeoR (5'-GTCCGCTTGCTCCCTA-3') (Okeoma et al., 2005). The conditions for the reactions were optimized for the primers and products obtained were analysed with a melting curve analysis. Standard curves were also generated to determine the sensitivity range of the assay. Products amplified from the initial run using *N. caninum* DNA from a pure cell culture were purified, sequenced, and subjected to BLAST to confirm its identity and specificity.

The 25 µl qPCR reaction mixture contained: 2 µl of sample DNA containing 1 ng DNA/µl , 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.1 µM primer NeoF, 0.15 µM primer NeoR, SYTO9 and 1 unit of Platinum Taq DNA polymerase (Invitrogen). The thermal cycler

conditions were as follows: 95°C for 10 minutes; 45 cycles of 94°C for 10 seconds (s), 65°C for 10 s and 72°C for 20 s. A melt curve analysis was visualized over the 80 - 90°C temperature range on the Rotor-Gene 6000 Series software 1.7 (Corbett Life Sciences, Sydney, Australia). DNA extracted from cell-cultured *N. caninum* was used as a positive control and was included with a negative control containing no DNA for each qPCR run. Each sample was prepared in triplicate.

A standard curve was generated for each run using a 10-fold dilution series of purified DNA from cell-culture adapted *N. caninum* with five known concentrations (1, 10, 100, 1000, 10000 tachyzoites/ml). The efficiency and standard curve of the qPCR cycle were calculated using the Rotor-Gene 6000 Series software 1.7. The standard curve was used to estimate the number of tachyzoites of *N. caninum* per ml of semen or mg of tissue.

2.3.7 Statistical analysis

All rams' S/P ratios for ELISA were tested for normality and all were found to be normally distributed. One way ANOVA were performed to compare the antibody responses between rams in Groups 1-4 at 1 month post-inoculation. The Fisher Exact test and the Pearson Chi-Square analysis were used to determine any association between the dose inoculated with the presence of proteins detected in western blot and presence of *N. caninum* DNA detected in semen. Statistical analyses were performed using Minitab (Minitab Inc., PA, USA) and all statistical analyses were considered significant when $p<0.05$.

2.4 Results

2.4.1 Controlled challenge study

2.4.1.1 Viability of tachyzoites

Upon returning from the farm after inoculating the rams, viability of the tachyzoites were determined using the trypan blue exclusion method and found that more than 50% were still viable. 2 other vials were inoculated onto Vero cells to confirm live tachyzoites were present. Within 48 hours post inoculation live tachzyoites were noted with lyses of Vero cells present in the flask.

2.4.1.2 IDEXX serology results

The serum IgG responses to *N. caninum* in all groups of ram at pre- and 4 weeks post-inoculation with *N. caninum* tachyzoites are shown in Figure 2.1. All experimentally infected rams were seropositive to *N. caninum* when tested by ELISA at one month post-inoculation when the mean S/P values were significantly higher than for the un-inoculated controls ($p<0.05$). Antibody responses in Group 4 were significantly higher than those in Groups 2 and 3 ($p<0.05$). Interestingly, there were no significant differences seen in the antibody responses between Groups 2 and 3 ($p > 0.05$). The S/P values for Groups 2, 3 and 4 ranged from 11% to 36.5%, 12% to 39.5% and 40% to 80% respectively. All the rams in the control group remained negative for *N. caninum* antibodies.

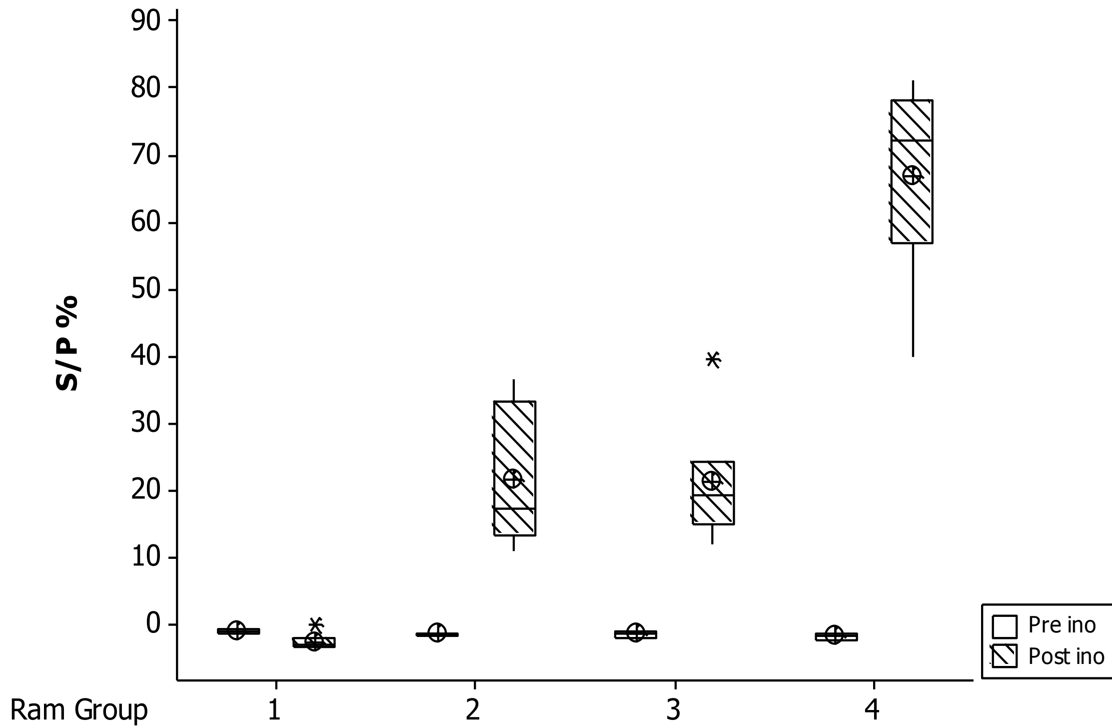

Figure 2.1: A box and whisker plot of the antibody responses to *N. caninum* at pre- and 4 weeks post-inoculation in control and experimentally infected rams. Rams were inoculated with various doses of *N. caninum* tachyzoites (Group 1 = control, Group2 = 50 tachyzoites, Group3 = 10^3 and Group4 = 10^7). Circle in the centre bar shows the mean S/P value.

2.4.1.3 Western blot serology results

Sera from rams at 1 month post-inoculation with *N. caninum* tachyzoites were analysed using western blot (Table 2.1). Antibodies were detected against a consistent range of known *N. caninum* tachyzoite IDAs (10, 17, 25-29, 30, 31, 33 and 37 kDa). In addition, 30/32 rams and one known *T. gondii* positive serum sample had reactive antibodies against a protein at 21 kDa. Due to the cross-reactive nature of this antigen, any antibody responses against the 21 kDa protein were dismissed as being not *N. caninum* specific. No other major bands were observed in the sera from Group 1 rams. In Group 2,

3 and 4, all 24 ram sera were noted to have responded to at least 3 IDAs with 23/24 ram sera recognising proteins at 17, 25-29 and 30 kDa.

Table 2.1: Proportion of IDAs recognised in western blot by ram sera 1 month post-inoculation with different doses of *N. caninum* tachyzoites.
(Group 1 = control, Group 2 = 50 tachyzoites, Group 3 = 10^3 and Group 4 = 10^7).

Immunodominant antigen kDa	Group 1	Group 2	Group 3	Group 4
10	NIL	2/8	2/8	5/8
17	NIL	8/8	8/8	8/8
21	8/8	8/8	7/8	7/8
25-29	NIL	7/8	8/8	8/8
30	NIL	8/8	8/8	8/8
31	NIL	3/8	2/8	4/8
33	NIL	4/8	2/8	7/8
37	NIL	6/8	1/8	6/8

2.4.1.4 qPCR results

QPCR results are shown in Table 2.2. *N. caninum* DNA was detected as early as one week post-inoculation in 1/8, 3/8 and 6/8 semen samples from rams in Groups 2, 3 and 4 respectively. *N. caninum* DNA was detected in four out six rams in Group 4 intermittently over a four week period. No *N. caninum* DNA was detected in the semen of the un-inoculated control rams (Group 1). Using Chi-Square analysis, no evidence of association (p=0.29) was found between dose of tachyzoites inoculated and detection of *N. caninum* DNA in the semen.

All positive samples were then quantified using the qPCR analysis (Table 2.2). If the quantity of DNA detected was less than the lowest range of the standards run, it was recorded as equivalent to <1 tachyzoite/ml (t/ml). The highest concentration of *N. caninum* DNA detected in the semen was from individual rams in Group 4 at Week 1 (Ram 46, 314 t/ml), Week 2 (Ram 37, 850 t/ml; Ram 46, 723 t/ml), or Week 4 (Ram 43, 889 t/ml). *N. caninum* DNA was detected in the brains of only 2/21 inoculated rams; one being from Group 2 given 50 tachyzoites and one from Group 4 given 10^7 tachyzoites. No *N. caninum* DNA was detected in the brain of the un-inoculated control rams (Group 1). All ram semen samples collected pre-inoculation were negative for *N. caninum* DNA.

2.4.2 Controlled exposure study

For the controlled exposure study, two out three rams (Rams 43 and 46), randomly selected from Group 4 were intermittently shedding *N. caninum* DNA during mating.
In this study, 16 out of 32 (50%) ewes were mated within the first week and 80% of the ewes were mated within the first oestrous cycle. Only one (from the control group) of 32 ewes was not pregnant at scanning 70 days post-mating but showed evidence of mating.

Another scan was conducted at 100 days post-mating to determine if there were any changes in the pregnancy status and revealed that 31 ewes remained pregnant. All ewes mated to infected rams subsequently lambed live and apparently healthy lambs. No further tests were conducted on these lambs.

All of the 32 ewes remained serologically negative with an S/P value of ≤ 11.8% at 4 and 8 weeks post-mating. Western blot was also conducted on these serum samples and confirmed these ewes were serologically negative for *N. caninum* antibodies.

Table 2.2: Detection and quantification of *N. caninum* DNA using qPCR in ram semen and brain tissue post inoculation with various doses of *N. caninum* tachyzoites.

ID	Dose	Pre – Inoc	1 week	2 week	3 week	4 week	5 week-	6 week	Brain
ALL	Con-trol		-	-	-	-		-	-
12	50	-	-	-	-	-	-	-	-
13	50	-	-	-	-	-	-	-	-
14	50	-	-	-	-	-	-	-	-
15	50	-	43 t/ml	-	-	-	-	-	-
19	50	-	-	-	-	-	-	-	3 t/mg
20	50	-	-	-	-	-	-	-	-
21	50	-	-	-	-	-	-	-	-
22	50	-	-	-	-	-	-	-	-
24	10^3	-	-	-	-	-	-	-	-
26	10^3	-	-	-	70 t/ml	-	-	-	-
27	10^3	-	10 t/ml	-	-	-	-	-	-
28	10^3	-	-	-	-	-	-	-	-
32	10^3	-	3 t/ml	-	-	-	-	-	-
33	10^3	-	-	-	-	-	-	-	-
35	10^3	-	-	-	-	-	-	-	-
36	10^3	-	-	-	-	-	-	-	-
37	10^7	-	-	850 t/ml	-	<1 t/ml	-	-	-
39	10^7	-	-	<1 t/ml	-	-	-	-	7 t/mg
40*	10^7	-	-	-	-	-	-	-	-
41	10^7	-	-	-	-	-	-	-	-
43*	10^7	-	<1 t/ml	<1 t/ml	-	889 t/ml	-	-	-
45	10^7	-	16 t/ml	<1 t/ml	1 t/ml	-	-	-	-
46*	10^7	-	314 t/ml	723 t/ml	-	314 t/ml	-	-	-
47	10^7	-	<1 t/ml	-	-	-	-	-	-

* indicates ram used for mating; t/ml = tachyzoite/ml semen; t/mg = tachyzoites/mg brain

2.5 Discussion

In this study, rams were experimentally infected with *N. caninum* tachyzoites. All inoculated rams seroconverted by four weeks post inoculation and *N. caninum* DNA was detected in the semen of 11/32 infected rams as early as one week PI. However, none of the ewes mated to the rams infected with the highest dose (10^7 tachyzoites) seroconverted after two oestrus cycles. In addition, no reproductive failure was observed. Thus, the results of this study, suggest that transmission via semen is not a significant transmission pathway in sheep.

These findings appear to be similar to the transmission pathway of *N. caninum* observed in cattle. While studies using AI had shown that cows were able to be infected using spiked semen (Serrano et al., 2006; Serrano-Martinez et al., 2007b), a natural mating study using experimentally infected bulls failed to induce seroconversion in the mated cows (Osoro et al., 2007) and these authors hypothesized that the parasite loads in the semen were too low to lead to infection. *N. caninum* DNA equivalent to 1 to 16 *N. caninum* t/ml has been detected in the semen of both naturally and experimentally infected bulls (Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004; Ferre et al., 2005; Serrano-Martinez et al., 2007a; Ferre et al., 2008). Assuming the mean volume of ejaculate is approximately 9-10 ml (Chenoweth, 1983) this represents <200 tachyzoites per ejaculate. Even in natural mating conditions where a bull can serve a female up to 10 times per oestrus cycle (Chenoweth, 1983) the total number of possible tachyzoites would still be less than 5000 tachyzoites which was reported by Serrano-Martinez et al. (2007b) as the minimum number needed to induce seroconversion in some cows.

In order to increase the possibility of getting infective semen, the rams used for mating in this study were inoculated with a high dose of *N. caninum* tachyzoites. Mating

was also commenced 1 week post-inoculation and continued for over two cycles in order to increase the chance of ewes being mated with infective semen. In the current study, electro-ejaculation was used instead of collecting semen with an artificial vagina (AV) due to time constraint in training the rams to serve into an AV. Studies have shown that total volume of semen obtained via electro-ejaculation is higher than those collected using artificial vagina due to increase in seminal plasma (Terrill 1940; Mattner et al., 1962; Salamon and Morrant, 1963; Moore 1985). In the current study, analysis was made using whole semen thus no conclusion could be made if tachyzoites were found in the cellular or seminal fraction of the semen. However, previous studies in bulls have indicated that *N. caninum* DNA was detected only in the cell fraction and not in the seminal fluid of the semen (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ortega-Mora et al., 2003; Serrano-Martinez et al., 2007a, b), thus suggesting that the method of collection would not have an effect on the number of tachyzoites found in the semen. Although no *N. caninum* DNA was detected in the semen of one of the rams, a concentration of DNA equivalent to a high number of tachyzoites was detected intermittently in the semen of the other two rams. Nevertheless, no ewes seroconverted in this study. It has been reported that an individual ram can breed 8-10 ewes daily and may mate with the same ewe several times (Chenoweth, 1981) with a mean of 0.8 ml of semen per ejaculate (Terrill, 1940; Hulet et al., 1964). Thus for the ram in the present study that was recorded as shedding DNA equivalent to approximately 900 t/ml, it is plausible that at least some ewes received DNA equivalent to >5000 tachyzoites during mating. If this DNA was from viable tachyzoites this would suggest that there is a high chance of *N. caninum* being transmitted to at least some of the ewes.

The qPCR using the primer set previously described by (Okeoma et al., 2005) showed a high degree of sensitivity and was able to detect the presence of *N. caninum* DNA from <1 *N. caninum* t/ml but was only able to measure concentrations accurately across the range of standards used which was from 1 to 10000 t/ml. It is unclear from this current study whether the parasite DNA detected in the semen represents viable tachyzoites as bioassay was not employed to determine if viable tachyzoites were present. In previous studies with experimentally infected cattle, viable tachyzoites could not be detected in bull semen by bioassay (Ferre et al., 2005; Ferre et al., 2008).

It was observed that there was intermittent shedding in some rams up to Week 4. Studies in bulls have shown a similar intermittent pattern of shedding of *N. caninum* DNA that has continued for more than a year (Serrano-Martinez et al., 2007a; Ferre et al., 2008). By comparison with the closely related protozoan *T. gondii*, DNA of this organism was also detected in the semen of experimentally infected rams up to 1 month post infection (Teale et al., 1982) which indicates a similar time frame to that seen with *N. caninum* in the present study. Whether long term shedding of *N. caninum* in the semen over a period of several months would occur has not been examined in sheep and remains an interesting question, especially if viable tachyzoites are present in the semen.

Interpreting the serological status of an animal can be difficult and thus in this study we opted to use a commercial ELISA for the detection of IgG against *N. caninum* in conjunction with a western blot analysis. IDEXX Chekit is an indirect ELISA which detects antibodies against *N. caninum* in ruminants and has been used in studies in sheep and goats (Reichel et al., 2008; Weston et al., 2009; Bishop et al., 2010; Czopowicz et al., 2011). According to the manufacturer's instructions an S/P value of 30% to 40% is considered as suspect positive and over 40% is considered as positive for *N. caninum*. However a cut-off

value of 11.8% was used in the present study as recommended by Reichel et al., (2008) after their evaluation using sera from experimentally infected sheep. In that study, this cut-off value gave a specificity of 98.8% and sensitivity of 100%. If the original threshold of 30% was used, 13 rams in the present study would have been considered as serologically negative. Five out of these 13 animals were positive for *N. caninum* DNA in the semen and 1 in the brain tissue. One of the animals inoculated with 10^3 tachyzoites was positive for *N. caninum* DNA in the semen at Week 3 and had an S/P value of only 11.9% at Week 4. These findings provide further evidence for the use of 11.8% as a cut-off point for the IDEXX ELISA and not 40% as recommended by the manufacturer. All experimentally infected rams, including the 3 rams used for mating, showed a high serum antibody response towards *N. caninum* at 4 weeks post-inoculation. Even rams that were inoculated with 50 tachyzoites were able to elicit a reactive immune response.

The use of western blot further confirms the serological status of these rams and has proven to be valuable in confirming the serological status of an animal (Atkinson et al., 2000) and validating serological assays (Alvarez-Garcia et al., 2002; Okeoma et al., 2004b; Schares et al., 1998). For *N. caninum,* different ranges of IDAs have been reported depending on the electrophoresis conditions as well as antigen solubilisation and extraction methods used (Barta and Dubey, 1992; Zintl et al., 2006). In this study reduced antigens were prepared and a consistent pattern of recognition of IDAs of 10, 17, 21, 25-29, 30, 31, 33 and 37 kDa were observed. These IDAs, except for the 21 kDa antigen, have been frequently reported by other studies examining *N. caninum* infection in cattle, goats and sheep (Barta et al., 1992; Bjerkas et al., 1994; Schares et al., 1998; Sondgen et al., 2001; Naguleswaran et al., 2004; Gaffuri et al., 2006; Rossi et al., 2011). No difference in band pattern was recognized between the sera from the experimentally infected animals and the

sera of the naturally infected animal that was used as a positive control in this study. All experimentally infected rams were positive by western blot using the criteria where at least 1 IDA was recognised. Indeed all the 24 inoculated ram sera recognised at least 3 IDAs. Of interest was that all animals recognized a 21 kDa protein not previously described in *N. caninum* studies. It is possible that this reactivity is a cross-reaction to other similar apicomplexan antigens such as *T. gondii*, *Hammondia hammondi,* or a 21kDa *Eimeria* merozoite protein (Riahi et al., 1998; Sasai et al., 1998).

Other factors such as breed and age may affect the susceptibility of animals to infection. Recent studies in cattle have shown lower *N. caninum* congenital infection and abortion rates when infected Holstein-Friesian dams were inseminated with semen from Limousin bulls compared to those inseminated with semen from Holstein- Friesian bulls (Lopez-Gatius et al., 2005; Almeria et al., 2009; Yaniz et al., 2010). The explanation for this effect is currently unclear but the authors suggested there was a genetic component involved. There have been no studies on breed effects in sheep but it is notable that the ewes and rams in the present studies were cross-bred animals of at least 3 recognised breeds. It has been suggested that seropositivity increased with age due to horizontal transmission via ingesting oocysts (Dubey et al., 2007) and factors such as management practises which includes replacement rates and selective culling of seropositive animals might have an effect on the age factor (Bartels et al., 2006). However the influence of these factors has not been fully examined and further studies comparing infection rates between breeds and reproductive maturity (primiparous or multiparous) are indicated in determining the outcome of the venereal route as a mode of transmission for *N. caninum*. It should also be noted that evidence of *N. caninum* transmission was only based on ewe

serology and none of these ewes were euthanized at the end of the study and no reproductive tissues or lambs born were examined for the presence of *N. caninum* DNA.

In conclusion, this is the first study to determine the presence of *N. caninum* DNA in the semen of experimentally infected rams and also to determine if transmission of *N. caninum* is possible via natural mating in sheep. The presence of *N. caninum* DNA found intermittently in semen would suggest the possibility of transmission via the venereal route. Nevertheless it should be noted that detecting DNA equivalent to a high number of *N. caninum* tachyzoites in the semen does not mean the disease is transmissible. In addition, no evidence of transmission of *N. caninum* to any of the mated ewes suggests that transmission via semen during natural mating is an unlikely possibility. This study has provided further insight into the epidemiology and dynamics of neosporosis in sheep and especially the mode of transmission.

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2.7 Authors' contributions to this study

S. Syed-Hussain contributed to the study design, conducted the experiment, sample collections, laboratory work and wrote the manuscript.

W. Pomroy provided help with the study design, sample collection and editorial advice.

L. Howe provided help with the study design, advice on laboratory work and editorial assistance.

N. Williamson provided help with the study design and editorial advice.

D. West provided help with the study design, help with sample collection and editorial advice.

S. Stefan provided help with sample collection

DRC 16

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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: Syed-Hussain, Sharifah

Name/Title of Principal Supervisor: William Pomroy

Name of Published Research Output and full reference:

Syed-Hussain, S.S., Howe, L., Pomroy, W.E., West, D.M., Smith, S.L., Williamson, N.B., 2013. Detection of Neospora caninum DNA in semen of experimental infected rams with no evidence of horizontal transmission in ewes. Vet. Parasitol. 197, 534-542

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

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

  contributed to the experimental design and planning in collaboration with supervisors, carried out experimental work and statistical analysis, prepared the manuscript in collaboration with supervisors.

Syed-Hussain, Sharifah — 21/08/2014

Candidate's Signature — Date

William Pomroy — 21/08/2014

Principal Supervisor's signature — Date

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CHAPTER THREE

Adaptation of a commercial ELISA to determine the IgG avidity in sheep experimentally and naturally infected with *Neospora caninum*

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3.1 Abstract

Recent reports indicate *N. caninum* has a possible role in causing abortions in sheep in New Zealand. Knowledge about the epidemiology of neosporosis in sheep is limited. This study aimed to adapt and validate a commercially available ELISA assay as an IgG avidity assay to discriminate between acute (primary and re-inoculated) and chronic *N. caninum* infections in sheep. In addition, it was used to compare the antibody avidity values between lambs from ewes inoculated with *N. caninum* either during the pregnancy or in the previous year. The avidity assay was undertaken by using 6M urea for the first wash after incubation with the primary antibody in the commercial ELISA (Chekit* *Neospora* Antibody Test Kit, IDEXX Laboratories, Australia). Sequential serum samples were obtained from naïve ewes (n=16) experimentally inoculated with live *N. caninum* tachyzoites. All ewes were seropositive by two weeks post-inoculation and remained seropositive for 20 weeks post-inoculation. There was a linear relationship between time after inoculation and avidity values ($p<0.05$) over the first 24 weeks. In Week 4, all animals had avidity values < 35% and by Week 8, 8/16 animals had avidity values of > 35%. These results suggest that an avidity value of < 35% indicates a recent primary infection while a value of > 35% is indicative of a chronic infection. The assay was then validated using samples from other groups of experimentally inoculated sheep as well as samples from naturally infected ewes. When comparing sample to positive ratio (S/P) and avidity values from lambs born from recently inoculated ewes with those from ewes inoculated the previous year and re-inoculated in the current year, it was possible to differentiate the lambs at 2 weeks of age. Lambs from recently inoculated ewes had low S/P and avidity values at 2 weeks of age which increased by 12 weeks of age. In comparison, lambs from

re-inoculated ewes had high S/P and avidity values at 2 weeks of age, due to maternal antibody influence but values were similar to those from lambs that were born from recently inoculated ewes at 12 weeks of age. Avidity values for four naturally infected ewes were all >60% indicating chronic infection. These results suggest that the assay is able to discriminate between recent and chronic infection in sheep as well as able to differentiate lambs with maternal immunity compared to their own *de novo* immunity. As such it can be utilized to understand the kinetics of *N. caninum* infection in sheep.

Keywords: *N. caninum*, serology, avidity IgG, ELISA, sheep

3.2 Introduction

Neospora caninum is an obligate intracellular apicomplexan protozoan that is recognised as a major cause of abortion in the dairy industry worldwide (Dubey, 2003). The disease can persist in cattle over generations due to the high efficiency of vertical transmission (Dubey, 2003). Serology plays an important role in investigating abortion outbreaks and commonly acts as a primary test undertaken to determine the cause or agent involved. Knowing when infection occurs is important for diagnostic purposes. Most serological assays for *N. caninum* can only determine that the animal has been infected but not when this occurred (Björkman and Uggla, 1999; Dubey et al., 2006). In addition, studies have shown that *N. caninum* antibody titres may fluctuate after infection for the duration of the animal's life and that magnitude of the antibody titres does not necessarily provide information on the duration of the infection (Stenlund et al., 1999; Okeoma et al., 2004b; Nogareda et al., 2007).

Avidity assays have been developed to determine the functional affinity of specific immunoglobulin G (IgG) antibodies for a range of pathogens including Rubella virus (Hedman and Seppala, 1988), *Toxoplasma* (Hedman et al., 1989) and cytomegalovirus (Bodeus and Goubau, 1999). Such assays enable the duration since exposure to the pathogens to be estimated in animals and are based on the principle that antibodies produced soon after a primary infection have a low affinity or binding strength towards the antigen as compared to those produced later in a chronic infection or as a B-cell memory response (Jenum et al., 1997). For estimation of IgG avidity, a hydrogen-bond disrupting agent is used, such as urea, that will lead to IgG antibodies with low avidity being dissociated while those with high avidity remain bound to the antigen. On this basis, the

avidity of the IgG can be determined to enable discrimination between acute and chronic infection.

In 1999, Björkman et al described an IgG avidity ELISA used to distinguish between acute and chronic *N. caninum* infections in cattle (Björkman et al., 1999). Since this initial report, a number of avidity assays for *N. caninum* infections in cattle have been described and the majority use ELISA technology. A variety of antigen preparations have been used for these including membrane antigens incorporated into iscoms (immunostimulating complexes) (McAllister et al., 2000; Guy et al., 2001; Dijkstra et al., 2002; Björkman et al., 2003; Björkman et al., 2005), somatic or whole tachyzoite sonicated lysate (Gottstein et al., 1998; Maley et al., 2001; Sager et al., 2003; Aguado-Martinez et al., 2005) and affinity purified surface antigen (Schares et al., 2002).

The aim of the present study was to adapt and validate a commercially available ELISA assay (Chekit* *Neospora* Antibody Test Kit, IDEXX Laboratories, Australia) as an IgG avidity assay for *N. caninum* infection in sheep. The study was conducted using sequential serum samples obtained from previously naïve sheep experimentally inoculated with live *N. caninum* tachyzoites. The results were subsequently validated with serum samples obtained from other experimentally and naturally infected sheep. Comparison of avidity values of lambs born from newly or re-inoculated dams with *N. caninum* was also undertaken.

3.3 Materials and method

3.3.1 Experimental design

This trial consisted of three parts. The first was a challenge study to determine the development of sample to positive ratio (S/P) and IgG avidity values of ewes experimentally inoculated with *N. caninum* tachyzoites. The second was to validate the IgG avidity assay by comparing the avidity values of serum samples between those obtained in the challenge study and from other experimentally and naturally infected sheep. The third was to compare the S/P and avidity values of lambs born from ewes experimentally inoculated with *N. caninum*. Details of the experimental groups are shown in Table 3.1.

3.3.1.1 Sequential serum samples from experimentally inoculated sheep used to develop the avidity assay.

Serum samples were collected from 16 ewes (Group 1) that were experimentally inoculated with 10^6 live *N. caninum* (NcNZ1) tachyzoites intravenously two months prior to natural mating in Year 1. The inoculums used were prepared as described previously (Syed-Hussain et al., 2013). In Year 2 of the study, the 16 ewes were mated again from Week 56 post initial inoculation for two oestrus cycles. At Week 72 (Day 120 of gestation in Year 2), 9 ewes in Group 1 were re-inoculated with 10^7 live *N. caninum* tachyzoites (now known as Group 3 from Week 72) while the other 7 were not re-inoculated and remained as Group 1. Five additional ewes were used as negative controls (Group 2) and were kept together with the inoculated animals to rule out the occurrence of horizontal transmission of *N. caninum* in the farm throughout the study. Blood samples were collected at Weeks 0, 2, 4, 8, 12, 16, 20, 24, 72, 76 and 84 post inoculation.

3.3.1.2 Serum samples from other experimentally and naturally infected sheep to validate the avidity assay

Samples were obtained from other studies where sheep had been experimentally inoculated and also from sheep naturally infected with *N. caninum*. Details of the these additional serum samples are: Group 4 - 8 rams (age 6 months) 2 weeks after inoculation with 10^7 live *N. caninum* tachyzoites as previously described by Syed-Hussain et al (2013); Group 5 – 6 mixed age ewes, 4 weeks post inoculation with 10^7 live *N. caninum* tachyzoites; Group 6 - 15 lambs with a mean age of 12 weeks born from naïve ewes in Group 5 that were experimentally inoculated with 10^7 live *N. caninum* tachyzoites on Day 120 of gestation in Year 2 (mean time post inoculation of 17 weeks); Group 7a - 5 lambs which were seropositive at 12 weeks of age that were born from ewes in Group 3 after re-inoculation in Year 2 (mean time post inoculation for these lambs is also 17 weeks); Four serologically positive ewes, naturally infected at an unknown time with *N. caninum* and included Unknown (UK) 1 (tested pre mating, during pregnancy and after an abortion), UK 2 tested soon after abortion at mid pregnancy, UK 3 and UK 4 both sampled immediately pre mating.

3.3.1.3 Comparison of S/P and avidity values of lambs

Only sera from lambs that were seropositive at both 2 and 12 weeks of age that were born to ewes that were either inoculated in Year 1 or inoculated in Year 1 and Year 2 were selected and compared. For the former, this included lambs from Group 6 (n=4) and for the latter this included lambs in Group 7a (n=5) together with the other lambs born from Group 3 ewes that were only ELISA seropositive at 2 weeks of age (Group 7b; n=7).

Table 3.1: Descriptions of experimentally and naturally infected sheep with *N. caninum* used in the IgG avidity assay

Groups	No of animals	Description
Group 1	16	ewes, inoculated with 10^6 live *N. caninum* tachyzoites intravenously two months prior to mating in Year 1
Group 2	5	control ewes not inoculated
Group 3	9	ewes in Group 1, re-inoculated with 10^7 live *N. caninum* tachyzoites at Day 120 of gestation in Year 2
Group 4	8	rams, 2 weeks after inoculation with 10^7 live *N. caninum* tachyzoites
Group 5	6	naïve ewes, 4 weeks post inoculation with 10^7 live *N. caninum* tachyzoites in Year 2
Group 6	15	lambs, 12 weeks of age that were born from naïve ewes in Group 5 that were experimentally inoculated with 10^7 live *N. caninum* tachyzoites on Day 120 of gestation in Year 2
Group 7a	5	lambs, 12 weeks of age that were born from ewes in Group 3 that were seropositive by ELISA at 2 and 12 weeks of age
Group 7b	7	lambs born from Group 3 ewes that were only ELISA seropositive at 2 weeks of age
UK	4	naturally infected ewes

3.3.2 IgG ELISA

All sera collected during this trial were tested using a commercial ELISA assay (Chekit* *Neospora* Antibody Test Kit, IDEXX Laboratories, Australia) and all the assay kits used in this study were from the same batch. This indirect assay utilises *N. caninum* antigen with anti-ruminant IgG conjugates and detects antibodies against *N. caninum* antibodies in samples from cattle, goats and sheep. Results were calculated as a corrected sample to positive ratio (S/P) and the value was expressed as a percentage. According to the manufacturer's instructions a value greater than 30% is considered as suspect and above 40% as positive for *N. caninum.* However in this study, the findings of Reichel et al. (2008) were adopted where an S/P value of ≥11.8% was regarded as positive.

3.3.3 IgG avidity ELISA

The IgG avidity ELISA was developed utilising the same commercial assay as in 3.3.2. Briefly, samples were assayed in duplicates on the same plate. The protocol was the same except for a modification of the first wash after incubation with the primary antibody. For one series, the first wash was with 200µl 6 M urea (Gibco BRL, Life Technologies, NY, USA) diluted in the wash provided by the manufacturer. For the second series, washing was with 200µl of the provided solution. This first wash for both series was conducted with mild agitation for 5 minutes at 37° C. The plate was then further washed twice without urea as per the manufacturer's instructions. Positive and negative controls provided by the manufacturer were also included in both duplicates. The avidity index was calculated by comparing the optical density (OD) ratios for samples either with or without the urea wash and expressed as a percentage as shown below. For Group 1, avidity values were

calculated for all samples from Week 2 onwards while for other groups only seropositive samples (S/P value ≥ 11.8%) were tested.

$$\boldsymbol{Avidity\ Index}\ (\%) = \frac{\boldsymbol{OD}\ (\boldsymbol{urea\ treated\ sample})}{\boldsymbol{OD}\ (\boldsymbol{untreated})}\ \boldsymbol{x}\ \mathbf{100}$$

3.3.4 Western blot

At least one serum sample from each animal was analysed using a western blot to confirm they were seropositive using the technique as described previously (Syed-Hussain et al., 2013).

3.3.5 Statistical analysis

S/P values for the commercial ELISA and the subsequent avidity values were found to be normally distributed. General linear model repeated measures with Greenhouse-Geisser correction were performed and *post hoc* tests using the Bonferroni method were undertaken to determine and compare the differences of mean S/P and avidity values between sampling times in Group 1. A One Way ANOVA was used to compare the avidity values between the other groups. The data spanning Week 2 to Week 24 for Group 1 was also described with a regression line. Statistical analyses were performed using SPSS (Version 20, IBM®, SPSS® Statistic) and all statistical analyses were considered significant when $p<0.05$

3.4 Results

3.4.1 Challenge study: Sequential serum samples from experimentally inoculated sheep used to develop the avidity assay

3.4.1.1 IgG ELISA

A summary of the S/P values is shown in Figure 3.1. All inoculated ewes in Group 1 were positive by western blot at 2 weeks post inoculation. In Group 1, all 16 ewes had seronegative ELISA S/P values in Week 0 which then increased to a peak in Week 4 followed by a gradual decline until Week 84. All ewes were serologically ELISA positive by Week 2 until Week 20 but from Week 24 onwards there were some ewes on each sampling occasion that were serologically negative. Two ewes were seronegative (S/P=10.3% and 11.5%) in Week 24 but were seropositive on all subsequent occasions. One ewe became seronegative from Week 72 onwards with S/P values ranging from 4.0% to 8.1%. In addition, in Week 76, 3 of 7 ewes were seronegative but all were found to be seropositive again in Week 84. The upper outlier shown in Week 12, 20 and 24 was the same ewe which had consistently high S/P values throughout the study.

The mean S/P values overall differed significantly between time points (F (2.95, 44.31) = 27.803, $P < 0.001$). *Post hoc* comparisons between weeks are also shown in Figure 3.1. In Week 2 post inoculation, mean S/P values were 26% and were significantly higher than Week 0 ($p<0.05$). S/P values in Week 4 ($p<0.05$) were significantly higher than in Week 2 but no significant differences were found between the S/P values from Week 4 until Week 16 ($p>0.05$). The mean S/P values in Week 20 to Week 84 were not significantly different from each other ($p>0.05$) but were significantly lower than the values from Week 4 to Week 16 ($p<0.05$).

Un-inoculated ewes in Group 2 (control) had S/P values lower than 11.8% and were all serologically negative in Week 0 and in Week 84. They were also negative on western blot at Week 84.

In Group 3, after re-inoculation of ewes (n=9) at Week 72, their mean S/P values increased to 49% and then 59% in Week 76 and Week 84 respectively as compared to those that were not re-inoculated in Group 1 (n = 7) which remained low with a mean S/P value of 17% and 19% respectively in these same two weeks. There was a significant difference in mean S/P values between Group 1 and 3 ($p<0.05$) in Week 76 and Week 84.

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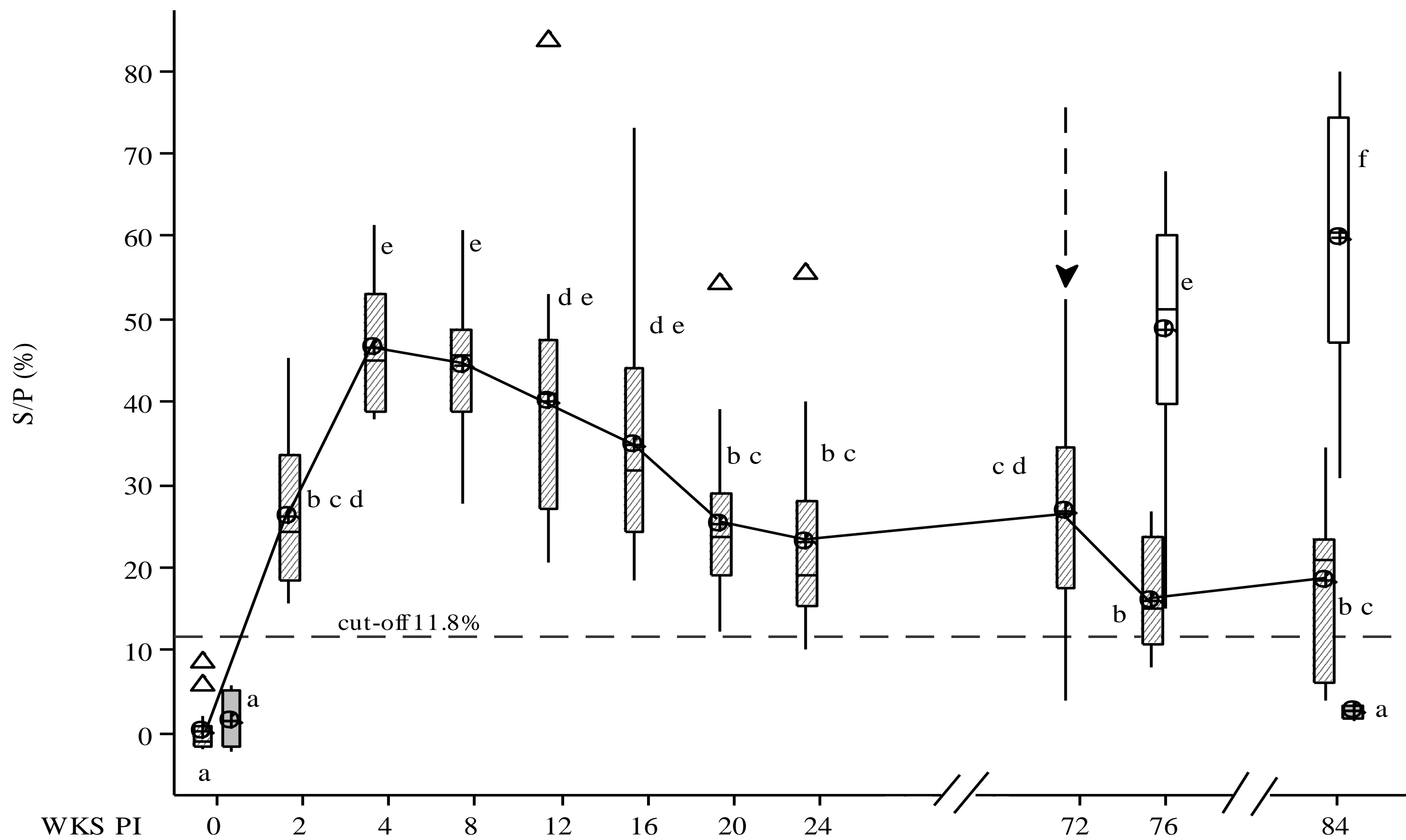

S/P (%)
80
70
60
50
40
30
20
10
0
cut-off 11.8%
a
a
b c d
e
e
d e
d e
b c
b c
c d
b
e
f
b c
a
WKS PI
0
2
4
8
12
16
20
24
72
76
84

Figure 3.1: Box and whisker plot of the S/P values of ewes in Group 1 (; Week 0 -72, n=16; Week 76 and 84, n=7) which were inoculated with *N. caninum* in Week 0. Group 2 are un-inoculated control ewes (; n=5). Group 3 (; n=9) represent ewes from Group 1 that were re-inoculated in Week 72 (arrow indicates day 120 gestation). The mean value (Ө) and outliers (Δ) are as indicated. Means that do not share a similar letter are significantly different ($p<0.05$).

3.4.1.2 IgG avidity assay of Group 1

The development of IgG avidity values for Group 1 was able to be determined from Week 2 post inoculation and is shown in Figure 3.2. The regression line from Week 2 to Week 24 is described below in Equation 3.1 and also shown in Figure 3.2 together with a 95% prediction interval.

Avidity % = 24.47 + 1.032 WEEKS PI (p<0.05; R^2 = 54%) Equation 3.1

Mean avidity values increased gradually and when analysed with repeated measures they differed significantly between time points (F (1, 110) = 130.86, p<0.001) as shown in Figure 2. In Week 2 the mean avidity values were not significantly different (p>0.05) from those in Week 4 but the values in Week 8 were significantly higher than those in Week 2 and 4 (p<0.05). In Weeks 2 and 4, all but 2 animals had avidity values < 35%. By Week 8, 8/16 animals had avidity values of ≥ 35%. All animals had avidity values of ≥ 35% at Week 24. In Week 8, the mean avidity value was lower than those in Weeks 12, 20 and 24 (p<0.05) although not different to those in Week 16 (p>0.05).

By Week 24, more than half of the group had avidity values of ≥ 45%. Subsequently all animals in Weeks 72, 76 and 84 had avidity values of ≥ 45%. In general, from Week 72 the mean avidity values plateaued for the remainder of the study.

3.4.2 Validation of IgG avidity assay: Serum samples from other experimentally inoculated and naturally infected sheep compared to Group 1.

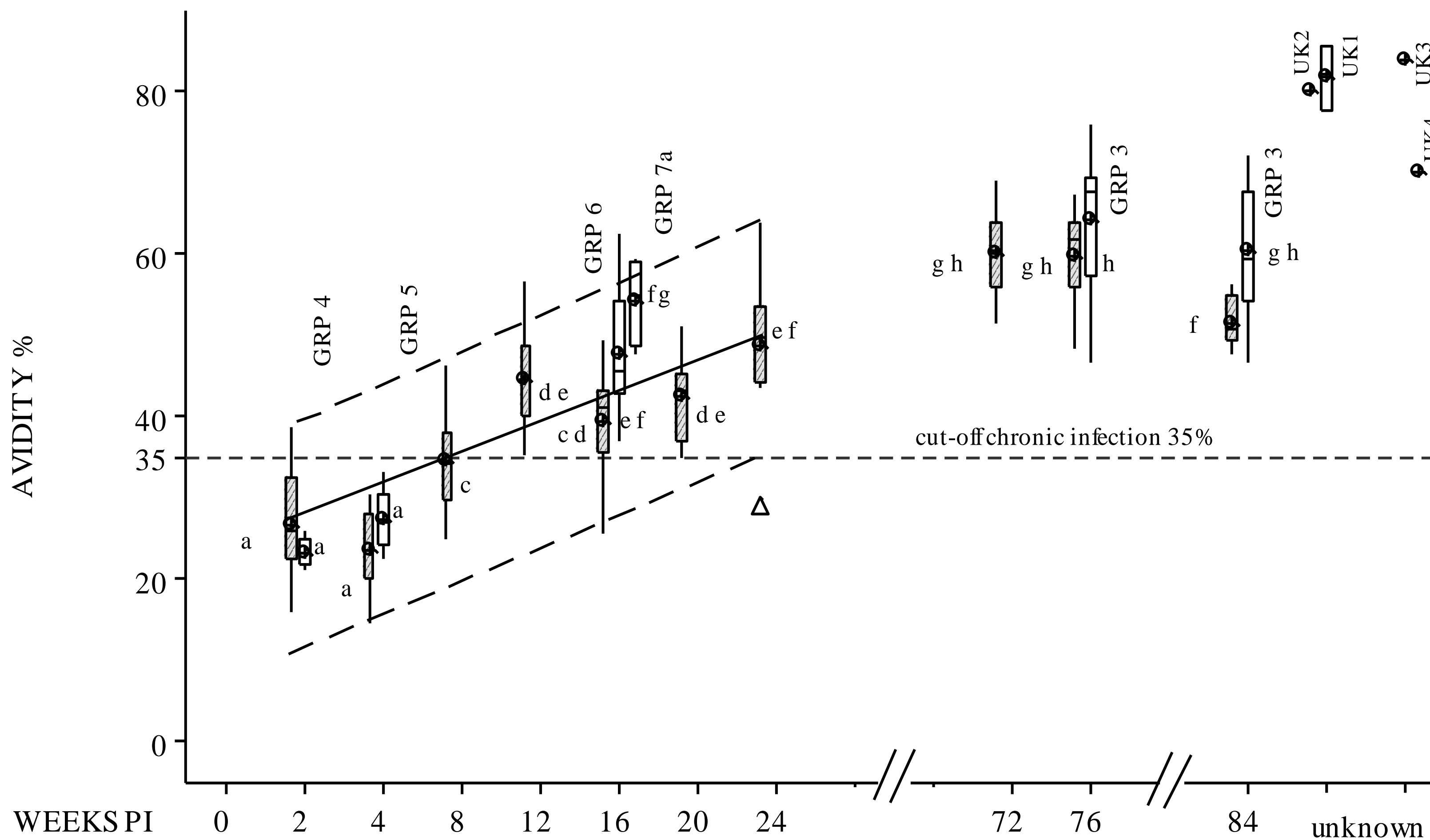
Figure 3.2 shows the avidity values for other groups compared to the values from Group 1. In Group 3, one month after re-inoculation (Week 76), no significant differences of avidity were detected between ewes in Group 1 and 3, although the mean avidity values for Group 1 were significantly lower than those of Group 3 ($p<0.05$) in Week 84.

Rams sampled two weeks post inoculation (Group 4) had a low mean avidity value of 23% (95% prediction interval; 11-41%) while ewes sampled 4 weeks post inoculation (Group 5) developed a mean avidity value of 27% (95% prediction interval; 14-43%). Avidity values for both these groups fell within the 95% prediction interval with avidity values consistent with recent infection of less than 8 weeks prior.

Mean avidity values for 12 week old lambs in Groups 6 (lambs from ewes inoculated once) and 7a (lambs from re-inoculated ewes) were 48% and 54% respectively and were not different ($p>0.05$) from each other. As these ewes were inoculated on Day 120 of gestation, these lambs presumably had been exposed for 16 to 17 weeks prior to the time of sampling. When their mean avidity values were compared to the prediction intervals for 17 weeks, the values of all but 1 lamb in Group 7a (which had a higher avidity value) fell within the 95% prediction interval (Figure 3.2) and are consistent with an infection of greater than 12 weeks prior.

Avidity profiles for four naturally infected ewes with *N. caninum* are also shown in Figure 3.2. UK1, bled on 3 occasions, consistently had high S/P and avidity values with the latter varying from 80-100%. UK2 was determined to have recently aborted at mid pregnancy and also had a high S/P of 100% with an avidity value of 72%. Both UK3 and UK4

were sampled pre-mating and had S/P values of 12% and 37% respectively with avidity values of 90% each.



AVIDITY %
WEEKS PI
0
2
4
8
12
16
20
24
72
76
84
unknown
80
60
40
35
20
0
GRP 4
GRP 5
GRP 6
GRP 7a
GRP 3
GRP 3
UK1
UK2
UK3
UK4
cut-off chronic infection 35%
a
a
a
a
c
d e
c d
e f
f g
d e
e f
g h
g h
h
f
g h

Figure 3.2: Box and whisker plot of mean avidity values of ewes in Group 1 (; n=16), Group 3 (n=9; ewes from Group 1 re-inoculated in Week 72), Group 4 (n=7; rams bled at 2 weeks post inoculation), Group 5 (n=6; ewes bled 4 weeks post inoculation), Group 6 (n=15; lambs at 12 weeks old from ewes inoculated at Day 120 of gestation), Group 7a (n=5; lambs at 12 weeks old from ewes re-inoculated at Day 120 of gestation) and values are also shown for four naturally infected ewes where the time of infection is unknown (UK1-4). Circle in the centre (Ө) of the bar indicates the mean value and the triangle sign (Δ) indicates outliers. Means that do not share a similar letter are significantly different ($p<0.05$). The regression line from Week 2 to Week 24 is shown (—) with 95% prediction intervals (---).

3.4.3 Comparison of S/P and avidity values of lambs born from experimentally inoculated ewes

Figure 3.3 shows the S/P and avidity values for lambs in Group 7a and those that were seropositive at both 2 and 12 weeks of age in Group 6 as well as values for Group 7b at 2 weeks of age. At 2 weeks of age, S/P and avidity values for lambs in Group 7a were significantly higher ($p<0.05$) than those in Groups 6 and 7b. Avidity values for Groups 7a and 7b at 2 weeks of age were all >60% and were significantly higher ($p<0.05$) than those in Group 6. However, at 12 weeks of age, no significant difference ($p>0.05$) in S/P or avidity values were found between Groups 6 and 7a. No avidity values are available for Group 7b as they were all ELISA seronegative at 12 weeks of age. Avidity values for Group 6 were significantly increased ($p<0.05$) between 2 and 12 weeks of age, whilst the values for Group 7a were significantly reduced ($p<0.05$) by 12 weeks of age. All lambs in Groups 6, 7a and 7b were positive by western blot when tested at 12 weeks of age.

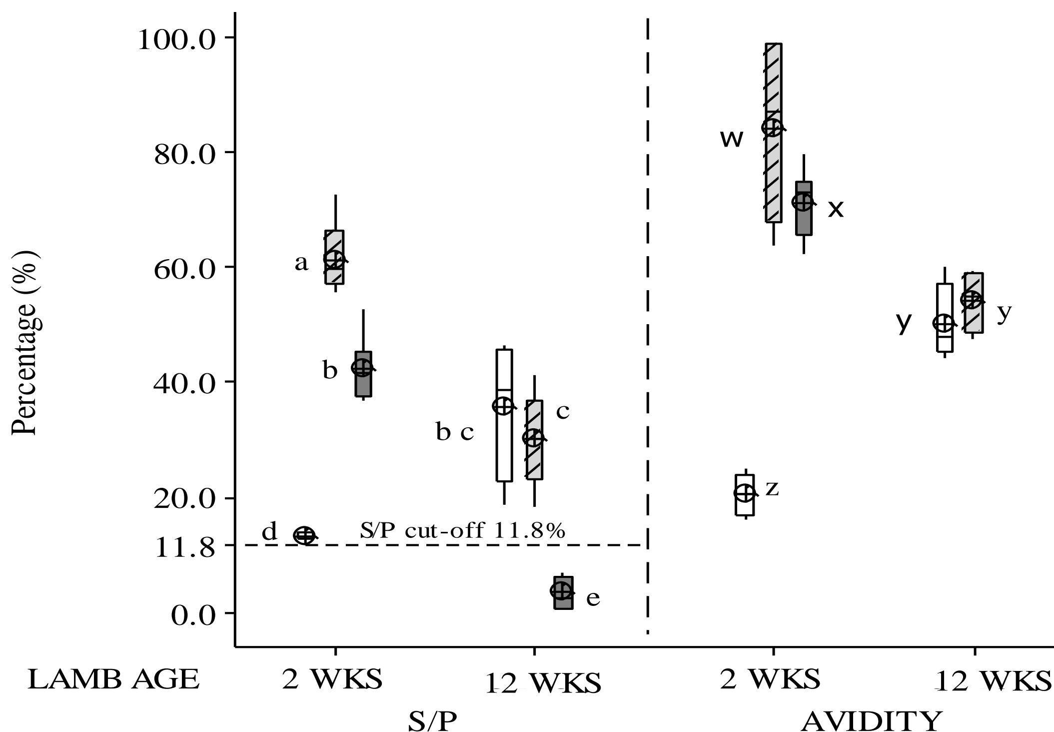


Figure 3.3: Box and whisker plot for mean S/P and avidity values at 2 and 12 weeks of age for lambs in Group 6 (; n=4), Group 7a (;n=5) and. Group 7b (; n=7). Group 7b were only seropositive at 2 weeks but seronegative at 12 weeks and not subjected to the avidity assay at 12 weeks. Means that do not share a similar letter are significantly different ($p<0.05$). Circle in the centre (ϴ) of the bar indicates the mean value.

3.5 Discussion

In the present study, a commercially available IgG ELISA test kit was adapted as an avidity assay to discriminate between acute and chronic *N. caninum* infection in sheep. The usefulness of this test was determined using sequential sera obtained from sheep experimentally inoculated with *N. caninum* tachyzoites and validated with sera obtained from other sheep either naturally infected or experimentally inoculated.

Using the ELISA, positive antibody titres were detected by 2 weeks post inoculation and this finding is consistent with other studies in sheep and cattle (Björkman et al., 1999;

Maley et al., 2001; Buxton et al., 2001; Sager et al., 2003). The specific IgG levels peaked at Week 4 post inoculation and then gradually declined throughout the study. This trend, with a peak titre one month post inoculation followed by a gradual decrease to 6 months post inoculation, correlates well to the findings of earlier infection studies in sheep by Buxton et al (1997; 2001). The S/P values at 28 days are similar to those reported by Weston et al (2009) using the same isolates and a similar infection protocol. However, the S/P values in these inoculated ewes were lower than those reported by Reichel et al (2008) but that may be due to an effect of a different isolate and/or breed of sheep. In the current study, the antibody responses increased in ewes re-inoculated in Week 72 and were greater in magnitude compared to those that were left un-inoculated. This is similar to the trend observed in sheep re-infected on Day 90 of gestation (Buxton et al., 2001). In the present study, some animals showed a fluctuating response, with some being considered seronegative especially beyond 6 months after inoculation. Fluctuating antibody titres which become seronegative in previously seropositive animals have been observed in experimentally infected calves (Maley et al., 2001) and especially in pregnant cows (Conrad et al., 1993a; Okeoma et al., 2004b). This finding emphasizes the obstacles to making a diagnosis using serology, especially in animals that were chronically infected with *N. caninum,* as it had been shown that being seronegative does not necessarily mean that the animal was never infected (Howe et al., 2012). It is also worth noting that the findings in this study suggest that infecting sheep with the NcNZ1 isolate of *N. caninum* originally isolated from cattle leads to persistent infection as most of the experimentally infected animals' antibody titres remained elevated above the seropositive cut-off value for more than 20 months post inoculation.

As rapid development of antibody titres were detected, it was possible to determine avidity values from 2 weeks post inoculation. A consistent increase in the avidity values over time was detected from Week 2, especially over the first 24 weeks and by Week 72 these values had effectively reached a plateau. While the linear relationship between time after inoculation and avidity is significant ($p<0.05$) over the first 24 weeks, the R^2 value was only 54% indicating variation in the development of high avidity values between animals. For the ewes experimentally inoculated for the first time (Group 1), higher values than expected were recorded at Week 12 which was inconsistent with the trend for other weeks where the mean value was generally near the regression line. The 95% prediction interval, spanning from Week 2 to 24 was quite wide suggesting that individual sampling might have a high variability but the assay would be more useful to assess the avidity of a flock.

Investigation of the avidity values in the current study has suggested that an avidity value of <35% is a good indicator of a recent primary infection while a value of >35% is indicative of a more long standing infection. The lower 95% prediction interval (Figure 3.2) was 35% at 24 weeks post-infection for Group 1 whilst the regression line intersected this value much earlier at 8 weeks. For ewes in Group 1, avidity values were <35% for all animals at 4 weeks post inoculation and for 14/16 animals at 2 weeks post inoculation. The choice of <35% as the cut off value indicating recent infection was validated by comparison with other recently inoculated animals (Group 4 and 5) which all had avidity values of <35%. This correlates well with the findings in recently infected cows which showed IgG avidity values between 21% to 40% during the initial outbreak and 9% to 18% in recently infected calves (Björkman et al., 1999; Björkman et al., 2003). Nevertheless, care has to be taken when interpreting low avidity results as it has been shown that low avidity persist longer in some individuals as seen in human cases of toxoplasmosis (Jenum et al., 1997). In the

present study, low avidities (<35%) were still detected in 4/16 animals in Week 16 and 1/16 animals in Week 24. Their avidity values did eventually increase to above 35% during the subsequent sampling. Therefore, although having a low avidity value may not conclusively indicate recent infection, higher avidity values can be used to rule out recent infection.

Different cut off values to determine the time point since exposure have been suggested for various avidity assays. These reports use either 2 or 3 categories such as acute, chronic or intermediate. Avidity values indicating recent infection have been reported as either <25 or <55% and values indicating chronic infection as either >35 or >55% (Björkman et al., 1999; Schares et al., 2002; Sager et al., 2003; Björkman et al., 2005; Aguado-Martinez et al., 2005). In the current study, at Week 12, all animals had avidity values of >35%. This suggests that an avidity value of >35% is a useful cut point indicative of chronic infection. Indeed the values for ewes inoculated for >6 months were all greater than 45% suggesting that, in some animals at least, the avidity will continue to increase to these higher levels. Avidity values of >45% for ewes inoculated for >6 months are also in agreement with those from experimentally infected cattle (Björkman et al., 1999; Sager et al., 2003). Interestingly, although there are some differences, the comparison of avidity values between different assays using different techniques and antigens do not have a marked effect on the extent of the resulting avidity values. In studies where whole antigen lysate of *N. caninum* was used, it was suggested that cut points of <25%, 25-35% and >35% were indicative of low, intermediate and high avidity values respectively (Sager et al., 2003; Aguado-Martinez et al., 2005). However, there were also variations in other aspects of the assay between this and other studies including types of urea, time of urea incubation and number of urea washes used (Hedman et al., 1989; Marcolino et al., 2000; Björkman et al., 2006).

The observation made after Week 72 on ewes in Groups 1 and 3, suggested that the combination of both S/P and avidity values could be useful to determine if chronically infected animals were experiencing recrudescence of an infection. Ewes which were not re-inoculated had low S/P values and a high avidity whilst those that were re-inoculated had much higher S/P ($p<0.05$) as well as high avidity values. However, to what extent this would be seen in natural infections in animals with oocysts remains to be determined, although similar trends have been observed in cattle when comparing results from experiments with infections with tachyzoites to those with oocysts (Björkman et al., 1999; Sager et al., 2003; Björkman et al., 2005). The avidity assay alone was not able to directly differentiate between those that were only inoculated in Year 1 (Group 1) and those that were re-inoculated (Group 3) as seen at Week 76 and 84. This limitation was also observed in other studies (Björkman et al., 2003; Sager et al., 2003; Aguado-Martinez et al., 2005). In the current study, in ewes where the antibody titres fluctuated to below the cut-off point (Figure 3.1; Group 1), the avidity values remained high thus making it a potentially useful diagnostic test with these low titre animals. This was also similar to the findings of other studies using the same method for determining avidity (Holliman et al., 1994; Maley et al., 2001). In field situations however, only samples with positive S/P values should be submitted to an avidity test. Unless other diagnostic methods such as western blots are used concurrently, interpreting test results for an individual animal with low antibody titres should be done cautiously as such low antibody titres could be due to non specific binding and lead to false positive results.

At 12 weeks of age, the avidity values for lambs in Groups 6 and 7a (Figure 3.2) ranged from 37% to 62%, which is within the 95% prediction interval for Week 20 to 24 onwards. This suggests that the lambs were chronically infected beyond their age, thus

indicating congenital transmission. These lambs were born from naïve dams that were either inoculated on Day 120 of gestation (Group 6) or dams that were re-inoculated (Group 7). Assuming the foetal immune system responded from the time of inoculation, these samples at 12 weeks of age would have been equivalent to 17 weeks post exposure. Although they generally fall within the 95% prediction interval, for the 17 week time point (Figure 3.2) there are some values that were slightly higher. Although pre-colostral blood was not collected, these lambs were kept as a mob together with control animals that did not seroconvert to *N. caninum* and these control animals were used to rule out the occurrence of post-natal infection that could have occurred on the farm. These control animals and their lambs remained seronegative throughout the study.

Comparison of S/P together with the avidity values can potentially enable differentiation between lambs born from recently inoculated (Group 6) and re-inoculated (Group 7) dams (Figure 3.3). At 2 weeks of age, a marked difference ($p<0.05$) was seen between these lambs in Group 6 and Group 7a. Lambs from Group 6 had low mean S/P (12%) and avidity (20%) values while Group 7a had high mean S/P (60%) and avidity values (80%). In contrast, at 12 weeks of age, there were no significant differences ($p>0.05$) in the mean S/P and avidity values between both groups. At 2 weeks of age, antibody responses would be a mixture of both maternal and those derived from the lambs themselves. The half-life of maternal IgG in lambs has been reported to vary from 14 to 25 days (Pearson and Brandon, 1976; Campbell et al., 1977; Whitelaw and Jordt, 1985; Watson, 1992). Thus by 12 weeks of age, the S/P and avidity values would overwhelmingly reflect the lamb's own responses. These values indicate these lambs had mounted their own humoral response. The seven lambs in Group 7b were positive by ELISA at 2 weeks of age with high avidity values but by 12 weeks of age their S/P values were negative indicating the

antibodies at 2 weeks of age were most likely of maternal origin and had waned to being undetectable with the ELISA by 12 weeks. Interestingly all 7 of these lambs were serologically positive by western blot at 12 weeks of age and may suggest that the western blot is sufficiently sensitive to detect low levels of the lambs own humoral response. As to whether this would also indicate the detection of low levels of maternal antibodies is unknown as no study on the duration of maternal antibodies of *N. caninum* in sheep has been reported. High levels of antibodies have been detected at 3-4 months of age in lambs congenitally infected with *T. gondii* (Dubey and Kirkbride, 1989) and these were considered to be due to an active infection as maternal antibodies will have disappeared by 3 months of age (Dubey, 2010). In this study, it shows that at 2 weeks of age, the avidity assay could be used as a tool to determine if seropositive lambs were born from dams that were recently infected with *N. caninum* or if they were born from chronically infected dams experiencing recrudescence. This study provides no information on lambs born from chronically infected ewes that were not re-inoculated.

In the current study, naturally infected animals had high avidity values indicating they were chronically infected when sampled. Interestingly, these ewes were the only individuals that tested seropositive for *N. caninum* from their particular mobs. The relevance of these infections could not be determined.

The results from the present study have shown this avidity assay was not only easy to use but was also able to discriminate between recent and chronic infection and could provide a useful method for determining the involvement of *N. caninum* in abortion cases on sheep farms. Although care and critical evaluation are required when examining samples from a single individual as compared to those in a mob, obtaining serial samples over time or from a group of animals would further improve the estimation of the exposure

time to the infection. This study has demonstrated that this avidity assay can be utilized to further understand the kinetics of this disease in sheep but it is essential to further evaluate its performance, especially in naturally infected sheep.

3.6 Acknowledgements

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3.7 Authors' contributions to this study

S. Syed-Hussain contributed to the study design, conducted the experiment, sample collections, laboratory work and wrote the manuscript.

W. Pomroy provided help with the study design, sample collection and editorial advice.

L. Howe provided help with the study design, advice on laboratory work and editorial assistance.

N. Williamson provided help with the study design and editorial advice.

D. West provided help with the study design, help with sample collection and editorial advice.

S. Stefan provided help with sample collection

DRC 16

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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: Syed-Hussain, Sharifah

Name/Title of Principal Supervisor: William Pomroy

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Three

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

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

  contributed to the experimental design and planning in collaboration with supervisors, carried out experimental work and statistical analysis, prepared the manuscript in collaboration with supervisors.

Syed-Hussain, Sharifah — 21/08/2014

Candidate's Signature — **Date**

William Pomroy — 21/08/2014

Principal Supervisor's signature — **Date**

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CHAPTER FOUR

Vertical transmission of *Neospora caninum* in experimentally infected sheep

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4.1 Abstract

Recent reports indicate *N. caninum* has a possible role in causing abortions in sheep in New Zealand. Knowledge about the mode of transmission of neosporosis in sheep in New Zealand is limited. This study aimed to determine the rate of vertical transmission that would occur in lambs born from experimentally inoculated ewes and to determine if previous inoculation would protect the lambs from *N. caninum* infection. A group of 50 ewes were divided into 2 groups with one group being inoculated with 5 X 10^6 *N. caninum* tachyzoites prior to pregnancy in Year 1. In Year 2, each of these groups were subdivided into 2 groups with one from each original group being inoculated with 1 X 10^7 *N. caninum* tachyzoites on Day 120 of gestation. Inoculation of *N. caninum* tachyzoites into ewes prior to mating resulted in no congenital transmission in lambs born in Year 1 but without further inoculation, 7 out of 11 lambs in Year 2 were positive for *N. caninum* infection. Ewes that were inoculated in both years resulted in all 12 lambs born in Year 2 being positive for *N. caninum* infection. This indicates that previous inoculation in Year 1 did not result in any vertical transmission in that year but did not provide any protection against vertical transmission in Year 2. These results suggest that vertical transmission occurs readily once a ewe is infected.

Keywords: *N. caninum*, vertical transmission, lambs, sheep

4.2 Introduction

Neospora caninum was first identified in 1984 and is now recognised as the leading cause of bovine abortion worldwide (Dubey and Schares, 2011; Thornton et al., 1991). It has been detected in wide range of animals including goats, sheep, deer and horses (Dubey, 2003). It was first described in sheep in 1990 (Dubey et al., 1990) and has been associated with congenital infection in lambs (Howe et al., 2012; Howe et al., 2008; Hassig et al., 2003; Kobayashi et al., 2001). Experimental studies have shown that sheep will seroconvert, and abort in a dose dependent manner and may have repeated abortions in subsequent pregnancies (Weston et al., 2009; Buxton et al., 2001; Jolley et al., 1999). Nevertheless, the role of *N. caninum* as a significant pathogen and abortifacient in sheep is still unclear and knowledge about its epidemiology, especially the mode of transmission of neosporosis in sheep, is limited (Helmick et al., 2002; Otter et al., 1997)

Congenital infection has been considered a major mode of transmission for *N. caninum* in cattle, resulting in foetal mortality or congenitally infected calves (Pare et al., 1996; Barr et al., 1993; Conrad et al., 1993a). Trees and Williams (2005) further defined transplacental infection (TPI) into two categories, endogenous and exogenous TPI. Endogenous TPI occurs when recrudescence of a pre-existing persistent maternal infection leads to foetal infection, while exogenous TPI occurs when a pregnant dam becomes infected during pregnancy. In sheep, experimental evidence to date indicates that exogenous TPI still results in congenital infections in subsequent pregnancies (Buxton et al., 2001; Jolley et al., 1999). Studies in cattle have suggested that when dams were experimentally infected with *N. caninum* tachyzoites before pregnancy, there was no infection of the foetuses in the following pregnancy (Innes et al., 2001b; Williams et al., 2000).

Recent reports suggest a role for naturally-acquired *N. caninum* infections as a cause of poor reproductive performance in sheep flocks in New Zealand (Howe et al., 2012; Howe et al., 2008; West et al., 2006). One experimental study found that using the local NcNZ isolates, sheep aborted in a dose dependent manner and at least 5000 tachyzoites were required to induce abortion when inoculated into ewes at Day 90 of gestation (Weston et al., 2009). An additional study has shown that transmission via semen is unlikely to occur, even when rams were inoculated with a large challenge (Syed-Hussain et al., 2013). Sheep, like cattle, can be infected by ingesting oocysts, however in the single report using the NC-2 isolate, the inoculation dose was high (10^4 oocysts) (O'Handley et al., 2002). Thus, the question remains whether vertical transmission plays an important role in the occurrence of the disease in sheep flocks in New Zealand.

This study was designed to investigate whether endogenous TPI using a local NcNZ isolate would lead to either abortion or vertical transmission or would protect the foetus following a challenge during the subsequent pregnancy. The two primary aims of this study were to determine the rate of vertical transmission that would occur in lambs born from experimentally inoculated ewes and to determine if previous inoculation would protect the lambs from *N. caninum* infection. The findings from this research will help to better understand the epidemiology of *N. caninum* among sheep flocks in New Zealand and worldwide.

4.3 Materials and methods

4.3.1 Experimental design

This was a controlled challenge experiment which consisted of two parts. The first aimed to determine if ewes inoculated with live *N. caninum* tachyzoites two months prior to mating were able to transmit the infection to their offspring born in that year (Year 1) and also in a subsequent pregnancy in Year 2. The second was to determine if ewes challenged by re-inoculation on Day 120 of pregnancy in Year 2 gave birth to congenitally infected lambs.

4.3.2 Animals

Fifty ewes, aged approximately 4 years old, were selected from a farm with no known history of reproductive problems. The ewes were a cross between Borderdale, Romney and Suffolk breeds. These ewes had successfully reared lambs the previous year. All ewes had been vaccinated for *Toxoplasma gondii* and *Campylobacter fetus fetus* and were free from *N. caninum* as indicated by negative serology using ELISA when sampled prior to removing them from their home farm.

For the first part of the trial in Year 1, the ewes were randomly divided into 2 groups with 25 ewes per group (Figure 4.1). Group A was inoculated with 5 X 10^6 *N. caninum* tachyzoites intravenously (i/v) and Group B (control) was left uninoculated. Inoculated ewes grazed in one mob in a paddock separated from the uninoculated control ewes in an adjacent paddock. Blood samples were collected prior to inoculation and one month post inoculation to determine serum antibody levels.

Two months after inoculation, ewes in Group A and B were combined and kept as one mob and two rams (seronegative for *N. caninum*) were introduced. These ewes were

then mated over 40 days (> 2 oestrous cycles). Ewes were ultrasound scanned on 3 occasions to determine their pregnancy status. Prior to lambing, Groups A and B were separated into different paddocks.

Lambs were tagged as soon after birth as possible and data on dam identification, lamb birth weight and sex were recorded. After lambing was completed, Groups A and B were again kept in the same paddock until the lambs had reached 8 weeks of age where they were bled and euthanized. For all lambs, brain and heart tissues were collected and portions kept at -20°C as well as in 10% formalin.

For the second part of the study in Year 2, fifty ewes from the Year 1 were mated over 40 days (> 2 oestrus cycle) with two rams which were seronegative for *N. caninum*. Animals were ultrasound scanned for pregnancy on 2 occasions to confirm pregnancy status.

On Day 120 of gestation, ewes that were pregnant and considered as in good body condition were chosen for the study in Year 2 with the remainder being removed from the study. Sixteen previously inoculated ewes from Group A were divided into 2 groups, Group C (n=9) were re-inoculated with 1 X 10^7 live *N. caninum* tachyzoites on Day 120 gestation while ewes in Group D (n=7) were not re-inoculated (Figure 4.1). Seventeen previously uninoculated ewes from Group B were also divided into 2 groups: Group E (n=12) were inoculated as for Group C whilst Group F (n=5) remained uninoculated and were used as control animals. Animals in Group D and F were kept in one paddock with Group C and E in a different paddock until lambing ended.

In Year 2, blood samples were collected from ewes before inoculation and 4 weeks post inoculation. Lambs were tagged as soon after birth as possible. In both Year 1 and 2,

ewes lambed outside in paddocks, thus obtaining pre-colostral blood from all new-born lambs was not feasible. Blood samples were collected from lambs at 12 weeks of age and lambs in Group C and E were also sampled at 2 weeks of age. All animal usage was approved by the Massey University Animal Ethics Committee.

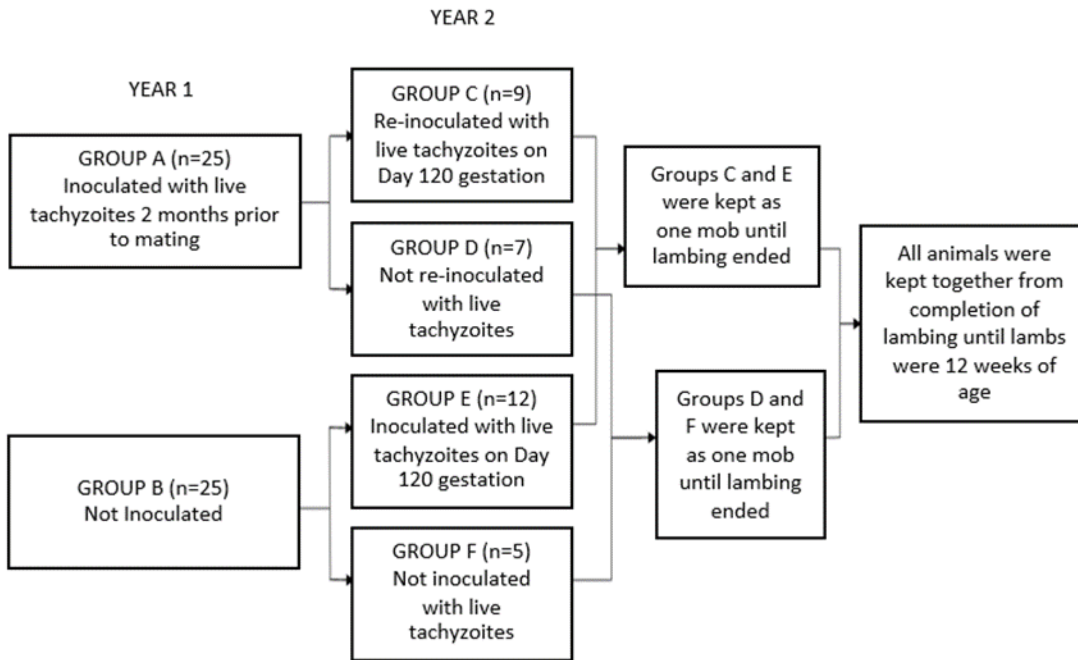

Figure 4.1: Flow diagram on the grouping of ewes in Year 1 and 2 of the study

4.3.3 Inoculums

The inoculums used were from the New Zealand *N. caninum* isolate NcNZ1. These were prepared and given as previously described (Syed-Hussain et al., 2013).

4.3.4 ELISA

All sera collected were tested as previously described (Syed-Hussain et al., 2013) using a commercial ELISA test kit (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories, Australia). An S/P ratio of ≥11.8% was regarded as positive as described by Reichel et al (2008).

4.3.5 Western blot

All sera from ewes were tested one month post inoculation to confirm their infectivity status while all sera from lambs in Year 1 and 2 respectively were also analysed using western blot as described previously (Syed-Hussain et al., 2013). For this study, sera that recognized at least one IDA with molecular weight (MW) of 17, 25-29, 30-33 or 37 kDa were considered positive for *N. caninum.*

4.3.6 Detection and quantification of *N. caninum* DNA in samples

4.3.6.1 DNA extraction

Five grams of each brain and heart sample previously stored at -20°C were homogenized using a sterile, single use, 50 ml conical tissue grinder (VWR, PA, USA) containing 10 ml of sterile PBS. DNA from these homogenized tissues was extracted using DNeasy Tissue Kit (Qiagen, Victoria, Australia) as previously described (Syed-Hussain et al., 2013). All extracted DNA samples were kept at -20°C until further analysis was performed.

4.3.6.2 Standard Polymerase Chain Reaction (PCR)

In Year 1, brain tissue samples from lambs euthanized at 8 weeks of age were tested using a standard PCR for the detection of *N. caninum* DNA. Single tube nested *N. caninum* PCR reactions were performed as described by Ellis et al. (1999) to amplify the ITS1 region using the internal (NR1 and NS2) and external (NF1 and SR1) primer pairs as described by Weston et al (2009). PCR products were run on a 1.5% (w/v) ultra-pure agarose gel (Invitrogen, CA, USA) containing ethidium bromide at 100 V for 45 minutes and visualized under UV light on a transilluminator. Water blanks were included as sample processing controls to confirm a lack of contamination during testing. Purified DNA from *N. caninum* NcNZ1 tachyzoites was used as a positive control. Selected positive PCR amplicon samples were purified (PureLink PCR purification kit, Invitrogen, California, USA) and subjected to automatic dye-terminator cycle sequencing with BigDyeTM Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems Inc., CA, USA). Sequencing results were submitted to NCBI BLAST to confirm *N. caninum* DNA amplification.

4.3.6.3 Real-time Polymerase Chain Reaction (qPCR)

In Year 2 of the study, all brain and heart tissue samples of lambs were subjected to qPCR for the detection of *N. caninum* DNA and only those that were positive were later quantified. The qPCR was designed to amplify the Nc-5 gene (Kaufmann et al., 1996) of *N. caninum* using the primers NeoF (5'-GTGAGAGGTGGGATACG-3') and NeoR (5'-GTCCGCTTGCTCCCTA-3') (Okeoma et al., 2005). The conditions for the reactions were optimized for the primers and products obtained were analysed with a melting curve analysis. Products amplified from the initial optimization run using *N. caninum* DNA from a

pure cell culture were purified, sequenced, and subjected to BLAST to confirm its identity and specificity.

The 10 µL qPCR reaction mixture contained: 2 µL of test sample containing 1ng DNA/ul, 2X Perfecta® SYBR® Green FastMix (Quanta Bioscience, Ca, USA) ready to use mix and 0.2µM of each primer. The thermal cycler conditions were as follows: 95°C for 10 minutes; 45 cycles of 95°C for 10 seconds (s), and 60°C for 30s. A melt curve analysis was visualized over the 80 - 90°C temperature range on the Illumina Eco™ Real-Time PCR software 3.1 (Illumina Inc, Ca, USA). DNA extracted from cell-cultured *N. caninum* was used as a positive control and was included with a negative control containing no DNA for each qPCR run. Each sample was prepared in triplicate.

A standard curve was generated in triplicates for each run using a 10-fold dilution series of purified DNA from cell-culture adapted *N. caninum* with five known concentrations (1, 10, 100, 1000, 10000 tachyzoites). The efficiency and standard curve of the qPCR cycle were calculated using the Illumina Eco™ Real-Time PCR software 3.1. The standard curve was used to estimate the number of tachyzoites of *N. caninum* per mg of tissue.

4.3.7 Brain histological examination

Histological examination of the lambs' brains was conducted for the detection of protozoal-associated lesions. Sections were examined from the frontal lobe, temporal lobe and the cerebellum. Tissues were prepared routinely and stained with Haematoxylin and Eosin (H&E). Each section was scored by the same observer. Sections were assessed for the presence or absence of lesions. When present, lesions of encephalitis were multifocal to coalescing and randomly distributed with no predilection for any anatomical structure. A total score for each slide was arrived at by counting the number of these lesions; coalescing

foci were treated as separate lesions if they overlapped by less than 33%. The severity of meningitis was graded from 1 to 4 (1=<5% of meninges affected; 2=5-25%; 3=25-50%; 4=50-100%) and this number was added to the total score for that section.

4.3.8 Statistical analysis

One way ANOVA were performed to compare the antibody responses in animals in Groups A to E between ewes at pre-inoculation and 1 month post-inoculation as well as lambs in Year 1 and 2. S/P values from ELISA tests for all ewes and lambs' were tested for normality and all were found to be normally distributed; one-way multiple comparison using Fisher's method was performed.

Brain lesion score values of lambs were not normally distributed and were analysed with the Mann-Whitney U test. A Chi-square method was conducted to determine if there was any association between the dams' previous inoculation statuses and the presence of brain lesions in the lambs. All statistical analyses were performed using Minitab (Minitab Inc., PA, USA) and all statistical analyses were considered significant when $p<0.05$.

4.4 Results

4.4.1 Controlled challenge study Year 1

4.4.1.1 Ewe serology

The serum IgG responses to *N. caninum* in ewes in Group A and B at pre and 4 weeks post inoculation with *N. caninum* tachyzoites are shown in Figure 4.2. The S/P values for all animals in Group A (inoculated) were seropositive and ranged from 15% to 48% while the ewes in Group B (control) remained negative for *N. caninum* antibodies. Mean S/P values were significantly higher in Group A than in the uninoculated controls in Group B ($p<0.05$).

4.4.1.2 Ewe pregnancy outcome

In Year 1, all 25 ewes in Group A gave birth to 44 live lambs. Two lambs died within 3 days of birth while the others appeared healthy. No abortion was detected in any of the inoculated animals. In Group B, 25 ewes gave birth to 34 live and apparently healthy lambs while 2 additional lambs were born dead. No signs of neurological disease were observed in any lambs prior to euthanasia.

4.4.1.3 Evidence of vertical transmission in lambs

In Year 1, no evidence of *N. caninum* infection at 8 weeks of age was detected in any of the lambs born from Group A. Although no pre-colostral serum samples were taken when the lambs were born, blood taken from lambs when euthanized at 8 weeks of age were all ELISA seronegative with S/P values ranging from -4% to 1.3% (Figure 4.3). These lambs were also seronegative when tested with western blot. No *N. caninum* DNA was detected using the standard PCR in any of the brain tissue of lambs that were born from ewes in Group A and there was no evidence of histological changes consistent with *N. caninum* infection in any of the brain sections from these lambs. In addition, no evidence of *N. caninum* infection was detected in lambs that died at or soon after birth. There was also no evidence of *N. caninum* infection in Group B by ELISA (Figure 4.3), histology or PCR.

4.4.2 Controlled challenge study Year 2

4.4.2.1 Ewe ELISA serology

The serum IgG responses to *N. caninum* in ewes in Group C, D, E and F sampled pre- and 4 weeks post-inoculation with *N. caninum* tachyzoites in Year 2 are shown in Figure 4.2. Prior to the second inoculation in Year 2, antibody responses in Group C (re-inoculated) and

D (not re-inoculated) were similar to each other with 8/9 and 6/7 ewes respectively being seropositive. One month after re-inoculation, the antibody titres of 9/9 ewes in Group C were significantly higher (S/P ratio 15-68%) than in Group D (4/7 seropositive, S/P ratio range 8-27%) ($p<0.05$). In addition, one month after inoculation, 10/12 ewes in Group E (inoculated only in Year 2) seroconverted (S/P ratio range 4-26%) and had similar antibody titres to those in Group D, such that the two groups were not significantly different from each other ($p>0.05$). All ewes (5/5) in the uninoculated control Group F remained negative for *N. caninum* antibodies by ELISA (Figure 4.2).

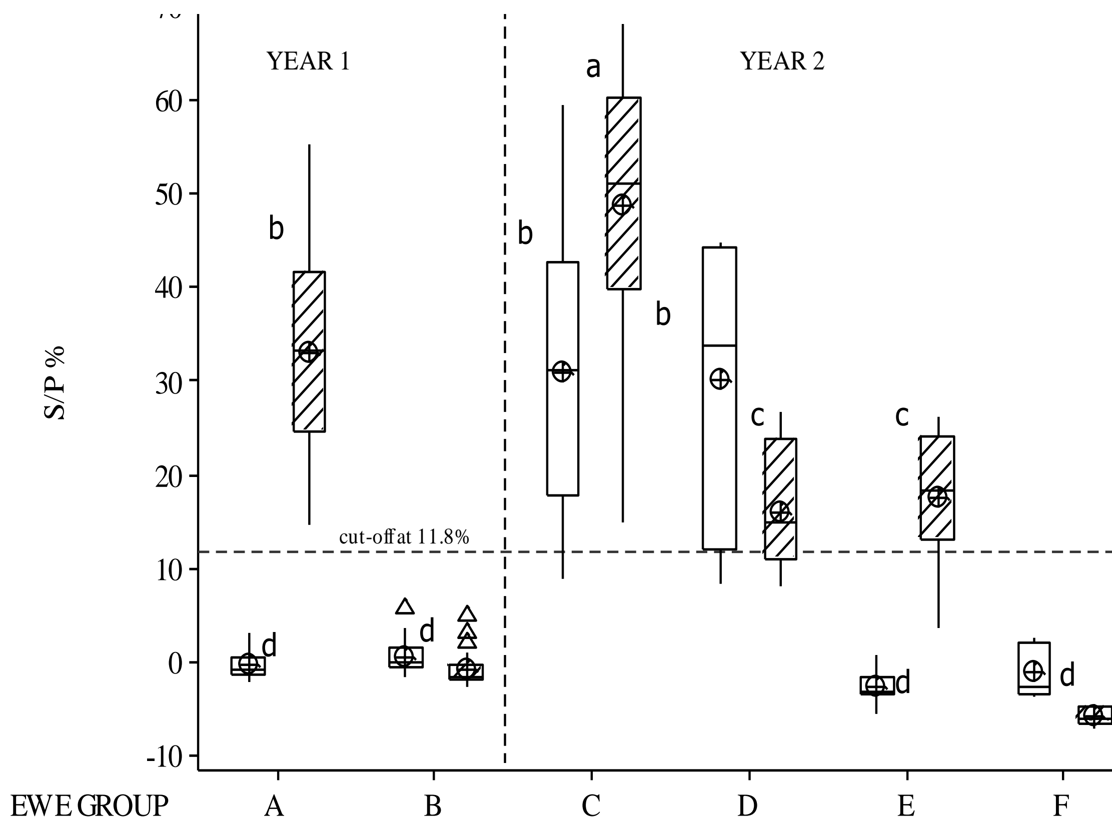

Figure 4.2: Box and whisker plot of S/P values for ewes before (□) and 4 weeks post inoculation (▨) with live *N. caninum* tachyzoites in Year 1 and 2. Group A (n=25) were experimentally inoculated ewes 2 months prior to mating; Group B (n=25) were uninoculated ewes; Group C (n=9) ewes from Group A re-inoculated in Year 2; Group D (n=7) ewes from Group A not re-inoculated in Year 2; Group E (n=12) ewes from Group B inoculated in Year 2 and Group F (n=5) un-inoculated control animals in Year 2. The mean value (Θ) and outliers (Δ) are as indicated. Means that do not share a similar letter are significantly different (p<0.05).

4.4.2.2 Ewe western blot result

As expected, none of the ewes in Group F (control) showed antibody reactivity to the tachyzoite antigens by western blot. However, antibodies to at least 2 IDAs were detected in all 28 ewe sera from Groups C, D, and E against a consistent range of known *N. caninum* tachyzoite IDAs (10, 17, 25-29, 30, 31, 33 and 37 kDa) one month post inoculation

4.4.2.3 Evidence of vertical transmission of *N. caninum* in lambs in Year 2

4.4.2.3.1 Pregnancy outcome of ewes in Year 2

Table 4.1 shows the details of *N. caninum* detection by ELISA, western blot, PCR or histology in neonatal lambs (n=11) that were stillborn or died within 1 week of birth as well as viable lambs (n=40) that were euthanased at 12 weeks of age. No ewes from any Year 2 group aborted.

4.4.2.3.2 Detection of *N. caninum* in non-viable neonates

In Year 2, the one neonate that died in Group C, 5 days after birth, was seropositive for *N. caninum* by western blot. Histological examination revealed presence of lesions consistent with *N. caninum* infection in the brain (Table 4.1). However, no *N. caninum* DNA was detected by qPCR. No evidence of *N. caninum* infection was detected serologically, histologically or with qPCR in the two neonates which died in Group D while in Group E, 5/7 neonate brain samples were positive for *N. caninum* DNA by qPCR and had lesions consistent with *N. caninum* infection in their brains (Table 4.1). None of these dead neonates from Group E were sero-positive by ELISA, but 4/7 were serologically positive by western blot (Table 4.1). Finally, no evidence of *N. caninum* infection was detected serologically, histologically or with qPCR in the one dead neonate in Group F (Table 4.1).

Table 4.1: Positive detection of *N. caninum* using ELISA, western blot, PCR and histological lesions consistent with *N. caninum* in neonates (died within 1 week of birth) and lambs at 12 weeks of age in Year 2 originating from ewes that were inoculated at different time points

	Groups‡	ELISA	Western Blot	qPCR	Histology		Total Infected Ŧ
					Number of lambs with lesions	Score range of positive animals	
Neonates that died within 1 week of birth	C	0/1	1/1	0/1	1/1	1	1/1
	D	0/2	0/2	0/2	0/2	-	0/2
	E	0/7	4/7	5/7	5/7	5-94	5/7
	F	0/1	0/1	0/1	0/1	-	0/1
TOTAL		0/11	5/11	5/11	6/11		6/11
Lambs at 12 weeks of age	C	5/12	12/12	0/12	5/12	3-43	12/12
	D	0/11	5/11	0/11	2/11	1-6	7/11
	E	9/10	10/10	2/10	9/10	1-30	10/10
	F	0/7	0/7	0/7	0/7	-	0/7
TOTAL		14/40	27/40	2/40	16/40		29/40

‡ neonates/lambs born from: Group C, ewes inoculated in Year 1 and re-inoculated in Year 2; Group D, ewes inoculated in Year 1 only; Group E, ewes only inoculated in Year 2 and Group F, uninoculated ewes used as control.

Ŧ animals are considered positive whenever detected positive by either ELISA, western blot, qPCR or by histological lesion.

4.4.2.3.3 ELISA results in viable lambs

The ELISA responses for *N. caninum* in lambs from all groups at 2 and 12 weeks of age in Year 2 are shown in Table 4.1 and Figure 4.3. All (12/12) lambs born to ewes in Group C were seropositive by ELISA at 2 weeks of age, however at 12 weeks of age only 5/12 (42%) were seropositive by ELISA (Table 4.1). Of the lambs born to ewes from Group E, only 4/10 were seropositive at 2 weeks, but this number increased to 9/10 (90%) by Week 12. No data are available for lambs in Groups D and F at 2 weeks of age. At 12 weeks of age, all lambs in Group D (n=11) and F (n=10) were seronegative and were not significantly different to each other ($p>0.05$). Antibody responses for lambs at 2 weeks of age in Group C were significantly higher (mean S/P value = 50%, $p<0.05$) than those in Group E (mean S/P values = 10%) (Figure 4.3). However, at 12 weeks of age, antibody responses for lambs in Group E were significantly higher ($p<0.05$) than in other lambs in Groups C, D and F with mean S/P values of 30%, 15%, -8% and -9% respectively (Figure 4.3). Antibody responses in Group C were also significantly higher ($p<0.05$) at 12 weeks when compared to those in Group D and F (Figure 4.3)

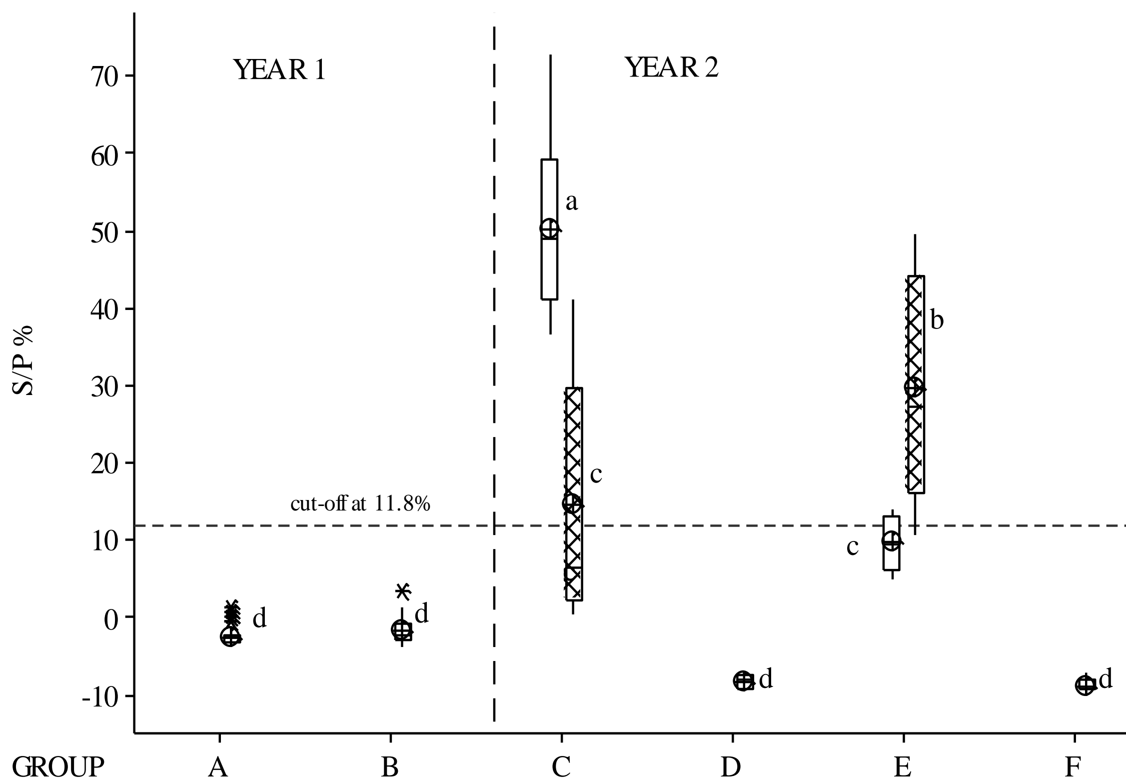

Figure 4.3: Box and whisker plot of S/P values of lambs in Year 1 and 2. In Year 1, values are shown for lambs at 8 weeks of age; Group A (n=44; born from ewes inoculated prior to mating) and Group B (n=34; born from uninoculated ewes). In Year 2, values are shown for lambs at 2 (☐) and 12 weeks of age (⊠) born from dams from Group C (n=12; born from ewes re-inoculated in Year 2), D (n=11; born from ewes not re-inoculated in Year 2), E (n=10; born from ewes newly inoculated in Year 2) and F (n=7; born from ewes uninoculated). No samples were obtained for Groups D and F at 2 weeks of age. The mean value (Θ) is as indicated. Means that do not share a similar letter are significantly different ($p<0.05$).

4.4.2.3.4 Western blot results for viable lambs

At 12 weeks of age, all lambs in Groups C (12/12) and E (10/10) plus 5/11 in Group D were positive by western blot including those that were seronegative by ELISA (Table 4.2). None of the seven lambs in Group F (control) were seropositive by western blot. Table 2 shows the summary of IDAs recognised by lambs' sera at 12 weeks of age. The protein at 10kDa was only recognised by 4/11 (40%) lambs sera in Group D while the 17kDa was recognized by 82% (27/33) of the sera from lambs born from infected ewes in Groups C, D and E. Proteins at 25-29 kDa were also recognised by lambs' sera in Group C (25%) and D (27%) but mostly by lambs' sera from Group E (70%). The 30 kDa proteins was recognised by 50% of lambs' sera in Groups C and 60% of lambs in Group E while proteins at 31 and 33 kDa were only recognised by one lamb sera in Group E. None of the lambs' sera from Group C, D or E recognised protein at 37 kDa.

Table 4.2: Proportion of IDAs recognised in western blot by lambs' sera at 12 weeks of age.

Immunodominant Antigen	‡			
kDa	Group C	Group D	Group E	Group F
10	0/12	4/11	0/10	0/7
17	12/12	5/11	10/10	0/7
25-29	3/12	3/11	7/10	0/7
30	6/12	0/11	6/10	0/7
31	0/12	0/11	1/10	0/7
33	0/12	0/11	1/10	0/7
37	0/12	0/11	0/10	0/7

‡ lambs born from: Group C, ewes inoculated in Year 1 and re-inoculated in Year 2; Group D, ewes inoculated in Year 1 only; Group E, ewes only inoculated in Year 2 and Group F, uninoculated ewes used as control.

4.4.2.3.5 qPCR results for viable lambs

At 12 weeks of age, *N. caninum* DNA was detected only in the brain samples of 2 lambs in Group E. Both positive samples were then quantified using qPCR which indicated less than 1 tachyzoite/mg of tissue was present.

4.4.2.3.6 Brain histopathology analysis for viable lambs

Table 4.1 shows the number of lambs positive for brain lesions consistent with *N. caninum* infection and the scores for all groups of lambs at 12 weeks of age. Lesions consisted of multifocal to coalescing, randomly arranged and variably sized area of gliosis, necrosis, non-suppurative perivascular cuffing and non-suppurative meningeal inflammatory infiltration.

A Chi-square test produced a p-value of 0.03, indicating evidence to suggest that the ewe's previous inoculation status (Group C and D; previously inoculated, Group E; newly inoculated) had an effect on the presence or observations of brain lesions seen in lambs. In Group E, most of the lambs were positive for brain lesions (9/10) while less than half (5/12) were positive in Group C and only 2/11 in Group D. No lesions were seen in any lambs in Group F.

The proportion of lambs with lesions was significantly higher ($p<0.05$) in Group E than in Groups C and D. However, whilst there was a significant difference ($p<0.05$) in lesion scores between Groups D and E, there was no difference between lesion scores between Groups C or D and C or E. This indicates that previous inoculation status of dams did not have an effect on reducing the severity of lesions seen in lambs.

4.5 Discussion

This study demonstrated that although inoculating ewes prior to pregnancy may not lead to any endogenous TPI in lambs born in that year, it did not provide sufficient protection to the foetus in the subsequent pregnancy to prevent exogenous TPI from occurring.

In the present study, ewes inoculated 2 months prior to mating did not produce any congenitally infected lambs in Year 1 but there was evidence that some lambs born in the subsequent year were congenitally infected indicating vertical transmission had occurred. There are inconsistencies between results from previous cattle and sheep studies as to whether such vertical transmission does occur following inoculation prior to pregnancy. In three cattle studies (Williams et al., 2007; Innes et al., 2001b; Williams et al., 2000), where the dams were inoculated with live tachyzoites prior to mating, there was no evidence of vertical transfer to the calves using serology, immunohistochemistry and PCR for the detection of *N. caninum* infection. In contrast, low levels of abortion or detection of *N. caninum* have been reported in 3 other studies where cows were inoculated prior to pregnancy (Weston, 2011; Hecker et al., 2013b; Weber et al., 2013). Thus, protection against vertical transmission in cattle following inoculation prior to pregnancy is not absolute. It should be noted that none of these cattle studies followed the dams through a further pregnancy the following year.

In sheep, two previous studies have shown that inoculation prior to pregnancy did not protect against vertical transmission in subsequent pregnancies (Buxton et al., 2001; Jolley et al., 1999) and this transmission rate was generally higher than in reports of similar studies in cattle. Buxton et al. (2001) reported the rate of vertical transmission was 10-12% in ewes that were inoculated prior to mating and from ewes that were chronically infected

whilst Jolley et al (1999) found 75% mortality rate in lambs born from ewes only inoculated in the previous year and 70% mortality rate (3/5 aborted) in those that were also re-inoculated in the second year.

In the current study, results in Year 1 were similar to most cattle studies where lambs born from ewes inoculated prior to mating were not infected. However, results in Year 2 were more consistent with earlier sheep studies where some lambs from ewes inoculated in Year 1 did show evidence of *N. caninum* infection although no abortions were recorded. All lambs in Group C (re-inoculated in Year 2) and more than half in Group D (not re-inoculated in Year 2) were serologically positive and/or had evidence of histological lesions in the brain at 12 weeks consistent with *N. caninum* infection. This suggests that the immune response triggered by the previous inoculation in the dams was insufficient to protect against vertical transmission in lambs born in the subsequent year. There were 5 out of 7 lambs in Group C that were seronegative by ELISA and histology. As to whether this would indicate some level of protection due to the dams previous inoculation status is still unknown as all the lambs were positive by western blot. Nevertheless, factors such as parasite strains, dose of inoculation used, pregnancy effect on maternal immunity, host susceptibility, foetal stage and time of inoculation play a role in determining the outcomes of pregnancy (Weston et al., 2009; Williams et al., 2000; Buxton et al., 1997; Conrad et al., 1993a; Innes et al., 2005).

Lambs in the current study were sampled at 12 weeks of age which increases the possibilities of horizontal transmission. Pre-colostral blood was not collected. These lambs were kept as a mob together with control animals that did not seroconvert to *N. caninum* and these control animals were used to rule out the occurrence of post-natal infection that

could have occurred on the farm. These control animals and their lambs remained seronegative throughout the study.

The S/P values of Group C (re-inoculated ewes) were going from high at Week 2 to lower values at Week 12 whilst those in Group E (recently inoculated) showed a reverse trend. At 2 weeks of age, antibody responses would be a mixture of both maternal and those derived from the lambs themselves. The half-life of maternal IgG in lambs has been reported to vary from 14 to 25 days (Watson, 1992; Whitelaw and Jordt, 1985; Campbell et al., 1977; Pearson and Brandon, 1976). Since the dams of lambs in Group C had been infected previously, the second inoculation would act as a booster to the humoral response, thus increasing the level of antibodies present and explaining the high values at 2 weeks of age. By 12 weeks of age, the S/P values would overwhelmingly reflect the lamb's own responses. As to whether this would indicate that lambs in Group E had more exposure to the parasite is unknown.

For lambs in Group D (not re-inoculated), at 12 weeks of age, all had seronegative S/P values, however 5 of these lambs were positive by western blot. This suggests that the western blot is sufficiently sensitive to detect low levels of the lambs own humoral response. ELISA is known as a quantitative test while western blot is a qualitative mode of diagnostic test. It is unknown whether this would also indicate the detection of low levels of maternal antibodies, as no study on the duration of maternal antibodies of *N. caninum* in sheep has been reported, although maternal antibodies have been suggested to disappear by 3 months of age in lambs (Dubey, 2010). No attempt was made to detect the presence of cysts or DNA in the inoculated ewes. Nevertheless, their antibody titres remained high throughout the study which is suggestive of chronic infection (Groups C and D).

In conclusion, the findings of this study were similar to those of other sheep studies, indicating vertical transmission occurs readily if infected during pregnancy and could occur even if infected prior to pregnancy. The inoculation dose used in the current study was high, which could have influenced the number of cysts that developed and may have influenced the occurrence of congenital transmission. How closely this resembles a natural infection has yet to be determined.

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4.7 Authors' contributions to this study

S. Syed-Hussain contributed to the study design, conducted the experiment, sample collections, laboratory work and wrote the manuscript.

W. Pomroy provided help with the study design, sample collection and editorial advice.

L. Howe provided help with the study design, advice on laboratory work and editorial assistance.

N. Williamson provided help with the study design and editorial advice.

D. West provided help with the study design and editorial advice.

M. Hardcastle provided help with scoring of histological lesions

DRC 16

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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: Syed-Hussain, Sharifah

Name/Title of Principal Supervisor: William Pomroy

Name of Published Research Output and full reference:

Syed-Hussain, S.S., Howe, L., Pomroy, W.E., West, D.M., Hardcastle, M., Williamson, N.B., 2014. Vertical transmission of Neospora caninum in experimentally infected sheep. Vet. Parasitol. XXX, XXX-XXX.

In which Chapter is the Published Work: Four

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 80%

  and / or

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

  contributed to the experimental design and planning in collaboration with supervisors, carried out experimental work and statistical analysis, prepared the manuscript in collaboration with supervisors.

Syed-Hussain, Sharifah	21/08/2014
Candidate's Signature	Date

William Pomroy	21/08/2014
Principal Supervisor's signature	Date

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CHAPTER FIVE

Study on the use of toltrazuril to eliminate *Neospora caninum* in congenitally infected lambs born from experimentally infected ewes

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5.1 Abstract

No chemotherapeutic approach has been shown to be safe and effective for the treatment of neosporosis in cattle or sheep. This study aimed to determine if toltrazuril was effective in eliminating *Neospora caninum* infection from congenitally infected lambs. Twenty-eight ewes were allocated to 3 groups where animals in Groups A and B were inoculated with 1 X 10^7 *N. caninum* tachyzoites on Day 120 of gestation and Group C was maintained as a negative control group. Lambs born from ewes in Group A were treated with toltrazuril (20 mg/kg) on Day 0, 7, 14 and 21 after birth. Groups B and C were untreated. All lambs in Groups A and B were seropositive at 12 weeks of age. At 12 weeks of age, no differences between lambs in Group A and Group B were observed in serological results (ELISA and western blot), presence of *N. caninum*-related brain histological lesions or the number of organisms detected by qPCR. Group C remained negative for serology, detection of *N. caninum* DNA as well as histopathology throughout the study. Results indicate that *N. caninum* congenitally-infected lambs had a continuing infection with *N. caninum* despite being treated with toltrazuril.

Keywords: *N. caninum*, toltrazuril, congenital transmission, lambs, sheep

5.2 Introduction

Neospora caninum is an important cause of abortion in cattle worldwide (Dubey et al., 2007) with an estimated national cost of USD$36 million/year in the dairy industry in New Zealand alone (Reichel et al., 2013). *N. caninum* infections have also been reported in a wide range of animals including horses, goats, deer, and sheep (Dubey and Schares, 2011). Where examined, the pattern of *N. caninum* infection in sheep parallels that which occurs in cattle although it has generally not been regarded as a significant cause of abortion in sheep (Helmick et al., 2002; Buxton et al., 1997; Otter et al., 1997; Dubey et al., 1990). Reports show that experimentally infected sheep seroconvert and abort in a dose-dependent manner (Weston et al., 2009; Buxton et al., 2001; Buxton et al., 1997). Sheep could act as a useful and cost effective model for the disease in cattle due to the similarities in infection in between them.

Chemotherapy has the potential to be an effective control measure for neosporosis (Hasler et al., 2006b) provided the drug used is effective. Various chemotherapeutically active substances including piritrexim, lasalocid, monensin, erythromycin, doxycycline, clindamycin, decoquinate, artemisinin and depudecin have been investigated for their potential use against *N. caninum* with both *in vitro* (Barna et al., 2013; Muller et al., 2008; Leepin et al., 2008; Esposito et al., 2005; Darius et al., 2004b; Kwon et al., 2003; Kim et al., 2002; Lindsay et al., 1997; Lindsay et al., 1996b; Lindsay et al., 1994) and *in vivo* testing, the latter principally using murine models and generally against the tachyzoite stage (Debache and Hemphill, 2012; Kropf et al., 2012; Debache et al., 2011; Strohbusch et al., 2009; Gottstein et al., 2005; Ammann et al., 2004; Gottstein et al., 2001; Lindsay and Dubey, 1990; Schorer et al., 2012). Some of these reported results were promising in reducing the

rate of infection but they did not give any indication of efficacy against the bradyzoite stage.

Toltrazuril, a symmetrical triazinone, is metabolized into ponazuril (toltrazuril sulfone) and although the specific mode of action of these two drugs is unknown, it was proposed that it affects the mitochondrial respiratory chain and the biosynthesis of pyrimidine in the parasite (Muller and Hemphill, 2011; Harder and Haberkorn, 1989).

Toltrazuril and ponazuril are effective against a broad spectrum of cyst-forming and non cyst-forming apicomplexan protozoa (Haberkorn, 1996; Mehlhorn et al., 1988). They are reported to be effective against all intestinal stages of *Eimeria* spp., *Isospora* spp., *T. gondii* and *N. caninum* (Haberkorn et al., 2001) and have been used to treat and prevent coccidiosis in poultry (Reynaud et al., 1999) and livestock such as in piglets (Mundt et al., 2003; Driesen et al., 1995), lambs (Mundt et al., 2009; Gjerde and Helle, 1986) and calves (Bohrmann, 1991) .

Both drugs have shown positive effects against *N. caninum in vitro* (Strohbusch et al., 2008; Darius et al., 2004b) and *in vivo*, both in murine studies (Strohbusch et al., 2009; Gottstein et al., 2005; Ammann et al., 2004; Darius et al., 2004a; Gottstein et al., 2001), and in experimentally and naturally infected calves (Haerdi et al., 2006; Kritzner et al., 2002). However, in order for toltrazuril to reach its full effectiveness in clearing *N. caninum* infection, one study has shown that a supportive T cell-immune response is necessary (Ammann et al., 2004). Regardless, no chemotherapeutic approach has been shown to be safe and fully effective for the treatment of neosporosis in cattle (Dubey and Schares, 2011). One approach has been to treat neonates to eliminate the organism following congenital infection. The aim of this present study was to determine if toltrazuril was

effective in eliminating *N. caninum* infection from congenitally infected lambs. If treating congenitally infected neonates proved to be an effective strategy, it would provide a cost effective means to control neosporosis.

5.3 Materials and methods

5.3.1 Experimental design and animals

This was a controlled challenge experiment involving 28 ewes restrictively randomized into three groups as shown in Figure 5.1. Group A (n=11) and Group B (n=12) were inoculated on Day 120 of gestation. Group C (n=5) was the un-inoculated control group. This study was run in conjunction with a previously reported study on vertical transfer in sheep (Syed-Hussain et al manuscript submitted concurrently with this one) and Group C was common to both studies. Lambs from ewes in Group A (n=11) were treated with toltrazuril (Baycox 5%, Bayer Healthcare, New Zealand) 20mg/kg orally on Days 1, 7, 14 and 21 after birth. Lambs in other groups were not treated. All animal usage was approved by the Massey University Animal Ethics Committee.

The ewes were approximately four years old and were selected from a farm with no known history of reproductive problems and had successfully reared lambs the previous year. They were a crossbred between Borderdale, Romney and Suffolk breeds. All had been vaccinated for *Toxoplasma gondii* and *Campylobacter fetus fetus* and were free from *N. caninum* as indicated by negative serology using ELISA (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories, Australia) when sampled prior to removing them from their home farm. These ewes were the control group from Year 1 in the previous study on vertical transfer (Syed-Hussain et al manuscript submitted concurrently with this one).

Ewes were mated over 40 days (> 2 oestrus cycles) with two rams which were seronegative for *N. caninum*. Animals were ultrasound scanned for pregnancy on two occasions (Day 40 and 100 of gestation) to confirm their pregnancy status. Twenty three ewes were inoculated intravenously with 1 x 10^7 *N. caninum* (NcNZ1) tachyzoites at Day 120 of gestation and five ewes were left untreated. The inoculums used were prepared as described in a previous study (Syed-Hussain et al., 2013). The inoculated ewes were further divided into two groups, Group A (n=11) and B (n=12) while the five un-inoculated animals (Group C) were used as control animals (Figure 5.1). Animals in Groups A and B were kept in one paddock while Group C animals were kept in a different paddock until lambing ended.

Blood samples were collected from ewes before inoculation and 4 weeks post-inoculation. Lambs born to ewes in Group A were weighed and treated with toltrazuril on Day 1, as soon after birth as possible and then as indicated above. In this study, ewes lambed outside in paddocks, thus obtaining pre-colostral blood from all new-born lambs was not feasible. Blood samples were taken from lambs at 12 weeks of age and in Groups A and B also at 2 weeks of age. All lambs were euthanased at 12 weeks of age and brain and heart tissues were collected, divided for storage at -20°C and fixed in 10% formalin.

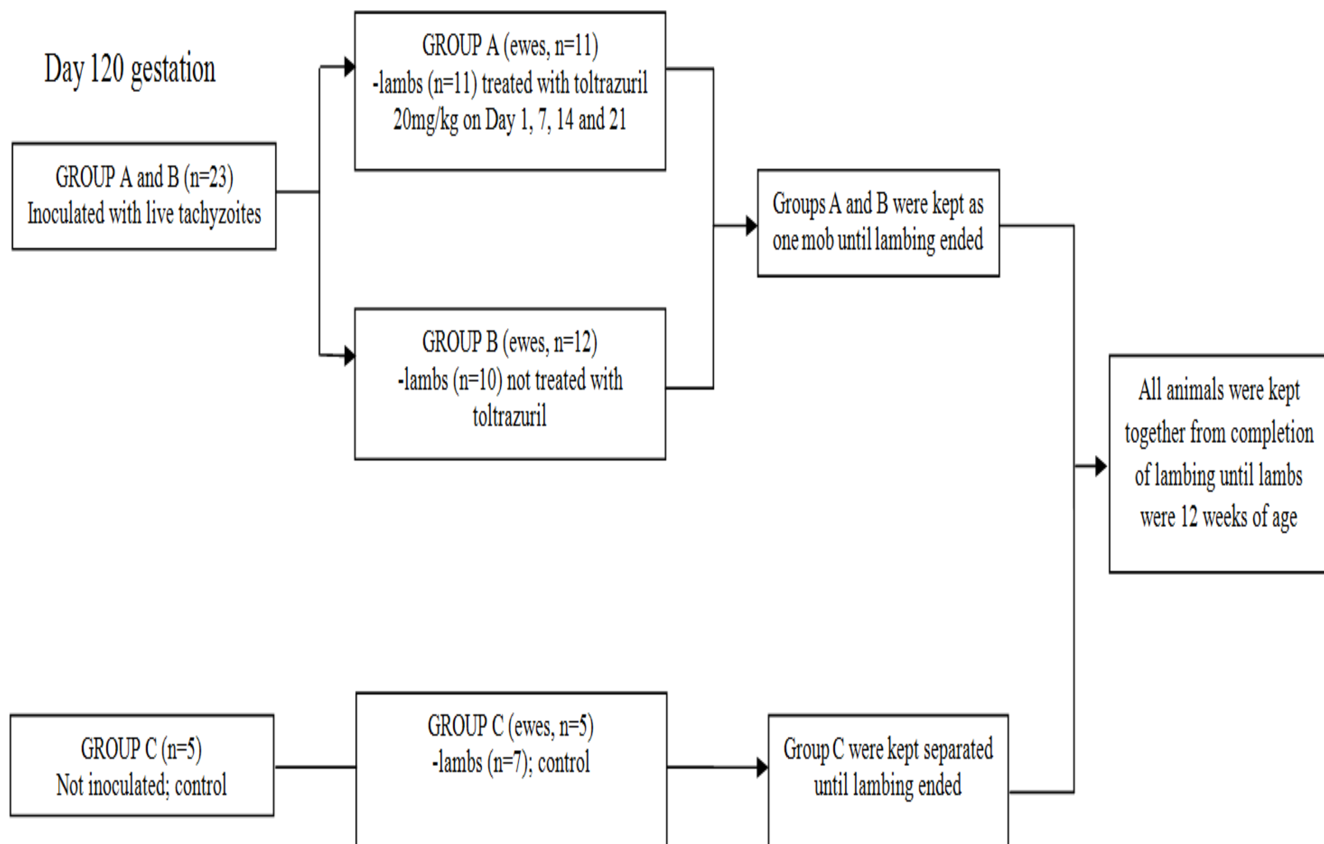

Figure 5.1: Flow diagram on the grouping of ewes and lambs in the study

5.3.2 ELISA

All sera collected were tested as previously described (Syed-Hussain et al., 2013) using a commercial ELISA test kit (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories, Australia). An S/P ratio of ≥11.8% was regarded as positive as described by Reichel et al. (2008).

5.3.3 Western blot

Sera from all ewes were tested one month post inoculation to confirm their infection status while all sera from lambs at 12 weeks of age were also analysed using western blot as described previously (Syed-Hussain et al., 2013). For this study, sera that recognized at least one immune dominant antigen (IDA) with molecular weight (MW) of 17, 25-29, 30-33 or 37 kDa were considered positive for *N. caninum.*

5.3.4 Detection and quantification of N. caninum DNA in samples

5.3.4.1 DNA extraction

Five grams of each brain and heart samples previously stored at -20°C were homogenized using a sterile, single use, 50 ml conical tissue grinder (VWR, PA, USA) containing 10 ml of sterile PBS. DNA from these homogenized tissues was extracted using DNeasy Tissue Kit (Qiagen, Victoria, Australia) as previously described (Syed-Hussain et al., 2013). All extracted DNA samples were kept at -20°C until further analysis was performed.

5.3.4.2 Real-time Polymerase Chain Reaction (qPCR)

All brain and heart tissue samples of lambs were subjected to qPCR for the detection of *N. caninum* DNA and those that were positive were later quantified. The qPCR was

designed to amplify the Nc-5 gene (Kaufmann et al., 1996) of *N. caninum* using the primers NeoF (5'-GTGAGAGGTGGGATACG-3') and NeoR (5'-GTCCGCTTGCTCCCTA-3') (Okeoma et al., 2005). The 10µL qPCR reaction mixture contained: 2 µL of test sample containing 1ng DNA/ul, 2X Perfecta® SYBR® Green FastMix (Quanta Bioscience, CA, USA) ready to use mix and 0.2 µM of each primer. The thermal cycler conditions were as follows: 95°C for 10 minutes; 45 cycles of 95°C for 10 seconds (s), and 60°C for 30 s. A melt curve analysis was visualized over the 80 - 90°C temperature range on the Illumina Eco™ Real-Time PCR software 3.1 (Illumina Inc, Ca, USA). DNA extracted from cell-cultured *N. caninum* was used as a positive control and was included with a negative control containing no DNA for each qPCR run. Each sample was prepared in triplicate. Products amplified from the initial run using *N. caninum* DNA from a pure cell culture were purified, sequenced and subjected to BLAST to confirm its identity and specificity.

A standard curve was generated in triplicate for each run using a 10-fold dilution series of purified DNA from cell-culture adapted *N. caninum* with five known concentrations (1, 10, 100, 1000, 10000 tachyzoites). The efficiency and standard curve of the qPCR cycle were calculated using Illumina Eco™ Real-Time PCR software 3.1. The standard curve was used to estimate the number of tachyzoites of *N. caninum* per mg of tissue.

5.3.5 Brain histological examination

Histological examination of the lambs' brains was conducted for the detection of lesions consistent with *N. caninum* infection in the brain as previously described (Syed-Hussain et al manuscript submitted concurrently with this one). In brief, a total pathological score was calculated for each slide from sections involving the frontal lobe, temporal lobe and the cerebellum.

5.3.6 Statistical analysis

One way ANOVA were performed to compare the antibody responses in animals in Groups A, B and C between ewes at pre-inoculation and 1 month post-inoculation.as well as all lambs at 2 and 12 weeks of age. All animals S/P values for ELISA were tested for normality and all were found to be normally distributed; one-way multiple comparison using Fisher's method was performed.

Brain lesion score values were not normally distributed and were analysed with the Mann-Whitney U test. A Chi-square method was conducted to determine if there was any association between a dam's previous inoculation status and the presence of brain lesions in her lamb(s). All statistical analyses were performed using Minitab (Minitab Inc., PA, USA) and all statistical analyses were considered significant when $p<0.05$.

5.4 Results

5.4.1 Ewe ELISA serology

The serum IgG responses to *N. caninum* in ewes in Group A and B at pre and 4 weeks post inoculation with *N. caninum* tachyzoites are shown in Figure 5.2. Twenty-one ewes from Groups A and B were seropositive after 4 weeks post inoculation. However, two ewes from Group A remained seronegative and these ewes and their lambs were excluded from further analysis. The S/P values for the inoculated ewes in Groups A and B ranged from 3% to 45% and 4% to 27% respectively while the ewes in Group C (control) remained negative (S/P<11.8%) for *N. caninum* antibodies.

5.4.2 Ewe western blot result

Antibodies to at least 2 IDAs were detected in all ELISA-positive ewe sera from Groups A and B one month post inoculation. None of the animals in Group C had antibodies that responded to any IDAs.

5.4.3 *N. caninum* status of lambs

Table 5.1 shows the details of *N. caninum* detection by ELISA, western blot, PCR and histology in neonatal lambs (n=10) that were stillborn or died within 1 week of birth as well as viable lambs (n=28) that were euthanased at 12 weeks of age. No abortions occurred in any ewes.

5.4.4 Detection of *N. caninum* in non-viable lambs

In Group A, one of two dead neonates was seropositive by western blot, had lesions consistent with *N. caninum* infection in the brain (score of 34) and *N. caninum* DNA was detected in the brain and heart. The other dead neonate had no signs of infection. At Week 3, one of the treated lambs died due to injury. It was serologically positive by ELISA (S/P=22%), western blot, had lesions consistent with *N. caninum* infection in the brain (score of 33), a tissue cyst in the brain and *N. caninum* DNA was detected in the brain. In Group B, there was evidence of *N. caninum* infection by western blot, qPCR and histopathology in 5/7 of the neonates. In Group C one ewe died in late pregnancy with one foetus that tested negative for *N. caninum* infection.

5.4.5 Detection of *N. caninum* in viable lambs

5.4.5.1 ELISA results

The ELISA responses to *N. caninum* in lambs from Groups A and B at 2 weeks and all groups at 12 weeks of age are shown in Table 5.1 and Figure 5.2. At 2 weeks of age, antibody responses for lambs in Groups A and B were not significantly different from each other ($p>0.05$) with mean S/P values (proportion seropositive) of 11% (5/11) and 10% (5/10) respectively. No data are available for lambs in Group C at 2 weeks of age. At 12 weeks of age, antibody responses for lambs in Group B were significantly higher than those in Group A ($p<0.05$) with mean S/P values (proportion seropositive) being 30% (9/11) and 21% (9/10) respectively. Lambs in Group C were seronegative.

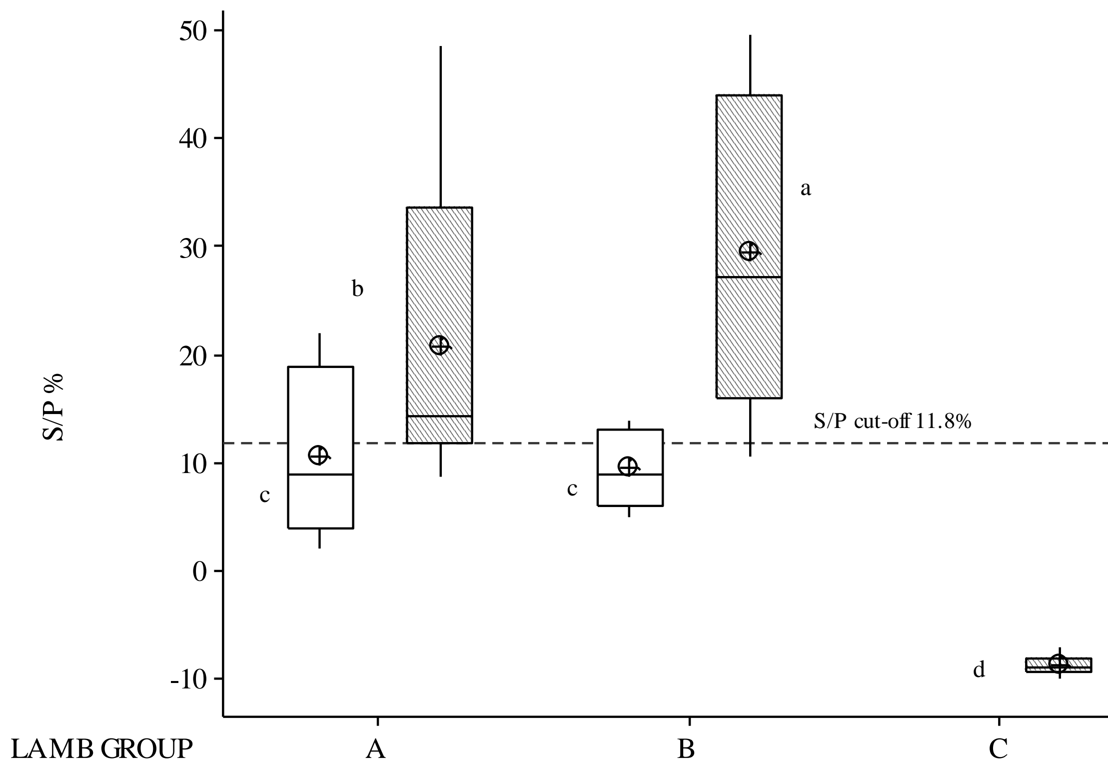

Figure 5.2: Box and whisker plot of S/P values of lambs at 2 ( ) and at 12 weeks of age ( ) for Group A (n=11); Group B (n=10) and Group C (n=7). The mean value (ϴ) is as indicated. Means that do not share a similar letter are significantly different ($p<0.05$). Lambs in Group A were treated with toltrazuril; Group B not treated; Group C control (ewes not inoculated).

Table 5.1: Positive detection of *N. caninum* using ELISA, western blot, PCR and histological lesions consistent with *N. caninum* in neonates (died within 1 week of birth) and lambs at 12 weeks of age.

	Groups‡	ELISA	Western Blot	PCR	Histology		Total Infected[Ŧ]
					Number of lambs	Score range of positive animals	
Neonates that died within 1 week of birth	A**	1/3	2/3	2/3	2/3	33-34	2/3
	B	0/7	4/7	5/7	5/7	5-94	5/7
	C*	0/1	0/1	0/1	0/1	0	0/1
Lambs at 12 weeks	A	9/11	11/11	1/11	8/11	2-22	11/11
of age	B	9/10	10/10	2/10	9/10	1-30	10/10
	C	0/7	0/7	0/7	0/7	0	0/7

Lambs in Group A were treated with toltrazuril at 20mg/kg (day 0, 7, 21 and 35) while lambs in Group B were not treated. Lambs in Group C were untreated control animals.

‡ Neonates/lambs born from: Group A, and B, ewes inoculated on Day 120 of gestation and Group C, uninoculated ewes used as control.

Ŧ Animals are considered positive whenever detected positive by either ELISA, western blot, PCR or by histological lesion.

*One ewe in Group C died at end of pregnancy with one foetus.

** this includes 1 lamb in Group A that died due to injury on Day 16 after the 3rd treatment with toltrazuril. This lamb had suckled

5.4.5.2 Western blot results for viable lambs

Table 5.2 shows a summary of the IDAs recognised by lambs' sera at 12 weeks of age. All lambs in Groups A and B were positive by western blot including those that were seronegative by ELISA (Table 5.1). None of the lambs in Group C (control) were seropositive by western blot.

Detection of specific IDAs were similar between Groups A and B, except for: the protein at 10kDa which was recognised by 7/11 lambs' sera in Group A but none in Group B; and the protein at 30kDa protein that was only recognised by 60% of lambs' sera in Group B and none in Group A.

Table 5.2: Proportion of IDAs recognised in western blot by lambs' sera at 12 weeks of age Group A (lambs treated with toltrazuril); Group B (lambs not treated with toltrazuril) and Group C (control).

Immunodominant Antigen kDa	Group A	Group B	Group C
10	7/11	0/10	0/7
17	11/11	10/10	0/7
25-29	8/11	7/10	0/7
30	0/11	6/10	0/7
31	0/11	1/10	0/7
33	0/11	1/10	0/7
37	0/11	0/10	0/7

5.4.5.3 qPCR results for viable lambs

At 12 weeks of age, *N. caninum* DNA was detected in the brain samples of 1/11 and 2/10 lambs in Group A and B respectively. All positive samples were then quantified using qPCR which indicated less than 1 tachyzoite/mg of tissue was present. No *N. caninum* DNA was detected in the brain of lambs from Group C (control).

5.4.5.4 Brain histopathology and analysis for viable lambs

Table 5.1 shows the number of lambs demonstrating brain lesions consistent with *N. caninum* infection in Groups A, B and C. Lesions consisted of multifocal to coalescing, randomly arranged and variably sized areas of gliosis, necrosis, non-suppurative perivascular cuffing and non-suppurative meningeal inflammatory infiltration. Most of the lambs in Group A (8/11) and B (9/10) were positive for brain lesions with no significant difference in the number of lambs with lesions or in the lesion scores ($p>0.05$) between these 2 groups. A comparison was also made between neonates (Groups A and B combined) and lambs at 12 weeks of age in Groups A and B. No significant differences ($p>0.05$) were seen in the number of neonates with brain histological lesions and lesion scores when compared with those of lambs in Groups A and B when killed at 12 weeks of age. No lesions were seen in any lambs in Group C.

The proportion of lambs with lesions and lesion scores were not significantly different ($p>0.05$) in Group A than in Group B. This indicates that treatment with toltrazuril did not have an effect on reducing the severity of lesions seen in these lambs.

5.5 Discussion

The results of this study indicate that treatment of congenitally infected newborn lambs with toltrazuril did not effectively eliminate the *N. caninum* infection from these lambs.

This study used congenitally infected lambs born from ewes inoculated on Day 120 of gestation to mimic a natural congenital infection where transmission is generally understood to occur during the last trimester of pregnancy. Previous studies indicate that inoculation of ewes before Day 120 of gestation may result in abortions or stillbirths, whereas inoculation on or after Day 120 is expected to lead to the birth of congenitally infected but otherwise healthy lambs (McAllister et al., 1996a; Weston et al., 2009; Buxton et al., 1997). A similar pattern has been observed in cattle, in that inoculation at about the beginning of the third trimester resulted in live congenitally infected calves (Williams et al., 2000).

Several questions can be raised about this experimental sheep model. The inoculation dose used in the current study was high and could have influenced the number of cysts which developed, therefore how closely this resembles a natural infection has yet to be determined. However, similar or higher inoculation doses or higher have been used in previous sheep and cattle studies to successfully produce experimentally infected animals (Weston et al., 2009; Kritzner et al., 2002; Jolley et al., 1999; Buxton et al., 1997). With the design of the present study, toltrazuril would be required to be effective against the bradyzoite stage and possibly the tachyzoite stage of *N. caninum*.

Inoculation of sheep 30 days before birth allows sufficient time for at least some tissue cysts to be formed in the lambs, presuming that foetal lambs are infected soon after

inoculation (McAllister et al., 1996a). To date there are no studies where toltrazuril treatment has been unequivocally directed against *N. caninum* bradyzoites within cysts although several studies have been conducted where it is not clear which stage of *N. caninum* is present at the time of treatment. Toltrazuril and its metabolites have been shown in a number of studies to be effective against tachzyoites (Strohbusch et al., 2008; Darius et al., 2004b; Kritzner et al., 2002; Harder and Haberkorn, 1989). Using a similar approach to that of the present study with a murine model, Strohbusch et al (2009) demonstrated that the treatment of neonatal mice with toltrazuril reduced, but did not eliminate, the infection. Presumably, both tachyzoites and bradyzoites would have been present in these treated animals. A related study treated pregnant mice with toltrazuril immediately after inoculation with *N. caninum* which significantly reduced foetal infection suggesting some efficacy against the tachyzoites inoculum (Gottstein et al., 2005). Similarly, in a study with the related protozoan *T. gondii*, treatment with toltrazuril was not totally effective in controlling infection in recently infected lambs (Kul et al., 2013). Considering these studies, the apparent failure of the toltrazuril treatment to eliminate *N. caninum* infection in the lambs in the present study therefore could not be considered to be unexpected.

In this study, the most compelling evidence to indicate that lambs were infected is the presence of histological lesions, although *N. caninum* DNA was detected in a small number of animals. In one reported study, similar lesions were observed in lambs at 2 months of age (90 days post challenge) and in aborted foetuses following inoculation of ewes at Day 120 of pregnancy (McAllister et al., 1996a) suggesting that there was little resolution of pathological changes over this time period. They observed that lesions were a more consistent finding than the presence of organisms. This is similar to the present study

where lesions were still present throughout all areas of the brain in 12 weeks old lambs that were euthanased (9 weeks after the last treatment). If the toltrazuril treatments were effective it was expected that these treated lambs would have fewer residual lesions and they would be more localised than were seen in these animals, as compared to untreated infected lambs. It is also noteworthy that there was no significant difference in the number of animals with histological lesions or in the lesion score between the neonates and lambs at 12 weeks of age although the mean score of lesions was lower in the latter. A possible explanation for this observation is that tachyzoites proliferation could still be occurring in the neonates but it is likely that only bradyzoites were present in lambs by 12 weeks of age. These are located within cysts that protect them from being detected by the lamb's immune response. It should be noted that the power to observe a difference was limited with the current experimental design.

Unfortunately, the impact of infection followed by toltrazuril treatment on the sero-status of young animals is less clear. For example, in this study although both challenged groups were seropositive at 12 weeks of age, treated lambs showed significantly lower antibody response compared to those that were not treated, suggesting higher antigenic stimulation in the non-treated lambs. This is similar to results in experimentally infected calves where one group was treated with toltrazuril (Kritzner et al., 2002). Conversely, in a different study by Haerdi et al (2006) no difference was observed in antibody titres between treated and non-treated congenitally infected newborn calves at 12 weeks of age. In that study, at 6 months of age, treated calves had higher antibody titres and it was suggested the successful treatment had resulted in higher humoral responses. However, no other diagnostic workup besides serology was undertaken as an indicator for treatment efficacy. In the current study, despite some of the lambs in Groups A and B being

seronegative by ELISA at 12 weeks of age, all of the congenitally infected lambs were positive by western blot. ELISA is known as a quantitative test while western blots will provide a more qualitative result. The most common IDA detected in Groups A and B were the 17 and 25-29 kDa proteins which is consistent with previous studies in sheep and goats (Bjerkas et al., 1994; Sondgen et al., 2001; Naguleswaran et al., 2004; Gaffuri et al., 2006; Rossi et al., 2011). The 30 kDa protein was only detected in the non-treated lambs and this protein has been associated with the surface protein (Björkman and Hemphill, 1998). In our previous study (Syed-Hussain et al manuscript submitted concurrently with this one) lambs born from ewes that were re-inoculated were also positive for this protein. Whether this would indicate that more viable organisms were present in these animals is unknown. The 10 kDa protein was observed only in the treated lambs (Group A). Although this protein has been previously reported (Zintl et al., 2006), its source remains unknown. In our previous study (data not shown) the 10 kDa protein was only detected in lambs that were born from ewes that were inoculated 18 months previously and not in lambs that were born from recently inoculated ewes. There is no obvious consistency between the two studies with regards to detection of this protein.

The use of qPCR was helpful in demonstrating congenital infection by *N. caninum* in neonates although *N. caninum* DNA was not found in all congenitally infected lambs. By 12 weeks of age, only 2 of 10 untreated animals in Group B were positive by qPCR which might reflect the wider dispersion of the organisms in the tissues. This particular qPCR assay (Okeoma et al., 2005) has shown a high degree of sensitivity and is able to detect the presence of *N. caninum* DNA from <1 *N. caninum* tachyzoite/ml. The one positive lamb in Group A provides some evidence that treatment was not totally effective in this group. If the parasite had been killed by Day 21 (last treatment), it would be expected that all *N.*

caninum DNA would have been cleared by the lamb's inflammatory system by the time of euthanasia.

This is the first study examining the efficacy of toltrazuril treatment to control neosporosis in sheep. The potential value of this study was to use sheep as a model for cattle. It is unclear from the previous studies in cattle if bradyzoites were actually present in the treated animals. In this study the expectation was that at least some of the organisms in the treated animals were bradyzoites within tissue cysts. The results suggest that toltrazuril treatment was not effective in eliminating *N. caninum* infection at this stage of infection. Future studies could investigate different treatment protocols including the treatment of the pregnant dams prior to birth. Effective treatment of infected young animals remains an elusive goal.

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5.7 Authors' contributions to this study

S. Syed-Hussain contributed to the study design, conducted the experiment, sample collections, laboratory work and wrote the manuscript.

W. Pomroy provided help with the study design, sample collection and editorial advice.

L. Howe provided help with the study design, advice on laboratory work and editorial assistance.

N. Williamson provided help with the study design and editorial advice.

D. West provided help with the study design and editorial advice.

M. Hardcastle provided help with scoring of histological lesions

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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: Syed-Hussain, Sharifah

Name/Title of Principal Supervisor: William Pomroy

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Please indicate either:

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  and / or

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

  contributed to the experimental design and planning in collaboration with supervisors, carried out experimental work and statistical analysis, prepared the manuscript in collaboration with supervisors.

Syed-Hussain, Sharifah — 21/08/2014

Candidate's Signature — Date

William Pomroy — 21/08/2014

Principal Supervisor's signature — Date

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CHAPTER SIX

Longitudinal investigation of *Neospora caninum* serodynamics and seroprevalence in naturally-infected pregnant ewes.

6.1 Abstract

Recent reports from New Zealand indicate *N. caninum* has a possible role in causing abortions in sheep. Previous experimental studies have shown that vertical transmission may play an important role in maintaining the disease in sheep. Thus, the aims of this study were to undertake a longitudinal study to investigate the dynamics of *N. caninum* infection in breeding sheep and their lambs on pasture and to investigate the seroprevalence of *N. caninum* infection on farms with a recent history of unexplained abortions in sheep.

Three farms with a history of foetal loss and abortion attributed to *N. caninum* infection were selected. Ewes were blood sampled at premating, at pregnancy scanning (mid pregnancy) and after lambing. Lambs born to the ewes were blood sampled at 12 week of age. Ewes from a further six farms with a recent history of unexplained abortions were also blood sampled opportunistically at a single time point. Ewe serology at pre-mating, scanning and lambing (Table 6.1) for Farms A, B and C revealed an average prevalence of 0.9% (4/430), 0.7% (3/451) and 1% (3/309) at pre-mating, ultrasound scanning for pregnancy and after lambing respectively. No ewes on the other 6 farms were seropositive although one lamb on one farm was seropositive. No lambs on Farms A, B and C were seropositive. This is the first study involving sheep farms in New Zealand to determine the serological status of sheep through pregnancy to attempt to better understand the dynamics of *N. caninum* infection. However, the low seroprevalence observed across all farms does not allow a meaningful interpretation to be made about the role of neosporosis on these farms.

Keywords: *N. caninum*, sheep, serological prevalence, pregnancy, lambing

6.2 Introduction

N. caninum is an obligate intracellular apicomplexan protozoan and has been recognised as a leading cause of bovine abortion worldwide which has been reported in horses, goats, deer and sheep (Dubey, 2003). A natural infection of sheep with *N. caninum* was first identified in 1990 in a congenitally infected lamb that showed signs of ataxia and weakness after birth (Dubey and Schares, 2011). Subsequently, naturally occurring ovine neosporosis and abortions have been reported worldwide including from Japan, South America, Australia, Switzerland, Italy, Spain, and New Zealand (Moreno et al., 2012; Moraes et al., 2011; Masala et al., 2007; Hassig et al., 2003; Hassig and Gottstein, 2002; Helmick et al., 2002; Kobayashi et al., 2001; Koyama et al., 2001; Otter et al., 1997). Repeated abortions in subsequent years have also been reported (Jolley et al., 1999). These reports suggest that *N. caninum* infections do occur and can cause disease in sheep, although generally it has not been regarded as a significant cause of abortion (Helmick et al., 2002; Jolley et al., 1999; Otter et al., 1997).

In New Zealand, the principal causes of ovine abortions, accounting for 80% of cases, were campylobacteriosis, toxoplasmosis and salmonellosis (*Salmonella enterica serovar Brandenburg)* (West, 2002). Progress in the development and extensive use of vaccines in New Zealand against these three most common agents of ovine abortion has greatly reduced the number of abortion cases, especially in maiden ewes (West et al., 2006; West, 2002). Yet in recent years, unexplained abortions in maiden ewes have been occurring in some sheep flocks and although not considered an important abortifacient in sheep, *N. caninum* has more recently been included as one of the possible differential diagnoses (Howe et al., 2012; Howe et al., 2008; West et al., 2006). Studies in sheep flocks from around the world have shown that the seroprevalence of *N. caninum* ranges from 2-14%

(Hecker et al., 2013a; da Silva Andrade et al., 2012; Moraes et al., 2011; Langoni et al., 2011; Panadero et al., 2010; Bishop et al., 2010; Ueno et al., 2009; Romanelli et al., 2007; Gaffuri et al., 2006; Figliuolo et al., 2004). Seroprevalence has been recorded to be as high as 30% and 92% in Pakistan and Jordan respectively (Abo-Shehada and Abu-Halaweh, 2010; Nasir et al., 2012). In New Zealand, a seroprevalence of 0.6% and 1.4% (Reichel et al., 2008; Howe et al., 2012) has been reported in sheep as compared to 9% and 2.8% in dairy and beef cattle respectively (Reichel, 2000; Tennent-Brown et al., 2000; Sanhueza et al., 2013).

This study was initiated following the results of previous studies (Howe et al., 2012; Howe et al., 2008; West et al., 2006) indicating that *N. caninum* might play an important role as an abortifacient agent in sheep in New Zealand. To date, no transmission studies have been undertaken under natural farming conditions in sheep. The aim of this study was to undertake a longitudinal study to investigate the dynamics of *N. caninum* infections in breeding sheep on pasture and to investigate the seroprevalence of *N. caninum* infection on farms with a recent history of unexplained abortions in sheep.

6.3 Materials and methods

6.3.1 Experimental design and farm selection

A longitudinal study was undertaken on three farms with a history of foetal loss and abortion, especially in young ewes. This study was conducted by collecting serological samples throughout one year.

A summary of the farms involved is shown in Table 6.1. The available information on the sheep reproductive performance on these farms is shown in Appendix C. In summary, Farms A, B and C had previously been shown to have *N. caninum* seropositive sheep. Farms

A and B were large commercial sheep farms and a subset of 200 ewes were randomly selected on each farm. All ewes were individually identified with ear tags. They were sampled at premating, soon after ultrasound scanning (mid pregnancy) and when lambs were at 12 weeks of age (ewes and lambs at this time). For the sampling soon after ultrasound scanning for pregnancy on Farms A and B, some additional ewes were included at that time which had been identified as having been pregnant but were now either non-pregnant or had dead foetuses in utero. Farm C was a small property and all available sheep were sampled (n=28). One ewe had consistently been observed to have an abortion or still-born lamb for 3 successive years where *Neospora* was identified in its tissues. One aborted foetus was submitted for necropsy from this property during the time of this study and the dam of this aborted foetus was available for necropsy the following year. Tissues from the aborted foetus and ewe were subjected to DNA extraction and analysed using qPCR for the detection and quantification of *N. caninum* DNA (Chapter 4-Section 4.3.6). Both the foetus and ewe were confirmed positive for the presence of *N. caninum* DNA as previously described in Chapter 4 (Section 4.3.6).

In addition to the 3 farms used in the longitudinal study, a further six farms with a history of unexplained abortions that year or the previous year were investigated. Serological samples were opportunistically taken, generally only on one occasion, from ewes premating (Farm E), at scanning with evidence of abortion (Farms F, G, H, I) or as ewe/lamb combinations (Farm D) when lambs were 12 weeks of age (Table 6.1).

6.3.2 Serology

All sera collected were tested as previously described (Syed-Hussain et al., 2013) using a commercial ELISA test kit (Chekit* *N. caninum* Antibody Test Kit, IDEXX Laboratories,

Australia). An S/P ratio of ≥11.8% was regarded as positive as described by Reichel et al (2008).

6.4 Results

Ewe serology at pre-mating, scanning and lambing (Table 6.1) for Farms A, B and C revealed an average prevalence of 0.9% (4/430), 0.7% (3/451) and 1% (3/309) at pre-mating, ultrasound scanning for pregnancy and after lambing respectively. None of the 418 lambs sampled, were seropositive.

Overall seroprevalence for individual ewes and lambs on Farm A was 0.4% (2/465) with one ewe seropositive at premating with an S/P value of 12.8%. However, this animal was later found seronegative at the time of scanning. In addition, at the time of sampling at scanning, 1/12 ewes showing evidence of recently having aborted was seropositive with an S/P value of 31%. Unfortunately, this ewe was not able to be re-sampled. No lambs were seropositive at 3 months of age.

Overall seroprevalence for individual ewes and lambs on Farm B was 0.8% (3/365). One ewe was seropositive for *N. caninum* with an S/P value of 37% at pre-mating and 27% at scanning. However no sample was obtained from this ewe after lambing as she disappeared from the mob. At the time of the scanning (Week 6 of gestation), 22 ewes were identified as recently having aborted or had a resorbing foetus but all were seronegative for *N. caninum* antibodies. All remaining pregnant ewes sampled were also seronegative. After lambing, two previously seronegative ewes had seroconverted with S/P values of 36% and 12.5% respectively. None of the lambs were seropositive.

Table 6.1: Serology results for ewes and lambs as indicated by ELISA on all farms at various sampling times.

FARM	EWES				LAMBS	
	Premating	Scanning		After Lambing	12 Weeks of Age	*TOTAL number of positive individual animals
		Pregnant	Dry/ Resorbing			
A	1/200	0/187	1/12	0/164	0/255	2/465 (0.4%)
B	1/202	1/202	0/22	2/117	0/141	3/365 (0.8%)
C	2/28	1/28	-	1/28	0/22	2/50 (4%)
TOTAL	4/430 (0.9%)	2/417 (0.5%)	1/34 (2.9%)	3/309 (1%)	0/418	7/880 (0.8%)
One-off sampling from farms with history of unexplained abortions						
D	-	-	-	0/201	1/205	1/406 (0.2%)
E	0/190	-	-	-	-	0/190 (0%)
F	-	-	0/16	-	-	0/16 (0%)
G	-	-	0/13	-	-	0/13 (0%)
H	-	-	0/13	-	-	0/13 (0%)
I	-	-	0/15	-	-	0/15 (0%)
TOTAL						1/653 (0.2%)

*total = animals found to be seropositive over the total number of animals at each sampling time including the lambs. If the same animal was found positive again, it was not included again in the total seropositive animals.

Overall seroprevalence for the individual ewes and lambs on Farm C was 4% (2/50). Two ewes were seropositive at pre-mating with S/P values of 15% and 80%. The former ewe was subsequently sold, however the later ewe was resampled throughout pregnancy and maintained her seropositive status with S/P values of 104% and 147% at scanning and after lambing. Of note, this ewe had a history of abortion attributed to *N. caninum* infection and aborted for a second time at pregnancy scanning during this study. Due to the history and sero-status of the ewe, the foetus was collected and *N. caninum* DNA was detected in the brain by qPCR (as described in Chapter 4-Section 4.3.6). The ewe was allowed to become pregnant the following year and again aborted but the foetus was not found. Subsequent necropsy of this ewe found *N. caninum* DNA in the tissues of the uterus, diaphragm, heart, skeletal muscle and kidney but none in the brain, liver, spleen or blood. Repeated DNA extractions of brain and blood samples confirmed the lack of *N. caninum* DNA. All other ewes, including two ewes that also aborted, were seronegative and no *N. caninum* DNA was detected by PCR in the brains of aborted foetuses.

Farm D animals were only sampled after lambing and none of the ewes were seropositive, however one lamb was seropositive at 3 months of age with an S/P value of 37% giving a sero-prevalence of 0.2%. For Farms E to I, none of the ewes with aborting or resorbing foetuses screened were seropositive, thus resulting in an overall sero-prevalence for animals on farms only sampled once as 0.2%

6.5 Discussion

This is the first report of a longitudinal study on the seroprevalence of *N. caninum* in sheep in New Zealand. The overall prevalence detected on farms followed throughout pregnancy was very low (0.8%; range 0.4% to 4%) thus forming any conclusions is difficult.

On farms A, B and C there was no real evidence of substantial horizontal or vertical transmission occurring. On Farms D-I where animals were only sampled once, an even lower overall prevalence of 0.2% was identified with no animals with reproductive failure seropositive for *N. caninum* antibodies.

The result in the current study was in line with the findings of previous studies using ELISA-based seroprevalence in New Zealand that detected 0.6% in 640 healthy rams (Reichel et al., 2008). It is also similar to a recent study by Howe et al (2012) involving 21 sheep farms with ongoing unexplained abortion problems that had an overall seroprevalence of 1.4% (5/403). Farm A and B in the current study were Farms No 2 and 31 as reported by Howe et al (2012). The initial investigation of these farms used an in-house IFAT which suggested a high seroprevalence for *N. caninum* on both Farms A and B with 18/40 and 10/21 aborted ewes found seropositive respectively. Subsequent use of the Chekit ELISA with the same serum found only 1 seropositive ewe on Farm A and none on Farm B. However, in that study using PCR, 5 out of 10 dead or dying foetuses examined on Farm A were positive for *N. caninum* DNA suggesting that it may have been playing a role in causing the foetal deaths although the seroprevalence was apparently low. The potential role of *N. caninum* in Farm B is less clear as the use of PCR found no *N. caninum* DNA in foetuses (n=16) or blood (n=21). In the report by Howe et al (2012) it is apparent that the specificity of the in-house IFAT was not high. A similar observation on the poor specificity of this IFAT was also made by Weston et al (2009). Given these results, the very low seroprevalence in ewes in the present study is not surprising and the absence of any seropositive lambs on these 2 farms is also not surprising.

In the current study, one of the possibilities for the low detection of seroprevalence in all sampled farms could be due to factors such as culling of ewes with reproductive

failures with the subsequent introduction of naive animals (hoggets) which are common farm practises. However, this is still largely similar to replacement practices on cattle properties although dairy cows may be kept for a subsequent pregnancy in some cases. It is known that in cattle naturally infected with *N. caninum*, vertical transmission occurs at a high rate of 81% to 95% (Davison et al., 1999; Schares et al., 1998; Pare et al., 1996). In cattle, it was reported that the proportion of seropositive cows in a herd is maintained despite the high risk of culling among those infected and aborting dams (Thurmond and Hietala, 1996). Similar observations were made in experimentally infected ewes which suggested that vertical transmission occurs readily at a high rate, including ewes that were chronically infected (Jolley et al., 1999) and this was also observed in ewes described in Chapter 4 suggesting that vertical transmission might play an important role in maintaining the infection in flocks. However, results in the current study suggest that in naturally infected sheep a different scenario appears to be occurring where the rate of vertical transmission was apparently close to nil although these farms had a previous history of *N. caninum* infection, especially Farm A. The difference between ewe serology and PCR of uterine and foetal tissues reported by Howe et al (2012) also raises questions about the usefulness of serology in these situations.

The reason for choosing Farm C for the longitudinal study was that the one ewe which aborted had also produced stillborn lambs in two subsequent years and each of these aborted lambs was shown to have *N. caninum* DNA in their brains. This ewe was also shown to have a consistently high titre of *N. caninum* antibodies over several years. The blood of this ewe collected in 2008 was used as a positive control serum in Chapter 2. She was euthanased within 1 week after aborting for the third time. Despite such a long history of abortions, although *N. caninum* DNA was detected in the uterus, diaphragm, heart,

skeletal muscle and kidney, there was surprisingly no evidence of *N. caninum* DNA in any of her brain tissue or even her blood. Repeated DNA extraction on various sections of the brain was conducted to confirm this. This result was unexpected as brain tissue has been suggested to be the main and most important organ for DNA detection (Nishimura et al., 2013; Dubey and Schares, 2006; Ho et al., 1997). Viable *N. caninum* has been isolated using brain tissues in most studies reported including in sheep (Dubey and Schares, 2011; Pena et al., 2007; Dubey et al., 2007; Koyama et al., 2001). In experimentally and naturally infected cattle, parasite DNA detection has been reported in other tissues such as the muscle, heart, lung, diaphragm, kidney, placenta and spinal cord (Kritzner et al., 2002; Ho et al., 1997) similar to some of the findings for this ewe in the current study. These findings suggest that it is critical to examine other tissues and not just brain sections as it could lead to false negative results in detecting *N. caninum* infections. Interestingly, there was no widespread occurrence of neosporosis on the farm despite having this particular positive ewe in the farm for the past three years. She was originally purchased as an adult sheep, so probably became infected prior to arrival on this farm.

In the present study, fluctuations of antibody titres were also observed in one seropositive ewe on Farm A. Fluctuating antibody titres which become seronegative in previously seropositive animals have been observed in experimentally infected calves (Maley et al., 2001) and in pregnant cows (Okeoma et al., 2004b; Conrad et al., 1993a). A similar situation was also observed in a previous study where experimentally infected ewes were seronegative 6 months post-inoculation (Syed-Hussain et al., 2014). It has been shown that being seronegative does not necessarily mean that the animal was never infected nor currently not infected (Howe et al., 2012). This finding thus emphasises the obstacles to making a diagnosis, particularly with serology alone and in animals that are

chronically infected with *N. caninum*. It has been suggested that a multi-faceted approach using various methods of diagnosis is important in determining the true involvement of *N. caninum* as an abortifacient agent in sheep (Howe et al., 2012). However, due to financial constraint and time limitation, additional assays such as PCR, were not performed in the current study.

In conclusion, this is the first study involving sheep farms in New Zealand to determine the serological status of sheep from pre-mating to weaning over one year to attempt to better understand the dynamics of *N. caninum* infection. Although ELISA is a useful tool in a seroprevalence study, it would be better if a multi-faceted approach was taken, which includes other serological and molecular assays, to capture the true involvement of *N. caninum* in naturally infected sheep as they could be experiencing fluctuations of antibody titres during pregnancy thus underestimating the true infection status.

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6.7 Authors' contributions to this study

S. Syed-Hussain contributed to the study design, conducted the experiment, sample collections, laboratory work and wrote the manuscript.

W. Pomroy provided help with the study design, sample collection and editorial advice.

L. Howe provided help with the study design, advice on laboratory work and editorial assistance.

N. Williamson provided help with the study design and editorial advice.

D. West provided help with the study design and editorial advice.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 Introduction

The aim of the work presented in this thesis was to better understand the epidemiology of *N. caninum* in sheep and to investigate if it plays an important role as an ovine abortifacient agent on New Zealand farms. This interest followed recent reports on the involvement of *N. caninum* as a cause of abortions and reproductive problems in sheep (Howe et al., 2012; Howe et al., 2008; West et al., 2006). With regards to sheep, one major question raised was how a large number of ewes could get infected over a short period of time. This includes the role of vertical transfer and venereal transmission. Other questions relate to the potential treatment and prevention of neosporosis. Previous investigations of neosporosis in sheep were largely focused on utilising sheep as a model system for investigating the disease in cattle and did not focus on neosporosis as a disease in sheep *per se*. Thus there are many issues around neosporosis in sheep that remain to be investigated.

There were five main objectives of the research involved in this thesis. The first objective was to determine if *N. caninum* could be transmitted to ewes via venereal transmission using experimentally infected rams. The second was to adapt a commercially available ELISA assay to determine the IgG avidity in experimentally and naturally infected sheep. The third objective was to determine the rate of vertical transmission from experimentally infected ewes. The fourth objective was to investigate the use of toltrazuril in treating congenitally infected newborn lambs to eliminate infection in these animals. This latter was principally intended to utilise sheep as a model to explore the usefulness of toltrazuril for treating neosporosis in cattle. The final objective was to determine the seroprevalence in animals from farms with a previous history of *N. caninum*-related

reproductive failures by comparing the serological dynamics of ewes from pre-mating through to lambing. This thesis was successful in meeting all the above objectives and the main findings are discussed under six general headings.

7.2 The role of venereal transmission as a possible mode of *N. caninum* transmission

To date, this is the only study that has investigated the possibility of venereal transmission of *N. caninum* in sheep. Results of this study indicate that *N. caninum* DNA was detectable in the semen of rams experimentally inoculated with *N. caninum*. However, similar to findings in cattle, the results suggested that venereal transmission in sheep is not a significant route of infection. The current work has provided further insight into the epidemiology and dynamics of neosporosis in sheep especially in understanding the possible mode of transmission.

In Chapter 2, all 24 experimentally inoculated rams became seropositive for *N. caninum* antibodies and *N. caninum* DNA was detected in the semen of 10 of the 24 inoculated rams. *N. caninum* DNA was detected in the semen of rams inoculated even at the lowest dose of 50 tachyzoites. However, this was only detected at the first semen collection on Day 7 post inoculation in rams given the lowest dose and the DNA concentration was only equivalent to 43 t/ml. By contrast, for those inoculated with the highest dose, DNA was detected intermittently up to Week 4 with a concentration of DNA equivalent to a maximum of 890 t/ml in their semen. This was particularly of interest as in cattle, DNA equivalent to only 1-16 *N. caninum* t/ml was detected either in the semen of both naturally or experimentally infected bulls (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ferre et al., 2008; Ortega-Mora et al., 2003; Serrano-Martinez et al., 2007a). This

higher concentration in rams suggests that it could be sufficient to produce an infection in the ewes if the tachyzoites were viable. However, ewes mated to these rams did not show any signs of infection and subsequently lambed live and apparently healthy lambs thus ruling out the possibility of venereal transmission. In cattle, natural mating with experimentally infected bulls failed to induce seroconversion in the mated cows (Osoro et al., 2007) implying there was no infection. In contrast, studies using AI with semen spiked with *N. caninum* did successfully infect cows (Serrano et al., 2006; Serrano-Martinez et al., 2007b). Interestingly, in a recent study with the closely related *T. gondii,* it was shown that venereal transmission was possible where experimentally infected rams which were naturally mated to ewes resulted in seroconversion of the ewes and detection of *T. gondii* DNA in both the mated ewes as well as their lambs (Lopes et al., 2013). Note that the present study did not examine the mated ewes or lambs born for the presence of DNA in their tissues or blood. This present study is only one experiment and does not rule out the possibility of venereal transmission occurring in other situations.

One of the limitations of the current study was that the viability of the parasites in the semen was not determined. As indicated above it should be noted that detecting DNA equivalent to a high number of *N. caninum* tachyzoites in the semen does not mean the disease is transmissible. In previous studies with experimentally infected cattle, viable tachyzoites could not be detected in bull semen using a mouse bioassay but the authors suggested that this could be due to the low number of parasites detected per ml of semen (Ferre et al., 2005; Ferre et al., 2008). In the current study no attempt was made to determine the viability of the tachyzoites. The results in cattle using spiked semen would indicate that if viable tachyzoites were present in the semen, it could lead to infection.

7.3 The role of vertical transmission as a possible mode of *N. caninum* transmission

To further understand the mode of transmission of *N. caninum* in sheep, the next study investigated vertical transmission. In cattle this is considered to be a major route of infection. Vertical transmission could occur via two ways from the dam: the first is when the dam is infected prior to pregnancy and vertical transmission follows recrudescence of a latent infection (also known as endogenous TPI) usually later in gestation; the second is when the dam is infected during pregnancy (also known as exogenous TPI) and the tachyzoites cross the placenta during the acute phase of the infection in the ewe (Trees and Williams, 2005).

The findings described in Chapter 4 showed that ewes inoculated prior to mating produced non-infected lambs in Year 1. This attempt at establishing endogenous TPI in the lambs was not successful. This is similar to the findings in three cattle studies (Williams et al., 2007; Williams et al., 2000; Innes et al., 2001b), where there was no evidence of vertical transfer from dams that were inoculated with live tachyzoites prior to mating, but is in contrast to low levels of abortion/detection of *N. caninum* that have been reported in 3 other cattle studies (Weston, 2011; Hecker et al., 2013b; Weber et al., 2013) where the cows were also inoculated prior to pregnancy. This also contrasts with what happens with naturally infected cows, many of which were infected in utero, where the rate of vertical transmission is generally reported as being as high as 81-95% (Anderson et al., 1997; Pare et al., 1996; Schares et al., 1998; Davison et al., 1999). To date there has been no explanation for this difference except that being initially infected in utero may make further vertical transmission more likely. It should be noted that none of these experimentally infected cattle studies followed the dams through a further pregnancy the following year.

In sheep there have only been 2 studies (Buxton et al., 2001; Jolley et al., 1999) that have investigated this general area and their results showed that vertical transfer did occur in the subsequent pregnancy and this transmission rate was generally higher than in reports of similar experimental studies in cattle. Buxton et al. (2001) reported the rate of vertical transmission was 10-12% in ewes that were inoculated prior to mating and from ewes that were chronically infected, whilst Jolley et al. (1999) found 75% mortality rate in lambs born from ewes only inoculated in the previous year and 70% mortality rate (3/5 aborted) in those that were also re-inoculated in the second year. Thus it was not unreasonable to expect that some lambs in the current study would have been infected in the 1st year but this did not happen. In contrast, in the present study, the results in Year 2 did show vertical transfer which is similar to these two previous sheep studies. The difference between the results in these two years is difficult to explain, especially for those ewes that were not re-inoculated in the second year.

When the ewes were re-inoculated in the second year the rate of congenital infection was higher ($p<0.05$) than those not re-inoculated, implying the initial inoculation did not provide effective protection. This observed lack of effective protection is consistent with other studies in sheep (Buxton et al., 2001; Jolley et al., 1999; McAllister et al., 1996a) where ewes re-inoculated had a high rate of vertical transmission. This failure to demonstrate protection has implications for the development of vaccines for neosporosis. To date, no vaccination is available to prevent neosporosis in animals. In 2001 a killed tachyzoite vaccine (Neoguard™) was registered in New Zealand to reduce and prevent abortion in cattle due to *N. caninum*. However this vaccine was later removed from the market due to reports of variable efficacy (Schetters, 2004) and some evidence of increased risk of early embryonic death (Weston et al., 2012). In comparison, a live attenuated

vaccine against *Toxoplasma* (Toxovax®) used in sheep has been successful in preventing ovine toxoplasmosis worldwide since its introduction in 1988 (Innes et al., 2007) although a recent report has indicated that it does not totally prevent the vaccinated sheep from getting infected (Katzer et al., 2014). This vaccine is the S48 strain of *T. gondii* which was originally isolated from New Zealand and is an incomplete strain due to prolonged passage through mice leading to the loss of its ability to form tissue cysts or oocysts (Buxton, 1993; Wilkins et al., 1987). The immunity induced by this vaccine will last at least 18 months post inoculation in sheep prior to mating (Buxton et al., 1993) and in New Zealand the claim is for a life-long immunity. There was original optimism that a similar approach could be taken with *N. caninum* but the results from the present study combined with those previously reported would indicate this is unlikely.

Results in the current study are suggestive that once a ewe is infected, the disease could be maintained in the flock. Whilst the rate of vertical transmission in sheep is still unclear the results of the present study and those previously published suggest that a similar pattern may occur with sheep as in cattle. This was the first attempt to investigate this phenomenon with a New Zealand isolate or within New Zealand.

7.4 The role of vertical transmission in sheep on farms with a previous history of *N. caninum*-related reproductive problems

To better understand vertical transmission of *N. caninum* in sheep in a natural infection setting, the study reported in Chapter 6 was initiated. Farms A and B were 2 farms with a history of reproductive problems where *N. caninum* was suspected of playing a role. Both of these farms had been included in the report by Howe et al (2012) and only a low seroprevalence was found although PCR positive foetuses were found on Farm A. In

the year prior to the study both Farms A and B were still experiencing unexplained foetal loss in hoggets although the numbers and dynamics were poorly defined. In contrast the reproductive performance of these farms in the year of the current study was considered acceptable. In consequence, no aborted samples were available. The ewes were bled and tested using an ELISA prior to mating, at pregnancy scanning and after lambing. The lambs were also sampled at 12 weeks of age. Overall a low detection rate was observed with only 2/465 animals positive on Farm A and 3/365 positive on Farm B. Samples were also examined from other farms experiencing reproductive problems at that time and overall the seroprevalence was only 0.5% (8/1533 animals). This is in accord with the findings of previous studies using ELISA-based seroprevalence in New Zealand that detected 0.6% in 640 healthy rams (Reichel et al., 2008) but slightly less than the 1.2% (5/403) in sheep farms with ongoing fertility problems (Howe et al., 2012). In the current study, due to financial and time constraints, it was not possible to undertake additional diagnostic procedures such as western blots and PCRs.

The identification of *N. caninum* by PCR in seronegative ewes (Castañeda-Hernández et al., 2014; Howe et al., 2012) suggests the sensitivity of serology is not as high as might be expected and can lead to an underestimation of the true infection status of the animals. In the study by Howe et al (2012), *N. caninum* DNA was detected in 7%, 4% and 2% of blood samples from aborting/non pregnant, pregnant and high fertility pregnant ewes respectively whereas the ELISA prevalence was only 1.2% using the same assay and cut points as in the present study. The study reported by Castaneda-Hernandez et al (2014) used a different ELISA (HerdChek* anti-*Neospora* ELISA) and maintained the same cut-off values for sheep as for cattle. They observed only 5.5% (18/324) of samples as being seropositive for *N. caninum* antibodies but 25% (84/324) of sheep were positive for *N.*

caninum DNA. Whether this is the appropriate cut-off value for this ELISA in sheep has not been investigated. In both studies however, it was shown that the agreement between the two diagnostic methods, PCR and ELISA was low. However, in the year of the current study, it was not feasible to be absolutely certain of the infection status of the two farms investigated. Nevertheless, the results indicated that there was a low incidence of neosporosis in these flocks. Given the differences between PCR and serology in detecting evidence of infection, the very low seroprevalence in ewes in the present study is not surprising and the absence of any seropositive lambs on these 2 farms is also not surprising. The study may have detected more infected animals if multiple diagnostic tests were used including PCR and western blot to determine the infection status of the animals.

The situation with cattle appears to be somewhat different than sheep. There is more reliance on serology in diagnosing neosporosis in cattle because more validation of different serological tests has been undertaken. Nevertheless, studies in cattle have shown that antibody titres fluctuate over the animal's life span especially during pregnancy with animals changing their sero-status at different times (Okeoma et al., 2004b; Maley et al., 2001; Stenlund et al., 1999; Hietala and Thurmond, 1999).

If *N. caninum* was causing problems on Farms A and B, then other possibilities for the low detection of seroprevalence in these farms could be due to management factors, including culling of ewes with reproductive failures and introduction of naive animals (hoggets). Interestingly, a different scenario is observed in naturally infected dairy cattle, where it has been reported that the proportion of seropositive cows in a herd is maintained despite the high risk of culling of infected and aborting dams (Thurmond and Hietala, 1996; Pabon et al., 2007).

The reason for choosing Farm C was the presence of one chronically infected ewe that had repeatedly aborted foetuses in successive pregnancies. She was an introduced sheep on that farm and her full reproductive history was unknown. This ewe aborted or produced stillborn lambs in the previous 2 years and each of these foetuses/lambs was shown to have *N. caninum* DNA in their brains. She was consistently seropositive with high titres. During the year of this study she aborted on the day of scanning. In the subsequent year she was euthanased within 1 week after aborting again in mid pregnancy. Despite such a long history of abortions there was surprisingly little evidence of *N. caninum* DNA in any of her brain tissues or even blood. Repeated DNA extractions on various sections of the brain were also conducted to confirm this. This was unexpected as brain tissues have been suggested to be the main and most important organ for DNA detection (Ho et al., 1997; Nishimura et al., 2013; Dubey and Schares, 2006).

The findings in the current study suggest that even using PCR at necropsy to confirm status of infection was not totally reliable. Interestingly, this contrasts with most published studies where the organism has either been seen or its DNA identified. Viable *N. caninum* has been isolated using brain tissues in most studies reported including in sheep (Koyama et al., 2001; Pena et al., 2007; Dubey and Schares, 2011; Dubey et al., 2007). In experimentally and naturally infected cattle, parasite DNA detection has been reported in other tissues such as the muscle, heart, lung, diaphragm, kidney, placenta and spinal cord (Ho et al., 1997; Kritzner et al., 2002) similar to some of the findings in the current study. These observations suggest that other tissues may need to be examined and not just the brain sections as it could lead to false negative results in detecting *N. caninum* infections but even then infection may be missed.

Based on the results obtained, Farms A, B and C were not ideal candidates for a longitudinal study on seroprevalence in sheep because of the low seroprevalence observed. Findings suggest that *N. caninum* was not playing a significant role in reproductive failures on these farms. Even in the report by Howe et al (2012) there were only a small number of positive foetuses identified on Farm A and even then it is not clear that *N. caninum* was the cause of foetal death. The original serological values obtained from Farms A and B were with IFATs and these showed a high seroprevalence but the subsequent use of ELISA on these farms produced different results. This is the first longitudinal study to look into the occurrence of vertical transmission on naturally infected sheep farms in New Zealand but with the low seroprevalence rate observed, further investigations are needed.

7.5 The efficacy of toltrazuril for treating congenitally-infected newborn lambs

The findings in the current study suggest that that treatment of congenitally infected newborn lambs with toltrazuril did not effectively eliminate *N. caninum* infection from these lambs. Inoculation of sheep 30 days before birth allows sufficient time for at least some tissue cysts to be formed in the lambs, presuming that foetal lambs are infected soon after inoculation (McAllister et al., 1996a). To date there are no studies where toltrazuril treatment has been unequivocally directed against *N. caninum* bradyzoites within cysts although several studies have been conducted where it is not clear which stage of *N. caninum* is present at the time of treatment (Haerdi et al., 2006; Kritzner et al., 2002). In contrast, toltrazuril and its metabolites have been shown to be effective against tachyzoites in a number of studies (Strohbusch et al., 2008; Darius et al., 2004b; Kritzner et al., 2002; Harder and Haberkorn, 1989). In the present study, this offers an explanation in that toltrazuril may not have been effective against the bradyzoite stage and hence was ineffective in eliminating the infection in these lambs. Using a similar approach to that of the present study with a murine model, Strohbusch et al (2009) demonstrated that the treatment of neonatal mice with toltrazuril reduced, but did not eliminate, the infection. Presumably, both tachyzoites and bradyzoites would have been present in these treated animals which could suggest that the treatment was effective against the tachyzoites but not the bradyzoites. Alternatively there is some evidence that toltrazuril may not be totally effective against tachzyoites as well. In a study pregnant mice were treated with toltrazuril immediately after inoculation with *N. caninum* which significantly reduced but did not eliminate foetal infection suggesting some but not total efficacy against the tachyzoites which would have been present at this time (Gottstein et al., 2005). Similarly, in a study with the related protozoan *T. gondii*, treatment with toltrazuril was not totally effective in

controlling infection in recently inoculated lambs (Kul et al., 2013). Considering these studies, the apparent failure of the toltrazuril treatment to eliminate *N. caninum* infection in the lambs in the present study therefore could not be considered to be unexpected.

In the present study a variety of diagnostic methods were used to indicate the lambs were infected. In this study, the most compelling evidence to indicate that lambs were infected is the presence of histological lesions since *N. caninum* DNA was detected in only a small number of animals. If the toltrazuril treatments were effective it was expected that these treated lambs would have fewer residual lesions and they would be more localised than were seen in these animals, as compared to untreated infected lambs. It is also noteworthy that there was no significant difference in the number of animals with histological lesions or in the lesion score between the neonates and lambs at 12 weeks of age although the mean score of lesions was lower in the latter. In a study by McAllister et al (1996a) there were no differences in lesions observed in the brain between lambs at 8 weeks of age (90 days post challenge) and in aborted foetuses following inoculation of ewes at Day 120 of pregnancy which is similar to the present study. They observed that there was little resolution of pathological changes over this time period and that lesions were a more consistent finding than the presence of organisms. This is similar to the present study where lesions were still present throughout all areas of the brain in 12 week old lambs that were euthanased (9 weeks after the last treatment). A possible explanation for this observation of histological lesions in the present study is that tachyzoites proliferation could still be occurring in the neonates but it is likely that only bradyzoites were present in lambs by 12 weeks of age. These are located within cysts that protect them from being detected by the lamb's immune response.

This is the first study examining the efficacy of toltrazuril treatment to control neosporosis in sheep. The potential value of this study was to use sheep as a model for cattle. It is unclear from the previous studies in cattle if bradyzoites were actually present when animals were treated. In this present study the expectation was that at least some of the organisms in the treated animals were bradyzoites within tissue cysts. The results suggest that toltrazuril treatment was not effective in eliminating *N. caninum* infection at this stage of infection. Future studies could investigate different treatment protocols including the treatment of the pregnant dams prior to birth. Effective treatment of infected young animals remains an elusive goal.

7.6 Diagnosis of *N. caninum* infection

One of the requirements of the present study was to adapt and validate a commercially available ELISA as an IgG avidity assay. Results suggest that the assay was able to discriminate between recent and chronic infections in sheep as well as being able to differentiate between lambs with maternal immunity compared to their own *de novo* immunity.

The results suggested that an avidity value of <35% indicated a recent primary infection while a value of >35% was indicative of chronic infection. The assay was then used with samples from other groups of experimentally inoculated sheep as well as samples from naturally infected ewes. Avidity values for all naturally infected ewes were >60% indicating chronic infection. The assay was also used to differentiate between lambs that were born from either recently inoculated or re-inoculated (chronically infected) dams. Lambs from recently inoculated ewes had low S/P and avidity values at 2 weeks of age which increased by 12 weeks of age. In comparison, lambs from re-inoculated ewes had

high S/P and avidity values at 2 weeks of age, due to the influence of the maternal antibodies. This assay as such can be utilized to better understand the kinetics of *N. caninum* infection in sheep.

Interpreting the serological status of an animal can be difficult and thus in the studies described in Chapters 2, 3, 4 and 5, a commercial ELISA for the detection of IgG against *N. caninum* was used in conjunction with western blot analysis. Previous studies using the IFAT in sheep produced unreliable results (Howe et al., 2012; Weston et al., 2009). The particular commercial ELISA used (IDEXX Chekit) is an indirect ELISA which detects antibodies against *N. caninum* in ruminants and has been used previously in studies in sheep and goats (Reichel et al., 2008; Weston et al., 2009; Bishop et al., 2010; Czopowicz et al., 2011). In the current study the use of an S/P value of 11.8% as a cut-off value in sheep proved to be reliable, as recommended by Reichel et al., (2008). For example, if the original threshold of 40% was used (as per the manufacturer's instructions), 13 rams in the study described in Chapter 2 would have been considered as serologically negative despite 5 out of these 13 animals having *N. caninum* DNA detected in their semen and 1 in the brain tissue. A more obvious example would be the ewes that were inoculated in the study described in Chapter 4 where 17 ewes in Group A (Year 1) and all 12 ewes in Group E (Year 2) would be seronegative if the 40% was used as the threshold.

The use of western blot throughout the studies in the current thesis has proven to be a valuable tool as it further confirms the serological status of the infected animals. Many of the animals used in the current studies were experimentally infected and their infection status was known, correlating well with the results of western blot assays. These western blot assays were found to be more sensitive than ELISA. For example, in studies described in Chapters 4 and 5, some of the previously infected ewes in Year 1 were found to have

negative S/P values with the ELISA but were positive by western blot. Similar situations were also seen in lambs born to Group D in Year 2 as described in Chapter 4, with 7 of the lambs seronegative by ELISA being positive by western blot. Other studies have also used western blot assays to confirm the serological status of an animal (Atkinson et al., 2000) and for validating other serological assays (Alvarez-Garcia et al., 2002; Okeoma et al., 2004; Schares et al., 1998). In the current studies, a consistent pattern of recognition of proteins at 10, 17, 26-29, 30, 31, 33 and 37 kDas was observed and these were consistent with other studies examining *N. caninum* infection in cattle, goats and sheep (Barta et al., 1992; Bjerkas et al., 1994; Schares et al., 1998; Sondgen et al., 2001; Naguleswaran et al., 2004; Gaffuri et al., 2006; Rossi et al., 2011) as summarized in Table 1.3 in Chapter 1. No difference in band pattern was recognized between the sera from the experimentally infected animals and the sera of the naturally infected animal that was used as a positive control in this study. This indicates that the western blot assay gives repeatable results regardless of how the animal was infected.

The qPCR used in the studies throughout this thesis were shown to be very sensitive and able to detect the presence of *N. caninum* DNA from <1 tachyzoite in semen or tissue which is consistent with the original report of this particular assay (Okeoma et al., 2005). The use of qPCR for detection and quantification of *N. caninum* DNA provided more information than the standard PCR.

Findings in the current studies suggest that histological methods are an important diagnostic measure and although the lesions are not pathognomonic, they are strongly suggestive of apicomplexan parasite infection (Dubey and Schares, 2006; Pescador et al., 2007). In the current study, the use of histopathology appeared to be more sensitive than the detection of *N. caninum* DNA using qPCR in brain tissues of lambs. For the studies

described in Chapters 4 and 5, using histopathology provided evidence to indicate that the lambs were infected even though *N. caninum* DNA was detected in only a small number of animals.

7.7 Limitations

Studies conducted in this thesis were all carried out under normal grazing conditions on farms at Massey University, thus it was not possible to guarantee that there was no horizontal transmission occurring as we could not confirm that the environment was free from *N. caninum* oocysts from dog faeces. However, throughout the study, the use of dogs on the premises was very limited. It was also not possible to obtain pre-colostral blood which is important in determining that the lambs were infected due to congenital transmission and not by horizontal transmission. However, the use of control sheep that were also kept at the same place was a good indicator to determine if any horizontal transmission had occurred. Throughout all the studies, none of these control sheep, including lambs born, showed any signs of *N. caninum* infection, thus it was safe to suggest that no horizontal transmission had occurred.

Despite detecting a large quantity of *N. caninum* DNA in the semen of rams (in Chapter 2), no bioassay was conducted to determine if the parasites detected were live and viable. In previous studies with experimentally infected cattle, viable tachyzoites could not be detected in bull semen by bioassay (Ferre et al., 2005; Ferre et al., 2008). Another limitation in this study was that, none of the ewes mated to the infected rams were euthanized and necropsied. If this had occurred, tissues could have been taken from the reproductive tract to see if any parasite DNA was present. As the ewes were only tested

using serology and given the limitations of serology indicated above, it is still not possible to say that they were truly uninfected.

Another limitation of the current study is that it did not progress to observe how congenitally infected lambs would grow and whether they would have any reproduction-related problems. Due to financial and time constraints, lambs born in Year 2 (Chapter 4 and 5) could not be kept longer to be bred in the next season. In the future, this could help to better understand if congenitally infected lambs would have any reproductive problems and whether the level of infection was different between those treated and not treated with toltrazuril. Further studies should also investigate the rate of vertical transmission through more generations.

7.8 Contributions to the field of knowledge from research presented in this thesis

One of the important findings in this study is that more information on the modes of transmission was gathered and this helps to better understand this disease in sheep. It was evident that venereal transmission did not occur from experimentally infected rams to the mated ewes, despite the high concentration of *N. caninum* DNA detected in the semen.

The inability of toltrazuril to eliminate N. caninum infection from lambs highlights the difficulty of effectively treating neonatal ruminants when the bradyzoites stage is present.

The dynamics of neosporosis in sheep and cattle appear to be reasonably similar and thus sheep should provide a useful model to investigate treatment protocols for cattle. Studies using sheep is likely to be more representative of the true situation when dealing with ruminant animals as compared to murine model studies. The use of sheep allows a larger number of animals in experimental studies than is possible with cattle.

An important tool that was developed during this project was the avidity assay for use with sheep serum. This makes it possible to discriminate between recent and chronic infection and could provide a useful method for determining the involvement of *N. caninum* in abortion cases on sheep farms.

The diagnosis of *N. caninum* infections and determining whether it is playing a role as an ovine abortifacient agent is not easy. In diagnosing neosporosis, the biggest obstacle is determining the true status of the animal and the difficulty in detecting the parasite. Thus the use of multiple diagnostic tests is recommended to more fully determine the involvement of *N. caninum*.

An important observation that has implications for vaccine development was the continued vertical transmission in ewes inoculated prior to pregnancy. In particular the observation of Group D (in Chapter 4) ewes inoculated prior to pregnancy in Year 1 clearly gave birth to vertically infected lambs in Year 2. The the Toxovax® vaccine in sheep is effective because it mimics the natural situation in sheep where infection prior to pregnancy prevents vertical transmission in subsequent pregnancies. It would seem that infection with *Neospora* does not follow this biological pattern and hence similar vaccines are unlikely to be effective.

7.9 Future studies

Results obtained have still not resolved the question as to whether *N. caninum* plays an important role in causing reproductive problems in sheep in New Zealand. Clearly a better understanding of the epidemiology of neosporosis in sheep is required. In particular, the risk factors which lead to neosporosis in sheep being a problem need to be defined. Abortion storms in cattle are commonly the results of oocyst ingestion by pregnant cows

and it is likely that the same situation occurs with sheep. Consequently, the potential role of dogs on sheep farms needs further investigation. For example, Information is required to determine how easily they are infected when ingesting ovine tissues. The most common way that cattle get infected is by the congenital route. Whilst the present studies investigated this aspect some of the results were unexpected and further studies are required to more fully explore this route of infection. Further studies should also include detection of parasite DNA in the reproductive tissues of the ewes mated as well as the infection status of the lambs born.

In this thesis the usefulness of toltrazuril was investigated in young lambs. This was ineffective but the problem remains as to which chemotherapeutic agents may be useful to allow clinicians to break the cycle of vertical transmission. Chemotherapeutic agents are also required for use with a group of ruminants suffering from *Neospora*-induced abortions and the sheep model provides a useful tool for investigating any likely drugs. Finally, as suggested by others, our studies have shown that sheep could be a good model for the disease in cattle although some aspects of the infection in sheep still remain to be defined.

The most likely therapeutic agents that would provide effective parasite control in cattle will be some form of a vaccine. The sheep model is likely to be a useful tool during this development and any resulting vaccine may also be useful in sheep.

In conclusion, the effort to study neosporosis in New Zealand sheep has proven beneficial to further understand the dynamics of this disease and opens more doors for further studies to be undertaken.

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Appendix 1: Chapter 2: IDEXX S/P values (%) for rams' serology response towards *N. caninum* at pre-inoculation and 1 month post inoculation with live *N. caninum* tachyzoites

ID	*N. caninum* tachyzoites dose	GRP	IDEXX S/P VALUE (%)	
			Pre-Inoculation	I month post inoculation
1	CTRL	1	-0.8	-0.1
3	CTRL	1	-1.2	-2.9
4	CTRL	1	-0.8	-2.8
5	CTRL	1	-0.8	-1.8
7	CTRL	1	-1.2	-3.3
8	CTRL	1	-1.5	-3.3
9	CTRL	1	-1.3	-3.3
11	CTRL	1	-0.7	-3.3
12	50	2	-1	16
13	50	2	-1.3	36.6
14	50	2	-1.3	11
15	50	2	-1.6	18.3
19	50	2	-1.6	12.4
20	50	2	-1.3	16
21	50	2	-2	34.9
22	50	2	-1.8	28.4
24	10^3	3	-2	19.1
26	10^3	3	-2.2	11.9
27	10^3	3	-1	24.3
28	10^3	3	-1.3	13.7
32	10^3	3	0.1	18
33	10^3	3	-1.7	39.5
35	10^3	3	-1.1	24.2
36	10^3	3	-1.6	19.6
37	10^8	4	-1.4	58.4
39	10^8	4	-0.3	39.8
40	10^8	4	-1.3	56.2
41	10^8	4	-2.1	76.1
43	10^8	4	-2.4	69.2
45	10^8	4	-1.5	78.9
46	10^8	4	-1.7	81
47	10^8	4	-2.6	75.2

Appendix 2: Chapter 2: IDEXX S/P values (%) for ewes at 1 and 2 months post mating with rams

EWE ID	GRP	IDEXX S/P VALUE (%)		
		PRE MATING	1 MO POST MATING	2 MO POST MATING
48	1	-0.7	-2.7	-0.6
49	1	-0.3	0.5	0.0
50	1	-1.4	-2.5	-1.1
51	1	-0.5	-1.6	0.0
52	1	-1.9	-2.9	-1.1
53	1	-1.0	-0.5	0.3
54	1	-0.3	-0.4	0.0
55	1	-1.2	-0.1	0.0
56	1	-0.9	-1.5	-0.3
57	1	0.0	-0.7	-0.6
58	1	-1.2	-2.2	-0.3
59	1	1.5	0.5	-0.8
60	1	-0.9	-2.2	-0.6
61	1	6.7	6.4	4.2
62	1	-1.2	-2.9	-2.2
63	1	-0.6	-2.1	-1.5
64	2	2.2	2.1	-0.5
65	2	6.9	6.6	2.0
66	2	-1.4	-2.7	-2.2
67	2	0.6	-1.9	-0.5
68	2	-1.6	-2.3	-2.2
69	2	0.0	-2.3	-1.0
70	2	-1.0	-3.2	-2.7
71	2	0.6	-2.2	-2.2
72	2	4.1	5.2	2.2
73	2	-1.2	-2.5	-1.5
74	2	-1.1	-2.1	-1.3
75	2	-1.7	-2.4	-1.0
76	2	5.4	5.7	6.0
77	2	0.0	-0.8	3.0
78	2	-1.5	-2.5	-0.7
79	2	0.7	-1.0	-0.3

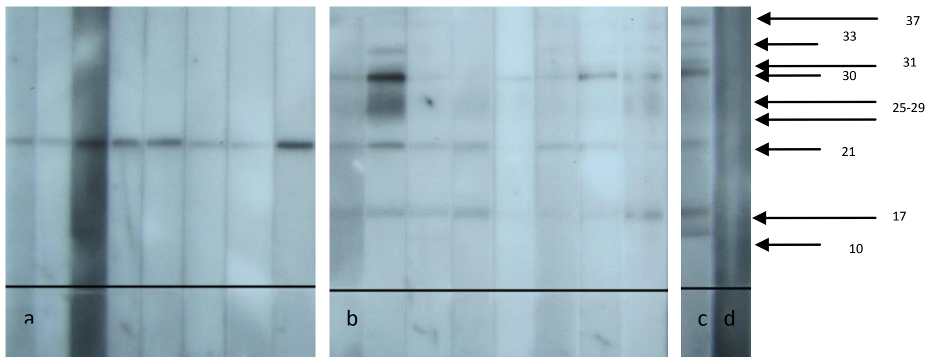
Group 1 mated with 3 rams from the control group

Group 2 mated with 3 rams from group 4 (infected with 10^7 Nc tachyzoites)

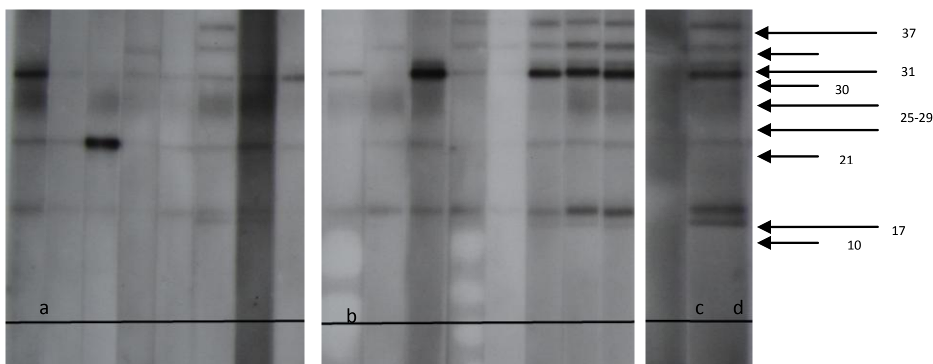
Appendix 3: Chapter 2: Western blot results of rams' sera against eight IDAs of *N. caninum*

PROTEIN BANDS GROUPS/ID	IDEXX S/P % VALUE	10	17	21	25-29	30	31	33	37
POSITIVE CONTROL	85	+++	+++	++	+	+++	++	++	++
GROUP 1									
RAM 1	-0.1			+++					
RAM 3	-2.9			+++					
RAM 4	-2.8			+++					
RAM 5	-1.8			+++					
RAM 7	-3.3			+++					
RAM 8	-3.3			++					
RAM 9	-3.3			++					
RAM 11	-3.3			+++					
GROUP 2									
RAM 12	16		++	++	+	++			+
RAM 13	36.6		++	+++	+++	+++	+	++	+
RAM 14	11	+	++	++		+			+
RAM 15	18.3		++	++	++	+			
RAM 19	12.4		+	+	+	++			
RAM 20	16		+	++	+	++		+	+
RAM 21	34.9		++	++	+	+++	++	+	+
RAM 22	28.4	+	+++	++	++	+++	+	++	++
GROUP 3									
RAM 24	19.1		++	++	++	+++	++		
RAM 26	11.9		+	++	+	+			
RAM 27	24.3		+	+++	++	+			
RAM 28	13.7		+	+	+	++		++	
RAM 32	18		++	++	+	++			
RAM 33	39.5	++	++	++	++	++		++	++
RAM 35	24.2	+	+	++	++	++			
RAM 36	19.6		+	+	+	++	+		
GROUP 4									
RAM 37	58.4		++		+	++			
RAM 39	39.8	+	++	+	++	+		++	
RAM 40	56.2	+	++	++	++	+++	++	+	+
RAM 41	76.1		++	+		++		++	++
RAM 43	69.2		+	+		+		++	+
RAM 45	78.9	++	++	++	+	+++	++	++	++
RAM 46	81	++	+++	++	++	+++	++	++	++
RAM 47	75.2	++	+++	++	++	+++	++	++	++

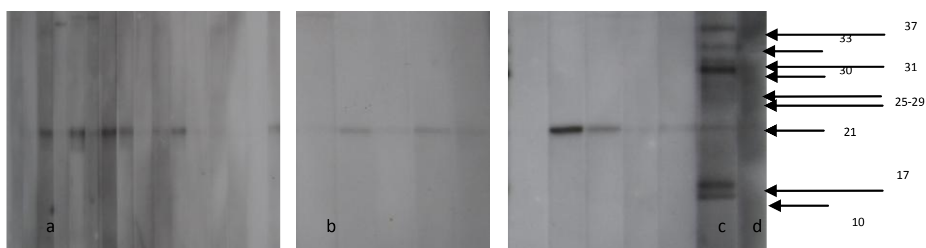
Appendix 4: Chapter 2: Western blot showing proteins recognized by antibodies in the sera of rams in (a) Group 1 (control rams) Lane 1-8, (b) Group 2 (inoculated with 50 Nc tachyzoites) Lane 9-16, (c) positive control sera and (d) negative control sera



Appendix 5: Chapter 2: Western blot showing proteins recognized by antibodies in the sera of rams in (a) Group 3 (inoculated with 10^3 Nc tachyzoites) Lane 1-8, (b) Group 4 (inoculated with 10^7 Nc tachyzoites) Lane 9-16, (c) positive control sera and (d) negative control sera



Appendix 6: Chapter 2: Western blot showing proteins recognized by antibodies in the sera of (a) Group 2 ewes mated with experimentally infected ram from Group 4, (b & c) Group 1 ewes mated with rams from the Group 1 (control)-8, (c) positive control sera and (d) negative control sera



Appendix 7: Trypan blue exclusion test to determine the viability of tachyzoites and vero cells.

(Strober, W., Trypan Blue Exclusion Test of Cell Viability, Appendix 3B, Current Protocols in Immunology, 1997 A.3B.1-A.3B.2, John Wiley & Sons, Inc.)

- Used to determine the number of viable cells present based on the principle that live cells possess intact cells membranes that will exclude certain dyes such as trypan blue, eosin or propidium while dead cells don't. Live viable cells have clear cytoplasm while non viable cells will uptake the dye and will have a blue cytoplasm
- Cell suspensions were centrifuged at 100xg for 5 minutes and the supernatant were discarded.
- The cell pellet were re-suspend in 1 ml of PBS or serum-free MEM (note: serum proteins stain with trypan blue and can produce misleading results)
- Make a 1/100 dilution of cell suspension (if not the count will be too high). Then take 5 ul of the diluted cell suspension and add 15 ul of trypan blue dye. The mixture was mixed thoroughly using the pipette.
- 10 ul of the mixture were placed onto the haemocytometer and were observed under the compound microscope (cells were counted within 3 to 5 min of mixing with trypan blue as longer incubation periods will lead to cell death and reduced viability).
- Viable cells (unstained) and non viable cells (stained) were counted and differentiated.
- Final number of viable cells were calculated as below:
  - Average number of cells counted (in 4 quadrants divide by 4) X 4 (dilution factor of trypan blue) X 100(dilution factor for cells) X 10^4
    -

Appendix 8: Protocol for IDEXX ELISA

- All reagents were allowed to come to room temperature and mixed gently before use.
- Wash solution were prepared by diluting the 10X Wash to a 1:10 with distilled water (100ml 10X + 900ml distilled water). The Chekit-Wash Solution can be stored for 1 week at 2^oC to 8^oC
- Using the U-bottom 96 wells plate,dispense 90ul of Chekit-Neospora into each well of the plate
- 10ul of the indiluted test samples were added into the appropriate wells including the positive and negative controls giving a final dilution of 1:10.
- Using a multi-channel pipette, transfer all mixture from the U-bottom plate to the IDEXX test plate.
- The plate was then covered with a parafilm and incubated for 60min (+/- 5 minutes) at 37^oC (in the 37^oC room). Contents of the plates were mixed by gentle shaking using a plate shaker.
- After the incubation, flick the plate to remove all solutions from the wells. Care must be taken not to contaminate the wells with flowing fluids. Washing was done for 3 times with 200ul of IDEXX 1X wash solution. Following each wash, the plate is tap gently on a piece of paper towel and care taken not to contaminate other wells.

- 100ul of CHEKIT-NEOSPORA-Anti-Ruminant IgG-PO Conjugate were added into each well
- Plate was covered with the parafilm and incubated for 60minutes (+/- 5 minutes) at $37^{o}C$.
- After incubation with the secondary antibody, washing was performed 3 times with 200ul of 1X CHEKIT wash solution at room temperature.
- 100ul of CHEKIT-TMB Substrate were added into each well
- The plate was incubated at room temperature ($18^{o}C$-$25^{o}C$) for 15 minutes. Kept the plate in a dark area.
- 100ul of CHEKIT–STOP solution TMB was added to stop the colour reaction. The stop solution was dispensed in the same order and at the same speed as the substrate.
- The results were read using a photometer at a wavelength of 450nm.
- Percentage of avidity was calculated by the following equation:
- The OD of the positive control and the OD of the test samples were corrected first by subtracting the OD of the negative covntrol.

Eg: $SP_{sample} = OD_{SAMPLE} – OD_{NEG\ control} / OD_{POS\ control} – OD_{NEG\ contro}$

Appendix 9: Protocol for western blot for the detection of *N. caninum* antibodies in sheep sera

Test Procedure

A. DAY 1:Sample preparation

- Using the 1 gel prep, calculate what volume of sample needs to be added to each lane. We are using 10ug of sample/well x 10 = 100ug/1 big prep. (max load is 800ul)
- Example: using N2 as antigen = 0.26mg/ml protein; therefore 100ug/260ug/ml = 0.3846ml = 384.5ul of N2 to be added in a micro tube.
- used 79ul of ag + 131dH2O + 200ul of 2X Laemli buffer
- If with a 10 well mini gel, a volume (including sample and buffer) of no more than 50 µl is recommended for each lane. Usually we do up to 40ul/well with the concentration of 10ug/well. If the volume is too much then try to reduce the concentration of the antigen used.
- If using the buffer 2X, add the same amount of buffer as the amount of antigen used. Therefore, add 385ul of buffer to the micro tube and 385ul of N2 and vortex.
- Put a cap on the micro tube to secure it and boil the micro tube at 100°C for 10 minutes. Boiling is done by placing the micro tube in a tray and immersing it in boiling water in a bicar. Another method is by placing the micro tube in a micro tube heater plate and set the temperature at 100°C for 10 minutes.
- Prepare the SDS-PAGE running buffer (add 100 ml of running buffer with 900 ml dH20)

B. Electrophoresis Separation (SDS-PAGE)

SETTING UP

- Pour the running buffer into the Western Blot tank.
- Take out the gel plate from the container. Carefully remove the comb from the plate and remove the tape as well. Flush the plate with dH_2O. Remove the tape from the bottom of the cassette.
- Insert the Criterion gel into one of the slots in the Criterion tank. Ensure that the upper buffer chamber of the gel is facing toward the centre of the cell.
- Fill the inner compartment (between the two gels) with the buffer till the full level.
- Fill the upper buffer chamber in each Criterion gel till full (up about 60 ml buffer)
- * Fill the tank to the line moulded into the sides of the tank or the lower edge of the gels upper buffer chamber (approximately 800ml). It is important to fill the tank to the proper level to prevent overheating during running. If the tank is over-filled, buffer will/may overflow onto the bench.
- * Must also remember not to fill the tank after loading the samples

SAMPLE LOADING

- Load the samples into the wells with a pipette using gel loading tips
- Add 10ul of ladder (pre-stained marker) on both sides of the gels as the marker
- Carefully load the samples into the wells (using a fine-tipped pipette). Careful not to penetrate/puncture the bottom of the well with the pipette tip.
- Place the lid on the tank and plug it into the power source. (Note: this procedure uses electric current to separate proteins – use caution when working with tank electrophoresis to avoid injury). Ensure that the red to red and black to black rods.
- Run the apparatus at 200V for 1 hour or 50 minutes and allow the samples time to separate; use a pre-stained molecular weight marker to determine the end-point of the electrophoresis (make sure the sample proteins do not run out of the gel).
- From 2010 run gel at 100MV for about 1.5 hours or till the blue lane reaches 2/3 of the gel and that the 10kDa marker has separated as well.

GEL REMOVAL

- After electrophoresis is complete, turn off the power supply and disconnect the electrical leads
- Remove the lids and carefully lift out the Criterion gel cassette. Pour off and discard the running buffer
- Use the cassette-opening tool built into the lid to break the weld-joint on the Criterion gel cassette. Place the Criterion gel cassette's upper buffer chamber over the built-in opening wedge of the lid. Push the cassette straight down until the upper edge of the upper buffer chamber contacts the top of the lid and weld-joint at the top of the cassette is broken. Pull the cassette halves apart. An alternative method is to run the comb down the sides of the cassettes to break the weld-joint and to pull the halves apart.
- It is best to remove the gel by floating it off the plate. Invert the gel and plate under the transfer solution and agitate gently until the gel separates from the plate.
- Rinse the Criterion tank and lid with distilled, deionized water after use
- After SDS-PAGE, we can either stain it with Comassie stain or do western blotting.

C. COMASSIE STAIN

- Staining with Comassie stain can be done by immersing the gel in comassie stain for a few hours on a shaker (up to an hour or till proteins can be seen).
- Then the solution is removed and adds in de-stain solution and placed on a shaker.
- Change the solution a few times until the comassie stain is totally removed from the gel. Prepare the gel-drying-drying apparatus.
- The gel is then placed on SDS PAGE cellophane gently.
- Ensure no bubble is present and put a lot of dH20 on the gel and cellophane.
- Put another layer of cellophane on top and ensure the frames are tightly stretched and no air bubbles present.
- Screw the frame and let it stand to dry. Cracking of the gel can occur due to presence of bubbles, lack of dH20 and cellophane not stretched properly.

D. Electrophoresis Transfer to PVDF Membranes

PREPARATION FOR BLOTTING

- Freeze ice block prior to preparation of blot assembly
- Prepare the transfer buffer (add 100ml of transfer buffer with 200 ml methanol and 700 ml dH20) and let it cool to 4o C overnight. Using buffer pre-chilled to 4°C improves heat dissipation
- Care must be taken when handling PVDF (polyvinylidene fluoride) membranes – use forceps and handle membranes by their edges so as not to damage the surface
- Cut filter paper (typical chromatography paper) in approximately 7 X 20 cm pieces (cut them earlier)
- Pre-wet the PVDF membrane using 100% methanol for 10 seconds and immerse in transfer buffer
- Soak the filter pads in PVDF Transfer Buffer
- Equilibrate gel in transfer buffer for 15 minutes
- Always wear clean gloves when handling the transfer cell, membranes, filter paper or gels to prevent contamination

SET UP OF THE TRANSFER APPARATUS

- Fill the Criterion Blotter tank with transfer buffer to about 50% of the fill volume
- Place a magnetic stir bar inside the tank
- Place the ice block in the ice block pocket at the back of the cell. Flip down the lever to hold the ice block down

SET UP OF THE GEL/MEMBRANE SANDWICH

- Pour chilled transfer buffer into each compartment of the gel/blot assembly tray
- Place the PVDF membrane in the front/small compartment of the tray. Let it soak while preparing the rest
- Place the cassette in the back/large compartment of the tray – open the cassette so that the red side with the handle is vertical (anode) and the black side (cathode) is laying horizontal and submerged in transfer buffer
- Place a fibre pad on top of the black side of the cassette, submerged in buffer. Push on the fibre pad with gloved finger tips to thoroughly soak the pad
- Place a piece of filter paper on top of that fibre pad
- Gently place the pre-equilibrated gel on top of the filter paper. Use the roller to remove any air bubbles that may be trapped underneath the gel (important step)
- Take the membrane from the front compartment and place it on top of the gel taking care not to trap any air bubbles. The membrane should not be moved or adjusted once it touches the gel because this can cause data ghost prints and artefacts. If need to adjust the membrane placement, use a fresh pre-wetted membrane. Use the roller to roll out bubbles.
- Place a piece of filter paper on top of the membrane. Run the roller gently over the top of the filter paper to remove any air bubbles trapped in the sandwich
- Wet a second fibre pad in the front compartment of the tray (where the membrane was soaking) again using finger tips to completely saturate the pad with transfer buffer. Then place the wet fibre pad on top of the second filter paper.
- Lower the clamp-side of the cassette and lock in the closed position.

TRANSFERRING

- Move the locked cassette into the groove in the blotter tank, aligning the red side of the card with the red electrode. Make sure the magnetic stirrer is free to move
- After cassette is in place, add the remaining transfer buffer to the fill level marked on the tank
- Put the lid on, plug the cables into the power supply and run the blot
- Transfer at a constant voltage at 100mv for 30 min. if voltage only reaches 70mv, then run it for 45-50 min
- transfer is set on 66mv for 55 min.
- Upon completion of the run, disassemble the blotting sandwich and remove the membrane for development. Clean the cell, fibre pads and cassette with multiple rinses of deionized water

E. WESTERN BLOT PROTOCOL

Staining with Ponseau Stain

- Soak the membrane in the ponseau stain solution for about 1 min to stain the membrane. Should be able to see the outline of the bottom and side lines of the membrane (outlines showing the demarcation of the bottom of the protein and standard markers)
- Rinse the membrane with running water till its clear of the ponseau stain
- Using a blue pencil (water colour pencil?) draw out the lines (to mark the bottom, left and right outlines)
- Place the membrane on a glass and cut the membrane into 24 strips (or as many as required) using a scalpel blade and a smaller glass slide as a ruler. Label each strip according to the sequence so that it can ease in rearranging them later during detection. Ensure the strips are kept moist.
- from 2010 just cut the membrane into two for blocking. Cutting of the strips can be done the next day – would ease placing them in the well-thorough the next day

Blocking

- After cutting the membrane into strips, block the membrane in 5% Blotto at 4° C room, on a shaker for overnight
- This can also be done at 37% for 1 hour
- Blotto is made using PAM's skim milk

DAY 2 : PRIMARY ANTIBODY

- After incubation overnight, placed the strips into the wells of the reservoir (8 slots reservoir). The strips with the standard markers on can be soaked with PBST solution till required later.
- Add in 1ml of sera sample (10ul of sera sample + 990ul blotto) at 1/100 diluted in 5% blotto (dilute the antibody with a 5% Non Fat Dry Milk (NFDM) in Tween PBS solution). Care to be taken not to contaminate the other wells.
- Incubate for 1 hour at 21°C (in the incubation box). Turn on the "see-saw" plate shaker. Important to do this or sera will not mix well with the strips. Ensure all plates are closed with the lids provided.
- Washing with PBST: Put each strip into a 50ml conical tube which had been filled with PBST earlier. Place these tubes on a rack (24 tubes/rack) which is placed on a plate shaker. Shake the rack vigorously 10 min. Carefully removed all the wash fluids and filled up again with PBST. Washing is done for 3 times, 10 minutes each.

Incubation with conjugated 2° antibody

- Place all strips into 1 container and add 2° ab
- 1/10000 dilution (5ul 2° ab in 50 ml blotto) in 5% blotto (may have to make up to 50 ml). Cover container with parafilm.
- Incubate at room temperature on a "see-saw" shaker or on a plate shaker to ensure that all strips were covered. Go to the xray room and turn on the machine. The machine needs about 30minutes to warm up.
- Washing with PBST: Remove the 2° ab from the container and filled up the container with PBST. Put all the strips into 1 reservoir and wash with PBST 3 times at 10 min interval between each wash.

Detection

- Prepare the chemi-luminescent reagents. It is important to prepare the ECL solution just prior to use in order to maximize its effectiveness
- Open the x-ray cassette and place a clean transparency plastic (cut the A4 size into 2 and just use half first) onto the x-ray cassette
- Add in 2 ml (1ml of solution 1 and 1 ml of solution 2) of the chemi-luminescent solution onto a reservoir
- Using a forceps, take one strip at a time and soak it into the chemi-luminescent and drain excess fluid by putting the tip of the strip on a piece of a clean tissue paper.
- Place the strip onto the transparency plastic. Ensure to lay the membrane face up on the transparency (acetate)
- Repeat this with all the other strips. Ensure to place the strips with the standard markers as well on both sides.
- Arrange the strips accordingly (with the help of the line made with the blue pen earlier)
- Place another piece of transparency plastic on top of the strips and ensuring excess fluids are dried out using a piece of tissue paper. Do not move the plastic or rough handling to ensure the strips are not misplaced or misaligned
- Close the cassette
- Go down to the dark room (bring along gloves, timer, marker pen and camera)
- In the dark room, remove one x-ray film and fold the end of the left top corner of the film as a marker
- Open the cassette and place the x-ray film gently. Close the cassette and time for 1 minute
- Quickly open the cassette and remove the film and put it in the developer
- If the exposure does not appear to be good, do a few more exposure with different time e.g.: 30 sec, 40 sec or 1 min
- Care should be taken as not to misaligned the strips on the transparency
- After the film has been developed, place the x-ray film back on the cassette and trace the lines where the markers are. This is to help with estimating the bands present.
- After making sure the lines are drawn accordingly, placed the film on the x-ray reader and take an image using a digital camera.

1. Solutions Preparations

    a. Ponceau S
    - 1g Ponceau S
    - 50 ml acetic acid
    - add both and add dH20 to make up 1 litre

    b. Running Buffer
    - 100 ml running buffer
    - 900 ml dH20

    c. Transfer buffer
    - 100 ml of transfer buffer
    - 200 ml methanol
    - add to 700 ml dH20
    - keep in the fridge overnight

    d. 5% Blotto (using Pam's skim milk powder) in PBST
    - Example to make 50mls, (5% X 50ml) = (100% X A); therefore A = 2.5 gm of milk powder and add into 50ml PBST
    - Make sure add a magnet ball on the stirrer and let it run for awhile.

    e. 0.4gm Comassie Stain
    - 200ml of 40% methanol (80ml methanol + 120 ml dH20 + 0.4gm of comassie)
    - 200ml of 20% acetic acid (40ml of acetic acid + 160ml dH20)
    - add both into 500 ml bottle

    f. De-stain solution
    - 500 ml methanol
    - 300 ml dH20
    - 100 ml acetic acid
    - add dH20 to make 1 litre

Appendix 10: SOP for DNA extraction of blood, semen and tissue samples

DNA extraction

- Tissues and semen 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 or whole blood.

- 100 ul of whole semen and blood diluted in 100ul of PBS were used for each sample DNA extraction.

- In the semen, most of the DNA is comprised in the sperm head, which is known to have inhibitory effects on PCR (van Engelenburg et al., 1993). When the semen samples were treated with a lysis buffer and proteinase K, we observed that the non-sperm cells and spermatozoon tails were lysed but the sperm head remained intact. Semen samples were diluted prior to use in the PCR assay to reduce any residual inhibitory components.

- Five grams of each brain sample were placed into a sterile, single use, 50 mL conical tissue grinder (VWR, PA, USA) containing 10 mL of sterile PBS. Brain tissues were homogenized and 100 µL used for DNA extraction using the DNeasy Tissue Kit.

- Water blanks were included as sample processing controls to confirm lack of contamination during sample manipulation. All extracted samples were kept at -20° C until further analysis was performed.

Appendix 11: SOP for histology slides

- At PM, cut tissues not more than 1 cm thickness to ensure all tissues would be fixed well.
- Get the accession number from the histology lab
- Cut the tissues to the size of about 0.5 cm square to that it could fit the tissue cassette.
- For the brain sections, cut into the frontal lobe, mid brain and cerebellum and another one section going horizontally at the midbrain section.
- Arranged the 4 cuts into the cassettes and closed it well.
- Label the cassette according to the accession number given.
- Submerged the cassette into the 10% formalin bucket
- Cassettes would be picked up by the histology lab

Histology Processing
Fixation = 10% Neutral Buffered Formalin
Processing = Leica TP1050 Tissue processor

PROGRAM 3 LARGE SAMPLES						
REAGENT	STATION	TIME HR:MIN	TEMP Deg C	P/V	DRAIN Secs	STIR
Formalin	1	1:15	40°C	AMB	120	ON
Ethanol 70%	2	:30	AMB	AMB	120	ON
Ethanol 95%	3	1:00	40°C	AMB	120	ON
Absolute Ethanol	4	1:15	40°C	AMB	120	ON
Absolute Ethanol	5	1:15	40°C	AMB	120	ON
Absolute Ethanol	6	1:15	40°C	AMB	120	ON
Ethanol/Xylene	7	1:20	40°C	AMB	120	ON
Xylene	8	:45	40°C	AMB	120	ON
Xylene	9	:45	40°C	VAC	120	ON
Paraffin Wax	LEFT	1:20	59°C	VAC	120	ON
Paraffin Wax	MIDDLE	1:20	59°C	VAC	120	ON
Paraffin Wax	RIGHT	1:20	59°C	VAC	120	ON

The processor is programmed to start at 5.30pm and finishes at 7.30am the following morning.

Embedding = Leica Histo Embedder

Merck Histosec Pastilles® Tissue Embedding Medium (Melting Point 56°C-58°C)

Cutting = Leica RM2235 Manual Rotary Microtome

S35 Feather Microtome Blade (stainless steel)

Slides = Menzel-Glaser Superfrost PLUS Slides 76 x 26mm Ground edge 90º (White)
Distributed by Lomb Scientific Pty Ltd New South Wales , Australia

Waterbath = Thermo Electron Corporation Circular Paraffin Section Floatation Bath

Water at 40°C

Staining = Leica Autostainer XL

Program 1 Haematoxylin and Eosin

STEP	STATION	REAGENT	TIME(min:sec)	EXACT
1		OVEN	10:00	Y
2	1	XYLENE	3:00	Y
3	2	XYLENE	2:00	N
4	3	ABSOLUTE ALCOHOL	1:30	Y
5	4	ABSOLUTE ALCOHOL	0:30	N
6	5	70% ALCOHOL	1:30	N
7		WASH 1	1:00	N
8	7	GILL'S (II) HAEMATOXYLIN	4:00	Y
9		WASH 2	0:30	Y
10	6	SCOTTS TAPWATER	0:30	Y
11		WASH 3	1:00	Y
12	8	EOSIN/PHLOXINE	2:00	Y
13		WASH 4	0:30	Y
14	13	70% ALCOHOL	0:10	Y
15	14	95% ALCOHOL	0:15	Y
16	15	ABSOLUTE ALCOHOL	0:45	N
17	16	ABSOLUTE ALCOHOL	1:00	N
18	17	XYLENE	1:00	N
19	18	XYLENE	1:00	N
20		EXIT		

Appendix 12: The protocol for modification of the IDEXX ELISA for determining IgG avidity values

- All sera samples positive with the IDEXX ELISA with S/P % of more than 11.8% are tested for avidity.
- All protocols are as described by the manufacturer with a modification of the first wash after the antibody-antigen incubation period.
- All reagents were allowed to come to room temperature and mixed gently before use.
- Wash solution were prepared by diluting the 10X Wash to a 1:10 with distilled water (100ml 10X + 900ml distilled water). The CHEKIT-Wash Solution can be stored for 1 week at 2°C to 8°C
- 6M Urea in CHEKIT-Wash solution was prepared earlier.
- Urea = 60.06 Molecular weight (MW) :to prepare use the formula:
- Molarity = moles salute/L; = weight of substances(gm) /MW X 1000/volume(ml)
- E.g. 6M urea = (60.06MW X 6 X100)/1000 = 36.04gm added to 100ml of CHEKIT wash solution
- Solution is prepared earlier and mixed using the electric stirrer until solution becomes clear. After use the solution can also be stored in the fridge at 2°C to 8°C for a week. Solution would appear cloudy when kept overnight but should be clear when mixed well at room temperature using the magnetic stirrer before use.

- Using the U-bottom 96 wells plate, dispense 90ul of CHEKIT-Neospora into each well of the plate
- 10ul of the undiluted test samples were added into the appropriate wells including the positive and negative controls giving a final dilution of 1:10. All samples were run in duplicates (lane 1 to 6 without urea wash, lane 7 to 12 with urea wash). For both sets of duplicates, positive and negative controls provided by the kit were added.
- Using a multi-channel pipette, transfer all mixture from the U-bottom plate to the IDEXX test plate.
- The plate was then covered with a parafilm and incubated for 60min (+/- 5 minutes) at 37°C (in the 37°C room). Contents of the plates were mixed by gentle shaking using a plate shaker.
- After the incubation, flick the plate to remove all solutions from the wells. Care must be taken not to contaminate the wells with flowing fluids. For the first wash, each 1 sample duplicate (eg from lane 7-12) was done with approximately 200ul of 6M urea solution while for the other duplicates (from lane 1 to 6) with 200ul of CHEKIT wash solution. Washing was done for 5 minutes with gentle shaking at 37°C. For the 2nd and 3rd wash, each well were washed with IDEXX 1X wash solution for 5 minutes at room temperature. Following each wash, the plate is tap gently on a piece of paper towel and care taken not to contaminate other wells.
- 100ul of CHEKIT-NEOSPORA-Anti-Ruminant IgG-PO Conjugate were added into each well

- Plate was covered with the parafilm and incubated for 60minutes (+/- 5 minutes) at 37°C

- After incubation with the secondary antibody, washing was performed 3 times with 200ul of 1X CHEKIT wash solution at room temperature.

- 100ul of CHEKIT-TMB Substrate were added into each well

- The plate was incubated at room temperature (18°C-25°C) for 15 minutes. Kept the plate in a dark area.

- 100ul of CHEKIT–STOP solution TMB was added to stop the colour reaction. The stop solution was dispensed in the same order and at the same speed as the substrate

- The results were read using a photometer at a wavelength of 450nm

- Percentage of avidity was calculated by the following equation:

$$\text{Avidity Index \%} = \frac{\text{OD (urea treated samples)}}{\text{OD (untreated)}} \times 100$$

Appendix 13: Chapter 4: S/P ratios for ewes in Groups A and B before and 1 month post inoculation in Year 1

NO	GROUP	S/P BEFORE INO	S/P AFTER INO
1	A	-1.34	29.5
2	A	2.18	25.96
3	A	1.01	25.1
4	A	1.85	32.85
5	A	-1.34	42.15
6	A	-0.84	21.17
7	A	-0.62	14.75
8	A	1.39	49.9
9	A	-0.15	20.5
10	A	1.39	40.33
11	A	-0.46	21.84
12	A	-1.24	35.73
13	A	3.1	40.13
14	A	-0.62	21.84
15	A	0.46	55.17
16	A	-0.15	29.02
17	A	0.46	34.58
18	A	-1.24	33.62
19	A	-1.08	34.39
20	A	-1.55	45.11
21	A	-0.77	47.99
22	A	-1.08	31.32
23	A	-1.86	24.62
24	A	-2.01	22.03
25	A	-0.93	47.13

NO	GROUP	S/P BEFORE INO	S/P AFTER INO
1	B	-1.3	-2.01
2	B	-0.37	-0.57
3	B	-1.48	-2.68
4	B	-1.48	-1.44
5	B	-0.37	-1.53
6	B	1.48	2.2
7	B	0	-0.19
8	B	0.51	-1.34
9	B	1.54	0.57
10	B	1.54	1.05
11	B	0.34	-2.2
12	B	-1.03	-2.39
13	B	-0.51	-1.82
14	B	0.17	-1.92
15	B	0	-1.92
16	B	-0.86	-1.92
17	B	2.4	-1.63
18	B	3.77	-2.3
19	B	0.34	-0.57
20	B	1.71	-1.15
21	B	-0.34	-1.63
22	B	-0.17	-0.67
23	B	-0.84	-1.72
24	B	2.52	3.26
25	B	5.7	4.9

Appendix 14: Chapter 4: S/P values (before and post inoculation) and western blot (post inoculation) results for ewes in Groups C, D, E and F in Year 2

				EWE: Immunodominant Antigen (kDa) detected by western blot								WESTERN BLOT RESULT
NO	GROUP	S/P BEFORE INO	S/P AFTER INO	10	17	21	26-29	30	31	33	37	
1	C	8.99	50.65	+	++		+++	++	+		++	POS
2	C	36.83	68.05	++	+++		+++	+	++	++	++	POS
3	C	16.96	51.17	++	+++		+++	++			++	POS
4	C	32.63	47.01	++	+++		+++	++	++	++	++	POS
5	C	48.59	32.73		++		++	+			++	POS
6	C	31.18	62.6	++	++		++	++	+	+	++	POS
7	C	24.16	15.06	++	++	++++	++	+			+	POS
8	C	18.88	53.77	++	+++		++	++	+		++	POS
9	C	59.43	57.92	++							++	POS

				EWE: Immunodominant Antigen (kDa) detected by western blot								WESTERN BLOT RESULT
NO	GROUP	S/P BEFORE INO	S/P AFTER INO	10	17	21	26-29	30	31	33	37	
1	D	11.94	8.05			++	+				+	POS
2	D	8.31	10.91			++	++			+		POS
3	D	36.1	15.06			+	+			+		POS
4	D	44.3	26.75			+	+	+		+	+	POS
5	D	44.76	23.9	++	++	+++	++	++			++	POS
6	D	33.82	16.36		+	++	+					POS
7	D	31.18	11.43		++	+++	+	+			+	POS

NO	GROUP	S/P BEFORE INO	S/P AFTER INO	EWE: Immunodominant Antigen (kDa) detected by western blot								WESTERN BLOT RESULT
				10	17	21	26-29	30	31	33	37	
1	E	-3.19	22.86		++	++	++	++	++	++	++	POS
2	E	-1.37	18.18		+	+++	++	+		+	+	POS
3	E	-2.19	25.19		+	++	+++	++++	++		++	POS
4	E	-5.39	20.78		++	++	++					POS
5	E	-3.15	18.44			++	++	+++	++	++	++	POS
6	E	-3.19	26.23		++	++	++	++++	+++	++	++	POS
7	E	-2.92	24.68	+	+	+++	++	+++	+	++	+	POS
8	E	-3.1	3.64		++	++	+	+++	++	++	++	POS
9	E	-4.04	14.55		++						++	POS
10	E	-3.37	18.44		++	+++	+	+++	++		+	POS
11	E	0.18	12.47					+			+	POS
12	E	0.73	4.94							+	++	POS

NO	GROUP	S/P BEFORE INO	S/P AFTER INO	EWE: Immunodominant Antigen (kDa) detected by western blot								WESTERN BLOT RESULT
				10	17	21	26-29	30	31	33	37	
1	F	-3.1	-7.01			+++						NEG
2	F	1.73	-4.42			+++						NEG
3	F	-2.64	-5.97			+++						NEG
4	F	-3.6	-5.97			+++						NEG
5	F	2.55	-5			+++						NEG

Appendix 15: Chapter 4: Results for ELISA, western blot, brain histology and lesion score as well as PCR for lambs in Groups C,D, E and F in Year 2

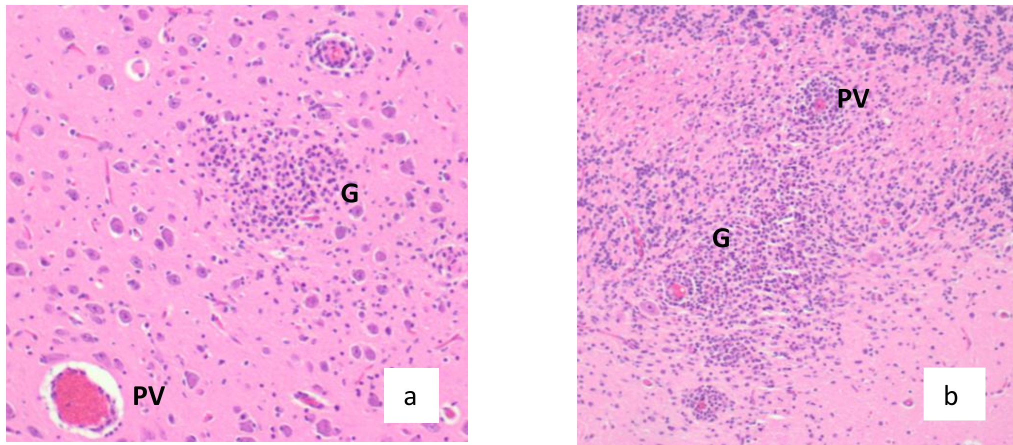
NO	LAMB GROUP	LAMB (2 WKS) ELISA S/P VALUE (%)	ELISA at 2 weeks	LAMB (12 WKS) ELISA S/P VALUE (%)	ELISA AT 12 WKS	LAMB WESTERN BLOT AT 3 MONTHS -Immunodominant Antigen (kDa)								LAMB WESTERN BLOT	HISTO RESULT	LAMB BRAIN HISTO SCORE	LAMB BRAIN PCR RESULT	LAMB HEART PCR RESULT	REMARK
1	C	59.75	POS	32.69	POS		++	++						POS	NEG	0	NEG	NEG	
2	C	41.63	POS	6.98	NEG		+	++						POS	POS	9	NEG	NEG	
3	C	58.64	POS	30.23	POS		++	++++	++	+				POS	NEG	0	NEG	NEG	
4	C	41.08	POS	6.33	NEG		++	++						POS	NEG	0	NEG	NEG	
5	C	72.89	POS	41.21	POS		+	+++	++	+				POS	POS	3	NEG	NEG	
6	C	55.60	POS	28.17	POS		+	++						POS	POS	13	NEG	NEG	
7	C	45.23	POS	5.81	NEG		+	++		+				POS	POS	24	NEG	NEG	
8	C	42.32	POS	2.07	NEG		+	++		+				POS	NEG	0	NEG	NEG	
9	C	60.30	POS	18.48	POS		++	++++	+++	+				POS	POS	43	NEG	NEG	
10	C	36.65	POS	0.52	NEG		+	+++		+				POS	NEG	0	NEG	NEG	
11	C	37.76	POS	0.52	NEG		+	++						POS	NEG	0	NEG	NEG	
12	C	NA		NA		++	++		++++	+++				POS	POS	1	NEG	NEG	DEAD
13	C	52.70	POS	2.58	NEG		+	++++						POS	NEG	0	NEG	NEG	

NO	LAMB GROUP	LAMB (2 WKS) ELISA S/P VALUE (%)	ELISA at 2 weeks	LAMB (12 WKS) ELISA S/P VALUE (%)	ELISA AT 12 WKS	LAMB WESTERN BLOT AT 3 MONTHS - Immunodominant Antigen (kDa)								LAMB WESTERN BLOT$^{\beta}$	HISTO RESULT	LAMB BRAIN HISTO SCORE	LAMB BRAIN PCR RESULT	LAMB HEART PCR RESULT	REMARKS
1	D	NA	NA	-9.30	NEG			+						NEG	NEG	0	NEG	NEG	
2	D	NA	NA	-9.04	NEG			++						NEG	POS	1	NEG	NEG	
3	D	NA	NA	NA										NEG	NEG	0	NEG	NEG	DEAD
4	D	NA	NA	-8.27	NEG			++++						NEG	NEG	0	NEG	NEG	
5	D	NA	NA	-7.11	NEG			+						NEG	NEG	0	NEG	NEG	
6	D	NA	NA	-9.43	NEG	+	++	++	+					POS	NEG	0	NEG	NEG	
7	D	NA	NA	-8.01	NEG		++	++						POS	NEG	0	NEG	NEG	
8	D	NA	NA	-7.62	NEG			++						NEG	NEG	0	NEG	NEG	
9	D	NA	NA	-8.14	NEG			++						NEG	POS	6	NEG	NEG	
10	D	NA	NA	-7.36	NEG	+	+	++						POS	NEG	0	NEG	NEG	
11	D	NA	NA	-7.24	NEG	+	+++	++++	++					POS	NEG	0	NEG	NEG	
12	D	NA	NA	NA										Blank/white	NEG	0	NEG	NEG	DEAD
13	D	NA	NA	-8.01	NEG	+	++	++	++					POS	NEG	0	NEG	NEG	

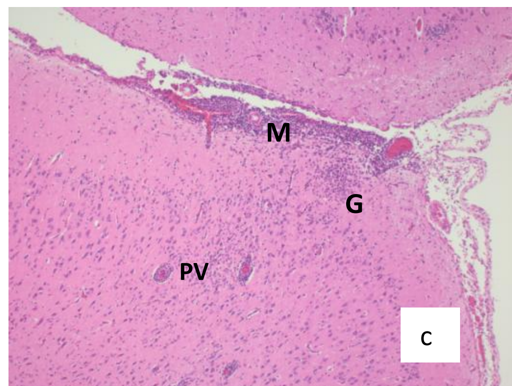
NO	LAMB GRP	LAMB (2 WKS) ELISA S/P VALUE (%)	ELISA at 2 weeks	LAMB (12 WKS) ELISA S/P VALUE (%)	ELISA AT 12 WKS	LAMB WESTERN BLOT AT 3 MONTHS -Immunodominant Antigen (kDa)								LAMB WESTERN BLOT$^{\beta}$	HISTO RESULT	LAMB BRAIN HISTO SCORE	LAMB BRAIN PCR RESULT	LAMB HEART PCR RESULT	REMARKS
						10	17	21	26-29	30	31	33	37						
1	E	NA		NA					++				+	POS	POS	20	POS	POS	DEAD
2	E	14.11	POS	18.99	POS		++	+++						POS	POS	1	NEG	NEG	
3	E	8.40	NEG	10.59	NEG		++	++		+				POS	POS	1	NEG	NEG	
4	E	6.47	NEG	16.54	POS		++	++						POS	NEG	0	NEG	NEG	
5	E	NA		NA					++	++		++	+	POS	POS	28	POS	POS	DEAD
6	E	NA		NA										Blank/white	POS	20	POS	NEG	DEAD
7	E	11.89	POS	NA			++	+++	++	++	+	++		POS	POS	94	POS	POS	DEAD
8	E	10.37	NEG	41.21	POS		++	++++	++	+				POS	POS	8	NEG	NEG	
9	E	4.98	NEG	49.61	POS		++	++++	+++	+				POS	POS	30	NEG	NEG	
10	E	NA		NA										Blank/white	NEG	0	NEG	NEG	DEAD
11	E	13.14	POS	43.28	POS		+	++	+++					POS	POS	10	NEG	NEG	
12	E	NA		NA										Blank/white	NEG	0	NEG	NEG	DEAD
13	E	NA		NA			+	+	++					POS	POS	5	POS	POS	DEAD
14	E	13.28	POS	46.51	POS		++		+	+				POS	POS	22	POS	NEG	
15	E	5.81	NEG	14.86	POS		++	++++	++	+	+	+		POS	POS	14	NEG	NEG	
16	E	12.86	POS	34.24	POS		++	+	+++	+				POS	POS	22	POS	NEG	
17	E	7.93	NEG	20.16	POS		++	++	+					POS	POS	14	NEG	NEG	

NO	LAMB GROUP	LAMB (2 WKS) ELISA S/P VALUE (%)	ELISA at 2 weeks	LAMB (12 WKS) ELISA S/P VALUE (%)	ELISA AT 12 WKS	LAMB WESTERN BLOT AT 3 MONTHS -Immunodominant Antigen (kDa)								LAMB WESTERN BLOT$^{\beta}$	HISTO RESULT	LAMB BRAIN HISTO SCORE	LAMB BRAIN PCR RESULT	LAMB HEART PCR RESULT	REMARKS
1	F	NA		-8.10	NEG			+						NEG	NEG	0	NEG	NEG	
2	F	NA		-9.04	NEG			+++						NEG	NEG	0	NEG	NEG	
3	F	NA		-9.95	NEG			+						NEG	NEG	0	NEG	NEG	
4	F	NA		-9.17	NEG			++						NEG	NEG	0	NEG	NEG	
5	F	NA		-6.98	NEG			++						NEG	NEG	0	NEG	NEG	
6	F	NA		-9.30	NEG			+++						NEG	NEG	0	NEG	NEG	
7	F	NA		-9.04	NEG			+++						NEG	NEG	0	NEG	NEG	

Appendix 16: Chapter 4: Images of H&E stained brain tissue from congenitally infected lambs

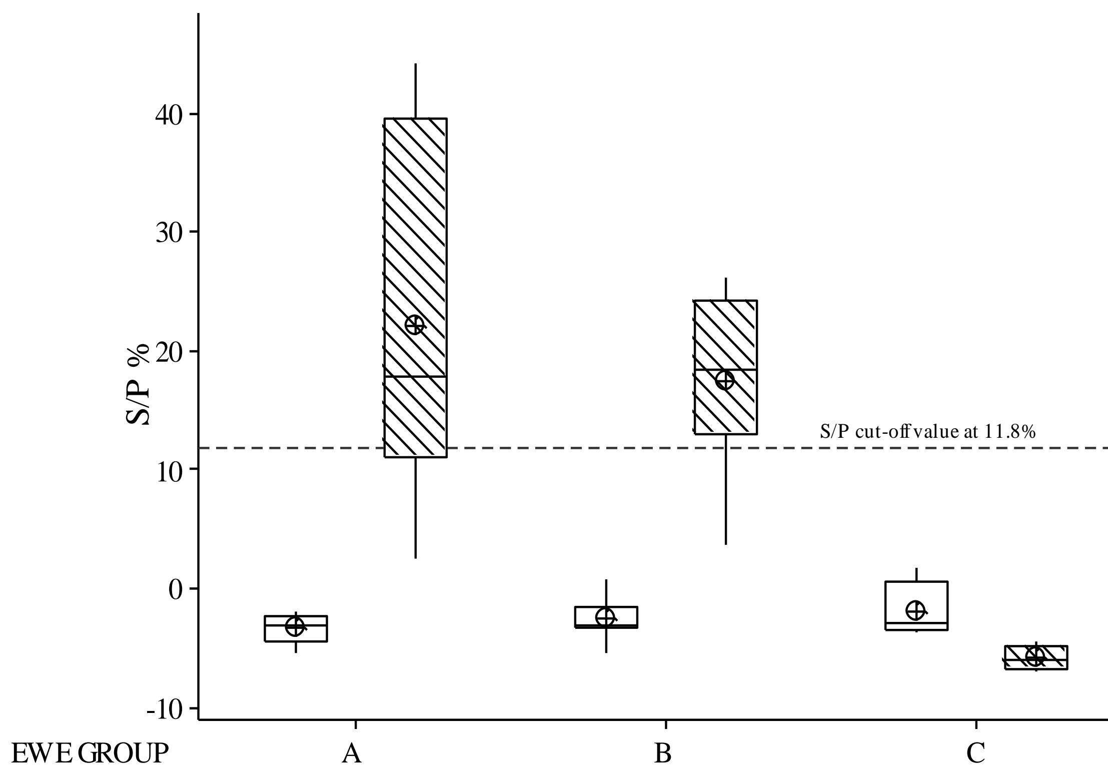


Histology of brain (a & b) from a lamb with encephalitis, showing foci of gliosis (G), coalescing areas of inflammation and perivascular cuffing (PV). H&E stain, 400X



Histology of brain (c) from a lamb with meningoencephalitis, showing multifocal gliosis (G), perivascular cuffing(PV) and involvement of the meninges (M). H&E stain, 400X

Appendix 17: Chapter 5: S/P values for ewes before and 1 month post inoculation with *N. caninum* tachyzoites



Box and whisker plot of S/P values for ewes before (☐) and 1 month post inoculation (▨) with live 10^7 *N. caninum* tachyzoites. The mean value (ϴ) and outliers (+) are as indicated.

Appendix 18: Chapter 5: ELISA S/P values for ewes in Groups A, B and C at pre and 1 month post inoculation with *N. caninum* tachzyoites

NO	GROUP	SP% PRE-INO	ELISA	SP% 1 MO POST INO	ELISA
1	A	-1.82	NEG	14.03	POS
2	A	-2.19	NEG	41.04	POS
3	A	-4.94	NEG	19.74	POS
4	A	-3.82	NEG	12.99	POS
5	A	-2.46	NEG	9.09	NEG
6	A	-5.39	NEG	2.60	NEG
7	A	-3.01	NEG	44.16	POS
8	A	-3.28	NEG	38.18	POS
9	A	-2.55	NEG	17.92	POS
1	B	-3.19	NEG	22.86	POS
2	B	-1.37	NEG	18.18	POS
3	B	-2.19	NEG	25.19	POS
4	B	-5.39	NEG	20.78	POS
5	B	-3.15	NEG	18.44	POS
6	B	-3.19	NEG	26.23	POS
7	B	-2.92	NEG	24.68	POS
8	B	-3.10	NEG	3.64	NEG
9	B	-4.04	NEG	14.55	POS
10	B	-3.37	NEG	18.44	POS
11	B	0.18	NEG	12.47	POS
12	B	0.73	NEG	4.94	NEG
1	C	-3.10	NEG	-7.01	NEG
2	C	1.73	NEG	-4.42	NEG
3	C	-2.64	NEG	-5.97	NEG
4	C	-3.60	NEG	-5.97	NEG

Appendix 19: Chapter 5: ELISA, western blot and histological lesions results for lambs in Groups A, B and C at 12 weeks. ELISA results for lambs in Groups A and B at 2 weeks old is included as well.

NO	GRP	S/P% at 2 wks	ELISA STATUS	S/P% at 12 wks	ELISA	PCR	WESTERN	HISTO	HISTO SCORE
1	A	19	POS	33.6	POS	NEG	POS	POS	22
2	A	4	NEG	21.3	POS	NEG	POS	NEG	0
3	A	22	POS	48.7	POS	NEG	POS	POS	9
4	A	19	POS	8.7	NEG	NEG	POS	POS	5
5	A	17	POS	35.1	POS	NEG	POS	POS	15
6	A	8	NEG	17.3	POS	NEG	POS	POS	2
7	A	4	NEG	14.1	POS	NEG	POS	POS	3
8	A	11	NEG	14.3	POS	NEG	POS	NEG	0
9	A	2	NEG	10.3	NEG	NEG	POS	NEG	0
10	A	9	NEG	11.8	POS	POS	POS	POS	10
11	A	2	NEG	13.2	POS	POS	POS	POS	3
1	B	14	POS	19	POS	NEG	POS	POS	1
2	B	8	NEG	10.6	NEG	NEG	POS	POS	1
3	B	6	NEG	16.5	POS	NEG	POS	NEG	0
4	B	10	NEG	41.2	POS	NEG	POS	POS	8
5	B	5	NEG	49.6	POS	NEG	POS	POS	30
6	B	13	POS	43.3	POS	POS	POS	POS	10
7	B	13	POS	46.5	POS	POS	POS	POS	22
8	B	6	NEG	14.9	POS	NEG	POS	POS	14
9	B	13	POS	34.2	POS	POS	POS	POS	22
10	B	8	NEG	20.2	POS	NEG	POS	POS	14
1	C	NA	NA	-8.1	NEG	NEG	NEG	NEG	0
2	C	NA	NA	-9	NEG	NEG	NEG	NEG	0
3	C	NA	NA	-9.9	NEG	NEG	NEG	NEG	0
4	C	NA	NA	-9.2	NEG	NEG	NEG	NEG	0
5	C	NA	NA	-7	NEG	NEG	NEG	NEG	0
6	C	NA	NA	-9.3	NEG	NEG	NEG	NEG	0
7	C	NA	NA	-9	NEG	NEG	NEG	NEG	0

Appendix 20: Chapter 6: History of farms

- This study was carried out between March 2010 and Feb 2011.

- All animals of 9 mobs were chosen from farms with a history of sporadic cases of *N. caninum* related abortion and the willingness of the owners to participate in this study.

- Farm A consisted of Waihora Romneys two-tooth and all animals were vaccinated for *Camplylobacter* and *Toxoplasma.* The first case of neosporosis from this farm was notified in 2006. With 380 two-tooth found to be dry (12%), 1133 two-tooth produced singles and 1631 two-tooth produced twins. Lambing rate at docking was 124%. Serology was conducted and revealed 4/10 (aborted dams) and 6/10 (full term dams) were positive for *N. caninum* antibody by IFAT (titres of 1:100). Five samples of cotyledon and placenta each were collected from the dams that had aborted from those diagnosed pregnant until full term. One sample from the aborted group was found to be positive for *N. caninum* DNA by PCR. In 2007, 291/3162 (9.4%) two-tooth only, 186/7666 (2.4%) from the mixed aged ewes were dry. Interestingly, all 200 two-tooth from 2006 that came back as 4-tooth were all found to be dry in 2007. These animals were isolated for investigation however no infectious agent could be found. Another 20 blood samples were taken and none of the 10 samples (aborted dams) were positive for *N. caninum* antibody by IFAT while 8/10 (full term dams) were positive with titres of 1:100. Interestingly, when re-tested using a commercially based ELISA (IDEXX) only 1/20 of the samples were positive and it was from one aborted ewe with a very high S/P ratio of 166%. No *N. caninum* DNA was detected by PCR in any of the blood samples. Five ewes were later euthanized and their uterine samples containing placentomes were submitted for PCR. Two foetuses from one ewe were positive when *N. caninum* DNA were detected in brains and cotyledons. In year 2008 scanning showed 15% (492/3600) of the two-tooth were dry. Twenty-one blood samples were tested with IDEXX ELISA from both dry and wet animals but all were negative for *N. caninum* antibody.

- At Farm B, the first case of *N. caninum* was notified in 2007 when 30% of 3811 animals were found dry during early scanning which consisted of 298/1181 two-tooth and 243/2630 of mixed aged ewes. Later another 10% dry animals were detected during the second scanning for those mated late in the season. Eighty of seven hundred and thirty-nine two-tooth and 62/876 mixed aged ewes were found dry. Five of the dry ewes were euthanased and their uteri were submitted for PCR. All were found to be negative for *N. caninum* DNA. Serology to detect antibodies of *N. caninum* was conducted on 10 blood samples taken from wet animals and 1 was positive by IFAT. Eleven blood samples were taken from dry animals and 9 were positive by IFAT with titres were ranging from 1:100, 1:1400 and 1:6400.

- Farm C is a “life-style” farm which consists of about 30 to 50 animals per mob. Animals were of mixed age and mixed breed. An isolated case of abortion in one of the ewes previously had occurred. In 2009 *N. caninum* DNA was detected in the brain of an aborted foetus and the dam was serologically positive when tested by IFAT.
- Farm D is from Northland which was included late in the trial. Samples were only obtained after lambing had taken place.

- Other farms: In an effort to detect problematic farms with possible involvement of *Neospora*, some samples were also collected from other farms within the North Island region with a history of unexplained abortions and previous cases of *Neospora*. Farm E (n = 190; has a previous report of *Neospora* and consisted of mixed aged group ewes); Farm F (n=16); Farm G (n=13); Farm H (n=13) and Farm I (n=15). All samples from Farm F, G, H and I were from dry ewes detected at scanning.