The application of immunological, molecular and epidemiological approaches to the study of Neosporosis in cattle

by

Craig Hall

Thesis submitted for the degree of
Doctor of Philosophy in Science

at the
University of Technology, Sydney
2006

Certificate of authorship and originality

I certify that the work in this thesis has not been previously submitted for a degree, nor has it been submitted as a part of requirements for a degree except as fully acknowledged in the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signed

_____

Acknowledgements

Firstly, I wish to acknowledge the support and assistance of the dairy farmer, Peter Williams without which this project could not have gone ahead.

I also wish to thank the farmhand, Adam Findlater and the herd veterinarian, Dr Bob Rheinberger for their assistance.

Thanks also to Rob Reeves for access to his herd and to Rani Sahay and Ken Phillips from Dairy Express for access to NSW milk samples.

I wish to acknowledge Kathleen Vanhoff, Dr Michael Reichel and Scott Hackett from the Novartis Animal Health research facility, "Yarrandoo", and Irene Sotirchos from UTS, for assistance with the experimental work. Thanks also to Peter Windsor from the University of Sydney for pathological examination of the mouse tissues.

I would also like to thank Novartis Animal Health for funding the PhD.

I would like to acknowledge the ongoing help, advice, support and encouragement of my supervisors Dr Michael Reichel and Professor John Ellis in all aspects of the experimental work and writing.

Lastly I would like to thank my family, Sharron, Jayden, Talicia and Kiara for their support and for allowing me the time to complete this thesis.

Publications arising from this thesis

Articles in refereed journals

CA Hall, MP Reichel, JT Ellis (2005) *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. Veterinary Parasitology 128, 213-241.
(This paper was the 11th most downloaded article from Veterinary Parasitology in the 2nd quarter of 2005. It also has a citation index of 3 (in only 3/4 of a year)).

CA Hall, MP Reichel, JT Ellis (2005) Performance characteristics of two enzyme-linked immunosorbent assays for the detection of antibodies to *Neospora caninum* in the serum of cattle. Accepted for publication in Veterinary Parasitology, March 2006.

CA Hall, MP Reichel, JT Ellis (2005) Validation and performance characteristics of an enzyme-linked immunosorbent assay for the detection of antibodies to *Neospora caninum* in the milk of cattle and subsequent application to determine prevalence of infection in NSW dairy cattle. Submitted to Veterinary Parasitology, December 2005.

Magazine article

CA Hall, MP Reichel, JT Ellis (2005) Control of *N. caninum* infection: a case study using test and cull and the value of serology. The Australian Cattle Veterinarian 35, 14-16.

Conference proceedings

C Hall, MP Reichel and JT Ellis (2003). Evidence of vertical transmission of *Neospora caninum* on a dairy farm in NSW. The 45th Annual Scientific Meeting of the Australian Society for Parasitology, 7-10 July, Darwin, Australia: abstract C24.

C Hall, MP Reichel, JT Ellis (2004) Control of *Neospora caninum* infection in a NSW dairy herd. Proceedings of the 1st Australian Dairy Science Symposium, 26-27 February, Shepparton, Australia: pg 42, poster 7.

C Hall, MP Reichel, JT Ellis (2004) Vertical transmission of *Neospora caninum* in a NSW dairy herd. Proceedings of the 1st Australian Dairy Science Symposium, 26-27 February, Shepparton, Australia: abstract pg 16.

C Hall, MP Reichel, JT Ellis, R Rheinberger (2004) Control of *Neospora caninum* infection in a NSW dairy herd. Proceedings of the COST 854 Workshop, Working Group 2 and 3, June, Bilbao, Spain.

CA Hall, MP Reichel, JT Ellis (2004) *Neospora caninum*: abortion and infection control in a dairy herd. The 46th Annual Scientific Meeting of the Australian Society for Parasitology, 26-30 September, Fremantle, Australia: abstract C13.

MP Reichel, CA Hall, JT Ellis (2004) *N. caninum*: abortion diagnosis and control of infection in a NSW dairy herd. New Zealand Society for Parasitology Annual Meeting No. 32, 2-3 November, Palmerston North, New Zealand.

CA Hall, MP Reichel, JT Ellis (2005) *Neospora caninum*: options for control Downunder. The 20th International Conference of the World Association for the Advancement of Veterinary Parasitology, 16-20 October, Christchurch, New Zealand: abstract N4.1.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AI	artificial insemination
ANGIS	Australian National Genomic Information Service
BHV	bovine herpes virus
bp	base pair
BVD	Bovine Viral Diarrhoea
BVDV	Bovine Viral Diarrhoea virus
CMDT	computational methods for diagnostic tests
d	days
DIM	days in milk
ELISA	enzyme linked immunosorbent assay
GHRL	Gore Hill Research Laboratories, Gore Hill, NSW, Australia
h	hours
IBR	Infectious Bovine Rhinotracheitis
IFAT	indirect fluorescent antibody test
IFN-γ	gamma interferon
IHC	immunohistochemistry
IL	interleukin
IP	Institut Pourquier
ITS	internal transcribed spacer
min	minute
mon	months
MQ	MilliQ
NCBI	National Centre for Biotechnology Information
NSW	New South Wales, (Australia)
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	persistently infected
rDNA	ribosomal DNA
RT	room temperature

SCC	somatic cell count
SCID	severe combined immunodeficient
Se	sensitivity
Sp	specificity
TG-ROC	two graph-receiver operating characteristics
TMB	tetramethylbendizine
UK	United Kingdom
USA	United States of America

Abstract

A prospective study was undertaken on a 260-head dairy herd in NSW, Australia to determine the modes of transmission, prevalence, effect on abortion and reproduction parameters and the impact on milk production of *Neospora caninum*. A test-and-cull method of control was also evaluated. The possible infectivity of milk from *N. caninum* infected cows was also investigated using a mouse model. As ELISAs form an important part of diagnosing *N. caninum* infection, studies were undertaken to evaluate two serum and one milk ELISA. Analysis was performed for each of the ELISAs to determine cut-off thresholds, Se and Sp. The milk ELISA was subsequently used to determine the overall prevalence of *N. caninum* in NSW dairy cows.

In the prospective study, 11.4% of the herd's cattle were seropositive to *N. caninum* by ELISA and the dominant route of transmission was vertical as the majority of the infected cattle were related and only a few seropositive cattle were born from seronegative dams (*i.e.* reflecting postnatal transmission). As 90% of offspring born from seropositive cows were also seropositive this suggests a high vertical transmission rate. *Neospora caninum* was found to be a major cause of abortion as cows seropositive to *N. caninum* had a 13-fold higher risk of abortion then seronegative cattle. Early foetal loss was also predicted to be associated with *N. caninum* infection as seropositive cows required a significantly greater number of inseminations and took longer to conceive than seronegative cattle. BVDV and IBR alone were not associated with causing abortion in this herd. This is the first report of an effective control strategy for *N. caninum* by either culling seropositives or not breeding from seropositive cows. This method was effective in reducing the number of infected cattle and was feasible due to the low prevalence of *N. caninum* on the farm and thus did not place too high a financial burden on the farmer. *Neospora caninum* DNA was also detected by PCR on milk samples from seropositive cows. This is the first Australian report demonstrating *N. caninum* DNA in milk. Of the serum ELISA evaluated, one was determined to have high Se and Sp at the cut-off recommended by the manufacturer while the other required modification of the cut-off value to gain the same high Se and Sp. After choosing the most suitable milk dilution, the milk ELISA was determined to have high

Se and Sp of 97%. Using this ELISA the prevalence of *N. caninum* in NSW dairy cattle was determined to be 21.1%.

Neospora caninum was found to be a significant cause of abortion and also foetal loss in the study herd and was vertically transmitted efficiently. Control efforts using a test-and–cull approach were successful without placing economic hardship on the farmer. The evaluation of several ELISAs was useful as these can be used in diagnosing infection. In particularly the milk ELISA will now make sampling easier so enabling whole herd sampling by the farmer. This ELISA may be of particular use in test-and cull programs or in epidemiological studies. The concept of detecting *N. caninum* DNA in milk could be of great importance to the dairy industry as it suggests a new route of infection. Although milk extracts from seropositive cows were not infective to SCID mice in this study this should be investigated further.

1. Introduction – The biology of *Neospora caninum*

Neospora caninum is a protozoan parasite related to *Toxoplasma gondii* and it was first associated with neuromuscular disease in dogs in the mid-1980's (Bjerkås *et al.* 1984). It was subsequently also identified as a major cause of abortion in cattle (Anderson *et al.* 1991, Thornton *et al.* 1991, Anderson *et al.* 1995, Davison *et al.* 1999b, Wouda *et al.* 1999a).

1.1 History

The disease in dogs was first recognised in 1984 in Norway but was not named (Bjerkås *et al.* 1984). The dogs showed clinical signs of encephalomyelitis and myositis. In 1988 a similar parasite was identified in dogs in the USA and a new genus, *Neospora* and the type species *N. caninum* were described (Dubey *et al.* 1988a). In these dogs the main lesions were meningoencephalitis and myositis. The organism present in dogs was isolated in cell culture and in mice. Koch's postulates were fulfilled by infecting another dog with tachyzoites previously isolated in cell culture. An IFAT was subsequently developed for serological diagnosis of neosporosis (Dubey *et al.* 1988b). Until 1988, *N. caninum* was misdiagnosed as *T. gondii* because of similarities in clinical signs and morphology (Dubey 1992, Dubey 1999b). Infection has now been reported retrospectively from stored dog tissues from the USA (Dubey 1992) and calf tissues from Australia (Dubey *et al.* 1990a) dating back to 1957 and 1974, respectively.

1.2 Biology and life cycle

There are three life cycle stages of *N.caninum* identified to date. These are tachyzoites, bradyzoites and oocysts, thus it has a coccidian life cycle. *Neospora caninum* is an intracellular parasite and the tachyzoites are able to penetrate the host cell by active invasion. They can become intracellular within 5 minutes of contact with host cells (Hemphill *et al.* 1996). Tachyzoites are usually found within a parasitophorous vacuole in the host cell cytoplasm (Lindsay *et al.* 1993). Tachyzoites have been found in many cell types and tissues including neural cells, macrophages, fibroblasts, vascular endothelial cells, myocytes, renal tubular epithelial cells and hepatocytes (Dubey *et al.*

1988a, Dubey and Lindsay 1996). The tachyzoites of *N. caninum* are ovoid and measure 7 x 2 μm when not dividing (Dubey *et al.* 2002a).

Bradyzoites are located in tissue cysts and are commonly found in neural tissues including brain, spinal cord, nerves and retina (Dubey *et al.* 1988a, Dubey *et al.* 1990c). A solitary cyst has also been found in the ocular muscle of a foal (Lindsay *et al.* 1996b) and cysts have recently been identified in the skeletal muscle of naturally infected dogs and cattle (Peters *et al.* 2001). Tissue cysts may contain 20-100 bradyzoites and the cyst walls are between 0.5 and 4 μm thick (Speer *et al.* 1999). Bradyzoites are elongate and measure approximately 8 x 2 μm (Dubey *et al.* 2002a). Although the length of time that tissue cysts can persist in tissues is not known, they remain viable in the brains of experimentally infected mice for at least 1 year (Lindsay *et al.* 1992). Bradyzoites and tachyzoites are the only life cycle stages found in the intermediate host (Peters *et al.* 2001) where only asexual duplication of the parasite occurs.

It has only recently been shown that experimentally infected dogs shed oocysts and that dogs are a definitive host where sexual reproduction occurs (McAllister *et al.* 1998, Lindsay *et al.* 1999). Oocysts have now also been isolated from dogs that had a naturally acquired infection (Basso *et al.* 2001). Even more recently coyotes have also been shown to be definitive hosts of *N. caninum* after experimental infection with infected bovine tissue (Gondim *et al.* 2002). *Neospora caninum* oocysts measure 11.7 x 11.3 μm and contain two sporocysts that each contain four sporozoites (Dubey *et al.* 2002a).

1.3 Taxonomy

Neospora caninum is a protozoan parasite in the phylum Apicomplexa. It belongs to the subfamily Toxoplasmatinae of the Sarcocystidae family. Of about 200 coccidia that belong to the Sarcocystidae family, around 180 belong to the Sarcocystinae subfamily and only a few species have been classified into the Toxoplasmatinae subfamily. The Toxoplasmatinae are tissue cyst forming coccidia that are divided into four genera: *Neospora* (two species), *Toxoplasma* (one species), *Hammondia* (two species) and *Besnoitia* (six species) (Mugridge *et al.* 1999, Heckeroth and Tenter 2000). As

previously stated, *T. gondii* is closely related and morphologically similar to *N. caninum*. However there have been many antigenic, morphological, biological and molecular differences described between *N. caninum* and *T. gondii* (Ellis *et al.* 1994, Holmdahl *et al.* 1994, Dubey and Lindsay 1996, Howe and Sibley 1999, Speer *et al.* 1999). Although both *N. caninum* and *T. gondii* can cause abortion, the former is an important cause of abortion in cattle and the latter is a cause of abortion in humans (Chowdhury 1986) and other animals, mainly sheep (Dubey 1996). Dogs are the only documented definitive hosts of *N. caninum* and cats are the definitive host of *T. gondii* (Dubey *et al.* 2002b).

Tachyzoites of *N. caninum* measure approximately 7.5 x 2 μm whereas those of *T. gondii* measure 6.8 x 1.5-3 μm. Tachyzoites of both species are found within a parasitophorous vacuole (Speer *et al.* 1999).

Hammondia hammondii and *H. heydorni* are also closely related but molecularly distinct from *N. caninum* (Ellis *et al.* 1999b, Dubey *et al.* 2002b). *Hammondia heydorni* has the dog as the definitive host while the definitive host of *H. hammondii* is the cat. *Hammondia* spp. are not known to have any clinical significance (Dubey *et al.* 2002b).

Figure 1 Life cycle of *N. caninum*

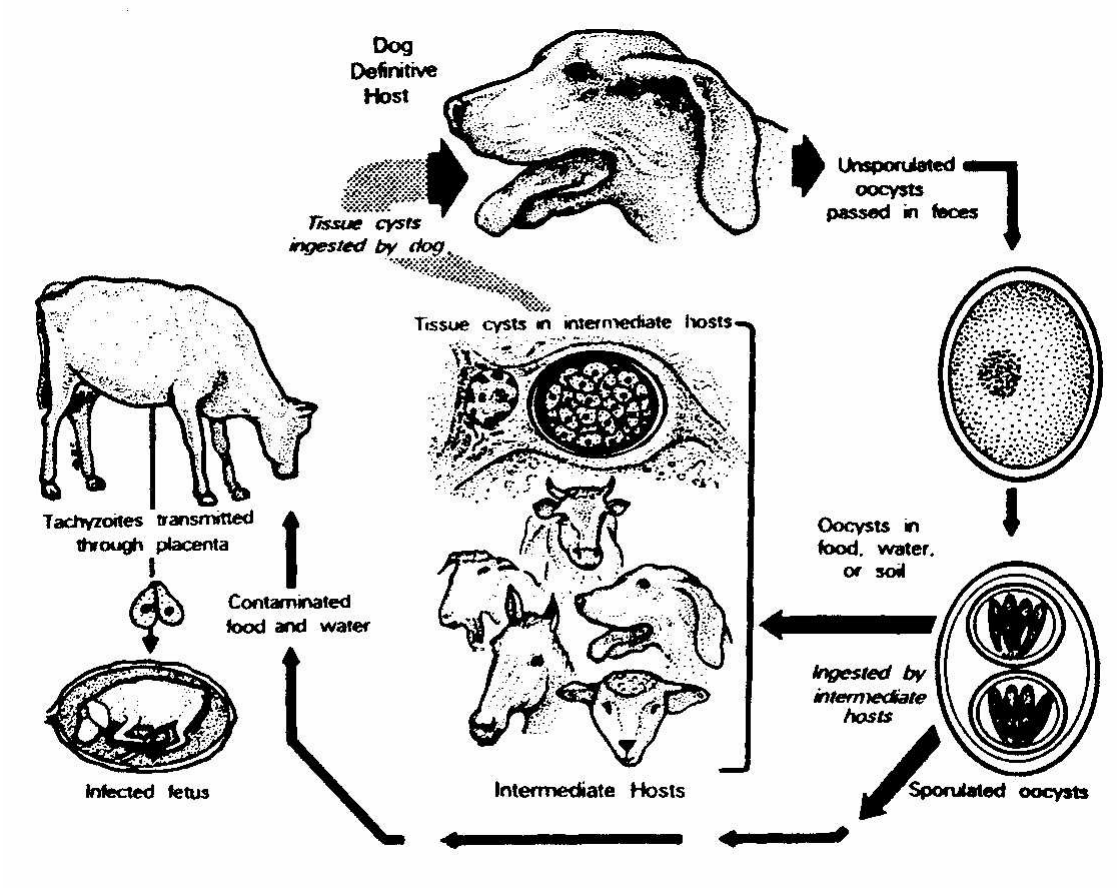


Figure 1 is reproduced from (Dubey 1999b).

1.4 Modes of transmission in cattle

Cattle can become infected with *N. caninum* by either vertical transmission or post-natal transmission (Figure 1). Vertical transmission occurs from cow to calf *in utero* (Paré *et al.* 1996, Anderson *et al.* 1997). The *N. caninum* parasite can cross the placenta during gestation and infect the developing foetus. Congenital infection has been shown to be important in the spread of *N. caninum* and appears to be the major mode of transmission in cattle (Paré *et al.* 1996, Schares *et al.* 1998). Vertical transmission is very efficient and the rate of vertical transmission has been found to be 81% in a Californian herd (Paré *et al.* 1996), 93% in a German study (Schares *et al.* 1998) and 95% in a UK study (Davison *et al.* 1999a). It has been found that most congenital infections result in the birth of a clinically normal, healthy, although chronically infected calf (Paré *et al.* 1996, Dubey 1999b).

Mathematical modelling studies have shown that vertical transmission alone cannot sustain an infection within a herd. For the disease to be maintained within the herd there must be some form of horizontal or postnatal transmission occurring (French *et al.* 1999).

Post-natal transmission results from infection of cattle by oocysts shed by dogs or possibly other definitive hosts (*i.e.* foxes). Dogs may become infected after ingesting infected tissues such as an aborted foetus, placenta (Dijkstra *et al.* 2001b) or other infected cattle tissue (Gondim *et al.* 2002). It has recently been found that coyotes are also definitive hosts of *N. caninum* (Gondim *et al.* 2004). Other canids such as foxes, dingoes and have been shown to be natural intermediate hosts of *N. caninum* by serological examination (Lindsay *et al.* 1996a, Barber *et al.* 1997, Schares *et al.* 2001) and it is possible that they are also definitive hosts. Postnatal infection also encompasses horizontal infection, which is cattle-to-cattle infection. This type of infection may be possible by feeding pooled milk or colostrum to calves or by ingestion of infected placentas, foetus or amniotic fluid however these routes of transmission have not been proven (French *et al.* 1999).

It has been demonstrated that calves can be orally infected with colostrum spiked with *N. caninum* tachyzoites (Uggla *et al.* 1998). *Toxoplasma gondii* has been transmitted to goats via milk (Dubey 1980) and humans have also been infected with *T. gondii* after milk consumption (Tenter *et al.* 2000). Considering the similar biology of *N. caninum* and *T. gondii,* it is quite possible that cattle and dogs could be infected by ingesting milk from seropositive cows (Dijkstra *et al.* 2001b).

Endemic abortions are usually associated with vertical transmission and evidence of this route is the equal distribution of seropositive cattle among age groups. However, age clustering of infected animals supports a point source of exposure and points to postnatal transmission (Dijkstra *et al.* 2001a). Abortion epidemics have been associated with point source infections (Dijkstra *et al.* 2001a) however it has also been hypothesised that they may be caused by reactivation of a latent infection due to factors causing immune suppression (Wouda *et al.* 1999a).

1.5 Disease in pregnant cattle

There are no prior signs of clinical illness in cattle that abort due to *N. caninum*. Serological diagnosis can identify cattle that are positive for *N. caninum* antibodies but cannot determine when or if that animal will abort.

Abortion due to *N. caninum* can occur at any time of gestation but the majority of abortions occur at 5-6 months (Anderson *et al.* 1991, Anderson *et al.* 1995, Wouda *et al.* 1999a). Calves can be stillborn, or a live deformed calf may be born. There may be early foetal death and resorption of the foetus (Dubey 1999c). The most common occurrence though is the birth of a clinically normal calf that is chronically infected (Paré *et al.* 1996, Schares *et al.* 1998, Dubey 1999c).

The time of exogenous infection of the foetus with *N. caninum* during pregnancy can affect the outcome of the pregnancy. When cows were infected 9 weeks before gestation there was no infection and the calves were born live at full term. Of six cows infected at 10 weeks gestation five had a resorbed foetus and one went to full term and bore a healthy *N. caninum*-free calf. Where cows were infected at 30 weeks gestation there was congenital infection of calves that were born healthy at full term (Williams *et al.* 2000). Other authors also found protection against vertical transmission of *N. caninum* in cows that were infected with *N. caninum* tachyzoites 6 weeks before mating and then challenged at mid-gestation (Innes *et al.* 2001).

1.6 Prevalence and abortions

Since the initial observations of *N. caninum* abortions in the USA there have been an increasing number of countries worldwide that have reported *N. caninum* associated abortion (Table 1). In non-aborting herds (*i.e.* those with no history of abortion problems) the mean seroprevalence was 19.6% and ranged from 5.5-44.9% whereas in aborting herds (*i.e.* those with a history of recent abortion) the mean seroprevalence was 38.0% and ranged from 18-72%.

Table 1 Prevalence of *N. caninum* in dairy cattle from various countries

Region	Country	Dairy prevalence (%)	
		Non-aborting	Aborting
Oceania	Australia [1]		31.0
	New Zealand [2]	6.8	30.0
North America	USA [3]	16	57.1
	Canada [4]	14.4	22.1
South America	Argentina [5]	16.6	43.1
	Costa Rica [6]	39.7	
	Mexico [7]	36.0	72.0
	Paraguay [8]	36.0	
Europe	Czech Republic [9]	1.9	13.6
	Spain [10]	35.9	
	UK [11]	6	18
	Italy [12]	13.6	
	Switzerland [13]	11.5	
	The Netherlands [14]	13.9	51.5
	Sweden [15]	2.1	6.9
	France [16]	5.6	
Asia	Taiwan [17]	44.9	
	Thailand [18]	5.5	
	Vietnam [19]	5.5	

References for Table 1: [a] and [b] refer to "non-aborting" and "aborting" respectively.
[1] (Atkinson *et al.* 2000b), [2a] (Reichel 1998), [2b] (Cox *et al.* 1998, Pfeiffer *et al.* 2002), [3a] (Rodriguez *et al.* 2002), [3b] (Paré *et al.* 1996, Jenkins *et al.* 2000), [4a] (Keefe and

VanLeeuwen 2000, Hobson *et al.* 2002), [4b] (Hobson *et al.* 2002) [5] (Moore *et al.* 2002), [6] (Romero *et al.* 2002), [7] (Morales *et al.* 2001), [8] (Osawa *et al.* 2002), [9] (Vaclavek *et al.* 2003), [10] (Pronost *et al.* 2000), [11] (Davison *et al.* 1999b), [12] (Otranto *et al.* 2003), [13] (Gottstein *et al.* 1998), [14] (Wouda *et al.* 1999a), [15] (Björkman *et al.* 2000), [16] (Ould Amrouche *et al.* 1999), [17] (Ooi *et al.* 2000), [18] (Kyaw *et al.* 2004), [19] (Huong *et al.* 1998).

By serological examination, many authors have found an increased risk of abortion amongst *N. caninum* seropositive cows. In Australia, Czech Republic, California, New Zealand, Britain, Netherlands and another Californian study there was a 8, 8, 7.4, 4.2, 3.5, 3 and 2-fold increased risk respectively of abortion in seropositive cows (Paré *et al.* 1997, Thurmond and Hietala 1997a, Wouda *et al.* 1998b, Davison *et al.* 1999b, Atkinson *et al.* 2000b, Pfeiffer *et al.* 2002, Vaclavek *et al.* 2003). In one *N. caninum* abortion outbreak, 33% of the breeding stock aborted within a few months (Thornton *et al.* 1994). In another study of aborted foetuses in New Zealand, it was found that 30% of abortions were caused by *N. caninum* (Thornton *et al.* 1991). Of 226 foetuses submitted for diagnosis from 50 herds experiencing epidemic abortions in The Netherlands, 77% were *N. caninum* infected (Wouda *et al.* 1999a). *Neospora caninum* was also the major cause (42.5%) of abortions in a Californian study of 26 dairy herds (Anderson *et al.* 1995).

1.7 Economic impact

It has been estimated that economic costs associated with *N. caninum* infection are AU$85 million per annum and AU$25 million for the dairy and beef cattle industry, respectively in Australia (Ellis 1997). In New Zealand the costs for the dairy industry are estimated at NZ$17.8 million (Pfeiffer *et al.* 1998).

In California, it is estimated that abortions due to *N. caninum* cost US$35 million per year (Dubey 1999a). As this calculation only takes into account the losses after abortion epidemics and not the costs associated with endemic abortion the actual losses are probably higher.

Whilst abortion is the major factor in determining the economic costs associated with *N. caninum* in cattle, there are others. These include early foetal death, increased culling of infertile or seropositive stock, the effect on milk production and reduced value of breeding stock (Trees *et al.* 1999). Early foetal death would increase the time to conception and cause a time delay in production in a beef and dairy setting. The increased time to conception and thus birth may reduce the milk production for a dairy cow however most likely the length of the current lactation may simply be extended. It is unknown how frequently early foetal death occurs naturally but experimental infection at 9 weeks gestation caused the death of five out of six foetuses as detected by ultrasound (Williams *et al.* 2000).

Reduced fertility in dairy cows is a reason for the culling of those animals. This is the most common reason for culling in the UK (Esslemont and Kossaibati 1997). Any effect that neosporosis may have on fertility, either through abortion or early foetal death will add to the economic losses. Other authors have found that seropositive cows were twice as likely to be culled for low milk production as seronegative cows (Thurmond and Hietala 1996). Beef and dairy cattle that test positive to *N. caninum* may also be culled early as means of controlling the spread of the parasite (Reichel and Ellis 2002).

1.8 Milk yield

Generally it would be reasonable to suggest that disease in cattle would have a negative impact on milk production rather than a positive effect. There is however, conflicting data on whether *N. caninum* infected cows produce more or less milk than uninfected cows. Two studies conducted in the USA, concluded that *N. caninum* seropositive cows produced less milk than seronegative cows (Thurmond and Hietala 1997b, Hernandez *et al.* 2001). In these studies *N. caninum* infected cows produced 1.3 kg/day/cow less milk. In contrast, two Canadian studies and one from New Zealand found that seropositive cows produced more milk than seronegative cows (Keefe and VanLeeuwen 2000, Hobson *et al.* 2002, Pfeiffer *et al.* 2002). The increased milk production amongst seropositive cows ranged from 0.5 to 1.1 kg/day/cow in these studies.

The calculated loss (based on 1.3 kg/day/cow and a price of AU$0.30/L) due to *N. caninum* would be AU$117 per cow for a 300-day lactation. For a herd with 30 *N. caninum* infected cows this would amount to losses of approximately AU$3500. If increased production of *N. caninum* infected cows is true then the calculated gains would range from AU$45-99 per cow for a 300-day lactation and AU$1350-2970 for a herd with 30 *N. caninum* infected cows. The latter calculations are based on a range of 0.5-1.1 kg/cow/day.

1.9 Methods of diagnosis and detection

There are many methods available for the detection of *N. caninum* although each has its limitations. Serology is a common method of diagnosing infection in cattle. Many ELISAs have been developed for the detection of serum antibodies to *N. caninum* (Paré *et al.* 1995a, Baszler *et al.* 1996, Björkman *et al.* 1997, Williams *et al.* 1997, Wouda *et al.* 1998a, Björkman *et al.* 1999). These tests are particularly useful for screening of many samples and especially in seroepidemiological studies. The IFAT is often used as the reference standard test (Dubey *et al.* 1988b, Paré *et al.* 1995b) although the drawback of this test is that it is subjective.

Histology is useful to find evidence of *N. caninum* infection in an aborted foetus. This evidence is typically multifocal non-suppurative encephalitis and myocarditis (Barr *et al.* 1991, Anderson *et al.* 1995, Wouda *et al.* 1997) Multifocal areas of necrosis surrounded by inflammatory cells are often also found in brain, spinal cord, heart, lung and placenta (Barr *et al.* 1991)**.** Immunohistochemistry may also be used to specifically diagnose *N. caninum* infection although there is no doubt that IHC is insensitive in diagnosing foetal neosporosis and that maternal serology may aid diagnosis (Dubey and Lindsay 1996). Foetal histology and IHC are useful as support but are not a reliable means of attributing *N. caninum* as the cause of the abortion (Thurmond *et al.* 1999).

PCR has also been used to detect *N. caninum* DNA extracted from foetal tissues. Different PCR methods have been developed by several researchers (Holmdahl and

Mattsson 1996, Payne and Ellis 1996, Ellis *et al.* 1999a, Liddell *et al.* 1999). The advantage of PCR is that it is both sensitive and specific (Ellis 1998) although as the majority of *N. caninum* infected calves are born healthy (Paré *et al.* 1996) the detection of *N. caninum* in a foetus does not prove that it was the cause of the abortion.

1.10 Methods of control

Many methods of control have been suggested or tested for *N. caninum*. These include culling seropositive cows, controlling access of dogs to cattle feed and foetal material, chemical control and attempts at vaccination.

1.10.1 Test and cull

In herds that have a high rate of vertical transmission and a low rate of post-natal transmission it has been suggested that the control of *N. caninum* infection could be achieved, by culling infected cattle or not breeding from infected cattle or a combination of the two (French *et al.* 1999, Reichel and Ellis 2002). However, this is only practical in herds with a low prevalence of infection due to otherwise high rates of culling. The method would prevent vertical transmission and so only a few new infections would be predicted from postnatal transmission.

1.10.2 Farm management practices

Farm management also appears important for the control of infection. Infected dogs may excrete oocysts (Basso *et al.* 2001) and contaminate feed sources. Dogs may also become infected after ingesting foetal material (Dijkstra *et al.* 2001b) or other bovine tissues (Gondim *et al.* 2002) and so it has been recommended that dogs be kept away from bovine tissues and cattle feed sources.

1.10.3 Chemical treatment

There has been successful treatment of some dogs with limb weakness due to neosporosis. Generally the dogs must be in the early stages of clinical disease and the response to the treatment is dependent on the stage of disease when treatment commences. Infections in dogs, if caught early enough can be treated with clindamycin

(Knowler and Skerritt 1994). Additionally, sulfonamides and pyrimethamine have been used with some success in treating canine neosporosis. Of 16 dogs that were treated with one or more of the treatments, five dogs made a full recovery and in another five a good response was seen (Barber and Trees 1996). Others have been successful in reversing the paralysis associated with *N. caninum* by treatment for 4 weeks with a combination of trimethoprim and sulfadiazine, and pyrimethamine (McGlennon *et al.* 1990, Mayhew *et al.* 1991).

At present there is no treatment for dogs that will prevent vertical transmission from mother to pup (Dubey and Lindsay 1996). There is also no known treatment that will prevent the production and excretion of oocysts to prevent farm dogs from continuing the life cycle of *N. caninum.*

At present there is no chemotherapeutic treatment that is practical for bovine neosporosis although one study showed efficacy. One day after calves were experimentally infected with *N. caninum* tachyzoites, they were treated with Ponazuril for either 1 day (five calves) or six consecutive days (four calves). The treatment was effective as *N. caninum* was not detected by PCR in calf brain or other organs (Kritzner *et al.* 2002). However the drawback of treating cattle would be the cost of long-term treatment and the possible milk residues and withdrawal period. It is also doubtful that bradyzoites in tissue cysts would be susceptible to chemical treatment (Reichel and Ellis 2002). However, vaccination is believed to be a potential form of control (Innes *et al.* 2002).

1.10.4 Vaccination strategies

At present there is no effective vaccine against *N. caninum* (Dubey 1999c). There have been several attempts to produce such a vaccine but none have succeeded to date. There is evidence that cattle develop protective immunity against vertical transmission (Innes *et al.* 2001) and abortion (Williams *et al.* 2003), which gives support to the development of a vaccine. Protection against vertical transmission has been achieved by experimentally infecting naïve cattle with *N. caninum* tachyzoites before pregnancy and then challenging the cattle at mid-gestation. Six live, seronegative calves were born and *N. caninum* DNA was not detected in any tissues with the exception of spinal cord in

one of the calves (Innes *et al.* 2001). Naturally, chronically infected pregnant cows have also shown to be protected against abortion after an *N. caninum* challenge infection at 10 weeks of gestation. However, the immune response produced did not protect against vertical transmission to the calves. In the control group, four previously uninfected pregnant cows that were challenged at 10 weeks into gestation had failed pregnancies (Williams *et al.* 2003). Field data also supports the theory of protective immunity as there is evidence that chronically infected cows were less likely to abort than previously uninfected cows on a farm with a likely point-source infection (McAllister *et al.* 2000).

Apicomplexan parasites have evolved mechanisms that allow them to evade or modulate the host immune response and allow them to survive within their host (Howe and Sibley 1999). Cell mediated immunity is likely to be the method of host defence against infection with *N. caninum* as it is an intracellular protozoan (Andrianarivo *et al.* 2000). This has been described for the closely related *T. gondii* (Gazzinelli *et al.* 1993). Generally, immunity to intracellular protozoa is cell mediated and dominated by type-1 T lymphocyte responses. During infection, IL-12 and IFN-γ mediate the development of antigen-specific T helper cells. These T helper cells then secrete the cytokines IL-2, IFN-γ and tumour necrosis factor. (Baszler *et al.* 1999). Type 1 cytokines **(**IFN-γ and IL-12) enhance the production of IgG2a antibodies (Cox 1997, Quinn *et al.* 2002a).

IFN-γ can inhibit intracellular multiplication of tachyzoites (Innes *et al.* 1995). Studies in mice confirm the role of IFN-γ in controlling infection by *N. caninum.* Interferon mediates protection against acute infection with *N. caninum* in BALB/c mice (Baszler *et al.* 1999). Several authors have found that PBMC from experimentally infected cattle can be stimulated by a *N. caninum* lysate to proliferate and produce IFN-γ (Lunden *et al.* 1998, Andrianarivo *et al.* 1999, Williams *et al.* 2000)

Some researchers tested the immunogenicity in cattle of a killed whole *N. caninum* tachyzoite preparation formulated with four different adjuvants (Andrianarivo *et al.* 1999). The immune responses of immunised cattle were compared with those of cattle that were experimentally infected with *N. caninum.* Serum IFAT titres were significantly higher in experimentally infected cattle than those from immunised cattle although after the third vaccination, antibody titres measured by IFAT were in the range of titres previously described for naturally infected cattle. Two of the adjuvant

formulations were also able to induce cell-mediated responses that were similar to those of the experimentally infected animals. The Havlogen preparation produced *N. caninum* specific proliferation of PBMC that was similar to that produced by the infected animals. Another preparation (Polygen-adjuvanted) stimulated animals to produce concentrations of IFN-γ that were similar to those from infected animals. This preparation was also found to produce one of the highest antibody responses as measured by the IFAT. In light of the importance of IFN-γ in host resistance to *N. caninum* in mice (Khan *et al.* 1997) and in the closely related *T. gondii,* it was considered worthy of further investigation.

The effectiveness of the POLYGEN^{TM}-adjuvanted killed *N. caninum* tachyzoite preparation in preventing foetal infection in pregnant cattle was studied further (Andrianarivo *et al.* 2000). Heifers were immunised with the POLYGEN-adjuvanted preparation on day 35 and 65 of gestation. Four weeks later (91 days gestation) they were challenged with two injections of $2x10^7$ *N. caninum* tachyzoites given intravenously and intramuscularly. The immunised heifers developed cellular immune responses specific to *N. caninum* that were characterised by increased IFN-γ production and lymphocyte proliferation. The heifers also developed a parasite specific humoral response shown by increased IFAT titres with a dominant IgG1 response. After a challenge with tachyzoites there was a significant humoral response with equal amounts of IgG1 and IgG2 antibodies detected. There was no anamnestic cellular response after challenge. The proliferative response and the production of IFN-γ after challenge was basically the same in all animals after challenge indicating there was no benefit to the immunised animals. Whether from immunised or non-immunised heifers, all foetuses had lesions typical of *N. caninum* infection and *N. caninum* parasites were detected by immunohistochemistry. It was concluded that this formulation did not prevent foetal infection in pregnant cattle.

Other authors (Choromanski and Block 2000) studied the safety of inactivated *Neospora* vaccine formulations and their humoral immune responses in cattle. *Neospora caninum* tachyzoites were formulated with Havlogen and Bay R1005 adjuvants. Cattle were vaccinated and then given a second booster injection 4 weeks later. Prior to vaccination all heifers had IFAT titres ≤160. After the second vaccination, all heifers had at least a four-fold increase in IFAT titre to *N. caninum* showing a great increase in

antibody production. These cattle were not challenged with *N. caninum* tachyzoites so it is not known whether these antibody tires would have resulted in producing a protective immunity.

Others have tried a live vaccine approach where a recombinant canine herpesvirus which expressed NcSRS2, was constructed and tested in dogs (Nishikawa *et al.* 2000). NcSRS2 is a surface protein of *N. caninum*, which is expressed in both the tachyzoite and bradyzoite stage but is down regulated in the bradyzoite stage (Hemphill and Gottstein 1996, Schares *et al.* 1999b, Vonlaufen *et al.* 2004). It is also involved in parasite invasion into host cells (Howe and Sibley 1999). Dogs were inoculated intranasally with this live virus and antibodies specific to SRS2 were produced.

Work with a live vaccinia virus was tried by others (Nishikawa *et al.* 2001). Recombinant vaccinia viruses, which expressed either NcSAG1 or NcSRS2 were constructed. These two proteins are surface proteins of *N. caninum* tachyzoites. A mouse model of *N. caninum* infection was used to test the efficacy of both recombinant viruses. BALB/c mice are immuno-competent so the efficacy of the vaccination was assessed by PCR detection of *N. caninum* in various tissues. It had previously been found that at 8 days post-infection, the most frequently parasitised organs were the lungs and brain and so these organs were collected and examined by *N. caninum* specific PCR. It was demonstrated that during the early stages of infection, the vaccination did inhibit the spread of *N. caninum* into lungs and brain. In the case of NcSRS2, this protective effect against *N. caninum* lasted at least until 26 days post-infection. This is the first report of protection against *N. caninum* after vaccination with a single protein. An advantage of using vaccinia virus and a live antigen delivery system is that since vaccinia virus has a wide host range this could be effective in many animals. Large-scale production is relatively easy, less expensive and has resistance to environmental extremes (Nishikawa *et al.* 2002). There is also no need for it to be formulated with an adjuvant as would be the case of a tachyzoite or recombinant antigen vaccine. Being a modified live virus, there are however, possible safety issues. A host may already have immunity to the virus vector and there may be interference with this immunity. The virus may also convert back to a virulent strain and cause further disease or even recombine with wild viruses (Reichel and Ellis 2002).

For a number of years now, Intervet has had a *N. caninum* vaccine, NeoGuard™ on the market in the USA and Canada. More recently this vaccine has been introduced into New Zealand. The vaccine contains a lysate of killed protozoa and a havlogen adjuvant. Since 1998, the license was conditional as efficacy and potency studies were in progress. A technical bulletin released by Intervet outlined the field safety study that involved 757 pregnant cattle and showed that the vaccine was safe for use in healthy, pregnant cattle, and did not adversely affect the pregnancy rates or the percentage of normal calves delivered. There were no excessive injection site reactions (Choromanski 2002b). In another study involving 38 pregnant cattle (two groups of 19 cattle) the vaccine was shown to reduce abortions caused by *N. caninum.* The cattle were subcutaneously injected with vaccinate on days 56 and 77 (*i.e.* in the first trimester) of gestation and challenged with a *N. caninum* preparation on day 95 of gestation. As a measure of the immune response, serum samples were tested in a commercially available *N. caninum* antibody ELISA from IDEXX Laboratories. Both at the time of challenge and 28 days after challenge there was a significant antibody response between the vaccinated group and the control group. The vaccinated heifers also mounted a marked anamnestic response after challenge. There were no abortions in the NeoGuard treated group while there were four abortions (22%) in the non-vaccinated control. Placental samples from three of the aborted foetuses were positive for *N. caninum* (Choromanski 2002a). In November 2001 full approval was granted by the United States Department of Agriculture to NeoGuard and is available in the USA. However, only one of the studies, which showed limited efficacy of NeoGuard in reducing abortion has been published in a peer reviewed journal (Romero *et al.* 2004).

There is a high cost associated with the labour intensive methods of *in-vitro* culture and purification of *N. caninum* tachyzoites to produce a vaccine based on live or killed tachyzoite preparations (Nishikawa *et al.* 2002, Reichel and Ellis 2002). Another approach, which may be less expensive, would be to use recombinant antigens of *N. caninum* as vaccine candidates. There have been many recombinant antigens produced, by different groups but these have mainly been used in serological diagnosis of the disease (Lally *et al.* 1996, Jenkins *et al.* 1997, Lally *et al.* 1997, Liddell *et al.* 1998). A recent study reported that mice immunized with recombinant antigens and plasmid DNA (containing sequences that encode *N. caninum* antigens) was protective against cerebral neosporosis whereas the recombinant antigens alone resulted in a lower degree

of protection (Cannas *et al.* 2003). Another study involving DNA vaccination (*i.e.* plasmid DNA containing sequences that encode *N. caninum* antigens) of mice found that this resulted in partial protection against congenital neosporosis (Liddell *et al.* 2003).

1.11 Other species affected

1.11.1 Natural infections

Neospora caninum has a wide host range and has been identified in natural infections of dogs (Dubey *et al.* 1988a, Trees *et al.* 1993, Reichel 1998, Sawada *et al.* 1998), cattle (Thilsted and Dubey 1989, Paré *et al.* 1996), sheep (Dubey *et al.* 1990b), goats (Barr *et al.* 1992, Dubey *et al.* 1996), horses (Dubey and Porterfield 1990) and deer (Woods *et al.* 1994, Dubey *et al.* 1999), Antibodies to *N. caninum* have also been found in naturally exposed water buffaloes (Huong *et al.* 1998), camels (Hilali *et al.* 1998), coyotes (Lindsay *et al.* 1996a), foxes (Lindsay *et al.* 2001, Schares *et al.* 2001), dingoes (Barber *et al.* 1997) and hares (Ezio and Anna 2003), which suggests that these animals are natural intermediate hosts of *N. caninum*. *N. caninum* DNA has also been detected in foxes, providing further evidence that foxes are intermediate hosts (Almeria *et al.* 2002).

1.11.2 Experimental infections

Experimental infections with *N. caninum* tachyzoites have also been induced in mice (Dubey *et al.* 1988b, Lindsay *et al.* 1995a), rats (Lindsay and Dubey 1990), dogs (Dubey *et al.* 1988b, Cole *et al.* 1995), goats (Lindsay *et al.* 1995b), cats (Dubey and Lindsay 1989), sheep (Dubey and Lindsay 1990), coyotes (Lindsay *et al.* 1996a), foxes (Schares *et al.* 2001), pigs (Jensen *et al.* 1998), gerbils (Cuddon *et al.* 1992), sheep (Dubey and Lindsay 1990) and cattle (Barr *et al.* 1994). Domestic pigeons have also been experimentally inoculated with *N. caninum* tachyzoites (McGuire *et al.* 1999) as have blackbirds and starlings (Packham *et al.* 1998).

1.12 Aims and Objectives

An opportunity arose to work with a farmer to explore the causes of abortion on his property particularly as he had just been informed by the veterinarian of the herd that the most recent abortion was caused by *N. caninum*. There are six chapters based around aspects of the on-farm investigation into abortion and *N. caninum*. Subsequently, there are a further two chapters involving the validation and performance characteristics of *N. caninum* ELISAs utilising milk and serum and also a prevalence study in NSW dairy cattle. These last two chapters are essentially presented in the form of the manuscripts submitted for publication to *Veterinary Parasitology*. Various sections of the on-farm investigation were published in an article in *Veterinary Parasitology*.

After the initial abortion the aim was to investigate the causes of abortion including *N. caninum* on the study property. The cattle found to be seropositive to *N. caninum* by ELISA were further confirmed as positive in other ELISAs before family trees were constructed to investigate the route of transmission. As the farmer also fed pooled milk to calves a further aim was to investigate this as a potential new route of transmission. Milk was collected from seropositive cows to firstly see if *N. caninum* DNA could be detected in milk by PCR and subsequently attempt to infect mice with milk from these cows. As it has been suggested that *Neospora caninum* may influence milk production and early foetal loss, parameters such as milk, protein, fat, number of inseminations and time to conception were analysed with an aim to confirm this. At present there is no treatment for control of *N. caninum* although it has been suggested that a test-and-cull program may be effective in herds with high levels of vertical transmission. Thus a further aim was to investigate the effectiveness of this method of control whereby *N. caninum* seropositive cattle were gradually culled over a period of one year.

The validation of reliable *N. caninum* ELISAs is essential for the diagnosis of causes of abortion, epidemiological studies and in test-and-cull control programs. Hence the aim of one study was to determine the performance characteristics of two ELISAs that detect serum antibodies. The final aim was to determine the performance characteristics of an ELISA modified for use with milk samples and subsequently apply the ELISA to determine the prevalence of *N. caninum* infection in NSW dairy cattle.

2. Infectious causes of abortion in Australian dairy cattle

2.1 Introduction

There are many causes of abortion in cattle ranging from genetic abnormalities of the foetus and toxic compounds in feed to infectious agents. In many cases the cause of the abortion is never determined but infectious agents are the most commonly diagnosed cause of abortion (Thornton 1992, Thornton 1996, Hovingh 2002).

Abortion in cattle is commonly defined as a loss of the foetus between the age of 42 and 260 days gestation. Pregnancies lost before implantation at around 42 days are usually described as early embryonic deaths. A stillbirth is defined as a calf that is born dead between 260 days and full-term (Hovingh 2002).

A low abortion rate of 3-5% per year is often accepted as normal in the dairy industry. A single abortion can cost the farmer approximately A$1000 depending on the cost of replacement stock, milk prices, stage of gestation and value of the aborting stock (Pfeiffer *et al.* 1997, Hovingh 2002). This seems a significant loss to the farmer and investigations as to the cause of the abortion should be undertaken so preventative action can be taken.

2.1.1 Abortion causes

Some infectious causes of abortion in Australia are BVD virus, IBR, *N. caninum*, *Leptospira interrogans* serovars (Hungerford 1990, Durham and Paine 1997, Reichel 2000).

2.1.1.1 BVDV

BVD is caused by a pestivirus (BVD virus) and may present with many different clinical signs in cattle. It may cause diarrhoea but may also cause abortion and mucosal disease. BVDV circulating in a cow can reach the foetus by crossing the placenta. Infection with BVDV during the pre-implantation period may result in embryonic death (Munoz-Zanzi *et al.* 2003). If a non-immune cow is exposed to BVDV at up to 125 days

gestation, this may result in foetal death, abortion or mummification of the foetus or the birth of a persistently infected calf (McGowan and Kirkland 1995). This time of infection of 42-125 days corresponds to a time when the calf's immune system has not developed sufficiently to recognise the virus as foreign. When the calf's immune system does develop, it then incorrectly identifies the BVD virus as a "self protein" and does not attack it. If foetal infection takes place during mid-gestation (125-180 days) there is a high incidence of congenital abnormality (Fray *et al.* 2000). A calf that is exposed to the virus in the last trimester of gestation is usually immunocompetent and will generally be unaffected (Fray *et al.* 2000) but will have produced antibodies to BVDV. In Australia, surveys have suggested that 60% of adult cattle are seropositive for BVD and that infection is widespread (St George *et al.* 1967, Littlejohns and Horner 1990). A more recent survey in South Australia found that 76% of cattle had antibodies to BVDV (Durham and Paine 1997).

2.1.1.2 IBR

IBR is an infectious disease of cattle that is caused by a type of bovine herpesvirus (BHV-1). The most common clinical symptom is respiratory disease where cattle get pneumonia. It is also a cause of abortion, which commonly occurs from 4 months of gestation to term (Hovingh 2002). BHV-1 is also the aetiological agent of a number of other diseases, being infectious pustular vulvovaginitis, infectious balanoposthitis, conjunctivitis, encephalomyelitis and mastitis (Straub 2001). IBR is widespread in Australia with prevalence rates of 28% (St George *et al.* 1967) while others more recently found that the prevalence in South Australia was 54% (Durham and Paine 1997).

2.1.1.3 Leptospirosis and brucellosis

Leptospirosis is caused by a bacterial infection and can result in mastitis and "red water" (blood in urine) in cattle. It is also a cause of abortion in cattle caused by several serovars of *L. interrogans* (*e.g. hardjo, pomona*) (Bureau of Animal Health: Attwood 1998). As the farmer in the present study vaccinates against leptospirosis in August of every year, this was not tested for. Brucellosis (*Br. abortus*) is a leading cause of

abortion in cattle in many countries but has been eradicated from Australia since about 1987 (Hungerford 1990).

2.1.1.4 Neospora caninum

Neospora caninum is a major cause of abortions in cattle in many countries including Australia (Dubey and Lindsay 1996). By serological examination, many authors have found an increased risk of abortion amongst *N. caninum* positive cows. In California, Britain and Netherlands there was a 7.4, 3.5 and 3 fold increased risk, respectively, of abortion in *N. caninum* seropositive cows (Thurmond and Hietala 1997a, Wouda *et al.* 1998b, Davison *et al.* 1999b). An Australian study found that there was an eight-fold increase in abortions amongst seropositive animals (Atkinson *et al.* 2000b). In one *N. caninum* abortion outbreak, 33% of the breeding stock aborted within a few months (Thornton *et al.* 1994). In another study of aborted foetuses it was found that 30% of the diagnosed abortions were caused by *N. caninum* (Thornton *et al.* 1991). The predominant route of transmission between cattle is congenital transmission where the foetus is infected *in utero* (Paré *et al.* 1996). It also has been suggested that cattle become infected postnatally, by ingesting oocysts from the environment (De Marez *et al.* 1999, Trees *et al.* 2002). Infected dogs (McAllister *et al.* 1998) and coyotes (Gondim *et al.* 2004) can excrete oocysts and contaminate the environment.

There have been some investigations into the risk of abortion in cattle with concurrent infections of BVDV and *N. caninum*. A Swedish study found a statistically significant association between the presence of *N. caninum* and BVDV among aborting cows (Björkman *et al.* 2000) indicating that concurrent effects of *N. caninum* and BVDV may increase the risk of abortion in cattle. A group in New Zealand also found a similar effect where concurrent infection of cattle with *N. caninum* and BVDV increased the risk of abortion greater that a single infection alone (Williamson *et al.* 2000). This may be due to the immunosuppressive effects of BVDV (Potgieter 1995). Others have also suggested that abortions due to *N. caninum* may follow immunosuppressive infections such as BVDV (Thurmond and Hietala 1995, Alves *et al.* 1996).

2.1.2 Abortion diagnosis

Diagnosis of the cause of abortion in cattle is difficult and costly and as there are answers in less than 50% of cases diagnosis is not always done. Of 527 aborted bovine foetuses sent in 1994-1995 to the Veterinary Laboratory, Ontario for pathology, the etiology was undetermined in 67% of cases (Alves *et al.* 1996) as was the case in a 10 year study of abortion in South Dakota where the cause of 67% of abortions was not determined (Kirkbride 1992). A similar story was found in New Zealand and Australia, where the cause of 75% and 69%, respectively of abortions remained undiagnosed (Norton *et al.* 1989, Thornton 1996).

Although *N. caninum* is a significant cause of abortion in cattle, it is often undiagnosed and unnoticed, as cattle infected with *N. caninum* show no prior clinical signs of disease. Abortion storms due to *N. caninum* can have a big impact however. When epidemics occur, 30% of pregnant cattle can abort in a period of only months (Thornton *et al.* 1994). Over time though, *N. caninum* can have a great impact even in endemically infected herds with sporadic abortions but in such instances the farmer may not even realise the cause of the abortions. An individual farmer will not usually attempt to follow up every cause of abortion and even if they do there is only a definitive answer in less than 50% of cases. With a low rate of diagnosis and high cost, many farmers will only submit foetuses for pathology after several abortions have occurred (Hobson *et al.* 2002). Based on a 100-cow dairy, where there are possibly only five sporadic abortions during the year, there is a low chance of farmers submitting foetuses and returning a positive finding. Therefore *N. caninum* is relatively unknown to Australian dairy farmers due to a low diagnosis rate and a low level of abortions in endemically infected herds. Thus in the view of most farmers in Australia, *N. caninum* is not seen as a great cause for concern. It is only those farmers who have experienced an abortion storm, who know of the massive impact of the disease.

2.1.3 Objective

At the time of identifying this case herd there had only been one recent abortion. *Neospora caninum* was suspected as the cause of the abortion by the cow's *N. caninum*

positive serology and the lack of other clinical signs of disease. The objective of this study was to investigate *N. caninum* in this herd by serologically testing for *N. caninum,* monitoring abortions and serologically testing for other abortifacients (*i.e.* BVDV and IBR).

2.2 Materials and Methods

After an initial abortion in November 2002, the cow was blood-sampled and serum sent for testing for anti-*N. caninum* antibodies by ELISA at the Elizabeth Macarthur Agricultural Institute (EMAI). This returned a positive result but other abortifacients were not tested for and so could not be completely ruled out as the cause of the abortion. The herd was composed of Holstein Friesian cattle and had been a closed herd with no foreign cows being introduced since at least 1980. In December 2002 the herd was composed of 140 cows, plus approximately 60 heifers and approximately 66 calves. The milking cows were on pasture on one side of the property and were generally kept separate from the rest of the herd. Calves were kept in calf rearing pens and when heifers were ready for breeding they were moved to another side of the farm to be joined with the bull. In subsequent years the maturing cows were artificially inseminated.

A total herd bleed was undertaken in December 2002, and the sera was tested by ELISA for antibodies to three known abortifacients. Over the 12 months following the initial abortion the herd was monitored by the farmer and farmhand, who reported all abortions. Aborted foetuses were collected where possible and submitted for pathology. The pathology was completed through EMAI in all cases except one through Sydney University's Veterinary Clinic, Camden. In two cases of abortion no foetus was found.

To be sure that all abortions were detected, only results of cows that were in the milking herd were included. These cows were milked twice daily and so were very closely monitored. They were also artificially inseminated so a precise date of conception was available from the farmer's records. In this herd the heifers were not monitored as closely as the milking cows and it is possible that an early abortion could have been missed, so they were not included in the analysis of abortions.

In each case of abortion, the serostatus of the aborting cow to *N. caninum*, BVDV and IBR was tabulated and the attributable risk of abortion, relative risk and probability were calculated. The attributable risk of abortion associated with a disease was calculated as the percentage of seropositive animals that aborted minus the percentage

of seronegative animals that aborted. The relative risk (RR) of abortion was calculated as the percentage of seropositive animals that aborted divided by the percentage of seronegative animals that aborted. The probability (P) that a disease caused the abortion was calculated by: P=(RR-1)/RR.

The farmer also gave details regarding the theoretical costs associated with an abortion. The loss of milk production was calculated by assuming that the aborting cow was sold for meat and a replacement milking cow was purchased. Other costs included the loss of a calf, veterinary fees and disease testing.

Figure 2 Photograph of study property (P. Williams, Kemps Creek)

2.2.1 Herd sampling

Blood samples were collected from all 266 cattle on the dairy in Kemps Creek, NSW, in December 2002. Serum was collected, transferred to 1 ml tubes in 96 well racks and stored in duplicate at -20ºC. Serum samples were screened for antibodies to three known abortifacients:

1) *N. caninum* in a blocking ELISA (Catalogue no. P00510/01, Institut Pourquier, Montpellier, France).

2) BVDV in a competition ELISA (Catalogue no. P00610/11E, Institut Pourquier, Montpellier, France). Evaluation of the BVDV ELISA has determined that the sensitivity and specificity were 97.6% and 97.3%, respectively as compared to the virus neutralisation test as the reference standard (Institut Pourquier, personal communication).

3) BHV-1 (Catalogue no. P03125/18E, Institut Pourquier, Montpellier, France). Evaluation of the indirect IBR ELISA determined that the sensitivity and specificity were 100% and 88.6% respectively (Institut Pourquier, personal communication).

This herd had been vaccinated against leptospirosis every August since 1992 and thus leptosporosis as a cause of abortion was not investigated.

2.2.2 *Neospora caninum* ELISA (Institut Pourquier, France)

This serum blocking or competition ELISA detects specific antibodies to *N. caninum.*
The test procedure supplied by the manufacturer was adhered to. All reagents, plates and serum were brought to RT before testing. The test serum was diluted 1:1 with dilution buffer by first dispensing 50 µl of buffer to each well then adding 50 µl of serum to the appropriate wells. 50 µl of positive and negative control was also added to the appropriate wells of the *Neospora* antigen coated plate. Test serum and controls were assayed in duplicate. The plate was covered with adhesive and then incubated for 2 h at 37°C. After incubation, the contents of the plate were discarded by flicking. The wells were washed three times with 300 µl of phosphate buffered wash solution. The

contents of the wells were discarded after each wash. After the final wash, the plate was tapped on dry paper towel to remove any residual wash fluid. Monoclonal anti-*N. caninum*/horseradish peroxidase conjugate (100 µl) was dispensed into each well and the plate was incubated for 30 min at 37°C. The plate was washed again as previously described. After washing, 100 µl of TMB chromogen solution was dispensed into each well. The plate was then incubated away from the light for 20 min at RT. Stop Solution (100 µl 0.5 M $H_2SO_{4)}$ was added to each well and the OD was read at 450 nm on a Microplate Reader (Model 3550, Bio-Rad Laboratories). The percentage of inhibition for each sample was calculated as compared to the negative control by the following formula:

Inhibition % = ((mean sample OD) / (Nc OD)) x 100

where: Nc = negative control
OD = optical density

Serum samples that gave an inhibition percentage equal to or less than 45% were considered as coming from an animal that carries specific antibodies to *N. caninum*. Samples with an inhibition of 45-50% were considered to be doubtful and an inhibition equal to or greater than 50% was considered to be negative to *N. caninum* antibodies.

The test was valid if the mean negative control OD was greater than or equal to 0.600 and the inhibition percentage of the mean positive control was equal to or lower than 30%. The actual values for the negative control OD ranged from 1.848-1.500 with a mean of 1.641. The mean inhibition of the positive control was 17.3% and ranged from 15.7-19.9%.

2.2.3 BVDV ELISA (Institut Pourquier, France)

This ELISA detects specific serum antibodies to the P80 protein of BVDV. The recommended test procedure supplied by the manufacturer was adhered to. All reagents, plates and serum were brought to RT. The test serum was diluted 1:1 with dilution

buffer by first dispensing 50 µl of buffer to each well then adding 50 µl of serum to the appropriate wells. Positive and negative control (50 µl) was also added to the appropriate wells of the P80 protein monoclonal antibody antigen coated plate. Test serum and controls were assayed in duplicate. The plate was covered with adhesive and then incubated for 1 h at 21°C. After incubation, the contents of the plate were discarded by flicking. The wells were washed three times with 300 µl of phosphate buffered wash solution. The contents of the wells were discarded between each wash. After the final wash the plate was tapped on dry paper towel to remove any residual wash fluid. Conjugate (100 µl of monoclonal anti-P80 monoclonal antibody coupled to horseradish peroxidase) was dispensed into each well and the plate was incubated for 1 h at 21°C. The plate was washed again as previously described. After washing, 100 µl of the enzyme substrate TMB was dispensed into each well. The plate was then incubated in the dark for 20 min at 21°C. Stop Solution (100µl of 0.5 M H_2SO_4) was added to each well and the OD was read at 450 nm on a Microplate Reader (Model 3550, Bio-Rad Laboratories). The percentage of competition for each sample was calculated as compared to the negative control. Competition percentages for each serum sample were calculated by the following:

Competition % = ((mean sample OD) / (Nc OD)) x 100

where: Nc = negative control
OD = optical density

Serum samples that gave a competition percentage equivalent or less than 40% were considered as coming from an animal that carries specific antibodies to BVDV. Samples with a competition of 40-50% were considered to be doubtful and a competition equal to or greater than 50% was considered to be negative to BVDV antibodies.

The test was valid if the mean negative control OD was greater than or equal to 0.800, and the percentage competition of the mean positive control was less than 20%. The actual values for the negative control OD ranged from 2.501-2.127 with a mean of 2.252. The mean percentage competition of the positive control was 12.0% and it ranged from 10.1-13.1% over the six plates.

2.2.4 IBR ELISA (Institut Pourquier, France)

This ELISA detects specific antibodies to BHV-1, which is the cause of IBR. The antigen is an ultra purified BHV-1 lysate and so it can be used to demonstrate the presence of various types of antibodies to the virus including capsid proteins and envelope proteins in sera. The recommended test procedure supplied by the manufacturer was adhered to. All reagents, plates and serum were brought to RT before testing. The test serum was diluted 1/20 by first dispensing 190 µl of dilution buffer to each well then adding 10 µl of serum to the appropriate wells of the BHV-1 antigen coated plate. Positive and negative control (10 µl) was also added to the appropriate wells. Test serum and controls were assayed in duplicate over four wells, as there were both specifically coated wells and non-coated wells. The plate was covered with adhesive and then incubated for 1 hr at 37°C. After incubation, the contents of the plate were discarded by flicking. The wells were washed three times with 300 µl of phosphate buffered wash solution. The contents of the wells were discarded between each wash. After the final wash the plate was tapped firmly on dry paper towel to remove any residual wash fluid. Monoclonal anti-bovine IgG peroxidase conjugate (100 µl) was dispensed into each well and the plate was incubated for 30 min at 37°C. The plate was washed again as previously described. After washing, 100 µl of TMB Substrate Solution was dispensed into each well. The plate was then incubated in the dark for 20 min at RT. Stop Solution (100 µl of 0.5 M H_2SO_4) was added to each well and the OD was read at 450 nm using a Microplate Reader (Model 3550, Bio-Rad Laboratories). The corrected OD for each serum sample was calculated by subtracting the OD value obtained from the uncoated well from the OD value obtained from the coated well. The S/P% for each serum sample was calculated by the following formula.

S/P = ((mean corrected OD sample) / (mean corrected OD Pc)) x 100

where: Pc = positive control
OD = optical density

Serum samples that gave an S/P% greater than or equal to 55% were considered positive for BHV-1 antibodies. A sample with an S/P% between 45-55% was considered doubtful. Serum samples with S/P% less than or equal to 45% were classified as negative for BHV-1 antibodies.

The test was valid if the uncorrected OD of the positive control was greater than 0.350 and the ratio between the corrected OD of the positive control and the corrected OD of the negative control (*i.e.* Pc OD/Nc OD) was greater than or equal to 3.5. The uncorrected OD of the positive control ranged from 0.596 to 1.123 and the mean was 0.827. The ratio between the corrected OD of the positive control and the corrected OD of the negative control ranged from 12.0 to 354 and the median and mean were 23.0 and 81.8, respectively.

2.3 Results

2.3.1 *Neospora caninum* ELISA

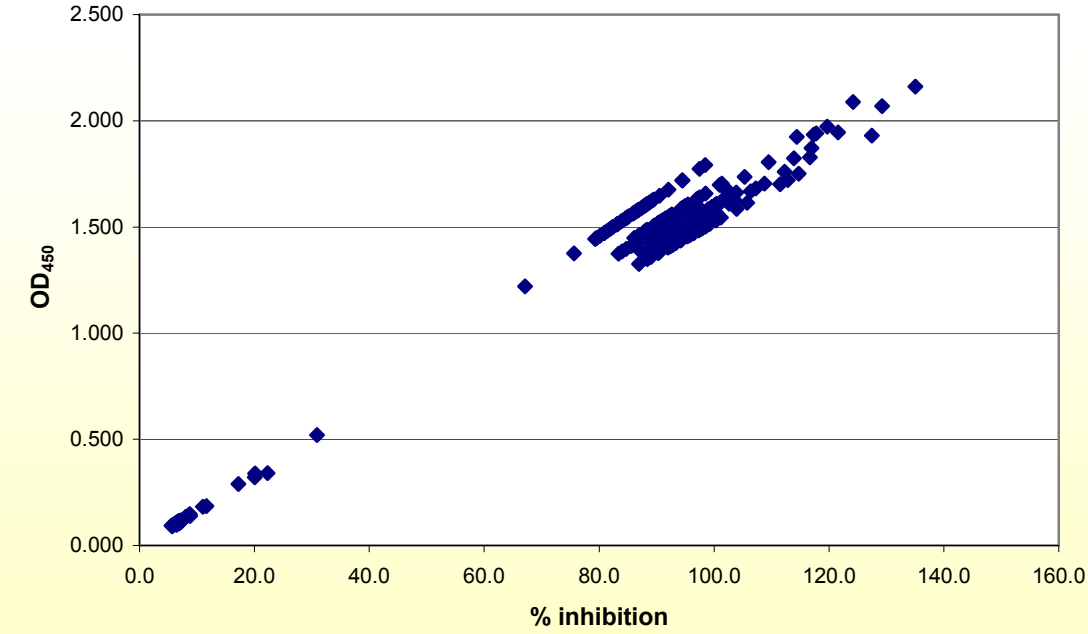
Twenty seven (27) out of 266 (10.2%) cattle had anti-*N. caninum* serum antibodies as determined by the Pourquier ELISA**.** Amongst the cows the prevalence was 11.4% (16/140) and in heifers there was a slightly lower prevalence of 8.7% (11/126). The two youngest seropositive cattle were 11 months old.

The seropositive animals with % inhibition ≤45%, are listed in Table 2. There was good discrimination between positive and negative samples by this ELISA (Figure 3). The % inhibition of the seropositive samples ranged from 5.6% to 30.9% with the median being 7.0%. Seronegative samples ranged from 67.1% to 135.1% inhibition and the median value was 93.6% inhibition. There were no samples determined to be doubtful, nor was any sample close to this cut-off as all samples were at least 15% from either side of this cut-off (Figure. 3).

Table 2 ***N. caninum*** **seropositive samples as determined by their % inhibition in the Pourquier ELISA.**

Cattle tag no.	Mean OD	% inhibition
325	0.290	17.2
339	0.136	8.1
289	0.114	6.7
303	0.339	20.1
338	0.520	30.9
261	0.116	6.9
273	0.147	8.7
246	0.111	6.6
267	0.104	6.2
284	0.094	5.6
274	0.138	8.8
333	0.106	6.7
55	0.096	5.8
109	0.118	7.1
200	0.094	5.7
178	0.182	11.0
237	0.186	11.6
43	0.321	20.0
81	0.091	5.7
234	0.120	7.5
914	0.098	6.4
222	0.110	7.2
77	0.104	6.8
231	0.099	6.5
68	0.107	7.0
225	0.340	22.3
211	0.105	6.8

Figure 3 **Percentage inhibition and corresponding OD exhibited by 266 individual cattle sera tested in the *N. caninum* ELISA (Institut Pourquier)**



2.3.2 BVDV ELISA

The overall prevalence of antibodies against BVDV in this herd of cattle was 44.0% (117/266). Amongst cows, there was a high seroprevalence of 71.4% (99/140) but in heifers and calves combined there was a much lower seroprevalence of 14.3%. (18/126).

The seropositive animals with % competition ≤40%, are listed in Table 3. The % competition of the seropositive samples ranged from 6.2% to 39.7% with the median being 17.0%. Seronegative samples ranged from 51.9% to 110.5% competition and the median value was 92.1%. There were four samples determined to be doubtful and these ranged from 41.8% to 49.9%. The doubtful range is indicated in Figure 4 by the area between the red and black bands. The discrimination between positive and negative samples was high in this ELISA (Figure 4). Around the recommended cut-off there was only a small number of samples (n=4) with values falling in the doubtful range. Aside

from these doubtful samples, there were also a total of eight other samples within 5% of each side of the doubtful region.

Figure 4 **Percentage competition and corresponding OD exhibited by 266 individual cattle sera tested in the BVDV ELISA (Institut Pourquier)**

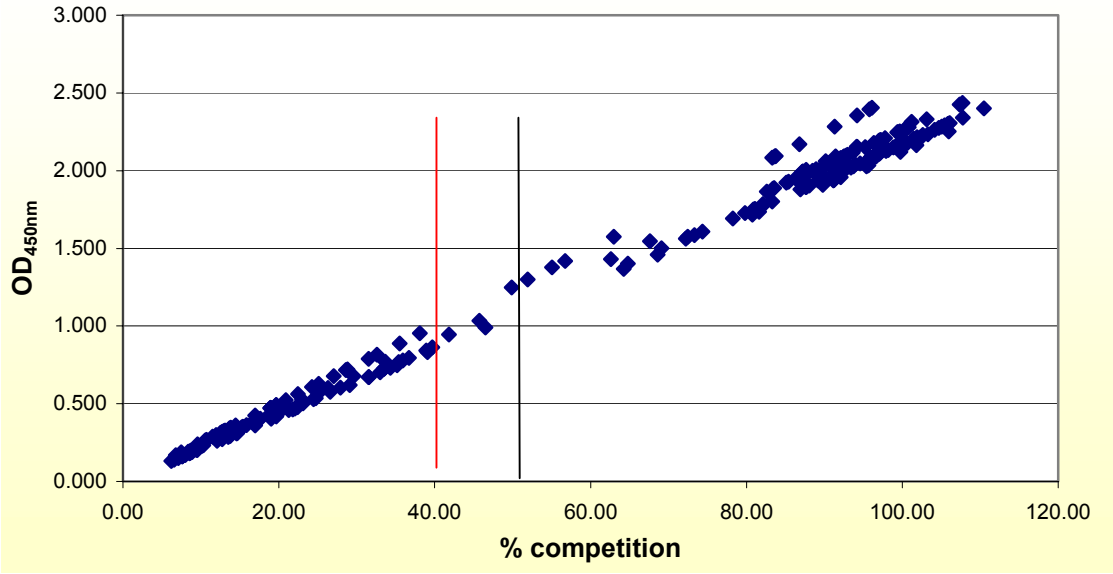


Table 3 **BVDV seropositive samples as determined by the % competition in the Pourquier ELISA.**

Sample ID	Mean OD	% competition
25	0.233	10.3
35	0.448	19.8
44	0.333	14.7
50	0.769	35.4
53	0.191	8.8
61	0.263	12.1
62	0.167	7.7
63	0.17	7.8
64	0.863	39.7
74	0.503	23.1
110	0.46	21.3
111	0.203	9.4
112	0.172	7.9
114	0.842	38.9
118	0.529	24.5
119	0.726	33.6
120	0.725	33.5
121	0.576	26.6
123	0.427	19.7
125	0.536	24.8
128	0.778	35.9
129	0.604	27.9
135	0.794	36.7
136	0.384	16.8
137	0.441	19.3
139	0.393	17.2
140	0.303	13.2
141	0.293	12.8
142	0.192	8.4
143	0.677	29.6

Sample ID	Mean OD	% competition
144	0.232	10.1
145	0.526	23
147	0.429	18.8
148	0.469	20.5
151	0.479	20.9
152	0.602	26.3
153	0.348	15.2
154	0.462	20.2
155	0.362	15.8
156	0.198	8.7
157	0.231	10.1
158	0.23	10
159	0.453	19.8
160	0.339	14.8
161	0.577	25.2
162	0.287	12.5
163	0.444	19.4
166	0.309	13.5
168	0.353	15.4
169	0.4	17.5
170	0.154	6.7
171	0.279	12.2
172	0.162	7.1
173	0.439	19.2
175	0.77	33.7
176	0.403	17.6
179	0.293	12.8
180	0.38	16.6
181	0.319	12.7
183	0.716	28.6

Sample ID	Mean OD	% Competition
185	0.888	35.5
186	0.342	13.7
188	0.607	24.3
189	0.313	12.5
190	0.953	38.1
191	0.628	25.1
194	0.347	13.9
195	0.288	11.5
196	0.721	28.8
198	0.322	12.9
199	0.361	14.4
200	0.523	20.9
201	0.424	17
203	0.472	18.9
205	0.677	27.1
206	0.492	19.7
207	0.324	12.9
208	0.562	22.5
209	0.239	9.6
210	0.169	6.8
211	0.816	32.6
212	0.264	10.6
214	0.299	12
215	0.268	10.7
216	0.491	19.6
219	0.473	18.9
221	0.328	13.1
223	0.187	7.5
224	0.789	31.5

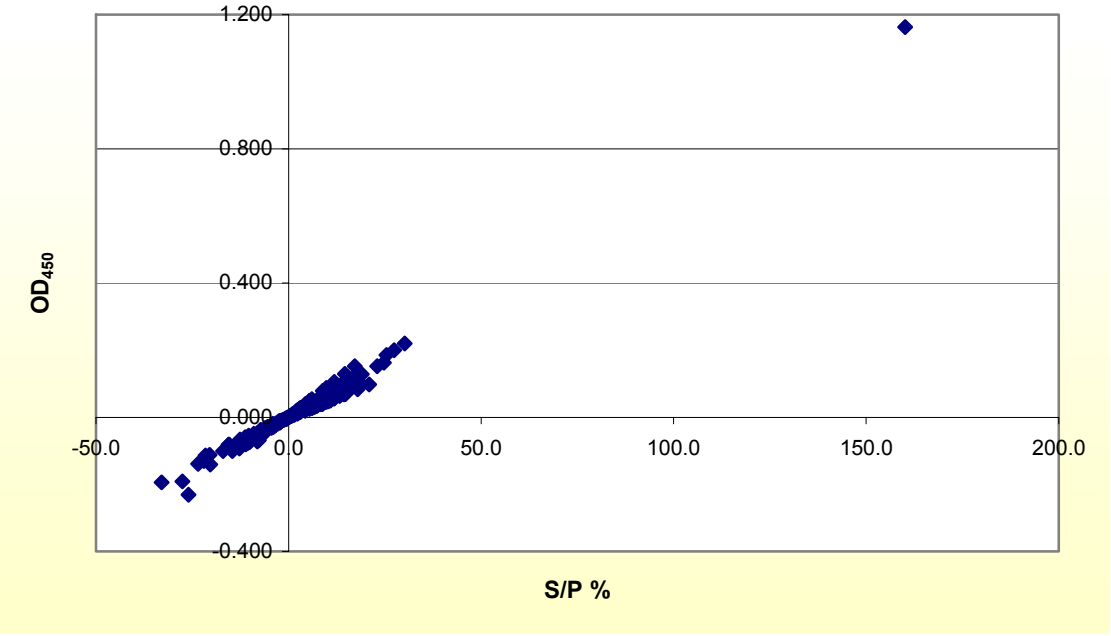
Sample ID	Mean OD	% Competition
226	0.619	29.1
227	0.189	8.9
228	0.171	8
233	0.47	22.1
235	0.184	8.6
237	0.203	9.5
239	0.312	14.6
240	0.73	34.3
242	0.162	7.6
244	0.671	31.5
245	0.477	22.4
246	0.132	6.2
247	0.831	39.1
249	0.271	12.7
251	0.702	33
252	0.14	6.6
253	0.671	31.6
254	0.162	7.6
255	0.151	7.1
256	0.748	35.2
257	0.405	19
258	0.419	19.7
259	0.31	14.6
260	0.287	13.5
261	0.464	21.8
263	0.361	17
265	0.293	13.8
266	0.182	8.5

A yellow highlighted sample ID indicates that the animal was also seropositive to *N. caninum*.

2.3.3 IBR ELISA

There was only one cow that had antibodies to BHV-1 virus, which gives an overall prevalence of 0.4% (1/266) in the herd studied. The sample from cow tagged #748 had a S/P of 160.1. This cow was 10.5 years old and thus one of the oldest in the herd. The S/P% of seronegative samples ranged from -33.0% to 30.2% and the median value was 5.1%. There were no samples determined to be doubtful. The negative sample with the highest S/P% was nearly 15% less than the doubtful cut-off of 45%. There was a clear distinction between the negative samples and the single positive sample as the positive sample was nearly three times the value of the positive cut-off (Figure 5).

Figure 5 **Percentage S/P and corresponding OD exhibited by 266 individual cattle sera tested in the IBR ELISA (Institut Pourquier)**



2.3.4 Abortions

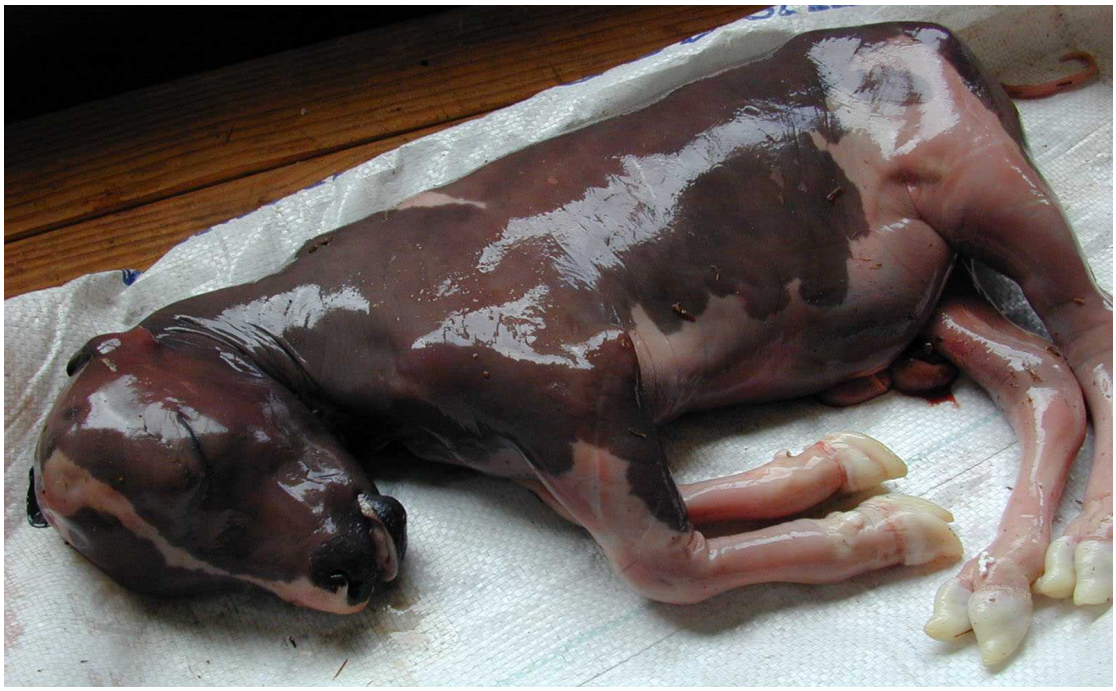
During the 12 months from November 2002 to October 2003 there was an overall abortion rate of 5.7% (8/140) amongst the cows. In five cases, a single foetus was found and submitted for pathology. A further cow aborted twins and both were submitted for pathology. The initial aborted foetus (dam #234) in this study was not submitted and the foetus of cow #109 was not found.

Overall results of aborted cows are presented in Table 4. Gestational ages at abortion ranged from 4-8 months and the median gestational age of aborted foetuses of *N. caninum* positive dams was 5.5 months. Of the eight aborting cows there were two with antibodies to *N. caninum* only, three with antibodies to BVDV only, and three animals had antibodies to both *N. caninum* and BVDV. No animal that aborted had antibodies to IBR.

Table 4 **Serostatus regarding *Neospora*, BVDV and IBR in cows that aborted from November 2002 to October 2003**

Cow tag #	*Neospora* status	BVDV status	IBR status	BVDV antigen	Date aborted	Gestation months (days)	Foetus collected Y/N
234	+	-	-	n/a	~7.11.02	4 (125)	N
237	+	-	-	n/a	27.2.03	5.5 (165-175)	Y
068	+	+	-	-	20.4.03	5.3 (150-165)	Y
211	+	+	-	n/a	14.7.03	8 (245)	Y
243	-	+	-	-	6.8.03	4.5 (138)	Y
008	-	+	-	-	11.8.03		Y (x2)
109	+	+	-	n/a	3.9.03	5.5m	N
224	-	+	-	n/a	22.10.03	7m	Y

Figure 6 **Aborted foetus from *N. caninum* seropositive cow at 5.5 months gestation**



2.3.5 Risk of abortion with *Neospora caninum*

Of the eight abortions in the 12-month period, five were from *N. caninum* seropositive dams. Sixteen (16) of the 140 cows were positive for *N. caninum* while 124 were negative. Of *N. caninum* seropositive cows 31.3% (5/16) aborted while only 2.4% (3/124) of *N. caninum* seronegative cows aborted. So the risk of abortion attributable to *N. caninum* in this herd was 28.9%. In this herd, a *N. caninum* seropositive cow was 13 (31.3/2.4) times more likely to abort than a seronegative (*i.e.* RR=13). The probability that *N. caninum* was the cause of the abortions was 92% (Table 5).

2.3.6 Risk of abortion with BVDV

Six of the eight abortions occurred in cows that were seropositive to BVDV. Of the 140 cows, 99 were seropositive while 41 were negative. Although 75% (6/8) BVDV seropositive cows aborted, the prevalence for the cows was 71.4%. Of BVDV seropositive cows 6.1% (6/99) aborted while 4.9% (2/41) of BVDV seronegative cows aborted in the same period. In this herd there was a 1.2% risk of abortion attributable to BVDV. The relative risk was 1.2 and the probability that BVDV caused the abortions was 17% (Table 5).

2.3.7 Risk of abortion with IBR

The single cow that was seropositive to BHV-1 did not abort. This cow was culled on 17 March 2003 due to chronic mastitis. She was one of the oldest cows in the herd at 10.5 years old.

Table 5 Risk of abortion

	Attributable risk of abortion (%)	**Relative risk of abortion**	**Probability of causing abortion (%)**
N. caninum	28.9	13	92
BVDV	1.2	1.2	17
IBR	0	0	0

2.3.8 Risk of abortion with concurrent infection

Table 6 Number of bovine sera with antibodies to *N. caninum* (*Nc*) and BVDV and abortions in these cows

	Nc+ and BVDV +	*Nc*+ and BVDV -	*Nc*- and BVDV+	*Nc*- and BVDV-
Aborting cattle	3	2	3	0
Non-aborting cattle	5	6	88	33

The attributable risk of abortion with seropositivity to both *N. caninum* and BVDV was 33.7% (3/8-5/132). If cows were only seropositive to *N. caninum* then the attributable risk of abortion was 20.5% (2/8-6/132). The attributable risk of abortion in cows that were only positive to BVDV was -6.9% (3/91-5/49). Where cows were seronegative to both *N. caninum* and BVDV the attributable risk of abortion was -7.5 (0/33-8/107.

It should be noted though, that one *N. caninum* positive cow was also rated as doubtful BVDV and for the previous calculations it was included as a BVDV negative. If this cow was considered as a BVDV positive then this would change the risk. The attributable risk of abortion with seropositivity to both *N. caninum* and BVDV would change to 29.5% (3/9-5/131) and the attributable risk of abortion with only *N. caninum* seropositive cows would change to 24.1% (2/7-6/133). There was still a greater attributable risk of abortion in cows with dual *N. caninum* and BVDV seropositivity although it was less than the previous calculations.

2.3.9 Economic impact

The dairy farmer gave estimates of costs of abortion for this dairy (Table 7).

Table 7 Estimated costs of abortion

Factor	Cost (A$)
Replacement milking cow	1200
Lost calf	50
Vet consultation and testing	150
Sale of cow for meat	-500
Total cost of abortion	**900**

The farmer experienced five abortions where *N. caninum* was implicated and the cost of these was calculated to be A$4,500.

2.4 Discussion

In this herd there was a low abortion rate of 5.7% over the 12-month period of observation. There was a strong association between dams seropositive to *N. caninum* and abortion, with 31% (5/16) positive cows aborting in the 12-month period and also 62.5% (5/8) of aborting cows were positive to *N. caninum*. The background rate of abortion in *N. caninum* negative cows was only 2.4%. The estimated risk of abortion attributed to *N. caninum* was 29%, with seropositive cows 13 times more likely to abort than seronegatives. These results support previous work where infection with *N. caninum* has increased the risk of abortion. A three-fold increased risk of abortion amongst seropositive cattle has been found (Wouda *et al.* 1998b) and others have reported a 7.4 fold-higher risk of abortion (Thurmond and Hietala 1997a). A study in Australia found an eight-fold increase in abortions amongst *N. caninum* positive cattle (Atkinson *et al.* 2000b).

The limitation of the BVDV antibody ELISA is that it only detects antibody and this does not mean that cattle are actually infected. In fact these cattle actually have a resistance to future BVDV infection. On the other hand *N. caninum* seropositivity is evidence of infection to *N. caninum*, which is expected to be long-term and lasting for life. In many situations the presence of specific antibodies to an organism indicates immunity. This is not the case with *N. caninum* where the presence of specific antibodies is indicative of infection (Paré *et al.* 1997, Stenlund *et al.* 1999).

In this study there was an trend towards increased attributable risk of abortion (33.7%) with concurrent infection with *N. caninum* and exposure to BVDV compared to a risk of 20.5% for infection with *N. caninum* alone and -6.9% for BVDV alone. This higher risk of abortion in cattle where there is concurrent infection with *N. caninum* and exposure to BVDV has been shown previously (Williamson *et al.* 2000). It has been suggested that abortion due to *N. caninum* is multi-causal as infection with *N. caninum* is not sufficient to cause abortion (Paré *et al.* 1997). In the latter study, 80% of infected cows did not abort and in the present study, 69% of infected cows did not abort. It is known that BVDV is a cause of immunosuppression (Potgieter 1995) and it has been suggested that an increase in infection with *N. caninum* could be due to the immunosuppressive

effects of BVDV (Alves *et al.* 1996). Others in Sweden have also found a significant association between infection with BVDV and *N. caninum*, indicating that there may be concurrent effects with these two organisms. In *N. caninum* positive cattle, there was a 2.1 times higher risk of infection with BVDV in randomly selected herds and a 2.8 times higher risk of infection with BVDV in herds with a history of abortion (Björkman *et al.* 2000). On the contrary, a group in The Netherlands found a negative relationship between seropositivity to BVDV and *N. caninum* in cows that had aborted (Bartels *et al.* 1999) and yet another study found that there was no substantial relationship between BVDV and *N. caninum* infection in cattle (Agerholm *et al.* 1997). A recent study of a dairy herd in Australia found a relationship between *N. caninum* and abortion but there was no association between presence of antibodies to BVDV and *N. caninum* due to the very high prevalence of BVDV (Quinn *et al.* 2004).

The importance of *Neospora* as a cause of abortion has been demonstrated in reports from New Zealand that suggest that approximately 30% of abortions were due to *N. caninum* (Thornton *et al.* 1991, Thornton *et al.* 1994). Reports from the UK estimate that 12.5% of abortions may be attributable to *N. caninum* (Davison *et al.* 1999b).

The prevalence of *N. caninum* (11.4%) in this herd is similar to that seen elsewhere. In Switzerland (Gottstein *et al.* 1998), New Zealand (Reichel 1998) and the UK (Davison *et al.* 1999b) herds with sporadic abortions had a seroprevalence of 11.5%, 6.8% and 6%, respectively. This is a relatively low rate of infection and elsewhere rates have been reported as high as 30-58% (Paré *et al.* 1996).

The median gestational age of the foetuses at the time of abortion was 5.5 months and this fits with the known pattern of abortions with *N. caninum*. Most abortions due to *N. caninum* occur at 5-6 months gestation (Anderson *et al.* 1991, Anderson *et al.* 1995, Wouda *et al.* 1999a). Of the five *N. caninum* seropositive cattle that aborted, three aborted at 5-5.5 months gestation while the other two aborted at 4 and 8 months gestation.

The prevalence of BVDV (71.4%) in this herd was similar to that found by others in Australia. In contrast, the prevalence of IBR (0.4%) in this herd was much lower than that found by others (Durham and Paine 1997). Although in this study there was no link

between infection with either BVDV or IBR and abortion, another study has found a significant correlation between simultaneous infection with BVDV and IBR in cows with reproductive disorders (Biuk-Rudan *et al.* 1999).

The set target of this herd was an annual abortion rate of 5% and as such, the actual abortion rate of 5.7% in the 12-month period would not be considered abnormal. Infection with *N. caninum* was implicated as the cause in five of these abortions and if eliminated there would possibly have been only three abortions. This is a 63% reduction in abortions to an overall rate of 2.1%. Due to the nature of *Neospora*, the farmer was not alerted to this fact that over 60% of aborting cows probably aborted due to *N. caninum* infection.

In this study of a herd with sporadic abortions it was found that the estimated risk of abortion attributable to *N. caninum* was 29% and seropositive cows were 13 times more likely to abort than seronegative cows. *Neospora caninum* was the major cause of the abortions, being implicated in 63% (5/8) of abortions. There has previously been suggestions that concurrent infection with BVDV may increase the risk of abortion (Pfeiffer *et al.* 2000). The current study also found that there was a trend towards increased risk of abortion (33.7%) attributable to dual exposure to BVDV and *N. caninum* and there was a 20.5% attributable risk of abortion in cows only infected with *N. caninum*.

3. Confirmation of cattle seropositive to *Neospora caninum* by ELISA

3.1 Introduction

There are a number of serological techniques that can be employed to diagnose *N. caninum* infection. The IFAT (Dubey *et al.* 1988b, Conrad *et al.* 1993, Paré *et al.* 1995b) is considered to be the reference standard of serological tests (Dubey and Lindsay 1996). The drawback with the IFAT is that it is not a standardised test. Laboratories use different antibody conjugates and the assessment is subjective, as it is done by eye and so it can be biased. Many laboratories have produced different ELISA's to detect anti *N. caninum* antibodies (Paré *et al.* 1995a, Baszler *et al.* 1996, Björkman *et al.* 1997, Williams *et al.* 1997, Wouda *et al.* 1998a, Björkman *et al.* 1999). Some of these ELISAs have been commercialised. The three commercialised *N. caninum* ELISA's used in this study were from: Institut Pourquier (Montpellier, France), IDEXX Laboratories (Westbrook, Maine, USA) and Cypress Diagnostics (Leuven, Belgium). The advantage of ELISA's is that they are standardised and are assessed optically by instruments. They are also rapid and therefore useful for large-scale screening.

Generally, when comparing ELISA's, all perform well when high titre sera are used but there are discrepancies when low titre sera are tested (Dubey *et al.* 1997, Wouda *et al.* 1998a) and there is evidence that individual cow serodiagnosis is difficult (Schares *et al.* 1999a). In the current study it was important to make the correct diagnosis on an individual animal basis as family trees were to be constructed based on this serology.

The IDEXX ELISA was first described (Paré *et al.* 1995a) and was later commercialised with a few changes. The antigen used is a sonicated bovine *N. caninum* (NC-1) tachyzoite lysate**.** This ELISA was shown to have high sensitivity and specificity of 89% and 97% respectively. When compared with the IFAT using a cut-off titre of ≥1:640, the ELISA was found to have superior sensitivity and specificity for the serodiagnosis of *Neospora* infection in cattle (Paré *et al.* 1995a). Others have also found

that the IDEXX ELISA has a higher sensitivity than the IFAT. Sera that were positive in the ELISA were also specifically positive in a Western blot (Immunoblot) which suggested that the ELISA was also specific (Schares *et al.* 1998). Other authors found that the sensitivity and specificity of the IDEXX ELISA was 98% and 87-92%, respectively (Wouda *et al.* 1998a) and that there was good correlation between the IDEXX ELISA and two other ELISA's: AHS ELISA – detergent tachyzoite lysate and MAST Diagnostics ELISA – whole fixed tachyzoites (Williams *et al.* 1997) when comparing abortion sera. There was also good correlation between the IDEXX and AHS ELISA's when using total herd samples.

Other authors compared four ELISA's, IFAT and Immunoblot using abortion outbreak sera (Schares *et al.* 1999a). They found that there was high correlation between the IDEXX ELISA and MAST Diagnostics ELISA (Williams *et al.* 1997) and also between the IDEXX and an in-house ELISA (Schares *et al.* 1999c). There was also high association detected between seropositivity and abortion with the IDEXX and MAST ELISA and they looked to be optimised for the purpose of detecting abortion. The correlation between IDEXX and MAST ELISA, using abortion sera is in agreement with others (Wouda *et al.* 1998a).

Researchers in Europe compared five commercial ELISA's and six in-house assays to detect *N. caninum* antibodies using 523 sera from experimentally infected animals (n=59), naturally infected dams without abortion (n=98) and dams that aborted (19 from endemic herds, 10 from epidemic abortion and 11 from herds with unknown pattern of abortion). There were also 326 negative sera. Included in this study were the IDEXX and Cypress ELISA's. The Cypress ELISA (Cypress Diagnostics) is an indirect test, which utilises a detergent lysate of *N. caninum* tachyzoites as antigen and is used for the detection of antibodies to *N. caninum* in bovine serum. This test has been found to have a sensitivity and specificity of 97.4-98.1% and 98.8-97.5%, respectively (von Blumröder *et al.* 2004). When using TG-ROC analysis of results compared to reference standards, the cut-off was found to be the same as that recommended by the manufacturer. The latter authors also found the IDEXX ELISA to have a sensitivity and specificity of 99.0-100.0% and 97.5-99.7%, respectively and the cut-off when using TG-ROC analysis was again determined to be the same as that recommended by the manufacturer. There was high correlation between the Cypress and IDEXX ELISA with

a kappa value of 0.96. The sera used were mainly from properties with endemic infection.

One group suggested that the cut-off for the IDEXX ELISA should be S/P =0.2 (Reichel and Pfeiffer 2002). They analysed epidemic abortion sera from New Zealand by TG-ROC with IFAT (>1:200 as positive) as the reference standard. The discrepancy between this cut-off and that suggested by others may be due in part to the use of sera from an abortion epidemic. It has been shown that IFAT-positive aborting dams from *N. caninum* associated epidemic abortions have significantly higher IFAT titres than those from endemic herds. Conversely, ELISA indices were higher in herds with endemic abortion rather than epidemic abortions (Schares *et al.* 1999c). This may cause sera from abortion epidemics to be determined as positive by IFAT but the S/P or ELISA indices may be low, thus causing a low cut-off.

As antibodies to *T. gondii* and *Sarcocystis* spp. have been found to cross react with multiple *N. caninum* antigens in the immunoblot assay, it has been suggested that any serological assay which utilises whole *N. caninum* organisms as the source of antigen, has the potential for reduced specificity (Baszler *et al.* 1996). Others though, did not find cross reactions between *Toxoplasma* sera in their ELISA (Paré *et al.* 1995a) and another group found that there was minimal binding of *N. caninum* antigens with anti-*Toxoplasma* sera in Western blots (Bjerkås *et al.* 1994).

The Pourquier ELISA is a competition or blocking ELISA and there are benefits to using this different type of test. Competition ELISA's are tests where a monoclonal antibody is allowed to compete with serum antibodies for epitopes on the assay antigen. One advantage of the competition ELISA is that it estimates the level of antibodies to one single epitope, and so is likely to be more specific and give less false positive results than conventional ELISA's (Björkman and Uggla 1999). Also, the secondary antibody is not species specific and so can allow for testing in many species.

3.1.1 Objective

After screening 266 cattle serum samples for antibodies to *N. caninum* by ELISA (Institut Pourquier), 27 positive animals were detected. To be sure that these were not false positives, the 27 positive sera and 10 sera that had not reacted were tested in a further two ELISA's.

3.2 Materials and Methods

3.2.1 Serum

Serum was collected from 266 cows on a farm in NSW and tested in the *N. caninum* ELISA (Institut Pourquier) as outlined in Chapter 2. The *N. caninum* positive sera (n=27) along with several negative sera (n=10) were further tested in two other *N. caninum* specific ELISA's (IDEXX and Cypress).

3.2.2 Anti-*Neospora caninum* ELISA (IDEXX Laboratories, USA)

Generally the test procedure supplied by IDEXX was adhered to. All reagents, plates and serum were brought to (RT). The test sera were diluted 1:100 with sample diluent in 1 ml microtubes of a 96 well rack (Bio-Rad Laboratories). Undiluted positive and negative control (100 µl) was dispensed in duplicate into the appropriate wells of the *Neospora* antigen coated plate. Diluted sample serum (100µl of 1/100) was dispensed in duplicate into the appropriate wells. The plate was then incubated for 30 min at RT. After incubation, the contents of the plate were discarded by flicking. The wells were washed four times with 300 µl of phosphate buffered wash solution. The contents of the wells were discarded between each wash. After the final wash the plate was drummed firmly on dry paper towel to remove any residual wash fluid. Anti-bovine horse radish peroxidase conjugate (100 µl) was dispensed into each well and the plate was incubated for 30 min at RT. The plate was washed again as previously described. After washing, 100 µl of TMB substrate solution (hydrogen peroxide and tetramethylbendizine) was dispensed into each well. The plate was then incubated for 15 min. at RT. Stop solution (100µl) was added to each well and the absorbance was read at 655 nm on a Microplate Reader (Model 3550, Bio-Rad Laboratories). S/P ratios for each serum sample were calculated by the following formula.

$$S/P = \frac{(\text{mean sample}) - (\text{mean NC})}{(\text{mean PC}) - (\text{mean NC})}$$

where: NC = OD negative control

PC = OD positive control

OD = optical density

Serum samples that gave S/P ratios greater than or equal to 0.50 were considered positive for *Neospora* antibodies. Serum samples with S/P ratios less than 0.5 were classified as negative for *N. caninum* antibodies.

The test was valid if the difference between the mean OD positive control (P) and the mean OD negative control (N) was greater than or equal to 0.15 (*i.e.* P-N) and the negative control OD was less than or equal to 0.2. The actual values were 0.640 (P-N) and 0.099 (N).

3.2.3 *Neospora caninum* Cypress ELISA (Cypress Diagnostics, Belgium)

In general, the test procedure supplied by the manufacturer was adhered to. All reagents, plates and serum were brought to RT. The test serum was diluted 1:100 in 1 ml microtubes of a 96 well rack (Bio-Rad Laboratories) with appropriately reconstituted Sample Diluent solution. Undiluted positive and negative control solution (50 µl) was dispensed in duplicate, into the appropriate wells of the *N. caninum* antigen coated plate. Diluted sample serum (100 µl) was dispensed in duplicate into the appropriate wells. The plate was then covered and incubated for 60 min at 37°C. The plate was then washed three times using 300 µl per well of reconstituted wash solution. After washing, the plate was drummed firmly on absorbent paper towel. Conjugate solution (50 µl containing a bovine monoclonal antibody conjugated to HRPO) was added to each well and the plate was incubated for 60 min at 37°C. The plate was washed again three times as previously. Substrate solution (50 µl) was added to each well and this chromogenic reaction was left to develop in the dark for 15 min at RT. Stop solution (50 µl) containing oxalic acid was added to each well. The OD was read at 405 nm on a Microplate Reader (Model 3550, Bio-Rad Laboratories) and the results recorded. IRPC values for each serum sample were calculated by the following formula.

$$IRPC = \frac{(\text{mean OD405 sample}) - (\text{mean OD405 NC})}{(\text{mean OD405 PC}) - (\text{mean OD405 NC})}$$

where: OD_{405} NC – optical density of negative control

OD_{405} PC – optical density of positive control

A serum sample that had an IRPC (Relative Index x 100) value greater than 5.0 was considered positive for *N. caninum* antibodies. Serum samples with IRPC values less than or equal to 5.0 were classified as negative for *N. caninum* antibodies.

The test was valid if the mean OD of the positive control (P) was greater than 0.9 and the mean OD of the positive control was greater than five times the mean OD of the negative control. The actual values were 1.3 (P) and P was 12 times greater than the negative control OD.

3.3 Results

The same 27 cattle serum samples were found to have antibodies to *N. caninum* as determined by both the IDEXX ELISA and Cypress ELISA at the cut-off recommended by the manufacturer. Ten sera that were negative in the Pourquier ELISA were also negative in the IDEXX and Cypress ELISA's.

With the IDEXX ELISA, there was good discrimination between positive and negative samples with a clear area around the cut-off of S/P=0.5 as visualised in Figure 7. There were no samples within 0.2 S/P of either side of the cut-off. The median S/P of positive samples was 1.74 (mean 1.69) and the range was 0.79-2.52 while the median S/P of negative samples was 0.21 and the range was 0.06-0.29.

The Cypress ELISA also showed good discrimination between the positive and negative samples. The median of the positive samples was 68.4 IRPC and the range was 5.4-126.7 IRPC while the median of the negative samples was 0.94 IRPC and the range was -0.08-1.8 IRPC. While there were two positive samples that were close to the cut-off, the remaining 25 positive samples had results that were at least five times higher than the cut-off in terms of IRPC (Figure 8).

There was high correlation (r^2=0.89) between the results of the IDEXX and Cypress ELISA (Figure 8). There was good agreement between all three of the ELISA's as these same 27 cattle were also shown to be seropositive by the *N. caninum* Institut Pourquier ELISA. The results of the 27 positive animals are shown in Table 8.

Figure 7 Distribution of samples by S/P of ELISA (IDEXX)

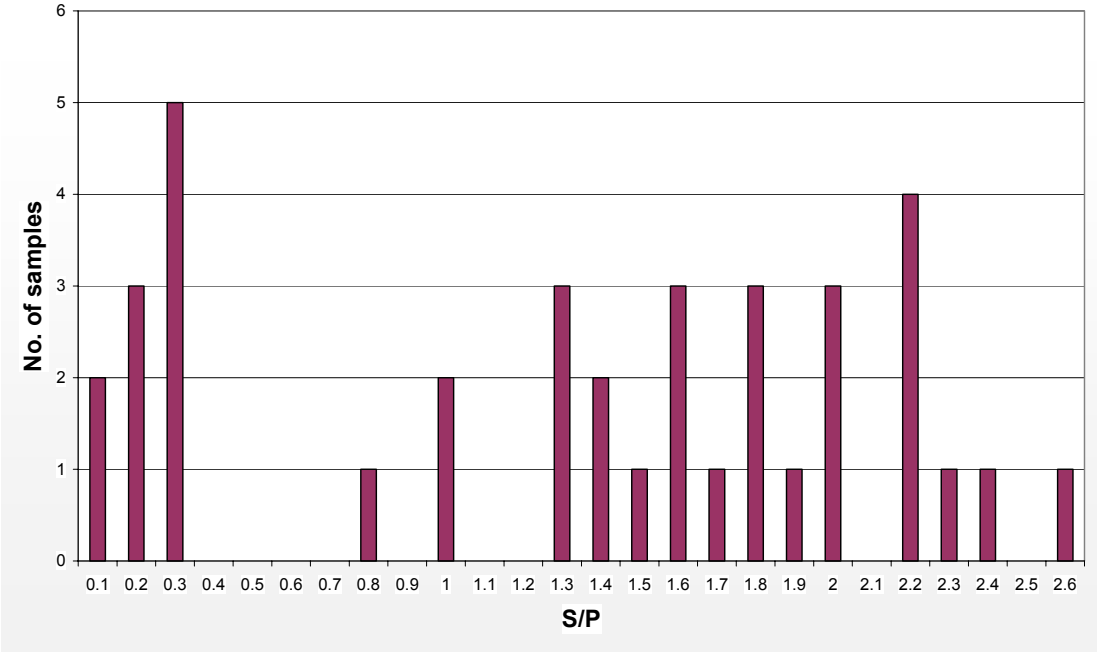


Figure 8 Comparison of two *N. caninum* ELISA's (IDEXX and Cypress)

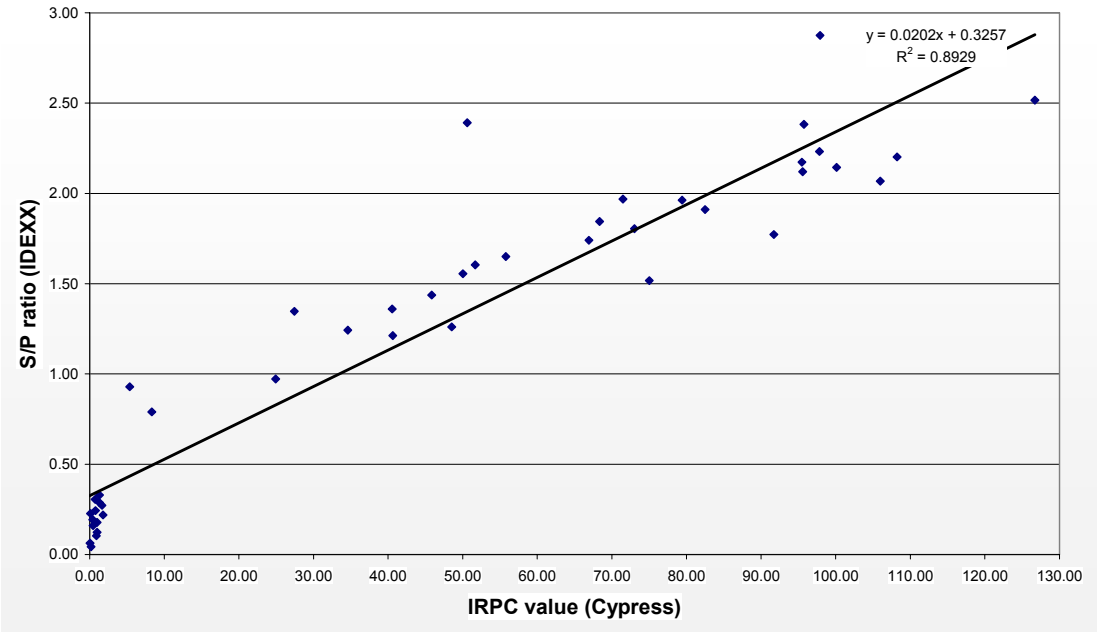


Table 8 Results of three *N. caninum* ELISA's for 27 positive cattle sera

Tag no.	S/P (IDEXX)	IRPC (Cypress)	% inhibition (Pourquier)
246	2.52	126.71	6.6
231	2.38	95.73	6.5
237	2.23	97.82	11.6
200	2.20	108.22	5.7
284	2.17	95.48	5.6
914	2.14	100.12	6.4
109	2.12	95.56	7.1
289	1.97	71.49	6.7
339	1.96	79.42	8.1
55	1.91	82.50	5.8
68	1.84	68.36	7.0
267	1.80	73.01	6.2
234	1.77	91.70	7.5
81	1.74	66.93	5.7
43	1.65	55.79	20.0
333	1.60	51.68	6.7
77	1.56	50.04	6.8
273	1.52	75.02	8.7
211	1.44	45.85	6.8
178	1.36	40.55	11.0
338	1.35	27.44	30.9
274	1.26	48.52	8.8
261	1.24	34.59	6.9
222	1.21	40.63	7.2
225	0.97	24.94	22.3
325	0.93	5.38	17.2
303	0.79	8.34	20.1

3.4 Discussion

The differences found between results of different ELISA's has been attributed in part to the antigens used (Wouda *et al.* 1998a, von Blumröder *et al.* 2004). The MAST ELISA uses intact tachyzoites and so there are mainly external surface antigens present. Other tests where lysates are used as antigen (*i.e.* IDEXX and Cypress), contain membrane antigens as well as cytoplasmic antigens. It has been suggested that antibodies to soluble cytoplasmic antigens are formed later in the infection whereas membrane antigens are expected to be recognized first (Wouda *et al.* 1998a, Schares *et al.* 1999a, Atkinson *et al.* 2000a). So a lysate based ELISA may be able to better detect antibodies from cows that have developed over a longer period than a membrane antigen based ELISA.

As the antigen used in both the Cypress and IDEXX ELISA is a lysate of *N. caninum* tachyzoites, it would be predicted that they would give similar results. The results presented here for these two ELISA's show a high correlation (r^2=0.89), which is similar to the results obtained by others (von Blumröder *et al.* 2004) where the level of agreement was high (kappa=0.96). These two ELISA's (IDEXX and Cypress) have previously been shown to have high sensitivity and specificity (von Blumröder *et al.* 2004).

The 27 samples identified as positive by the Institut Pourquier ELISA were confirmed as positive by the two lysate based ELISA's suggesting high agreement between all three of these tests. It may be expected that the monoclonal antibody of the Pourquier ELISA should increase the specificity for *N. caninum* antibodies.

Even though there was excellent agreement in distinguishing between positive and negative sera, correlation was poor between percentage inhibition values of the competition ELISA (Pourquier) and the S/P of the indirect ELISA (IDEXX). The reason behind this is that maybe, in a competition ELISA, once the maximum inhibition is reached, the addition of more competing antibody does not significantly affect the % inhibition (Baszler *et al.* 2001). This may also be due to the fact that the S/P of the IDEXX ELISA is affected mostly by the quantity of antibody present in a sample whereas the competition ELISA is affected by both the quantity and also the quality or

binding affinity of the serum antibody in order to effectively compete with the monoclonal antibody.

The agreement between all three of these *N. caninum* ELISA's is of importance to the next chapter of this study. The cattle identified as being positive to *N. caninum* were analysed for any family relationship that they may have to one another in order to assess the importance of vertical transmission as a route of infection in this herd.

4. Evidence of vertical transmission of *Neospora caninum* on a dairy farm in NSW

4.1 Introduction

Cattle can become infected with *N. caninum* by either vertical or postnatal transmission. The most predominant route of transmission of *N. caninum* in cattle is from cow to calf *in utero*. The *N. caninum* parasite can cross the placenta during gestation and infect the developing foetus. It has been shown that most congenital infections result in the birth of a clinically normal, healthy calf although chronically infected (Paré *et al.* 1996, Dubey 1999b).

Vertical or congenital transmission is very efficient, with 81-95% of infected cows giving birth to infected calves (Paré *et al.* 1996, Paré *et al.* 1997, Wouda *et al.* 1998b, Davison *et al.* 1999a). This seems to be the main method of maintaining the infection in the herd. The above studies used pre-colostral blood samples from calves born to seropositive dams to demonstrate vertical transmission of *N. caninum.* In two Californian drylot dairies, the congenital transmission rate was determined to be 81% and 89% (Paré *et al.* 1996, Paré *et al.* 1997). A study in the Netherlands of 50 dams that had previously aborted, found that 89% transmitted the infection to their offspring in their next pregnancy (Wouda *et al.* 1998b). In Britain, a study of six Holstein Friesian dairy herds found that there was 95% probability of vertical infection (Davison *et al.* 1999a). A German study (Schares *et al.* 1998) found 93% of descendants of seropositive cows were also seropositive. This study did not use pre-colostral serum, rather it used calves older than 6 months to eliminate the potential interference of maternal antibodies. Antibodies in cows do not pass the placenta, so the source of antibodies for newborn calves is colostrum (Black *et al.* 1995).

At least two studies used serological testing of cattle for *N. caninum* to form family trees in order to identify family associations of *N. caninum* infections. A Swedish study of a dairy herd showed there was familial clustering of seropositives in successive generations, which supports the role of congenital transmission (Björkman *et al.* 1996).

All seropositive cattle were the progeny of two of the original cows, which shows that these two cows were already infected when they were purchased. There were limited numbers of animals analysed and so the extent of vertical transmission could not be quantified. A study in Germany (Schares *et al.* 1998) also analysed family trees for evidence of congenital transmission. They found that seropositive animals were found in 38% (8/21) of the families. In five families all members were positive, while in three families, 25-69% of cattle analysed were positive.

Mathematical modelling studies have suggested however, that vertical transmission alone cannot sustain an infection within a herd. For the disease to be maintained within the herd there must also be some form of postnatal transmission occurring (French *et al.* 1999).

Postnatal transmission is generally used as a term to describe infection of cattle after ingesting oocysts from the environment. Dogs as the definitive host may shed these oocysts. It has been shown that dogs experimentally fed mouse brain or tissue containing *N. caninum* cysts excrete oocysts in their faeces although they shed them in low numbers and intermittently (McAllister *et al.* 1998, Lindsay *et al.* 1999, Basso *et al.* 2001). *Neospora caninum* oocysts have also been identified in a naturally infected dog. The oocysts from the dog were identified as *N. caninum* by gerbil bioassay and PCR (Basso *et al.* 2001).

Dogs may become infected after ingesting infected aborted material. Dogs have been fed placenta from *N. caninum* positive cattle and they shed *N. caninum* oocysts, although low in number and the shedding only lasted a few days (Dijkstra *et al.* 2001b). After feeding with small amounts of placenta there was no shedding but after a 6-10 fold increase in the amount of placenta fed, the dogs shed oocysts. After repeated feeding of the dogs with large amounts of placenta the dogs did not shed oocysts again. This is in line with the findings that dogs can be infected after being fed tissue cysts from *N. caninum* infected mice although again there was low and inconsistent shedding (McAllister *et al.* 1998, Lindsay *et al.* 1999, Basso *et al.* 2001). Recently, others have hypothesised that since cattle are natural intermediate hosts of *N. caninum*, they would be a more efficient source of infection for dogs than infected mice. Dogs fed tissue from infected calves produced nearly 30 times more oocysts than those fed infected mice

(Gondim *et al.* 2002). That dogs have been found to excrete high numbers of oocysts increases the argument that dogs are important in the transmission to cattle.

Seroepidemiologic data also support the role of the dog in the life cycle of *N. caninum.* An association has been found between postnatal infection of cattle on farms and the introduction of a new dog to the property in the previous 18 months (Dijkstra *et al.* 2002b). It was also found that there is a link between postnatal infection of cattle with *N. caninum* and certain activities of dogs on these farms. These activities being: dogs consuming placenta or licking uterine discharge, feeding colostrum or milk to dogs, and dogs defecating in the feeding alley (Dijkstra *et al.* 2002a). Farm dogs have been found to have a greater prevalence of anti-*N. caninum* antibodies than urban dogs and high seropositivity to *N. caninum* in cattle is correlated with seropositivity of farm dogs (Sawada *et al.* 1998, Wouda *et al.* 1999b).

Other definitive hosts may include other canids such as foxes. Serological evidence of naturally occurring infection with *N. caninum* has been found in foxes (Barber *et al.* 1997, Lindsay *et al.* 2001, Schares *et al.* 2001), coyotes (Lindsay *et al.* 1996a) and dingoes (Barber *et al.* 1997). This indicates that these animals are at least intermediate hosts of *N. caninum*. It is quite possible that canids other than dogs could be definitive hosts of *N. caninum*. It has recently been found that coyotes are also definitive hosts of *N. caninum* (Gondim *et al.* 2004). One of four coyote pups fed tissues from infected calves excreted *N. caninum*-like oocysts which were later determined to be *N. caninum* by PCR.

Domestic pigeons have also been experimentally inoculated with *N. caninum* tachyzoites (McGuire *et al.* 1999). These authors speculated that birds are common prey of foxes and other canids, and they may be a natural source of infection for foxes. There are large numbers of pigeons on the property studied here and crows have also been observed consuming the remains of aborted calves. Blackbirds and starlings have also been experimentally infected with *N. caninum* tachyzoites (Packham *et al.* 1998)

Postnatal infection also encompasses horizontal infection, such as cattle-to-cattle infection. This type of infection may be possible by feeding pooled milk or colostrum to

calves or from ingesting infected placentas, foetus or amniotic fluid but these routes of transmission have not been proven (French *et al.* 1999).

Toxoplasma gondii has been transmitted to goats via milk (Dubey 1980) and humans have also been infected with *T. gondii* after milk consumption (Tenter *et al.* 2000). Considering the similar biology of *N. caninum* and *T. gondii* it is quite possible that cattle and dogs could be infected by ingesting milk from seropositive cows (Dijkstra 2001). It has previously been demonstrated that newborn calves can be orally infected with colostrum spiked with *N. caninum* tachyzoites (Uggla *et al.* 1998). The latter authors have referred to the possible infection of calves by their dams' milk, as pseudo-vertical transmission. The offspring would likely be already infected by *in uteru* transmission. The more important point though is that farmers often feed pooled milk to calves. If the milk originates from *N. caninum* infected cows and contains *N. caninum* tachyzoites then there is the possibility that these calves will be postnatally infected.

Endemic abortions are usually thought to be associated with vertical transmission whereas abortion epidemics have been associated with point source infections (Thornton *et al.* 1994, McAllister *et al.* 1996, Dijkstra *et al.* 2001a). Evidence for vertical transmission comes from the equal distribution of seropositives cattle among age groups whereas age clustering of infected animals supports a point source of exposure and points to post natal transmission (Dijkstra *et al.* 2001a).

The objective of this study was to investigate the route of transmission of *N. caninum* in a herd of dairy cattle.

4.2 Materials and Methods:

Herd details and serology have been previously described in Chapters 2 and 3. It is also noted that the farmer feeds pooled milk to newborn calves.

4.2.1 Genealogy

Using a database (DairyCHAMP Pastoral) supplied by the consultant veterinarian of the herd and the farmer's own records, family relationships between *N. caninum* seropositive cattle were investigated. All seropositive cattle were traced back five generations and family trees were constructed. Cattle were concluded to have been found along family lines if either of the following were found: 1) a dam and daughter were positive 2) a grand-dam and daughter were positive (where dam not tested) 3) two offspring from a non-tested dam were positive or 4) two non-tested but related dams had positive offspring.

The age of cattle was also extracted from the DairyCHAMP database and the farmer's records. Seropositive cattle were grouped in age brackets of 12 months, and the frequency of *N. caninum* infection according to age was determined. The mean S/P ratio (from IDEXX ELISA) was also compared with age. The S/P of the IDEXX ELISA was used rather than the % inhibition from the Pourquier ELISA because as previously stated, in a competition ELISA, once the maximum inhibition is reached the addition of more antibody does not significantly affect the % inhibition. Cattle were grouped in age brackets of 12 months, and the mean S/P was calculated.

All positive cattle were followed up and where possible blood was collected from seropositive dams at calving or abortion. There were no blood samples collected from newborn calves of seropositive cows before they had ingested colostrum. In two cases though, the calf of a seropositive cow died during delivery, which allowed the collection of serum. These calves had not ingested colostrum and as such would not have received antibodies from the dam. The serum was tested for antibodies to *N. caninum* by ELISA (IDEXX).

Foetuses from cows that aborted were collected where possible and were sent to Elizabeth Macarthur Agricultural Institute or Sydney University, Veterinary Centre for pathological examination. Serology was also performed on the pericardial fluid or serum from these foetuses by ELISA (IDEXX).

4.2.2 Dog serology

4.2.2.1 IFAT

There were four dogs on the property in December 2002. Serum from these dogs was collected, stored at -20ºC and later tested in an IFAT (VMRD Inc, Pullman USA). Two of the dogs belonged to the property owner and had come on to the property as young pups. There was a female kelpie (Bessie) aged 3 years and a female cattle dog (Flea) aged 18 months. Two other dogs were owned by the farm worker who had moved onto the property in April 2002. These dogs were a female cattle-kelpie cross (Sheba) aged 3 years, and a female maltese (Mischief) aged 18 months. Prior to working on this property, the farm worker and his two dogs had been working on another dairy farm.

The four dogs were tested in the IFAT, initially at a 1:50 dilution. It is generally accepted that for dog serum, a positive result at 1:50 dilution is regarded as a specific titre for *N. caninum* (Dubey and Lindsay 1996). Any positive serum was retested in a titration. Slides and reagents were brought to RT. Dog serum was serially diluted in doubling dilution from 1:25 to 1:800 in serum diluting buffer (VMRD). Diluted serum (20µl) was placed in the appropriate wells and then incubated at 37°C for 30 min. Using a wash bottle, the slides were gently washed in Rinse Buffer (VMRD) and then soaked for 10 min in Rinse Buffer. The slide was drained and then dried by blotting with paper to the front surface (without touching wells). FITC-labelled anti-dog IgG conjugate (Sigma) was diluted to 1:100 and then 20 µl was added to each well. The slides were incubated in a humid chamber for 30 min at 37ºC and rinsed as before. The slides were drained and the back and edges were dried with paper towel. Three drops of Flurosave reagent (Calbiochem) were added to each slide and a large coverslip placed on top. The wells were viewed at 400X, under a fluorescent microscope (Olympus BX51) using a burner (Olympus U-RFL-T) and converter (Olympus U-LH100HG). Serum was considered to be positive to *N. caninum* antibodies if tachyzoites displayed peripheral

fluorescence only and was regarded as negative if there was no fluorescence or it displayed apical (non-specific) fluorescence (Paré *et al.* 1995b).

4.2.2.2 Blocking ELISA (Institut Pourquier)

Serum from the four dogs was also tested in the *N. caninum* blocking ELISA (Institut Pourquier) as described in Chapter 2. This ELISA has not been validated for serum other than cattle serum but as it is a blocking ELISA, theoretically it should be applicable to serum from any animal.

4.2.3 Pigeon serology

Serum was also collected from 84 pigeons processed as part of a pigeon control program. Serum from 53 pigeons from the study property and 31 pigeons from an adjacent property were tested in the *N. caninum* blocking ELISA (Institut Pourquier).

4.3 Results

4.3.1 Vertical transmission

Of the 27 *N. caninum* seropositive cattle, 20 (74%) were found to have family associations. This is to say that they were dam/daughter positives, daughter/grand-dam positives, two positive offspring from a non-tested dam or that two related dams had seropositive offspring. Genealogical trees are shown in Figure 9.

In family one, a grand-dam (born 1996) gave birth to three seropositive dams (1999 and twins in 2000) and one dam (1998) that was not tested. The latter gave birth to a seropositive daughter (2000).

In family two, a grand-dam (born 1986) gave birth to two seropositive dams (1994 and 1997) that each gave birth to seropositive daughters (respectively 1999 and 2000).

In family three, a grand-dam (born 1994) gave birth to a seropositive dam (1996) and one dam (1998) that was not tested. The former gave birth to two seropositive daughters (1999 and 2001) and the latter gave birth to a seropositive daughter (2000).

In family four, a grand-dam (born 1989 and who had an abortion in 1994) gave birth to one dam (1993) that was not tested and a seropositive dam (1996). The former gave birth to a seropositive daughter (1998) who in turn gave birth to a seropositive daughter (2001).

Family five had a dam (born 1996) that was not tested but gave birth to two seropositive daughters (1999 and 2000). The latter aborted in late 2002. In family six, a seropositive dam (born 1996) gave birth to a seronegative daughter (1998) and a seropositive daughter (2001). The dam aborted in April 2003.

In families 7-10, the dams (born 1996, 1997, 1997 and 1999) were found to be seronegative. These dams all gave birth to daughters (1999, 2001, 2001 and 2002) that were later found to be seronegative. None of the grand-dams were tested.

In families 11 and 13, two untested dams (born 1988 and 1999) each gave birth to a seropositive daughter (2000 and 2002).

In family 12, an untested dam (born 1994) gave birth to a seropositive daughter (1996) and a seronegative daughter (1999).

When looking at direct descendants (*i.e.* dam/daughter) on the property at the time of sampling, there were six positive cows, which had given birth to nine seropositive and one seronegative offspring. This equates to a vertical transmission rate of 90% (9/10) of *N. caninum* in this herd.

Three cows (see family trees 11-13) that returned positive results to *N. caninum* antibodies had neither offspring, dam or grand-dam available for testing, thus no family history of the infection could be identified. One of the above cows calved 2 months after the survey was conducted. This calf died during delivery and so did not ingest colostrum but was identified as being seropositive for *N. caninum*.

In the follow up after the initial survey there were a total of two seropositive cows that had calves that died during delivery. These calves did not ingest colostrum, and were also shown to be seropositive.

In the 12-month period after the initial survey there were five seropositive cows that aborted. Of three foetuses that were able to be collected, only two foetuses were tested for antibodies to *N. caninum.* One was serologically positive and the other negative.

In summary, of four calves or foetuses from *N. caninum* positive dams, three (75%) were themselves serologically positive. The seronegative calf was aborted at 170 days gestation and so should have been immunocompetent. As seropositivity is an indicator of infection, there is a vertical transmission rate of 75% amongst these animals.

4.3.2 Postnatal transmission

Four cattle (family trees 7-10) that tested positive to *N. caninum* were born from dams that had tested negative, *i.e.* 15% (4/27) of seropositive cattle were born from seronegative cattle. These seropositive cattle are most likely to have been infected postnatally.

4.3.3 Dog serology

4.3.3.1 IFAT

Of the four dogs on the property, one dog (Flea - 18 mths old) was determined to have a titre of 1:200 in the IFAT. Serum from another dog (Bessie) gave unclear results, as there was whole peripheral staining in some tachyzoites and apical only staining in other tachyzoites in the same slide. This occurred at all dilutions (1:25 to 1:100) and on two different occasions. The two dogs belonging to the farm hand tested negative to *N. caninum* in the IFAT at 1:50 dilution.

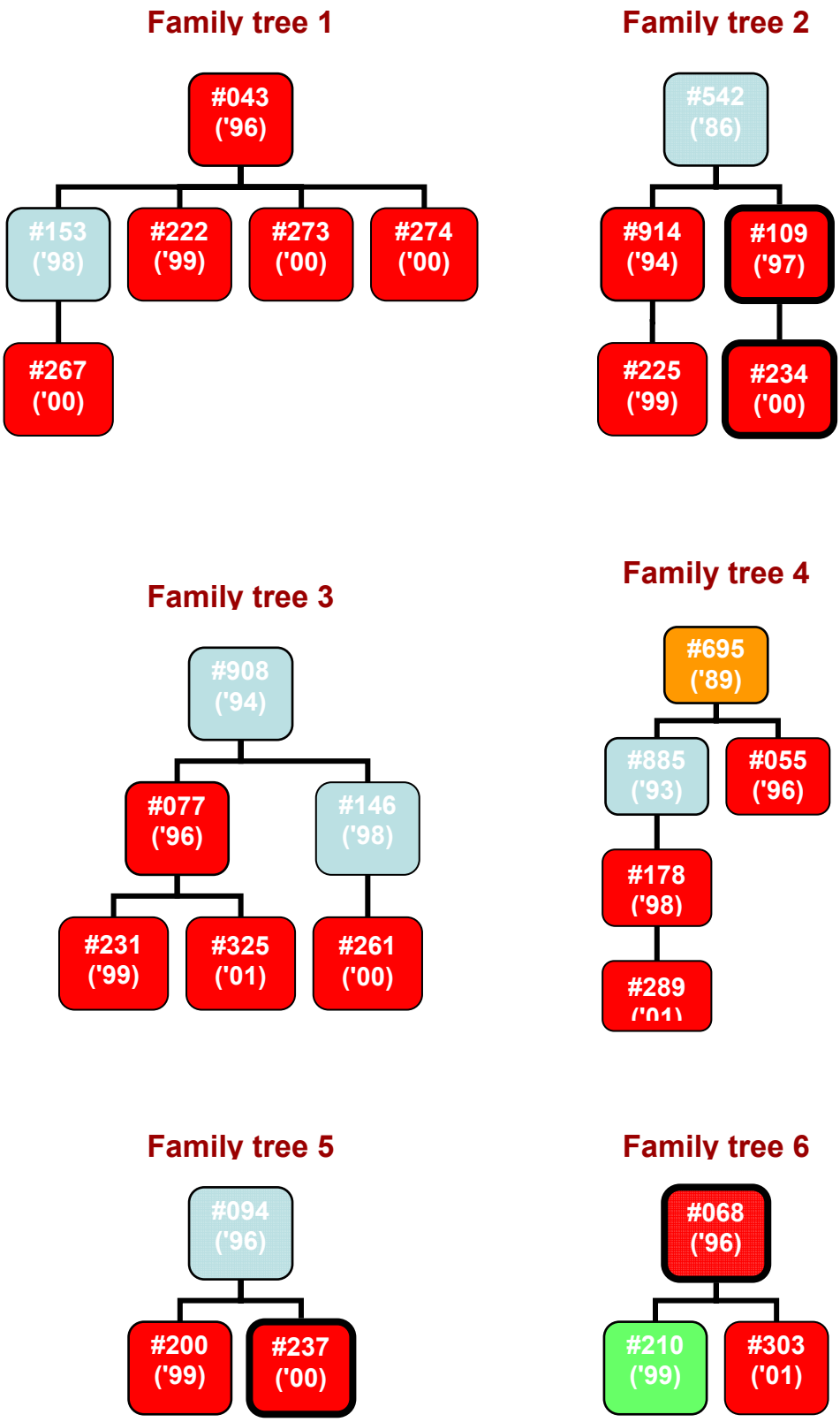
4.3.3.2 ELISA

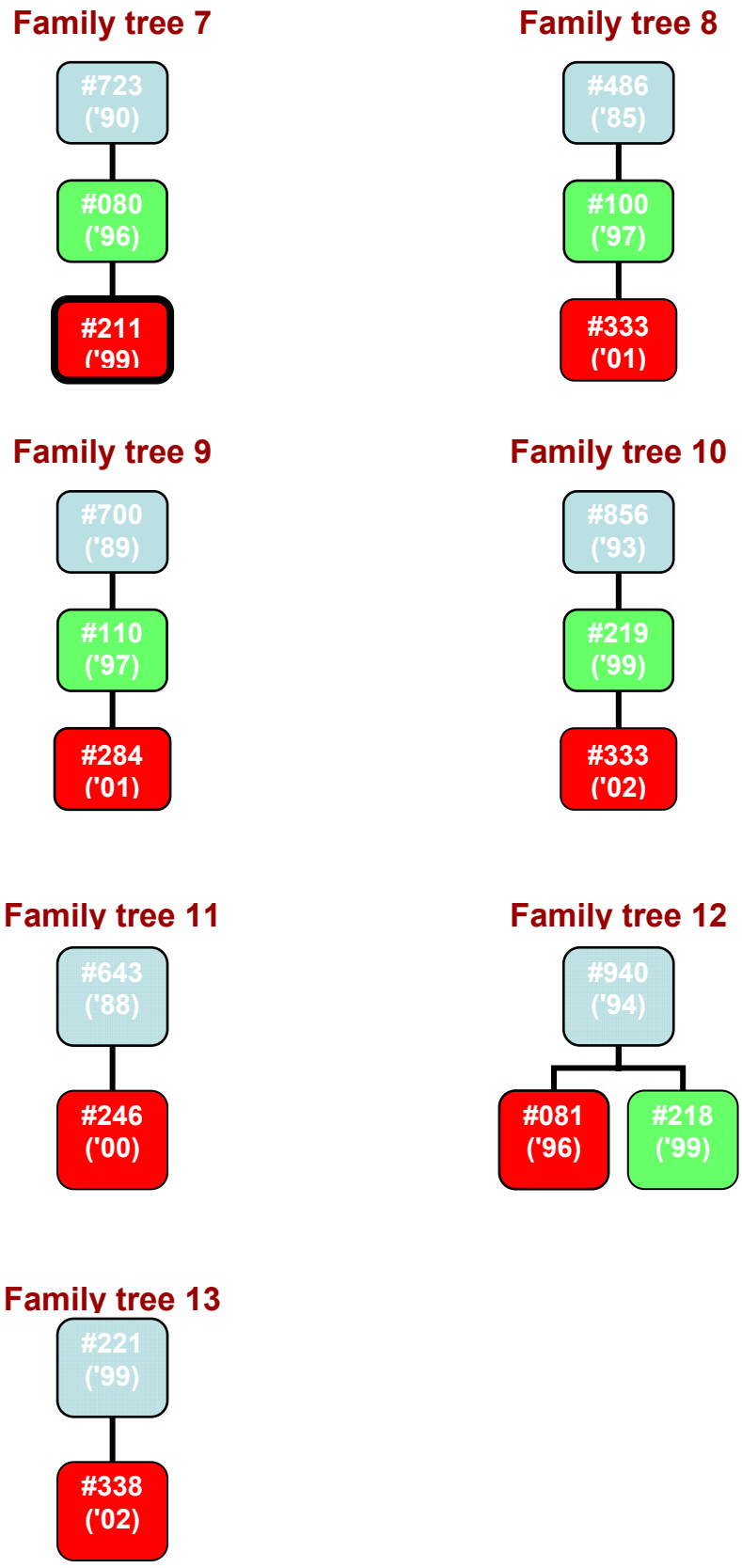
When tested in the *N. caninum* blocking ELISA (Institut Pourquier) the serology results were similar. One dog (Flea) was regarded as seropositive and the other three dogs were negative for *N. caninum* antibodies.

4.3.4 Pigeon serology

Of the 84 pigeons tested in the blocking ELISA, none were determined to be infected with *N. caninum.*

Figure 9 **Family trees of *N. caninum* seropositive cattle**





The colours red, green and blue indicate a seropositive, seronegative and untested cow respectively. Orange indicates a serologically untested cow, but evidence of abortion due to *N. caninum*. Bold border indicates a cow that aborted in the past 12 months. The cattle tag number is indicated (#) and the year of birth is shown in brackets

Figure 10 Frequency of *Neospora* infection according to age

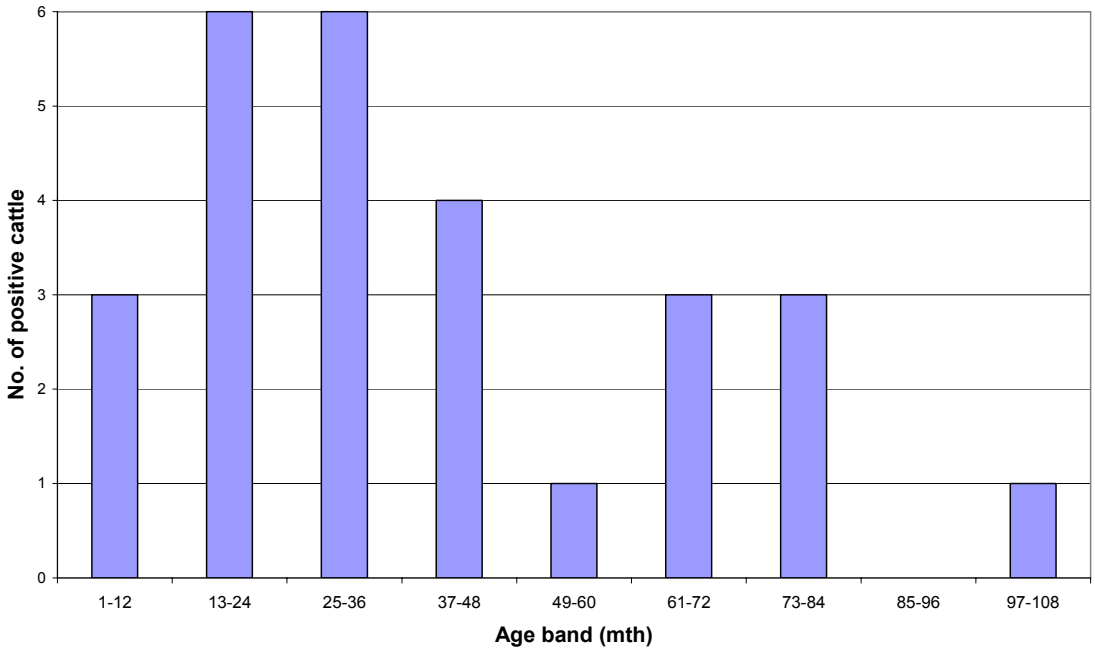
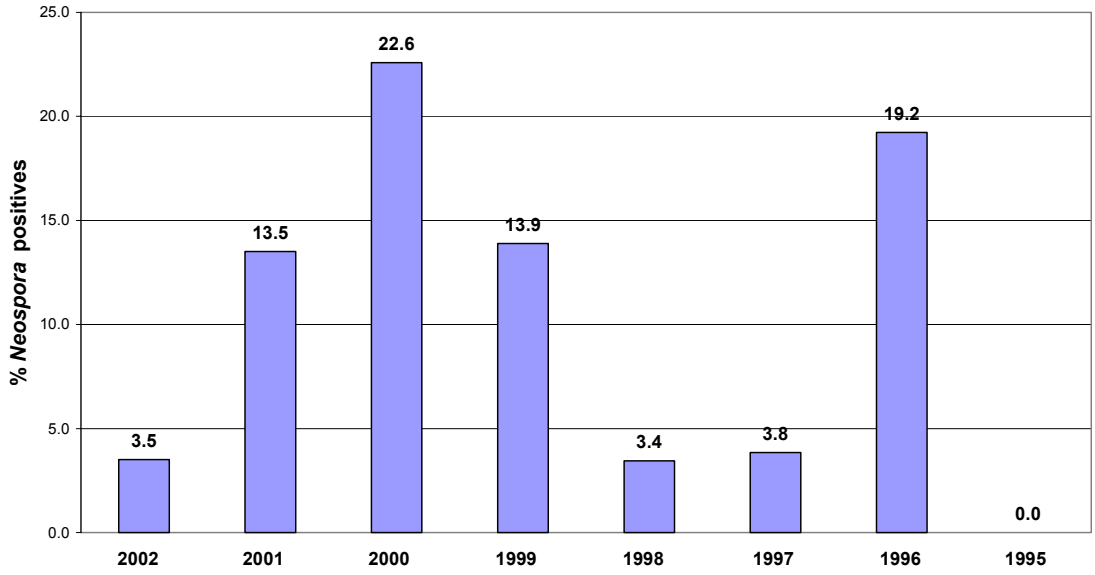


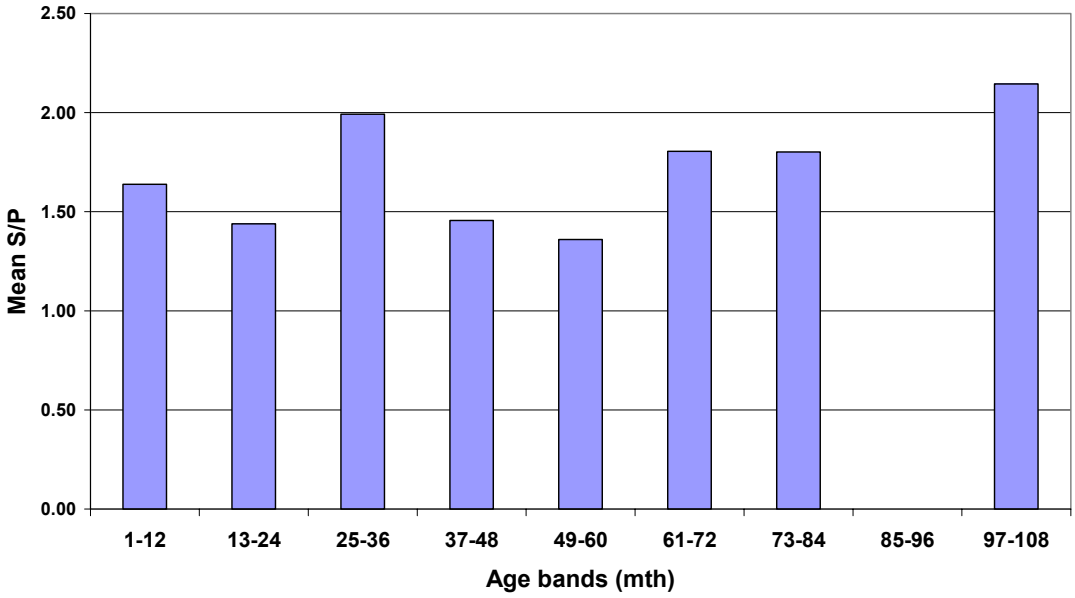
Figure 11 Percentage of *Neospora* infection according to year of birth



The age-specific frequency of infection with *N. caninum* is shown in Figure 10. There are two peaks in the frequency of infection. The highest frequency of infection was in the younger cattle (*i.e.* from 13-36 months) although there were no cattle infected that were 10 months or younger. There were also high numbers of cattle infected in the surrounding age brackets. The other peak of infection was in cattle aged 61-84 months. In Figure 11 the year of birth of positive cattle is compared to the total number of cattle in these age brackets. This again suggests that infection was not uniform over the years as there were two peaks. These occurred in the years 1996 and 2000. Although this age clustering of positive cattle points to postnatal transmission in this herd, the cattle born actually came from family trees and thus were probably vertically infected. Of the 17 infected animals that were born in 1999-2001, 13 had strong family ties to other positives, one had no relation to other tested cattle and three were born from seronegative cattle. Of the five infected animals that were born in 1996, none had a dam that could be serologically tested, as these are some of the oldest cows on the property. One cow was the offspring of an infected cow (aborted in 1994) as determined by pathology.

The mean S/P of seropositive cattle in 12 month age brackets are shown in Figure 12. The overall mean S/P of seropositive cattle was 1.7 and the mean S/P of the age bands ranged from 1.4 (at 13-24 mon and 49-60 mon) to 2.1 (at 97-108 mon). The highest mean S/P was found in 2-3 and 8-9 year old cattle. The lowest mean S/P was found in the 1-2 and 4-5 year old cattle. There was no statistical difference between S/P amongst age groups. Some authors have shown that increased antibody titre is more related to the stage of pregnancy or proximity to abortion (Atkinson *et al.* 2000b) and others have found that high dam ELISA values at calving were significantly associated with an increased probability of congenital infection in calves. There is no trend with age but this graph suggests that even cattle up to 8 years of age maintain a high antibody level to *N. caninum* and also suggests that titres are life-long. This is shown by the age of cattle and the inferred time of infection by epidemiology and diagnosis, *i.e.* #695 was diagnosed to have had a *N. caninum* abortion in 1994 and then had a seropositive offspring in 1996.

Figure 12 Comparison of mean S/P (IDEXX ELISA) and age of cattle



4.4 Discussion

There is a high rate of vertical transmission of *N. caninum* in this herd with 74% (20/27) of the seropositive animals found to have family associations. The 20 positive cattle were distributed in six family trees. Evidence of vertical transmission is the familiar clustering of seropositive animals (Dijkstra et al. 2001a) and is usually associated with endemic infection of the herd. Authors in Sweden (Björkman et al. 1996) and Germany (Schares et al. 1998) have found similar familial distributions of seropositive cattle after constructing family trees.

Six positive cows that had descendants on the property at the time of sampling gave birth to nine seropositive and one seronegative offspring. This equates to a vertical transmission rate of 90% (9/10) of *N. caninum* in this herd and again demonstrates that this is the main mode of transmission.

There was also a high vertical transmission rate of 75% (3/4) identified after serologically testing four calves or foetuses from *N. caninum* positive dams.

Of the seropositive cattle there were 15% (4/27) that were born from seronegative cattle, which indicates that they were most probably infected postnatally. This could have been by ingestion of oocysts (excreted by a dog, fox or other species), ingesting infected foetus or animal tissues, or by ingesting infected milk or colostrum. This is a low rate of postnatal transmission for *N. caninum* and agrees with reports from the UK (Davison *et al.* 1999a) and California (Paré *et al.* 1996, Thurmond and Hietala 1997a).

There have been reports that suggested that the postnatal transmission rate was high as 33% of the breeding stock aborted in a few months due to *N. caninum* (Thornton *et al.* 1994). Although these abortion epidemics could be due to a point source exposure of *N. caninum*, they could also be due to recrudescence of the parasite due to immune suppression by BVDV infection, mycotoxicosis or other (Alves *et al.* 1996, Bartels *et al.* 1999, Atkinson *et al.* 2000a). Rates of postnatal transmission as high as 31% have been reported by comparing seropositivity of dam and daughter pairs especially in herd with age clustering of positives (Dijkstra *et al.* 2001a).

Postnatal transmission is usually associated with abortion epidemics and age clustering of infected animals supports a point source of exposure of these animals (Dijkstra *et al.* 2001a). Age clustering of infected cattle can indicate postnatal infection, but in this herd, although there were high numbers of infected cattle that were born in 1999-2001, the majority (13/17) of these cattle were actually offspring of seropositive dams and so are more likely to have been infected vertically. Others have suggested that age clustering of positive animals is evidence for postnatal transmission but the evidence presented here shows that this is not necessarily true for *N. caninum* infected herds. There are limitations in this study, as there were only 27 positives out of 266 cattle screened, but it is possible to find clusters of seropositive animals without postnatal infection occurring. In the four years (1999-2002) there were also four suspected cases of postnatal transmission, as four positive cattle were offspring of seronegative cattle. This gives a mean of one postnatal infection per year.

The age clustering peak of seropositive animals born in 1996 could also point to postnatal infection. Of the five animals, none had a dam that was tested serologically but one had a dam that aborted in 1994 and *N. caninum* was implicated by histopathology. As the dam was most likely infected by *N. caninum,* its offspring would probably have been vertically infected.

The oldest seropositive cow was 8 years old (born 1994) at the time of sampling but it is possible that this cow was only recently infected. According to the DairyCHAMP database, the earliest record of a *Neospora* infection in this herd is from a grand-dam (family four) that aborted in 1994. All four dogs on the property were younger than 8 years old, and so they were not the cause of the primary infection in this herd. This is aside from the fact that the majority of infections are explained by vertical transmission. It cannot be ascertained whether the oldest member of each family tree was infected postnatally or vertically.

One of these seropositive cows gave birth to a seronegative calf in her first pregnancy and a seropositive calf in her second pregnancy. During her third pregnancy this cow aborted and the foetus was seropositive. It is quite possible that this dam was not infected during the first pregnancy but was infected prior to the birth of her second calf.

It is likely that the dam was postnatally infected, after the first pregnancy but it is also possible that the dam was herself vertically infected but that it did not pass on the infection to her first offspring.

It is possible that the seronegative cattle that gave birth to seropositives were actually infected but were immunotolerant to *N. caninum*. Immunotolerance is known to occur with BVDV if the infection occurs before 100 days gestation, when the foetal immune system has not fully developed (Roeder and Harkness 1986) It is plausible that this immunotolerance effect occurs with infectious diseases other than BVD. This means that there may well be infected animals in the herd that do not produce antibodies to *N. caninum* and so are not detected by serological techniques. Due to the sensitivity of the ELISA's used (less than 100%), there are also animals that would not have been detected.

Dogs can become infected by ingestion of tissues of infected intermediate hosts. These could include birds (pigeons, crows), dead cows, aborted foetuses or placenta. Dogs may also become infected by ingesting colostrum or milk that has come from infected cows. In the current study there was no evidence of *N. caninum* infection of pigeons. Although the blocking ELISA used in this case has not been validated for pigeon serum it has been validated for cattle serum and as a blocking ELISA it should theoretically be valid for use with serum other than cattle.

The one *N. caninum* seropositive dog (18 months old) that came to the property as a pup, may well have been excreting oocysts since it's arrival and so could have been the cause of the low level postnatal transmission to cattle. The three seronegative dogs may also have contributed to the postnatal infection of these cattle as it is known that some dogs can excrete *N. caninum* oocysts but remain seronegative (McAllister *et al.* 1998). All four of these dogs on the property had originated from other farms and so there is a higher probability that they were previously infected as compared to dogs originating from domestic properties. Foxes have also been found on the property and there is a possibility that as canids they may also excrete *N. caninum* oocysts when infected. It is known that foxes are natural intermediate hosts of *N. caninum*, but whether they can

also act as definitive hosts is unproven (Barber *et al.* 1997, Lindsay *et al.* 2001, Schares *et al.* 2001).

In this herd the majority of *N. caninum* infected cattle are explained by vertical transmission as seen in family trees. There also seems to be a low rate of postnatal transmission. Postnatal infection of cattle may be due to excretion of oocysts by infected dogs or foxes on the property. Cows may also be infected horizontally by ingesting aborted material from other cows. Calves fed pooled milk from *Neospora*-infected cows may become infected.

Since vertical transmission is the main route of infection on this property, it would be prudent to cease breeding from *N. caninum* seropositive animals. This would reduce the number of seropositive animals in the herd and also reduce further infection to dogs and foxes, as there would be less infected abortive material. These seropositive animals could be gradually culled as they cease milk production. Since there is only a low prevalence of *N. caninum* infected animals in this herd this should not place too great a financial burden on the farmer. The postnatal infection rate is already low and could be lowered further with a few steps. Dogs and cattle should be kept away from aborted material and cattle carcases so as not to become infected. Dogs should also be kept away from cattle feed as a means of avoiding the infection of the cattle feed source. Foxes on the property are harder to manage but it may be possible to control these also. Calves should not be fed pooled milk unless it comes from known *N. caninum* negative cows. With these steps it should be possible to limit the spread of *N. caninum* on this property.

5. Detection by PCR of *Neospora caninum* in milk from seropositive cattle

5.1 Introduction

It has been postulated that colostrum or milk from *N. caninum* infected cows may contain an infective stage of *N. caninum.* Milk and colostrum are often fed to calves and this may be a route of horizontal infection in a herd, although this has not been proven yet (French *et al.* 1999). This may result in infection of new families of cattle.

Swedish researchers have demonstrated that calves can be orally infected with colostrum spiked with *N. caninum* tachyzoites (Uggla *et al.* 1998). *Toxoplasma gondii* has been transmitted to goats via milk (Dubey 1980) and humans have also been infected with *T. gondii* after milk consumption (Tenter *et al.* 2000). Considering the similar biology of *N. caninum* and *T. gondii* it is quite possible that cattle and dogs could be infected by ingesting milk from seropositive cows (Dijkstra *et al.* 2001b).

Others have reported that several attempts to demonstrate *N. caninum* in colostrum of seropositive cows by PCR have failed (Dijkstra *et al.* 2001b). The methods though were unpublished. Only recently was the first report of *N. caninum* DNA being detected in milk from seropositive cattle (Moskwa *et al.* 2003).

There are also reports, that *N. caninum* DNA has been detected in the semen of bulls (Ortega-Mora *et al.* 2003, Ferre *et al.* 2005) and this may be another transmission route.

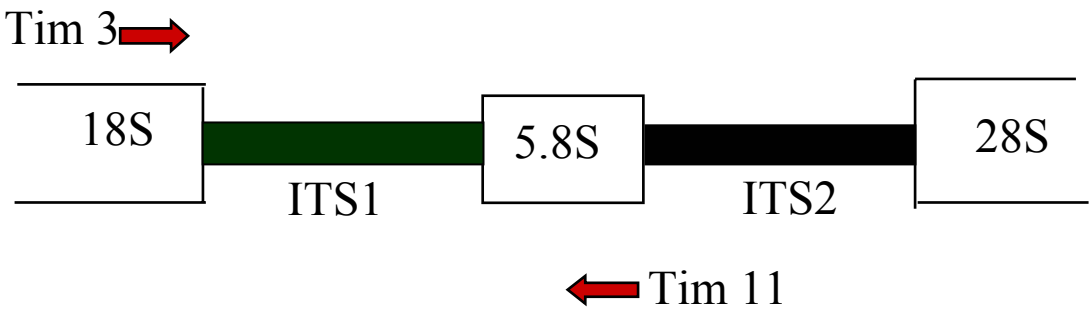
PCR has proven to be a highly specific and sensitive method for diagnosing protozoal infections in both tissue samples and body fluids (Holmdahl and Mattsson 1996, Ellis *et al.* 1999a).

There is much evidence that the ITS1 is a valuable species-specific marker for distinguishing between members of the Toxoplasmatinae (Holmdahl and Mattsson 1996, Payne and Ellis 1996, Homan *et al.* 1997, Ellis 1998, Ellis *et al.* 1999b, Dubey *et al.* 2002a).

The ITS regions of rDNA are ideal targets for PCR as they are present at high copy number (Ellis 1998). The repeat unit of rDNA is present approximately 110 times in *T. gondii* and a high gene copy number has been suggested to increase the sensitivity of PCR amplifications (Holmdahl and Mattsson 1996). Detection of *N. caninum* by PCR is therefore a highly sensitive technique.

The PCR used in the current study for the detection of *N. caninum* DNA in milk samples utilises primers (Tim 3 and Tim 11) that amplify a region of the ITS1 of *N. caninum* (Figure 13). They have been previously described (Payne and Ellis 1996). CR3/CR4 primers, which target the large subunit rDNA were also used in the current study (Quinn *et al.* 2002b, Mohammed *et al.* 2003).

Figure 13 Region of amplification of ITS1 by Tim 3/Tim 11 primers



In this study of a farm with endemic *N. caninum* infection, a low rate of postnatal transmission was detected. It was noted that on this dairy the farmer fed pooled milk to calves and it is postulated that milk from infected cows may be the source of this postnatal infection. To investigate this, milk from *N. caninum* seropositive cows was collected and PCR was used to detect *N. caninum* DNA. Milk extracts from *N. caninum* seropositive cows were also injected into immuno-compromised mice to determine whether milk was infective. SCID mice were utilised as they lack both T and B cells and so could be infected more easily.

5.2 Materials and Methods

5.2.1 Detection of *N. caninum* by PCR

Neospora caninum seropositive cows from the dairy herd previously described in Chapter 2 were selected and 10 ml milk samples were collected. Four milk samples were collected: from three cows within a few days post-calving and from one cow approximately 24 hours after calving. Milk samples were kept refrigerated for 4-6 days until they underwent extraction.

5.2.1.1 Genomic DNA extraction from milk

Milk samples were centrifuged at 3000 rpm for 5 min. The top layer of "congealed" milk was scraped away and the remaining liquid supernatant was poured off. Two ml of lysis buffer (10 mM Tris pH 9, 100 mM EDTA) was used to resuspend the pellet by pipetting up and down. SDS (100 µl of 20%) and 10 µl Proteinase K (20 mg/ml) was added to each tube and gently swirled. These tubes were then incubated for 4 h at 65°C. Phenol (1 ml) and chloroform (1 ml) was added to each tube and then mixed on a rotary shaker for 5 min. To break the phase the tubes were then centrifuged for 5 min at 5000 rpm and the aqueous phase (top layer) was removed and placed in a fresh tube. This phenol:chloroform extraction was repeated another two times. To each tube was added 40 µl of 5M NaCl (100 mM) and 3 ml of ice cold 100% ethanol. The tubes were then incubated overnight at -20°C. DNA was pelleted by centrifuging at 10000 rpm for 10 min and the supernatant was poured off. The pellet was then washed in 5 ml of 70% ethanol and centrifuged for 10 min at 10,000 rpm. The ethanol was removed and the tube was inverted for 20 min to allow the pellet to air-dry. The pellet was then resuspended in 500 µl TE buffer (10mM Tris pH 9.6, 1 mM EDTA) by gently flicking the side of the tube. The solutions were left overnight at 4°C to fully resuspend before they were run on a gel.

5.2.1.2 Quantifying DNA in extracts

The amount of DNA in the extracted milk samples was quantified before running on a gel. A small aliquot of each extract was diluted (1/5) in MQ water. The Gene Quant machine (Pharmacia) was firstly zeroed using MQ water and then the concentration of DNA in each sample was determined. From this determination a minimum of 100 ng of each sample was loaded onto the initial gel and 100 ng of DNA was also used in each PCR.

5.2.1.3 Gel electrophoresis of extracts

The extracted DNA samples were first run on a 1% agarose gel to check the success of the DNA extraction method and to confirm its integrity.

A 1% agarose gel was prepared using TBE buffer (0.089M Tris base, 0.089M borate and 0.002M EDTA). TBE buffer was poured into the electrophoresis box and allowed to cover the well. DNA samples (5 µl and 20 µl) were combined with 2 µl and 4 µl of 6x loading dye (10 µg bromophenol blue, 10 µg xylene cyanol, 4 g sucrose and 9.5 ml MQ H_20), respectively. Each of these samples was then loaded into the appropriate well of the gel and allowed to run on the gel for 1 h at 120 V. The gel was then moved to a staining tray and covered in MQ H_2O containing 12 µl ethidium bromide (10 mg/ml). This was placed on a gentle rocker and allowed to stain for 20 min. The ethidium bromide solution was carefully poured off and the tray was filled with enough MQ H_2O to cover the gel. This was allowed to destain for 10 min while gently rocking. The gel was then placed on the transluminator and a photo of the gel was taken with a Kodak U/V camera using a Kodak 1D program (Kodak).

5.2.1.4 PCR of DNA extracted from milk samples

To determine whether *N. caninum* genomic DNA was present in the extracted DNA two different PCRs were set up. Primers Tim 3 and Tim 11 were used in one PCR and primers CR3 and CR4 were used in another. Tim 3/Tim 11 targets the ITS1 whereas CR3/CR4 targets the large subunit rDNA.

A standard mix of PCR reagents was used as below:

1) 5 µl DNA polymerase 10x reaction buffer (Fisher)
2) 4 µl dNTP mix (2 mM) (Fisher)
3) 3 ul $MgCl_2$ (25 mM) (Fisher)
4) 0.2 µl Taq Polymerase (250units, 5.5U/µl) (Fisher)
5) 1 µl Primer A (*e.g.* Tim 3) (Sigma, Genosys)
6) 1 µl Primer B (*e.g.* Tim 11) (Sigma, Genosys)
7) 30.8 µl H_2O

Two PCR mixes were made: one with Tim 3/Tim 11 (50 ρmol/µl) (Sigma, Genosys) and the other containing CR3/CR4 primers (50 ρmol/µl) (Sigma, Genosys). Each mix was made up in seven times quantity to allow for the number of replicates and samples. Approximately 100 ηg DNA (either 2 µl or 5 µl) from each extract was added to 45 µl of each PCR reaction mix in PCR tubes. A negative control was included by adding 5 µl H_2O to 45 µl of each reaction mix. The PCR tubes containing Tim 3/Tim 11 primers were run using a PCR protocol of: 1 cycle of 95 °C for 5 min, 34 cycles of: 94 °C for 1 min, 50 °C for 1 min and 72°C for 1 min. There was then 1 cycle of 72 °C for 5 min. The PCR tubes containing CR3/CR4 primers were run using a PCR protocol of: 1 cycle of 95 °C for 2 min, 35 cycles of: 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1.5 min. There was then 1 cycle of 72 °C for 5 min. The PCR machine used was a PTC-200, Peltier Thermal Cycler (M. J. Research Inc., Watertown, Maine, USA). Each of the PCR products (20 µl) and a 100 bp ladder were then run on a 1% agarose gel as previously described in 5.2.1.3.

5.2.1.5 Scale-up of PCR for sequencing

To conclusively prove that *N. caninum* DNA was detected, the sequence of the PCR products was determined and compared to that of known *N. caninum* sequences. To do this the PCR was scaled-up to produce more DNA product. After the products were run on a gel and the appropriate bands cut out, the PCR product was purified and sent for sequencing.

A standard PCR mix containing the primers Tim 3/Tim 11 was prepared as previously described in 5.2.1.4. Aliquots of the original PCR products were diluted 1/10. PCR mix

(45 µl) and 5 µl of each diluted PCR product was added to a PCR tube. This was repeated to give seven replicates for each sample. Similarly, this was done for a negative control containing water and a positive control containing *N. caninum* DNA. A PCR was performed using the protocol described for Tim 3/Tim 11 primers. A 1% agarose gel containing ethidium bromide (1 µl/100 ml TBE) was prepared with large wells able to hold 300 µl of product. The replicates of each sample were combined and the appropriate amount of loading dye added before loading into the wells. The gel was run at 120 V for 1 h. The DNA bands were visualised under a transluminator emitting UV light, which enabled them to be carefully cut away from the gel using a scalpel. Each band was placed in separate tubes that were weighed to give an approximate weight of the gel.

5.2.1.6 QIAquick gel extraction

This procedure purifies the PCR product from the gel. A commercial kit, QIAquick Gel Extraction Kit Protocol (using microcentrifuge) (Qiagen, Clifton Hill, Victoria) was used for this procedure.

Buffer (300 µl) was added to each tube for every 100 mg of gel present. The tubes were then incubated for 10 min in a 50 °C water bath until the gel dissolved. Isopropanol 1 ml was added to each tube. A QIAquick column was taken for each sample and a maximum of 800 µl at a time was added to the column and centrifuged in a microcentrifuge for 1 min. The flow through was discarded and another 800 µl was added to the column and centrifuged again. This process was continued until the whole sample had gone through the column. The DNA should bind to the column. QC buffer (0.5 ml) was added to each column and spun again in a microcentrifuge for 1 min. The flow through was discarded. Buffer PE (0.75 ml) was added to each column and left for 4 min to wash before centrifuging for 1 min. The flow through was discarded and then centrifuged at 1300 rpm for 1 min. The QIAquick column was placed in a 1.5 ml centrifuge tube and 25 µl of EB buffer was added to the centre of the column. This was allowed to stand for 1.5 min before being centrifuged at 1300 rpm for 1 min. Each sample (10µl) was added to 1 µl of the forward primer (Tim 3) and 5 µl of H_2O. Similarly, 10 µl of the each sample was added to 1 µl of the reverse primer (Tim 11)

plus 5 µl of H_2O. These 16 µl aliquots were sent to SUPAMAC, Royal Prince Alfred Hospital, Camperdown, NSW, Australia for sequencing.

The sequences produced by the forward primers from each of the four samples were aligned using Clustal W. The sequences produced by the reverse primers from each of the four samples were aligned and then the bases were changed to the complement using Clustal W. A BlastN analysis of 360 bp of the forward sequence was carried out as was a BlastN analysis of the reverse sequence using ANGIS and NCBI. The sequences were analysed separately as they overlapped by only 8 bp. The sequences were then combined to create a 543 bp sequence, which was blasted as above. All Blast N analyses were performed using default settings.

5.2.2 SCID mice - experiment 1a

5.2.2.1 Preparation of milk extracts for injection into SCID mice

Milk (10 ml) was collected from each of nine *N. caninum* seropositive cows from the dairy described in Chapter 2. The same four cows whose milk was previously sampled (5.2.1) and tested by PCR for *N. caninum* were re-sampled for this experiment. Milk from another five seropositive cows was also collected for this experiment. After collection the milk samples were stored for 24 h at 4 °C. Milk samples were centrifuged for 5 min at 3000 rpm and the supernatant and fat layer was discarded to leave a pellet. The pellet was then resuspended in 2 ml of PBS using a vortex. The pellet was washed again by centrifuging (as before), discarding the supernatant and adding 2 ml of PBS. To break up the cells the solution was then drawn through a 26 G needle attached to a syringe. This was then washed again by centrifuging and the supernatant discarded. The pellet was resuspended in 250 µl PBS and transferred to 1.5 ml eppendorf tubes ready for injection into mice.

5.2.2.2 Mouse trial 1a

Ten male SCID mice (15 weeks old) were equally allocated and housed in two standard mouse cages with constant access to mouse chouda and water at GHRL. A different number of bands were marked on the tail of each mouse for identification and the mice were weighed before injection of extract. Using a 23 G needle nine mice were injected subcutaneously at the base of the neck with 100 µl of one extract each. One mouse was similarly injected with 100 µl of PBS to act as the negative control. The mice were monitored daily for symptoms such as ruffled coat, lethargy, circling, head tilting and limb paralysis and they were weighed every second day. On day 33, all mice were euthanased by CO_2 asphyxiation and the brain removed.

5.2.3 SCID mice - experiment 1b

5.2.3.1 Mouse brain homogenate

Each brain from mice in Experiment 1a was dissected into small pieces and 3 ml of PBS added in a biohazard cabinet. To homogenise the brain, this mixture was progressively drawn through 19 G, 21 G and 23 G needles. Once the brain homogenate could be drawn relatively smoothly through the 23G needle the homogenate was dispensed into a 5 ml vial and placed on ice ready for injection into mice.

5.2.3.2 Mouse trial 1b

Ten male SCID mice (6 weeks old) were equally allocated and housed in two standard mouse cages with constant access to mouse chouda and water at GHRL. A different number of bands were marked on the tail of each mouse for identification and the mice were weighed before injection of extract. Each mouse was injected with a single brain homogenate from experiment 1. Mice 1 and 2 received 500 µl and mice 3-10 received 700 µl of the appropriate homogenate. The homogenate was injected into the intra-peritoneal cavity of the mice using a 23 G needle. The mice were monitored daily for symptoms such as ruffled coat, lethargy, circling, head tilting and limb paralysis and they were weighed regularly. On day 31, all mice were euthanased by CO_2 asphyxiation.

5.2.4 SCID mice - experiment 2

5.2.4.1 Preparation of milk extracts

Milk (50 ml) was collected from a *N. caninum* seropositive cow that had aborted 2 weeks earlier. Five tubes containing 10 ml of freshly collected milk were centrifuged for 5 min at 3000 rpm. The supernatant and fat layer was discarded to leave a pellet that was then resuspended in 1.5 ml of PBS. The contents of each tube was combined and then centrifuged for 5 min at 3000 rpm. The supernatant was discarded and the pellet was resuspended in 2 ml of PBS. The pellet was washed again by centrifuging, the supernatant discarded and the pellet resuspended in 2 ml of PBS. The pellet was washed a final time but was resuspended in 1 ml of PBS. To break up the cells the solution was first drawn through a 23 G needle attached to a syringe and then a 25 G needle. The solution was washed twice more by centrifuging, discarding the supernatant and resuspending in PBS. After the first wash it was resuspended in 2 ml of PBS and after the second wash the pellet was resuspended in 1 ml of PBS. This 1 ml milk extract was then transferred to a 1.5 ml eppendorf tube ready for injection into mice.

5.2.4.2 Positive control

Tachyzoites of a laboratory strain of *N. caninum* (NC-1) were harvested from vero cell culture. Tachyzoites were counted under a microscope using a Neubauer counting chamber and diluted to $5x10^7$ parasites/ml in PBS. Aliquots of $1x10^6$, $1x10^5$ and $1x10^4$ parasites/ml in PBS were prepared giving $1x10^5$, $1x10^4$ and $1x10^3$ parasites/100 µl.

5.2.4.3 SCID mice treatment

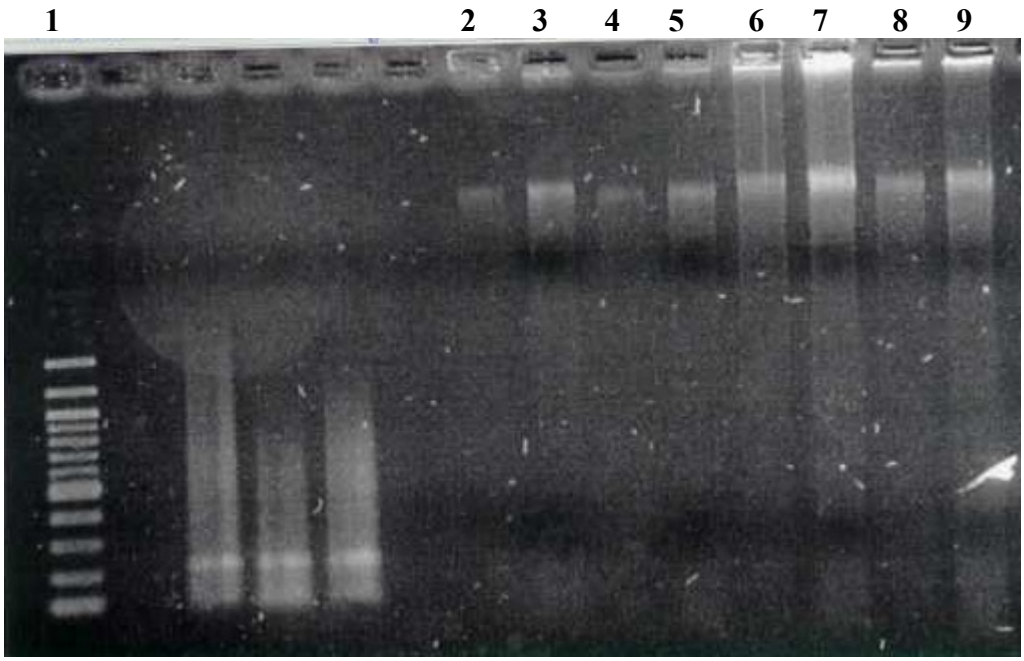
Fifteen male SCID mice (9 weeks old) were housed in three standard mouse cages with constant access to mouse chouda and water at GHRL. A different number of bands were marked on the tail of each mouse for identification and the mice were weighed before injection of extract, infective dose or PBS. All injections were performed subcutaneously at the base of the neck using a 26 G needle. Mice 1-6 were housed together and received 100 µl of freshly prepared milk extract. Mice 7-8, 9-10 and 11-12 were housed together and were injected with 100 µl of NC-1 tachyzoites and received $1x10^3$, $1x10^4$ and $1x10^5$ parasites, respectively. These mice acted as a positive control. Mice 13-15 were housed together and acting as the negative control they received an injection of PBS only. The mice were monitored daily for symptoms such as ruffled coat, lethargy, circling, head tilting and limb paralysis and they were weighed regularly. On day 67, all mice were euthanased by CO_2 asphyxiation. The brain was collected and placed in 10% formalin. The chest cavity of each mouse was opened, the head removed and both were placed in 250 ml of 10% formalin. These preserved samples were sent for pathology by Dr Windsor at the University of Sydney. Brain and spleen samples were examined for lesions typical of *N. caninum* infection including non-suppurative encephalitis and focal splenic necrosis.

5.3 Results

5.3.1 PCR detection of *N. caninum* in milk

After extraction of genomic DNA from milk samples the DNA was quantified and run on a gel. Figure 14 shows that the DNA extracted from milk was genomic because of the molecular weight. The bands detected in lanes 2-9 did not run far through the gel indicating that they are relatively large.

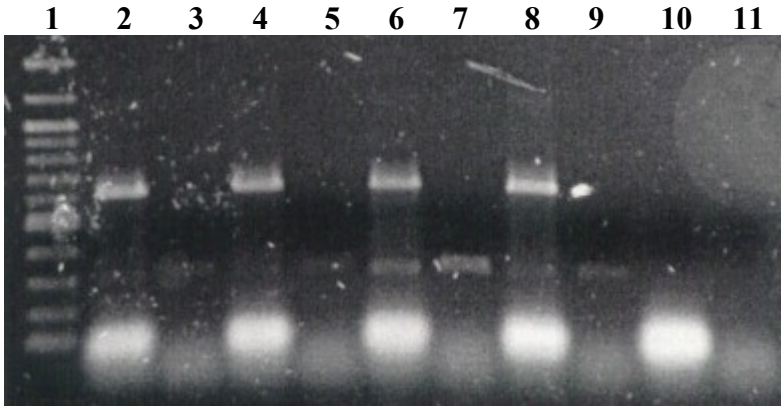
Figure 14 Gel electrophoresis of genomic DNA from milk extracts



Lane 1 contains 1200 bp ladder (in 100 bp units). Lanes 2, 4, 6 and 8 contain 5 µl of milk extract from cow #237, #284, #055 and #261, respectively. Lanes 3, 5, 7 and 9 contain 20 µl of milk extract from cow #237, #284, #055 and #261, respectively.

PCR amplification with primers Tim 3/Tim 11 or CR3/CR4 gave PCR products. After running the PCR products on an agarose gel, bands were detected at positions equivalent to 300 bp and 600 bp (Figure 15). This is consistent with the detection of *N. caninum* DNA and so it is likely that *N. caninum* DNA was present in the milk samples.

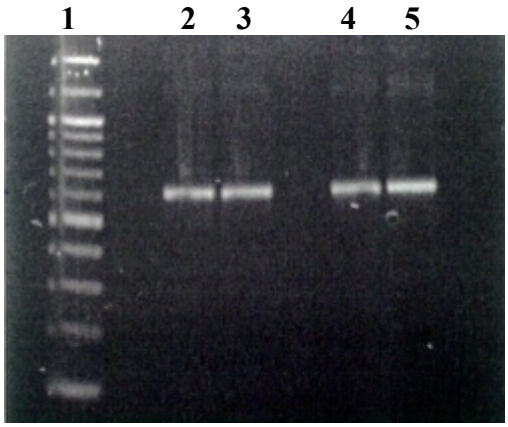
Figure 15 Gel electrophoresis of PCR products from milk extracts



Lane 1 contains a 1200 bp ladder (in 100 bp units). Lanes 2, 4, 6 and 8 contain PCR products using Tim3/Tim11 primers on genomic DNA extracts from cow #237, #284, #055 and #261, respectively. Lanes 3, 5, 7 and 9 contain PCR products using CR3/CR4 primers on genomic DNA extracts from cow #237, #284, #055 and #261, respectively. Lane 10 and 11 contain PCR products of water using Tim 3/Tim 11 and CR3/CR4 primers, respectively as a negative control on the PCR.

The scale-up of the PCR utilising Tim 3 and Tim 11 primers also detected *N. caninum* DNA by the presence of a 600 bp band. Before sequencing, the products were run on a gel (Figure 16). This shows that a 600 bp product was produced and purified.

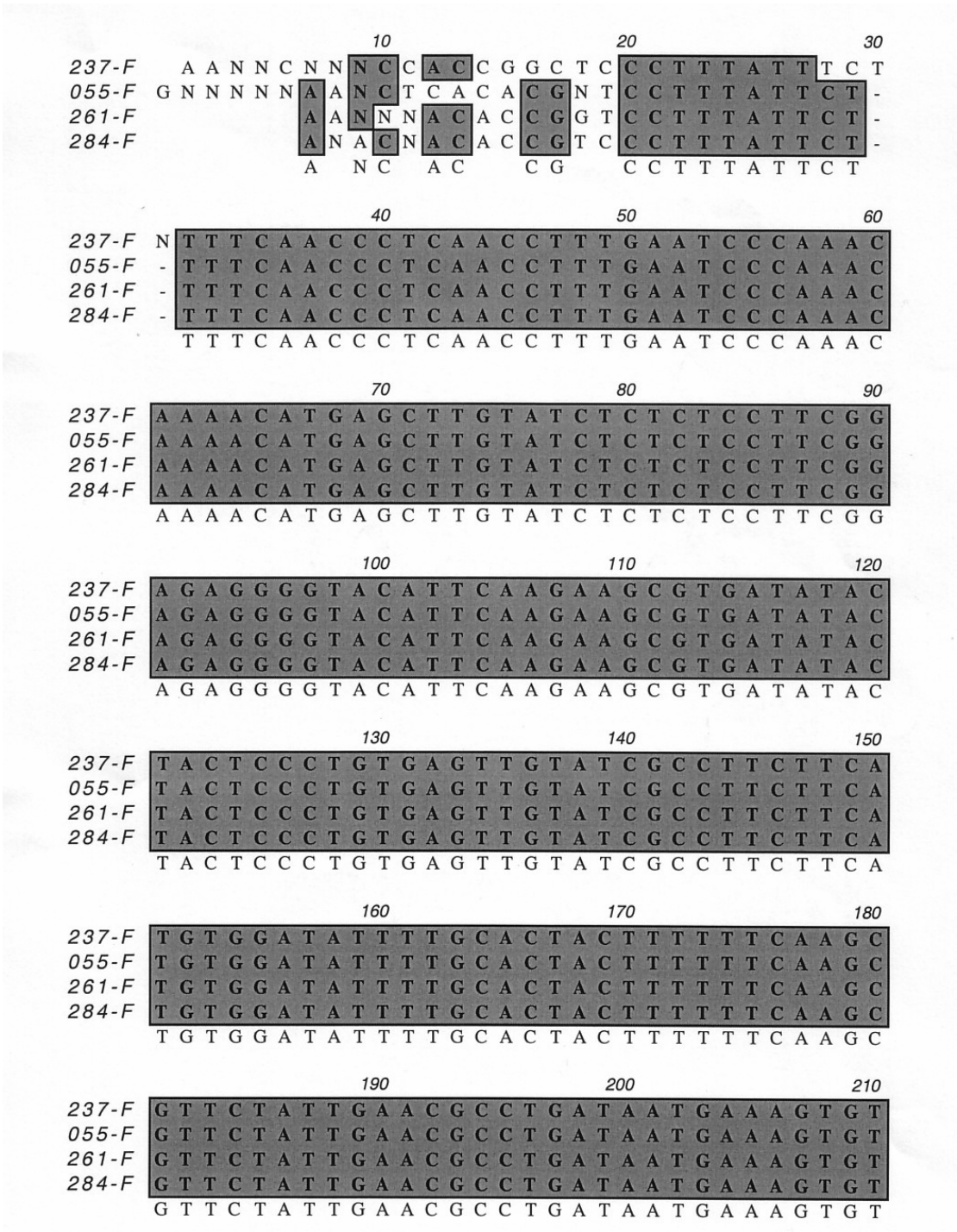
Figure 16 Gel electrophoresis of PCR products sent for sequencing



Lane 1 contains a 1200 bp ladder (in 100 bp units). Lanes 2, 3, 4 and 5 contain purified DNA extracts after QIA extraction from cows #237, #284, #055 and #261, respectively.

Figures 17 and 18 depict the Clustal W aligned sequences obtained using the forward and reverse primers, respectively.

Figure 17 Clustal W aligned sequences (forward primer)



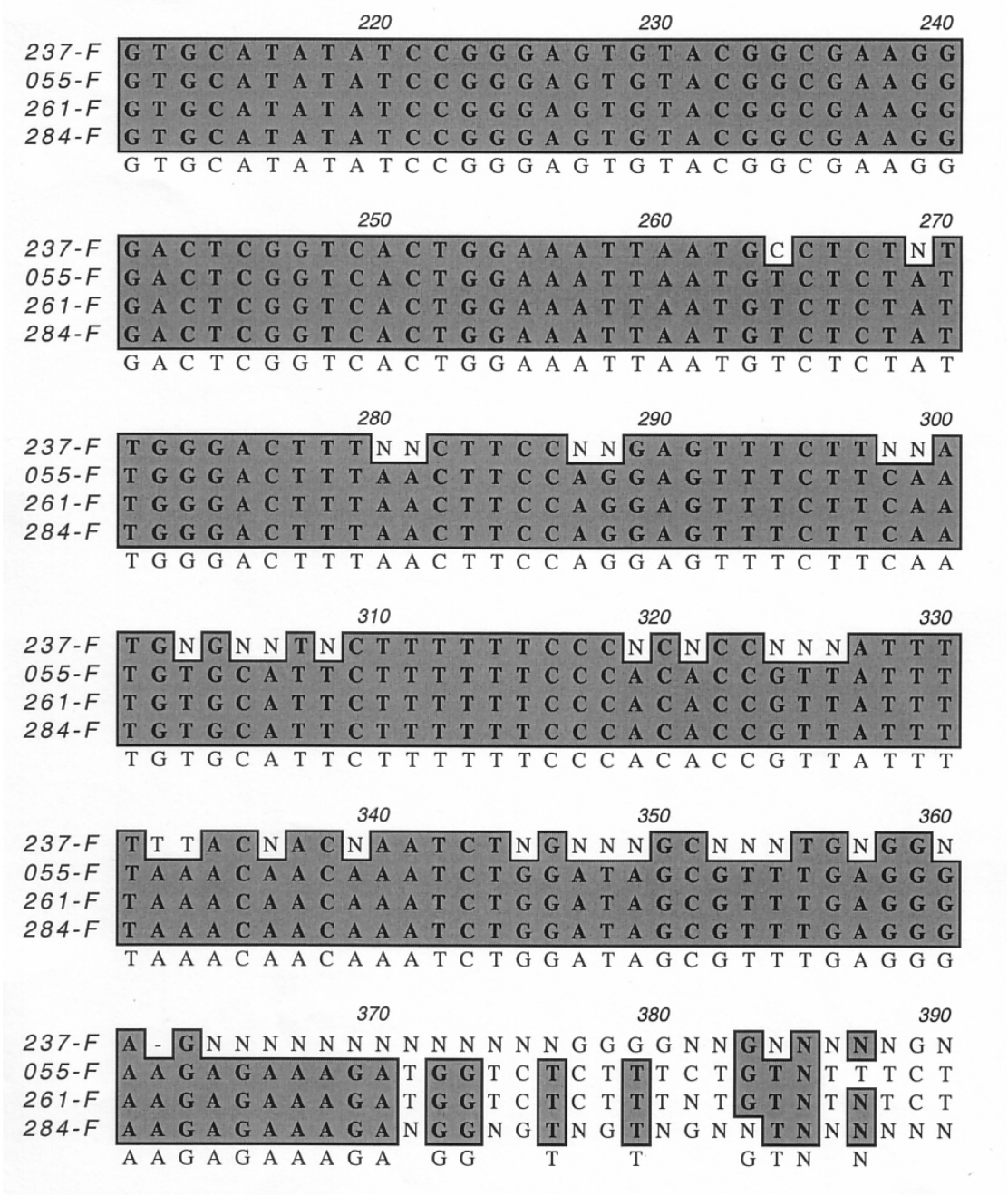
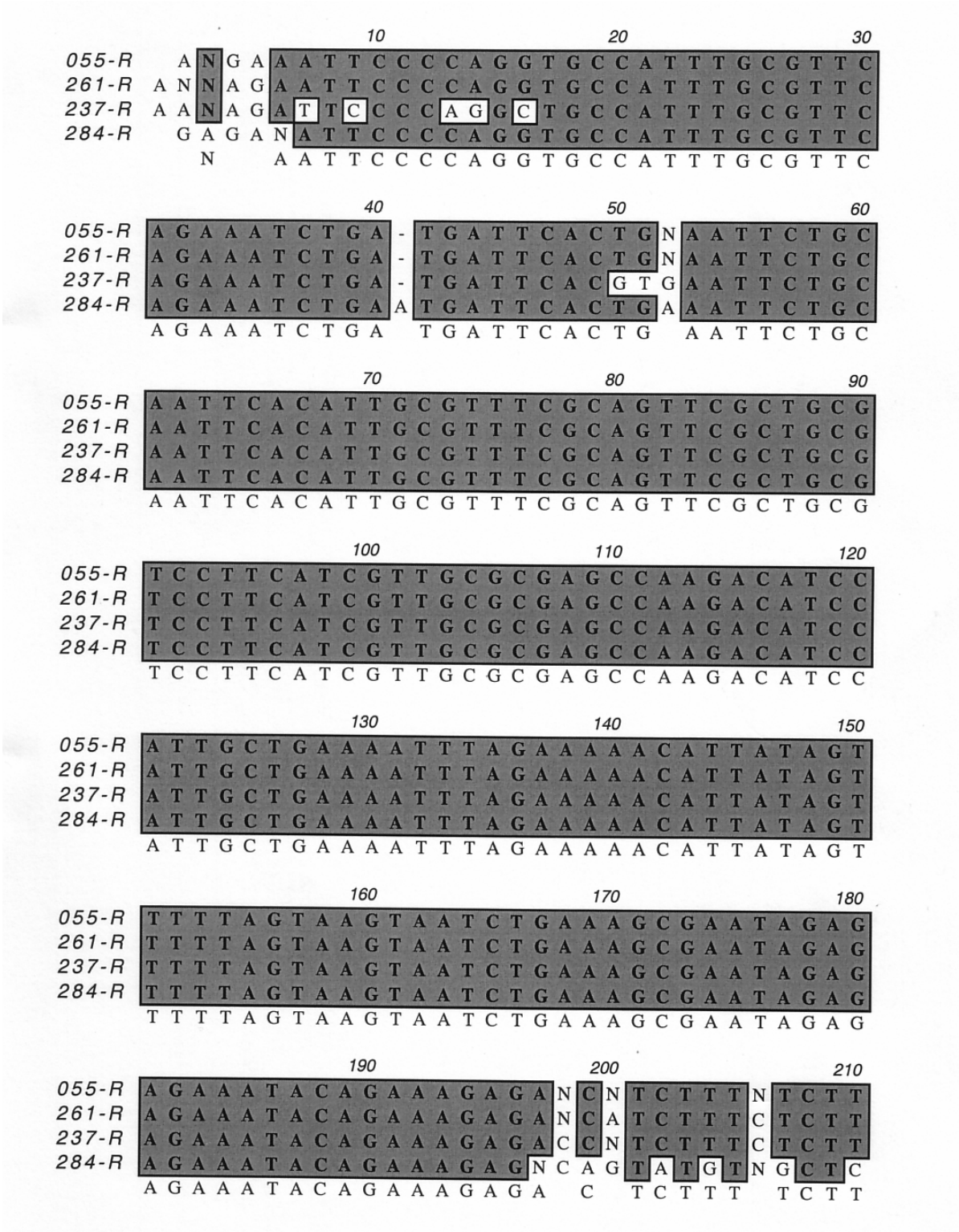
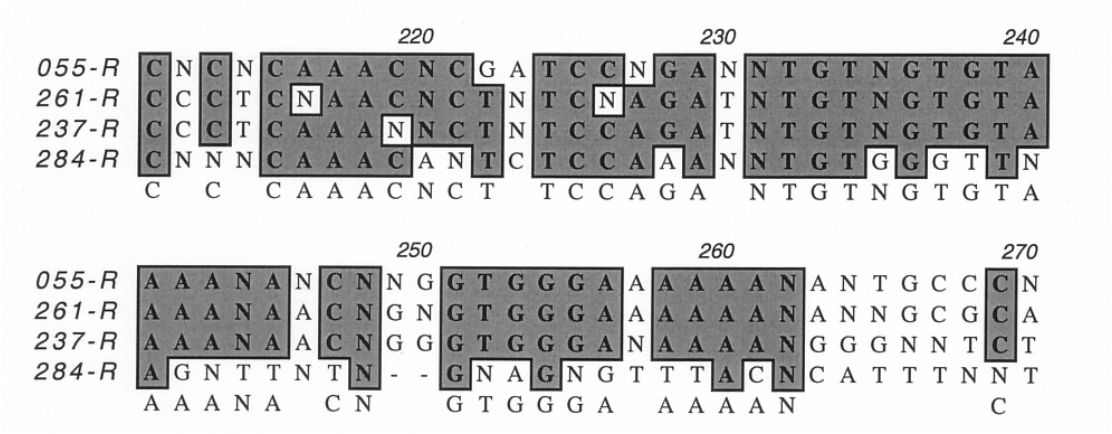

220 230 240
237-F GTGCATATATCCGGGAGTGTACGGCGAAGG
055-F GTGCATATATCCGGGAGTGTACGGCGAAGG
261-F GTGCATATATCCGGGAGTGTACGGCGAAGG
284-F GTGCATATATCCGGGAGTGTACGGCGAAGG
GTGCATATATCCGGGAGTGTACGGCGAAGG
250 260 270
237-F GACTCGGTCACTGGAAATTAATGCCTCTNT
055-F GACTCGGTCACTGGAAATTAATGTCTCTAT
261-F GACTCGGTCACTGGAAATTAATGTCTCTAT
284-F GACTCGGTCACTGGAAATTAATGTCTCTAT
GACTCGGTCACTGGAAATTAATGTCTCTAT
280 290 300
237-F TGGGACTTTNNCTTCCNNGAGTTTCTTNNA
055-F TGGGACTTTAACTTCCAGGAGTTTCTTCAA
261-F TGGGACTTTAACTTCCAGGAGTTTCTTCAA
284-F TGGGACTTTAACTTCCAGGAGTTTCTTCAA
TGGGACTTTAACTTCCAGGAGTTTCTTCAA
310 320 330
237-F TGNGNNTNCTTTTTTCCCNCNCCNNNATTT
055-F TGTGCATTCTTTTTTCCCACACCGTTATTT
261-F TGTGCATTCTTTTTTCCCACACCGTTATTT
284-F TGTGCATTCTTTTTTCCCACACCGTTATTT
TGTGCATTCTTTTTTCCCACACCGTTATTT
340 350 360
237-F TTTACNACNAATCTNGNNNGCNNNTGNGGN
055-F TAAACAACAAATCTGGATAGCGTTTGAGGG
261-F TAAACAACAAATCTGGATAGCGTTTGAGGG
284-F TAAACAACAAATCTGGATAGCGTTTGAGGG
TAAACAACAAATCTGGATAGCGTTTGAGGG
370 380 390
237-F A-GNNNNNNNNNNNGGGGNNGNNNNGN
055-F AAGAGAAAGATGGTCTCTTNTTTCT
261-F AAGAGAAAGATGGTCTCTTNTNTCT
284-F AAGAGAAAGANGGNGTNGTNGNNTNNNNN
AAGAGAAAGA GG T T GTN N

Figure 18 Clustal W aligned sequences (reverse primer)





The region of overlap of the forward and reverse sequences is GGTCTCTTT. This region is highlighted in Figure 19.

Figure 19 Full sequence of products produced by Tim 3/Tim 11 primers

CCTTTATTCTTTTCAACCCTCAACCTTTGAATCCCAAACAAAACA

TGAGCTTGTATCTCTCTCCTTCGGAGAGGGGTACATTCAAGAAGC

GTGATATACTACTCCCTGTGAGTTGTATCGCCTTCTTCATGTGGAT

ATTTTGCACTACTTTTTTCAAGCGTTCTATTGAACGCCTGATAATG

AAAGTGTGTGCATATATCCGGGAGTGTACGGCGAAGGGACTCGG

TCACTGGAAATTAATGTCTCTATTGGGACTTTAACTTCCAGGAGT

TTCTTCAATGTGCATTCTTTTTTCCCACACCGTTATTTTAAACAAC

AAATCTGGATAGCGTTTGAGGGAAGAGAAAGAT**GGTCTCTTT**CT

GTATTTCTCTCTATTCGCTTTCAGATTACTTACTAAAAACTATAAT

GTTTTTCTAAATTTTCAGCAATGGATGTCTTGGCTCGCGCAACGAT

GAAGGACGCAGCGAACTGCGAAACGCAATGTGAATTGCAGAATT

CAGTGAATCATCAGATTTCTGAACGCAAATGGCACCTGGGGAATT

5.3.1.1 BlastN analysis

BlastN analysis of 359 nucleotides from the forward sequence showed 100% homology to 15 other *N. caninum* ITS1 sequences. There was also 99% homology with four other *N. caninum* ITS1 sequences with the level of sequence similarity ranging from 354/355 to 358/359 bases.

BlastN analysis of 192 nucleotides from the reverse sequence showed 100% homology (184/184 bases) to one other *N. caninum* ITS1 sequence. The next two highest ranking alignments were also *N. caninum* sequences with homology of 99% (183/184 bases) and 98% (150/153 bases).

BlastN analysis of 542 nucleotides from the combined sequence produced by forward and reverse primers showed 100% homology to 12 other *N. caninum* ITS1 sequences. This ranged from 534/534 bases to 415/415 bases. There was also 99% homology with four other *N. caninum* ITS1 sequences with the level of sequence similarity ranging from 495/499 bases to 411/415 bases. These alignments are shown in Figure 20

Figure 20 Top 17 alignments after blasting the combined sequence (542 bp)

Sequences producing significant alignments:	Score (bits)	E Value
gb|AF432123.1| Neospora caninum isolate CZ-4 18S ribosomal RNA g...	1059	0.0
gb|L49389.1|NSPRGEBN Neospora caninum 18S ribosomal RNA (18S rRN...	995	0.0
gb|AY463245.1| Neospora caninum internal transcribed spacer 1, c...	950	0.0
gb|AF029702.1|AF029702 Neospora caninum 18S ribosomal RNA gene, ...	910	0.0
gb|AF038861.1|AF038861 Neospora caninum strain CN1 internal tran...	856	0.0
gb|AF038860.1|AF038860 Neospora caninum strain BPA1 internal tra...	856	0.0
gb|U16160.1|NCU16160 Neospora caninum N.C.-1 5.8S rRNA gene, par...	846	0.0
gb|U16159.1|NCU16159 Neospora caninum N.C.-Liverpool 18S rRNA ge...	846	0.0
gb|AY259041.1| Neospora caninum strain NC-Illinois internal tran...	823	0.0
gb|AY259040.1| Neospora caninum strain NC-beef internal transcri...	823	0.0
gb|AY259039.1| Neospora caninum strain Nc-SweB1 internal transcr...	823	0.0
gb|AY259038.1| Neospora caninum strain NC-Liverpool internal tra...	823	0.0
gb|AY259037.1| Neospora caninum strain NC-2 internal transcribed...	823	0.0
gb|AF249970.1|AF249970 Neospora caninum isolate NC-5 internal tr...	823	0.0
gb|AF249969.1|AF249969 Neospora caninum isolate NC-2 internal tr...	823	0.0
gb|AF249968.1|AF249968 Neospora caninum isolate NC-beef internal...	823	0.0
gb|AY508811.1| Neospora caninum isolate NC-deer1 internal transc...	823	0.0

5.3.1 SCID mice experiments

5.3.2.1 Experiment 1a and 1b

None of the mice involved in experiment 1a showed any signs of infection with *N. caninum* after injection with milk extracts. The negative control mouse also remained in good health. The live weights of the mice are found in Figure 21. One reason for the lack of virulence in mice was possibly that the strain of *N. caninum* had been passaged in cows for a long time. If there was any sub-clinical infection of the mice, brain tissue would likely be infected. Thus the brain tissue was passaged back into further SCID mice.

None of the mice involved in experiment 1b showed any signs of infection with *N. caninum* after injection with brain homogenate. The negative control mouse also remained in good health. The live weights of the mice are found in Figure 22.

Figure 21 Live weights of mice in experiment 1a

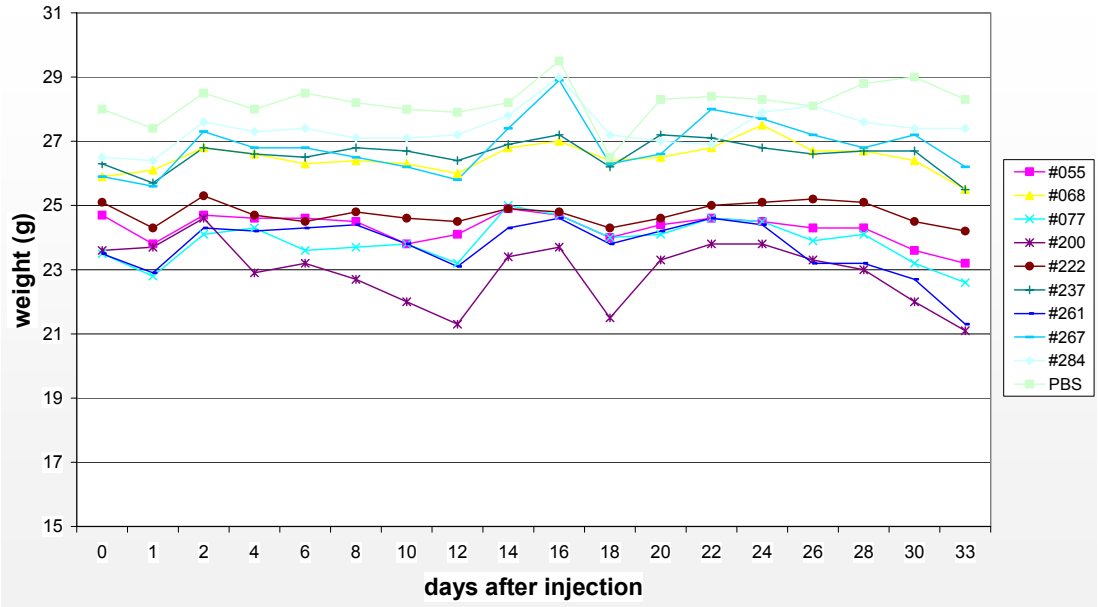
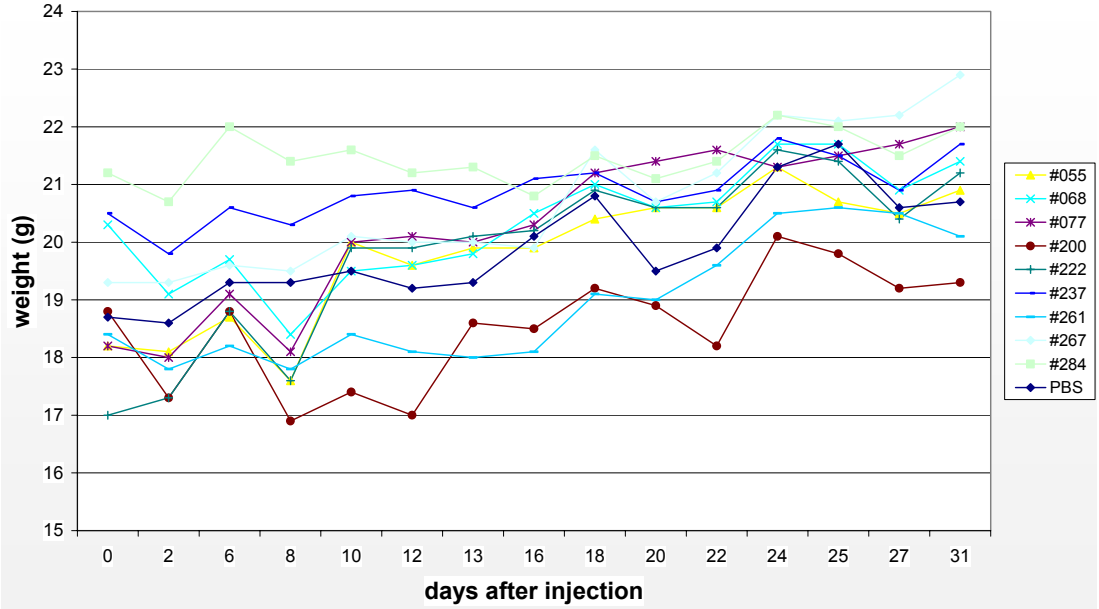


Figure 22 Live weights of mice in experiment 1b



5.3.2.2 Experiment 2

Four of the mice (mice 1,3,4 and 5) injected with milk extract from a recently aborted *N. caninum* seropositive cow remained healthy and generally put on weight throughout the 67 days post-injection. These four mice increased in weight by an average of 5.0 g and ranged from 3.1-6.5 g over the study period. Mouse 2 generally put on weight throughout the study until day 53 when he lost 3.5 g over 11 days. Mouse 6 was euthanased on day 25 due to a rectal prolapse. This mouse had also lost 3.8 g over a 10-day period.

Of the two mice that were injected with the lowest dose (1×10^3) of NC-1 tachyzoites one (mouse 7) remained healthy throughout the 67 day period showing no weight loss while the other (mouse 8) lost 3.1 g after day 27 and was dead on day 32. Mouse 9 and mouse 10 were injected with 1×10^4 tachyzoites and both lost 5.3 g in weight from days 25 and 32, respectively and died on days 29 and 35, respectively. Of the mice that received the highest dose (1×10^5) of NC-1 tachyzoites, mouse 11 showed only a small weight loss of 1.6 g at day 13 and died at 15 days post-infection while mouse 12 died at 11 days post-infetion with no weight loss detected.

The three mice injected with PBS remained healthy throughout the study with an average weight gain of 3.4 g and a range of 3.0-4.2 g over the 67-day period. The live weights of the mice throughout the study are shown in Figures 23, 24 and 25.

There were no significant lesions found by pathological examination of the brain or spleen tissues of any of the mice that were injected with milk extracts.

Figure 23 Live weights of mice injected with milk extract (Exp 2)

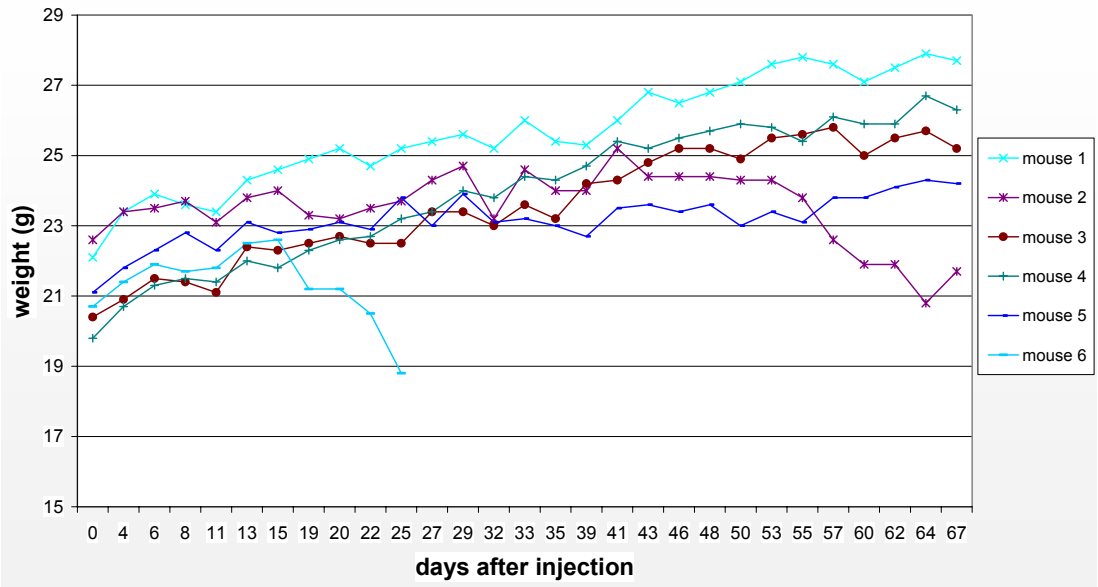


Figure 24 Live weights of mice injected with NC-1 tachyzoites (Exp 2)

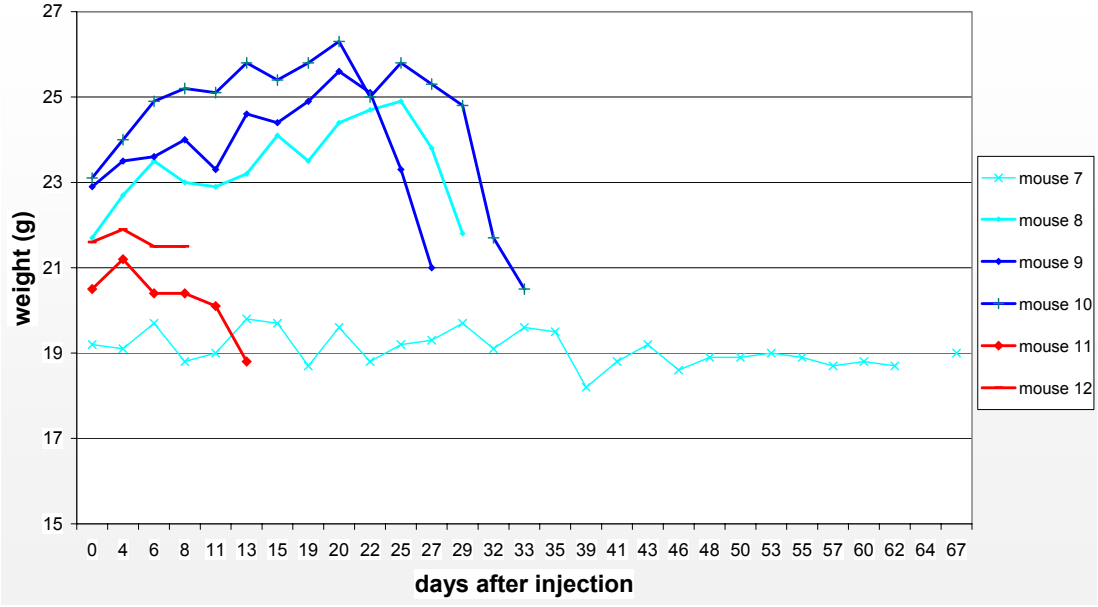
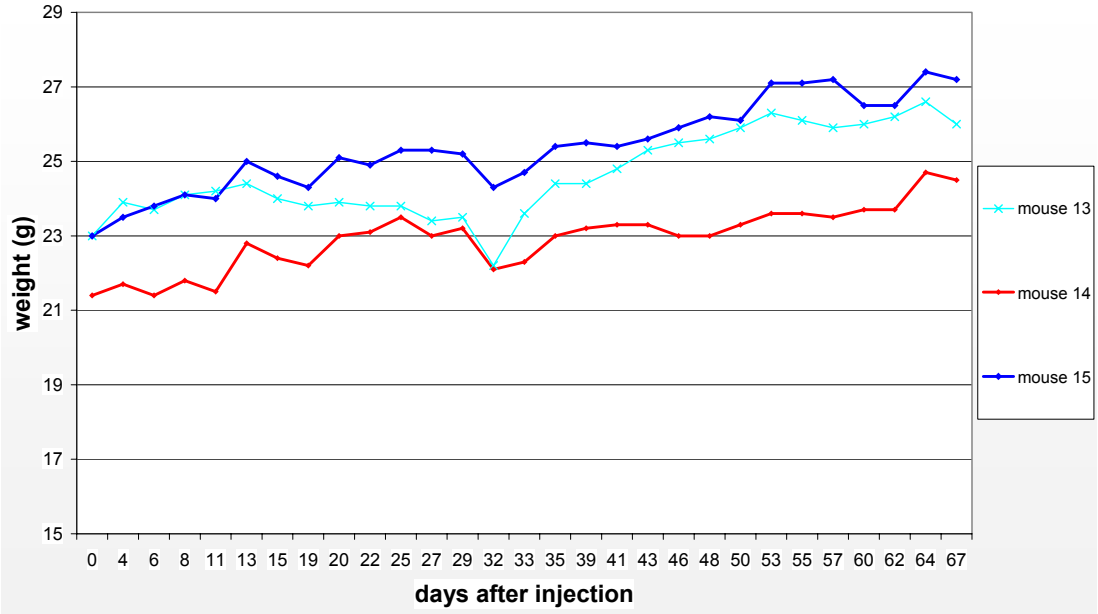


Figure 25 Live weights of mice injected with PBS (Exp 2)



5.4 Discussion

This is the first study where *N. caninum* DNA has been detected in milk from cows in Australia. This finding corroborates a recent report from Poland of *N. caninum* DNA detected in milk from seropositive cows (Moskwa *et al.* 2003). This would be a concern for farmers as it possibly indicates a new route of transmission of *N. caninum*. Where farmers feed pooled milk from their herd to calves there appears to be a risk of infecting further stock if this milk has originated from *N. caninum* infected cows. In the additional studies though it was not demonstrated that milk from *N. caninum* seropositive cows was infectious to mice. Considering that others have previously failed to demonstrate *N. caninum* in milk or colostrum from infected cows this most likely indicates that this is not a dominant route of transmission. If it were a major route there would probably be greater numbers of postnatal infections occurring. Even if it is assumed that milk can be infectious via the oral route it may be that this is not an efficient method of transmission. Tachyzoites may be susceptible to degradation in the acidic stomach environment as a previous study indicated that *N. caninum* tachyzoites were not resistant to HCl-pepsin (Dubey *et al.* 1988c). As vertical transmission is very efficient, there is a great probability that the offspring of a *N. caninum* infected cow would be infected even without ingesting infected milk.

In the first PCR there seems to have been cross contamination of the Tim primers with CR3/CR4 primer pair. In Figure 15 where the Tim 3/Tim 11 primers were used (lanes 2, 4, 6 and 8) the expected 600 bp molecule is produced. There is also a 300 bp molecule in these lanes and it is possible that the Tim 3/Tim 11 primers became contaminated with CR3/CR4 primer and thus also produced the 300 bp product. Bands equivalent to 300 bp were produced by CR3/CR4 primers in all four milk extracts and are found in lanes 3, 5, 7 and 9. There were no bands detected in the water controls when using either primer pair as seen in lanes 10 and 11. In later PCRs there was no evidence of contamination of primers.

Mice were injected with milk extracts rather than infected via the oral route as it is possible that tachyzoites would degrade in the stomach. This possibly occurred in dogs fed colostrum spiked with *N. caninum* tachyzoites as they did not induce infection (Dijkstra *et al.* 2001b). Others though, found that calves orally fed *N. caninum*

tachyzoites added to colostrum could be infected (Uggla *et al.* 1998). Two calves that were dosed via a stomach tube showed no signs of infection while two other calves that were bottle fed the inoculum developed a significant immune response and *N. caninum* DNA was isolated from the brains. These authors hypothesised that the calves feeding on the bottle stimulated the oesophageal groove to direct the milk straight into the abomasum whilst tube feeding might have caused the bulk of the inoculum to end up in the rumen where the parasites may have degraded. These authors also hypothesised that bottle fed calves may have been infected by the parasite penetrating the buccal or pharyngeal mucosa (Uggla *et al.* 1998). This method of transmission has been demonstrated in mice orally infected with *T. gondii* (Raisanen and Saari 1978).

It is also not known whether *N. caninum* antibodies in the colostrum have a negative effect on any tachyzoites present in the milk. This may be another reason for the lack of infection in the mouse experiments.

The dose of *N. caninum* in the milk extracts may also have been too low to cause infection, even in SCID mice. In the first experiment mice were injected with the equivalent of 4 ml of milk (*i.e.*10 ml of milk was concentrated into 250 µl but only 100 µl was injected into mice). In the second experiment, 50 ml of milk was concentrated into 1 ml and then 100 µl was injected into each mouse meaning that each mouse received the equivalent of 5 ml of milk.

Neospora caninum has been detected in milk samples from seropositive cows as shown by PCR and sequencing but injection of extracts from seropositive cows into immuno-compromised mice could not show that the milk contained viable *N. caninum*. Further analysis of milk samples by PCR is necessary to determine at what stage of pregnancy or milking that *N. caninum* can be detected in milk. Further mouse studies should be undertaken to be certain that *N. caninum* is not viable within milk samples or if the concentration of *N. caninum* in milk is important. Until this issue is resolved it remains a potentially major issue for the dairy industry.

6. *Neospora caninum* and the effect on milk production and reproduction parameters

6.1 Introduction

6.1.1 Milk production

In dairy herds, the economic importance of *N. caninum* is often thought of in terms of costs associated with abortion (*i.e.* loss of calf, fewer milking cows) and subsequent culling of cows (Chi *et al.* 2002). To date there have been only a few studies that attempted to find a link between *N. caninum* infection and milk production. Two studies conducted in dry-lot dairies in the USA, concluded that *N. caninum* seropositive cows produce less milk than seronegatives (Thurmond and Hietala 1997b, Hernandez *et al.* 2001). Conversely, two Canadian studies and one from New Zealand have data that show that seropositive cows produce more milk than seronegative cows (Keefe and VanLeeuwen 2000, Hobson *et al.* 2002, Pfeiffer *et al.* 2002).

A study involving 372 first-lactation Holstein dairy cows on a Californian dairy (the seroprevalence to *N. caninum* in this herd was 32%), demonstrated that *N. caninum* seropositive cows produced 2.5 lb/cow/day less than seronegative cows using daily mean milk weights (Thurmond and Hietala 1997b). The daily milk production was significantly less amongst seropositive cows for 18 of the first 19 weeks of lactation and was consistently less for the first 43 weeks of lactation. Using Dairy Herd Improvement Association records, they also demonstrated that seropositive cows produce less milk (3.1 lb/cow/day) and less fat (0.14 lb/cow/day) than seronegative cows. The estimated 305-day mature equivalent milk production of seropositive cows was significantly less than that of seronegative cows with seropositive cows producing 2.8 lb/cow/day (1.3 kg/cow/day) less.

In Florida, a study of 565 multiparous Holstein cows from a single dairy was undertaken. In this study, *N. caninum* seropositive cows (seroprevalence was 23%) produced significantly less milk (2.8 lb/cow/day or 1.3 kg/cow/day) than seronegative cows based on the 305-day mature equivalent milk production data (Hernandez *et al.*

2001). After adjusting for lactation number, calving season, clinical mastitis and lameness, milk weights of seropositive cows were 2.5 lb/cow/day less than seronegative cows. In their first lactation, seropositive cows produced 2.1 lb/cow/day less milk than seronegative cows, which is in line with the previous study in California where positive cows in their first lactation produced 2.5 lb/cow/day less than seronegative cows. In their second and later lactations, seropositive cows produced 3.2 lb/cow/day less than seronegative cows.

A case control study of 3702 Holstein cows in 83 herds (*N. caninum* seroprevalence was 22%) in Ontario, Canada found that there was significantly less milk produced by seropositive cows in herds that had abortions due to *N. caninum* and also in herds that had abortions due to pathogens other than *N. caninum*. In herds without evident abortion problems, there was no significant difference in milk production between seropositive and seronegative cows. The average seroprevalence to *N. caninum* in these herds was 22% (Hobson *et al.* 2002).

In the observational portion of this study (seroprevalence was 10.5%) involving 3162 Holstein cows in 57 herds, researchers analysed the 305-day milk production and found that seropositive cows produced 0.5 kg/day more milk, and seropositive first lactation cows produced 0.6 kg/day more milk than seronegative cows. However, these results were not statistically significant. From the projected 305-day milk production of cows that were culled during this study, seropositive cows produced significantly more milk (0.9 kg/day) than seronegative cows (Hobson *et al.* 2002).

A study of 2425 cows on 90 randomly selected farms in Canada, showed a *N. caninum* seroprevalence of 19.2%. The 305-day projected milk production was analysed and showed that on average, *N. caninum* seropositive cows produced 0.6 kg/day more milk than seronegative cows. Seropositive cows in their first, second and third lactation produced 0.5 kg/day, 0.7 kg/day and 1.1 kg/day/cow more milk respectively than seronegative cows. Again, these results were not significantly different (Keefe and VanLeeuwen 2000).

A New Zealand study (Pfeiffer *et al.* 2002) of a 600-cow dairy herd (seroprevalence to *N. caninum* in cows was 30%) demonstrated that seropositive cows produced

significantly more milk (0.4 kg/day/cow) than seronegative cows. There was also a significantly greater amount of protein (0.01 kg/day/cow) produced by seropositive cows than seronegative cows. There was however no difference in other production parameters, such as fat and solids between positive and negative cows.

6.1.2 Reproduction parameters

It is likely that *N. caninum* is responsible for some early foetal loss aside from abortion, but there are no estimates available. This may represent an important although unquantified economic loss. Early foetal death would present as a return to service, increased time to conception or infertility. This would increase the calving interval (Trees *et al.* 1999). A Swedish study compared the number of inseminations per confirmed pregnancy but found that there was no significant difference between seropositive and seronegative cows (Björkman *et al.* 1996). A recent study found that *N. caninum* infection was associated with additional services per conception (Munoz-Zanzi *et al.* 2004). After one AI, *N. caninum* infected heifers had 1.8 times higher odds of not conceiving compared to non-infected heifers.

6.1.3 Objective

The objective of this study was to determine whether cows seropositive to *N. caninum* in the study herd produced more or less milk than seronegative cows. It was also to determine the number of inseminations required for a confirmed pregnancy and the time taken from insemination to conception amongst *N. caninum* seropositive and seronegative cows.

6.2 Materials and Methods

6.2.1 Milk production

In December 2002, a herd of 266 Friesian cattle on a dairy in Kemps Creek, NSW, Australia, was blood sampled and serologically tested by ELISA for antibodies to *N. caninum* as described previously (Chapters 2 and 3). Of these 266 cattle, 140 were cows that were pregnant or had previously lactated. A database (Dairy CHAMP Pastoral) was accessed and projected 305-day milk production data was collected for 128 of these cows. Data was not collected for 12 cows as they were either dry at the time of sampling or had not been milking for long enough to be able to estimate a projected milk output. Of the 128 cows, 15 (11.7%) were seropositive for *N. caninum* antibodies and 113 (88.3%) were seronegative. The data collected was projected 305-day milk production, fat and protein. The lactation number and the number of DIM at the time of projection were also recorded. The cows in this herd were tested regularly for milk, protein and fat production and also for SCC. Milk production of seropositives cows was compared with that of seronegative cows after allowing for the lactation number. The individual cow 305-day milk production data corresponded to the lactation at the time of blood collection. For logistical reasons the 305-day projected milk production data was collected at either of two time points: late October 2002 (60 cows - 6 weeks before blood collection) or July 2003 (68 cows). The earliest time-point in the lactation that these projected milk estimates were taken was 127 days in milk. The projected 305-day milk production of seronegative cows in different lactations was also compared to see any effect that parity had on milk production. Projected 305-day milk data was statistically analysed by using single factor Anova (Excel, Microsoft Office XP, Enterprise Edition).

6.2.2 Reproduction parameters

6.2.2.1 The number of inseminations to conception

The Dairy CHAMP Pastoral database was accessed and the number of services to conception was obtained regarding the two most recent pregnancies of each cow. For the most recent lactation there was data from 137 cows with 10.9% of these being seropositive to *N. caninum.* For the previous pregnancy there were 87 cows included

with 6.9% of these being seropositive for *N. caninum*. There were reduced numbers of cows in the latter pregnancy as only cows that had conceived in the previous 3 years were included and there were also many younger cows that had only conceived once. The data was statistically analysed by using single factor Anova (Excel, Microsoft Office XP, Enterprise Edition).

6.2.2.2 Time to conception

The "time from calving to first service" and the "time from calving to conception" was also obtained from the database and subtracted from one another to give the "number of days from first service to conception". There was data from 134 cows with 10.4% of these being *N. caninum* seropositive. Again, data was statistically analysed by using single factor Anova (Excel, Microsoft Office XP, Enterprise Edition).

6.3 Results

6.3.1 Effect of *N. caninum* infection on milk production parameters

The following tables (9-11) show the results of the mean projected milk, protein and fat of both *N. caninum* seropositive and seronegative cattle. Table 12 shows the difference that lactation number (parity) has on milk production amongst cows.

Table 9 The mean projected 305-day milk production of *N.caninum* seropositive and seronegative cattle

	***Neospora* positive**		***Neospora* negative**				
Lactation no.	**n**	**Milk/cow (kg)**	**n**	**Milk/cow (kg)**	**Difference (kg)**	**Diff. (kg/cow/ day)**	***P* value**
1	6	6834.8	20	6194.7	640.1	2.1	0.15
2	5	7958.0	41	7720.2	237.8	0.8	0.66
3	1	6948.0	26	8531.8	-1583.8	-5.0	0.29
4	3	9850.0	12	8321.2	1528.8	2.4	0.48
5-8	0	-	14	8836.8	-	-	na
mean 1-4	15	7819.8	99	7698.0	121.8	0.4	0.77
mean 1,2 & 4	14	7882.1	73	7401.0	481	1.6	0.24

Abbreviations used in Tables 9-11

Lactation no. – categorises cows by their number of lactations.

n – number of cows.

Milk/cow (kg) – the projected 305-day milk output per cow in kg.

Difference – the difference in milk production between seropositive and seronegative cows in projected 305-day lactations.

Table 10 **The mean projected 305-day protein production by *N. caninum* seropositive and seronegative cattle**

	Neospora positive		*Neospora* negative				
Lactation no.	**n**	**Protein/ cow (kg)**	**n**	**Protein/ cow (kg)**	**Difference (kg)**	**Diff. (kg/cow/ day)**	***P* value**
1	6	218.5	20	204.9	13.6	0.045	0.28
2	5	254.0	41	246.9	7.1	0.023	0.69
3	1	199.0	26	267.4	-68.4	-0.224	0.15
4	3	303.0	12	259.3	43.7	0.143	0.16
5-8	0	-	14	264.7	-	-	na
mean 1-4	15	245.9	99	245.3	0.6	0.002	0.96
mean 1,2 & 4	14	249.3	73	237.4	11.9	0.039	0.34

Table 11 **The difference in projected 305-day fat produced by *N. caninum* seropositive and seronegative cattle**

	Neospora positive		*Neospora* negative				
Lactation no.	**n**	**Fat/cow (kg)**	**n**	**Fat/cow (kg)**	**Difference (kg)**	**Diff. (kg/cow/ day)**	***P* value**
1	6	215.2	20	206.8	8.4	0.028	0.61
2	5	242.8	41	260.6	-17.8	-0.058	0.46
3	1	208.0	26	289.9	-81.9	-0.269	0.22
4	3	313.0	12	280.7	32.3	0.106	0.41
5-8	0	-	14	291.6	-	-	na
mean 1-4	15	243.5	99	259.8	-16.3	-0.053	0.32
mean 1,2 & 4	14	246.0	73	249.2	3.2	0.010	0.84

6.3.2 Effect of parity on milk production

It is known that cows generally produce more milk as their lactation number increases. Second lactation cows produce more milk than first lactation cows, third lactation cows produce more milk than second lactation cows and so on. The following table shows the projected 305-day milk production of *N. caninum* seronegative cows in different lactations.

Table 12 The projected 305-day milk produced by *N. caninum* seronegative cattle compared over different lactations

			***P* value**			
Lactation number	**Mean proj305milk**	**n**	**2^{nd} lactation**	**3^{rd} lactation**	**4^{th} lactation**	**$5^{th} – 8^{th}$ lactation**
1	6195	20	<0.001	<0.001	<0.001	<0.001
2	7720	41	-	0.009	0.110	0.001
3	8532	26	-	-	0.678	0.504
4	8321	12	-	-	-	0.322
5-8	8837	14	-	-	-	-

n - number of cows

6.3.3 Number of inseminations for conception

Table 13 shows the number of inseminations for conception to occur. After analysing the most recent pregnancy it was found that there were more inseminations required for conception amongst seropositive cows compared to seronegative cows and this was significant at a 90% confidence level (*P*=0.06). For the previous pregnancy there were also more inseminations required for conception of seropositive animals. This difference was statistically significant (*P*=0.01).

Table 13 The number of inseminations for conception of *N. caninum* infected and non-infected cows

	Mean number of inseminations		
Pregnancy	***N. caninum* positive**	***N. caninum* negative**	***P*-value**
Most recent pregnancy	3.5 (15)	2.5 (122)	0.06
Previous pregnancy	4.0 (6)	2.2 (81)	0.01
Total (two most recent pregnancies)	3.7 (21)	2.4 (203)	0.003

Number in brackets signifies *n*

6.3.4 Time to conception

Table 14 shows the time to conception comparing *N. caninum* seropositive and negative cows. Although it took 22.5 days more for conception to occur in *N. caninum* positive cows compared to seronegative cows this difference was not statistically significant.

Table 14 The number of days from the first service till conception of *N. caninum* infected and non-infected cows

	Mean days to conception (from 1st service)		
Pregnancy	***N. caninum* positive**	***N. caninum* negative**	***P* value**
Most recent pregnancy	88.1 (14)	65.6 (120)	0.349

Number in brackets signifies *n*

6.4 Discussion

Previous studies have shown conflicting data with regard to the effect of *N. caninum* infection on milk production. It is generally assumed that a disease will have a negative impact on the general health of the animal and it has been hypothesised that the organ system function of cows infected with *N. caninum* would be sufficiently compromised to adversely affect the cows ability to produce large quantities of milk (Hernandez *et al.* 2001). In the present study, *N. caninum* seropositive cows in their first, second and fourth lactation on average produced more milk than that of seronegative cows in the corresponding lactation. This increase was not statistically significant because of the low number of seropositive animals and the small difference in milk production. There was only one seropositive cow in the third lactation group and this animal produced less milk than the seronegative third lactation cows. There were no *N. caninum* seropositive cows in their fifth or higher lactation.

Seropositive cows in the first to fourth lactation produced on average 7820 kg of milk compared to seronegative cows in the same lactation periods that produced only 7698 kg milk. The difference was 122 kg milk (1.6% less) in a 305-day lactation period (or 0.4 kg/cow/day). Due to fact that there was only one seropositive cow in the third lactation, it is possible to exclude this result and other third lactation negative cows from calculations. When only including first, second and fourth lactation cows, seropositive cows produced 1.6 kg more milk/cow/day than seronegative cows.

This increase in milk production amongst seropositive cows agrees with studies in New Zealand and eastern Canada where they found an increase of 0.4 kg/cow/day and 0.6 kg/cow/day, respectively. The observational study of cows in Ontario, Canada also agrees with these findings as they found that seropositives produced 0.5 kg/cow/day more milk. Of these three studies though, only the New Zealand one found that the difference statistically significant.

In contrast, studies from California and Florida (Thurmond and Hietala 1997b, Hernandez *et al.* 2001) determined that seropositive cows produce around 1.3 kg/cow/day less milk that that of seronegative cows. They hypothesised that organ

system function (brain, heart, liver, kidney, adrenal gland) of clinically normal cows, infected with *N. caninum* could be sufficiently compromised to negatively impact a cow's ability to produce large quantities of milk.

When calculating milk production the lactation number must be taken into account, as there is usually an increase in milk production as lactation number increases. This was shown in the present study where there was a trend of increasing milk output from first to third lactation cows. There was a significant difference in the amount of milk produced by first lactation cows when compared with second, third, fourth and combined fifth-eight lactation cows. There was also a significant difference in milk production between second and third lactation cows and second and combined fifth-eight lactation cows. There was no difference detected between second and fourth lactation cows at a 95% confidence level probably due to the fact that n was small; but there was a difference detected at an 89% confidence level. When comparing milk production of third and fourth lactation cows there was actually more milk produced by the third lactation cows. There was more milk produced by combined fifth-eight lactation cows than fourth lactation cows. These results affirm that in this study it was necessary to take the number of lactations into account when comparing milk production.

It has previously been reported from New Zealand that *N. caninum* seropositive cows produced significantly (P=0.01) more protein (0.01kg/cow/day) than seronegative cows (Pfeiffer *et al.* 2002). The current study also found that there was a greater amount of protein produced (0.04 kg/cow/day) by *N. caninum* infected cows than non-infected cows over lactations one, two and four although this was not statistically significant. There was less protein produced by the one seropositive cow in the third lactation compared to seronegative cows but this is not considered to be a true representation due to sample size. Thus the third lactation cows were omitted from further analysis as they would otherwise bias results due to the greater amount of milk produced by cows of high parity.

When evaluating total milk fat produced by *N. caninum* infected cows, there was more fat produced by cows in lactations one and four and less fat in lactations two and three compared to non-infected cows. Overall, in lactations one to four there was less fat

produced by seropositive cows than seronegative cows although this was not statistically significant.

In this study it was difficult to demonstrate statistical significance as the number of cows per lactation were low. The greatest number of *N. caninum* infected animals in a lactation group was only six.

There are many reasons that could account for the differences in milk production between *N. caninum* infected and non-infected cattle. Factors such as seroprevalence, management practices, cattle genotypes, experimental design and diagnostic test performance have been suggested (Hernandez *et al*. 2001). New Zealand and Australia are pasture-based herd management systems and it is in these two countries that increased milk production has been found amongst *N. caninum* seropositive cattle. This includes results from this study and a previous study (Pfeiffer *et al.* 2002). These results are in contrast to those from California (Thurmond and Hietala 1997b, Hernandez *et al.* 2001) where the dry-lot system of herd management is used.

With regard to milk production in this study there is a theoretical benefit to the farmer with *N. caninum* infected cattle. On average this farmer receives AUS$0.30/L of milk and so with an increase of 0.4L milk/cow/day (or 122L/cow/305days) this amounts to a gain of $549 from the 15 infected cows. When using the data from the first, second and fourth lactation cows only (*i.e.* 1.6L/cow/day) then this amounted to a gain of $2196 during a 305-day milking of the 15 infected cows. This though, is a small benefit to the farmer especially when considering that two abortions due to *N. caninum* could cost $2000.

In this herd *N. caninum* infected cows required more inseminations per confirmed pregnancy than their seronegative herd mates. With regard to the most recent pregnancy, seronegative cows were inseminated 2.5 times while seropositive cows were inseminated 3.5 times on average. There was 6% risk of this occurring by chance as P=0.06. There was a significant difference (P=0.01) in the previous pregnancy with seropositive cows again requiring 4.0 inseminations compared with 2.2 inseminations for seronegative cows. A previous study found no significant difference between the number of inseminations per confirmed pregnancy with regard to seropositive and

seronegative cattle (Björkman *et al.* 1996). However a recent study found that *N. caninum* infected heifers had a 1.8 times greater chance of not conceiving after one AI than their non-infected herd mates (Munoz-Zanzi *et al.* 2004). In the current study there was also an increased time to conception amongst seropositive cows. Seronegative cows took 65.6 days for conception while seropositive cows took an extra 22.5 days (34% longer).

The increased time to conception and the greater number of services both indicate that early foetal loss may have occurred. It has been suggested that *N. caninum* is likely to be a cause of early foetal death but there has previously been no data to support this (Trees *et al.* 1999).

It may be difficult to appreciate that *N. caninum* could have a positive effect on milk production and as suggested elsewhere (Pfeiffer *et al.* 2002) it may be that selective culling by the farmer has contributed to the increase in milk production by seropositive cows in this study. A farmer may select high milking cows when it comes to culling. Any cows that have aborted or are difficult to get pregnant have a higher chance of being culled unless they have a redeeming quality. *Neospora caninum* infected cows are more likely to abort and take longer to get pregnant, but the high milk producing cows stand a greater chance of being retained. In this case we would see selective retention of high milk producing *N. caninum* positive cows rather than low milking seropositive cows. One example from the present herd of possible selective retention is where a *N. caninum* infected cow took six services to become pregnant but had produced 15,000L of milk. In the following lactation the same cow produced 18,000L of milk, was serviced seven times before becoming pregnant and then aborted. Also, as milk production may be determined in part by genetic makeup and *N. caninum* infection is predominantly transferred from dam to daughter, the farmer may be unknowingly maintaining *N. caninum* infection in the herd.

7. Control of *Neospora caninum* infection on a dairy farm

7.1 Introduction

Cattle become infected with *N. caninum* by either vertical or postnatal transmission. The most predominant route of transmission in cattle is from cow to calf *in utero*. Vertical or congenital transmission is very efficient, with 81-95% of infected cows giving birth to infected calves (Paré *et al.* 1996, Paré *et al.* 1997, Wouda *et al.* 1998b, Davison *et al.* 1999a).

It has been suggested that where herds have a high rate of vertical transmission and low postnatal transmission, control of *N. caninum* infection could be achieved by culling and/or not breeding from infected cattle (French *et al.* 1999, Reichel and Ellis 2002). This would break the vertical transmission cycle and where the rate of postnatal infection is low, few new infections would be expected. This would also decrease the number of abortions and decrease the possibility of infecting other cows or definitive hosts. This is only a practical method of control where there is a low prevalence of infection so few animals would need to be culled or not bred.

Mathematical modelling studies (assuming a high vertical transmission rate) have shown that a policy of annual culling of infected cattle would reduce the prevalence of infection rapidly (French *et al.* 1999). Not breeding replacements from infected cattle would also be effective in the short term. In both cases the level of postnatal infection was the key to the degree of control obtained.

A detailed description of possible modes of infection in this herd is found previously in Chapter 4. Briefly, *N. caninum* infected dogs (McAllister *et al.* 1998, Basso *et al.* 2001) and coyotes (Gondim *et al.* 2004) can excrete oocysts which contaminate the environment and subsequently infect cattle (De Marez *et al.* 1999). Dogs can become infected after ingesting infected bovine tissue (Dijkstra *et al.* 2001b). It has been suggested that dogs may become infected after ingesting milk from infected cows (Dijkstra *et al.* 2001b). Foxes can act as intermediate hosts (Barber *et al.* 1997) and

there is speculation that as canids they may also act as definitive hosts (Schares *et al.* 2001).

Calves have been orally infected with colostrum spiked with *N. caninum* tachyzoites and there has been speculation that milk or colostrum may be a natural source of infection for calves (Uggla *et al.* 1998).

7.1.1 Herd background

A whole herd bleed had previously been undertaken in December 2002 at a dairy farm in Kemps Creek, NSW. There was a seroprevalence of 10.2% (27/266) of *N. caninum* infection. Of the 27 *N. caninum* seropositive cattle, 20 (74%) were found to have family associations and they were found to belong to six family trees. When looking at direct descendants (*i.e.* dam/daughter pairs) on the property at the time of sampling, there were six seropositive cows, which had given birth to nine seropositive and one seronegative offspring. This equates to a vertical transmission rate of 90% (9/10) of *N. caninum* in this herd. Of four calves or foetuses from *N. caninum* positive dams, three (75%) were themselves serologically positive. As seropositivity is an indicator of infection, we see a vertical transmission rate of 75% amongst these animals. This is evidence for vertical transmission of *N. caninum* in this herd.

There was a low level of postnatal transmission detected in this herd. Four cattle that tested positive to *N. caninum* were born from dams that had tested negative *i.e.* 15% (4/27) of seropositive cattle were born from seronegative cattle. These seropositive cattle were most likely to have been infected postnatally.

The farmhand's two dogs had been on the property since only March 2002 and left in October 2003. The farmer's two dogs remained on the property throughout the study period. All four dogs were tested in November 2002 using IFAT and ELISA and one of the farmer's dogs was positive in both tests.

As the majority of infections were due to vertical transmission and the overall infection rate was low, it was proposed that infected cattle should not be rebred, and culling

should take place when milking ceased, in order to break the vertical transmission cycle. With the low observed rate of postnatal infection, only few new infections were expected.

7.2 Materials and Methods

7.2.1 Cattle

Twelve of the original 27 seropositive cattle were retained with 15 being selectively culled during the course of the year (December 2002 to December 2003) without causing a serious financial burden. Seropositive heifers that had never been bred were culled, and seropositive cows were culled when they ceased milking, or gave additional reason to be culled (*e.g.* abortion, mastitis). One year after the initial survey a whole herd bleed was undertaken. Cattle serum samples (n=207) were collected and tested for *N. caninum* antibodies by ELISA (IDEXX Laboratories) as previously described in 3.2.2. Three border-line samples were re-sampled 1 month later and retested in the same ELISA. The positive samples and three borderline samples were also tested in a *N. caninum* blocking ELISA (Institut Pourquier) as previously described in 2.2.2.

7.2.2 Dogs

Serum from the two dogs was also tested in the *N. caninum* ELISA (Institute Pourquier as described in 2.2.2).

7.3 Results

7.3.1 Cattle serology

Thirteen (13) the 207 cattle were shown to have high levels (S/P>2.0) of anti-*N. caninum* serum antibodies and three cattle were found to have a borderline level (0.4<S/P<0.6) of *N. caninum* antibodies as determined by the IDEXX ELISA. Two border line samples were re-tested in the IDEXX ELISA and one was confirmed as seronegative and another remained borderline (S/P=0.59). The S/P of the seropositive samples ranged from 0.59-4.1 with a mean value of 2.9. The S/P of seronegative samples ranged from -0.04-0.43 and the mean value was 0.13. The results are shown graphically in Figure 26. The seropositive animals with S/P ≥0.5 (IDEXX) along with the corresponding results of the Pourquier ELISA are listed in Table 15.

Of the 16 cattle sera tested in the *N. caninum* blocking ELISA (Insitut Pourquier), 13 were considered positive. The % inhibition of the seropositive samples ranged from 4.7% to 10.9% with a mean value of 6.7%. The three seronegative samples ranged from 85.9% to 109.4% inhibition and the mean value was 94.4% inhibition. There were no samples determined to be doubtful, nor was any sample close to this cut-off as all samples were at least 30% from either side of this cut-off Figure 27.

Thirteen (6.3%) of the cows were *N. caninum* positive in both ELISAs while 193 were negative in both. One cow was borderline positive in the IDEXX ELISA but negative in the Pourquier ELISA. Only one of the 13 seropositive cattle presented a new infection as 12/13 were previously (2002) known positives.

7.3.2 Dog serology

Of the two dogs on the property, the same dog that had previously been determined to be positive for *N. caninum* remained positive after testing in the *N. caninum* blocking ELISA (Insitute Pourquier). The other dog was negative by ELISA.

Table 15 ***N. caninum*** **seropositive cattle samples as determined by the IDEXX and Pourquier ELISA**

	IDEXX ELISA		Pourquier ELISA	
Cattle tag no.	**S/P ratio**	**Result**	**% inhibition**	**Result**
211	4.06	Pos	5.91	Pos
225	3.94	Pos	8.11	Pos
273	3.86	Pos	5.44	Pos
077	3.13	Pos	5.08	Pos
200	3.10	Pos	8.79	Pos
261	3.07	Pos	5.69	Pos
267	3.01	Pos	4.67	Pos
122	2.92	Pos	7.20	Pos
274	2.83	Pos	7.36	Pos
068	2.70	Pos	5.08	Pos
231	2.56	Pos	5.55	Pos
284	2.29	Pos	7.23	Pos
055	2.02	Pos	10.94	Pos
236	0.59	Pos	85.88	Neg

Pos- positive, Neg- Negative

Figure 26 **S/P ratio and corresponding OD exhibited by 207 individual cattle sera tested in the *N. caninum* ELISA (IDEXX)**

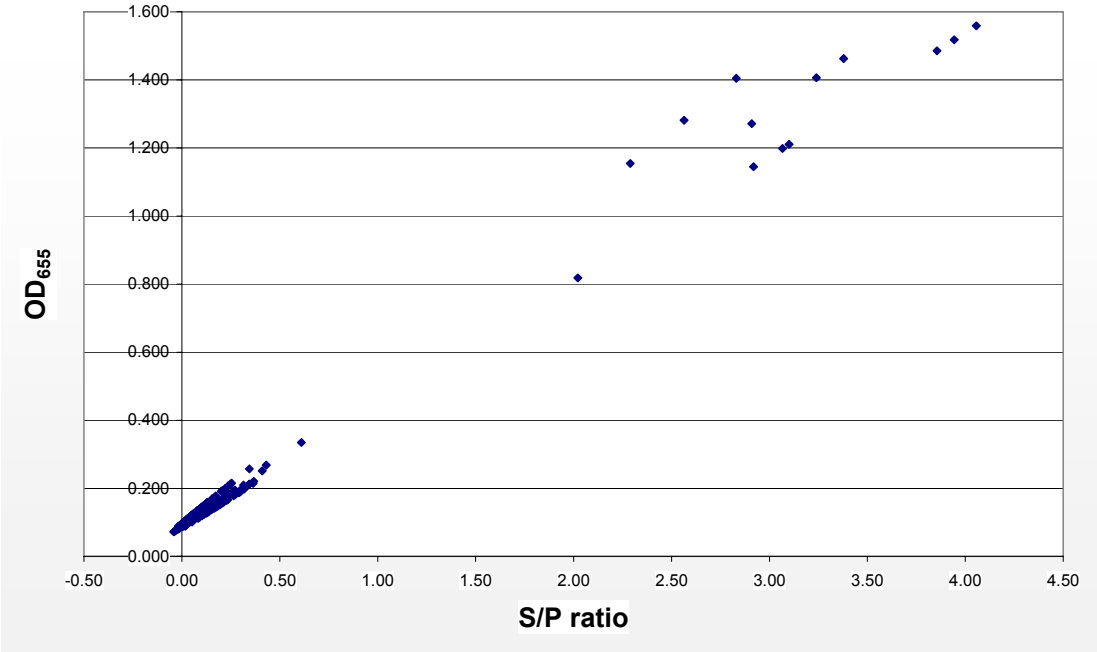
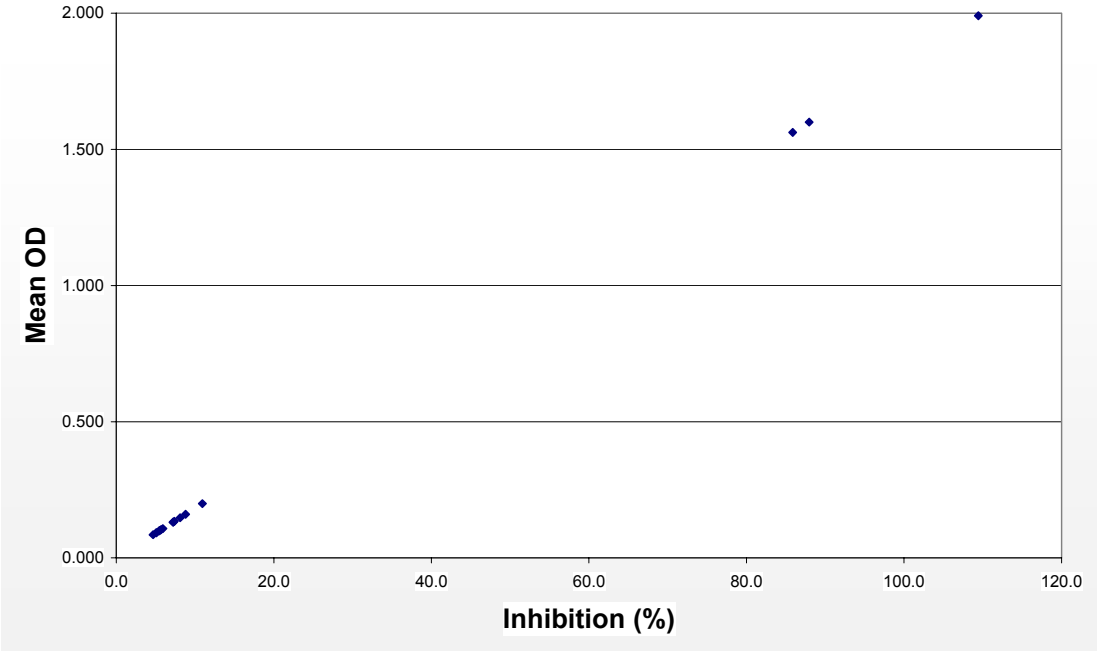


Figure 27 **Percentage inhibition and corresponding OD exhibited by 16 individual cattle sera tested in the *N. caninum* ELISA (Institut Pourquier)**



7.4 Discussion

This study demonstrates that infection with *N. caninum* can be controlled in a herd that has a low level of postnatal transmission. Only one recently infected cow was detected after 12 months while 12 previously known positive cattle remained positive. The one new infection was determined to have been infected postnatally as it was negative 12 months prior. If the 12 previously known positive cattle had also been culled then the prevalence of *N. caninum* infection would have been 0.5% (1/207) compared to 10.2% prevalence the previous year. This is the first time that control of *N. caninum* infection in a herd has been reported by using a test-and-cull strategy.

A high rate of vertical transmission of *N. caninum* had previously been detected in this herd with 74% (20/27) of the seropositive animals found to have family associations. The 20 positive cattle were found in six family trees. Evidence for vertical transmission is the familiar clustering of seropositive animals (Björkman *et al.* 1996, Schares *et al.* 1998, Dijkstra *et al.* 2001a)**.**

Six positive cows, which had descendents on the property at the time of sampling, had given birth to nine seropositive and one seronegative offspring. This equates to a vertical transmission rate of 90% (9/10) of *N. caninum* in this herd and again shows that this is the main mode of transmission. There was also a high vertical transmission rate of 75% (3/4) identified after serologically testing four calves and foetuses from *N. caninum* positive dams.

There was a low rate of postnatal transmission previously detected in this herd with 15% (4/27) of seropositive cattle born from seronegative cattle, which indicates that they were most probably infected postnatally. Three of the cows that were postnatally infected were born in the years from 1999-2001, which gives a mean rate of one postnatal infection per year. Postnatal infection of cattle may have occurred by ingesting oocysts (excreted by a dog, fox or other species), ingesting infected foetus or animal tissues, or by ingesting infected milk or colostrum. This low rate of postnatal transmission of *N. caninum* agrees with reports from UK (Davison *et al.* 1999a) and California (Paré *et al.* 1996, Thurmond and Hietala 1997a).

The method of culling or not breeding from seropositive cows in a herd where vertical transmission is the main route of infection was extremely effective in this study. Only one newly infected cow was detected and infection was probably postnatal. As there was a low rate of postnatal infection detected previously, the low rate was expected to continue. A rate of one new postnatal infection per year may have been expected considering that previously it had been found that there was a mean rate of one postnatal infection per year. Since there was a low prevalence of *N. caninum* infected animals in this herd it did not place too great a financial burden on the farmer when having to cull seropositive cows.

In herds where prevalence is low and vertical transmission is predominant, it would appear to be prudent to cease breeding from *N. caninum* seropositive animals. This would reduce the number of seropositive animals in the herd by blocking vertical transmission and also reduce further infection to dogs and foxes, as there would be less infected abortive material. These seropositive animals should be gradually culled as they cease milk production. Again, as there was a low prevalence of *N. caninum* infected animals in a herd it should not place too great a financial burden on the farmer. The postnatal infection rate could possibly be lowered further with a few proactive steps. Dogs and cattle should be kept away from aborted material and cattle carcases to avad them becoming infected. Dogs should also be kept away from cattle feed so as to not infect the cattle feed source. Foxes on the property are harder to control but some control measures may be practical. Calves should not be fed pooled milk unless it comes from known *N. caninum*–negative cows. With these steps it should be possible to limit the spread of *N. caninum* on a property.

8. Performance characteristics of two enzyme-linked immunosorbent assays for the detection of antibodies to *Neospora caninum* in the serum of cattle

8.1 Introduction

Neospora caninum is known to infect both dairy and beef cattle and has been described as a cause of bovine abortion in many countries around the world, including New Zealand and Australia (Reichel 2000). The ELISA has assumed an important, central role in the development of knowledge about the distribution, prevalence and importance of neosporosis. Several ELISA tests have now been described in the literature (Paré *et al.* 1995a, Björkman *et al.* 1997, Williams *et al.* 1997, Schares *et al.* 2000) and some are available commercially.

A recent study (Hall *et al.* 2005) showed that a commercially available ELISA was able to diagnose *N. caninum* infection with a high degree of sensitivity (Se) and specificity (Sp) and that, by using a test-and-cull strategy, it was possible to effectively eliminate *N. caninum* infection from a dairy herd. In the present study, the performance of this inhibition (or blocking) ELISA on serum samples was further analysed as was an indirect *N. caninum* ELISA. The performance characteristics of these two assays, including Se, Sp and cut-off thresholds are further described.

If test-and cull approaches are to be used on a larger scale, serological assays that lend themselves to quickly and efficiently testing large numbers of samples are required (*i.e.* for testing whole-herd bleeds). ELISA technology appears to fit this premise well, more so if these tests can be demonstrated to be reliable and shown to produce accurate and reproducible results. The correct diagnosis of cattle infected with *N. caninum* is also important when undertaking epidemiological studies and diagnosing abortion.

ELISAs are widely used in this process and so it is important that they are validated and their performance characteristics and limitations are known. A recent study comparing

11 ELISAs to detect *N. caninum* antibodies found that the IDEXX ELISA had excellent Se and Sp (100% and 99.7%, respectively) when the results of the majority of tests were used as the reference standard (von Blumröder *et al.* 2004).

When TG-ROC analyses were performed using the computer program CMDT (Free University Berlin, Germany), this also produced cut-offs similar to that of the manufacturer with high Se and Sp (99.6% and 98.1%, respectively). The IDEXX ELISA was shown to have good agreement with the IFAT. It has also been subject to comparisons with an 'in-house' ELISA and TG-ROC analysis in Australasia (Reichel and Pfeiffer 2002), which suggested high specificity (95%) at the manufacturer's suggested cut-off of 0.5 (SP%). The IDEXX ELISA is based on the ELISA by others (Paré *et al.* 1995a) who demonstrated that their ELISA was highly sensitive and specific when compared to the IFAT. Thus the IDEXX ELISA is an excellent choice as a reference standard test when comparing serological tests.

8.2 Materials and Methods

8.2.1 Herd sampling

The serum samples came from two dairy herds located in New South Wales, Australia. There were 266 samples from Herd 1 (discussed in Chapter 2) (Hall *et al.* 2005) and 260 samples were collected from a whole herd bleed undertaken on Herd 2 in February 2005. The latter herd had experienced a *N. caninum* abortion storm five years earlier (April – July 2000) during which approximately 70 foetuses were lost. In September 2001 the whole herd was blood sampled and the prevalence of *N. caninum* was 24% (53/224) (personal communication, P. Windsor).

8.2.2 Sera

Serum samples (n=526) were available from individual animals with naturally acquired *N. caninum* infections and from non-infected cattle.

The serum samples were assayed for anti-*N. caninum* antibodies using three commercially available ELISAs. Of the two ELISA manufactured by Institut Pourquier (Montpellier, France), one was a blocking ELISA (P00510/01) in its configuration, the other one an indirect ELISA (P00511/01). Samples and controls were assayed in duplicate for the blocking ELISA and singly for the indirect ELISA unless there was a discrepancy between qualitative results, in which case repeat analysis occurred. The third ELISA (5N05.00) was manufactured by IDEXX Laboratories (Westbrook, Maine, USA) and was used to determine the 'reference standard' status of the sera.

8.2.3 IDEXX ELISA

The *N. caninum* IDEXX ELISA was performed on all sera (in duplicate) according to the manufacturer's specifications (refer section 3.2.2) and the *N. caninum* infection status of each serum determined. Sera where the S/P was ≥ 0.5 was regarded as positive and those where the S/P was <0.5 were regarded as negative for *N. caninum* antibodies.

8.2.4 Indirect ELISA (Institut Pourquier)

This serum ELISA detects specific antibodies to *N. caninum*. The test procedure supplied by the manufacturer was adhered to. All reagents, plates and serum were brought to room temperature (RT) before testing. The test serum was diluted 1:20 with dilution buffer by first dispensing 190 µl of sample dilution buffer to each well then adding 10 µl of serum to the appropriate wells. Positive and negative control (10 µl) was also added to the appropriate wells of the *Neospora caninum* antigen coated plate. The plate was covered with adhesive and then incubated for 1 h at 21 °C. After incubation, the contents of the plate were discarded by flicking. The wells were washed three times with 300 µl of phosphate buffered wash solution. The contents of the wells were discarded after each wash. After the final wash, the plate was tapped on dry paper towel to remove any residual wash fluid. Anti-bovine IgG peroxidase conjugate (100 µl) was dispensed into each well and the plate was incubated for 30 min at 21 °C. The plate was washed again as previously described. After washing, 100 µl of TMB chromogen solution was dispensed into each well. The plate was then incubated away from the light for 20 min at RT. Stop Solution (100 µl of 0.5 M H_2SO_4) was added to each well and the OD was read at 450 nm on a Microplate Reader (Model 3550, Bio-Rad Laboratories). The serum to positive percentage (S/P%) was calculated for each sample as compared to the positive control by the following:

S/P % = ((mean sample OD) / (Pc OD)) x 100

where: Pc = positive control
OD = optical density

The manufacturer recommended that sera with a S/P% ≤30% should be regarded as negative, a S/P% of between 30-50% regarded as suspicious and S/P% ≥ 50% regarded as positive.

8.2.5 Blocking ELISA (Institut Pourquier)

The *N. caninum* blocking ELISA was also performed according to the manufacturer's instructions (refer section 2.2.2). The results for any sample were expressed as the degree of inhibition compared to the positive control expressed as a percentage (inhibition %). The manufacturer's cut-off recommendation for discrimination of negative from positive was given as a value where ≤45% inhibition was regarded as positive, ≥50% inhibition considered as negative and between 45-50% regarded as doubtful.

8.2.6 Analysis of serological data

The computer programme CMDT was used for the computation of the performance characteristics of the two Institut Pourquier ELISAs while using the qualitative results of the IDEXX ELISA as the reference standard. Characteristics, such as the Se, Sp and optimal cut-off thresholds were determined. Two graph-receiver operating characteristic analysis (Greiner 1995) was used to assess the diagnostic performance of the ELISA assays. This calculates and plots the effects of varying cut-off values on both Se and Sp. A cut-off value was derived from the TG-ROC plot, using parametric and non-parametric methods, as the S/P% (or inhibition)-value (d0) where Se and Sp were equal (theta0). The S/P percentages were $\log_{10}$-transformed before being analysed in the CMDT programme. The inhibition percentage results were firstly subtracted from 150 (to make them suitable for CMDT-analysis) before they were analysed in the CMDT programme. Frequency distribution histograms were also produced using CMDT.

8.3 Results

8.3.1 IDEXX serum sample results

Of the 526 sera assayed in this evaluation, 56 were positive in the IDEXX ELISA and 470 were classified as negative.

8.3.2 Optimising the performance characteristics of the Institut Pourquier indirect *N. caninum* ELISA

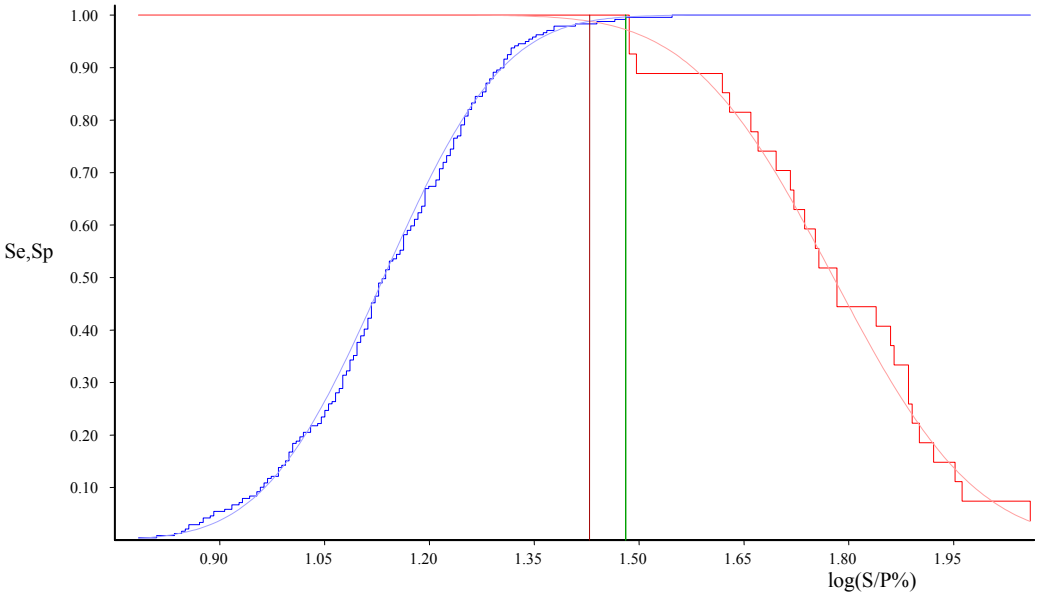
After $\log_{10}$-transformation, the results of the indirect ELISA were subjected to TG-ROC analysis, initially for the two herds separately and then as a combined dataset. For the analysis of Herd 1, non-parametric methods suggested a cut-off threshold of 30.3% (S/P%) whereas the parametric method suggested a S/P ratio of 26.9% as an appropriate threshold (Figure 28a). For Herd 2, non-parametric methods suggested a cut-off threshold of 26.5%, and the parametric method suggested a S/P ratio of 28.8% as the appropriate threshold (Figure 28b).

For the combined data set, non-parametric methods suggested a cut-off threshold of 26.9%, whereas the parametric method suggested 27.8% as an appropriate threshold. The estimated Se and Sp at those points were (theta0) 98.3% and 98.4%, respectively (Figure 28c). The frequency distribution of the log-transformed S/P ratios showed good discrimination around the cut-off threshold (Figure 29).

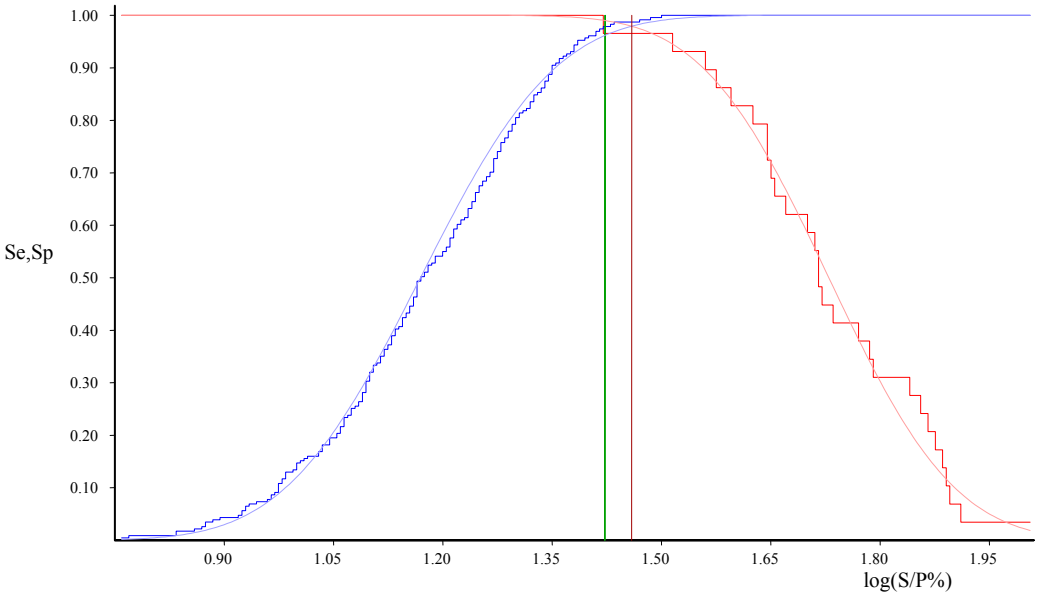
Figure 28 **TG-ROC analysis of bovine sera assayed in the Institut Pourquier indirect *N. caninum* ELISA**

(Se, red lines; Sp, blue lines; green = non-parametric threshold value, brown = parametric threshold value).

(a) **Herd 1 (n=266)**



(b) **Herd 2 (n=260)**



(c) **Combined herds (n=526)**

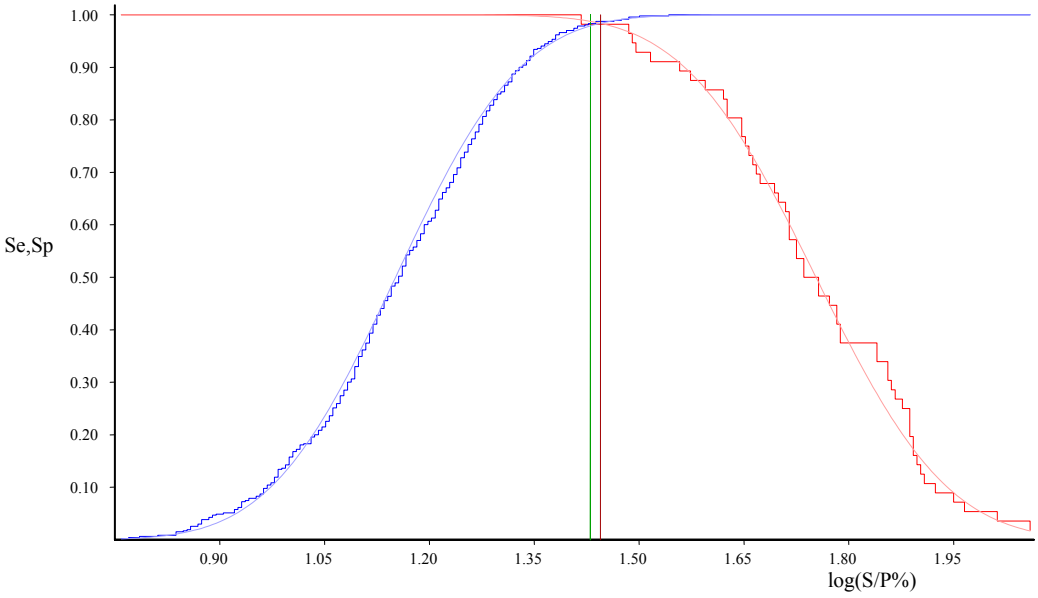
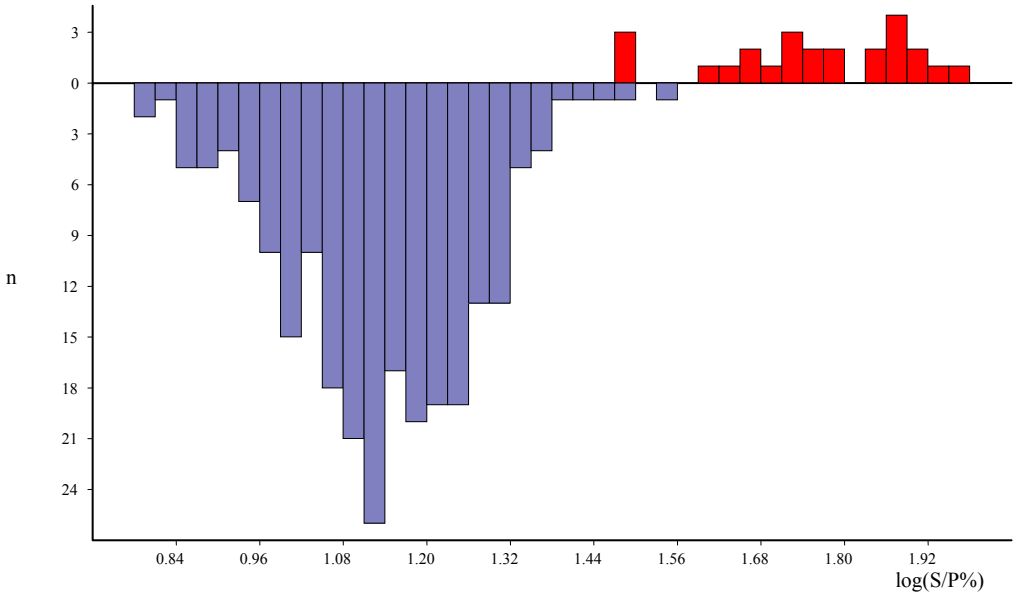


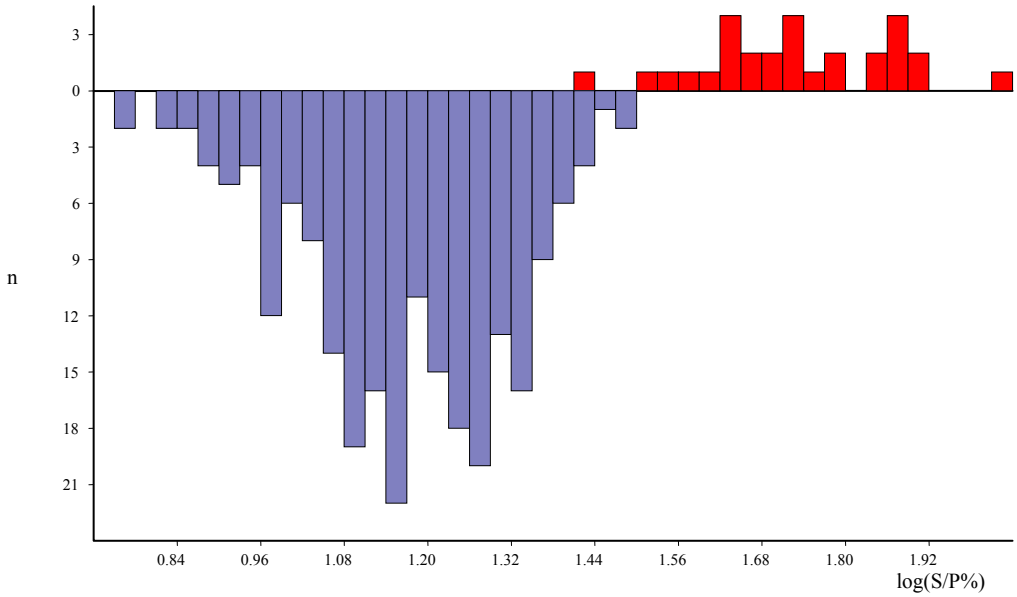
Figure 29 **Frequency distribution of bovine sera assayed in the Institut Pourquier indirect *N. caninum* ELISA**

(Blue bars = negative sera; Red bars = positive sera).

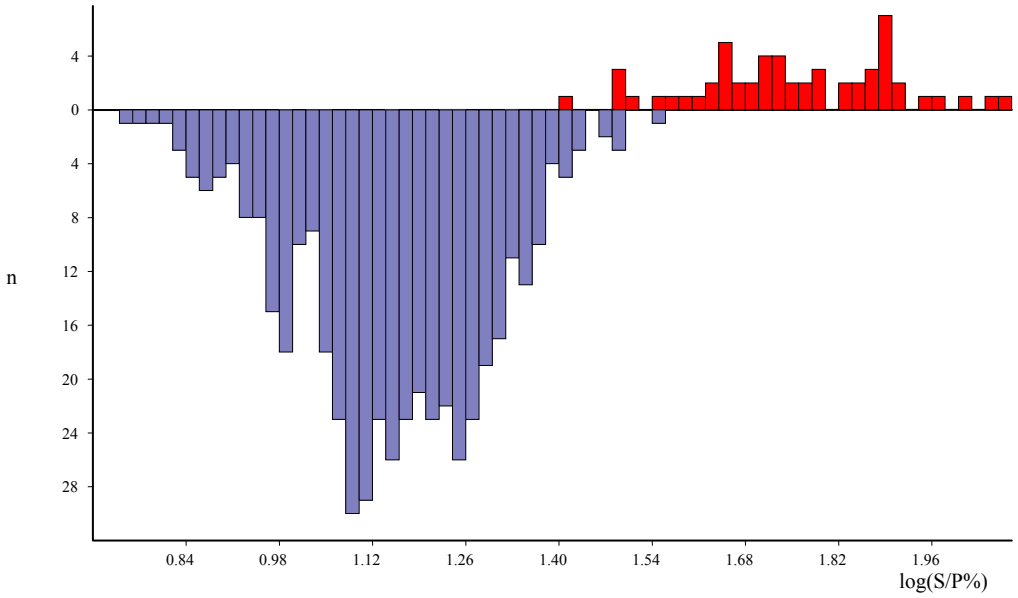
(a) **Herd 1 (n=266)**



(b) : Herd 2 (n=260)



(c): Combined herds (n=526)



8.3.3 Performance characteristics of the Institut Pourquier *N. caninum* blocking ELISA

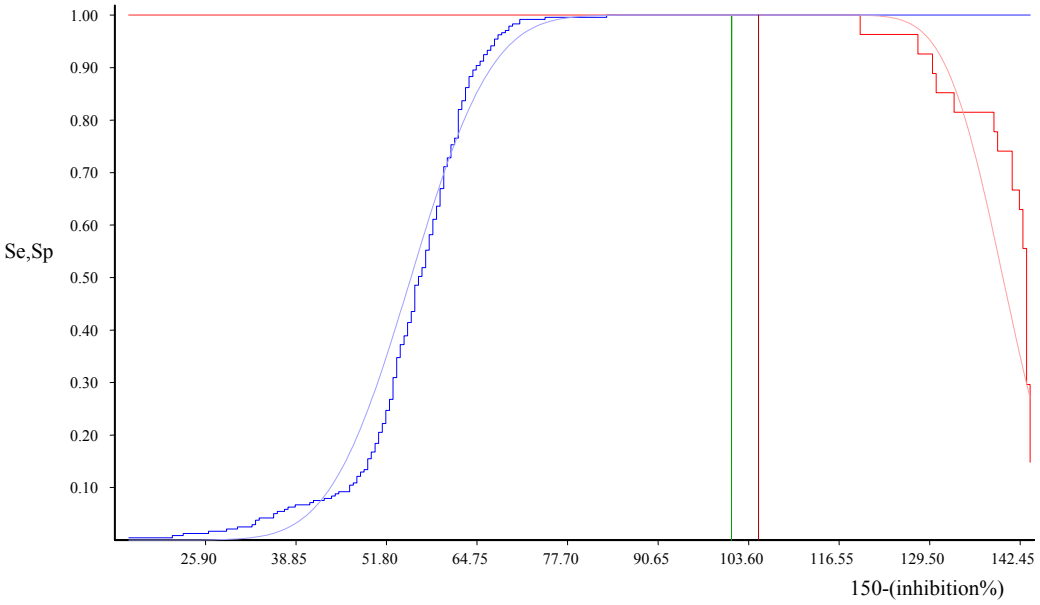
After subtracting the inhibition percentages from 150 (150-inhibition %) to make them suitable for analysis by CMDT, the serum results of the blocking ELISA were subjected to TG-ROC analysis as individual herds and then as combined herds. For the separate analysis of Herd 1, non-parametric methods suggested a cut-off threshold of an inhibition % of 48.8%, whereas the parametric method suggested an inhibition % of 45.0% (Figure 30a). For Herd 2, TG-ROC analysis of 258 sera (two excluded as outliers because of discordant results in the other two ELISA's) suggested a cut-off threshold of 46.4% inhibition using non-parametric methods while the parametric method suggested an inhibition % of 48.9% as an appropriate threshold (Figure 30b).

For the combined data set (two excluded as outliers), non-parametric methods suggested a cut-off threshold of an inhibition % of 46.4%, whereas the parametric method suggested an inhibition % of 47.5% The estimated Se and Sp at those points were (theta0) 100% and 99.9%, respectively (Figure 30c). The frequency distribution of the transformed inhibition percentages showed very good discrimination around the cut-off threshold (Figure 31).

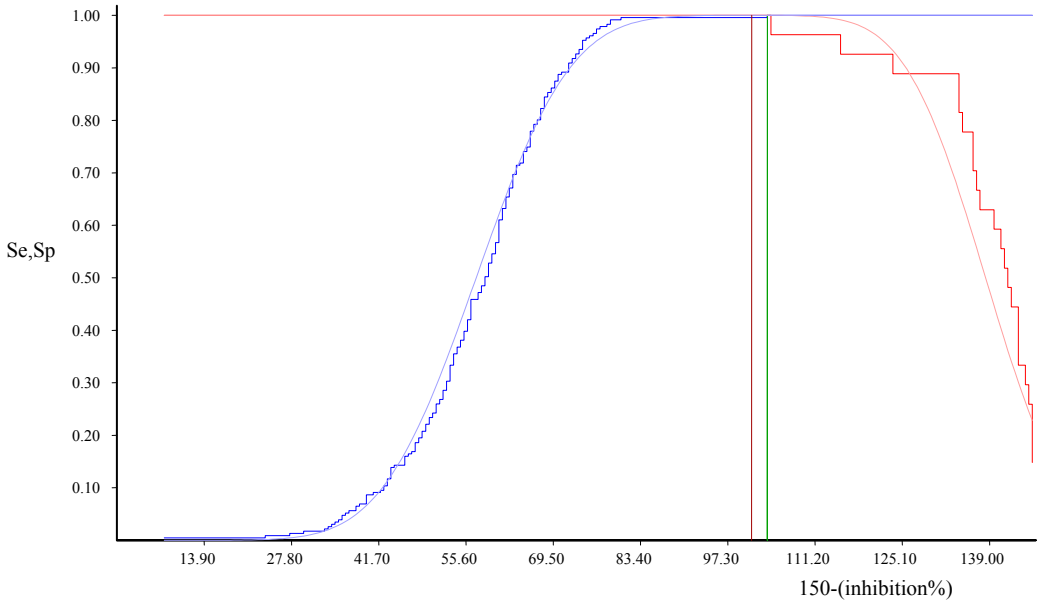
Figure 30 **TG-ROC analysis of bovine sera assayed in the Institut Pourquier blocking *N. caninum* ELISA**

(Se, red lines; Sp, blue lines; green = non-parametric threshold value, brown = parametric threshold value).

(a) **Herd 1 (n=266)**



(b) **Herd 2 (n=260)**



(c) **Combined Herds (n-524)**

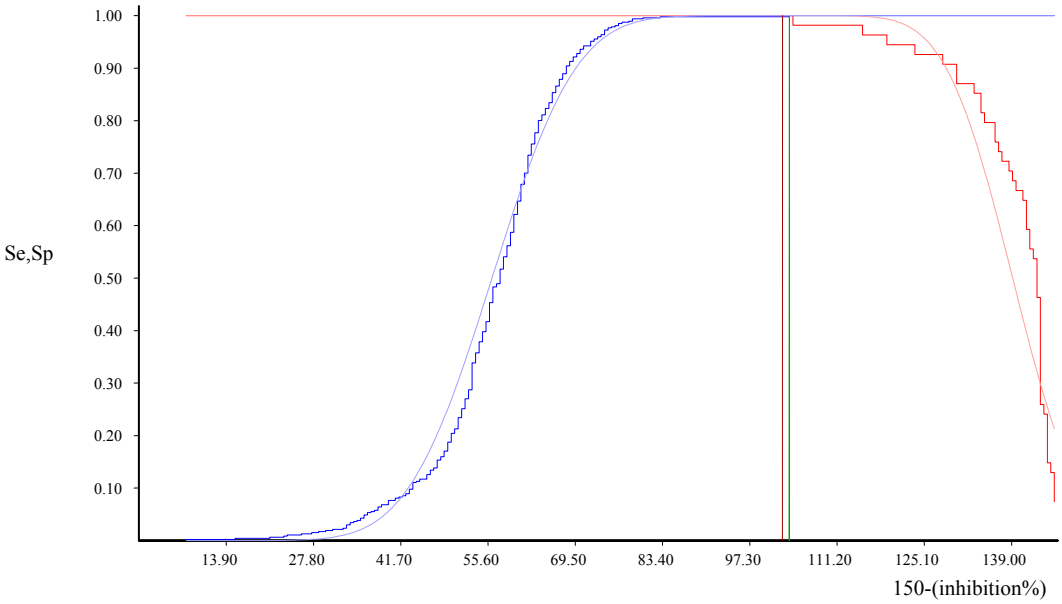
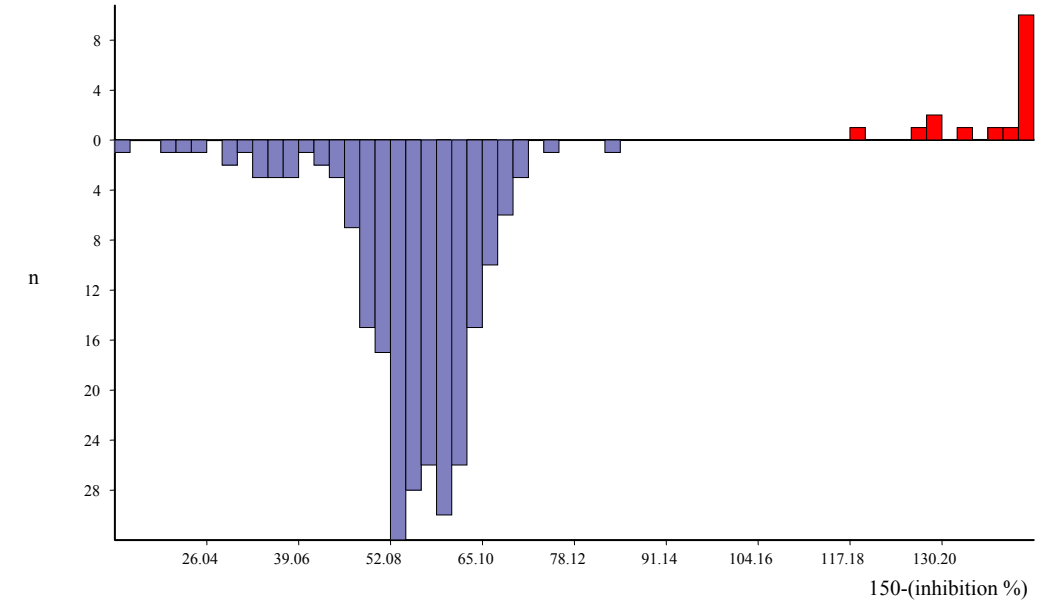


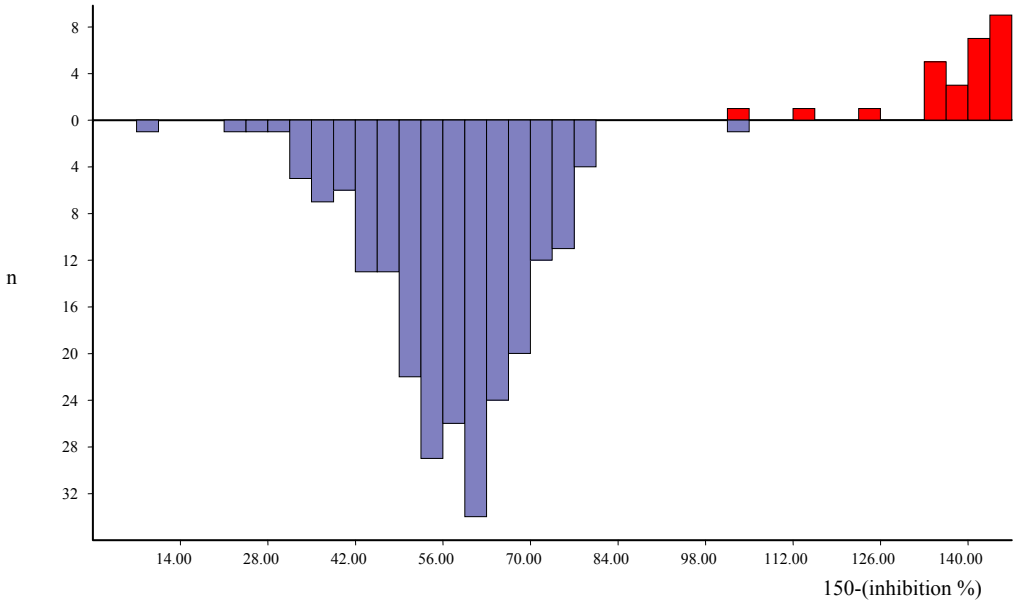
Figure 31 **Frequency distribution of bovine sera assayed in the Institut Pourquier blocking *N. caninum* ELISA**

(Blue bars = negative sera; Red bars = positive sera).

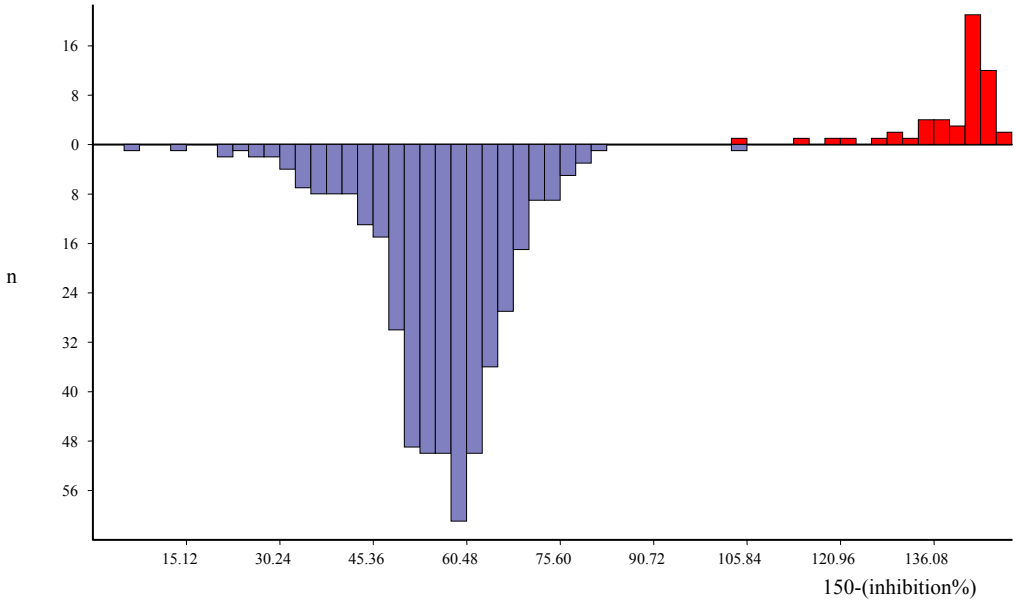
(a) **Herd 1 (n=266)**



(b) **Herd 2 (n=260)**



(c) **Combined herds (n=524)**



8.4 Discussion

Two commercially available ELISAs for the detection of antibodies to *N. caninum* were evaluated in the current study for their performance on the sera of dairy cattle. In a previous study (Hall *et al.* 2005) one of the assays, the blocking ELISA, was used in a test-and cull approach for *N. caninum* eradication in cattle. In the present study the aim was to further optimise the cut-off thresholds by TG-ROC analysis (Greiner 1996). This would allow further understanding of the assay characteristics thereby allowing rational decisions to be made on the infection status of the animals tested.

The TG-ROC analysis, in the case of the blocking ELISA, arrived at cut-off thresholds (46% and 48%) that were very close to the threshold suggested by the manufacturer (45%), and thus provides additional confidence in this value. In fact, these values lie between the doubtful range suggested by the manufacturer (*i.e.* 45-50%). The very good discrimination of negative and positive populations, as identified by the frequency distribution of the inhibition values, as well as the large range of values over which both Se and Sp remain above 99% (Figure 31) suggest that this ELISA is well suited for the accurate identification of *N. caninum*-infected and non-infected cattle. However it should be noted that two outliers were excluded from the analysis. These two samples were positive in both the other ELISAs (IDEXX and indirect Institut Pourquier). In addition, the configuration of this ELISA as an inhibition assay suggests that it might also be able to be used on serum samples of other species that are affected by *N. caninum* infection, such as dogs (Barber *et al.* 1997) or sheep (Dubey *et al.* 1990b). Further validation of the assay in those species, however, has not yet been reported.

The TG-ROC analysis of the thresholds for the indirect *N. caninum* ELISA arrived at cut-off values that were considerably lower (at 27-28%) than those suggested by the manufacturer. Thresholds derived from the TG-ROC analysis were very similar in both herds; hence it seemed justified to combine the sera from both herds for a combined analysis. As they were close, but still lower than the suspicious range suggested by the manufacturer they suggest that the suspicious range classification could be abolished. The discrimination between negative and positive samples can accurately be achieved at

a cut-off value of around 25%, with good discrimination between positive and negative populations, as observed in the frequency distribution of sample results (Figure 29c).

As the taking of serum samples is time-consuming and often expensive, further work should be directed at evaluating the utility of ELISAs for the detection of antibodies to *N. caninum* in milk, as was demonstrated with a number of ELISA assays for other diseases (Beaudeau *et al.* 2001, Reichel *et al.* 2005). While this type of approach would be limited to cows in milk, it would simplify the procedure considerably, provided that accurate diagnosis of the *N. caninum* infection status is possible through that medium.

9. Validation and performance characteristics of an enzyme-linked immunosorbent assay for the detection of antibodies to *Neospora caninum* in the milk of cattle and subsequent application to determine prevalence of infection in NSW dairy cattle

9.1 Introduction

Neospora caninum is well known as a cause of bovine abortion in many countries around the world. It has also been described in New Zealand and Australia (Reichel 2000) where it often causes abortions of epidemic ("storm"-like) proportions (Thornton *et al.* 1994, Atkinson *et al.* 2000b). Diagnosis in these cases has been based largely on the serological testing of all or parts of the affected dairy herds. However, for many other infectious diseases of economic importance in dairy cattle, available serological assays have also been adapted to use on milk samples, such as in the case of testing for liver fluke (*Fasciola hepatica*) (Reichel *et al.* 2005) or bovine viral diarrhoea virus (Beaudeau *et al.* 2001), and others. A similar adaptation of a serum-based ELISA for testing milk samples is described here as a further step towards control of *N. caninum*.

In the present study, the performance of an indirect ELISA (IP) on milk samples was analysed, and the results compared with an indirect *N. caninum* ELISA (IDEXX) on corresponding sera. From this the performance characteristics of the milk assay, including cut-off thresholds and Se and Sp were derived. Test-and-cull programs have previously been described (Hall *et al.* 2005) (also refer to Chapter 7) and this ELISA to detect antibodies to *N. caninum* in milk may also be useful in such control programs. Milk samples are cheaper and easier to collect than blood samples and this may lower the barrier for whole herd testing for *N. caninum.* It would enable farmers to collect samples rather than an experienced person required for blood collection.

When validating a new ELISA an appropriate reference standard test is often required for comparison. In the current study, the IDEXX ELISA was used as the "reference standard" test and evidence of its suitability has been reviewed previously in Chapter 8.

9.2 Materials and Methods

9.2.1 Sampling

The serum samples came from three dairy herds (Herd 1, 2 and 3) located in New South Wales, Australia. Herd 1 (Hall *et al.* 2005) and Herd 2 were the subject of *N. caninum* investigations that involved whole herd bleeds (refer to Chapter 8). Only a few samples were sourced from Herd 3. A total of 93 corresponding individual milk samples were collected from both seropositive and seronegative cows.

9.2.2 IDEXX ELISA

The serum samples were assayed in duplicate for anti-*Neospora* antibodies using a commercially available indirect ELISA (5N05.00) (IDEXX Laboratories, Westbrook, Maine, USA). The results of this ELISA were used as the "reference standard" to determine the *N. caninum* infection status of the cows.

9.2.3 Indirect Institut Pourquier (IP) ELISA

9.2.3.1 Determination of optimal dilution

A subset of 55 milks from cows with established *N. caninum* infection status (see 9.2.2) were assayed in the indirect IP ELISA (P00511/01) with slight modification. Milk samples were assayed undiluted (neat), 1/2 and 1/3 in dilution buffer supplied with the ELISA kit and the rest of the assay was run as suggested by the manufacturer (refer section 8.2.4). Positive and negative control sera delivered with the ELISA kit were used as controls in the milk ELISA. Data were analysed to determine the optimal dilution of milk for this ELISA.

9.2.3.2 Optimisation of cut-off values

Milk samples (n=93) from dairy cows for which the infection status had been determined in the IDEXX ELISA (see 9.2.2) were assayed in the IP indirect *N. caninum*

ELISA. The milks were tested at the optimal dilution as determined in 9.2.3.1. To correct for plate-to-plate variation, results were expressed as the ratio of the mean absorbance values of the Sample (S) to the mean absorbance value of the Positive (P) control sample provided with the diagnostic kit. The resultant S to P ratio (S/P%) was expressed as a percentage. The manufacturer recommended that sera with S/P% ≤30% be regarded as negative, S/P% of between 30-50% regarded as suspicious and S/P% ≥ 50% regarded as positive sera. However, there was no specific cut-off threshold set for milk samples.

9.2.4 Analysis of serological data

The computer program CMDT was used for the computation of the performance characteristics of the IP *N. caninum* ELISA when applied to milk samples. Characteristics, such as Se, Sp and the optimal cut-off threshold were determined. Two graph-receiver operating characteristic analysis (Greiner 1995) was used to assess the diagnostic performance of the ELISA. This calculates and plots the effects of varying cut-off values on both Se and Sp. A cut-off value was derived from the TG-ROC plot, using parametric and non-parametric methods, as the S/P%-value (d0) where Se and Sp were equal (theta0).

9.2.5 Milk prevalence survey

Milk samples (n=398) from individual cows were obtained at random from 203 dairy farms across NSW. Two samples were collected from 195 properties and in another eight cases only one sample was tested as the other had curdled. The majority of the samples were collected by regular herd samplers at the time of herd recording. The samples were collected with no preservative, stored at 4 °C overnight and then sent express to a laboratory where they were stored at -20 °C until tested. Samples were centrifuged at 1000 *g*, skim milk collected and assayed as per the optimal ELISA conditions determined in step 9.2.3.1., applying the cut-off value obtained in step 9.2.3.2.. The results were displayed as a frequency distribution of S/P% values and prevalence by shire. A map displaying the distribution of positive and negative farms was also produced to allow comparison with the main dairying areas. Farms where one

or both of the samples were positive were indicated by a red dot and those where both samples were negative were indicated by a blue dot.

9.3 Results

9.3.1 IDEXX serum sample results

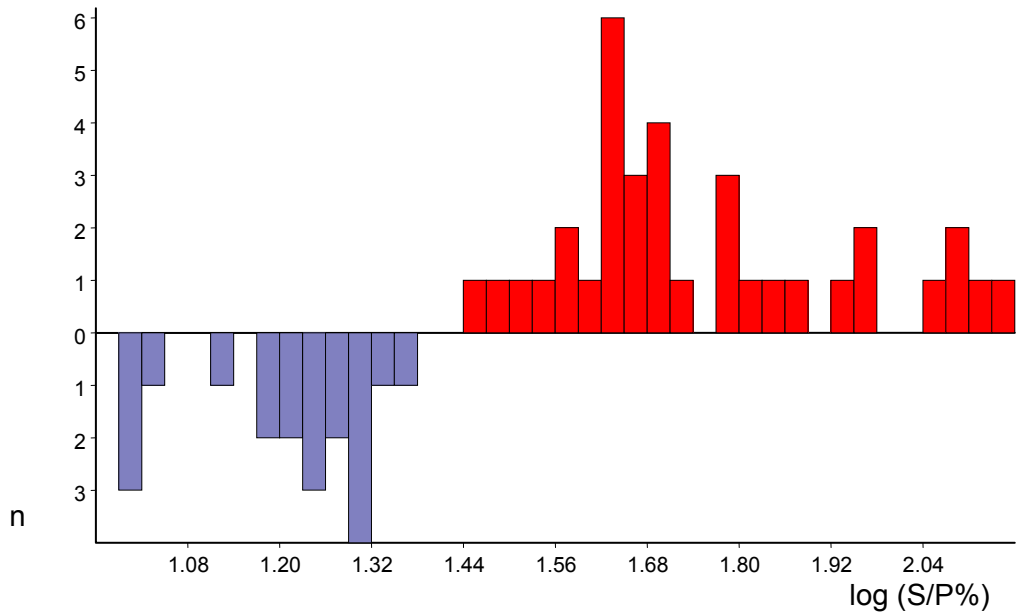
Of the total of 93 sera assayed, 36 were positive in the IDEXX ELISA and 57 were classified as negative.

9.3.2 Optimal dilution of the IP indirect *N. caninum* ELISA

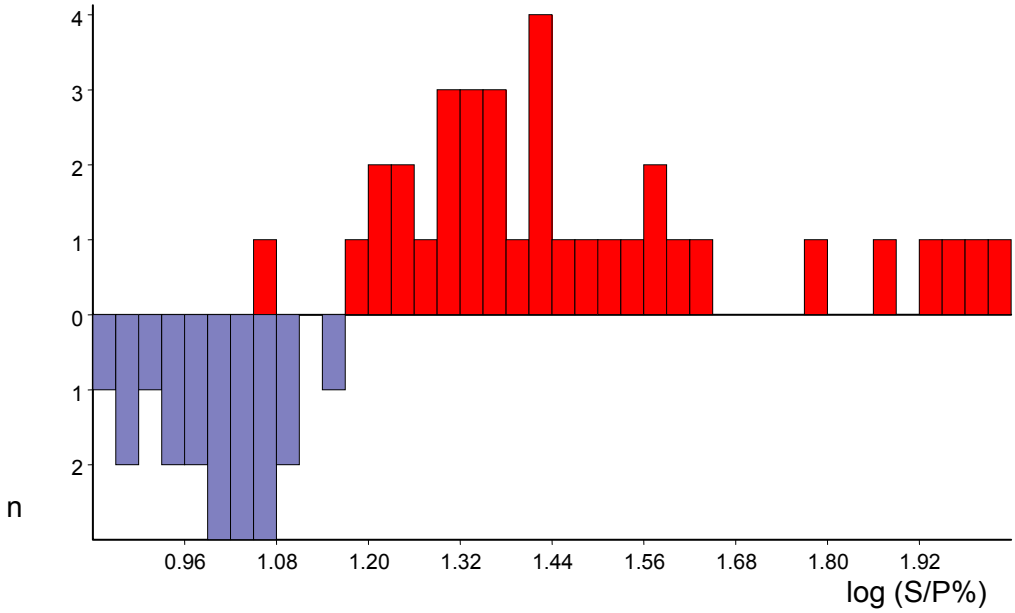
A subset of 55 milks were diluted to find the optimal dilution compared to the serum results. The milks were diluted neat, 1/2 and 1/3. The frequency distributions (Figure 32) show a greater separation between positive and negatives when the milk was undiluted than when it was diluted 1/2 or 1/3. The undiluted milk also showed a higher exponential correlation (R^2=0.686) with serum results than the 1/2 dilution (R^2=0.550) and the 1/3 dilution (R^2=0.481)(Figure 33). Thus the milk was used undiluted for further testing of all samples.

Figure 32 **Frequency distribution of milk samples (n=55) assayed in the IP indirect *N. caninum* ELISA:**
(a) undiluted (neat), (b) diluted 1/2 and (c) diluted 1/3.

(a)



(b)


4
3
2
1
0
1
2
n
0.96
1.08
1.20
1.32
1.44
1.56
1.68
1.80
1.92
log (S/P%)

(c)

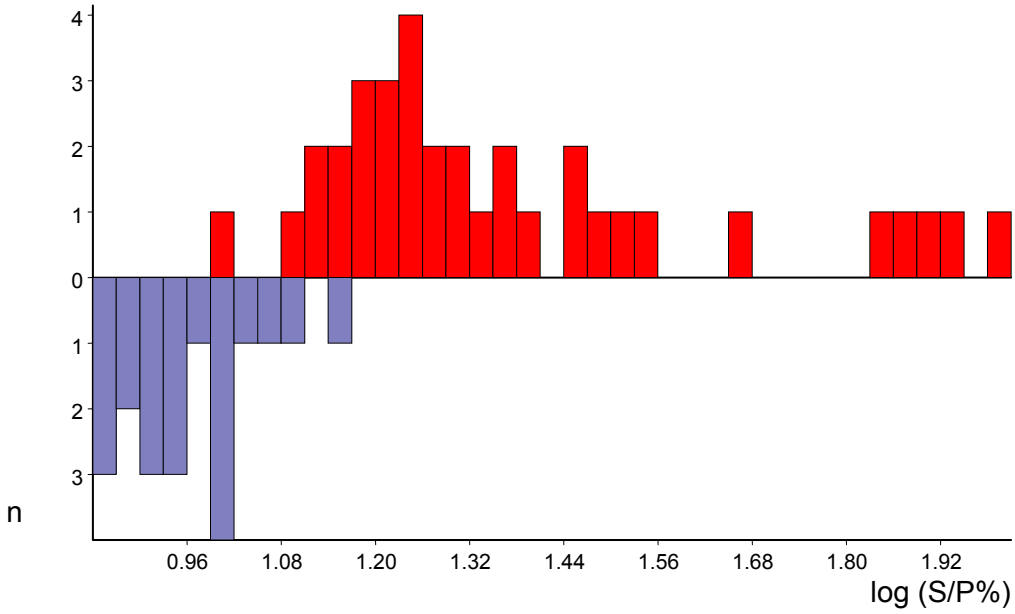
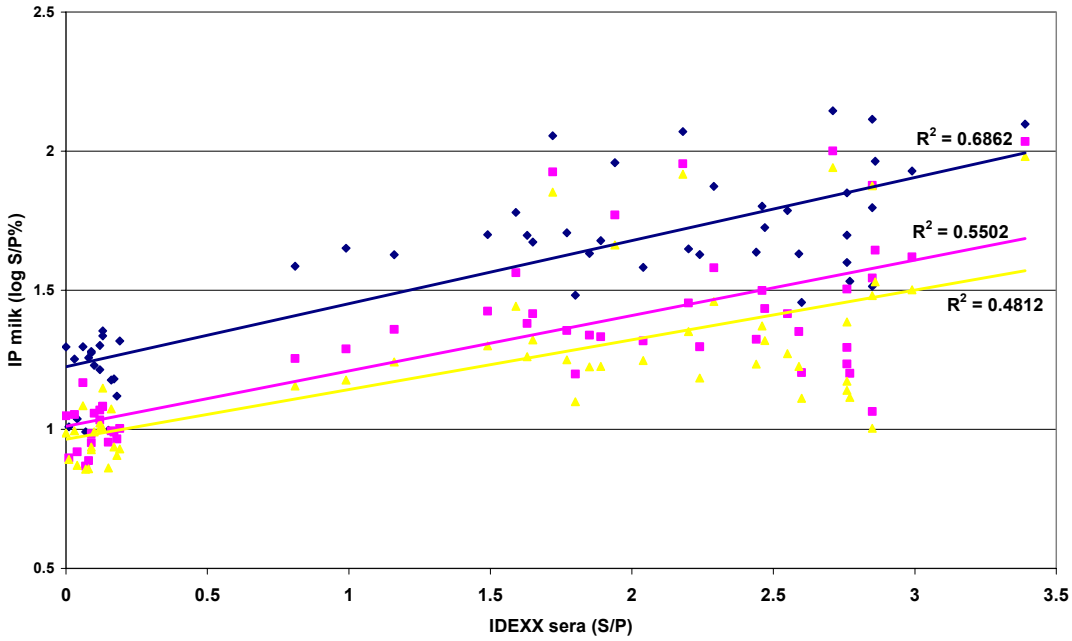

4
3
2
1
0
1
2
3
n
0.96
1.08
1.20
1.32
1.44
1.56
1.68
1.80
1.92
log (S/P%)

Figure 33 Regression of milk samples from 55 individual cows



Milk diluted neat, 1/2 and 1/3 denoted by diamonds, squares and triangles respectively.

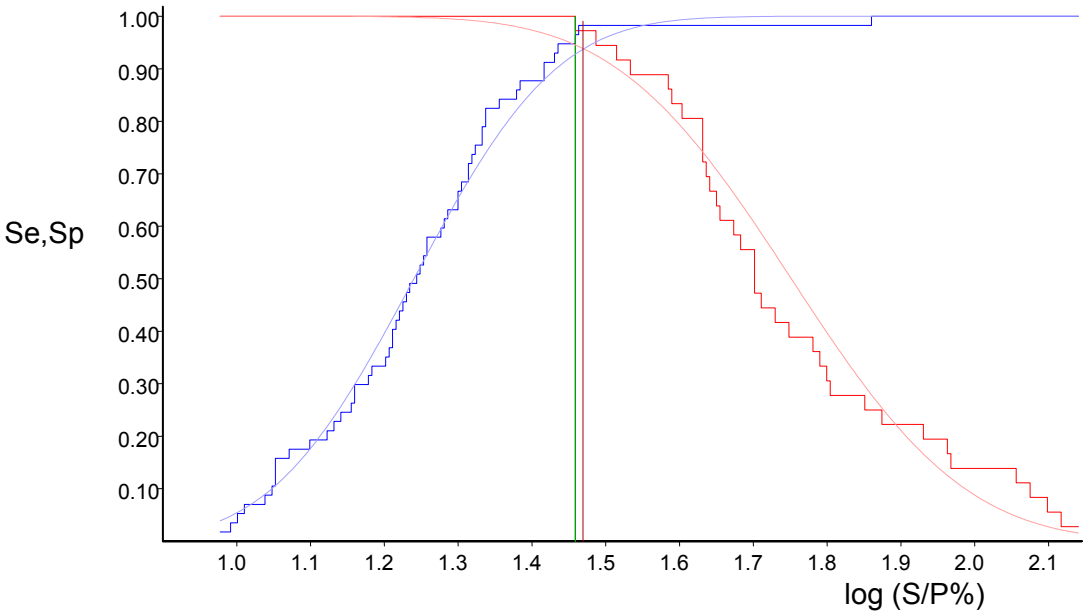
9.3.3 Optimising the performance characteristics of the IP indirect *N. caninum* ELISA

After $\log_{10}$-transformation, the 93 results of the indirect IP ELISA for the corresponding neat milk samples were subjected to TG-ROC analysis in CMDT. Non-parametric methods suggested a cut-off threshold of an S/P ratio of 28.8%, whereas the parametric method suggested 29.5%. The estimated Se and Sp at those points were theta0 = 96.9%, and 93.8%, respectively (Figure 34a). The frequency distribution of the log-transformed S/P% ratios showed good discrimination around the cut-off threshold (Figure 34b).

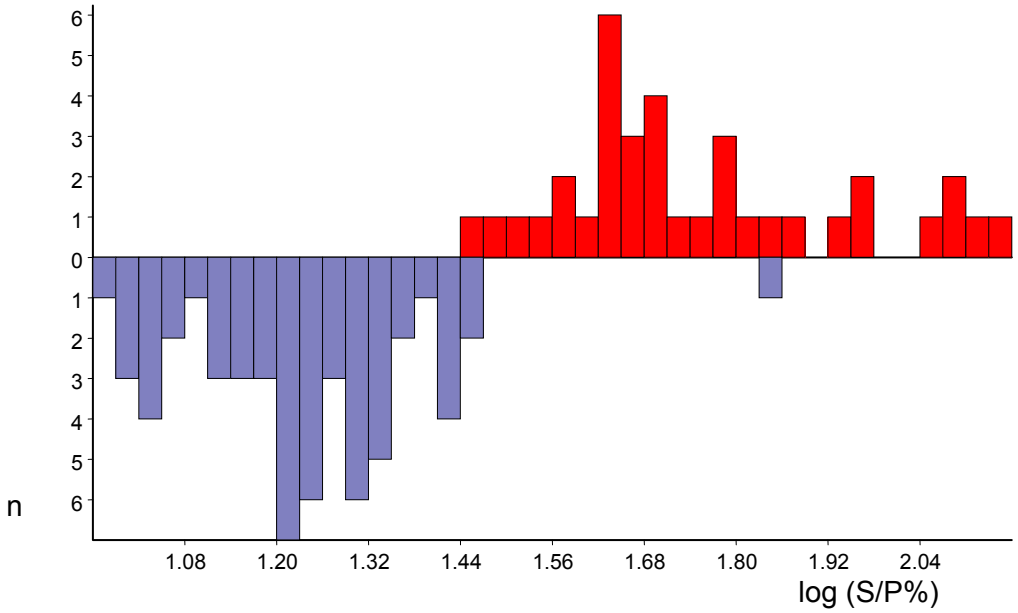
Figure 34 **Results of 93 bovine milk samples, assayed in the Institut Pourquier indirect *N. caninum* ELISA:**

(a) TG-ROC analysis (Se, red lines; Sp, blue lines; green = non-parametric threshold value, brown = parametric threshold value), (b) Frequency distribution (blue bars = negative sera; red bars = positive sera)

(a)



(b)



9.3.4 Milk prevalence survey

The 398 bovine milk samples, randomly obtained from 203 dairy farms across NSW were assayed under the optimised conditions in the indirect IP *N. caninum* ELISA. In the majority (96%) of cases, two samples were collected from each property. The frequency distribution of the NSW milk samples shows what appears to be a relatively normal population distribution (Figure 35). Milk prevalence ranged from 0% to 100% per shire with a median shire prevalence of 17%. The statewide prevalence was 21.1% (Table 16). Of 198 farms there were 69 (34.8%) where there was at least one *N. caninum* positive cow. The distribution of the positive and negative farms (Figure 36a) shows that *N. caninum* was found in most areas. The areas of sampling compare well with the areas of highest dairy cow density in NSW (Figure 36b).

Figure 35 Frequency distribution of NSW milk samples (n=398) assayed in the IP indirect N. caninum ELISA

(blue bars = negative sera; red bars = positive sera)

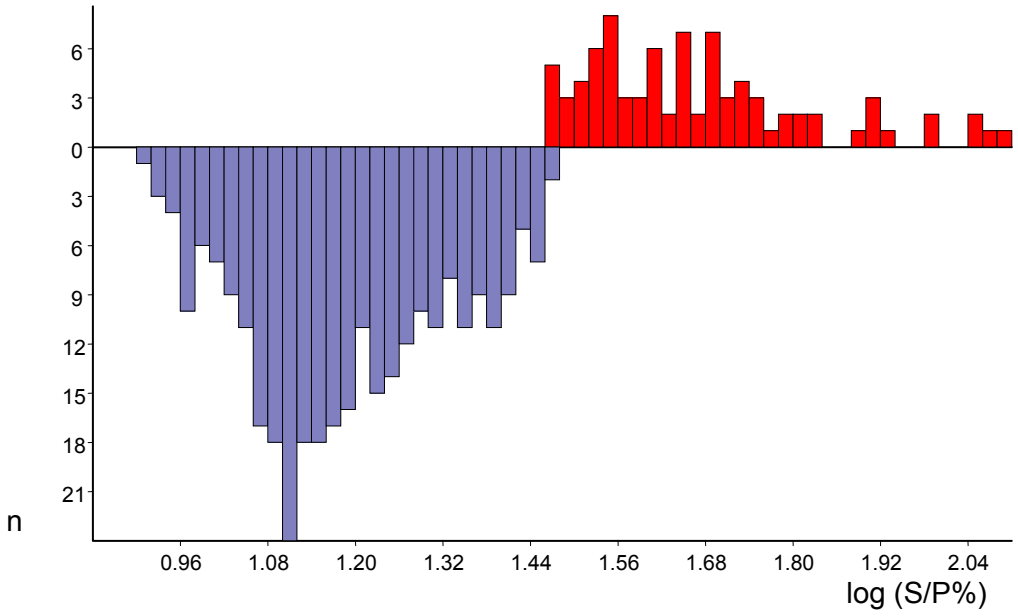
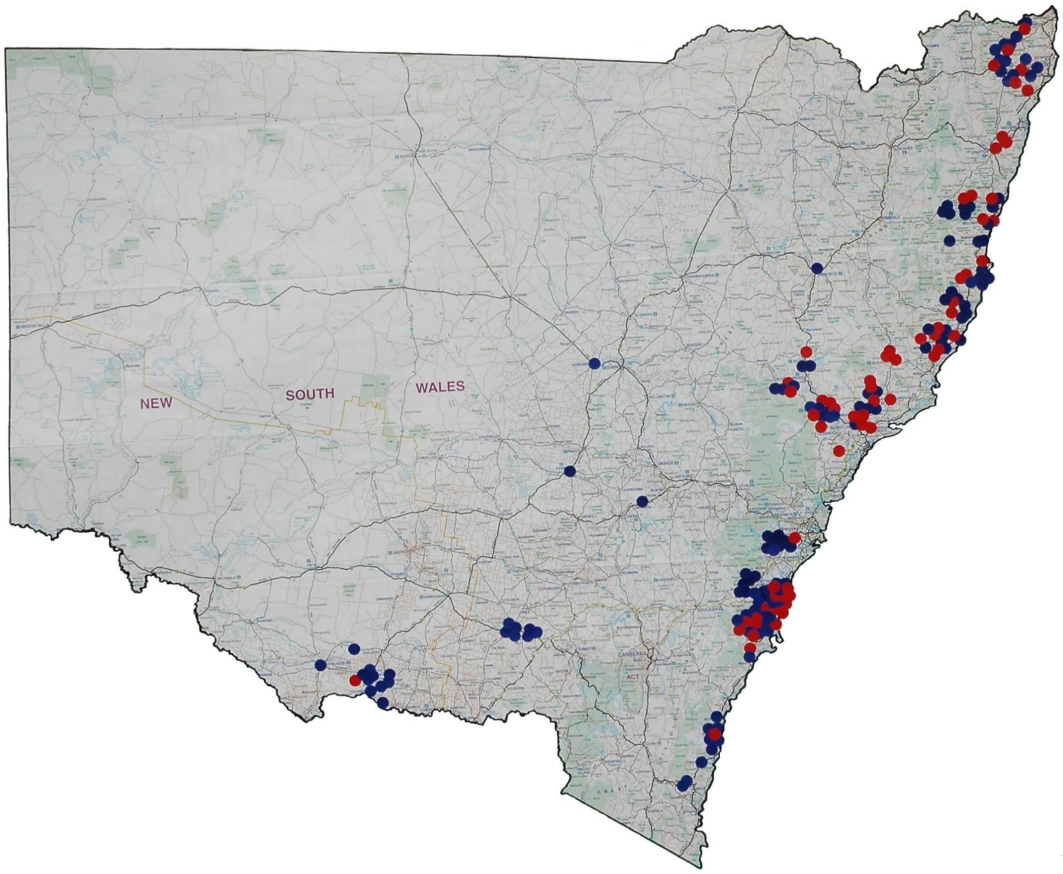


Table 16 Prevalence of *N. caninum* antibodies in milk samples from 398 dairy cows in NSW (in 44 Shires)

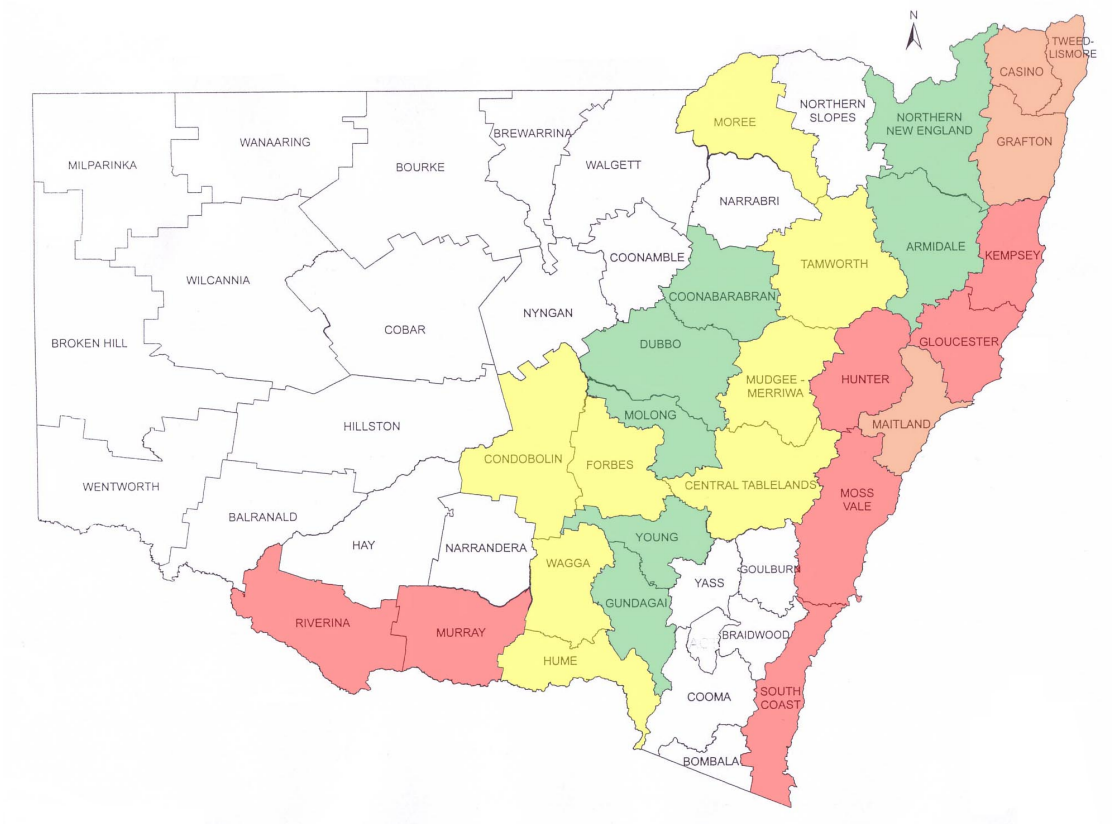
Shire	Positive samples (n)	Total samples (n)	Prevalence (%)
Shoalhaven	12	48	25
Manning	6	28	21
Singleton	8	27	30
Bellingen	5	22	23
Dungog	6	20	30
Hastings	4	20	20
Berrigan	0	18	0
Richmond River	5	16	31
Kempsey	3	16	19
Kiama	6	14	43
Eurobodalla	1	14	7
Denman	2	10	20
Mittagong	0	10	0
Wingecarribee	0	10	0
Port Stephens	5	8	63
Wollondilly	0	8	0
Kyeamba	0	8	0
Conargo	1	7	14
Camden	0	7	0
Coffs Harbour	2	6	33
Kyogle	1	6	17
Lismoe	1	6	17
Tweed	1	6	17
Scone	1	6	17
Numbulla	0	6	0
Mitchell	0	6	0
Gloucester	3	5	60
Ulmarra	3	4	75
Wollongong	1	4	25
Casino	0	4	0
Nambucca	0	4	0
Shellharbour	2	2	100
Nymboida	1	2	50
Great Lakes	1	2	50
Lake Macquarie	1	2	50
Maitland	1	2	50
Campbelltown	1	2	50
Parry	0	2	0
Forbes	0	2	0
Talbragar	0	2	0
Waugoola	0	2	0
Mulwaree	0	2	0
Condobolin	0	1	0
Jemalong	0	1	0
TOTAL	**84**	**398**	**21.1**

Figure 36 Distribution of *N. caninum* positive and negative cows in NSW



Each dot represents a sampled farm, red indicates that at least one sample was positive, blue indicates both samples were negative

Figure 37 Distribution of dairy cows in NSW



Areas defined by the Rural Lands Protection Board. Red indicates 20,000-50,000, orange indicates 8,000-20,000, yellow indicates 4,000-8000, green indicates 1,000-4000 and white indicates less than 1,000 head of dairy cattle).

Modified from source: State Council of Rural Lands Protection Boards 2005 Annual Report (Anon. 2005b).

9.4 Discussion

As the taking of serum samples is time-consuming and often expensive, an ELISA for the detection of antibodies to *N. caninum* in milk would be a useful tool. The utility of testing milk has been demonstrated with a variety of ELISAs for other diseases (Beaudeau *et al.* 2001, Reichel *et al.* 2005) including the IDEXX *N. caninum* ELISA (Schares *et al.* 2004). One limitation of milk ELISAs is that only samples from lactating cows can be tested and thus dry cows or calves need to tested by other methods (*e.g.* serum testing). In the current study, milk was determined to be a useful alternative to serum when attempting to detect antibodies to *N. caninum* using the indirect Institut Pourquier ELISA. This ELISA may be a useful tool in diagnosing *N. caninum* abortion, assessing in-herd prevalence, for use in possible test-and cull programs and subsequent monitoring. It may also be used in future NSW surveillance programs and possibly in other states of Australia and further a field.

In the current study the greatest discrimination between positive and negative milks was found with the undiluted milk and this also had the highest correlation with the IDEXX ELISA. After modifying the procedure of the indirect IP ELISA to allow testing of neat bovine milk samples, the test was found to have high sensitivity and specificity (97%, non-parametric methods) at a cut-off threshold of 29% (S/P%). Non-parametric assessment seems the most suitable in light of the low numbers of positive samples involved. Using this IP ELISA, the cut-off determined in this study for the testing of undiluted milk samples was similar to that previously determined (Chapter 8) for serum samples diluted 1/20 (as recommended by the manufacturer).

Bulk milk ELISAs are also useful as one sample may be collected to give information about the herd prevalence. Bovine viral diarrhoea virus is also a cause of abortion in cattle and bulk milk ELISAs have been developed and in use to control this disease (Lindberg and Alenius 1999). Bulk milk ELISAs have also been reported for *N. caninum* but in comparison to individual serum or milks none perform as well at present. One bulk milk ELISA is reported to be able to detect as low as 10-15% within herd prevalence (Björkman and Lunden 1998, Uggla *et al.* 2000).

Although in the present study this ELISA is accurate in diagnosing infection from individual milks, it seems to be of little use for bulk milk testing as even at the 1/3 dilution there was not clear delineation between positive and negative samples. Further TG-ROC analysis was performed on these 1/3 diluted samples (data not shown) which produced a cut-off of 13% (S/P%) but with less than 95% (94.6) Se and Sp. As a 1/3 dilution is comparable to a herd prevalence of 33%, this effectively means that only herds with prevalence of 33% or greater would be positive in a bulk milk test while having 94% confidence in the result. This is not practical as herds frequently have prevalence of lower than 33% (Schares *et al.* 1998, Hall *et al.* 2005) while still presenting with reproductive problems. Some other ELISAs have shown similar low Se when herd prevalence is not high. Three ELISAs were examined to determine their Se and Sp using a bulk milk sample from herds with 15% with-in herd prevalence. While one ELISA lacked Sp due to different antigen batches, two other ELISAs had low Se of 61% and 47% while having 92% and 94% Sp, respectively (Bartels *et al.* 2005). In another study the p38 ELISA was determined to have a low Se of 40% and 80% at 10% and 20% prevalence, respectively, although this increased to 90% and 100% at 30% and 40% prevalence, respectively (Schares *et al.* 2003). Although at present these bulk milk ELISAs have limited sensitivity in low prevalence herds, they may be of use as a tool in large epidemiological studies or for farmers to monitor *N. caninum* prevalence over time. Recent work from the Netherlands to control *N. caninum* in cattle makes use of serological testing of aborting cows and regular bulk milk screening (3 times per year) using an ELISA validated to detect ≥15% herd prevalence (Dijkstra *et al.* 2005). Herds with a positive bulk milk test and abortion problems then undergo whole herd screening to identify seropositive dams.

Until this study there has been no previous estimate of the prevalence of *N. caninum* in NSW cattle, dairy or beef. The only prevalence data available has been from individual dairy herds which have ranged from 10%-31% (Atkinson *et al.* 2000b, Quinn *et al.* 2004, Hall *et al.* 2005). In other Australian states, estimates of *N. caninum* prevalence have ranged from 4% in Western Australian beef and dairy cattle (Palmer *et al.* 2001) (3000 sera from 95 farms) to 15% in central Queensland beef cattle (Stoessel *et al.* 2003) (1673 sera from 45 farms). The prevalence in other countries has ranged from 2% to 45% among non-aborting herds (Ooi *et al.* 2000, Vaclavek *et al.* 2003)and 7% to 72% among aborting herds (Björkman *et al.* 2000, Morales *et al.* 2001).

The 203 farms included in the present study are representative of NSW and are located mainly on the eastern coast where the majority of dairy cows in NSW are found. The areas of sample collection (Figure 36) correlate to the main dairy areas (Figure 37). It was estimated that there are approximately 247,000 dairy cows and 1000 registered dairies in NSW (Anon. 2005a) and thus this study corresponds to a sampling of nearly 20% of those farms.

The *N. caninum* prevalence of 21.1% in dairy cows detected in this study is relatively high and could pose a problem for many NSW dairies. It has been shown that *N. caninum* infection as detected by the ELISA described here is widespread throughout the areas sampled. Although only two samples were taken from each farm, 35% (69/198) of farms sampled had at least one *N. caninum* positive cow. The median shire prevalence was 17% in the 44 shires sampled. Although the prevalence in shires ranged from 0-100% this does not give a true representation as in some cases only a few samples were collected from these shires.

This ELISA presented here was demonstrated to have high Se and Sp when used with undiluted milk samples. It could be a useful tool when applied to abortion diagnosis, control efforts or, epidemiological studies. The prevalence component of this study showed that *N. caninum* could be a wider problem in NSW and so this ELISA could now be used further on a larger scale. More work should be done on ELISA technology using milk as it is reliable, cheap and easy to use.

10. Discussion

10.1 Abortion

In this study of a dairy herd experiencing sporadic abortions *N. caninum* was implicated as the major cause of the abortions. This herd was endemically infected with *N. caninum* and the majority of infections were vertically transmitted. During the 12-month study, the risk of abortion attributable to *N. caninum* was 29% with seropositive cows being 13 times more likely to abort than seronegative cows. This is one of only three reported Australian studies quantifying the risk of abortion due to *N. caninum* in an endemically aborting herd. These results support previous work where *N. caninum* was shown to increase the risk of abortion. Two previous studies in Australia, in dairy herds that had experienced abortion epidemics, had detected a five and eight-fold increase in abortion risk for seropositive cows (Atkinson *et al.* 2000b, Quinn *et al.* 2004). Reports from other countries have also found increased risk of abortion (from 7.4 to 2-fold) amongst seropositive cows (Paré *et al.* 1997, Thurmond and Hietala 1997a, Wouda *et al.* 1998b, Davison *et al.* 1999a, Pfeiffer *et al.* 2002).

In the current study there was a high prevalence (71.4%) of BVDV antibodies in cattle although there was no increased attributable risk of abortion amongst BVDV seropositive cows (RR=1.2). However in cows seropositive to both *N. caninum* and BVDV there was a higher attributable risk of abortion (37.5%) than in cows that were only seropositive to *N. caninum* (27.5%) or BVDV (3.3%) alone. Other authors have demonstrated that concurrent infection with *N. caninum* and BVDV may increase the risk of abortion compared to *N. caninum* infection alone (Björkman *et al.* 2000, Williamson *et al.* 2000). It has been suggested that this may be due to the immunosuppressive effects of BVDV (Potgieter 1995, Thurmond and Hietala 1995, Alves *et al.* 1996). In the current study although BVDV by itself was not found to be a major cause of the abortions it did increase the risk of abortion in *N. caninum* infected cows. Therefore, in order to reduce the risk of *N. caninum* abortions, it may be prudent to vaccinate against BVDV. Previously there has been no BVDV vaccine available in Australia but recently one has entered the market, so this option would now be feasible.

Cows that are seropositive to *N. caninum* are thought to have a long-term infection (Hietala and Thurmond 1999) whereas cows seropositive to BVDV only show that these animals have been previously exposed to the virus. Detection of BVDV antibodies does not mean that the animal is presently infected or only recently exposed. A more valuable test would be a BVDV avidity ELISA that could discriminate between animals with recent exposure and those infected sometime ago. Although PI animals are important for the spread of BVDV it is estimated that only 1% of a herd are PI. Persistently infected animals can be detected by using an antigen test.

10.2 Control

This is the first study to demonstrate effective control of *N. caninum* infection in a farm environment. With gradual culling of *N. caninum* seropositive cows over a 12-month period and not breeding from positive cows there seemed to be only one new infection in the herd. It was assumed that the newly infected animal had been infected postnatally as it was negative in the ELISA the previous year.

It has been suggested by others that this method of culling or restricting breeding of *N. caninum* seropositive cows could be successful in reducing infection in a herd (Thurmond and Hietala 1995, Reichel and Ellis 2002). Mathematical modelling studies have also concluded that both culling positive cattle and not breeding replacements from positive cattle would be effective methods of control (French *et al.* 1999). The long-term effectiveness of both of these methods is dependant on the level of postnatal infection in the herd. This method of control works by reducing further congenital infections that would be otherwise certain to occur due to the high efficiency of vertical transmission of *N. caninum* (86% in this herd). It would also be expected to decrease the abortions due to *N. caninum* and thus reduce the potential for further infection of dogs or other definitive hosts on the property.

A recent simulation study of several control strategies in endemically infected herds with various prevalence, has concluded that the strategy of testing the whole herd and excluding daughters from seropositive dams as potential replacements provided the best economic return (Larson *et al.* 2004). The model was somewhat different to the approach undertaken in this study where seropositive cows were gradually culled as

well as excluding daughters from seropositive dams so as to lower the prevalence more quickly. The modelling study suggests that cows in their third or higher pregnancy abort less than those in their first or second pregnancy and so these cows were retained although in this situation the prevalence is only halved after 5 years. In this present study half of the seropositive cattle were culled in the first year and the remaining seropositive cattle are expected to be culled in the second year.

The current study provides evidence that farmers of dairy herds with low prevalence of *N. caninum* infection do not have to wait for a vaccine for the control of *N. caninum.* Herds with 10% prevalence can control *N. caninum* infection with little economic hardship by culling and or not breeding from seropositive cattle.

A particularly useful tool in a test-and-cull program for *N. caninum* would be the milk ELISA described here. This would allow farmers to easily and cheaply collect milk samples from their herd for subsequent testing, thus lowering the cost barrier for farmers wanting to control *N. caninum.*

10.3 Vertical transmission

A high rate of vertical transmission was demonstrated by clustering of seropositive cattle in family trees. This has been seen by others (Björkman *et al.* 1996, Schares *et al.* 1998) but this is the first report from Australia where the family relationships between dam and daughter have been studied in detailed family trees. An overall vertical transmission rate of 86% (12/14) on this property was calculated by a combination of nine seropositive daughters born from 10 seropositive dams (90%) and three seropositive foetuses/calves born from four seropositive dams (75%).

10.4 Postnatal transmission

In the study herd there seemed to be a low rate of postnatal infection as only four seropositive cows were descendents of seronegative dams. These four cows were born over four consecutive years suggesting a mean postnatal infection rate of one per year. As mentioned previously there seemed to be only one new infection identified in the one-year period that this herd was monitored. This was expected as the calculated mean

rate of postnatal infection was also one per year. The one new infection also equates to a postnatal infection rate of 0.5% (*i.e.* 1/195) when all naïve cattle are included and 0.8% (1/124) when only cows are included. Low rates of post-natal transmission, as suggested in this study, have also been demonstrated elsewhere (1.9% and <1%) (Davison *et al.* 1999a, Hietala and Thurmond 1999) although others have reported slightly higher rates (8.5%) (Paré *et al.* 1997). It is generally assumed that the postnatal infection rate is higher in herds with an epidemic abortion pattern as this is often due to a point source infection. So the low level of postnatal transmission in this herd fits with an endemic infection pattern.

10.5 DNA in milk

Previously *N. caninum* has not been detected in milk from infected cows and this is the first Australian report to demonstrate *N. caninum* DNA in milk. It corroborates a recent report from Poland of *N. caninum* DNA detected in milk from seropositive cows (Moskwa *et al.* 2003). This observation could be of great importance to the dairy industry and should be further investigated. Although *N. caninum* DNA was detected in the milk of four seropositive cows in the current study, milk extracts from seropositive cows were not infective to SCID mice, so a new route of transmission was not proven.

There are several explanations for the lack of infectivity in mice. *Neospora caninum* tachyzoites in the milk may not have been viable due to the effect of antibodies in the milk. It may also be due to the number of tachyzoites in the milk. Aliquots of the milk extracts were examined under the microscope but none were found. The milk extracts injected into each mouse were approximately equivalent to 4 ml of whole milk. This volume of milk may not have contained sufficient tachyzoites to be infective. This may be especially true when comparing the volume ingested by a newborn calf. A newborn calf (approximately 35 kg) ingests approximately 15 L of milk in one day, which calculates to be 0.43 ml milk/g bodyweight. The mice (20 g) in the study were given the equivalent of 4 ml of milk, which equates to only 0.2 ml milk/g bodyweight. So in only one day of feeding, a calf ingests two times the quantity of milk that was given to mice in proportion to body weight. Even if a calf was fed milk for only 5 days it would still ingest 10 times the amount of milk proportionally given to mice. In a farm situation, a calf will be fed milk for many days thus increasing the likelihood of becoming infected.

In the mouse study, 10^3 tachyzoites caused death in 50% of SCID mice and 10^4 tachyzoites caused death in 100% of SCID mice. It could be argued that 1000 tachyzoites in 4 ml of milk (*i.e.* 250 tachyzoites/ml) would have had a significant effect on mice. As no effect was visible the concentration of *N. caninum* tachyzoites in milk is probably lower than 250 tachyzoites/ml (*i.e.* less that 10^3 in the inoculum).

If a seropositive dam gives birth to a calf then the calf is highly likely (86% in this study) to become congenitally infected so any milk fed to that calf does not add significantly to that calf's risk of infection. If a seropositive mother does not congenitally infect a calf however, the ingestion of milk may provide for a new route of infection and may in effect boost (*i.e.* top-up) the "vertical" transmission rate.

Milk may also be a potential source of postnatal infection to calves. When calves are fed pooled milk from unknown dams there is a chance that this milk contains tachyzoites if some of the pool comes from seropositive cows. Even if a calf is only fed milk from an infected dam on one occasion, there is still a chance of infection. On the study property calves were fed pooled milk or several calves were fed milk from a single cow. If the milk was derived from an infected cow then this may lead to new infections. Thus, pooled milk should only come from known seronegative cows otherwise there is a risk of infecting further cattle.

It may be that the colostrum contains higher concentrations of tachyzoites. The stage of gestation may also be important in determining the concentration of tachyzoites in milk. In a chronically infected cow, tachyzoites in serum and milk are possibly highest at the time of recrudescence of the chronic infection. This should be further investigated with cows at various stages of gestation and post-gestation.

10.6 Dogs

Dogs are known definitive hosts as are coyotes (McAllister *et al.* 1998, Gondim *et al.* 2004). There is a high possibility that foxes are also definitive hosts of *N. caninum* although this remains to be shown. On the study property there were many foxes, which may represent a potential vector for postnatal infection. The control of foxes is a difficult task but aborted material and bovine tissue should be collected and disposed of so foxes do not become infected from such sources. One in four dogs residing on the farm was seropositive to *N. caninum* when the first survey was undertaken in this herd and that same dog remained positive one year later. This dog may have been the source of infection that caused the low level of postnatal transmission.

10.7 Production parameters

In the current study a significantly greater number of inseminations required for pregnancy was found in *N. caninum* seropositive cows than seronegative cows. Along with this was a longer time from first service to conception. The number of inseminations required for conception has recently been found to be higher in cows with *N. caninum* infection (Munoz-Zanzi *et al.* 2004).

Whether milk production is an important effect in all cases is undetermined as some studies have found differing results. Although the current report detected an increased milk production in *N. caninum* seropositive cows the differences were not significant. However, this increase is in line with other reports from this part of the world (Pfeiffer et al, 2002).

10.8 ELISAs

Selection of valid cut-off values for ELISAs and knowing their limitations is important before being used for diagnosis. Depending on what the ELISA is to be used for the sensitivity and specificity can be chosen by selecting a different cut-off value. If abortion diagnosis is required then a cut-off with a high specificity may be chosen where as if diagnosing for prevalence then a cut-off with high sensitivity may be chosen. Obviously the best ELISA is that which has both high sensitivity and

specificity. In any particular ELISA the sensitivity and specificity at a particular cut-off are fixed but depending on the prevalence the costs of misclassification differ. Thus a useful tool is the Misclassification Cost Term in CMDT which produces a cut-off to minimise the misclassification at a particular prevalence. This tool can be used in conjunction with TG-ROC to determine an appropriate cut-off.

When a test-and-cull program is undertaken the reliability of the test results are essential. As no test is 100% perfect there may be a need to retest doubtful samples or indeed test in a different medium.

Two graph-receiver operating characteristic analysis of sera was found to be a useful tool in determining the sensitivity and specificity of both serum and milk based ELISAs. The cut-off of the IP blocking ELISA as determined by TG-ROC in this study, was found to agree with that suggested by the manufacturer. This provides support for the use of this cut-off as high Se and Sp was also obtained. TG-ROC analysis of serum results from testing in the IP indirect ELISA suggested changes to the cut-off threshold recommended by the manufacturer With that change the ELISA performed with high sensitivity and specificity (in excess of 98% for individual sera). The manufacturer suggested a doubtful range although in this study there were many known positive sera that were found to be in this range and so this range should be abolished. It is possible that the reason for the manufacturers high cut-off is that the sera used were from aborting cattle which have high antibody titres or that limited numbers of sera were used.

10.9 Milk ELISA

As the cost of serological testing for *N. caninum* is high, methods to reduce the cost would be valuable. The evaluation here of an ELISA to detect antibodies to *N. caninum* in milk has proven to be useful. A reliable assay has now been described for diagnosis of infection and it has also been utilised to give the first overall estimates of *N. caninum* prevalence in NSW.

After determining the optimal milk dilution for use in the IP indirect ELISA, TG-ROC analysis suggested an appropriate cut-off that would allow milk testing with high

sensitivity and specificity of 97%. This ELISA may be used in test-and-cull programs as each individual cow can be sampled with relative ease and cheaply as the farmer could do this for themselves, without requiring assistance.

The 1/20 dilution difference between milk and serum samples in the IP indirect ELISA (using similar cut-off thresholds) seems reasonable given that there is an approximate 20-fold difference between the level of IgG antibodies in milk and serum (Tizard 2004). It is unlikely that there will be correlation between milk and serum results as cattle show significant seasonal differences in serum IgG (Tizard 2004) and others have found that the stage of lactation is a significant factor (Schares *et al.* 2004). In the latter study, there was an increase in the milk ELISA result compared to the serum result as the lactation progressed. This was explained by a decrease in milk yield as the lactation progressed thus leading to a higher level of IgG in milk.

At present this ELISA does not seem to be of use as a bulk milk ELISA. The ELISA has a low sensitivity at a 1/3 dilution which means that it will only detect a within herd prevalence of 33%. Thus using this ELISA with bulk milk samples would not be practical as many herds are known to have *N. caninum* seroprevalence of lower than 33% and this report also demonstrated that the mean prevalence in NSW is 21.1%. The majority of *N. caninum* bulk milk ELISAs described to date also have a limitation of only being able to detect a within herd prevalence of 15% or greater, thus many infected herds would go undetected.

10.10 Prevalence

The prevalence study undertaken here is the first of its kind in NSW. The data gained from this study is valuable as it demonstrates the overall level of infection and that *N. caninum* was detected over a wide region. The prevalence of 21.1% is quite high and so neosporosis may be a greater problem for dairy farmers than previously thought. The main dairy areas in NSW are found on the coast and also the southern/central region bordering with Victoria. *Neospora caninum* infected animals were found to be distributed throughout all these main dairy areas of NSW.

10.11 Conclusion

This is the first time that the seroprevalence of *N. caninum* in NSW dairy cattle has been estimated. The validation of the ELISA to detect *N. caninum* antibodies in milk was particularly beneficial as it made the prevalence study possible. This ELISA may also be used for further epidemiological studies. Of all the states in Australia, the greatest number of dairy cattle are found in Victoria, thus it would be worthwhile to utilise the milk ELISA to determine the prevalence and scope of infection of *N. caninum* in that state.

The case study demonstrated that the economic cost due to *N. caninum* related abortion was significant. In this study the effect on early foetal loss was demonstrated by the increased number of inseminations required however the increased time required for successful conception was not significant. There was also no significant effect on milk production. However, as only low numbers of animals were utilised in the current study, further studies need to be performed which include greater numbers of animals.

The present study also represents only the second reported detection of *N. caninum* DNA in bovine milk. Although in this study mice were not successfully infected when injected with milk extracts from seropositive cows, further work should be done to determine if milk from infected cattle is infective to mice or calves when ingested. Until this issue is resolved it remains a potentially major issue for the dairy industry.

At present there is no proven treatment for control of *N. caninum* in cattle. The test-and-cull control effort implemented in this study is the first of its kind anywhere in the world to be reported in the literature and proved to be successful on this farm. Although this method of control does not prevent postnatal transmission (which was demonstrated to be low in the present case), there are no other viable options for control at present until possibly a more effective vaccine is produced.

The work presented here has developed (control methods) and validated (ELISAs) the tools for further detailed studies of the epidemiology of *N. caninum* in the Australian dairy industry and opens the door for further studies into routes of transmission (milk), the economics (reproductive and milking performance) and control of the infection.

References

1. **Agerholm JS, Willadsen CM, Nielsen TK, Giese SB, Holm E, Jensen L, Agger JF**. Diagnostic studies of abortion in Danish dairy herds. *Journal of Veterinary Medicine. Series A* 44,551-558,1997
2. **Almeria S, Ferrer D, Pabon M, Castella J, Manas S**. Red foxes (*Vulpes vulpes*) are a natural intermediate host of *Neospora caninum*. *Veterinary Parasitology* 107,287-294,2002
3. **Alves D, McEwen B, Hazlett M, Maxie G, Anderson N**. Trends in bovine abortions submitted to the Ontario Ministry of Agriculture, Food and Rural Affairs, 1993-1995. *Canadian Veterinary Journal* 37,287-288,1996
4. **Anderson ML, Blanchard PC, Barr BC, Dubey JP, Hoffman RL, Conrad PA**. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. *Journal of the American Veterinary Medical Association* 198,241-244,1991
5. **Anderson ML, Palmer CW, Thurmond MC, Picanso JP, Blanchard PC, Breitmeyer RE, Layton AW, McAllister M, Daft B, Kinde H, Read DH, Dubey JP, Conrad PA, Barr BC**. Evaluation of abortions in cattle attributable to neosporosis in selected dairy herds in California. *Journal of the American Veterinary Medical Association* 207,1206-1210,1995
6. **Anderson ML, Reynolds JP, Rowe JD, Sverlow KW, Packham AE, Barr BC, Conrad PA**. Evidence of vertical transmission of *Neospora* sp infection in dairy cattle. *Journal of the American Veterinary Medical Association* 210,1169,1997
7. **Andrianarivo AG, Choromanski L, McDonough SP, Packham AE, Conrad PA**. Immunogenicity of a killed whole *Neospora caninum* tachyzoite preparation formulated with different adjuvants. *International Journal for Parasitology* 29,1613-1625,1999
8. **Andrianarivo AG, Rowe JD, Barr BC, Anderson ML, Packham AE, Sverlow KW, Choromanski L, Loui C, Grace A, Conrad PA**. A POLYGEN TM - adjuvanted killed *Neospora caninum* tachyzoite preparation failed to prevent foetal infection in pregnant cattle following i.v/i.m experimental tachyzoite challenge. *International Journal for Parasitology* 30,985-990,2000
9. **Anon.** Australian Dairy Industry in Focus 2004. Dairy Australia:9-10. 2005a
10. **Anon.** Rural Lands Protection Boards 2004 Annual Report. Orange, NSW: State Council of Rural Lands Protection Boards:103. 2005b:103
11. **Atkinson R, Harper PAW, Reichel MP, Ellis JT**. Progress in the serodiagnosis of *Neospora caninum* infections of cattle. *Parasitology Today* 16,110-114,2000a
12. **Atkinson RA, Cook RW, Reddacliff LA, Rothwell J, Broady KW, Harper PAW, Ellis JT**. Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. *Australian Veterinary Journal* 78,262-266,2000b
13. **Barber JS, Gasser RB, Ellis J, Reichel MP, McMillan D, Trees AJ**. Prevalence of antibodies to *Neospora caninum* in different canid populations. *Journal of Parasitology* 83,1056-1058,1997
14. **Barber JS, Trees AJ**. Clinical aspects of 27 cases of neosporosis in dogs. *Veterinary Record* 139,439-443,1996

15. **Barr BC, Anderson ML, Woods LW, Dubey JP, Conrad PA**. *Neospora*-like protozoal infections associated with abortion in goats. *Journal of Veterinary Diagnostic Investigation* 4,365-367,1992
16. **Barr BC, Conrad PA, Dubey JP, Anderson ML**. Neospora-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. *Journal of Veterinary Diagnostic Investigation* 3,39-46,1991
17. **Barr BC, Rowe JD, Sverlow KW, BonDurant R, Ardans AA, Oliver MN, Conrad PA**. Experimental reproduction of bovine fetal *Neospora* infection and death with a bovine Neospora isolate. *Journal of Veterinary Diagnostic Investigation* 6,207-215,1994
18. **Bartels CJM, van Maanen C, van der Meulen AM, Dijkstra T, Wouda W**. Evaluation of three enzyme-linked immunosorbent assays for detection of antibodies to *Neospora caninum* in bulk milk. *Veterinary Parasitology* 131,235-246,2005
19. **Bartels CJM, Wouda W, Schukken YH**. Risk factors for *Neospora caninum*-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). *Theriogenology* 52,247-257,1999
20. **Basso W, Venturini L, Venturini MC, Hill DE, Kwok OC, Shen SK, Dubey JP**. First isolation of *Neospora caninum* from the feces of a naturally infected dog. *Journal of Parasitology* 87,612-8,2001
21. **Baszler T, Long M, McElwain T, Mathison B**. Interferon gamma and interleukin-12 mediate protection to acute *Neospora caninum infection* in BALB/c mice. *International Journal for Parasitology* 29,1635-1646,1999
22. **Baszler TV, Adams S, Vander-Schalie J, Mathison BA, Kostovic M**. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. *Journal of Clinical Microbiology* 39,3851-7,2001
23. **Baszler TV, Knowles DP, Dubey JP, Gay JM, Mathison BA, McElwain TF**. Serological diagnosis of bovine neosporosis by *Neospora caninum* monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology* 34,1423-1428,1996
24. **Beaudeau F, Belloc C, Seegers H, Assié S, Sellal E, Joly A**. Evaluation of a blocking ELISA for the detection of bovine viral diarrhoea virus (BVDV) antibodies in serum and milk. *Veterinary Microbiology* 80,329-337,2001
25. **Biuk-Rudan N, Cvetnic S, Madic J, Rudan D**. Prevalence of antibodies to IBR and BVD viruses in dairy cows with reproductive disorders. *Theriogenology* 51,875-881,1999
26. **Bjerkås I, Jenkins MC, Dubey JP**. Identification and characterization of *Neospora caninum* tachyzoite antigens useful for diagnosis of neosporosis. *Clinical and Diagnostic Laboratory Immunology* 1,214-21,1994
27. **Bjerkås I, Mohn SF, Presthus J**. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Zeitschrift für Parasitenkunde* 70,271-274,1984
28. **Björkman C, Alenius S, Emanuelsson U, Uggla A**. *Neospora caninum* and bovine virus diarrhoea virus infections in Swedish dairy cows in relation to abortion. *Vet J* 159,201-206,2000
29. **Björkman C, Holmdahl OJM, Uggla A**. An indirect enzyme-linked immunoassay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. *Veterinary Parasitology* 68,251-260,1997

30. **Björkman C, Johansson O, Stenlund S, Holmdahl OJM, Uggla A**. *Neospora* species infection in a herd of dairy cattle. *Journal of the American Veterinary Medical Association* 208,1441-1444,1996
31. **Björkman C, Lunden A**. Application of iscom antigen preparations in ELISAs for diagnosis for *Neospora* and *Toxoplasma* infections. *International Journal for Parasitology* 28,187-193,1998
32. **Björkman C, Naslund K, Stenlund S, Maley SW, Buxton D, Uggla A**. An IgG avidity ELISA to discriminate between recent and chronic *Neospora caninum* infection. *Journal of Veterinary Diagnostic Investigation* 11,41-44,1999
33. **Björkman C, Uggla A**. Serological diagnosis of *Neospora caninum* infection. *International Journal for Parasitology* 29,1497-1507,1999
34. **Black L, Francis MJ, Nicholls MJ**. Protecting your domesticated animals from infectious disease. *Veterinary Annual* 25,46-61,1995
35. **Bureau of Animal Health: Attwood**. Leptospirosis in cattle pigs, sheep, horses and humans. *Agriculture Notes*,1-3,1998
36. **Cannas A, Naguleswaran A, Muller N, Eperon S, Gottstein B, Hemphill A**. Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. *Parasitology* 126,303-312,2003
37. **Chi J, VanLeeuwen JA, Weersink A, Keefe GP**. Direct production losses and treatment costs from bovine viral diarrhoea virus, *Mycobacterium avium* subspecies *paratuberculosis*, and *Neospora caninum*. *Prevetative Veterinary Medicine* 55,137-153,2002
38. **Choromanski L, Block W**. Humoral immune responses and safety of experimental formulations of inactivated *Neospora* vaccines. *Parasitology Research* 86,851-853,2000
39. **Choromanski LJ**. The evaluation of a new vaccine, NeoGuard ™ *Neospora caninum* vaccine as an aid in reducing abortions in healthy, pregnant heifers challenged with *Neospora caninum*. *Intervet Bulletin*,1-4,2002a
40. **Choromanski LJ**. *Neospora caninum* vaccine field safety evaluation. *Intervet Bulletin*,1-3,2002b
41. **Chowdhury MN**. Toxoplasmosis: a review. *Journal of Medicine* 17,373-396,1986
42. **Cole RA, Lindsay DS, Blagburn BL, Sorjonen DC, Dubey JP**. Vertical transmission of Neospora caninum in dogs. *Journal of Parasitology* 81,208-211,1995
43. **Conrad PA, Sverlow K, Anderson M, Rowe J, BonDurant R, Tuter G, Breitmeyer R, Plamer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B**. Detection of serum antibody response in cattle with natural or experimental *Neospora* infections. *Journal of Veterinary Diagnostic Investigation* 5,572-578,1993
44. **Cox BT, Reichel MP, Griffiths LM**. Serology of a *Neospora* abortion outbreak on a dairy farm in New Zealand: a case study. *New Zealand Veterinary Journal* 46,28-31,1998
45. **Cox FEG**. Designer vaccines for parasitic diseases. *International Journal for Parasitology* 27,1147-1157,1997
46. **Cuddon P, Lin DS, Bowman DD, Lindsay DS, Miller TK, Duncan ID, DeLahunta A, Cummings J, Suter M, Cooper B, King JM, Dubey JP**. *Neospora caninum* infection in English Springer spaniel littermates. *Journal of Veterinary Internal Medicine* 6,325-332,1992

47. **Davison HC, Otter A, Trees AJ**. Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. *International Journal for Parasitology* 29,1683-1689,1999a
48. **Davison HC, Otter A, Trees AJ**. Significance of *Neospora caninum* in British dairy catttle determined by estimation of seroprevalence in normally calving cattle and aborting cattle. *International Journal for Parasitology* 29,1189-1194,1999b
49. **De Marez T, Liddell S, Dubey JP, Jenkins MC, Gasbare L**. Oral infection of calves with *Neospora caninum* oocysts from dogs: humoral and cellular responses. *International Journal for Parasitology* 29,1647-1657,1999
50. **Dijkstra T, Barkema HW, Eysker M, Hesselink JW, Wouda W**. Natural transmission routes of *Neospora caninum* between farm dogs and cattle. *Veterinary Parasitology* 105,99-104,2002a
51. **Dijkstra T, Barkema HW, Eysker M, Wouda W**. Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. *International Journal for Parasitology* 31,209-15,2001a
52. **Dijkstra T, Barkema HW, Hesselink JW, Wouda W**. Point source exposure of cattle to *Neospora caninum* consistent with periods of common housing and feeding and related to the introduction of a dog. *Veterinary Parasitology* 105,89-98,2002b
53. **Dijkstra T, Bartels CJM, Wouda W**. Control of bovine neosporosis: experiences from the Netherlands. The 20th International Conference of the World Association for the Advancement of Veterinary Parasitology. Christchurch, New Zealand:191 2005
54. **Dijkstra T, Eysker M, Schares G, Conraths FJ, Wouda W, Barkema HW**. Dogs shed *Neospora caninum* oocysts after ingestion of naturally infected bovine placenta but not after ingestion of colostrum spiked with *Neospora caninum* tachyzoites. *International Journal for Parasitology* 31,747-752,2001b
55. **Dubey JP**. Neosporosis - a newly recognized protozoal infection. *Comparative Pathology Bulletin* 24,4-6,1992
56. **Dubey JP**. Neosporosis in cattle: biology and economic impact. *Journal of the American Veterinary Medical Association* 214,1160-1163,1999a
57. **Dubey JP**. Neosporosis-the first decade of research. *International Journal for Parasitology* 29,1485-1488,1999b
58. **Dubey JP**. Persistence of incysted *Toxoplasma gondii* in caprine livers and public health significance of of *Toxoplasmosis* in goats. *Journal of the American Veterinary Medical Association* 177,1203-1207,1980
59. **Dubey JP**. Recent advances in *Neospora* and neosporosis. *Veterinary Parasitology* 84,349-367,1999c
60. **Dubey JP**. Strategies to reduce transmission of *Toxoplasma gondii* to animals and humans. *Veterinary Parasitology* 64,65-70,1996
61. **Dubey JP, Barr BC, Barta JR, Bjerkas I, Bjorkman C, Blagburn BL, Bowman DD, Buxton D, Ellis JT, Gottstein B, Hemphill A, Hill DE, Howe DK, Jenkins MC, Kobayashi Y, Koudela B, Marsh AE, Mattsson JG, McAllister MM, Modry D, Omata Y, Sibley LD, Speer CA, Trees AJ, Uggla A, Upton SJ, Williams DJ, Lindsay DS**. Redescription of *Neospora caninum* and its differentiation from related coccidia. *International Journal for Parasitology* 32,929-46,2002a

62. **Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A**. Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medical Association* 192,1269-1285,1988a
63. **Dubey JP, Hartley WJ, Lindsay DS**. Congenital *Neospora caninum* infection in a calf with spinal cord anomaly. *Journal of the American Veterinary Medical Association* 197,1043-1044,1990a
64. **Dubey JP, Hartley WJ, Lindsay DS, Topper MJ**. Fatal congenital *Neospora caninum* infection in a lamb. *Journal of Parasitology* 76,127-130,1990b
65. **Dubey JP, Hattel AL, Lindsay DS, Topper MJ**. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medical Association* 93,1259-1263,1988b
66. **Dubey JP, Hill DE, Lindsay DS, Jenkins MC, Uggla A, Speer CA**. *Neospora caninum* and *Hammondia heydorni* are separate species/organisms. *Trends in Parasitology* 18,66-69,2002b
67. **Dubey JP, Hollis K, Romand S, Thulliez P, Kwok OCH, Hungerford L, Anchor C, Etter D**. High prevalence of antibodies to *Neospora caninum* in white-tailed deer (*Odocoileus virginianus*). *International Journal for Parasitology* 29,1709-1711,1999
68. **Dubey JP, Jenkins MC, Adams DS, McAllister MM, Anderson Sprecher R, Baszler TV, Kwok OCH, Lally NC, Bjorkman C, Uggla A**. Antibody responses of cows during an outbreak of neosporosis evaluated by indirect fluorescent antibody test and different enzyme-linked immunosorbent assays. *Journal of Parasitology* 83,1063-1069,1997
69. **Dubey JP, Koestner A, Piper RC**. Repeated transplacental transmission of *Neospora caninum* in dogs. *Journal of the American Veterinary Medical Association* 197,857-860,1990c
70. **Dubey JP, Lindsay DS**. Fatal *Neospora caninum* infection in kittens. *Journal of Parasitology* 75,148-151,1989
71. **Dubey JP, Lindsay DS**. *Neospora caninum* induced abortion in sheep. *Journal of Veterinary Diagnostic Investigation* 2,230-233,1990
72. **Dubey JP, Lindsay DS**. A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* 67,1-59,1996
73. **Dubey JP, Lindsay DS, Speer CA**. Structures of *Toxoplasma gondii* tachyzoites, bradyzoites, and sporozoites and biology of tissue cysts. *Clinical Microbiology Revue* 11,267-299,1988c
74. **Dubey JP, Morales JA, Villalobos P, Lindsay DS, Blagburn BL, Topper MJ**. Neosporosis-associated abortion in a dairy goat. *Journal of the American Veterinary Medical Association* 208,263,1996
75. **Dubey JP, Porterfield ML**. *Neospora caninum* (Apicomplexa) in an aborted equine fetus. *Journal of Parasitology* 76,732-734,1990
76. **Durham PJK, Paine GD**. Serological survey for antibodies to infectious agents in beef cattle in northern South Australia. *Australian Veterinary Journal* 75,139-143,1997
77. **Ellis J, Luton K, Baverstock PR, Brindley PJ, Nimmo KA, Johnson AM**. The phylogeny of *Neospora caninum*. *Molecular and Biochemical Parasitology* 64,303-311,1994
78. **Ellis JT**. *Neospora caninum*: prospects for diagnosis and control using molecular methods. In: Shirley MW, Tomley FM, Freeman BM, eds. *Control of Coccidiosis into the next millennium*. Compton Newbury: Institute for Animal Health, 1997:80.

79. **Ellis JT**. Polymerase chain reaction approaches for the detection of *Neospora caninum* and *Toxoplasma gondii*. *International Journal for Parasitology* 28,1053-1060,1998
80. **Ellis JT, McMillan D, Ryce C, Payne S, Atkinson R, Harper PAW**. Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. *International Journal for Parasitology* 29,1589-1596,1999a
81. **Ellis JT, Morrison DA, Liddell S, Jenkins MC, Mohammed OB, Ryce C, Dubey JP**. The genus *Hammondia* is paraphyletic. *Parasitology* 118,357-362,1999b
82. **Esslemont RJ, Kossaibati MA**. Culling of 50 dairy herds in England. *Veterinary Record* 140,36-39,1997
83. **Ezio F, Anna T**. Antibodies to *Neospora caninum* in European brown hare (*Lepus europaeus*). *Veterinary Parasitology* 115,75-78,2003
84. **Ferre I, Aduriz G, Del-Pozo I, Regidor-Cerrillo J, Atxaerandio R, Collantes-Fernandez E, Hurtado A, Ugarte-Garagalza C, Ortega-Mora LM**. Detection of *Neospora caninum* in the semen and blood of naturally infected bulls. *Theriogenology* 63,1504-18,2005
85. **Fray MD, Paton DJ, Alenius S**. The effects of bovine viral diarrhoea virus on cattle reproduction in relation to disease control. *Animal Reproduction Science* 60-61,615-627,2000
86. **French NP, Clancy D, Davison HC, Trees AJ**. Mathematical models of *Neospora caninum* infection in dairy cattle: transmission and options for control. *International Journal for Parasitology* 29,1691-1704,1999
87. **Gazzinelli RT, Denkers EY, Sher A**. Host resistance to *Toxoplasma gondii* : model for studying the selective induction of cell-mediated immunity by intracellular parasites. *Infectious Agents of Disease* 2,139-149,1993
88. **Gondim LF, Gao L, McAllister MM**. Improved production of *Neospora caninum* oocysts, cyclical oral transmission between dogs and cattle, and in vitro isolation from oocysts. *Journal of Parasitology* 88,1159-1163,2002
89. **Gondim LFP, McAllister MM, Pitt WC, Zemlicka DE**. Coyotes (*canis latrans*) are definitive hosts of *Neospora caninum*. *Internationla Journal of Parasitology* 34,159-161,2004
90. **Gottstein B, Hentrich B, Wyss R, Thur B, Busato A, Stark KDC, Muller N**. Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. *International Journal for Parasitology* 28,679-691,1998
91. **Greiner M**. Two-graph receiver operating characterisic (TG-ROC): update version supports optimisation of cut-off values that minimise overall misclassification costs. *Journal of Immunological Methods* 191,93-94,1996
92. **Greiner M**. Two-graph receiver operating characteristics (TG-ROC): a Microsoft-EXCEL template for the selection of cut-off values in diagnostic tests. *Journal of Immunological Methods* 185,145-146,1995
93. **Hall CA, Reichel MP, Ellis JT**. *Neospora* abortions in dairy cattle: diagnosis, mode of transmission and control. *Veterinary Parasitology* 128,231-241,2005
94. **Heckeroth AR, Tenter AM**. Evaluation of cross-reactivity between tachyzoites of *Neospora caninum* and *Toxoplasma gondii* by two-dimensional electrophoresis. In: A.Hemphill, B. Gottstein eds. A European perspective on *Neospora caninum*. *International Journal for Parasitology* 30,913-919,2000
95. **Hemphill A, Gottstein B**. Identification of a major surface protein on *Neospora caninum* tachyzoites. *Parasitology Research* 82,497-504,1996

96. **Hemphill A, Gottstein B, Kaufmann H**. Adhesion and invasion of bovine endothelial cells by Neospora caninum. *Parasitology* 112,183-197,1996
97. **Hernandez J, Risco C, Donovan A**. Association between exposure to *Neospora caninum* and milk production in dairy cows. *Journal of the American Veterinary Medical Association* 219,632-5,2001
98. **Hietala SK, Thurmond MC**. Postnatal *Neospora caninum* transmission and transient serologic responses in two dairies. *International Journal for Parasitology* 29,1669-1676,1999
99. **Hilali M, Romand S, Thulliez P, Kwok OCH, Dubey JP**. Prevalence of *Neospora caninum* and Toxoplasma gondii antibodies in sera from camels from Egypt. *Veterinary Parasitology* 75,269-271,1998
100. **Hoar BR, Ribble CS, Spitzer CC, Spitzer PG, Janzen ED**. Investigation of pregnancy losses in beef cattle herds associated with *Neospora* sp. infection. *Canadian Veterinary Journal* 37,364-366,1996
101. **Hobson JC, Duffield TF, Kelton D, Lissemore K, Hietala SK, Leslie KE, McEwen B, Cramer G, Peregrine AS**. *Neospora caninum* serostatus and milk production of Holstein cattle. *Journal of the American Veterinary Medical Association* 221,1160-1164,2002
102. **Holmdahl OJM, Mattsson JG**. Rapid and sensitive identification of *Neospora caninum* by in vitro amplification of the internal transcribed spacer 1. *Parasitology* 112,177-182,1996
103. **Holmdahl OJM, Mattsson JG, Uggla A, Johansson KE**. The phylogeny of *Neospora caninum* and *Toxoplasma gondii* based on ribosomal RNA sequences. *FEMS Microbiology Letters* 119,187-192,1994
104. **Homan WL, Limper L, Verlaan M, Borst A, Vercammen M, van Knapen F**. Comparison of the internal transcribed spacer, ITS 1, from *Toxoplasma gondii* isolates and *Neospora caninum*. *Parasitology Research* 83,285-289,1997
105. **Hovingh E**. Abortions in Dairy Cattle - I : Common Causes of Abortions.Publication:404-288,2002
106. **Howe DK, Sibley LD**. Comparison of the major antigens of *Neospora caninum* and *Toxoplasma gondii*. *International Journal for Parasitology* 29,1489-1496,1999
107. **Hungerford TG**. Diseases of Livestock. (9th ed.) Sydney: McGraw-Hill Book Company, 1990
108. **Huong LTT, Ljungstrom BL, Uggla A, Björkman C**. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes in southern Vietnam. *Veterinary Parasitology* 75,53-57,1998
109. **Innes EA, Andrianarivo AG, Bjorkman C, Williams DJL, Conrad PA**. Immune responses to *Neospora caninum* and prospects for vaccination. *Trends in Parasitology* 18,497-504,2002
110. **Innes EA, Wright SE, Maley S, Rae A, Schock A, Kirvar E, Bartley P, Hamilton C, Carey IM, Buxton D**. Protection against vertical transmission in bovine neosporosis. *International Journal for Parasitology* 31,1523-34,2001
111. **Innes EW, Panton WRM, Marks J, Trees AJ, Holmdahl J, Buxton D**. Interferon gamma inhibits the intracellular multiplication of *Neospora caninum*, as shown by incorporation of 3H uracil. *Journal of Comparative Pathology* 113,95-100,1995
112. **Irwin PJ**. Companion animal parasitology: a clinical perspective. *International Journal for Parasitology* 32,581-93,2002

113. **Jenkins MC, Caver JA, Bjorkman C, Anderson TC, Romand S, Vinyard B, Uggla A, Thulliez P, Dubey JP**. Seroloical investigation of an outbreak of *Neospora caninum*-associated abortion in a dairy herd in southeastern United States. *Veterinary Parasitology* 94,17-26,2000
114. **Jenkins MC, Wouda W, Dubey JP**. Serological response over time to recombinant *Neospora caninum* antigens in cattle after a neosporosis-induced abortion. *Clinical and Diagnostic Laboratory Immunology* 4,270-274,1997
115. **Jensen L, Jensen TK, Lind P, Henriksen SA, Uggla A, BilleHansen V**. Experimental porcine neosporosis. *APMIS Acta Pathologica, Microbiologica et Immunologica Scandinavica* 106,475-482,1998
116. **Keefe G, VanLeeuwen J**. *Neospora caninum* in maritime Canada: historic prevalence and influence on milk production. *Proceedings. 52nd Annual Convention of the Canadian Veterinary Medical Association*,377-380,2000
117. **Khan IA, Schwartzman JD, Fonseka S, Kasper LH**. *Neospora caninum*: role for immune cytokines in host immunity. *Experimental Parasitology* 85,24-34,1997
118. **Kirkbride CA**. Etiologic agents detected in a 10-year study of bovine abortion and stillbirths. *Journal of Veterinary Diagnostic Investigation* 4,175-180,1992
119. **Kritzner S, Sager H, Blum J, Krebber R, Greif G, Gottstein B**. An explorative study to assess the efficacy of Toltrazuril-sulfone (Ponazuril) in calves experimentally infected with *Neospora caninum*. *Annals of Clinical Microbiology and Antimicrobials* 1,1-10,2002
120. **Kyaw T, Virakul P, Muangyai M, Suwimonteerabutr J**. *Neospora caninum* seroprevalence in dairy cattle in central Thailand. *Veterinary Parasitology* 121,255-263,2004
121. **Lally N, Jenkins M, Liddell S, Dubey JP**. A dense granule protein (NCDG1) gene from *Neospora caninum*. *Molecular and Biochemical Parasitology* 87,239-243,1997
122. **Lally NC, Jenkins MC, Dubey JP**. Evaluation of two *Neospora caninum* recombinant antigens for use in an enzyme-linked immunosorbent assay for the diagnosis of bovine neosporosis. *Clinical and Diagnostic Laboratory Immunology* 3,275-9,1996
123. **Larson RL, Hardin DK, Pierce VL**. Economic considerations for diagnostic and control options for *Neospra caninum*-induced abortions in endemically infected herds of beef cattle. *Journal of the American Veterinary Medical Association* 224,1597-1604,2004
124. **Liddell S, Jenkins MC, Dubey JP**. A competitive PCR assay for quantitative detection of *Neospora caninum*. *International Journal for Parasitology* 29,1583-1587,1999
125. **Liddell S, Lally NC, Jenkins MC, Dubey JP**. Isolation of the cDNA encoding a dense granule associated antigen (NCDG2) of *Neospora caninum*. *Molecular and Biochemical Parasitology* 93,153-158,1998
126. **Liddell S, Parker C, Vinyard B, Jenkins M, Dubey JP**. Imunization of mice with plasmid DNA coding fro NcGRA7 of NcsHSP33 confers partial protection against vertical transmission of *Neospora caninum*. *Journal of Parasitology* 89,496-500,2003
127. **Lindberg A, Alenius S**. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. *Veterinary Microbiology* 64,197-222,1999
128. **Lindsay DS, Blagburn BL, Dubey JP**. Factors affecting the survival of *Neospora caninum* bradyzoites in murine tissues. *Journal of Parasitology* 78,70-72,1992

129. **Lindsay DS, Dubey JP**. *Neospora caninum* (Protozoa: Apicomplexa) infections in rats. *Canadian Journal of Zoology* 68,1595-1599,1990
130. **Lindsay DS, Dubey JP, Duncan RB**. Confirmation that the dog is a definitive host for *Neospora caninum*. *Veterinary Parasitology* 82,327-333,1999
131. **Lindsay DS, Kelly EJ, McKown RD, Stein FJ, Plozer J, Herman J, Blagburn BL, Dubey JP**. Prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in coyotes (*Canis latrans*) and experimental infections of coyotes with *Neospora caninum*. *Journal of Parasitology* 82,657-659,1996a
132. **Lindsay DS, Lenz SD, Cole RA, Dubey JP, Blagburn BL**. Mouse model for central nervous system *Neospora caninum* infections. *Journal of Parasitology* 81,313-315,1995a
133. **Lindsay DS, Rippey NS, Powe TA, Sartin EA, Dubey JP, Blagburn BL**. Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of *Neospora caninum*. *American Journal of Veterinary Research* 56,1176-80,1995b
134. **Lindsay DS, Speer CA, Toivio Kinnucan MA, Dubey JP, Blagburn BL**. Use of infected cultured cells to compare ultrastructural features of *Neospora caninum* from dogs and *Toxoplasma gondii*. *American Journal of Veterinary Research* 54,103-106,1993
135. **Lindsay DS, Steinberg H, Dubielzig RR, Semrad SD, Konkle DM, Miller PE, Blagburn BL**. Central nervous system neosporosis in a foal. *Journal of Veterinary Diagnostic Investigation* 8,507-510,1996b
136. **Lindsay DS, Weston JL, Little SE**. Prevalence of antibodies to *Neospora caninum* and *Toxoplasma gondii* in gray foxes (*Urocyon cinereoargenteus*) from South Carolina. *Veterinary Parasitology* 97,159-64,2001
137. **Littlejohns IR, Horner GW**. Incidence, epidemiology and control of bovine pestivirus infections and disease in Australia and New Zealand. *Revue of Science and Technology* 9,195-205,1990
138. **Lunden A, Marks J, Maley SW, Innes EA**. Cellular immune responses in cattle experimentally infected with *Neospora caninum*. *Parasite Immunology* 20,519-526,1998
139. **Mayhew IG, Smith KC, Dubey JP, Gatward LK, McGlennon NJ**. Treatment of encephalomyelitis due to *Neospora caninum* in a litter of puppies. *Journal of Small Animal Practice* 32,609-612,1991
140. **McAllister MM, Bjorkman C, Anderson-Sprecher R, Rogers DG**. Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. *Journal of the American Veterinary Medical Association* 217,881-887,2000
141. **McAllister MM, Dubey JP, Lindsay DS, Jolley WR, Wills RA, McGuire AM**. Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* 28,1473-1478,1998
142. **McAllister MM, Huffman EM, Hietala SK, Conrad PA, Anderson ML, Salman MD**. Evidence suggesting a point source exposure in an outbreak of bovine abortion due to neosporosis. *Journal of Veterinary Diagnostic Investigation* 8,355-357,1996
143. **McGlennon NJ, Jefferies AR, Casas C**. Polyradiculoneuritis and polymyositis due to a toxoplasma-like protozoan: diagnosis and treatment. *Journal of Small Animal Practice* 31,102-104,1990
144. **McGowan MR, Kirkland PD**. Early reproductive loss due to bovine pestivirus infection. *British Veterinary Journal* 151,263-270,1995

145. **McGuire AM, McAllister M, Wills RA, Tranas JD**. Experimental infection of domestic pigeons (*Columbia livia*) and Zebra finches (*Poephila guttata*) with *Neospora caninum* tachyzoites. *International Journal for Parasitology* 29,1525-1529,1999
146. **Mohammed OB, Davies AJ, Hussein HS, Daszak P, Ellis JT**. *Hammondia heydorni* from the Arabian Mountain gazelle and red fox in Saudi Arabia. *Journal of Parasitology* 89,535-539,2003
147. **Moore DP, Campero CM, Odeon AC, Posso MA, Cano D, Leunda MR, Basso W, Venturini MC, Spath E**. Seroepidemiology of beef and dairy herds and fetal study of *Neospora caninum* in Argentina. *Veterinary Parasitology* 107,303-316,2002
148. **Morales E, Trigo FJ, Ibarra F, Puente E, Santacruz M**. Seroprevalence study of bovine neosporosis in Mexico. *Journal of Veterinary Diagnostic Investigation* 13,413-415,2001
149. **Moskwa B, Cabaj W, Pastusiak K, Bien J, Wojdan J, Sloniewski K**. The suitability of milk in detection of *Neospora caninum* infection in cows. COST 854 - Protozoal repoduction losses in farm ruminants. Rheinsberg, Germany:9 2003
150. **Mugridge NB, Morrison DA, Heckeroth AR, Johnson AM, Tenter AM**. Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. *International Journal for Parasitology* 29,1545-1556,1999
151. **Munoz-Zanzi CA, Hietala SK, Thurmond MC, Johnson WO**. Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea in dairy calves. *American Journal of Veterinary Research* 64,358-364,2003
152. **Munoz-Zanzi CA, Thurmond MC, Hietala SK**. Effect of bovine viral diarrhea virus infection on fertility of dairy heifers. *Theriogenology* 61,1085-1099,2004
153. **Nishikawa Y, Ikeda H, Fukumoto S, Xuan X, Nagasawa H, Otsuka H, Mikami T**. Immunisation of dogs with a canine herpesvirus vector expressing *Neospora caninum* surface protein, NcSRS2. *International Journal for Parasitology* 30,1167-1171,2000
154. **Nishikawa Y, Inoue N, Xuan X, Nagasawa H, Igarashi I, Fujisaki K, Otsuka H, Mikami T**. Protective efficacy of vaccination by recombinant vaccinia virus against *Neospora caninum* infection. *Vaccine* 19,1381-1390,2001
155. **Nishikawa Y, Mikami T, Nagasawa H**. Vaccine development against *Neospora caninum* infection. *Journal of Veterinary Medical Science* 64,1-5,2002
156. **Norton JH, Tranter WP, Campbell RS**. A farming systems approach of abortion in dairy cattle on the Atherton Tableland. 2. The pattern of infectious diseases. *Australian Veterinary Journal* 66,163-167,1989
157. **Ooi HK, Huang CC, Yang CH, Lee SH**. Serological survey and first finding of *Neospora caninum* in Taiwan, and the detection of its antibodies in various body fluids of cattle. *Veterinary Parasitology* 90,47-55,2000
158. **Ortega-Mora LM, Ferre I, del-Pozo I, Caetano-da-Silva A, Collantes-Fernandez E, Regidor-Cerrillo J, Ugarte-Garagalza C, Aduriz G**. Detection of *Neospora caninum* in semen of bulls. *Veterinary Parasitology* 117,301-308,2003

159. **Osawa T, Wastling J, Acosta L, Ortellado C, Ibarra J, Innes EA**. Seroprevalence of *Neospora caninum* infection in dairy and beef cattle in Paraguay. *Veterinary Parasitology* 110,17-23,2002
160. **Otranto D, Llazari A, Testini G, Traversa D, di Regalbono AF, Badan M, Capelli G**. Seroprevalence and associated risk factors of neosporosis in beef and dairy cattle in Italy. *Veterinary Parasitology* 118,7-18,2003
161. **Ould Amrouche A, Klein F, Osdoit C, Mohammed HO, Touratier A, Sanaa M, Mialot JP**. Estimation of *Neospora caninum* seroprevalence in dairy cattle from Normandy, France. *Veterinary Research* 30,531-538,1999
162. **Packham AE, Sverlow KW, Conrad PA, Loomis EF, Rowe JD, Anderson ML, Marsh AE, Cray C, Barr BC**. A modified agglutination test for *Neospora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* 5,467-473,1998
163. **Palmer D, Forshaw D, Payne C**. *Neospora caninum* infection of Western Australian cattle has a low prevalence and causes sporadic abortions only. *VIII International Coccidiosis Conference and Annual Scientific Meeting of the Australian Society for Parasitology*,124-125,2001
164. **Paré J, Hietala SK, Thurmond MC**. An enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of *Neospora* sp. infection in cattle. *Journal of Veterinary Diagnostic Investigation* 7,352-359,1995a
165. **Paré J, Hietala SK, Thurmond MC**. Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. *Journal of Veterinary Diagnostic Investigation* 7,273-275,1995b
166. **Paré J, Thurmond MC, Hietala SK**. Congenital *Neospora caninum* infection in dairy cattle and associated calfhood mortality. *Canadian Journal of Veterinary Research Revue Canadienne de Recherche Veterinaire* 60,133-139,1996
167. **Paré J, Thurmond MC, Hietala SK**. *Neospora caninum* antibodies in cows during pregnancy as a predictor of congenital infection and abortion. *Journal of Parasitology* 83,82-87,1997
168. **Payne S, Ellis J**. Detection of *Neospora caninum* DNA by the polymerase chain reaction. *International Journal for Parasitology* 26,347-351,1996
169. **Peters M, Lutkefels E, Heckeroth AR, Schares G**. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. *International Journal for Parasitology* 31,1144-1148,2001
170. **Pfeiffer DU, Wichtel JJ, Reichel MP, Williamson NB, Teague WR, Thornton RN**. Investigations into the epidemiology of *Neospora caninum* infection in dairy cattle in New Zealand. Dairy Cattle and Industry Sessions. 75th Jubilee New Zealand Veterinary Association Annual Conference. Rotorua: Continuing Education, Massey University:279-292 1998
171. **Pfeiffer DU, Williamson NB, Reichel MP**. Long-term serological monitoring as a tool for epidemiological investigation of *Neospora caninum* infection in a New Zealand dairy herd. *9th Symposium of the International Society for Veterinary Epidemiology and Economics*,2000
172. **Pfeiffer DU, Williamson NB, Reichel MP, Wichtel JJ, Teague WR**. A longitudinal study of *Neospora caninum* infection on a dairy farm in New Zealand. *Preventative Veterinary Medicine* 54,11-24,2002
173. **Pfeiffer DU, Williamson NB, Thornton RN**. A simple spreadsheet simulation model of the economic effects of *Neospora caninum* abortions in dairy cattle in

New Zealand. 8th Symposium of the International Society for Veterinary Epidemiology and Economics. Paris: Special Issue of *Epidemiologie et santé animale*:10.12.1-3 1997
174. **Potgieter LND**. Immunology of Bovine Viral Diarrhea virus. *Veterinary Clinics of North America: Food Animal Practice* 11,501-520,1995
175. **Pronost S, Pitel P, Foucher N, Collobert C, Fortier G**. Equine neosporosis. *Point Veterinaire* 31,299-304,2000
176. **Quinn HE, Ellis JT, Smith NS**. *Neospora caninum*: a cause of immune-mediated failure of pregnancy? *Trends in Parasitology* 18,391-394,2002a
177. **Quinn HE, Miller CMD, Ryce C, Windsor PA, Ellis JT**. Characterization of an outbred pregnant mouse model of *Neospora caninum* infection. *Journal of Parasitology* 88,691-696,2002b
178. **Quinn HE, Windsor PA, Kirkland PD, Ellis JT**. An outbreak of abortion in a dairy herd associated with *Neospora caninum* and bovine pestivirus infection. *Australian Veterinary Journal* 82,99-101,2004
179. **Raisanen SA, Saari KM**. The importance of trophozoites in transmission of toxoplasmosis: survival and pathogenity of *Toxoplasma gondii* trophozoites in liquid media. *Medical Hypotheses* 4,367-375,1978
180. **Reichel MP**. *Neospora caninum* infections in Australia and New Zealand. *Australian Veterinary Journal* 78,258-261,2000
181. **Reichel MP**. Prevalence of *Neospora* antibodies in New Zealand dairy cattle and dogs. *New Zealand Veterinary Journal* 46,38,1998
182. **Reichel MP, Ellis JT**. Control options for *Neospora caninum* infections in cattle - current state of knowledge. *New Zealand Veterinary Journal* 50,86-92,2002
183. **Reichel MP, Pfeiffer DU**. An analysis of the performance characteristics of serological tests for the diagnosis of *Neospora caninum* infection in cattle. *Veterinary Parasitology* 107,197-207,2002
184. **Reichel MP, Vanhoff K, Baxter B**. Performance characteristics of an enzyme-linked immunosorbent assay performed in milk for the detection of liver fluke (*Fasciola hepatica*) infection in cattle. *Veterinary Parasitology* 129,61-66,2005
185. **Rodriguez I, Choromanski L, Rodgers SJ, Weinstock D**. Survey of *Neospora caninum* antibodies in dairy and beef cattle from five regions of the United States. *Veterinary Theriogenology* 3,396-401,2002
186. **Roeder PL, Harkness JW**. BVD virus infection: prospects for control. *The Veterinary Record* 118,143-147,1986
187. **Romero JJ, Perez E, Dolz G, Frankena K**. Factors associated with *Neospora caninum* serostatus in cattle of 20 specialised Costa Rican dairy herds. *Preventative Veterinary Medicine* 53,263-273,2002
188. **Romero JJ, Perez E, Frankena K**. Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions. *Veterinary Parasitology* 123,149-159,2004
189. **Sawada M, Park CH, Kondo H, Morita T, Shimada A, Yamane I, Umemura T**. Serological survey of antibody to *Neospora caninum* in Japanese dogs. *Journal of Veterinary Medical Science* 60,853-854,1998
190. **Schares G, Barwald A, Staubach C, Wurm R, Rauser M, Conraths FJ, Schroeder C**. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. *Veterinary Parasitology* 120,55-63,2004
191. **Schares G, Barwald A, Staubach C, Ziller M, Kloss D, Wurm R, Rauser M, Labohm R, Drager K, Fasen W, Hess RG, Conraths FJ**. Regional

distribution of bovine *Neospora caninum* infection in the German state of Rhineland-Palatinate modelled by Logistic regression. *International Journal for Parasitology* 33,1631-40,2003
192. **Schares G, Conraths FJ, Reichel MP**. Bovine neosporosis: comparison of serological methods using outbreak sera from a dairy herd in New Zealand. *International Journal for Parasitology* 29,1669-1676,1999a
193. **Schares G, Dubremetz JF, Dubey JP, Barwald A, Loyens A, Conraths FJ**. *Neospora caninum*: identification of 19-, 38-, and 40-kDa surface antigens and a 33-kDa dense granule antigen using monoclonal antibodies. *Experimental Parasitology* 92,109-119,1999b
194. **Schares G, Peters M, Wurm R, Barwald A, Conraths FJ**. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. *Veterinary Parasitology* 80,87-98,1998
195. **Schares G, Rauser M, Söndgen P, Rehberg P, Bärwald A, Dubey JP, Edelhofer R, Conraths FJ**. Use of purified tachyzoite surface antigen p38 in an ELISA to diagnose bovine neosporosis. *International Journal for Parasitology* 30,1123-1130,2000
196. **Schares G, Rauser M, Zimmer K, Peters M, Wurm R, Dubey JP, de Graaf DC, Edelhofer R, Mertens C, Hess G, Conraths FJ**. Serological differences in *Neospora caninum*-associated epidemic and endemic abortions. *Journal of Parasitology* 85,688-694,1999c
197. **Schares G, Wenzel U, Muller T, Conraths FJ**. Serological evidence for naturally occurring transmission of *Neospora caninum* among foxes (*Vulpes vulpes*). *International Journal for Parasitology* 31,418-423,2001
198. **Speer CA, Dubey JP, McAllister MM, Blixt JA**. Comparative ultrastructure of tachyzoites, bradyzoites, and tissue cysts of *Neospora caninum* and *Toxoplasma gondii*. *International Journal for Parasitology* 29,1509-1519,1999
199. **St George TD, Snowdon WA, Parsonson IM, French EL**. A serological survey of mucosal disease and infectious bovine rhinotracheitis in cattle in Australia and New Guinea. *Australian Veterinary Journal* 43,549-557,1967
200. **Stenlund S, Kindahl H, Magnusson U, Uggla A, Bjorkman C**. Serum antibody profile and reproductive performance during two consecutive pregnancies of cows naturally infected with *Neospora caninum*. *Veterinary Parasitology* 85,227-234,1999
201. **Stoessel Z, Taylor LF, McGowan MR, Coleman GT, Landmann JK**. Prevalence of antibodies to *Neospora caninum* within central Queensland beef cattle. *Australian Veterinary Journal* 81,165-166,2003
202. **Straub OC**. Advances in BHV1 (IBR) research. *Dtsch Tierarztl Wochenschr* 108,419-422,2001
203. **Tenter AM, Heckeroth AJ, Weiss LM**. *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology* 30,1127-1158,2000
204. **Thilsted JP, Dubey JP**. Neosporosis-like abortions in a herd of dairy cattle. *Journal of Veterinary Diagnostic Investigation* 1,205-209,1989
205. **Thornton R**. Bovine abortion diagnoses in 1995. *Surveillance Wellington* 23,21-22,1996
206. **Thornton R**. Bovine abortions - laboratory diagnosis 1991. *Surveillance Wellington* 19,24,1992
207. **Thornton RN, Gajadhar A, Evans J**. *Neospora* abortion epidemic in a dairy herd. *New Zealand Veterinary Journal* 42,190-191,1994

208. **Thornton RN, Thompson EJ, Dubey JP**. *Neospora* abortion in New Zealand cattle. *New Zealand Veterinary Journal* 39,129-133,1991
209. **Thurmond M, Hietala S**. Strategies to control *Neospora* infection in cattle. *Bovine Practitioner* No. 29,60-63,1995
210. **Thurmond MC, Hietala SK**. Culling associated with *Neospora caninum* infection in dairy cows. *American Journal of Veterinary Research* 57,1559-1562,1996
211. **Thurmond MC, Hietala SK**. Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. *American Journal of Veterinary Research* 58,1381-1385,1997a
212. **Thurmond MC, Hietala SK**. Effect of *Neospora caninum* infection on milk production in first-lactation dairy cows. *Journal of the American Veterinary Medical Association* 210,672,1997b
213. **Thurmond MC, Hietala SK, Blanchard PC**. Predictive values of fetal histopathology and immunoperoxidase staining in diagnosing bovine abortion caused by *Neospora caninum* in a dairy herd. *Journal of Veterinary Diagnostic Investigation* 11,90-94,1999
214. **Tizard IR**. Veterinary Immunology: An introduction. (7th ed.) Philadelphia: Saunders, Elsevier, (Merchant T, ed. 2004
215. **Trees AJ, Davison HC, Innes EA, Wastling JM**. Towards evaluating the economic impact of bovine neosporosis. *International Journal for Parasitology* 29,1195-1200,1999
216. **Trees AJ, Guy F, Tennant BJ, Balfour AH, Dubey JP**. Prevalence of antibodies to *Neospora caninum* in a populaton of urban dogs in England. *Veterinary Record* 132,125-126,1993
217. **Trees AJ, McAllister MM, Guy CS, McGarry JW, Smith RF, Williams DJL**. *Neospora caninum*: oocyst challenge of pregnant cows. *Veterinary Parasitology* 109,147-154,2002
218. **Uggla A, Mattsson JG, Lunden A, Jakubek EB, Naslund K, Holmdahl OJM, Stenlund S, Frossling J, Bjorkman C**. *Neospora caninum* in Sweden. In: Hemphill, A., Gottstein, B. (Eds.), A European perspective on *Neospora caninum*. *International Journal for Parasitology* 30,877-924,2000
219. **Uggla A, Stenlund S, Holmdahl OJM, Jakubek EB, Thebo P, Kindahl H, Björkman C**. Oral *Neospora caninum* inoculation of neonatal calves. *International Journal for Parasitology* 28,1467-1472,1998
220. **Vaclavek P, Koudela B, Modry D, Sedlak K**. Seroprevalence of *Neospora caninum* in aborting dairy cattle in the Czech Republic. *Veterinary Parasitology* 115,239-245,2003
221. **von Blumröder D, Schares G, Norton R, Williams DJL, Esteban-Renondo I, Wright S, Björkman C, Frössling J, Risco-Castillo V, Fernandez-Garcia A, Ortega Mora LM, Sager H, Hemphill A, van Maanen C, Wouda W, Conraths FJ**. Comparison and standardisation of serological methods for the diagnosis of *Neospora caninum* infection in bovines. *Veterinary Parasitology* 120,11-22,2004
222. **Vonlaufen N, Guetg N, Naguleswaran A, Muller N, Bjorkman C, Schares G, von Blumroeder D, Ellis J, Hemphill A**. In vitro induction of *Neospora caninum* bradyzoites in vero cells reveals differential antigen expression, localization, and host-cell recognition of tachyzoites and bradyzoites. *Infection and Immunity* 72,576-583,2004
223. **Williams DJL, Guy CS, McGarry JW, Guy F, Tasker L, Smith RF, MacEachern K, Cripps PJ, Kelly DF, Trees AJ**. *Neospora caninum*-

associated abortion in cattle: the time of experimentally-induced parasitaemia during gestation determines foetal survival. *Parasitology* 121,347-358,2000

224. **Williams DJL, Guy CS, Smith RF, Guy F, McGarry JW, McKay JS, Trees AJ**. First demonstration of protective immunity against foetopathy in cattle with latent *Neospora caninum* infection. *International Journal for Parasitology* 33,1059-1065,2003
225. **Williams DJL, McGarry J, Guy F, Barber J, Trees AJ**. Novel ELISA for detection of *Neospora*-specific antibodies in cattle. *Veterinary Record* 140,328-331,1997
226. **Williamson NB, Antony A, Pfeiffer DU, Pomroy WM, Reichel MP, Walton JK**. Recent research into *Neospora caninum* in New Zealand. *XXI World Buiatrics Conference*,95,2000
227. **Woods LW, Anderson ML, Swift PK, Sverlow KW**. Systemic neosporosis in a California black-tailed deer (Odocoileus hemionus columbianus). *Journal of Veterinary Diagnostic Investigation* 6,508-510,1994
228. **Wouda W, Bartels CJM, Moen AR**. Characteristics of *Neospora caninum*-associated abortion storms in diary herds in the Netherlands (1995 to 1997). *Theriogenology* 52,233-245,1999a
229. **Wouda W, Brinkhof J, van Maanen C, de Gee ALW, Moen AR**. Serodiagnosis of neosporosis in individual cows and dairy herds: A comparative study of three enzyme-linked immunosorbent assays. *Clinical and Diagnostic Laboratory Immunology* 5,711-716,1998a
230. **Wouda W, Dijkstra T, Kramer AMH, van Maanen C, Brinkhof JMA**. Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. *International Journal for Parasitology* 29,1677-1682,1999b
231. **Wouda W, Moen AR, Schukken YH**. Abortion risk in progeny of cows after a *Neospora caninum* epidemic. *Theriogenology* 49,1311-1316,1998b
232. **Wouda W, Moen AR, Visser IJR, van Knapen F**. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. *Journal of Veterinary Diagnostic Investigation* 9,180-185,1997