STUDIES ON *NEOSPORA CANINUM* AND NEOSPOROSIS IN NEW ZEALAND CATTLE

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General Abstract

The objective of this research was to investigate neosporosis in New Zealand cattle using experimental and molecular tools. The research also aimed to isolate and characterise an indigenous New Zealand isolate of *Neospora caninum*. A series of discrete projects were conducted to achieve the set objectives. A pilot study was first conducted and a wild-type strain of non-cytopathic bovine viral diarrhoea virus (BVDV) type 1 virus was isolated *in vitro* from a persistently infected heifer. The isolate was used to challenge heifers and the effect of experimental BVDV infection on heifers naturally infected with *N. caninum* was investigated. Results showed that heifers that were both BVDV and *N. caninum* seropositive produced 44.4% (8/18) dam-calf pairs that were both BVDV and *N. caninum* seropositive. Serologically, 66.7% (12/18) dam-calf pairs were seropositive to *N. caninum* antibodies, while 80% (8/10) dam-calf pairs were BVDV seropositive. A Polymerase Chain Reaction (PCR) study was also conducted and *N. caninum* DNA was detected in the blood of naturally infected aborting and pregnant heifers. Real-time quantification of *N. caninum* DNA in the blood of infected heifers showed a decrease of *N. caninum* DNA after abortion in the aborting group and an increase through gestation in the pregnant group. A study of antigenicity recognised 7 immunodominant (~18, ~25, ~33, ~35-36, ~45-46, ~47 and 60-62 kDa) and 5 minor antigens of *N. caninum* by cow sera. Three isolates of *N. caninum* (NcNZ 1, NcNZ 2 and NcNZ 3) were isolated from the brains of an infected cow, her calf and a stillborn calf. These isolates were confirmed as *N. caninum* by PCR, immunofluorescence antibody test and immunohistochemistry and were pathogenic to BALB/c mice.

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Dedication

This work is dedicated to my mother-in-law, Madam Iheanahuru Enyo Okeoma, who passed on as this work was being compiled and father-in-law, Mazi Obierezie Okeoma, who also did not live to see the award of this degree. To my parents, Mr. Pius and Mrs. Felicia Egbujiobi, your hard work and sacrifices will always be appreciated.

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Abbreviations

aa	Amino acid
BELU	Bovine embryonic lung
BLAST	Basic local alignment search tool
Bp	Base pairs
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CO_2	Carbon dioxide
cp	Cytopathic
DAB	Diaminobenzidine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides triphosphates
ELISA	Enzyme linked immunosorbent assay
ERMA	Environmental Risk Management Authority
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GMO	Genetically modified organism.
IFAT	Imunofluorescent antibody test
IFN	Interferon
IgE	Immunoglobulin E
IgG	Imunoglobulin gamma
IHC	Immunohistochemistry
IGS	Intergenic spacer
IL	Interleukin
ITS 1	Internal transcribed spacer 1
kDa	Kilodalton
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
NCP	Non-cytopathic
NcNz	*Neospora caninum* New Zealand
NeoF	*Neospora* forward

NeoR	*Neospora* reverse
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PV	Parasitophorous vacoule
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SAS	Statistical analysis system
SDS-PAGE	Sodium dodecyl sulphate polyacrilamide gel electrophoresis
Se	Sensitivity
Sp	Specificity
S/P ratio	Sample/positive ratio
TBE	Tris-Borate-EDTA
TEM	Transmission electron microscopy
T. gondii	*Toxoplasma gondii*
Th	T helper
TNF	Tumour necrosis factor
UNG	Uracil N-glycosylase
UTR	Untranslated region
UV	Ultra violet
VMRD	Veterinary medical research and development
VNT	Virus neutralisation Test

Chapter 1:

Introduction

1.0 Introduction

Neospora caninum is a protozoan parasite of the Kingdom Protista; Phylum Apicomplexa; Order Eucoccidiida; Family Sarcocystiidae. *N. caninum* was placed in the Family Sarcocystiidae because of its close similarity in morphology to other cyst forming coccidia. *N. caninum* is structurally similar to, but antigenically and phylogenetically distinct from *Toxoplasma gondii* (Lindsay and Dubey 1989; Dubey *et al.* 2002). *N. caninum* and *N. hughesi* also share some structural similarities. However, phenotypic differences in their immunoreactive proteins exist. Molecular analysis of the 18S rRNA gene revealed no differences in the nucleotide sequence between *N. caninum* and *N. hughesi*, but their ITS 1 region has 7 nucleotide base differences (Marsh *et al.* 1996). The similarity between *N. caninum* and *N. hughesi* may have caused the misidentification of *N. hughesi* in the past because there were quite a few diagnostic techniques to differentiate between the two species. The serum antibody test used to diagnose *N. caninum* infection can not distinguish between the two *Neospora* species because of high antigenic cross reactivity (Walsh *et al.* 2000). Presently, both *Neospora* species can be distinguished by sequence differences in the ITS-1, SAG1 and SRS2 genes and by differential expression of several undefined antigens by Western blot (Marsh *et al.* 1998; Marsh *et al.* 1999).

Infection due to *N. caninum* (neosporosis) occurs in cattle and several other species of ruminants, dogs and horses. Neosporosis was first reported in dogs to cause neuromuscular disorders and mortality (Bjerkas *et al.* 1984; and Dubey *et al.* 1988). In horses, it causes myeloencephatis (Marsh *et al.* 1996), although this reported occurrence of neosporosis in horses may have been of *N. hughesi.* In cows, neosporosis is the major cause of abortion in many countries including New Zealand (Thornton *et al.* 1991; Thobokwe and Heuer 2004). One of the major reasons for testing cattle for *N. caninum* infection is to identify the potential cause of abortion. However, determining the cause of bovine abortion is difficult as abortions are caused by numerous infectious and non-infectious disease causing agents. Mostly, bovine abortions occur due to endemic infectious pathogens such as *N. caninum.* Abortions due to *N. caninum* can be endemic and/or epidemic and have been described in herds of cattle (Thurmond and Hietala, 1999). Endemic abortion is characterised by an elevated abortion rate which can persist for years. The epidemic pattern of *N. caninum* induced abortion is not

common and is characterized by abortions in a high proportion of pregnant cattle in a brief period of time. There are instances where over 30% of pregnant cattle have aborted due to neosporosis within several months (Thilsted and Dubey 1989). In addition to abortion, *N. caninum* may cause congenital disease in calves infected *in utero* (Barr *et al.* 1990). At birth, such calves may have neurological signs, be underweight, unable to rise, or have no clinical signs (Dubey and Lindsay 1996; Barr *et al.* 1993). *N. caninum* infection may also cause reduced milk production and shortened production life due to early culling (Thurmond and Hietala 1996; Thurmond and Hietala 1997). All these put together, make *N. caninum* an important pathogen in the cattle industry.

Chapter 2:

Literature Review

2.0 Literature Review

This review will concentrate on the factors that predispose to neosporosis in cattle, the epidemiology of *N. caninum* and the molecular tools used in characterising *N. caninum.*

2.1 Life cycle of *N. caninum*

N. caninum is an obligate intracellular parasite with the dog as a definitive host and dogs, cattle and other ruminants as the intermediate hosts (McAllister *et al.* 1998). The *N. caninum* life cycle is comprised of three distinct stages; namely, bradyzoites, tachyzoites and sporozoites. Bradyzoites are a dormant, slowly proliferating stage, with parasites encysted in the host's tissue. Tissue cysts of *N. caninum* can survive for up to 14 days at 4°C, but are rendered non-infective after 1 day at -20°C (Lindsay *et al.* 1992). Bradyzoites in tissue cysts are resistant to acid pepsin solution, indicating that carnivorism is a likely part of the *N. caninum* life cycle. Oral ingestion of bradyzoites leads to an as yet undefined sequence involving gametogomy and production of oocysts. Oocysts are excreted along with faeces of the host (Lindsay *et al.* 1999; McAllister *et al.* 1998). Excreted oocysts sporulate to form sporozoites (Lindsay *et al.* 1999). Sporulated oocysts contain 2 sporocysts, each containing 4 sporozoites and may be infective if ingested by both definitive and intermediate hosts (McAllister *et al.* 1998). Oocysts may excyst inside the intemediate host, liberating infective sporozoites into the intestinal tract which penetrate cells to become tachyzoites. These divide rapidly causing tissue damage and infecting other tissues of the host (Hemphil 1999). Tachyzoites can infect and destroy a variety of cell types including but not limited to neural cells, macrophages, fibroblasts, vascular endothelial cells, hepatocytes and other cells of the body (Dubey and Lindsay 1996). They can also be identified in the placenta of pregnant cattle (Shivaprasad *et al.* 1989). Under the influence of the immune response of the host, tachyzoites are transformed into bradyzoites and the cycle continues. Figure 2.1 is a summary of the life cycle of *N. caninum.*

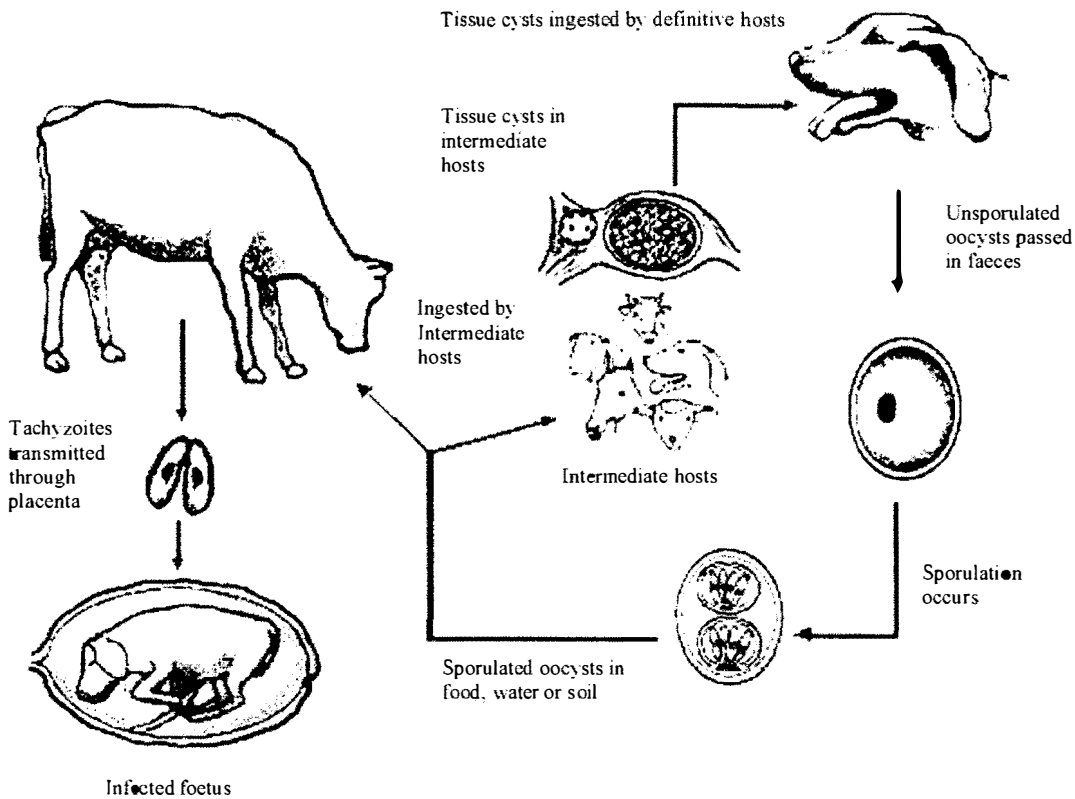

Figure 2.1: *N. caninum* lifecycle showing the definitive host (dog) ingesting a tissue cyst; the definitive host may also shed unsporulated oocysts via its faeces. The intermediate host (cattle) may feed on contaminated food and water containing sporulated oocyst. Sporulated oocysts form tachyzoites and these may be transmitted from dam to foetus *in utero* (vertical transmission). Diagram modified after Dubey *et al.* (1999).

2.2 Biology of *N. caninum*

N. caninum tachyzoites are ovoid, lunate or globular and measure between 3-7 x 1-5 μm, depending on the stage of division (Dubey and Lindsay 1993). They proliferate by endodyogeny, producing several hundreds of new parasites by a few days post-inoculation (Hemphill *et al.* 1999). As tachyzoites proliferate, they form a new membrane bound pseudocyst. As soon as the pseudocyst reaches a critical mass, the host cell lyses, releasing tachyzoites that subsequently infect neighbouring cells (Hemphill 1999).

N. caninum tachyzoites have a 3 layered plasmalemma, 22 subpellicular microtubules, 2 apical rings, a conoid, a polar ring, mitochondria, about 150 micronemes, rhoptries, dense granules, a golgi complex, rough and smooth endoplasmic reticulum, a nucleus and a nucleolus which are all structures that are found in other closely related apicomlexan parasites (Lindsay and Dubey 1989; Dubey *et al.* 2002). In infected animals, tachyzoites are found in a number of cells, including neural cells, macrophages, fibroblasts, vascular endothelial cells, myocytes, renal tubular epithelial cells and hepatocytes (Dubey *et al.* 1988; Lindsay *et al.* 1993). *N. caninum* tachyzoites were once thought to reside directly in the host cell cytoplasm (Dubey *et al.* 1988). However, Lindsay *et al.* (1993) later demonstrated the presence of a parasitophorous vacuole.

N. caninum bradyzoites are slender (6-8 x 1-1.80 μm) and have a cell wall that is up to 4 μm thick. They contain the same organelles found in tachyzoites except that they have fewer rhoptries and more periodic acid schiff (PAS) positive (amylopectin) granules (Lindsay and Dubey 1989). Tissue cysts are delineated by a cell wall that protects bradyzoites from physiological and immunological reactions by the host. Thus, bradyzoites can persist in their hosts for years without causing any significant clinical manifestation (Dubey and Lindsay 1996).

2.3 Predisposing factors to neosporosis in cattle and the epidemiology of neosporosis

Factors associated with neosporosis in cattle include: congenital transmission; presence of possible host animals on the farm e.g. the dog and domestic fowl; lactogenic transmission; immunosuppressive effect of pregnancy; some feed materials like mouldy fodder; disease organisms such as bovine viral diarrhoea virus (BVDV) infection.

2.3.1 Congenital Transmission

Congenital transmission of *N. caninum* is the most important route of infection of cattle with *N. caninum* and is thus an important risk factor for abortion due to *N. caninum*. Evidence of congenital transmission has been well documented by researchers in different countries (Anderson *et al.* 1997; Bergeron *et al.* 2000; Bjorkman *et al.* 1996; Davison *et al.* 1999; Dubey *et al.* 1992; Liddel *et al.* 1998; Paré *et al.* 1996; Waldner *et al.* 1999; Romero and Frankena 2003; Okeoma *et al.* 2004). Neosporosis can be maintained by means of vertical transmission which can occur during consecutive pregnancies in the same cow and for several generations (Anderson *et al.* 1997; Schares *et al.* 1998) without the aid of an external vector (Paré *et al.* 1996; Schares *et al.* 1998). Cows infected congenitally may have higher risk of abortion during their initial pregnancy than non-infected cows. Such cows have substantially more abortions, predominantly as heifers and in the second pregnancy during the first lactation. The relative risk of *N. caninum* induced abortion in congenitally infected cows may decrease with each subsequent pregnancy and could be related to dairy management factors (Thurmond and Hietala 1997). Furthermore, the relative risk of congenital infection may decrease with increasing parity which could be due to increasing immunity to transplacental infection with increasing age. *N. caninum* seropositive heifers may produce about 80% of congenitally infected offspring, while 66% of the offspring of older cows may be congenitally infected (Dijkstra *et al.* 2003).

2.3.2 Presence of animals as definitive hosts on the farm

The dog (McAllister *et al.* 1998) is a definitive host of *N. caninum*. The presence on farms of dogs is a risk factor for *N. caninum* associated abortion and congenital infection in cattle (Bartels *et al.* 1999; Paré *et al.* 1998). Cattle most likely acquire the infection from dogs because the dog is a definitive host of *N. caninum* and canine oocysts are the most likely source of infection/re-infection of cattle herds (Wouda 2000). The presence of *N. caninum* seropositive dog on farms has been associated with a high prevalence (7-31%) of *N. caninum* infection and abortion in cattle in many countries (Barber *et al.* 1997; Reichel 1998; Sawada *et al.* 1998; Wouda *et al.* 1999; Cañón-Franco *et al.* 2003). Antony and Williamson (2003) reported a much higher percentage: 96.8% in sheep/beef-farm dogs, 74.5% in dairy-farm dogs and 30.7% in urban dogs.

Seroepidemiological data and the results of experimentation support the role of dogs in the life cycle of *N. caninum*. There is significant relationship between the number of dogs on a farm and the assessed risk of an abortion (Paré *et al.* 1998; Sawada *et al.* 1998; Bartels *et al.* 1999). However, there were some farms without dogs that had a high prevalence of antibodies in their cattle (Wouda 2000). This is consistent with the existence of sources of infection other than dogs. It has been shown that other canids (Almeria *et al.* 2002; Gondim *et al.* 2004) may have a place in the epidemiology of *N. caninum* infection. *N. caninum* DNA was detected in 10% (13/122) of red foxes in North Eastern Spain (Almeria *et al.* 2002) and also in coyotes (Gondim *et al.* 2004).

2.3.3 Lactogenic transmission

There is some evidence that calves can be experimentally infected via contaminated colostrum (Cole *et al.* 1995; Uggla *et al.* 1998; Davison *et al.* 2001), but not by foster nursing to an infected dam (Davison *et al.* 2001). Calves infected *in utero* may present antibodies to *N. caninum* at calving. Uninfected calves born to infected dams may present colostrum derived antibodies, which decrease to negligible levels within 3 to 6 months, whereas their congenitally infected counterparts remain seropositive for much longer periods (Wouda *et al.* 1998). It has been demonstrated that culture-derived *N.*

caninum tachyzoites added to milk were able to infect newborn calves when given orally (Uggla *et al.* 1998). Furthermore, seroconversion may occur if calves are fed pooled colostrum/milk (Davidson *et al.* 1999). Evidence from other species suggests that lactogenic transmission of *N. caninum* may occur in them. *N. caninum* infection was demonstrated in one of 51 mouse pups which were suckled by an experimentally infected mouse (Cole *et al.* 1995). *T. gondii,* an organism that is similar in many ways to *N. caninum* was isolated from the colostrum of infected women (Langer 1963).

2.3.4 Contaminated tissue/fluids from seropositive animals

Other possible sources of *N. caninum* infection that have been suggested include peroral or transmucosal infection by *N. caninum* tachyzoites present in foetal membranes and uterine fluids (Wouda 2000). Calves inoculated subcutaneously or orally with fluids containing *N. caninum* tachyzoites may seroconvert within 5 weeks post-inoculation (Uggla *et al.* 1998; Lunden *et al.* 1998). Peripheral blood lymphocytes from calves inoculated orally may mount *in vitro* proliferative responses to crude *N. caninum* antigen extract as early as 1 week post-inoculation (De Marez *et al.* 1999). The occurrence of transmucosal infection in cows may be suggestive of horizontal *N. caninum* transmission.

2.3.5 Immunosuppression

The cow's immune system can be suppressed by a number of factors including bovine virus diarrhoea virus (BVDV) and pregnancy. *N. caninum* elicits both humoral and cell-mediated immune responses in infected animals (Lundén *et al.* 1998; De Marez *et al.* 1999). In non-pregnant cows, *N. caninum* infection is asymptomatic indicating that the immune system is able to control the infection. This may not be the case in pregnant cows because the immune system of the cow may be suppressed by pregnancy. In the same way, BVDV infection may suppress the immune system of a cow, rendering her susceptible to opportunistic pathogens like *N. caninum* (Reggiardo 1979; Reggiardo and Kaeberle 1981; Kahrs 1981; Nagele 1984; Potgieter *et al.* 1984a and b; Edwards *et al.* 1986).

2.3.5.1 Bovine viral diarrhoea virus (BVDV) infection

BVDV infection may cause a recrudescence of latent *N. caninum* infection. Most postnatal BVDV infections of cattle are subclinical (Ames 1986; Duffell and Harkness 1985) and many primary postnatal infections, whether sub-clinical or clinical, render the animal transiently immunosuppressed (Edwards *et al.* 1986; Kahrs 1981; Nagele 1984; Potgieter *et al.* 1984a and b; Reggiardo 1979; Reggiardo and Kaeberle 1981). As a result, BVDV has the potential to enhance disease caused by other pathogens or precipitate illness by opportunistic pathogens (Bohac and Yates 1980; Malmquist 1985; Potgieter 1977).

BVDV infection results in specific B and T lymphocyte immunotolerance. These lymphocytes are target cells for the replication of BVDV (Coria and McClurkin 1978; Donis and Dubovi 1987; Larsson and Fossum 1992; McClurkin *et al.* 1984). In addition, the pathological lesions induced by BVDV in cattle suggest that immunosuppression is the consequence of infection by this virus (Tyler and Ramsey 1965). Lymphoid destruction results in varying degrees of lymphocyte depletion in infected calves, even in those that remain clinically normal (Bolin *et al.* 1985; Reggiardo and Kaeberle 1981). It is this depletion of immunocompetent cells in cattle caused by BVDV that results in reduced resistance to pathogens.

A statistically significant association between the presence of antibodies to *N. caninum* and bovine viral diarrhoea was demonstrated in Sweden by Bjorkman *et al.* (2000). Thus, they confirmed that *N. caninum* infection is associated with bovine abortion in Sweden and also indicated that there might be concurrent effects of *N. caninum* and BVDV in causing abortion. In contrast, a negative relationship between seropositivity to *N. caninum* and BVDV was demonstrated in 117 aborting cows belonging to 50 Dutch dairy herds experiencing abortion storms (Bartels *et al.* 1999). The immunosuppressive potential of BVDV can increase susceptibility to and cause more severe clinical effects from other infectious agents (Potgieter 1995). It has also been speculated that pathological changes in the placenta induced by BVDV may allow other pathogens to more easily cross the feto-maternal barrier (Murray 1991). The risk of abortion and congenital infection with *N. caninum* may increase as a result of a

synergistic effect of infection with *N. caninum* and BVDV above the risks for infections with each of the pathogens alone.

2.3.5.2 Pregnancy

Pregnancy can compromise the immune response of a cow. This has serious consequences for pregnant animals because T-helper cell 1 cytokine responses are highly incompatible with pregnancy (Quinn *et al.* 2002). The production of T-helper cell 1 cytokine could result in either the pregnancy being compromised or resistance to *N. caninum* being compromised which may both lead to undesirable consequences. Th1 cytokine [IL-12, IFNγ, Tumour necrosis factor (TNF) α that activates the production of free oxygen radicals (FOR) and nitric oxide (NO)] are detrimental to pregnancy because they destroy the foeto-placental unit and can cause abortion and or foetal resorption (Raghupathy 1997). Thus, during pregnancy the body down-regulates the production of Th1 cytokine, but Th2 cytokines (IL4, IL5 and IL10) are produced (Quinn *et al.* 2002). Th2 cytokine have consequences for immune responses to parasitic infection during pregnancy (Scott and Farrell 1998). Thus lack of Th1 cytokine (which control intracellular parasitic infection) and abundance of Th2 cytokines during pregnancy help explain why *N. caninum* induces abortion in cows. *N. caninum* induces a T-cell immune response in the host. Interferon-gamma (IFNγ) may be involved in host immunity as was suggested by *in vitro* studies (Innes *et al.* 1995). The host cellular response to neosporosis is believed to be mediated by the production of IL12 and IFNγ. Infection with *N. caninum* results in a significant decrease in IL12 and a slight increase in IL10 in infected calves (De Marez *et al.* 1999; Khan *et al.* 1997). The gene that encodes IL10 (IL-10) inhibits the synthesis of a number of cytokines, including IFNγ and IL12. This could increase the host's susceptibility to neosporosis since IL10 has potent immunosuppressive properties. Humoral and cell mediated immune response (IFNγ) may be elicited in pregnant cows infected with *N. caninum* tachyzoites (Andrianarivo *et al.* 2001).

If *N. caninum* infection occurs during pregnancy, tachyzoites invade the uterus and may cross the foeto-maternal barrier to infect the uterus depending on the gestational age. During pregnancy, foetal trophoblast cells produce IL10 creating a Th2 cytokine

environment at the foeto-maternal interface and down regulate the production of IL12. IL12 and IFNγ generate a T helper type 1 response in animals and limit the multiplication of *N. caninum* but they are damaging during pregnancy and might cause abortion or rejection of the foetus (Raghupathy 1997). It can be assumed that foetal infection follows a maternal parasitaemia, although most foetal infections occur in cattle already harbouring a persistent infection before pregnancy was established (Buxton *et al.* 2002). The outcome of neosporosis during pregnancy may depend on all or a combination of the following: the gestational age at the time of infection, the virulence of the particular *N. caninum* including the magnitude and time of occurrence of parasitaemia (Innes *et al.* 2002; Williams *et al.* 2000).

2.3.5.3 Oestrogen

Oestrogen concentrations in plasma, urine and faeces of healthy cows increase at approximately 4 months of gestation (Hoffman *et al.* 1997) with a second increase at 8 months of gestation. A rise in oestradiol concentration may suppress cell-mediated immunity and enhance the formation of systemic antibodies to infectious agents; thus, may result in a suppressed cellular immune system (Styrt and Sugarman 1991). This may lead to infection or recrudescence of latent infection since it has been shown that *N. caninum* elicits both humoral and cell-mediated immune responses in infected animals (Lunden *et al.* 1998; De Marez *et al.* 1999).

2.3.5.4 Mouldy fodder

The feeding of mouldy fodder is considered to be a factor that may induce recrudescence of a latent *N. caninum* infection through mycotoxins causing immune suppression (Bartels *et al.* 1999). Mycotoxins are produced when fodder is contaminated by fungi. Several mycotoxins have been shown to cause immunosuppression after repeated ingestion at low doses (Corrier 1991; Sharma 1993). Immunosuppression may lead to tissue cyst rupture, as has been demonstrated in mice with chronic toxoplasmosis (Venturini *et al.* 1996). Such tissue cyst rupture in the

presence of immunosuppression could allow liberated tachyzoites to replicate and recrudesce.

2.3.6 The influence of management practices

Grazing of young stock on commercial pasture has been reported to be a risk factor for a *Neospora* abortion storm (Bartels 1999). Similarly, the presentation of young stock at a local fair is thought to be a predisposing factor to *N. caninum* infection (Bartels 1999). Both have been suggested as an indication of horizontal transmission but direct evidence for this is lacking in both reports. Seronegative animals kept in contact with seropositive animals demonstrated a low rate of seroconversion (Waldner *et al.* 1999) which was also suggested as evidence of horizontal transmission but other reports have found no such evidence when infected and non infected heifers were housed together (Anderson *et al.* 1997). Hygiene around the calving area seems to play a role as a predisposing risk factor for neosporosis (Bartels *et al.* 1999). Exposure to placenta and uterine effusions might serve as a source of infection as *N. caninum* tachyzoites have been demonstrated in the placenta (Shivaprasad *et al.* 1989; Wouda *et al.* 1992). Similarly, the use of a calving pen for hospitalising sick cows may predispose neosporosis in cattle (Bartels *et al.* (1999). These potentially contradictory observations seem to suggest that neosporosis may be minimally contagious; however, empirical evidence for horizontal transmission is lacking.

2.4 Isolation of *N. caninum*

Indigenous isolates of *N. caninum* have been made in many different countries and regions where neosporosis occurs (Table 2.1). Researchers have had limited success isolating *N. caninum* around the world. Conrad *et al.* (1993) only succeeded in isolating *N. caninum* from two out of more than 100 foetuses suspected to have neosporosis. Workers at Massey University (Palmerston North, New Zealand) have attempted isolations on many occasions without success. One reason for this could be problems with cell culture/animal model sensitivity, since different cell lines and animal models might interact differently with different *N. caninum* strains. Therefore, it is important to

do further research into the interactions between host cells [*in vitro* (cell cultures) and *in vivo* (animal models)] and *N. caninum*. This will help in developing the optimum *in vitro* and *in vivo* environments for *N. caninum* tachyzoite and bradyzoite isolation and cultivation in New Zealand; thereby, enhancing effective diagnosis, prevention and control of neosporosis in New Zealand.

N. caninum has been isolated and cultivated by *in vitro* and *in vivo* techniques. *In vitro* isolation has been done in many cell lines, both primary and established (Dubey *et al.* 1988; Hay *et al.* 1990; Cuddon *et al.* 1992; Conrad *et al.* 1993; Barber *et al.* 1995; Stenlund *et al.* 1997; Dubey and Lindsay 2000; Miller *et al.* 2002). *In vivo* techniques using immunosuppressed, immunodeficient or knockout mice have also been used for the isolation of *N. caninum* and for the production of tissue cysts (Dubey *et al.* 1998; McGuire 1997; Yamane 1997), but immunocompetent laboratory mice are generally resistant to infection although there is strain variation. On the contrary, gerbils (*Meriones unguiculatus*) are susceptible to infection with *N. caninum* without prior immunosuppression. Different *N. caninum* isolates have different proliferation rates and also the proliferation rate is dependent on the host cell type used (Hemphill *et al.* 1999). It has been observed that extracellular maintenance of *N. caninum* tachyzoites in the presence of growth medium for more than 4 hours may result in a rapid loss of infectivity (Hemphill *et al.* 1996).

Table 2.1: A summary of *N. caninum* isolates from different countries, hosts and sources

N. caninum Strain	Source	Host	Country	Reference
NC-WTDVA-1	Brain	Deer	USA	Vianna *et al.* (2005)
NC-WTDVA-2	Brain	Deer	USA	Vianna *et al.* (2005)
NC-WTDVA-3	Brain	Deer	USA	Vianna *et al.* (2005)
Nc-MalB1	Brain	Cattle	Malaysia	Cheah *et al.* (2004)
NcNZ 1	Brain	Cattle	New Zealand	Okeoma *et al.* (2004)
NcNZ 2	Brain	Cattle	New Zealand	Okeoma *et al.* (2004)
NcNZ 3	Brain	Cattle	New Zealand	Okeoma *et al.* (2004)
NcBrBuf-1	Brain	Water Buffaloes	Brazil	Rodrigues *et al.* (2004)
NcBrBuf-1	Brain	Water Buffaloes	Brazil	Rodrigues *et al.* (2004)
NcBrBuf-1	Brain	Water Buffaloes	Brazil	Rodrigues *et al.* (2004)
NcBrBuf-1	Brain	Water Buffaloes	Brazil	Rodrigues *et al.* (2004)
NcBrBuf-1	Brain	Water Buffaloes	Brazil	Rodrigues *et al.* (2004)
NC-6	Brain	Dog	USA	Dubey *et al.* (2004)
NC-7	Brain	Dog	USA	Dubey *et al.* (2004)
NC-8	Brain	Dog	USA	Dubey *et al.* (2004)
NC-Nowra	Brain	Cattle	Australia	Miller *et al.* (2002)
NC-Porto1	Foetus	Cattle	Portugal	Canada *et al.* (2002)
NC-Illinois	Brain	Cattle	USA	Gondim *et al.* (2002)
NC-6-Argentina	Faeces	Dog	Argentina	Basso *et al.* 2001
NC-Bahia	Tissues	Dog	Brazil	Gondim *et al.* (2001)
NC-PG1	Brain	Cattle	Italy	Floretti *et al.* (2000)
BT-3	Brain	Cattle	Japan	Sawada *et al.* (2000)

Table 2.1: A summary of *N. caninum* isolates from different countries, hosts and sources (contd)

KBA-1	Brain	Cattle	Korea	Kim *et al.* (2000)
KBA-2	Foetus	Cattle	Korea	Kim *et al.* (2000)
NC-LIV-B2	Foetus	Cattle	UK	Trees and Williams (2000)
NC-Beef	Brain	Cattle	USA	McAllister *et al.* (2000)
NC-Ger1	Brain	Dog	Germany	Peters *et al.* (2000)
NC-PV1	Brain	Cattle	Italy	Magnino *et al.* (1999)
NC-LIV-B1	Brain	Cattle	UK	Davison *et al.* (1999)
CN-1	Brain	Dog	USA	Marsh *et al.* (1998)
NC-4	Foetus	Dog	USA	Dubey *et al.* (1998)
NC-5	Foetus	Dog	USA	Dubey *et al.* (1998)
JPA-1	Brain	Cattle	Japan	Yamane *et al.* (1997)
NC-SweB1	Brain	Cattle	Sweden	Stenlund *et al.* (1997)
NC-Liv	Brain	Dog	UK	Barber *et al.* (1995)
BPA-1	Foetus	Cattle	USA	Conrad *et al.* (1993)
BPA-2	Foetus	Cattle	USA	Conrad *et al.* (1993)
BPA-3	Brain	Cattle	USA	Barr *et al.* (1993)
BPA-4	Brain	Cattle	USA	Barr *et al.* (1993)
NC-3	Brain	Dog	USA	Cuddon *et al.* (1992)
NC-2	Brain	Dog	USA	Hay *et al.* (1990)
NC-1	Brain	Dog	USA	Dubey *et al.* (1988)

2.4.1 *In vitro* Isolation

N. caninum has been isolated using *in vitro* techniques. Dubey *et al.* (1988) were the first to isolate and characterise *N. caninum.* Following the isolation of *N. caninum* by Dubey and his colleagues, other workers (Hay *et al.* 1990; Cuddon *et al.* 1992; Conrad *et al.* 1993; Barber *et al.* 1995; Stenlund *et al.* 1997; Dubey and Lindsay 2000; Miller *et al.* 2002) have isolated this organism around the world. Sawada *et al.* (2000) isolated *N. caninum* from the brain of a cow. A cow's brain was bioassayed into 3 nude mice. All mice developed paralysis and were euthanased 39-days pi. Parasites isolated from the brain of the nude mice were inoculated into Vero cell culture. *N. caninum* tachyzoites were discovered in the cell culture 39 days pi. *N. caninum* DNA was detected in the cell culture using *N. caninum*-specific PCR assay with primer pair Np21 and Np6.

Some further examples where *N. caninum* has been "isolated" include the following. Kim *et al.* (2000) reported two isolations of *N. caninum* from bovine foetuses. Foetal brains were bioassayed into Vero cell cultures. Tachyzoites of *N. caninum* were observed in the Vero cells 45 and 56 days pi respectively. Also, *N. caninum* DNA was detected in the cell culture using *N. caninum*-specific PCR assay with an oligonucleotide primer (COC-1 and COC-2) set (Ho *et al.* 1996). In addition, a *N. caninum*-specific internal probe (5'-AGTCAAACGCG-3') hybridised to PCR products while a *T. gondii*-specific internal probe failed to hybridise.

Stenlund *et al.* (1997) also characterised a bovine isolate of *N. caninum.* Brain homogenate of a calf was inoculated onto a Vero cell monolayer. Tachyzoites were observed in the cell culture 8 weeks pi and electron microscopy revealed parasites of approximately 5 x 1.3 μm.

Furthermore, Dubey *et al.* (1998) homogenised the brain and spine of mice previously infected with *N. caninum.* The homogenate was inoculated into human foreskin fibroblast (HS68) and tachyzoites were observed 58 days pi. *N. caninum* DNA was detected in cell culture using two *N. caninum*-specific PCR assays: Nc14-3-3 gene nested PCR assay (Lally *et al.* 1996) and Nc-5 PCR assay (Yamage *et al.* 1996) with improved primers Np21+ and Np6+ (Muller *et al.* 1996).

Also, Davison *et al.* (1999a) isolated *N. caninum* designated NC-LivB1 in Vero cell culture from the brain of a stillborn calf. The isolate was confirmed as *N. caninum* by immunofluorescence with specific antibodies and by internal transcribed spacer 1 (ITS1) sequence analysis. Likewise, McGuire *et al.* (1999) bioassayed the brains of domestic pigeons experimentally inoculated with *N. caninum* into Vero cell culture. Tachyzoite growth was noticed 28-30 days pi in the cell culture.

2.4.2 *In vivo* Isolation

Many researchers have isolated *N. caninum in vivo* using animal models. Sawada *et al.* (2000) isolated and characterised *N. caninum* from the brain of a 2-year-old dairy cow. Serologically, the cow had an IFAT titre of 1:1600. The brain of the cow was bioassayed for *N. caninum* in 3 nude mice. All 3 mice became emaciated and developed paralysis. Microscopically, these mice revealed systemic *N. caninum* infection with tachyzoites found in different organs when necropsied on the 46th day pi. The brain homogenates from these mice were subsequently inoculated into Vero cell culture.

Similarly, Canada *et al.* (2002) isolated and characterised *N. caninum* from the brain of a 4-month-old foetus from a dairy herd with endemic neosporosis. The brain homogenate of the foetus was inoculated intraperitoneally (1 ml/mouse), first into out-bred Swiss Webster mice given dexamethasone (10 μg/ml) in drinking water for 10 days. These mice were euthanased 4-days after inoculation and their peritoneal exudates were mixed with mouse sarcoma cells 2 ml Tg 180 ($2x10^5$ cell/μl) and inoculated intraperitoneally (1 ml/mouse) into another group of Swiss Webster mice also given dexamethasone (10 μg/ml). Mice were euthanased 4-days after inoculation and *N. caninum* tachyzoites were seen in peritoneal exudates of all 4 mice. Tachyzoites from the exudates reacted positively with anti-*N. caninum* antibodies and contained *N. caninum* specific DNA.

McGuire *et al.* (1999) experimentally inoculated domestic pigeons and Zebra finches intraperitoneally with *N. caninum* tachyzoites ($1x10^6$ NC-2 and NC-Liverpool). All pigeons and finches were euthanased 6-weeks after inoculation. They reported a

positive result for tissue culture, serology and histology for one or more pigeons while all Finches resisted infection. Also, tachyzoites from one or more pigeon(s) contained *N. caninum* specific DNA.

N. caninum tachyzoites were isolated and characterised by Dubey *et al.* (1998) from canine brain and spine homogenates. They inoculated Swiss Webster and Balb/c mice intraperitoneally with 0.5 ml of homogenate/mouse. Swiss Webster and Balb/c were given 2.5 mg of medroxyprogesterone acetate (MPA)/mouse prior to inoculation. In addition, Swiss Webster mice were given 2.5 mg of MPA/mouse 3 days prior to inoculation. Mice were euthanased 62-days pi and tachyzoites were observed in all animals. Furthermore, *N. caninum* DNA was detected in the brain tissues using two *N. caninum*-specific PCR assays: Nc14-3-3 gene nested PCR assay (Lally *et al.* 1996) and Nc-5 PCR assay (Yamage *et al.* 1996) with improved primers Np21+ and Np6+ (Muller *et al.* 1996).

Gondim *et al.* (2001) isolated and characterised *N. caninum* from the brain of a dog using cerebral tissue inoculated intraperitoneally and/or subcutaneously into Mongolian gerbils. Gerbils were euthanased 3-4 months later and brain removed for squash preparation and cell culture inoculation. Brain homogenate was inoculated into COS-1 Cell and tachyzoites observed at 7-15 days pi. Parasites were passaged into Vero and COS-1 cell cultures. DNA was isolated and amplified from tachyzoites in Vero cell using Np21/NP6 primer pair. Similarly, Basso *et al.* (2001) fed gerbils the faeces of a naturally infected dog. These authors' isolated *N. caninum* in cell culture and gamma-interferon knockout mice inoculated with brain homogenates of infected gerbils. Isolation was confirmed by detecting *N. caninum* specific DNA by PCR reaction. Also, Peters *et al.* (2000) isolated *N. caninum* from an infected dog by inoculating mice and cell culture with the brain and spinal cord of an infected dog in Germany. The isolate was designated NC-GER1.

Koyama *et al.* (2001) isolated *N. caninum* from the brain of a naturally infected sheep. They inoculated immunodeficient mice with the brain homogenate of the sheep. Isolation was confirmed by immunohistochemical staining with anti-*N. caninum* antibodies and by detecting *N. caninum* specific DNA by PCR reaction.

2.5 Molecular aspects of *N. caninum*

2.5.1 Genes and gene expression

Researchers have constructed and used *N. caninum* cDNA libraries (Lally *et al.* 1996; Payne and Ellis 1996; Asai *et al.* 1998; Louie and Conrad 1999; Ellis *et al.* 2000; Sonda *et al.* 2000; Atkinson *et al.* 2001; Keller *et al.* 2002; Bruno *et al.* 2004). cDNA facilitates the isolation and molecular characterisation of *N. caninum* genes.

A cDNA clone encoding a 14-3-3-protein homologue has been isolated and characterised (Lally *et al.* 1996). The 14-3-3 proteins are a class of proteins that may show a high degree of amino acid sequence conservation across several eukaryotic taxa. *N. caninum* cDNA may be used to isolate genes coding for antigens of *N. caninum* which may be recognised by the host immune system. A cDNA encoding a subtilisin-like serine protease (NC-p65) of *N. caninum* has been isolated (Louie and Conrad 1999) using full-length cDNA by 5'- and 3'- rapid amplification of cDNA ends (RACE). The NC-p65 consisted of 865 amino acids with a predicted signal sequence, a proposed pro-domain and an internal region of conserved repeats (Louie and Conrad 1999). Further analysis of these amino acids revealed that NC-p65 had homology to serine proteases of the subtilisin-like superfamily (subtilases) and a predicted active site made up of the catalytic residues, aspartate (Asp) 253, histidine (His) 309 and serine (Ser) 484. Antibodies to recombinant NC-p65 recognised multiple bands on *N. caninum* lysate immunoblots, but most intensely stained a 65 kDa band (Louie and Conrad 1999).

A GRA2 homologue from *N. caninum* has been isolated and characterised by screening a cDNA libraries derived from mRNA of tachyzoites of *N. caninum* (NC-Liverpool strain) with antisera from a cow naturally infected with *N. caninum* (Ellis *et al.* 2000). The DNA sequence of the GRA2 recombinant isolate predicted a significant protein sequence homology of the gene product to the 28 kDa (GRA2) antigen of *T. gondii,* indicating the antigenic similarity with *N. caninum.* The *N. caninum* gene coding for this 28 kDa antigen has an intron flanked by 2 exons and may be highly expressed in culture-derived tachyzoites (Ellis *et al.* 2000). Similarly, GRA1 (homologous to the GRA1 of *T. gondii*) and NCP20 genes encoding small polypeptides were isolated (Atkinson *et al.* 2001). NCP20 induces an IgG response in mouse and may be

recognised by IgG from a cow chronically infected with *N. caninum* (Atkinson *et al.* 2001).

Two cDNA clones named NcPI-H and NcPI-S (Bruno *et al.* 2004) were obtained from the *N. caninum* expressed sequence tag (EST) project. These clones have homology with the *T. gondii* serine proteinase inhibitor (serpin) gene, TgPI-1 and TgPI-2. NcPI-H may show premature stop codons, while NcPI-S may encode a 79 amino acid protein characterised by putative signal peptide and non-classical Kazal domain (Bruno *et al.* 2004). Interaction of recombinant NcPI-S and rabbit antiserum has been reported to recognise antigenic bands of 20, 30, 40 and 66 kDa in SDS-PAGE when whole parasite homogenate is used (Bruno *et al.* 2004). Type 1 nucleoside triphosphate hydrolase (NTPase; Ec 3.6.1.3) activity, previously thought to be restricted to the virulent strains of *T. gondii* has been identified in the cell extracts of *N. caninum* tachyzoites using cDNA (Asai *et al.* 1998). Southern blot of this genomic DNA and sequence analysis of the two independent NTP clones from the NC-1 strain revealed the presence of multiple genes.

N. caninum microneme protein (NcMIC1) that interacts with sulphated host cell surface glycosaminoglycans has been identified (Keller *et al.* 2002). NcMIC1 polypeptide sequence contains an N-terminal signal peptide of 20 amino acids (aa) followed by two tandem internal repeats of 48 and 44 aa, respectively. Integrated into each repeat is a CXXXCG sequence motif reminiscent of the thrombospondin-related family of adhesive proteins (Keller *et al.* 2002). Immunohistochemistry of mouse brains infected with tissue cysts showed that expression of NcMIC1 may be reduced in the bradyzoite stage (Keller *et al.* 2002). Elevation of host temperature to about 37°C may result in the secretion of NcMIC1. The removal of glycosaminoglycans from the host cell surface and modulation of host cell surface glycosaminoglycan sulfation has been reported to reduce the binding of NcMIC1 to the host cell surface (Keller *et al.* 2002).

N. caninum cDNA has also enabled the characterisation of the primary structures of Internal Transcribed Spacer (ITS) 1, ITS 2 and the 5.8S rRNA genes from *N. caninum* (Payne and Ellis 1996). The structure of the transcribed spacer region in *N. caninum* is typical of that found in most eukaryotic cells. The rDNA of a eukaryotic cell contains a tandem, head to tail repetitive sequence with the structure '5-IGS-18S rDNA-ITS1-5.8S

rDNA-ITS2-28S rDNA-IGS-3' (Payne and Ellis 1996). RNA polymerase 1 transcribes the repeat to produce a pre rRNA which is processed in the nucleolus to remove the internal transcribed spacer regions (ITS 1 and ITS 2, which are noncoding regions of DNA sequence) and intergenic spacer regions (IGS). The ITS regions may be involved in the processing of the pre rRNA (Campbell *et al.* 1987; van der Sande *et al.* 1992). This region has been successfully used to distinguish between closely related species and strains of *Neospora*, *Toxoplasma*, *Hammondia* and *Eimeria* (Ellis *et al.* 1998; Ellis *et al.* 1999; Mugridge *et al.* 2000; Dubey *et al.* 2001).

A plastid is a non-photosynthetic chloroplast-like organelle found in a wide variety of apicomplexan parasites, including *N. caninum* (Lang-Unnash *et al.* 1998; McFadden *et al.* 1997). The plastid is believed to be the target of a number of agents, including Rifampicin, that inhibit the growth of apicomplexans (Wilson and Williamson 1997). Rifampicin inhibits bacterial DNA-dependent RNA polymerase by binding to the beta subunit, which is encoded by the *rpo*B gene. *N. caninum* plastid DNA has been characterised (Gleeson and Johnson 1999) using pulsed-field gel electrophoresis and transmission electron microscopy (TEM). The unit length of the plastid of *N. caninum* is approximately 35 kb; however, there is evidence for the formation of oligomeric molecules, which may migrate as linear molecules in approximate multiples of the unit length (Gleeson and Johnson 1999). Four other plastid genes encoding the ssrRNA, lsrRNA, *rpo*C and tufA were isolated (Gleeson and Johnson 1999).

Gene expression has been studied in humans and yeasts using transcriptome analysis. Transcription of DNA into RNA and the subsequent translation of messenger RNA (mRNA) into protein are the basic mechanisms by which cells mediate their growth, function and metabolism. Transcriptome analysis is efficient in characterising the full complement of genes involved in the expression of specific biological functions. One of them is the Serial Analysis of Gene Expression (SAGE) technique. SAGE is a powerful tool that allows the analysis of overall gene expression patterns with digital analysis. It does not require a pre-existing clone and can be used to identify and quantitate new genes as well as known genes. SAGE technique consists of the construction of transcript libraries for a quantitative analysis of the entire genes expressed or inactivated at a particular step of cellular activation. Several authors' have applied SAGE in gene expression (Buckhaults *et al.* 2001; Zhang *et al.* 1997). The

SAGE approach could be used in comparing the total expressed genes in *N. caninum* infected and uninfected cows/calves; infected-aborting and infected-non-aborted cows; and the interaction between BVDV and *N. caninum*.

2.5.2 Host-Parasite Interaction

The study of host parasite interaction involves understanding the different types of parasite antigens and their interactions with the host. Knowledge of the interaction between *N. caninum* and its host will help improve our understanding of the ability of *N. caninum* to establish infection, survive within the host, be transmitted to new hosts especially *in utero* and to cause disease and abortion.

2.5.2.1 Antigens

Studies have been undertaken to identify and characterise specific antigenic components of *N. caninum* at the molecular level in order to enhance vaccine production, improve serological diagnosis as well as to enhance current views on the many unanswered questions concerning the cell biology of this parasite and its interactions with the host at the immunological and cellular level (Hemphill *et al.* 1999). Four antigens have been identified by immunoblot as predominant targets for the humoral immune response of *N. caninum*-infected animals. These antigens are 17, 29, 30 and 37 kDa (Barta and Dubey 1992; Bjerkas *et al.* 1994; Paré *et al.* 1995).

2.5.2.1.1 Surface Antigens (SAG)

Several antigens have been identified which could participate in host cell adhesion and/or invasion. These antigens are either expressed on the outer plasma membrane, or are only briefly localised on the surface, as they are expulsed from the secretory vesicle before, during, or after host cell invasion (Fuchs *et al.* 1999). Surface proteins of *N. caninum* are important because they start the interactions between the pathogen on one hand and host cell surface molecules and the host immune system on the other. The surface of the tachyzoite stage of *N. caninum* might be covered by a family of

glycosylphosphatidylinositol (GPI)-anchored antigens as for *T. gondii* (Hemphill *et al.* 1997b; Howe *et al.* 1998; Sonda *et al.* 1998). These antigens called SAGs (Surface antigens) and SRSs (SAG1-related sequences) are identified using *in vivo* [^{3}H] ethanolamine labelling followed by autoradiography (Fuchs *et al.* 1999). SAGs and SRSs antigens migrate at approximately 29 and 35 kDa respectively under native SDS-PAGE and have been referred to as p29 and p35 respectively (Howe *et al.* 1998). Furthermore, these antigens have been called Nc-p36 and Nc-p43 respectively because of their altered migration in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) when reduced with β-mercaptoethanol (Hemphill and Gottstein 1996; Hemphill *et al.* 1997b).

It has been reported that both p29 and p35 are tethered to the parasite surface membrane via a GPI anchor and that they are highly immunogenic because they elicit strong antibody responses in *N. caninum* infected animals (Howe *et al.* 1998). This immunogenic potential makes them ideal markers for diagnostic purposes (Howe *et al.* 1999). Similarly, SAGs can confer partial immunity in an animal model (Bulow and Boothroyd 1991; Khan *et al.* 1991), suggesting they play an important role in the parasite-host immune defence.

In addition, TgSAG1 has been shown to provide protective responses against fatal toxoplasmosis in mice (Bulow and Boothroyd 1991; Khan *et al.* 1991). Thus, NcSAG1 and NcSRS2 are possible vaccine candidates to protect against neosporosis (Howe *et al.* 1999). Vaccination with recombinant vaccinia virus carrying the NcSRS2 gene prevented infection with and vertical transmission of *N. caninum* in Balb/c mice (Nishikawa *et al.* 2000; Nishikawa *et al.* 2001 and Nishikawa *et al.* 2001). Furthermore, Howe *et al.* (1999) compared p29 (Nc-p36) and p35 (Nc-p43) primary sequences with *T. gondii* surface antigens and concluded that they are direct homologues of SAG1 (Burg *et al.* 1988) and SRS2 (Manger *et al.* 1998). Immunofluorescence of viable tachyzoites, as well as the immunoprecipitation of antigens extracted from tachyzoites revealed 19 kDa, 38 and 40 kDa surface antigens and that these antigens are in the outer surface membrane of *N. caninum* tachyzoites (Schares *et al.* 1999). Immunohistochemical analysis of infected tissue sections indicated that the 38 kDa surface antigens from tachyzoites appear to be absent in bradyzoites.

2.5.2.1.2 Dense Granule Antigens (GRA)

Dense granules are found in all apicomplexan parasites. They are secretory organelles whose contents are secreted into the lumen of the parasitophorous vacuole (PV) once they have completely invaded the host (Dubremetz *et al.* 1993). The dense granule protein is important for the establishment of proper functioning of the vacuole (Cesbron-Delauw 1994). The dense granule proteins are collectively involved in modifying the PV to allow for nutrient (purines and tryptophan) acquisition (Cesbron-Delauw 1994). Recombinant forms of the dense granule proteins GRA6 and GRA7 are the antigens used in an ELISA used to diagnose *N. caninum* infection (Lally *et al.* 1996).

By screening a cDNA expression library with bovine antisera, Lally *et al.* (1997) identified NCDG1 as a dense granule antigen of *N. caninum.* Immunogold labelling with polyclonal antiserum against recombinant NCDG1 indicates that the antigen is contained within the dense granules of *N. caninum* tachyzoites. In addition, Western blot analysis shows that the native protein migrates at approximately 33 kDa in native SDS-PAGE (Howe *et al.* 1999). NCDG1 is homologous to TgGRA7; 42% identity (Jacobs *et al.* 1998; Fischer *et al.* 1998) thus, NCDG1 is referred to as GRA7 (Howe *et al.* 1999). Liddell *et al.* (1998) identified NCDG2 in a similar manner to NCDG1. A comparison of NCDG2 with *T. gondii* proteins indicates an amino acid sequence identity of 34% (Lecordier *et al.* 1995); thus, NCDG2 is referred to as GRA6 (Howe *et al.* 1999). *N. caninum* nucleoside triphosphate (NcNTP) is a dense granule that was identified based on its similar enzymatic activity and antigenic cross reactivity to the nucleoside triphosphate hydrolase (NTPase) of *T. gondii* (Asai *et al.* 1998). In *T. gondii*, the dense granule proteins have been found to provide some protection against experimental *T. gondii* infection (Darcy *et al.* 1988; Duquesne *et al.* 1990).

2.6 *N. caninum*: Host cell recognition, attachment and invasion

N. caninum as an obligate intracellular parasite must gain entry into the host to cause infection (Figure 2.2). Tachyzoites are the invasive stage of *N. caninum.* They multiply and rupture cells and can infect and destroy a variety of cell types. In contrast to other

intracellular pathogens, which gain entry by inducing phagocytic/endocytic events, *N. caninum* enters the host cell by an active process (Dobrowolski and Sibley 1996; Hemphill *et al.* 1996). Once intracellular, they reside and proliferate within a membrane-bound parasitophorous vacuole (PV) that serves as an interface for nutrient acquisition while preventing interactions with host cell vesicular traffic (Howe *et al.* 1999). These functions are accomplished with the help of a large number of unique proteins. It is these parasite-specific proteins that provide form and function to the cytological structures and mechanisms that are important for their life style as intracellular parasites (Howe *et al.* 1999).

Host cell invasion is divided into adhesion onto the host cell surface and the host cell entry process. To invade the host cell, *N. caninum* attaches itself to the host membrane with its apical tip. This results in the secretion of adhesion proteins from micronemes which facilitates parasitic penetration of the host cell (Carruthers and Sibley, 1997). Adhesion is mediated largely through proteins from micronemes that are secreted at the onset of establishing contact with the host cell surface (Keller *et al.* 2002). NcMIC3 is a microneme associated-protein found in *N. caninum* tachyzoites and bradyzoites and a large portion of this protein is comprised of a stretch of four consecutive epidermal-growth-factors (EGF)-like domains.

This step is followed by the discharge of lipids and proteins from the rhoptries to form the parasitophorous vacuole. Together with lipids and proteins derived from the host cell membrane, they contribute to the formation of a new membrane, which encases the parasite in the parasitophorous vacuole compartment. After the parasite is fully enclosed in the parasitophorous vacuole membrane, dense granule proteins (GRA) are released from the dense granule organelles. These GRA proteins modify the lumen of the vacuole and its delimiting membrane. Within this parasitophorous vacuole, the parasite develops and replicates and having reached a critical mass within the parasitophorous vacuole; parasites egress the vacuole (which results in host cell lysis) to infect adjacent cells and spread to other tissues *via* body fluids and migrating cells (Hemphill 1999).

NcMIC3 may be secreted onto the tachyzoite surface immediately following host cell lysis in a temperature dependent manner (Naguleswaran *et al.* 2001). Following

secretion onto the surface, NcMIC3 may be translocated towards the posterior end of the parasite, which requires a functional actin microfilament system. NcMIC3 is a prominent component of Triton X-100 lysates of tachyzoites and co-sedimentation assays employing prefixed Vero cells shows that the protein binds to Vero cell surfaces (Naguleswaran *et al.* 2001).

N. caninum tachyzoites can bind to sulphated proteoglycans, which naturally occur on the surface of mammalian cells, including heparin/heparan sulphate, chondroitin sulphates as well as to the artificially sulphated glycosaminoglycan dextran sulphate. Removal of heparan sulphate from the host cell surface does not affect the binding of *N. caninum* tachyzoites whereas enzymatic removal of chondroitin sulphate A, B and C decreases *N. caninum* adhesion to Vero cells (Naguleswaran *et al.* 2002). Furthermore, experiments employing Triton X-100 solubilised NcSRS2 and NcMIC3 showed that NcSRS2 binds to the host surface cells but not through sulphated glycosaminoglycans. In contrast, Triton X-100 dramatically influences NcMIC3 binding to host surface cell.

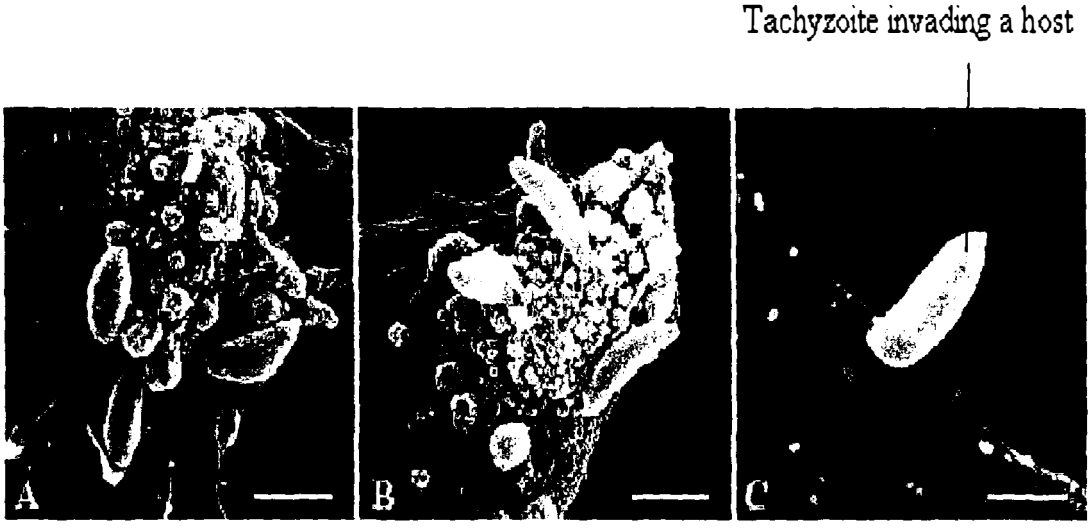

Figure 2.2: Electron micrograph of adherent and invading *N. caninum* tachyzoites in cell culture adapted from Hemphill *et al.* 2004. A: Tachyzoites adhering to the host cell; B: Reorientation of tachyzoites with the apical complex in contact with the host cell; and C: Tachyzoite invasion of the host cell. Photos shown to scale: Bars in A, B and C are 1400 nm, 1260 nm, 900 nm, respectively.

2.7 Pathogenesis of *N. caninum*

It is crucial to understand the pathogenesis of *N. caninum*. The outcome of neosporosis could be influenced by the host's genetic background. Research with CBA/CA and Swiss-White mice showed that these mouse strains were both resistant to *N. caninum* infection and that Th1 response was likely generated in both strains without a visible pathological finding (Chantal Rettigner *et al.* 2004). Some of the pathological effects induced by *N. caninum* include pancreatitis, radiculoneuritis, encephalitis, primary pneumonia and myositis (Lindsay *et al.* 1995).

The occurrence of cell destruction and subsequent disease would depend on the lack of ability of the host to defend itself from invading and penetrating parasites and a subsequent parasitaemia. Several authors' have reported some dissimilarity in pathogenesis among isolates from different regions. An isolate designated NC-LivB1 from the United Kingdom was found to be different from other bovine and canine isolates of *N. caninum* (Davison *et al.* 1999). Moreover, McGuire *et al.* (1997) showed that NC-Liverpool (UK) produced more tissue cysts than the NC-2 strain. In addition, Lindsay and Dubey (1990) reported differences in pathogenicity between NC-1 and NC-2 that are both isolates from the United States of America. They further stated that bradyzoites of the NC-2 isolate were more able to withstand treatment with pepsin-hydrochloride solution than were those of the NC-1 isolate.

Magnino *et al.* (2000) observed that pathological differences may exist between NC-PV1 (Italy) and other *N. caninum* isolates. In addition, Atkinson *et al.* (1999) reported that a mouse model for central nervous system (CNS) infection demonstrated marked differences in pathogenicity between isolates [NC-SweB1 (Sweden) and NC-Liverpool (UK)] of *N. caninum.* Differences observed in pathology, in response to infection in animal species, in *N. caninum* isolates and in different countries could in part be as a result of differences in the mode and route of infection, such as vertical versus horizontal infection; and different challenge routes such as oral; intranasal; intravenous; intraperitoneal; or subcutaneous.

N. caninum infection during pregnancy can lead to abortion, transmission or congenital disease. It has been shown that the first trimester of gestation is the time when the

foetus is most vulnerable. At this time, the thymus, spleen and peripheral lymph nodes are just beginning to form but during the second trimester, these tissues start to react to the presence of micro-organisms (Osburn 1986). In a pregnant cow, *N. caninum* infection in the first trimester of gestation could be fatal to the foetus but during the second and third trimesters, the foetus can begin to mount an immunologic defence (Williams *et al.* 2002; Andrianarivo *et al.* 2001; Barr *et al.* 1994). Also, abortion induced by *N. caninum* appears to be common in the second trimester of pregnancy because the foetus is not sufficiently immunocompetent and thus abortion or foetal mummification ensues.

2.8 Diagnosis

Numerous researchers (Thornton *et al.* 1991; Reichel and Drake 1996; Dubey *et al.* 1997; Cox *et al.* 1998; Patitucci *et al.* 1999; Reichel 1999; Tennent-Brown *et al.* 2000; Antony and Williamson 2003; Chahan *et al.* 2003; Hässig *et al.* 2003; Caetano-da-Silva *et al.* 2004; Canada *et al.* 2004; Naguleswaran *et al.* 2004; Lasri *et al.* 2004; Cabaj *et al.* 2005; Hall *et al.* 2005) have used serology to study the seroprevalence of *N. caninum* in cattle and dogs. Serology has been the main diagnostic tool for neosporosis. However, serology alone may not be adequate in all cases. Molecular techniques may complement serological tests for the diagnosis of *N. caninum* infection. Some of the diagnostic tools available for the diagnosis of neosporosis are PCR, Western blot, ELISA and IFAT.

In the study of neosporosis using serology, the IFAT (Dubey *et al.* 1988) is often used as a gold standard because infection studies using different *N. caninum* hosts have shown that the *N. caninum* IFAT shows little or no cross-reactivity with other apicomplexan parasites (Trees *et al.* 1993; Dubey *et al.* 1996). The IFAT was the first serological assay used for the demonstration of antibodies to *N. caninum.* The principle of the IFAT involves attaching intact *N. caninum* tachyzoites to microscopic slides, which are then incubated with diluted test serum, followed by incubation with fluorescein-labelled antibodies directed against immunoglobulins of the test animal which in the case of the present study is the cow. Positive and negative control sera samples are treated in the same way as the test sera and evaluated along with the test

sera under a fluorescence microscope. A sample is positive if it has unbroken peripheral fluorescence while cap staining or sole fluorescence of the apical part of the tachyzoites is regarded as a non-specific reaction. IFAT reagents are commercially available. The IFAT detects surface antigens thus requiring the use of whole and intact tachyzoites as the antigen for the assay. It has been shown that the surface antigens of the apicomplexian parasites are more specific than intracellular antigens (Hughes 1982; Björkman 1994; Packham 1998). A test is said to be sensitive if it correctly identifies infected animals and specific if it correctly identifies uninfected animals (Smith 1995). The presence of *N. caninum* antibodies in serum of animals indicates that the animal is, or has been infected. Exposure to neosporosis may also trigger antibody response from the infected animal.

The first ELISA for the study of neosporosis was developed for the analysis of canine sera (Björkman 1994). The ELISA is a specific and sensitive assay for the detection of *N. caninum* in sera of infected animals. This assay can use any antigen of choice (Harlow 1988) which is coated onto the plastic surface of multiwell microtitre plates and incubated for attachment. Samples to be analysed are added to the wells and incubated followed by the application of an enzyme-labelled, species-specific anti-immunoglobulin antibody called the conjugate. After incubation, a substrate is added which transforms to a coloured product in the presence of the conjugate. This is followed by another incubation period after which a stop solution is added to stop the enzyme-substrate reaction. Finally, the absorbance or O.D. is measured by a spectrophotometer. There are tests which apply "the change in absorbance". This change is measured and a maximum slope of O.D. over time (V_{max}) is calculated (Paré 1995). The ELISA test has been applied to the study of neosporosis by different workers (Björkman 1994; Paré 1995; Baszler *et al.* 1996; Lally *et al.* 1996; Dubey *et al.* 1997; Williams *et al.* 1997). Three ELISA formats exist and each format is described below:

Indirect format: In this format, the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labeled, anti-species globulin conjugate. The addition of an enzyme substrate-chromogen reagent causes colour to develop. This colour is directly proportional to the amount of bound sample antibody. The more

antibodies present in the sample, the stronger the colour development in the test well. This format is suitable for determining total antibody level in samples.

Blocking (Competitive) Format: Here, the specific sample antibody competes with and or blocks the enzyme-labelled, specific antibody in the conjugate. The addition of an enzyme substrate-chromogen reagent causes colour to develop. This colour is inversely proportional to the amount of bound sample antibody. The more antibodies present in the sample, the less colour development in the test well.

Antigen-Capture (Direct) Format: Here, the antigen in the sample is sandwiched between antibodies coated on the plate and an enzyme-labelled conjugate. The antibody conjugate can be either monoclonal or polyclonal. The addition of an enzyme substrate-chromogen reagent causes colour to develop. This colour is directly proportional to the amount of the target antigen present in the sample.

In addition to serology, diagnosis of neosporosis can be achieved by detecting parasite specific antigens using Western blot (Hamir *et al.* 1998; Schares *et al.* 1999; Chahan *et al.* 2003; Naguleswaran *et al.* 2004; Cabaj *et al.* 2005). Whereas ELISA and IFAT can measure antibody to whole parasite and give a "positive", "negative" or indeterminate test result, Western blotting is a more specific test. It allows one to visualize antibodies directed against each parasite antigen. For this reason, it may be a confirmatory test for a positive IFAT or ELISA test as is the case in the present study. In Western blotting, antigens are electrophoresed into a gel (SDS-PAGE). As the antigens migrate through the gel they are separated based upon size and charge. Characteristically, smaller antigens migrate through the gel faster than larger antigens. Separated antigens are then transferred to a solid membrane (Nylon membrane or PVDF) for Western blot analysis. For this procedure, an electric current is applied to the gel so that the separated antigens transfer through the gel and onto the membrane in the same pattern as they separate on the SDS-PAGE. All sites on the membrane which do not contain blotted antigen from the gel can then be non-specifically "blocked" so that antibody will not non-specifically bind to them, causing a false positive result. Often the membrane is cut into strips to facilitate testing of a large number of samples for antibodies directed against the blotted antigen.

To detect the antigen blotted on the membrane, a primary antibody is added at an appropriate dilution and incubated with the membrane. If there are any antibodies present which are directed against one or more of the blotted antigens, those antibodies will bind to the antigen(s) while other antibodies will be washed away at the end of the incubation. In order to detect the antibodies which have bound, anti-immunoglobulin antibodies coupled to a reporter group such as the enzyme alkaline phosphatase are added. This anti-Ig-enzyme is commonly called a "secondary antibody" or "conjugate". Finally after excess secondary antibody is washed free of the blot, a substrate is added which will precipitate upon reaction with the conjugate resulting in a visible band where the primary antibody bound to the antigen.

Researchers (Barta and Dubey 1992; Björkman and Hemphill 1998; Cole *et al.* 1994; Bjerkas *et al.* 1994; Baszler *et al.* 1996; Howe *et al.* 1998; Álvarez-García *et al.* 2002; Paré *et al.* 1995; Sonda *et al.* 1998; Hemphill and Gottstein 1996; Hemphill *et al.* 1996; Hemphill *et al.* 1997; and Sonda *et al.* 1998; Tomioka *et al.* 2003; Atkinson *et al.* 2000; Schares *et al.* 1998; Ashburn *et al.* 1998; Cazabonne *et al.* 1994) have applied Western blot techniques in the study of neosporosis in animals. *N. caninum* immunodominant antigens ranging between 16 and 80 kDa were identified by Western blot analysis (Barta and Dubey 1992) using polyclonal antibodies (pAbs). Sera from different animal species such as cattle, dogs, mice, rabbits, goats, sheep, pigs and foxes have reacted with *N. caninum* antigens of 17, 29, 30, 37 and 46 kDa (Barta and Dubey 1992; Bjerkas *et al.* 1994). Western blot has also been used in the diagnosis of *N. caninum* induced abortion. Sera from cows with confirmed *N. caninum* induced abortion have been shown to recognise *N. caninum* antigens with a molecular weight of 25, 65 and 116 kDa (Baszler *et al.* 1996).

Besides the detection of parasite specific antigens, parasite specific DNA can also be detected in infected samples using polymerase chain reaction (Table 2.2). PCR is a powerful method used to exponentially amplify *in vitro* a small amount of a specific nucleotide sequence in the presence of template sequence (region of interest). Also required for the conduct of PCR are two oligonucleoetide primers that hybridize to opposite strands and flank the region of interest, thermostable DNA polymerase, deoxynucleotide triphosphate (dNTP), salts and water. The PCR reaction is cycled in a thermocycler. The reaction is a three step process (cycles) with each occurring at a

different temperature. The cycles involve; DNA (template) denaturation, which separates the double DNA strands into single stands. The second step is primer annealing, which enables the primers to hybridize to their complementary sequence on the target DNA. The last step is the extension of annealed primer by DNA polymerase. The PCR cycle is repeated 20 to 40 times, resulting in about 1 million fold amplification of the DNA fragment of interest. Different types of PCR (Standard, Multiplex, Nested, Reverse transcription and real-time) have been used by research workers in the study of neosporosis and some of these were applied in this research.

Standard PCR methodology involves the enzymatic amplification of the DNA sequence of interest that is less than 5 Kb in length. This method uses only one oligonucleotide primer pair. This system is useful for a variety of applications like DNA sequencing, cloning and detection of mutation. Multiplex PCR method enables the simultaneous amplification of more than one target region of interest in one reaction by using more than one pair of oligonucleotide primers. This PCR variant was first described by Chamberlain *et al.* (1988). Reverse transcription PCR (RT-PCR) is a technique used to detect the degree to which genes are expressed. This PCR variant uses the same principles as the standard PCR but the template is a complimentary copy of mRNA called cDNA. By starting with mRNA, RT-PCR measures only the expressed DNA. Real-time PCR is yet another variant of PCR that can perform relatively accurate and reliable measurements to quantitatively determine the presence of a specific nucleic acid (DNA, RNA) sequence. This technique enables a researcher to monitor in real-time the amplification of PCR products. Fluorescent monitoring of DNA amplification is the basis of real-time PCR. Reactions are characterised, based on the point in time during cycling when amplification of a PCR product is first detected rather than the amount of PCR product accumulated after a fixed number of cycles. The real-time PCR assay uses different formats like the SYBR Green 1 and *Taq*Man Probes (5' Nuclease Assay) as explained below.

The *Taq*Man probe (Holland *et al.* 1991; Livak *et al.* 1995) is a technology (ABI patented) that uses 5' Nuclease activity of the *Taq* enzyme to cause the dissociation of a fluorescent molecule from a specific quencher. Usually, the probe is a sequence of 25-30 nucleotides in length that is labelled with a reported dye at the 5' end and a quencher at the 3' end. During amplification, the probe binds to its target sequence and is cleaved

by the 5' nuclease activity of *Taq* DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. The cleavage separates the quencher from the reporter dye and restores fluorescence. The advantage of the *Taq*Man probes over SYBR Green is that specific hybridisation between probe and target DNA sequence is required to generate a fluorescent signal. In addition, the fluorescence probe can be labelled with different reporter dyes for use in a multiplex PCR assay without melting curve analysis. The *Taq*Man technology is provided with a primer and probe design software for quantitative PCR. However, it is more expensive than SYBR Green 1 and more time consuming.

The SYBR Green method (Morrison *et al.* 1998) is based on the use of dyes (SYBR Green 1) that emits fluorescent light when intercalated into double-stranded DNA (dsDNA). SYBR Green 1, when unbound, exhibits very little fluorescence which is enhanced upon intercalating with DNA. The SYBR Green technique is advantageous because it is a cheap, simple and reliable method for monitoring PCR amplicons. However, it does not discriminate between the different dsDNA molecules in a PCR reaction and as a result, the formation of non-specific amplicons, as well as primer dimers, must be prevented by accurate primer design and reaction condition optimisation. At the end of the reaction, products of different length and/or sequence can be observed as distinct fluorescent peaks by heating and cooling the reaction from 30°C to 95°C whilst continuously monitoring the fluorescence (melting curve analysis). The melting curve is plotted as derivative of fluorescence versus temperature and the point at which dsDNA melts will appear as a peak. A single symmetric peak indicates an optimized reaction, which in turn represents the specific PCR product. Melting curve analyses enables the use of SYBR Green 1 in multiplex PCR assays since different amplicons have different melting points and different melting curves. On the whole, with proper optimization, both SYBR Green 1 and *Taq*Man probes have the same detection range and accuracy (Nitsche *et al.* 1999).

The choice of target region (sequence) and method of PCR amplification depends on the questions to be addressed. For the purposes of diagnosis, nuclear ribosomal DNA (rDNA) is a useful target to amplify (Gasser 1999). Researchers have used PCR for the study of *N. caninum* and neosporosis (Table 2.2). A real-time PCR assay that uses the double-stranded DNA-binding dye SYBR Green 1 to monitor product formation using

oligonucleotide primers that amplify a 76-bp DNA fragment corresponding to the Nc5 sequence of *N. caninum* has been developed (Collantes-Fernandez *et al.* 2002). This technique is used for quantitative detection of *N. caninum* in infected host cells and tissues. A similar method could be used to quantify the 28S rRNA host (cattle, dog and mouse cell cultures) gene for comparison of parasite load in different cell cultures and animal models and to correct for the presence of potential PCR-inhibiting compounds in DNA samples.

Yamage *et al.* (1996) designed 5 sense (Np1, Np3, Np5, Np7, Np21) and 4 antisense (Np2, Np4, Np6, Np8) oligonucleotides for a sensitive and specific PCR based on an *N. caninum*-specific DNA fragment they cloned. They reported that among 19 combinations of sense and antisense primers, the Np21/Np6, Np21/Np4 and Np7/Np6 primer pairs were found to generate specific single bands in the presence of at least 10 pg genomic parasite DNA as a template. The Np21/Np6 primer pair was able to detect a single tachyzoite in the background of DNA derived from 2 mg of brain tissue. This therefore indicates that PCR with the primer pair Np21/Np6 could provide an efficient tool for epidemiological studies using brain tissues obtained at necropsy (Yamage *et al.* 1996). Similarly, Ho *et al.* (1996) described a PCR approach that uses universal primers in amplification of the 18S rDNA. *N. caninum* and *T. gondii* products were identified by the use of species-specific, chemiluminescent DNA probes. Subsequently, Ho *et al.* (1997) used this PCR to investigate the tissue distribution of *N. caninum* in experimentally infected cows and rhesus macaques (Ho *et al.* 1997).

Ellis *et al.* (1998) compared the Isu rDNA sequences of *N. caninum* and *T. gondii* and reported that the D2 expansion was the most variable in nucleotide sequence. Subsequently, they developed a PCR based on the Isu rDNA. This PCR is specific for *N. caninum* but not for *T. gondii* or *H. hammondi* because the Isu rDNA sequences of *H. hammondi* and *T. gondii* are similar in the D2 domain. In addition, a PCR based on the ITS region of the rDNA was developed (Holmdahl and Mattsson 1996) for the differentiation of *N. caninum* from *T. gondii*.

Šlapeta *et al.* (2002) reported molecular isolation of *N. caninum* DNA from dog faeces in the Czech Republic. A diagnostic PCR with primers Np6+ and Np21+ was performed and the yield was ~350 bp abundant product. An additional band of ~370 bp

that was significantly less abundant was also amplified. The 350 bp amplicon is characteristic for the presence of the *N. caninum* DNA with this primer pair. Brindley *et al.* (1993) and Ellis *et al.* (1994) demonstrated that diagnosis by *Dde1* of PCR products obtained from 18S rDNA of *N. caninum* after riboprinting (to establish the real existence of nucleotide differences in the 18S rDNA) produced profiles of restriction fragments that could be used to identify it. The *Dde1* site is located at the 5' end of the gene and therefore rDNA can be PCR amplified by primers such as AP1 and A (Ellis *et al.* 1994). Subsequently, the PCR product is purified, digested by *Dde1* and the products resolved on a gel. This protocol is limited in its usefulness as a diagnostic tool because of the generation of PCR products from DNA of dogs, cattle and other host organisms (Ellis 1998).

Ho *et al.* (1996) described an alternative PCR approach using universal primers that amplify 18S rDNA. A *N. caninum* PCR product was identified by species-specific, chemiluminescent DNA probes. This protocol was subsequently used to investigate the tissue distribution of *N. caninum* in naturally and experimentally infected cows (Ho *et al.* 1997). PCR based on 18S rDNA detection have found use in investigations on the biology of *N. caninum* but are not suitable for veterinary research because of the high sequence homology that exist between rDNAs of *N. caninum* and *T. gondii* (Ellis *et al.* 1994; Holmdahl *et al.* 1994). Ellis *et al.* (1997) investigated the development of a single tube, nested PCR using internal transcribed spacer (ITS) targeted primers. This approach employs amplification by first external and then nested primers in a single tube in order to enhance the sensitivity and specificity of an ITS-based PCR for the detection of *N. caninum*. This protocol should reduce the number of false positives or negatives produced during PCR diagnosis (Ellis 1998).

Louie *et al.* (1999) cloned, characterised and expressed two recombinant *N. caninum* proteins (N54 and N57) for the development of an enzyme-linked-immunosorbent assay (ELISA) for use in the serodiagnosis of neosporosis in cattle. These proteins were 29 and 20 kDa respectively when expressed as histidine fusion proteins from the pRSET expression vector. These recombinant-protein-based ELISAs have higher sensitivities (95% for N54 and 82% for N57) and higher or similar specificities (96% for N54 and 93% for N57) when compared with a whole-tachyzoite lysate-based ELISA thus suggesting that recombinant-protein-based ELISAs are a better tool for serodiagnosis of

N. caninum infection. Recombinant NcSRS2 expressed in insect cells by a baculovirus was used to establish a highly specific and sensitive ELISA method for the serodiagnosis of neosporosis (Nishikawa *et al.* 2001). This is because, NcSRS2 is a predominant antigen recognised by antisera from *N. caninum* infected animals and it is conserved in all isolates of *N. caninum* (Howe *et al.* 1998).

Table 2.2: Some primers and probes used in the study of *N. caninum*

Organism	Amplification Site/amplicon	PCR Type	Primer name and sequence	Reference
N. caninum	76-bp	Real-time PCR	Nc5 sequence forward primer (5'-AACAATGCTTCGCAAGAGGAA-3') nucleotide 248-257 and reverse primer (5'-AACAATGCTTCGCAAGAGGAA-3') nucleotide 303-323.	Collantes-Fernandez *et al.* 2002
Cattle, dog, mouse	28S rRNA gene	Real-time PCR	28S-PF (5'-TGCCATGGTAATCCTGCTCA -3') and 28S-PR (5'-CCTCAGCC AAGCACATACACC-3')	Collantes-Fernandez *et al.* 2002
N. caninum	DNA	Standard PCR	Sense primer Np1(5'-CCGGAGAATGAGAGCGATT-3'), Antisense primer Np2 (5'-CGCTCTGCAAACCCATCTA-3')	Kaufman n *et al.* 1996
N. caninum	DNA	Standard PCR	Np21+ (5'CCCAGTGCGTCCAATCCTGTAA C3') Np6+ (5'CTCGCCAGTCAACCTACGTCTT CT3') primer pair	Šlapeta *et al.* 2002, Yamage *et al.* 1996, Müller *et al.* 1996

Table 2.2: Some primers and probes used in the study of *N. caninum* (contd)

Organism	Amplification Site/amplicon	PCR Type	Primer name and sequence	Reference
Bovine	156-bpDNA fragment for the identification of false –ves	Multiplex PCR	Np4-Np7 and PRL HL033-HL035 primer pair, HL033 (5' CGAGTCCTTATGAGCTTGATT CTT3') HL035 (5' GCCTTCCAGAAGTCGTTTGTT TTC3')	Baszler *et al.* 1999
N. caninum	213 bp fragment of the Internal transcribed spacer 1(ITS1)	Standard PCR	Oligo primers for 1st ampli. = NN1 (5'TCAACCTTTGAATCCCAA) and NN2 (5'CGAGCCAAGACATCCATT). Oligo primers to amplify a 213 bp fragment for 2nd amplify NP1 (5'-TACTACTCCCTGTGAGTTG) and NP2 (5'-TCTCTTCCCTCAAACGCT)	Pereira-Bueno *et al.* 2003
N. caninum	Nuclear small-subunit rRNA (nss-rRNA) gene	Standard PCR	Oligo primers (COC-1[5'AAGTATAAGCTTTTATACG GCT-3'] and COC-2 [5'-CACTGCCACGGTAGTCCAATA C-3']); PROBE specific for N.c on the basis of nucleotide at position 254 = (5'AGTCAACGCG-3') = thymine base.	Ho *et al.* 1996

Table 2.2: Some primers and probes used in the study of *N. caninum* (contd)

N. caninum	NCGRA1 = 20 kDa, 125 bp(intron), open reading frame (ORF) was PCR amplified		P24F (5'TCAAGCGCCTACCTTGTT 3') and P24R (5'CCGTCTACACATAATGC G3'), PTrcHisp24F (5'ACGGATGGATCCTATGC TAGGTGGCGGGCG3') and pTrcHisp24R (5'TACCGAGAATTCCGCTA ACCCATGCCGTCG3'), pTrcHisFwd (5'GAGGTATATATTAATGT ATCG3')	Atkinson *et al.* 2001
N. caninum	18S rDNA gene	*Dde1*	AP1 (5'-CCATCCTAATACGACTCAC TATAGGGC –3') and A	Ellis *et al.* 1994
	18S rDNA gene	Chemilumines-cent DNA probes	Universal primers	Ho *et al.* 1996
N. caninum	NcSRS2	Standard PCR	5'TCGAATTCAAACATGGCG ACGCATGCTTG-3' and 5'-AAGGATCCAATGTTTCCTC GGGCAGTG-3'	Nishikawa *et al.* 2001

Table 2.2: Some primers and probes used in the study of *N. caninum* (contd)

N. caninum	NcSAG1	Standard PCR	5'GCGAATTCTCAGATGTTTCCTC GGGCAGTG-3' and 5'-TTGGATCCTCACGCGACGCCAGC CGCT-3'	Nishikawa *et al.* 2001
N. caninum	RNA polymerase C subsection (1013bp)	Standard PCR	rpoCR2:5'-CCCATTTGATCTCCATCAAAATC AGC-3', rpoCRVS:5'-AGCTACTCCTATTGCCCATAAAT GGT-3'	Fichera and Roos, 1997; Gleeson and Johnson 1999
N. caninum	Plastid encoded ssrRNA gene subsection (1020 bp)	Standard PCR	MG1: 5'-GTGCCAGCAGCCGCGGTAATAC-3', MG2:5'-TACGGCTACCTTGTTACGACTTC A-3'	Egea and Lang-Unnasch 1995.
N. caninum	Nuclear encoded ssrRNA gene subsection (64 bp)	Standard PCR	8: 5'-TTTGACTCAACACGGG-3', AP2: 5'-CCCGGGATCCAAGCTTGATCCTT CTGCAGGTTCACCTAC-3'	Sogin and Gunderson 1986; Medlin *et al.* 1988

2.9 Vaccination

One of the most effective means of preventing and controlling neosporosis would be by vaccination but developing an effective vaccine presents several challenges. The principal mechanism for protection against neosporosis appears to be via the induction of IL-12 and IFNγ (Khan *et al.* 1997; Lundén *et al.* 1998; De Marez *et al.* 1999; Nishikawa *et al.* 2001; Teixeira *et al.* 2005) so effective vaccines may be aimed at stimulating these 2 cytokines. Antigens of molecular weight ≤30 kDa would be suitable candidates for incorporation into a *N. caninum* vaccine because this group of antigens stimulates the proliferation and production of IFNγ by $CD4^+$ T cell lines raised from *N. caninum*-infected cattle (Lundén *et al.* 1998). Furthermore, *N. caninum* surface antigens, dense granule antigens, micronemes and rhoptries antigens are also prospective vaccine candidates because they may be responsible for parasite survival and development in the host (Nishikawa *et al.* 2001). In mice, endogenous IL12 is important for host protection against neosporosis (Khan *et al.* 1997). Furthermore, administration of antibody to IL12 and/or *in vivo* depletion of IFNγ was able to reverse natural protection such that the host succumbed to infection with *N. caninum* (Khan *et al.* 1997). Similarly, IL-12 and IFNγ are important for host immunity to *T. gondii* (Gazzinelli *et al.* 1993; Khan *et al.* 1994; Hunter *et al.* 1995).

There are many different types of vaccines to prevent infectious diseases. Some may be broadly categorised as: killed, modified live and chemically or physically altered vaccines with each having advantages and disadvantages. A killed vaccine has been developed for the prevention of *N. caninum*-induced abortion. It has the following advantages over all others. It cannot revert to the virulent form of the disease, has little risk of inducing abortion and the vaccine organism does not spread to other animals. The killed vaccine is stable in storage and is an excellent stimulant of passive antibodies in colostrum. Despite the listed advantages, the killed vaccine has its pitfalls. It may not provide a long-lasting immunity to the animal and can cause allergy and vaccination reactions. The killed vaccine may not work in the presence of passive colostral immunity.

Modified live and chemically altered vaccines share most of their advantages and disadvantages. They provide more rapid protection and are generally less expensive than killed vaccines. Modified live vaccines have the potential to revert to virulent

forms and this may be exacerbated in immunosuppressed animals. Both the modified live and altered vaccines, being live organisms, have some risk of inducing abortion in animals.

Several researchers (Choromanski and Block 2000; Nishikawa *et al.* 2001) have reported on the use of *N. caninum* vaccine in preventing infection, abortion and congenital infection. The *N. caninum* killed vaccine derived from tissue culture grown *N. caninum* tachyzoites was shown to be safe. The vaccine produced significant antibody titres in vaccinated cows. Peak antibody titre was also detected 28 days after a booster vaccination among cows vaccinated with *N. caninum* vaccine formulated with Bay R1005 adjuvant (Choromanski and Block 2000). The first commercial licence for *N. caninum* vaccine called NeoGuard (a havlogen-adjuvanted killed vaccine) was issued to Intervet Inc. (Merriam, KS, USA) by the United State Department of Agriculture (USDA) in 1998. This vaccine was developed to reduce abortion in cows. When applied to pregnant cows that had been experiencing multiple abortions, it was reported to reduce the rate of abortion in the herd (Choromanski *et al.* 2001).

A live vaccine developed from recombinant vaccinia viruses carrying NcSRS2 and NcSAG1 (vv/Nc-p43 and vv/Ncp36, respectively) genes, was tested in a mouse model for the prevention of *N. caninum* infection. More effective protection against vertical transmission was recorded when mice were vaccinated with vv/Nc-p43 than with vv/Nc-p36. Moreover, cellular immune response and antibody production against *N. caninum* were enhanced using vv/Nc-p43 (Nishikawa *et al.* 2001). In their study, (Andrianarivo *et al.* 1999) reported higher ($P<0.05$) IFAT titres in experimentally infected cattle than in cattle immunised with vaccines formulated with different adjuvants.

2.10 Neosporosis in New Zealand

N. caninum has been identified as a major cause of abortion in cattle in a number of countries including New Zealand. A major route of transmission of *N. caninum* is transplacental, resulting in abortion or birth of congenitally-infected progeny (Dubey and Lindsay 1996). Most investigations of outbreaks of neosporosis in cattle herds in

New Zealand and some from overseas suggest an external point-source of infection (Thornton *et al.* 1991; Thornton *et al.* 1994; McAllister *et al.* 1996a; Pfeiffer *et al.* 1998; Thornton 1998; Wouda *et al.* 1998; Patitucci *et al.* 1999). Horizontal transmission through ingestion of oocysts causes infection in cattle and dogs (De Marez *et al.* 1999; Trees *et al.* 2002). Dogs can act as definitive hosts for *N. caninum* (McAllister *et al.* 1998; Lindsay *et al.* 1999) and natural infection has now been confirmed (Basso *et al.* 2001a). Researchers in New Zealand have reported the occurrence of neosporosis in both bovine and canine families. In a retrospective study, Patitucci *et al.* (1997) reported three cases of *N. caninum* infection in dogs that showed ataxia and progressive hind limb paralysis due to multifocal non-suppurative meningoencephalomyelitis which was most severe in the spinal cord and base of the brain stem. They concluded that although *N. caninum* abortion in cattle has only recently been recognized in New Zealand, neosporosis has been present in dogs since at least 1972.

Riechel *et al.* (1998) reported neosporosis in a 13-week-old female boxer pup suffering from rigidity of the left hind leg. They stated that the pup had an IFAT titre of 1:51,200 when tested for anti *N. caninum* antibodies. Antony and Williamson (2003) investigated the seroprevalence of *N. caninum* infection in populations of dogs from dairy farms, sheep/beef farms and urban areas in the central part of New Zealand. They observed that the relative risk of having a titre of ≥1:200 to *N. caninum* was 2.43 for dairy-farm dogs and 3.16 for sheep/beef-farm dogs. At a titre of ≥1:200, the seroprevalence of *N. caninum* infection in dogs was 30.7%, 74.5% and 96.8% in urban, dairy-farm and sheep/beef-farm dogs respectively (Antony and Williamson 2003). In another study, the seroprevalence of *N. caninum* among randomly selected canine serum samples at a titre of 1:200 was 9% (Reichel 1998). Neosporosis appears to have a lower prevalence in the New Zealand beef cattle population than in the dairy cattle population since the prevalence of *N. caninum* seropositive beef cattle was 2.5% (n = 120), 3.6% (n = 166) and 2.3% (n = 213) at 3 different slaughter sites with the overall prevalence being 2.8% (Tennent-Brown *et al.* 2000).

Thobokwe and Heuer (2004) investigated the causes of abortion in dairy herds across New Zealand and reported that *N. caninum* caused 35% of abortions in New Zealand dairy farms. They further stated that *N. caninum* was suspected to have caused 33% of

abortions in herds with low abortion rates and 46% in herds with high abortion rates. Abortion risks are 2.2 times higher in seropositive cows in New Zealand than their seronegative counterparts (Pfeiffer *et al.* 2002). Percentages of *N. caninum* seropositive animals may reach 32% in calves, 3% in heifers, 31% in younger cows and 27% in older cows (Patitucci *et al.* 1999). High serological titres were observed in cows within weeks of *N. caninum* induced abortions (Cox *et al.* 1998; Reichel and Drake 1996). Laboratory investigation of the cause of abortion has shown that *N. caninum*-like lesions were seen in the brains of 28% of 320 aborted foetuses, 10% of the hearts and 34% of the placentas (Thornton *et al.* 1991).

The economic impact of neosporosis has been estimated in California to be US$35 million/year (Dubey 1999). In Australia, neosporosis costs the dairy industry an estimated AUS$85 million per annum and AUS$25 million/annum for the beef cattle industry (Ellis 1997). In New Zealand, the economic impact of neosporosis to the dairy industry was reported to be NZ$17.8 million/annum (Pfeiffer *et al.* 1998). Economic losses due to neosporosis have mostly resulted from abortion which impacts on cattle population, milk and meat production.

Research on *N. caninum* and neosporosis in New Zealand has been impeded by lack of isolated parasites in culture. Absence of a local isolate has restricted the opportunity to conduct *in vivo* studies, especially in the field. A New Zealand isolate is necessary to undertake studies to elucidate the pathogenesis of *N. caninum*. A local strain would help to further study the identification and characterisation of tachyzoite antigen(s) that may be useful for the diagnosis of *N. caninum*. The characterization of the genes and proteins of a New Zealand isolate of *N. caninum* would provide insight into factors that determine *N. caninum* host restriction, cell preference and virulence. This would assist in answering the question "Does *N. caninum* in New Zealand differ from existing overseas isolates?"

2.11 Conclusion

Infection with *N. caninum* in New Zealand appears to be endemic, widespread and subject to infrequent herd epidemics. This makes isolation and characterisation of *N.*

caninum in this country a matter of great significance. Research into *N. caninum* and its effects on New Zealand's animals and the economy has been impeded due to lack of an isolate. One of the benefits of obtaining a local isolate is the ability to conduct *in vivo* studies and compare a New Zealand isolate with those from overseas. A local isolate would also enable studies that may provide insight into factors that determine host restriction, cell preference and virulence. Once these factors are known, efficient strategies for control, prevention and diagnosis of neosporosis will be more readily developed. Control and prevention techniques for neosporosis will depend mainly on knowledge of infection risk experienced by cattle: beginning with exposure as a foetus and continuing through calfhood to productive adulthood through postnatal infection.

An improved knowledge of the factors that predispose to infection of cattle neosporosis will better enable farmers to manage the risk from neosporosis in order to help protect farm profitability. Better knowledge of some of the risks as reviewed above will help researchers to develop efficient control and prevention modules. Above all, the application of molecular techniques in the study of *N. caninum* will not only enhance our knowledge of the parasite but will also shed more light on what happens in the cow during the different stages of infection. Molecular techniques will also help in the development of better diagnostic tools for *N. caninum* infection like the use of blood samples for the PCR amplification of tachyzoite DNA.

The objectives of the research described in this thesis were:

- To experimentally investigate the dynamics of *N. caninum* infection in naturally infected cows challenged with BVDV.
- To use molecular techniques to study the dynamics of neosporosis in naturally infected cattle.
- To isolate and characterise a New Zealand isolate of *N. caninum.*

This project was performed as a sequence of studies and as a result is presented in the form of a series of discrete papers combined to form the thesis. The references in all papers have been combined into a single list of references. Published papers are in the appendix.

Chapter 3

Bovine viral diarrhoea virus: Isolation, infection and amplification of viral 5' untranslated region (UTR) by Reverse Transcription-Polymerase Chain Reaction from acutely infected calves

3.0 Abstract

Blood from an 8-month-old heifer that was persistently infected with bovine virus diarrhoea virus (BVDV) was used for the isolation of a wild-type strain of non-cytopathic BVDV type 1 virus. The isolate was identified in the source heifer and in cell culture by ELISA and PCR and was thereafter used to challenge two 8-month-old female calves. These calves were inoculated intravenously with 10 ml of a cell suspension containing 2.7 x 10^5/ ml of BVDV grown on bovine embryonic lung cells (BELU). Blood samples were collected from both calves for antigen ELISA and reverse transcription polymerase chain reaction (RT-PCR) tests.

Clinical observations were carried out daily for 13 days. On days 1 and 2 post-challenge, both calves had a BVDV sample to positive ratio on BVDV antigen ELISA test that was close to the cut-off value that was indicative of infection, but were PCR positive for BVDV and continued to be PCR positive up to day 13 post-inoculation. PCR products were sequenced and the BLAST returned the best score with BVDV.

3.1 Introduction

BVDV is a pathogen that is involved in many disease processes that affect cattle. BVDV is in the family *Flaviviridae* (Wengler *et al.* 1995) and is a small positive stranded RNA virus with a genome of approximately 12.5 kb (Collett *et al.* 1988). Related viruses include the hog cholera virus and border disease virus. BVDV can be classified into two main genotypes namely genotype 1 (BVDV1) and (BVDV2) genotype 2 (Donis 1995). This classification is based on the nucleotide sequence of the BVD virus (Dubovi 2002). There are strain differences within each genotype of BVDV that are expressed in their ability to cause disease (Donis 1995). The genotypes express variable virulence and have different antigenic properties. Genotype 2 isolates may differ from Genotype 1 isolates in the processing of the E2 glycoprotein. Variation in the 5' UTR region is similar for each genotype (Ridpath *et al.* 2000).

There are two biotypes of BVDV termed cytopathic (CP) and non-cytopathic (NCP). Most BVDV wild-type strains are non-cytopathic (Donis 1995). The biotypes are distinguished from each other by a phenotypic characteristic (the ability to cause cell lysis), which can be identified either *in vivo* or *in vitro* (Dubovi 2000). The CP biotype is thought to be a mutant of the NCP biotype. It was once thought that the CP biotype was more virulent; however Dubovi (2000) concluded that the biotypic classification of BVDV has nothing to do with virulence. The perceived virulence of the CP biotype was based on its activity on cultured cells. While the CP biotype can cause cell lysis *in vitro*, the NCP biotype does not. The NCP biotype produces a protein referred to as p125. This viral protein is cleaved in CP biotypes to give two protein products namely p54 and p80. The p80 protein is responsible for its cytopathic effects in mucosal disease (Misra 1998).

Bovine Viral Diarrhoea is caused by the infection of an immunocompetent animal with BVD virus. BVDV infection can cause one or a combination of the following; immuno-suppression, abortion, congenital infection and/or persistent infection (PI), repeat breeding, thrombocytopenia, enteric diseases, respiratory diseases, lameness and fever (up to 41°C). Type 1 BVDV infection is usually mild and sub-clinical and infection resolves as the animal's immune system mounts an appropriate response. This makes diagnosis of acute infection difficult and farmers are often unaware that infection

has occurred. BVDV type 2 is more virulent, can cause peracute and fatal diarrhoea with a morbidity rate of about 40% and mortality rate of about 20% (Radostits 2000). BVDV of both types has a marked immunosuppressive potential that may enhance the pathogenicity of other diseases like *N. caninum* (Bjorkman 2000) and pneumonia (Baker 1995). BVDV can be diagnosed by serology, PCR and virus isolation throughout.

The aim of this study was to test the infectivity of a wild-type strain of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer to confirm its suitability for use as an inoculum in a longitudinal challenge experiment aiming to test for interactions between BVDV and *N. caninum* in causing abortion. This test of infectivity was carried out by challenging BVDV antibody and antigen negative heifers with the isolate, then monitoring the heifers clinically and using RT-PCR and antigen ELISA for evidence of infection.

3.2 Materials and methods

3.2.1 Virus isolation

Two tubes of EDTA blood were collected from a previously diagnosed 8-month-old persistently infected heifer. One was sent to Gribbles Veterinary Pathology Laboratory for BVDV antigen and antibody tests using ELISA. The other tube was used for Virus isolation. For virus isolation, EDTA blood was processed on the day of collection. Blood was centrifuged at 2000 rpm for 20 minutes; the buffy coat collected and diluted 1:10 in transport medium (minimum essential medium without foetal calf serum) and frozen at -70°C until required.

3.2.2 Cell inoculation

Bovine embryonic lung (BELU) cells that were BVDV virus free were grown at 37°C under 5% CO_2 in cell culture flasks (T_{75}) for 7 days using Minimum Essential Media (MEM) in the following concentration; 400 ml milliQ sterile water, 50 ml MEM 10X,

50 ml adult bovine serum (ABS), 10 ml sodium bicarbonate, 5 ml L- glutamine (Glutamax), 5 ml antibiotic-antimycotic and 5 ml non-essential amino acids. Cells were trypsinised and counted using a Neubauer chamber, depth 0.1mm and divided into stock and seed flasks. The stock flask was incubated (at 37°C under 5% CO_2) while the seed flask was inoculated with BVDV at 4 x 10^4 cells/ml in suspension. Inoculated cells were incubated for 6 days and subjected to two cycles of freezing-thawing before sub-culturing. Five ml aliquots from the infected cells were used to inoculate new BELU (subculture) cells in suspension to increase virus concentration. The infected cells were screened at each passage for the presence of antigen using an Institut Pourquier BVDV antigen ELISA kit. The cells were sub-cultured 6 times to achieve a final viral titre of 2.7 x 10^5/ml assessed using immuno peroxidase staining. Aliquots of 10 ml were frozen at -70°C ready for use as an inoculum.

3.2.3 Immuno peroxidase: Fixing and staining cells

BVDV infected BELU cells were sub-cultured in a 24-well plate, incubated at 37°C under 5% CO_2 for 4 days. On the 5th day, plates were emptied carefully into a 1% Virkon discard jug. Cell sheets were washed gently using 100 µl of 0.15M NaCl /well. A 20% acetone fixative was then added to the plates, incubated at room temperature for 10 minutes and discarded. Plates were incubated overnight at 37°C under 5% CO_2 to dry.

Monoclonal antibody (CF 10; TropBio, JCU, Townsville, Australia) was diluted to a final working volume of 1/1000. Antibody (50 µl) was added to each well and plates were incubated at 37°C for 1 hour in a humidified box. Plates were washed 3 times with PBS-Tween 20. Conjugate (50 µl of affinity-purified HRPO conjugate-goat anti-mouse IgG, Dakopatts PO 447) diluted appropriately in casein diluent (1/500) was added to each well and then plates were incubated at 37°C for 1 hour in a humidified box. Plates were washed 3 times with PBS-T_{20} then 100 µl of freshly prepared substrate (1ml 3-*A-9EC* solution, 19 ml acetate buffer and 10 µl H_2O_2) was added to each well. Plates were monitored under an inverted microscope for about 30 minutes and reaction was stopped by discarding the substrate and washing plates under running tap water.

Plates were incubated at 37°C for 20 minutes to dry and reading was done using the inverted microscope.

3.2.4. Experimental animals

Two 8-month-old calves were challenged intravenously with 10 ml of cell suspension containing 2.7 x 10^5/ ml of BVDV grown on bovine embryonic lung cells (BELU). These animals were housed and fed together in an isolation unit of the Massey University Veterinary Teaching Hospital. Fresh clean water and hay were provided daily. All animal usage was approved by the Massey University Animal Ethics Committee, protocol number 02/29.

3.2.5 Specimen collection and animal observation

Pre-inoculation blood samples were collected and tested for BVDV antibody and antigen. Temperature, respiratory and heart rates were also taken pre-inoculation. Blood samples were collected and clinical observations were carried out daily after inoculation for 14 days. Blood samples were processed for serology, Virus isolation and RT-PCR. All samples were processed within 12 hours of collection. An Institut Pourquier kit for BVDV was used for antigen ELISA test, following the manufacturer's instructions. Blood was processed for Virus isolation for use in ELISA on the day of collection. Virus isolation was carried out as stated above in the initial isolation. Blood samples for serology were allowed to clot at ambient temperature for 4 hours, refrigerated for another 4 hours and then centrifuged for 10 minutes at 600 g. Serum was removed and aliquots of 2 ml were stored at -20°C until required for analysis.

For RNA isolated from blood for use in RT-PCR analysis, leucocytes were extracted from blood as follows; 2 ml of EDTA blood was pipetted into a 15 ml tube, containing 3 ml of haemolysis buffer (Institut Pourquier, Montpellier, France) following the manufacturer's instructions. The mixture was incubated, pelleted and re-suspended in 100 µl PBS. An equal volume (100 µl) of RNA*Later* (Ambion, Austin, Texas USA),

was added to the cell suspension, shaken and stored at -20°C till required for RNA extraction.

3.2.6 Virus neutralisation assay (VNT)

Virus neutralization assays were performed in 96 well plates containing a confluent monolayer of bovine embryonic lung cells (BELU). Briefly, 50 ml of viral suspension containing 2.7 x 10^5/ ml BVDV was mixed with 50 ml of heat-inactivated (56°C for 30 min.) sera and incubated for 2 hours. The mixture was then seeded on 96 well cell culture plates and incubated at 37°C under 5% CO_2 for seven days. Sera from the test heifers were tested in triplicate at a dilution of 1: 4. Un-neutralized virus was detected by indirect immunoperoxidase staining as described in section 3.2.3. Each well was scored as positive or negative for virus growth. The inhibition of viral growth by VN antibodies was determined according to the method of Reed and Muench (1938) by calculating the 50% end point titre or 50% effective dilution (ED_{50}), expressed in $\log_2$.

3.2.7 RNA extraction and RT-PCR

Reverse Transcription-PCR for the detection of BVDV RNA in calves was conducted on the blood of all calves. It is worth noting that time zero samples were not analysed by RT-PCR in this experiment. Briefly, total RNA extraction was carried out using Qiagen RNA extraction kit (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia) according to the manufacturer's instructions. Formaldehyde gel electrophoresis was carried out to test the integrity of RNA. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (La Jolla, California, USA) and stored at -20°C until required for RT-PCR.

The 5′ UTR of BVDV was amplified using the following oligonucleotide pair; PI-U (5'-AGAGGCTAGCCATGCCCTTAGT -3') as the forward primer and PEST 2L (5'-TCAACTCCATGTGCCATGTAC -3') as the reverse primer. This primer pair was previously described by Grom and Barlic-Maganja (1999). In summary, a one-step RT PCR (superscript one step RT-PCR with platinum *Taq*, Invitrogen, Auckland, New

Zealand) was set up using the following reagents: 1 µl (10 µM) each of forward and reverse primers (PI-U and PEST), 1 µl of RT/*Taq*, 25 µl of 2X reaction buffer, 3 µl of template and 19 µl of autoclaved Milli Q water. The reaction conditions for the PCR were as follows: cDNA synthesis and pre-denaturation - 1 cycle of 42°C for 15 minutes, 99°C for 5 minutes; PCR amplification - 35 cycles of 94°C for 45 seconds, 60°C for 1 minute and 72°C for 1 minute; Final extension - 1 cycle of 72°C for 7 minutes. Electrophoretic separation of PCR product was carried out with 3% Nusieve agarose gel (Cambrex, Hallam, Victoria, Australia) and 1 X TBE, run at 100V for 1 hour. PCR products were visualised after staining with ethidium bromide for 15 minutes.

3.2.8. Sequencing

PCR products were purified using a High Pure™ PCR product purification kit (Boehringer Mannheim Corp., Indianapolis, IN, USA) and quantified using a NanoDrop ND-1000 Spectrophotometer. Purified products were sequenced using BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit. Sequencing was done from both directions and was primed by the RT-PCR primers.

3.3 Results

Blood from the 8-month-old PI heifer was antigen positive on ELISA. BVDV was isolated from the blood and the isolate used to experimentally challenge 2 heifers to test the infectivity of this isolate. Figure 3.1 shows the 300 bp PCR product of the inoculum. Pre-inoculation blood samples from the two challenged calves were both BVDV antibody and antigen negative when tested. Pre-inoculation RT-PCR was not done on the samples. On days one and two after challenge, both calves were RT-PCR positive for BVDV and continued to be RT-PCR positive up to day 13 after challenge (Figure 3.2). Table 3.1 summarizes the results of the antigen ELISA test for both calves. Figure 3.2 shows the 300 bp fragment of the 5'UTR of BVDV from the 2 calves. When the 5'UTR sequence was subjected to a BLAST search, using the nucleotide-nucleotide BLAST (blastn) at the National Centre for Biotechnology Information USA (http://www.ncbi.nlm.nih.gov/BLAST/) the best score was returned

with BVDV (AF026777, AF026787, AF026785, AF026782, AF026772, AF091605, AF041040, AF026786, AF026784, AF026783, AF026773, AF026775). The nucleotide sequences of both isolates were identical when compared using the Align two sequences (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) at the National Centre for Biotechnology Information USA (http://www.ncbi.nlm.nih.gov/BLAST/).

The antibody levels of the calves (Table 3.2) were negative on virus neutralization test (VNT). A slight increase was observed on day 13 after challenge but the experiment was ended on day 14 pi for one calf and day 16 pi for the other calf. The results of the clinical observations on both calves are presented in Table 3.3. At the end of the experiment, the isolation unit was disinfected and washed and calves taken to one of the University's paddocks.

Table 3.1: Result of BVDV Antigen ELISA test of calves (n = 2) experimentally challenged with a wild-type strain of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer using bovine embryonic lung cells *in vitro*.

		Calf 1		Calf 2	
Day pi	Positive	Sample	Sample/Positive	Sample	Sample/Positive
0	1.46	0.23	0.16	0.26	0.18
1	1.46	0.37	0.25	0.4	0.27
2	1.46	0.37	0.25	0.41	0.28
3	2.25	0.81	0.36	0.71	0.31
4	1.41	0.78	0.55	0.69	0.49
5	1.41	0.79	0.56	0.71	0.5
6	1.41	0.82	0.58	0.85	0.6
7	1.68	0.99	0.59	1.01	0.6
8	1.68	1.02	0.6	1.22	0.72
9	1.68	1.21	0.72	1.22	0.73
10	1.68	1.21	0.72	1.5	0.89
11	1.68	1.21	0.72	1.55	0.92
12	1.68	1.41	0.83	2.01	1.19
13	1.68	1.46	0.87	2.26	1.34

Table 3.1 summarises the results of two 8-month-old calves that were experimentally challenged with a wild-type strain of non-cythpathic strain of BVDV type 1 virus isolated from a persistently infected heifer. Blood samples were collected from days 0 to 13 post-inoculation and were analyzed for the presence of BVDV antigen using an enzyme linked immunosorbent assay (ELISA). Sample: positive ratios were calculated based on the ELISA results. Both calves were suspected of being infected with BVDV on days 1 and 2 post-inoculaton. From day 3 to day 13 post infection, both calves tested positive to BVDV antigen. Note that an S/P ratio of < 0.25 is considered negative to BVDV antigen, an S/P ratio of ≥ 0.25 means that the animal could be infected, while an S/P ratio of ≥ 0.30 is interpreted as a definite positive.

Table 3.2: Result of BVDV Antibody (virus neutralisation test) test of calves (n = 2) experimentally challenged with a wild-type strain of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer using bovine embryonic lung cells *in vitro.*

Day pi	Calf 1 VNT titre	Calf 2 VNT titre
0	<1:4	<1:4
1	<1:4	<1:4
2	<1:4	<1:4
3	<1:4	<1:4
4	<1:4	<1:4
5	<1:4	<1:4
6	<1:4	<1:4
7	<1:4	<1:4
8	<1:4	<1:4
9	<1:4	<1:4
10	<1:4	<1:4
11	<1:4	<1:4
12	<1:4	<1:4
13	<1:4	1:4
14	1:4	1:6
15	1:6	N/A
16	1:6	N/A

Table 3.2 summarises the results of two 8-month-old calves experimentally infected with a wild-type strain of non-cytopathic strain of BVDV type 1 virus isolated from a persistently infected heifer. Blood samples were collected from days 0 to 16 post-inoculation and were probed for antibodies to BVDV using a virus neutralisation test (VNT). From days 0 to 12 pi, both calves were BVDV antibody negative with a slight increase in antibody titre observed from day 13 and day 14 for calves 2 and 1 respectively. Note that the titre of <1:4 is interpreted as a negative result to serum BVDV antibodies while a VNT titre of 1:64 (low titre) is interpreted as evidence of a recent infection.

Table 3.3: Clinical parameters observed in calves (n = 2) experimentally inoculated with a wild-type strain of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer using bovine embryonic lung cells *in vitro*.

Days pi	Calf 1					Calf 2				
	Temp-erature	Respira-tory rate	Heart rate	Diarr-hoea	Others	Temp-erature	Respira-tory rate	Heart rate	Diarr-hoea	Others
0	38	60	100	-		38	52	80	-	
1	38.2	52	60	-		38.2	80	60	-	
2	38.7	69	66	-	↑LS	39	60	80	-	↑LS
3	40.1	60	70	-	↑LS	39.1	60	73	-	↑LS
4	40.1	71	71	-	↑LS	40	80	80	-	↑LS
5	40	92	84	+	↑LS SLN	40	80	80	-	↑LS
6	40	92	80	+	↑LS, SLN, Sa	40.4	82	68	+	↑LS
7	41.1	112	100	+	Md	40.6	86	70	+	↑LS SLN
8	41.4	114	95	+	↑LS, SLN, Sa	41.1	100	100	+	↑LS, SLN, Sa
9	41	111	95	+	↑LS, SLN, Sa	41.1	125	89	+	Md
10	40.8	98	64	+	↑LS, SLN, Sa	40.9	88	82	+	↑LS, SLN, Sa
11	40.5	93	64	+	Md, Sa	41	88	79	+	↑LS, SLN, Sa
12	39.3	70	75	+	Md, Sa	39.7	57	68	+	↑LS, SLN, Sa
13	39.2	60	60	+	Md, Sa	38	52	80	+	Md, Sa
14	39.2	62	60	-	Md, Sa	38.2	80	60	+	Md, Sa
15	39.2	60	62	-	Md, Sa	N/A	N/A	N/A	N/A	N/A
16	39	61	60	-	Md, Sa	N/A	N/A	N/A	N/A	N/A

Calves' temperatures, respiratory rates and heart rates were taken daily during the course of this experiment. Lymph nodes were also checked by palpation and calves were monitored for visible discharges. Note: ↑LS = increase in lung sounds, SLN = swollen lymph node, Sa = salivation, Md = mucopurulent discharge, N/A = not applicable, + and - denote respectively the presence and absence of diarrhoea.

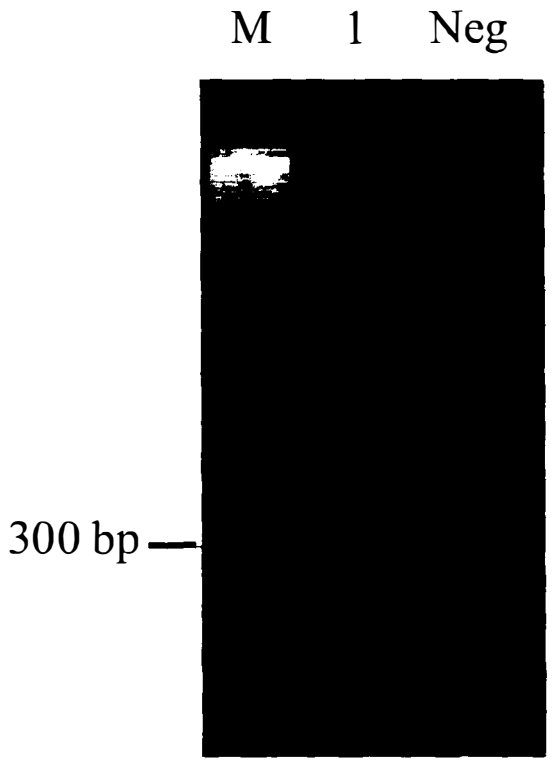

Figure 3.1: 300 bp RT-PCR product of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer using bovine embryonic lung cells *in vitro* and used as an inoculum in a challenge experiment. Lane M is 1 kb+ molecular weight marker, Lane 1 is the isolate and Lane Neg is a negative control.

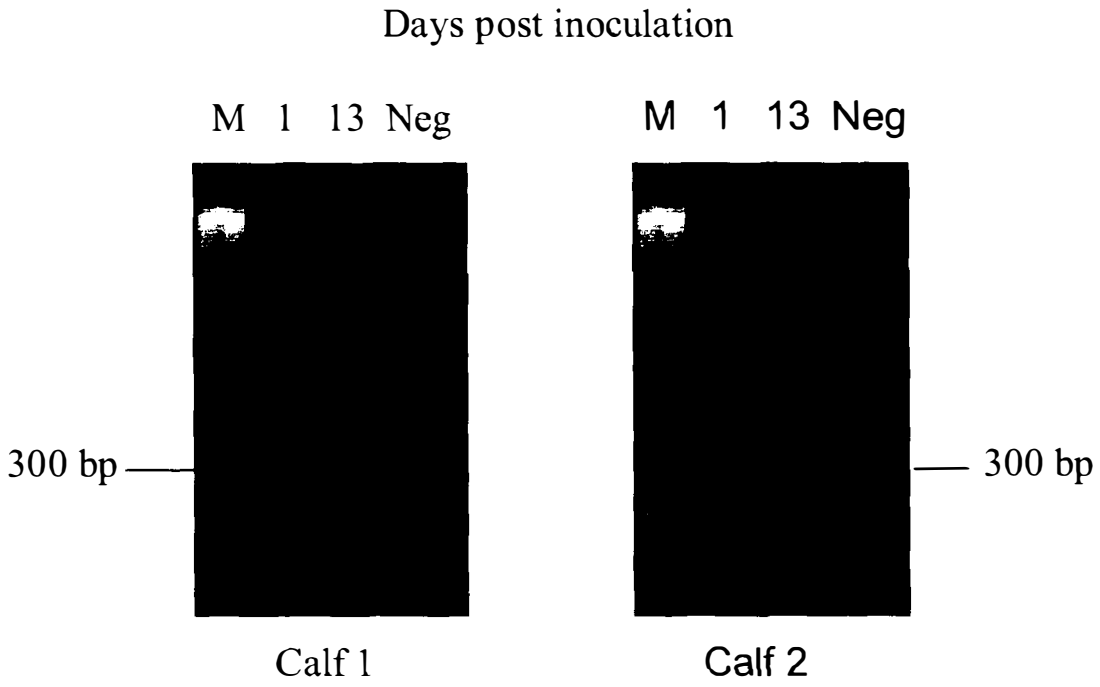

Figure 3.2: 300 bp RT-PCR products of non-cytopathic BVDV type 1 virus amplified from the blood of experimentally challenged heifers (n = 2) on days 1 and 13 post inoculation with a viral culture isolated from a persistently infected heifer onto bovine embryonic lung cells *in vitro*. Lanes marked M are 1 kb+ molecular weight marker, Lanes 1 are products amplified on day 1 post inoculation. Lanes 13 are products amplified on day 13 post inoculation. Lanes Neg are negative controls.

3.4 Discussion

A wild-type strain of NCP BVDV type1 virus was isolated from a persistently infected heifer. Virus infectivity was tested by experimentally challenging BVDV negative calves. PCR amplification of the 5' UTR using blood samples from the experimentally infected calves was conducted to confirm infectivity of the isolate. The NCP biotype of BVDV was used, because it is the predominant circulating biotype in the field in New Zealand.

Acute infection of immunocompetent cattle with BVDV can result in a wide range of clinical syndromes. Following inoculation, virus is able to spread through the bloodstream as was confirmed in this study. Spread can occur through free virus in the serum and virus infected leucocytes, particularly lymphocytes and monocytes. Once the virus becomes intracellular, viral RNA is released into the cytosol. RNA translation then begins with the 5' UTR serving as an internal ribosomal entry site. Viral proteins can be detected as early as 3 hours after cell infection. Once the RNA-dependent RNA polymerase is produced, new genomic RNA is produced and packaged. Viral packaging occurs in either the Golgi apparatus or endoplasmic reticulum where viruses acquire their lipid envelope through budding into the vesicle lumen. Mature virus packages are then released from the cell by exocytosis. New virus can be released as early as 10 hours after cell infection and detected either by ELISA or RT-PCR. In this study, viral antigen was detected 24-hours post-inoculation using RT-PCR and ELISA. On day 1 post-challenge, BVDV isolates from blood could possibly either still be inoculum virus or could be replicating virus.

The persistently infected heifer from which the inoculum was isolated was not tested by RT-PCR because antigen ELISA was the virus detection method chosen when the experiment was planned. In addition to this, the blood sample from which the virus was isolated was a generous gift from Dr Peter Anderson (a veterinarian working with this animal) and as such, the animal was not very accessible to us. Time zero samples were not analysed by RT-PCR in this experiment. The reason was that antigen ELISA was the virus detection method chosen for this experiment. PCR detection was only incorporated when it was demonstrated that PCR could be used. At this stage time zero samples had been used. Quantitative RT-PCR was not done as the experiment was

focused on using PCR to confirm infection and not on transcript levels, or expression patterns of the virus in the host tissue.

The incubation period of BVDV in this study was approximately 3 days. Viraemia occurred 4-5 days after challenge and persisted up to 13th day post challenge. Viraemia was followed by a biphasic fever (40°C), which was then followed by diarrhoea, increased respiratory and heart rates, oculonasal discharge and excessive lachrymation. Increase in serum antibodies were not recorded in this study because acutely infected animals normally produce serum antibodies 3 to 4 weeks post infection but this study was terminated before then. In this experiment, antibody neutralization test was only conducted for 16 days because from day 1 to day 13 pi, it was evident that these animals were not infected with BVDV prior to the commencement of this experiment. It takes about 3-4 weeks for an infected animal to mount antibody to BVDV as a result, the experiment could not be stretched due to lack of resources especially as it was only a pilot experiment and it was obvious that these animals were not infected prior to the experiment but were infected with the inoculum.

There is controversy over whether acute infection or persistent infection poses the greatest risk of infection to non-infected stock (Cherry *et al.* 1998; Houe 1999; Nettleton and Entrican 1995). It seems that replication by non-lytic (non-cytopathogenic virus) infection could result in persistent infection while replication by lytic infection (cytopathogenic virus) results in acute infection. However, acute BVDV may play a role in the epidemiology (Moerman *et al.* 1993) of BVDV. Voges *et al.* (1998) reported a persistent BVDV infection in the testicles of a bull that had acute infection previously. They reported that the bull was not viraemic, had high levels of anti-BVDV antibody while shedding approximately $10^3 CCID_{50}$ of virus/ml of semen. Grooms *et al.* (1998) also identified BVDV antigen present in the ovaries of heifers 60 days post-infection. It seems that persistent BVDV infections may follow acute infections because the establishment of chronic infections would provide an additional mechanism for BVDV to persist in cattle populations. This is an indication that acute infection might be just as important as the persistent infection carrier state in maintaining the virus in populations. Circulation of BVDV in cattle populations can also be influenced by acute infections. Although persistent infections represent a major source of virus spread, acutely infected animals may be the primary source of virus

introduction into cattle herds and may contribute to continued circulation of BVDV within infected herds.

Acute infections with BVDV are most important in pregnant cattle because of the ability of the virus to cross the placenta and cause intrauterine infections of the foetus. Foetal infections can result in early embryonic death, abortion, congenital defects and the birth of calves persistently infected with BVDV. Acute infections can result in reproductive and respiratory disease, which in turn result in major economic losses to the cattle industry. Due to the constant movement of animals, acute infections can be very important in the spread and maintenance of BVDV in cattle.

Use of RT-PCR in the detection of BVDV infected cattle overcomes the limitations of ELISA because PCR overcomes the masking problem when maternal antibodies are present in young colostrum-fed animals. Although PCR has a high risk of amplifying contaminants, nevertheless, it is a useful complement to other screening methods. Most RT-PCR protocols for BVDV isolation involve cell culture extraction of BVDV RNA. This is as labour intensive as other routine diagnostic methods and cell culture assays can take several days to complete. Detectable levels of BVDV are found in most commercial foetal calf serum (Andre *et al.* 1995), so cell culture assays have a risk of increasing the number of false positive diagnoses. A two-step RT-PCR assay, which is most commonly used, involves separate cDNA synthesis that can increase the risk of contamination. Using a single buffer one-tube RT-PCR (as was used in the present study), which utilises an uninterrupted thermo-cycling program, reduces the risk of cross contamination.

In conclusion the BVDV isolated in this study was found to be infective to calves at the dosage administered and was found to be adequate for use as an inoculum in an experimental challenge study.

Authors' contributions to this experiment

N.B. Williamson helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the experiments as well as providing editorial advice.

C.M. Okeoma helped with animal inoculation, sample collection and clinical observations. She conducted the experiments and wrote the manuscript.

Chapter 4

The effect of experimental bovine virus diarrhoea virus (BVDV) challenge in heifers naturally infected with *Neospora caninum*.[1]

[1] Submitted as: Okeoma CM, Williamson NB, Pomroy WE, Stowell KM, Okeoma CB, Gillespie L, Alley M. New Zealand Veterinary Journal

4.0 Abstract

The effect of experimental BVDV infection on heifers naturally infected (n = 12) and non-infected (n = 12) with *N. caninum* was investigated using a wild-type strain of non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer. Each heifer was intravenously challenged with 10 ml of an inoculum containing 2.7 x 10^5/ml of BVDV of this isolate. Results showed that concurrent infection of BVDV and *N. caninum* produced 44.4% (8/18) dam-calf pairs that were both BVDV and *N. caninum* seropositive. Seven of the 8 pairs were experimentally challenged with BVDV while 1 was not challenged. Twenty-three of the 24 heifers (95.8%) were positive for *N. caninum* by PCR test on blood while 1 was negative. The brains of 77.7% (14/18) dam-calf pairs were positive for *N. caninum* DNA using PCR. Serologically, 66.7% (12/18) dam-calf pairs were positive for *N. caninum* antibodies using the IFAT. To confirm the results obtained with IFAT for anti-*N. caninum* antibodies, Western blot analysis was conducted on all serum samples with titres at or above the 1:80 dilution. Results were consistent with IFAT test results, thus confirming the presence of anti-*N. caninum* antibodies. When tested for antibodies against BVDV, 80% of dam-calf pairs were BVDV seropositive. All calves were negative on reverse transcription-PCR test, indicating that viral RNA was not present in the blood of these calves and that they were not persistently infected with BVDV.

4.1 Introduction

N. caninum is an intracellular protozoan parasite that is structurally similar to, but antigenically and phylogenetically distinct from *T. gondii* (Lindsay and Dubey 1989). *N. caninum* infection (neosporosis) occurs in cattle and several other species of ruminants, dogs and horses. Neosporosis was first reported as a cause of neuromuscular disorders and mortality in dogs (Bjerkas *et al.* 1984; Dubey *et al.* 1988a). In horses it can cause myeloencephalitis (Marsh *et al.* 1996), while it is the major cause of abortion in cattle in many countries including New Zealand (Thornton *et al.* 1991; Reichel and Drake 1996; Cox *et al.* 1998; Pfeiffer *et al.* 2002; Thobokwe and Heuer 2004). Although congenitally infected calves with neosporosis may show clinical disease in the form of posterior paresis or paralysis (Barr *et al.* 1990), other neurological signs, low birth weight, inability to rise or stillbirth, most calves may be born congenitally infected and have no clinical signs (Barr *et al.* 1993; Dubey and Lindsay 1996).

Many factors are associated with neosporosis in cattle. Some are: seropositivity of the dam (Anderson *et al.* 1997; Bergeron *et al.* 2000); presence of possible definitive host animals on the farm e.g. dogs (Paré *et al.* 1998; Bartels *et al.* 1999) and domestic fowl (Bartels *et al.* 1999); and the immunosuppressive effect of pregnancy (Quinn *et al.* 2002). It has also been reported that bovine virus diarrhoea virus (BVDV) is associated with bovine neosporosis (Bjorkman *et al.* 2000).

Most postnatal BVDV infections of cattle are sub-clinical (Ames 1986; Duffell and Harkness 1985) and many primary postnatal infections, whether sub-clinical or clinical render an animal transiently immunosuppressed (Edwards *et al.* 1986; Kahrs 1981; Nagele 1984; Potgieter *et al.* 1984a; Potgieter *et al.* 1984b; Reggiardo 1979; Reggiardo and Kaeberle 1981). As a result, BVDV has the potential to enhance disease caused by other pathogens or precipitate illness in association with other pathogens (Bohac and Yates 1980; Malmquist 1985; Potgieter 1977).

Previously Coria and McClurkin (1978), Donis and Dubovi (1987), Larsson and Fossum (1992) and McClurkin *et al.* (1984) have reported that persistence of BVDV in cattle seems to be the consequence of specific B-lymphocyte and T-lymphocyte immuno-tolerance and the target cells for the replication of BVDV are lymphocytes and

macrophages. In addition, lesions induced by BVDV in cattle suggest that immuno-suppression is a consequence of infection with this virus (Tyler and Ramsey 1965). Lymphoid destruction results in varying degrees of lymphocyte depletion in infected calves, even in those that remain clinically normal (Reggiardo and Kaeberle 1981). The mechanism by which BVDV induces immunosuppression is generally understood to result from the depletion of immunocompetent cells in cattle. This in turn leads to reduced resistance to other pathogens.

Bjorkman *et al.* (2000) reported a statistically significant association between the presence of antibodies to *N. caninum* and BVDV. Thus, they confirmed that *N. caninum* infection is associated with bovine abortion in Sweden and that there were apparent interactive effects of *N. caninum* and BVDV. Murray (1991) speculated that pathological changes in the placenta induced by BVDV may allow other pathogens to more easily cross the foeto-maternal barrier. Conversely, Bartels *et al.* (1999) reported a negative relationship between seropositivity to *N. caninum* and BVDV in 117 aborting cows belonging to 50 Dutch dairy herds that experienced abortion storms. Concomitant *N. caninum* and BVDV infections may result in an increased risk of abortion and congenital disease than infection with each agent alone. This study investigated the effect of experimental BVDV inoculation of pregnant cows when half were naturally infected with *N. caninum* in order to examine the possible interaction of BVDV and *N. caninum* in causing abortion.

4.2 Materials and methods

4.2.1 Pilot trial

A pilot study to test the infectivity of the BVDV isolated from a persistently infected calf was carried out as described in Chapter 3. This trial showed the virus to be capable of infecting two previously seronegative calves, as demonstrated by a febrile response and the maintenance of a positive RT-PCR.

4.2.2 Main study

4.2.2.1 Animals and experimental design

Figure 4.1 is a flow chart that describes the experimental design. Four pregnant *N. caninum* seropositive, 5 aborted *N. caninum* seropositive and 6 pregnant *N. caninum* seronegative Friesian and Friesian-Jersey cross heifers were assigned to a BVDV-challenged experimental group. The control group was housed at a different experimental site and comprised 2 pregnant *N. caninum* seropositive, 1 aborted *N. caninum* seropositive and 6 pregnant *N. caninum* seronegative Friesian and Friesian-Jersey cross heifers. Heifers underwent blood sampling and pregnancy testing prior to and on arrival at the experiment sites. The blood samples collected on arrival were used as pre-challenge blood samples. At arrival, the pregnant heifers were in their 5th month (~150 days) of gestation.

The selection of animals was based on historical repeated serological results of testing with an ELISA for *N. caninum* (IDEXX, Wörrstadt, Germany). Historically, the dams of these heifers were serologically monitored (4 times/annum) for antibodies to *N. caninum* and BVDV as well as BVDV antigen. Once the heifers were born in 2001, they were also monitored using IDEXX ELISA serology. As a result, all *N. caninum* seropositive heifers had been consistently seropositive on ELISA from birth and had seropositive dams, while the *N. caninum* seronegative heifers were consistently seronegative on ELISA from birth and their dams were also seronegative. The same is true for BVDV, as all heifers had maintained their negative BVDV antibody and antigen status from birth to the start of the experiment.

After experimental challenge, all inoculated heifers were grazed together with a known persistently infected (PI) heifer, monitored daily and blood samples were collected on a weekly basis for 4 weeks and then biweekly until calving. Immediately after calving, blood samples were collected from all dams and precolostral blood samples were taken from the calves. Control heifers were not inoculated but were subjected to a similar sampling and monitoring regime conducted at a different site. All calves from both groups were slaughtered within 2 days of birth and their brains were collected and divided to be processed immediately for parasite isolation, PCR and histo-pathological

examination. Heifers that were seropositive to *N. caninum* were slaughtered with their calves whilst seronegative heifers were milked for up to 4 months before being slaughtered for the same series of examinations as the calves.

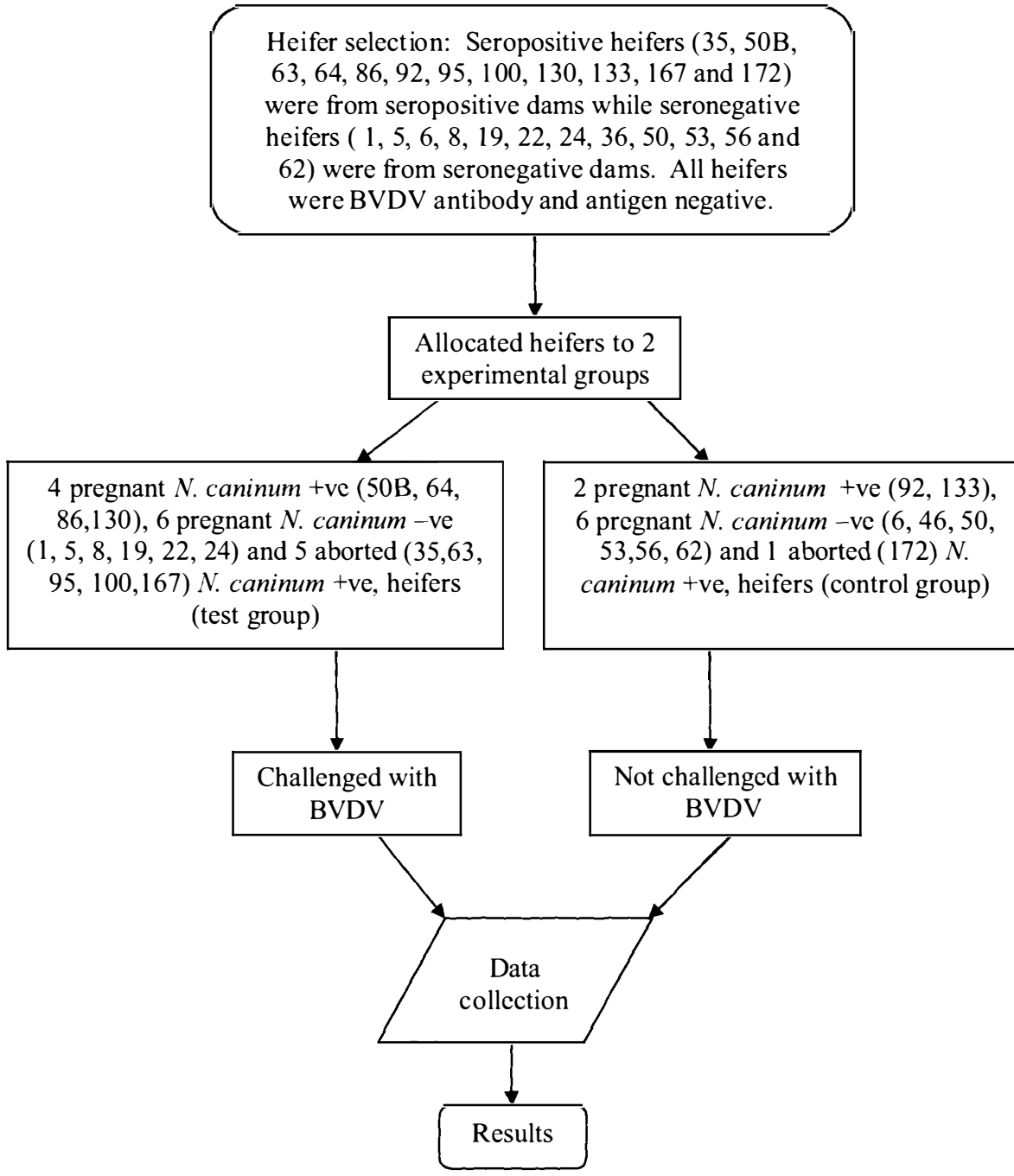

Figure 4.1: A flow chart of experimental design to study the effect of experimental bovine virus diarrhoea virus (BVDV) challenge in heifers naturally infected with *N. caninum*. The chart shows the sequence of events from animal selection, allocation to experimental groups, experimental challenge and data collection.

4.2.2.2 Animal inoculation with BVDV and sample collection

Pregnant heifers in the BVDV-challenged group were inoculated intravenously at approximately 150 days of gestation with 10 ml of an inoculum containing 2.7 x 10^5/ml of BVDV isolated from the pilot study and were grazed together with a persistently infected heifer. Heifers in the control group were sham inoculated with 10 ml of minimum essential medium (MEM). Blood samples were collected weekly for 7 weeks and fortnightly on 4 more occasions. Whole blood samples collected into EDTA were used for PCR analysis while serum samples were analysed for *N. caninum* and BVDV antibodies. Serum samples were also used for the detection of *N. caninum* specific antigens by Western blot. At the end of the experiment, all calves and heifers were sacrificed and brain samples collected. These samples were used for parasite isolation, PCR and histopathological examinations.

4.2.2.3 Analysis of blood samples

Prior to this study, serology had been the gold standard for the detection of *N. caninum* infection in animals. In the present study, PCR was used as a gold standard for the detection of circulating *N. caninum* in animals infected with *N. caninum*. PCR assays were conducted on all blood samples from the experiment, as previously described by Okeoma *et al.* (2004a). Sera were tested for *N. caninum* antibody using IFAT (VMRD, Washington, USA), while BVDV antibody ELISA (HerdChek, Wörrstadt, Germany) was conducted on serum samples for antibodies to BVDV. BVDV ELISA and *N. caninum* IFAT tests (1:80 dilution) were also conducted on calves' serum samples for the detection of antibodies. All serological tests were run and assessed according to manufacturers' instruction by Gribbles Pty Ltd Animal Health Laboratory (Palmerston North, New Zealand) which conducts commercial *N. caninum* and BVDV testing. All BVDV and IFAT tests were run together as one batch.

Antigen preparation, SDS PAGE and Western blot analysis were conducted on all serum samples including those with low IFAT titres (≤ 1:80) as described by Okeoma *et al.* (2004). Methodology for SDS PAGE is presented on Chapter 7, sections 7.2.3, 7.2.4 and 7.2.5. After blotting, membranes were developed using the opti-4CN^{TM} substrate

kit (Bio-Rad Cat #170-8235), then washed with water for 20 minutes. After washing, bands were recorded manually and with a digital camera. Images were scanned into a Microsoft word document, exported to the Microsoft 'Paint' software program and saved as tiff files. This process resulted in Figures that provide a high contrast image of the blots and very clear geometrical protein bands. These images were annotated and/or labelled using the Paint program. Reverse Transcription-PCR (RT-PCR) for the detection of BVDV RNA in persistently infected calves was conducted on the blood of all calves as described in the pilot study in Chapter 3, section 3.2.7.

4.2.2.4. Analysis of brain samples

The left-brain hemisphere of the first *N. caninum* seropositive heifer to calve, her calf and a stillborn calf from another *N. caninum* seropositive heifer were used for parasite isolation as described by Okeoma *et al.* (2004b). Samples from the left-brain hemisphere of all calves and their dams were also used in PCR analysis. The right-brain hemisphere was fixed in 10% neutral buffered formalin for histo-pathological examinations.

For PCR analysis, a sample from the left-brain hemisphere was separately homogenised in serum-free minimum essential medium (MEM from Gibco, Invitrogen Corp. NZ, Cat number 4109-036) and 30 mg of homogenate was used for DNA isolation as described in Okeoma *et al.* (2004a). Electrophoresis of each DNA sample on a 2% agarose gel in 1X TBE buffer was undertaken to check integrity. The PCR for detection of *N. caninum* was performed using a *N. caninum* species-specific primer pair Np21+ (5'-CCAGTGCGTCCAATCCTGTAAC-3') and Np6+ (5'-CTCGCCAGTCAACCTA-CGTCTTCT -3') that anneals to the Nc-5 region (Müller *et al.* 1996). PCR reactions were performed in a 20 µl volume containing 1 µl of 0.2 µg of sample DNA, 0.4 µl of 0.2 mM dNTPs mix (Gibco, Invitrogen Corp. NZ, Cat number 18427-013), 1.4 µl of 3.5 mM MgCl, 2 µl of 1X PCR Mg-free buffer, 0.12 µl of 0.6 units of Platinum *Taq* polymerase (Gibco, Invitrogen Corp. NZ, Cat number 10966-026), 1 µl of dUTP/uracil N-glycosylase (UNG) and 0.4 µl of 0.2 mM of each primer. PCR was performed in a Perkin Elmer GeneAmp PCR Thermocycler System 2400 (Applied Biosystems, USA)

with the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, with a final extension of 72°C for 10 min. A negative control (water blank) and a positive control DNA from an Nc Liverpool isolate (Barber *et al.* 1993), donated by Prof. J. Ellis of the University of Technology, Sydney with acknowledgement to Prof. A.J. Trees, of the University of Liverpool were included in each reaction. Amplified product of the Nc 5 fragment was separated by electrophoresis on a 2% Nusieve 3:1 agarose (Cambrex) gel and stained with ethidium bromide for 15 min, destained in water and viewed under UV light.

For histo-pathological examination of the right brain-hemisphere, 5 sections of 2 μm thick were cut from each brain sample: 1 section from cerebral cortex (caudatus nucleus), 1 section from hippocampus, 1 section from mid brain, 1 section from cerebral peduncles and 1 section from cerebellum. Sections were stained with haematoxylin and eosin and changes in the brain were classified by a simple grading system based on a lesion severity score of none (-), mild (+), or moderate (++).

4.2.2.5 Transmission study

Pre-colostral whole blood and serum samples were obtained from calves and their dams immediately after calving for PCR, IFAT, BVDV antibody ELISA and Western blot analysis. Samples of brains from dams and calves were used for PCR and histo-pathological analysis. Results for each dam and her calf were compared.

4.3 Results

4.3.1 Analysis of blood samples

PCR was used to detect *N. caninum* DNA in the blood of the experimental animals. For the PCR assay, an animal was considered infected if it tested positive once. Of the 24 experimental heifers, 23 (95.8%) had *N. caninum* DNA detected in their blood at least once by the end of the study (Table 4.1, Appendix 3). The remaining heifer was

negative on PCR test throughout the study period. All aborted heifers were consistently PCR positive until the 7th week of observation which occurred at approximately 8 to 13 weeks after the abortions occurred. Thereafter, the blood of these aborted heifers was PCR negative. All of the heifers that were originally pregnant at the start of the experiment and were *N. caninum* seropositive (IDEXX ELISA) remained PCR positive from the start to the end of the observations with a few negative results observed in-between. Interestingly, the blood of all heifers but 1 (heifer #50) that were *N. caninum* seronegative (IDEXX ELISA) originally, were found to be positive for *N. caninum* DNA at some time before the end of observations at calving time, again with some negative observations in-between (Table 4.1, Appendix 3).

The results of IFAT analysis of serum samples over the course of the study is also presented in Table 4.1, Appendix 3. The dilution rate used for the lower cut-off for screening for positive IFAT tests was 1:80. All heifers were positive to *N. caninum* antibodies at this dilution at some time in the study. To confirm the results obtained with the IFAT, Western blot analysis was conducted on all serum samples (Table 4.3). There was agreement of the results obtained between the IFAT and Western blot test results on all except 2 of 125 sample pairs tested. Both of the disagreements were in samples from one heifer where the IFAT titre was 1:80 and the Western blot showed no antibody bands at weeks 2 and 13 of sampling. The results of IFAT and Western blot tests on this heifer were negative at all other test weeks. Figure 4.2 is a composite figure showing Western blot analyses of serum samples with titres of <1:80, 1:80, 1:200, 1:600, 1:1000, 1:2000 and >1:2000 and for the control samples from *T. gondii* as the negative control and *N. caninum* Liverpool isolate as the positive control.

Fluctuations in antibody titre were also observed (Table 4.1, Appendix 3). Antibody fluctuations occurred across all heifers irrespective of their initial serological status on IDEXX test.

Table 4.1: *N. caninum* antibody and DNA as observed using Immuno fluorescent antibody test (IFAT) and Polymerase chain reaction (PCR) analysis respectively on blood samples taken over 15 weeks of observation from heifers (n = 24) naturally infected with *N. caninum* and experimentally challenged with non-cytopathic BVDV type 1 virus isolated from a persistently infected heifer.

	Pre-Experimental status				One week pre-challenge	IFAT titre and PCR status (+/-)										
						Period in weeks after experimental challenge										Result
Heifer ID	*N. caninum*	BVDV	Pregnancy status	Inoculation status	1	2	3	4	5	6	7	9	11	13	15	summary
1	Neg	Neg	Preg	Challenged	2000+	6000+	80+	80+	200+	600-	80-	0-	0+	80+	80+	Positive+
5	Neg	Neg	Preg	N/C	80+	80+	80+	80+	80+	80+	80-	80-	80-	200+	80+	Positive+
6	Neg	Neg	Preg	Challenged	0-	2000+	80+	80+	0-	1000-	80+	80+	0+	600+	600+	Positive+
8	Neg	Neg	Preg	Challenged	80-	2000+	80+	80+	80-	0-	0+	80+	0+	600+	600+	Positive+
19	Neg	Neg	Preg	Challenged	80-	80-	80-	80-	0-	80-	0-	0-	0-	80+	600-	Positive+
22	Neg	Neg	Preg	Challenged	0-	1000+	80+	80+	0-	80-	80+	0+	0+	80+	200+	Positive+
24	Neg	Neg	Preg	Challenged	0-	600-	0-	2000+	80-	80-	80+	0+	80+	0+	600+	Positive+
46	Neg	Neg	Preg	N/C	0-	80-	0-	0-	80-	600-	0+	80+	0+	0+	80+	Positive+
50	Neg	Neg	Preg	N/C	0-	80-	0-	0-	0-	0-	0-	0-	0-	80-	N/A	Negative-
53	Neg	Neg	Preg	N/C	0-	200-	0-	N/A	0-	200-	0+	0+	0+	0+	N/A	Positive+
56	Neg	Neg	Preg	N/C	0-	80-	80-	2000+	0-	2000+	0-	0+	80+	80+	200+	Positive+
62	Neg	Neg	Preg	N/C	80-	1000+	80+	600+	80+	1000+	80+	80+	0+	80+	80+	Positive+
50B	Pos	Neg	Preg	Challenged	0+	1000+	80+	0+	80+	2000+	80+	80+	80+	80+	80+	Positive+
64	Pos	Neg	Preg	Challenged	0+	2000+	2000+	600+	2000+	2000+	2000+	2000+	2000+	2000+	2000+	Positive+
86	Pos	Neg	Preg	Challenged	1000+	1000+	2000+	600+	2000+	2000+	2000-	2000+	2000+	2000+	2000+	Positive+
92	Pos	Neg	Preg	N/C	80+	0+	80+	600+	80+	600+	0-	80-	0+	2000+	0-	Positive+
130	Pos	Neg	Preg	Challenged	0+	80+	80+	80+	0-	0-	0-	80-	600+	0+	N/A	Positive+
133	Pos	Neg	Preg	N/C	200+	600+	600+	80+	200+	600+	200-	600-	200+	600+	N/A	Positive+
35	Pos	Neg	Abort	Challenged	200+	600+	600+	600+	600+	600+	600+	600+	600-	600-	600-	Positive+
63	Pos	Neg	Abort	Challenged	200+	600+	600+	600+	1000+	2000+	1000-	600+	600-	2000-	1000-	Positive+
95	Pos	Neg	Abort	Challenged	200+	600+	80+	2000+	600+	600+	600-	600-	200-	1000-	600-	Positive+
100	Pos	Neg	Abort	Challenged	200+	1000+	200+	600+	1000+	1000+	1000-	1000+	1000-	600-	1000-	Positive+
167	Pos	Neg	Abort	Challenged	200+	600+	80+	600+	1000+	600+	1000-	600-	600-	600-	1000-	Positive+
172	Pos	Neg	Abort	N/C	2000+	600+	0+	600+	600+	80+	80-	600-	600-	600-	600-	Positive+

Four pregnant *N. caninum* seropositive, 5 aborted *N. caninum* seropositive and 6 pregnant *N. caninum* seronegative heifers were experimentally challenged with BVDV, while 2 pregnant *N. caninum* seropositive, 1 aborted *N. caninum* seropositive and 6 *N. caninum* seronegative heifers were sham inoculated with MEM. All heifers were at ~ 150 days of gestation at time of challenge. Note: Week 1 samples were collected prechallenge; Pos = positive; Neg = negative; Preg = pregnant; N/C = not challenged; + and – are used to denote PCR results displayed next to IFAT.

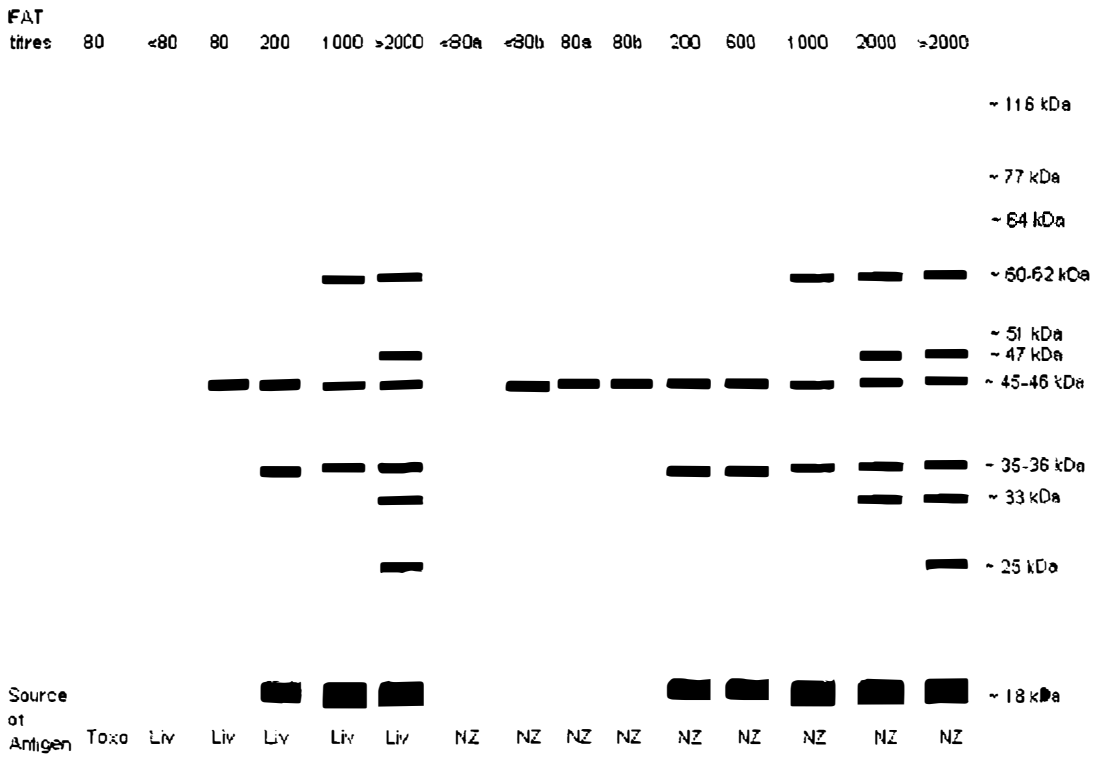

Figure 4.2: Results of Western blot showing binding pattern of *N. caninum* antigen at different immunofluorescent antibody test titres of <1:80, 1:80, 1:200, 1:600, 1:1000, 1:2000 and >1:2000. *N. caninum* tachyzoites of New Zealand isolate 1 (NcNZ 1) were grown on Vero cell culture. Tachyzoites were harvested and used for the preparation of antigen used in this experiment. Sera samples were obtained from heifers (n = 24) that were either *N. caninum* seropositive or seronegative and challenged or not challenged with BVDV. Antigens were separated by SDS PAGE, blotted and probed with cow sera. Control antigens were Liv for Liverpool isolate of *N. caninum* used as the positive control, *Toxo* for *T. gondii* used as a negative control. NZ is for New Zealand isolate of *N. caninum*. Samples labelled a and b are the same IFAT titre from different samples.

Sera from all heifers were analysed for antibodies to BVDV throughout the 15-week period and the results are summarised in Table 4.3 with the raw data presented in Appendix 1. Among the 6 aborted heifers, 5 were positive to BVDV antibodies while 1 had an antibody titre that was close to the cut-off point. None of these heifers was experimentally challenged by inoculation with BVDV. However, the 5 aborted heifers that became BVDV positive were grazed with the experimentally challenged group and a persistently infected heifer and thus were subject to challenge in this way. All 10 heifers experimentally challenged with BVD virus seroconverted. Four heifers in the control group seroconverted while the remaining 4 heifers (including the aborted heifer mentioned above) in this group had antibody titres that were close to the cut-off point. A heifer in the unchallenged group was the only one to remain negative when tested for BVDV antibodies (Table 4.3, Appendix 1).

4.3.2. Analysis of brain samples

Figures 4.3a and 4.3b present the results of PCR on the brains of the 24 heifers used in this study. *N. caninum* nucleic acid was demonstrated in the brains of 20 of 24 (83.3%) heifers after calving, 19 of 20 (95%) had *N. caninum* DNA (presumably from tachyzoites) detected in their blood and 45% (9/20) were pregnant, challenged with BVDV and produced anti-BVDV antibodies. Six heifers were pregnant, not challenged with BVDV and were either seropositive to BVDV or had antibody titres that were close to the cut-off point; 25% (5/20) aborted, were challenged with BVDV and were seropositive to BVDV antibodies; while 1 heifer aborted, was not challenged with BVDV but had antibodies to BVDV close to the cut-off margin (Table 4.3). Further, on brain analysis, *N. caninum* tachyzoites were isolated from the brains of 2 calves and a heifer (Figure 4.4, Table 4.3 [133*] and Table 4.3 [92*, 133*]). Histo-pathological examination of brain sections revealed brain lesions in 8 heifers and 2 calves (Table 4.3).

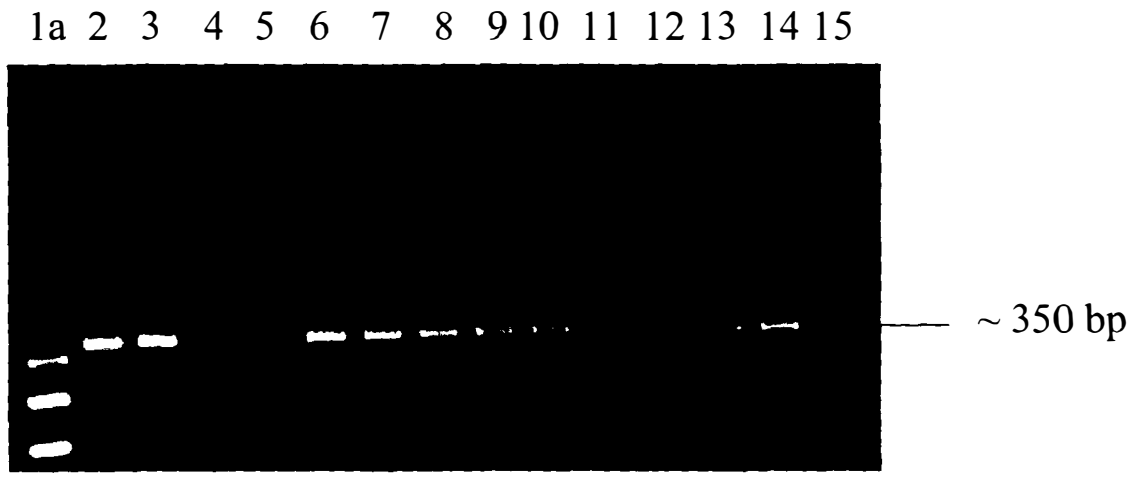

Figure 4.3a: A 350 bp PCR product from the brains of heifers (n = 24) that are either *N. caninum* seropositive or *N. caninum* seronegative and challenged or not challenged with BVDV. DNA was isolated from the brains of heifers and used in PCR at the end of the experiment after sacrifice. Lane 1a is 1 kb+ molecular weight marker. Lanes 2 to 15 are cows 1, 5, 6, 8, 19, 22, 24, 35, 46, 50, 50B, 53 and 56 respectively. Note: All controls are presented in Figure 4.3b.

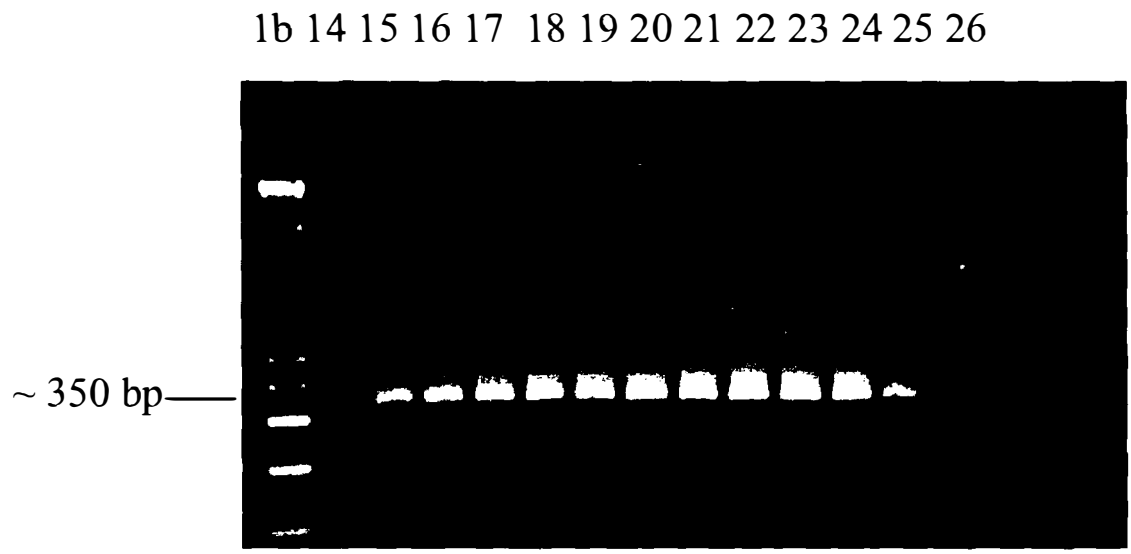

Figure 4.3b: A 350 bp PCR product from the brains of heifers (n = 24) that are either *N. caninum* seropositive or *N. caninum* seronegative and challenged or not challenged with BVDV. DNA was isolated from the brains of heifers and used in PCR at the end of the experiment after sacrifice. Lane 1b is 1 kb+ molecular weight marker. Lanes 14 to 24 are dams 62, 63, 64, 86, 92, 95, 100, 130, 133, 167 and 172 respectively. Lane 25 is a positive control (Nc Liverpool DNA) and Lane 26 is a no DNA negative control of saline.

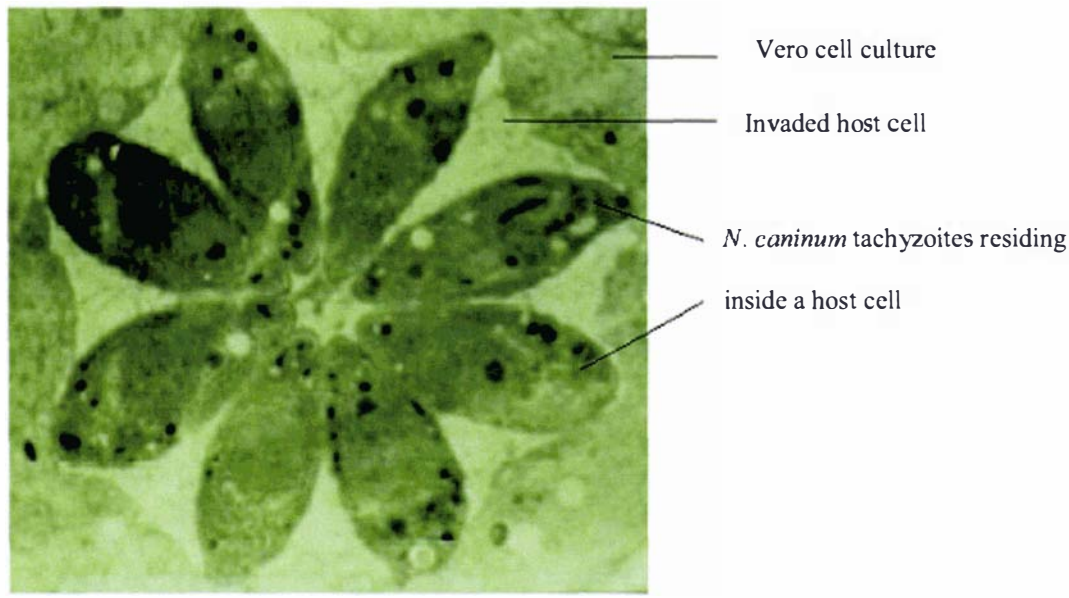

Figure 4.4: A phase contrast photo of tachyzoites of *N. caninum* isolated from the brain of an infected calf (calf #133). The photo shows 8 tachyzoites inside a host cell with their conoids pointing towards the centre. As we were watching the flower-like tachyzoites, the host cell lysed and all 8 tachyzoites were released into the culture. These tachyzoites immediately attached to other healthy cells initiating the process of host cell invasion. For details of parasite isolation, see Chapter 8 or Okeoma *et al.* Isolation and molecular characterisation of *N. caninum* in cattle in New Zealand, *New Zealand Veterinary Journal*, 52, 6, 364-370, 2004b.

4.3.3. Transmission study

In order to study the vertical transmission of infection from dams to calves, 18 of the 24 heifers were available because 6 had previously aborted and their foetuses had not been recovered. A summary of the results of the tests conducted on the dams at parturition and their calves is presented in Table 4.2 and Figures 4.3a, 4.3b and 4.4. Of the 18 heifers available, 12 (66.7%) dam and calf pairs were IFAT positive; 14 (77.7%) dam and calf pairs were positive on brain PCR tests (Figures 4.3a, 4.3b and 4.4.); and 1 dam and calf pair tested positive on blood PCR. Of the 18 dams used in the transmission study, 10 were challenged with BVDV while 8 were used as controls. Amongst the 10 challenged dams, 8 (80%) dams along with their calves were positive to BVDV antibody test and out of this 8, 7 (70 %) were *N. caninum* brain PCR positive (Table 4.3). Of the 8 control dams, 1 dam and calf pair tested negative to BVDV and was also negative to all *N. caninum* assays used. Another dam and calf pair in this group tested positive to BVDV antibodies and was also positive to *N. caninum.* All calves were negative on reverse transcription-PCR test for BVDV (data not shown), indicating that BVDV viral RNA was not present in their blood.

Table 4.3 summarises the results for *N. caninum* and BVDV assays over the duration of the trial including the results for *N. caninum* DNA in the blood and brain, serology using IFAT or Western blot and lesions in the brain due to *N. caninum.*

Table 4.2: Evaluation of transmission rates of *N. caninum* and BVDV in heifers (n = 18) by *N. caninum* natural infection status and BVDV experimental challenge group using blood samples obtained immediately after parturition and brain samples taken immediately after slaughter.

Heifer ID	Dams historical sero-status based on IDEXX ELISA	Dams BVDV challenge status	IFAT (1:80)		Western blot		Blood PCR		Brain PCR		Histo pathology		Antibody against BVDV		Dam/Calf transmission data	
			Dam	Calf	Dam	Calf	Dam	Calf	Dam	Calf	Dam	Calf	Dam	Calf	*N. caninum*	BVDV
1	Neg	Challenged	2000	4000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Pos	Pos/Pos	Pos/Pos
5	Neg	N/C	2000	4000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Pos	Pos/Pos	Pos/Pos
6	Neg	Challenged	80	INS	Pos	Pos	Pos	INS	Neg	Neg	-	-	Neg	JNS	Neg/Neg	Pos/INS
8	Neg	Challenged	200	80	Pos	Pos	Pos	Neg	Neg	Neg	-	-	Pos	Pos	Neg/Neg	Pos/Pos
19	Neg	Challenged	INS	0	INS	Neg	INS	Neg	Pos	Pos	+	-	INS	Neg	Pos/Pos	Pos/Neg
22	Neg	Challenged	600	1000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Pos	Pos/Pos	Pos/Pos
24	Neg	Challenged	1000	100	Pos	Pos	Pos	Neg	Pos	Pos	+	-	Pos	Pos	Pos/Pos	Pos/Pos
46	Neg	N/C	80	0	Pos	Neg	Pos	Neg	Pos	Pos	+	-	Neg	Neg	Pos/Pos	Pos/Neg
50	Neg	N/C	0	0	Neg	Neg	Neg	Neg	Neg	Neg	-	-	Neg	Neg	Neg/Neg	Neg/Neg
53	Neg	N/C	600	80	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Neg	Pos/Pos	Bdl/Neg
56	Neg	N/C	80	80	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Neg	Pos/Pos	Pos/Neg
62	Neg	N/C	1000	2000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Pos	Pos	Pos/Pos	Pos/Pos
50B	Pos	Challenged	1000	0	Pos	Neg	Pos	Neg	Neg	Neg	+	-	Neg	Neg	Pos/Pos	Bdl/Neg
64	Pos	Challenged	8000	4000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Neg	Pos	Pos/Pos	Pos/Pos
86	Pos	Challenged	4000	8000	Pos	Pos	Pos	Neg	Pos	Pos	-	++	Pos	Pos	Pos/Pos	Pos/Pos
92*	Pos	N/C	2000	8000	Pos	Pos	Neg	Pos	Pos	Pos	-	++	BDL	Neg	Pos/Pos	Bdl/Neg
130	Pos	Challenged	0	0	Neg	Neg	Pos	Neg	Pos	Pos	+	-	BDL	Pos	Pos/Pos	Pos/Pos
133**	Pos	N/C	2000	2000	Pos	Pos	Pos	Neg	Pos	Pos	-	-	Neg	Neg	Pos/Pos	Pos/Neg

Four pregnant *N. caninum* seropositive and 6 pregnant *N. caninum* seronegative heifers were experimentally challenged with BVDV, while 2 pregnant *N. caninum* seropositive and 6 *N. caninum* seronegative heifers were sham inoculated with MEM. All heifers were at ~150 days of gestation at time of challenge. Note: N/C = not challenged: Bdl = borderline result = around cut-off point; N/A = not available; Calf 92 was stillborn and heart blood used for analysis; INS = insufficient serum sample; - = no brain lesion; + = mild brain lesion; ++ = moderate brain lesion; * = *N. caninum* isolated from brain of calf; ** = *N. caninum* isolated from brains of both dam and calf.

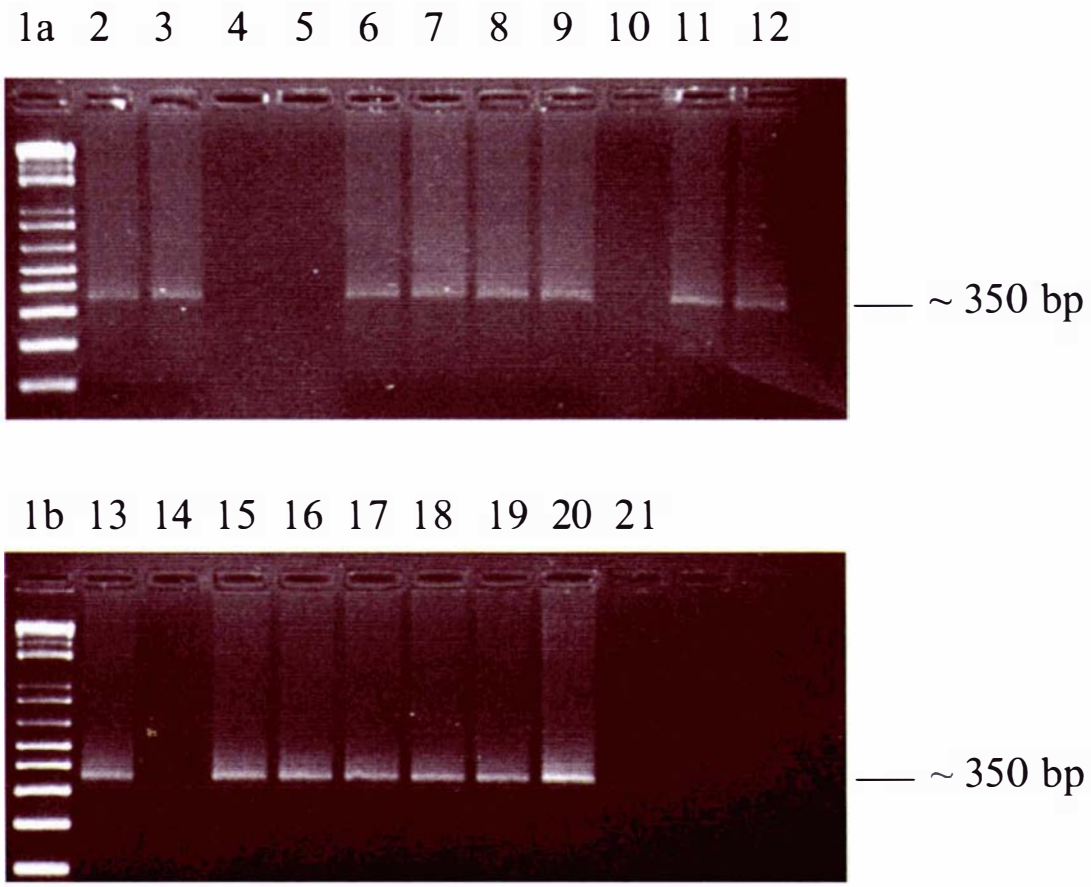

Figure 4.5: A 350 bp PCR product from the brains of calves (n = 18) born to heifers that were either *N. caninum* seropositive or seronegative. Calves were sacrificed ~2 days after birth and DNA was isolated from their brains and used in a PCR analysis. Lanes 1a and 1b are 1 kb+ molecular weight marker. Lanes 2 to 19 are calves 1, 5, 6, 8, 19, 22, 24, 46, 50, 50B, 53, 56, 62, 64, 86, 92, 130 and 133 respectively. Lane 20 is a positive control (Nc Liverpool DNA) and Lane 21 is a no DNA negative control of saline.

Table 4.3: Summary of *N. caninum* and BVDV assays on blood samples taken over 15 weeks of observation and brains taken after slaughter from heifers (n = 24) by natural infection status with *N. caninum* and experimental group for challenge with noncytopathic BVDV type 1 virus isolated from a persistently infected heifer.

Heifer ID	Dams historical sero-status based on IDEXX	Dams BVDV challenge status	*N. caninum* DNA (PCR)		Lesions in brain (Histopathology)	*N. caninum* antibodies		BVDV antibodies (ELISA)	*N. caninum* assay aggrement
			Blood PCR	Brain PCR		IFAT (1:80)	Western blot		
1	Neg	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
5	Neg	N/C	Pos	Pos	Neg	Pos	Pos	Pos	4/5
6	Neg	Challenged	Pos	Neg	Neg	Pos	Pos	Pos	3/5
8	Neg	Challenged	Pos	Neg	Neg	Pos	Pos	Pos	3/5
19	Neg	Challenged	INS	Pos	Neg	Pos	Pos	Pos	5/5
22	Neg	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
24	Neg	Challenged	Pos	Pos	Pos	Pos	Pos	Pos	5/5
46	Neg	N/C	Pos	Pos	Pos	Pos	Pos	Pos	5/5
50	Neg	N/C	Pos	Neg	Neg	Pos	Neg	Neg	1/5
53	Neg	N/C	Pos	Pos	Neg	Pos	Pos	Bdl	4/5
56	Neg	N/C	Pos	Pos	Neg	Pos	Pos	Pos	4/5
62	Neg	N/C	Pos	Pos	Pos	Pos	Pos	Bdl	4/5
50B	Pos	Challenged	Pos	Neg	Neg	Pos	Pos	Pos	4/5
64	Pos	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
86	Pos	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
92*	Pos	N/C	Neg	Pos	Neg	Pos	Pos	Bdl	4/5
130	Pos	Challenged	Pos	Pos	Pos	Pos	Pos	Pos	5/5
133*	Pos	N/C	Pos	Pos	Neg	Pos	Pos	Pos	4/5
35	Pos	Challenged	Pos	Pos	Pos	Pos	Pos	Pos	5/5
63	Pos	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
95	Pos	Challenged	Pos	Pos	Neg	Pos	Pos	Pos	4/5
100	Pos	Challenged	Pos	Pos	Pos	Pos	Pos	Pos	5/5
167	Pos	Challenged	Pos	Pos	Pos	Pos	Pos	Pos	5/5
172	Pos	N/C	Pos	Pos	Neg	Pos	Pos	Bdl	4/5

Four pregnant *N. caninum* seropositive, 5 aborted *N. caninum* seropositive and 6 pregnant *N. caninum* seronegative heifers were experimentally challenged by I/V inoculation with BVDV, while 2 pregnant *N. caninum* seropositive, 1 aborted *N. caninum* seropositive and 6 *N. caninum* seronegative heifers were sham inoculated with MEM. All heifers were at ~ 150 days of gestation at time of challenge. Note: Pos = positive; Neg = negative; Bdl = borderline result = around cut-off point; INS = insufficient serum; N/C = not challenged; *N. caninum* assay agreement: numerator = positive results and denominator = total number of assay; * = *N. caninum* isolated from brain.

4.4 Discussion

An experimental challenge study was conducted during the second trimester of pregnancy in heifers that were naturally infected with *N. caninum* or not, were pregnant or previously aborted and that were experimentally challenged by intravenous inoculation with BVDV or not. Rectal palpation and serology conducted on sera obtained from these heifers at their time of arrival showed that 6 previously pregnant seropositive (IDEXX *Neospora caninum* ELISA) heifers had aborted before arrival. On arrival at the experimental sites these and 6 other previously seropositive (IDEXX *N. caninum* ELISA) heifers that remained pregnant and 1 previously seronegative (IDEXX *N. caninum* ELISA) pregnant heifer were seropositive to *N. caninum* using the IFAT at a cut-off titer of 1:80. Repeated serology for the duration of their gestations, or for the equivalent time for those that had aborted, was conducted on these heifers using the IFAT with the lowest test dilution of 1:80. This cut-off point was used because at an initial cut-off of 1:200, parasite DNA was detected in samples that were being interpreted as seronegative. There was thus a need to increase IFAT sensitivity by decreasing the cut-off dilution. Researchers have discussed the effect of the cut-off dilution chosen on IFAT sensitivity. Some have suggested IFAT cut-off titres of 1:25 and 1:40 for adult cows (Conrad *et al.* 1993; Dubey 1999; Schares *et al.* 1999) and 1:80 for foetal serology (Dubey 1999). Others have also used low IFAT cut-offs in foetal serology. Barr *et al.* (1995) found IFAT titres of 1: 80 in 50% of foetuses with confirmed neosporosis. Wouda *et al.* (1997) found low IFAT titres (1: 25) in 65% (31 of 48) of foetuses with confirmed neosporosis.

An interesting observation in this study was the proportion (44.4%) of vertical transmission in previously consistently seronegative dams that were also born to dams that were consistently seronegative based on the ELISA. It is not known that seronegative dams transmit neosporosis to their calves *in utero*. However, it is plausible that the ELISA used in screening these animals may not have been sensitive enough to detect infection since PCR assay on the ELISA-seronegative dams and their calves showed circulating *N. caninum* DNA presumably associated with infection. This observation may also be true for the IFAT at 1:200, because the same dams tested negative at this IFAT dilution. However, when IFAT sensitivity was increased by reducing the titre accepted as positive to 1:80, 7/18 (38.8%) were positive to *N.*

caninum. Western blot analysis of sera from these animals corroborates the PCR and IFAT results since all 8 IDEXX-seronegative dams were positive on Western blot analysis. Reichel and Pfeiffer (2002) had compared IDEXX-ELISA with IFAT and recommended that the ELISA cut-off be reduced from the manufacturer's recommended value of S: P ratio of 0.5 to 0.21 or 0.22 in order to increase the sensitivity of the ELISA. In the present study, the manufacturer's recommended cut-off of 0.5 was used. Lowering the ELISA cut-off to an S:P ratio of 0.21 may have produced similar results as were obtained with the IFAT (1:80), PCR and Western blot.

Despite the reduction in IFAT cut-off, antibody fluctuations occurred in IFAT titres across all heifers irrespective of their physiological status and whether they were challenged with BVDV or not (Table 4.1). A heifer challenged with BVDV (19) and an unchallenged one (50) fluctuated mostly between <1:80 and a 1:80 titre. Despite this, heifer 19 with a low titre gave birth to an infected calf as shown by PCR of the brain (Figure 4.5). Other interesting observations are for heifers 5 and 130 (Tables 4.1 and 4.2) that were both challenged with BVDV and delivered calves that were infected but yet had *N. caninum* titres that were generally low or were seronegative throughout the study. The reason for the observed antibody fluctuations in these animals is not known but the results obtained indicate that these animals were infected and that even a low IFAT titre of 1: 80 may occur in infection with *N. caninum*. Reports on antibody fluctuations in *N. caninum* infected cattle have been documented previously (Conrad *et al.* 1993; Dubey *et al.* 1996; Dubey *et al.* 1997; Dannatt 1997; Hietala and Thurmond 1999). In the present study, antibody titres rose, fell and sometimes deteriorated below IFAT sensitivity resulting in some negative results (Table 4.1). This implies that if the IFAT cut off titre was set at 1:200, many apparently negative results would be false negative results.

The PCR assay conducted on blood samples obtained from all heifers on their arrival at the beginning of the experiment, showed circulating DNA in all (12) previously seropositive animals, including the 6 heifers which had aborted, plus in two out of 12 previously seronegative heifers (Table 4.1). In addition, serial PCR on the blood of the 12 previously seronegative heifers (IDEXX *Neospora caninum* ELISA) revealed *N. caninum* DNA associated with low or intermittently high IFAT titres to *N. caninum* in at least 10 heifers (Table 4.1). This was in contrast to the consistency observed in the

results of previous serology (data not shown) using the IDEXX *N. caninum* ELISA in the herd of origin and may indicate that recent horizontal transmission and parasitaemia had occurred.

The consistent presence of parasite DNA in the blood of naturally infected heifers that went full term showed that during pregnancy and around the time of abortion in these heifers, parasitaemia had occurred, presumably from the release of tachyzoites into the blood. Hence, *N. caninum* DNA was amplified in blood using PCR. These heifers were in their 2nd trimester of pregnancy at the start of this study. It appears that parasitaemia occurred and tachyzoites invaded the placenta resulting in vertical transmission, as their calves were infected. In contrast, no parasite DNA was detected in the blood of the aborted heifers at the time of expected parturition although it had been detected earlier. This time corresponds to approximately the 17th week after abortion had occurred in the aborted heifers. This suggests the heifers had resumed control over their *Neospora* infections and whatever conditions that had allowed parasitaemia in the pregnant heifers had disappeared by this time. Okeoma *et al.* (2005) semi-quantified the amount of parasite DNA present in the blood of aborted and pregnant cows and concluded that DNA concentration increased in the pregnant group but decreased with time in the aborted group. Although parasitaemia cleared in the group that had aborted, their brains remained positive on PCR for parasite DNA when they were tested after their cohorts had calved, presumably from tissue cysts containing bradyzoites. The presence of parasite DNA in the brains of these heifers indicates that they remained chronically infected with *N. caninum.* The absence of parasite DNA in the brains of 4 heifers of which 3 had parasite DNA in their blood, suggests that these heifers were either not infected from a natural challenge or were transiently infected via horizontal routes and that their immune systems cleared the infection before tissue cyst formation. If horizontal infection occurred, a source of infection could be through the ingestion of contaminated pasture, supplementary feed, or water from the farm where these heifers were raised before the start of the experiment. This is because the majority of heifers involved were raised and grazed together. Another plausible explanation of the positive DNA in the blood of heifers is that the tests for *N. caninum* in the brain were insufficiently sensitive to demonstrate infection or that parasite load in the brain was below the test sensitivity.

It is assumed that circulating tachyzoites will be found in the blood of cows but Dubey *et al.* (1988) noted that bradyzoites in tissue cysts are found in neural tissues. It appears that during pregnancy, bradyzoites are reactivated causing recrudescence of a latent infection. Tachyzoites are produced which proliferate by endodyogeny (Hemphill *et al.* 1999), producing several hundred new parasites in a few days. Tachyzoites form a membrane-bounded pseudocyst on proliferation and at a critical mass; host cell lysis releases tachyzoites which then infect other cells and tissues (Hemphill 1999). Thus, during tachyzoite movement from cell to cell and between tissues including during placental invasion, tachyzoites appear in blood. PCR on blood does not discriminate between free tachyzoites and those within leucocytes. These events are more likely during pregnancy because of the down-regulation of the immune response to parasitic pathogens observed in animals and man (Quinn *et al.* 2002). In the present study, parasitaemia was cleared from the cows that aborted, from the 11th week of observations.

The release of tachyzoites and their presence in the circulation of aborted heifers could mean that recrudescence of latent infection occurred in the 1st trimester of pregnancy resulting in foetal infection and death. At this stage of pregnancy, the foetal immune system is not sufficiently developed to mount an immune response against the parasite. Williams *et al.* (2000) and Guy *et al.* (2001) both observed that infection in the 1st trimester of pregnancy resulted in foetal death. By contrast Williams *et al.* (2000); Guy *et al.* (2001); Innes *et al.* (2002) and Piergili Fioretti *et al.* (2003) reported that infection with *N. caninum* in mid and late pregnancy results in the birth of a clinically healthy but congenitally infected calf. This suggests that parasitaemia with *N. caninum* tachyzoites in mid and late pregnancy allows exposure and infection of immunocompetent foetuses that results in the birth of chronically infected but healthy calves, as were seen in this study. In the present study, all observations commenced in the second trimester, so it was not possible to study this effect of timing.

No heifer challenged intravenously with BVDV aborted. This could be a result of the time of inoculation and stage of gestation which was at approximately 150 days and designed to be after the danger of a primary BVD abortion had passed. Repeated serology on all 24 heifers revealed that at some point, 19 heifers were seropositive to BVDV, 4 heifers had antibody titres at the cut-off margin, while 1 heifer remained

seronegative. One heifer had BVDV antibodies on arrival at the experimental site while 5 aborted heifers showed antibodies to BVDV only one week after arrival and sampling began. Even though they were grazed with a PI heifer, the early appearance of antibodies indicates that these cows were apparently infected prior to arrival for the start of the experiment, since acutely infected animals are recorded to produce serum antibodies 3 to 4 weeks after infection. After experimental challenge with BVD virus, heifers did not show clinical signs indicative of BVDV infection; apart from moderate temperature increases to ≤ 40°C. Virus isolation was not attempted. As a consequence, it is difficult to interpret any likely effect of BVDV as it appears most were naturally challenged by the time of the inoculation and would have at least a modest immune response.

In the transmission study, 14 of 18 (77.8%) available dam-calf pairs had *N. caninum* DNA in their brains (Table 4.2, Figures 4.3a, 4.3b and 4.5). This showed that the calves were infected *in utero* as reported by Anderson *et al.* (1997); Bergeron *et al.* (2000); Bjorkman *et al.* (1996); Davison *et al.* (1999); Dubey *et al.* (1992); Liddel *et al.* (1998); Paré *et al.* (1996) and Waldner *et al.* (1999). No *N. caninum* DNA was detected in the blood of these 14 calves except for in one stillborn calf. The presence of parasite DNA in the blood of the stillborn calf showed that it might have suffered from acute neosporosis before death. Blood samples tested from other calves were PCR negative while their brains were PCR positive. This suggests that they did not have an active parasitaemia at the time of sampling but had become chronically infected with tissue cyst formation having occurred by the time of birth. Serologically, 11 of these 14 dam-calf pairs had antibodies to *N. caninum* in both dam and calf confirming calf infection *in utero* because pre-colostral sera were used. One pair (heifer 19 Table 4.2) could not be assessed because a sample was not available for the dam although the antepartum sample was positive, but the titre for the calf was negative. Three calves had tissue cysts in the brain but no antibodies were demonstrable. One of these calves (calf 19, Table 4.2) had a dam with antibodies antepartum on 7 of 11 occasions but the dam's serum could not be tested at calving because of leakage of the serum tube prior to testing. Another calf (calf 46, Table 4.2) was PCR positive in the brain sample but did not have a positive antibody titre and also had a dam with a fluctuating antepartum antibody titre (heifer 46, Table 4.1) and a postpartum antibody titre of 1:80 (heifer 46, Table 4.2). The remaining dam-calf pair (heifer and calf 130) both had no antibodies

postpartum (Table 4.2) but their brains were PCR positive. This suggests that the sensitivity of precolostral antibody titres as a means of identifying infected calves is less than 100%, at an IFAT titre of 1:80 as a cut-off. In addition there was one dam-calf pair in which both were strongly serologically positive but the brain of neither was positive, possibly indicating that PCR of the brain is also not 100% sensitive.

Nine calves were born with detectable antibodies to BVDV in their serum before they ingested colostrum providing evidence that the virus had infected these immune competent foetuses via the placenta. Others have found BVDV antibodies in foetuses of colostrum-deprived calves. Zimmer *et al.* (2002) reported active immunity in colostrum-deprived calves from dams treated with BVDV vaccines. Similarly, Stokstad *et al.* (2003) reported the presence of BVDV antibodies in calves born to cows experimentally infected with BVDV between days 74 and 81 of pregnancy. In addition, Scherer *et al.* (2001) reported the presence of BVDV antibodies and absence of BVD virus in lambs born to ewes inoculated with BVDV-2 at days 65-70 of gestation. None of the calves in this study were born with evidence of persistent infection with BVDV since no viral RNA was detected in their blood. An explanation is that the time of inoculation and infection of the dam was at ≥ 150 days of gestation, when the foetal immune system is sufficiently developed to prevent the carrier state from occurring. Persistent foetal infection may result if the dam is viraemic between days 45 and 125 of gestation when the foetus is unable to recognise the virus as foreign and is thus unable to produce serum neutralising antibodies against it. As the immune system develops during the second trimester of gestation, the foetus is able to mount an immune response to BVDV infection. However, congenital defects and/or abortion may still occur but rarely. Infection late in pregnancy usually results in the birth of clinically normal calves because at this stage, the immune system of the foetus has fully developed resulting in the presence of pre-colostral antibodies to BVDV.

Detection of *N. caninum* DNA in the blood of the heifers indicates that they were subjected to active horizontal infection, or recrudescence of latent infection either just before or during the observations. This appears to have caused some to abort and others to vertically transmit infection to their calves. Heifers challenged with BVDV produced calves with active immunity to BVDV because heifers were challenged when the foetal immune system was sufficiently developed to allow foetal survival with immunity.

However, *N. caninum* which was believed to be present in half of the dams and also appears to have been present in others or acquired horizontally was shown to be vertically transmitted in this study. Antibody fluctuations were observed in both aborted and pregnant heifers and antibody levels were frequently below what would be taken as a lower cut off screening level for infection.

Experimental BVDV challenge of pregnant and aborted heifers that were *N. caninum* seropositive or seronegative lead to seroconversion. None of the pregnant heifers aborted after BVDV challenge indicating that their foetuses were immunocompetent at the time of challenge. It is important to note that heifers that were previously *N. caninum* seronegative reacted by producing anti *N. caninum* antibodies after BVDV challenge. These heifers showed signs of parasitaemia because *N. caninum* DNA was amplified from their blood. The ramifications of synergistic infection of *N. caninum* and BVDV are still not yet completely understood. However, BVDV is believed to potentially enhance diseases caused by other pathogens or precipitate illness by opportunistic pathogens (Bohac and Yates 1980; Malmquist 1985; Potgieter 1977). The present study supports this hypothesis since results show that *N. caninum* in cows that are also BVDV positive produced 44.4% (8/18) dam-calf pairs that were both BVDV and *N. caninum* seropositive. Bjorkman *et al.* (2000) had previously reported a significant association between BVDV and *N. caninum* infection in cattle. Although the dams and their calves in the present study had antibodies to BVDV, none of the calves was positive to reverse transcription PCR test indicating that there was no viral RNA in their blood. Thus, they were not persistently infected.

N. caninum antibody fluctuation has been well documented (Conrad *et al.* 1993; Dubey *et al.* 1996; Dubey *et al.* 1997; Dannatt 1997; Hietala and Thurmond 1999). The current study revealed that in cows, antibody to *N. caninum* can rise and fall and sometimes can deteriorate to below the detection limit of IFAT and Western blot within a brief period. It is surprising that this observation was made on cows labelled as seronegative by long term monitoring using an ELISA test. This observation was true regardless of the pregnancy status of the cow. The observation was also supported by concurrent positive PCR responses demonstrating *N. caninum* DNA. As a result of these observations, it is important to consider redefining what constitutes a *N. caninum* positive cow by critically examining and considering the cut-off values applied for different assays used

in the study of neosporosis. Correct use of cut-off titres and their appropriate interpretation along with the use of DNA determination by PCR will help to reveal and identify animals that are suffering from exposure or transient infection, as well as chronically infected animals whose immunologic system is masking infection.

The original experimental design (Appendix 2) was compromised by abortions which occurred in 6 of the 12 *N. caninum* seropositive heifers. This necessitated redesign of the experiment (Figure 4.1) and subsequent loss of statistical power. Nevertheless, the longitudinal study of *N. caninum* infections during the second half of pregnancy allowed several interesting observations to be made which have been discussed above.

Author's contributions to this experiment

N.B. Williamson helped with designing and redesigning the experiment, animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the molecular analyses in these experiments as well as providing editorial advice.

C.M. Okeoma helped with project design and redesign, animal inoculation, sample collection and clinical observations. She conducted the experiments and wrote the manuscript.

C.B. Okeoma read the manuscript and provided editorial advice.

L. Gillespie helped with animal observation and sample collection.

M. Alley carried out pathological analysis of the brain samples.

Chapter 5

The use of polymerase chain reaction to detect *Neospora caninum* deoxyribonucleic acid in the blood of naturally infected cows[2]

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5.0 Abstract

Twelve 2-year-old heifers in their 5th month of gestation when pregnancy tested were the subjects of this study. Six heifers aborted at approximately 4 months of gestation and had blood samples drawn less than 6 weeks after the abortions were identified. Blood samples were also drawn from three seropositive pregnant and three seronegative pregnant heifers. DNA was isolated from the samples and a 350 bp fragment of the Nc-5 gene was PCR amplified using primer pair Np21+ and Np6+. Also, the Internal Transcribed Spacer 1 (ITS1) was PCR amplified using Tim 3 and Tim 11 primer pair. The Nc-5 gene fragment was cloned, sequenced and the sequence BLAST-tested. Similarly, the ITS1 product was sequenced and subjected to BLAST analysis. The BLAST test results revealed that *N. caninum* DNA was present in these blood samples indicating that Polymerase Chain Reaction can be used in the detection of *N. caninum* DNA in the blood of parasitaemic cows.

5.1 Introduction

N. caninum is an apicomplexan parasite first associated with bovine abortion in the USA in 1989 (Thilsted and Dubey 1989). Since this observation *N. caninum* has been implicated as one of the major causes of infectious abortion of cattle worldwide (Dubey 1999; Trees 1999; Hattel *et al.* 1998). Abortion due to neosporosis can occur at any stage of pregnancy but is most likely to occur at 5 to 6 months of gestation (Dubey 1999). Cows that have aborted in a previous pregnancy due to neosporosis can abort again (Obendorf *et al.* 1995). They can also give birth to diseased calves, calves with a sub-clinical *N. caninum* infection, or uninfected calves (Anderson *et al.* 1995). *N. caninum* induced abortions may be endemic, epidemic or sporadic in cattle (Dubey and Lindsay 1996; Schares *et al.* 1999; Atkinson *et al.* 2000; Thobokwe and Heuer 2004).

N. caninum antibodies can be detected by serology, specific *N. caninum* antigens are detected by Western blot and antigen-ELISA, while polymerase chain reaction (PCR) is used to detect *N. caninum* DNA. Detection of *Neospora* DNA in brains and lungs of animals has been reported (Lally *et al.* 1996; Holmdahl *et al.* 1996). Other authors' have inferred the presence of circulating *N. caninum* tachyzoites from circulating antibody levels (Williams *et al.* 2003). Recently *N. caninum* DNA was detected from 7 to 49 days after inoculation in the blood of ewes inoculated orally with *N. caninum* oocysts (O'Handley *et al.* 2002). Furthermore, *N. caninum* was detected in the semen of naturally infected bulls (Ortega-Mora *et al.* 2003). We report the successful and confirmed detection of *N. caninum* deoxyribonucleic acid (DNA) in the blood of naturally infected heifers, providing a direct demonstration of the presence of parasite DNA in blood.

5.2 Materials and methods

5.2.1 Animals

Twelve 2-year-old heifers in their 5th month of gestation when pregnancy tested were used in this study. Historically, nine heifers out of the twelve were seropositive while three were seronegative. This serological history was based on IDEXX ELISA tests

conducted 4 times with an interval of 3 months per test on these heifers. When pregnancy tests were repeated on all heifers 43-days later, 6 seropositive heifers were no longer pregnant. No aborted material from these heifers was recovered. Blood samples for Immunofluorescent Antibody Tests (IFAT) and PCR were collected from all 12 heifers (6 seropositive-aborting heifers, 3 seropositive pregnant heifers and 3 seronegative pregnant heifers). Serologists at the Gribbles Pty Ltd Animal Health Laboratory (Palmerston North, New Zealand) conducted IFAT. All animal usage was approved by the Massey University Animal Ethics Committee, protocol number 02/29.

5.2.2 DNA isolation

Blood was collected into EDTA tubes and processed immediately or stored at 4°C for later processing. Total DNA was isolated from blood using a Qiagen DNAeasy blood kit according to manufacturer's instructions. Electrophoresis of each DNA sample on 2% agarose gel in 1X TBE buffer was undertaken to check the integrity of the DNA. A 100 µl aliquot of total DNA was produced from each sample and stored at -20°C until required for analysis. As a control to detect contamination, a water blank isolation was always performed alongside the DNA isolation.

5.2.3 PCR Primers

Two different primer pairs were used for the PCR. A *N. caninum* species-specific primer pair Np21+ (5'-CCCAGTGCGTCCAATCCTGTAAC-3') and Np6+ (5'-CTCGCCAG-TCAACCTACGTCTTCT-3') that anneals to the Nc-5 region (Muller *et al.* 1996), Tim 3 (5'- CCGCTGCAGAGGTGAACCTGCGGAAGGATC -3') and Tim 11 (5'- CACTGA-AAACAGACGTACC-3") primer pair (Payne and Ellis 1996) that target the ITS 1 region were used. A multiplex PCR using Np21+, Np6+ (Muller *et al.* 1996) and HL033-HL035 (Baszler *et al.* 1999) primer pairs was carried out as a control against false positives. HL033 (5' CGAGTCCTTATGAGCTTGATTCTT3') and HL035 (5' GCCTTCCAGAAGTCGTTTGTTTTC3') target part of the bovine prolactin gene.

5.2.4 PCR Amplification

All PCR reactions were performed in a 20 µl volume containing 2 µl of sample containing 0.2 µg DNA, 0.4 µl (0.2 mM) dNTPs mix, 1.4 µl (3.5 mM) $MgSO_4$, 2 µl of 1X PCR Mg-free buffer, 0.12 µl of 0.6U of Platinum *Taq* polymerase, 0.4 µl of 0.2 mM of each primer and 0.25 µl of 1X PCR enhancer (Invitrogen Cat #11495-017). PCR was performed in a Perkin Elmer GeneAmp PCR Thermocycler System 2400 with the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, with a final extension of 72°C for 10 min. A negative control (water blank) and a positive control DNA from an Nc-Liverpool isolate were also included in each reaction. The amplified product of the Nc-5 region was separated by electrophoresis on a 2% NuSieve 3:1 agarose gel while the product of the ITS 1 region was separated on a 2% agarose gel. PCR products from the multiplex reaction were separated with 3% NuSieve 3:1 agarose gel. Separated products were stained with ethidium bromide and viewed under UV light.

5.2.5 Cloning of Nc-5 fragment

To clone the PCR products of the Nc-5 fragments, samples from 2 heifers were selected for PCR amplification. Using the first PCR product as a template for a second PCR increased the DNA concentration of the PCR products. Briefly, a first PCR was performed in a 20 µl volume after which 10 µl of the product was used as a template for a second PCR performed with the same primer pair under the same reaction conditions in a 50 µl volume. Amplified products were separated on 2% NuSieve agarose gel in 1X TBE buffer. The remaining PCR products were column purified using a High Pure PCR purification kit (Roche Diagnostics Corporation, Indianapolis, USA) according to the manufacturer's instructions. The purified products were quantified using a NanoDrop ND-1000 Spectrophotometer and ligated into plasmid vector pGEM-T Easy (Promega, Madison, USA). The ligated mixture was used to transform chemically competent *E. coli* XL 1 Blue according to Sambrook *et al.* (2001). A positive and a negative control were included. Three clones from each sample were obtained, purified and recombinant products sequenced and analysed. The New Zealand Environmental

Risk Management Authority (ERMA) approved the protocols used in cloning these products and the approval code is GMO 03/MU/12.

5.2.6 Sequencing of recombinant DNA and PCR products

The recombinant DNA was sequenced using BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit and T7 sequencing primer. Also, the PCR product of the ITS region was sequenced from both ends using the above reaction kit and the PCR primer as the sequencing primer. The DNA sequence databases were searched using the nucleotide-nucleotide BLAST (blastn) at the National Centre for Biotechnology Information USA (http://www.ncbi.nlm.nih.gov/BLAST/). Comparison of the Nc-5 fragments and ITS-1 sequences with previously described sequences was done using the Align two sequences (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) at the National Centre for Biotechnology Information USA (http://www.ncbi.nlm.nih.gov/BLAST/).

5.2.7 Fractionation of blood

Leucocytes, lymphocytes and serum were isolated from blood samples of 2 seropositive and PCR positive heifers (1 aborting heifers and 1 pregnant heifer). DNA was isolated from whole blood, leucocytes, lymphocytes and serum samples as described above using Qiagen DNAeasy blood kit. All DNA samples were PCR amplified using primer pair Np21+ and Np6+ and the reaction conditions described above.

5.3 Results

Table 5.1 summarises the IFAT and PCR results from the 12 heifers. Products of ~350 bp were amplified from blood obtained approximately 1-5 weeks after abortion in six aborting heifers, three seropositive pregnant heifers and three seronegative pregnant heifers using primer pair Np21+ and Np6+ (Figure 5.1). The size of the bands matched the positive control (Nc-Liverpool). The PCR products were sequenced from both ends.

When the Nc-5 sequence was subjected to a BLAST search, the best score of 100% to 97% identity was returned with *N. caninum* (AF190701.1, X84238.1, AY497042, AY497044, AY497043 and AY497041) in listed order. The nucleotide sequences of all clones were identical when compared and can be viewed in GeneBank with the accession number AY459289. Similarly, using the Tim 3 and Tim 11 primer pair, a product of ~620 bp was obtained from all seropositive heifers (Figure 5.2). The ITS BLAST search returned the best score of 100% to 97% identity for *N. caninum* (AF432123.1, NSPRGEBN, AF029702 and AF038861). The GeneBank accession number is AY463245.

The multiplex PCR reaction (Figure 5.3) gave products of ~350 bp (Np21+ and Np6+) and ~156 bp (HL033 and HL035) in all lanes containing samples from seropositive aborting and pregnant heifers, a product of ~156 bp from seronegative pregnant heifers, a product of ~350 bp in the positive control lane which contained no bovine DNA and no product in the negative control. Figure 5.4 shows the result of the fractionation experiment. PCR Products of 350 bp were obtained from leucocytes, lymphocytes and whole blood samples while no product was obtained from serum samples.

Table 5.1: Summary of heifers' (n = 12) serological (IFAT) and polymerase chain reaction product status during the experimental period.

S/N	Heifer ID	Initial serology status	Physiologic status	IFAT at 1:80	PCR
1	35	Positive	Aborted	1:200	Positive
2	63	Positive	Aborted	1:200	Positive
3	95	Positive	Aborted	1:200	Positive
4	100	Positive	Aborted	1:200	Positive
5	167	Positive	Aborted	1:200	Positive
6	172	Positive	Aborted	1:2000	Positive
7	46	Negative	Pregnant	0	Negative
8	50	Negative	Pregnant	0	Negative
9	53	Negative	Pregnant	0	Negative
10	86	Positive	Pregnant	1:1000	Positive
11	92	Positive	Pregnant	1:80	Positive
12	133	Positive	Pregnant	1:200	Positive

The initial serological status of heifers was established using an IDEXX *N. caninum* ELISA test. All seronegative heifers came from consistently seronegative dams, while all seropositive heifers came from consistently seropositive dams. All pregnant heifers were approximately 150 days pregnant, while abortion occurred in the group that aborted between days 100 and 150 of gestation. Blood samples were collected from all heifers at approximately 150 days of gestation or up to 6 weeks after abortion which was when pregnancy diagnosis had occurred.

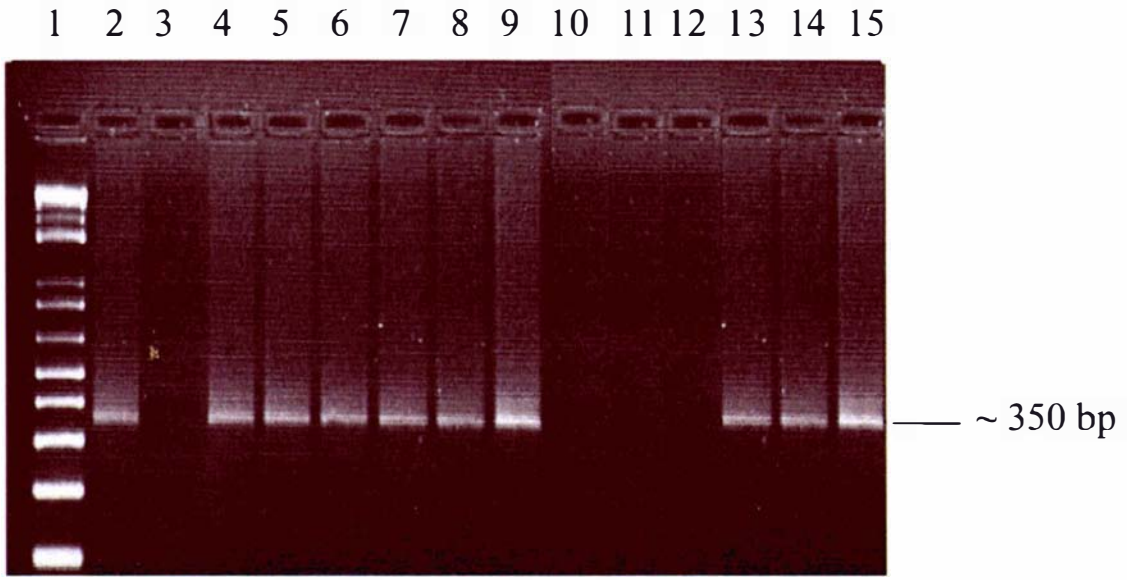

Figure 5.1: PCR product of Nc-5 fragment amplified with primer pair Np21+ and Np6+ from heifers (n = 12) infected or not infected with *N. caninum*. Lane 1 = 1 kb+ ladder, Lanes 2 and 3 = positive and negative controls respectively, Lanes 4 to 9 = products from seropositive heifers after abortion, Lanes 10 to 12 = products from seronegative pregnant heifers, Lanes 13 to 15 = products from seropositive pregnant heifers. All pregnant heifers were approximately 150 days pregnant, while abortion occurred in the group that aborted between days 100 to 150 of gestation. Sampling started around 150 days of gestation in the pregnant group and up to 6 weeks after abortion in the aborting group.

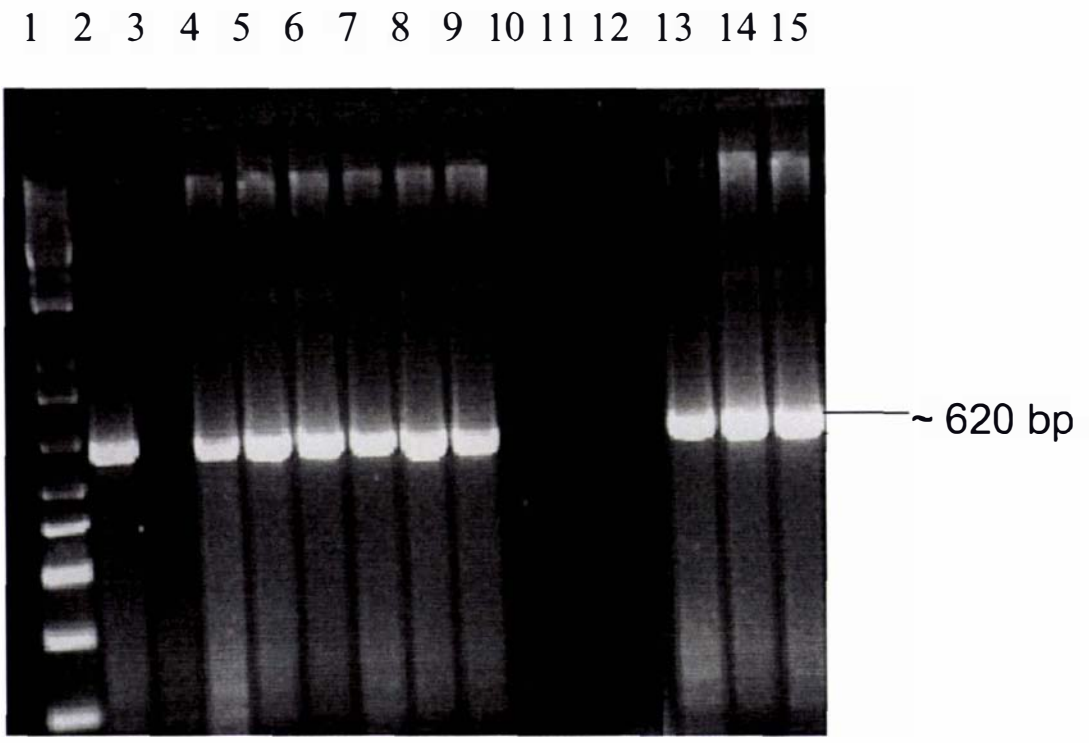

Figure 5.2: PCR product of *N. caninum* 18S rRNA and part of 5.8S rRNA gene amplified with primer pair Tim 3 and Tim 11 from heifers (n = 12) infected or not infected with *N. caninum* Lane 1 = 1 kb+ ladder, Lanes 2 and 3 = positive and negative controls respectively, Lanes 4 to 9 = products from seropositive heifers after abortion, Lanes 10 to 12 = products from seronegative pregnant heifers, Lanes 13 to 15 = products from seropositive pregnant heifers. All pregnant heifers were approximately 150 days pregnant, while abortion occurred in the group that aborted between days 100 to 150 of gestation. Sampling started at approximately 150 days of gestation in the pregnant group and up to 6 weeks after abortion in the aborting group.

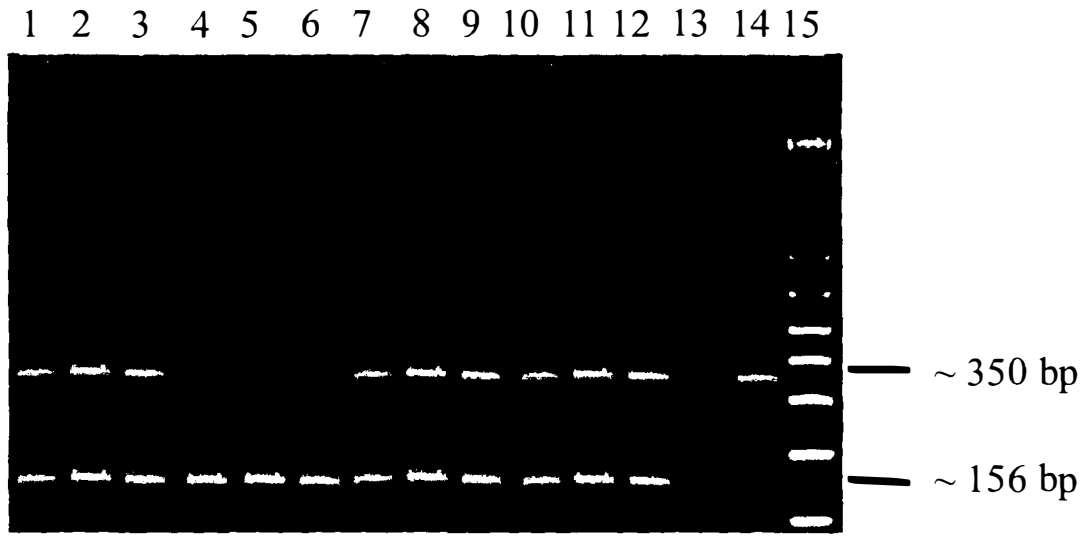

Figure 5.3: Multiplex PCR products amplified with primer pairs Np21+, Np6+ and PRL HL033-HL035. Lanes 1 to 3 = 350 bp and 156 bp products from seropositive pregnant heifers, lanes 4 to 6 = 156 bp products from seronegative pregnant heifers, lanes 7 to 12 = 350 bp and 156 bp products from seropositive heifers after abortion, lane 13 = negative control, lane 14 = 350 bp from positive control, lane 15 = 1 kb+ ladder. All pregnant heifers were approximately 150 days pregnant, while abortion occurred in the group that aborted between days 100 to 150 of gestation. Sampling started around 150 days of gestation in the pregnant group and up to 6 weeks after abortion in the aborting group. Oligonucleotide pair PRL HL033-HL035 was used as an internal control to identify true *N. caninum* positive heifers.

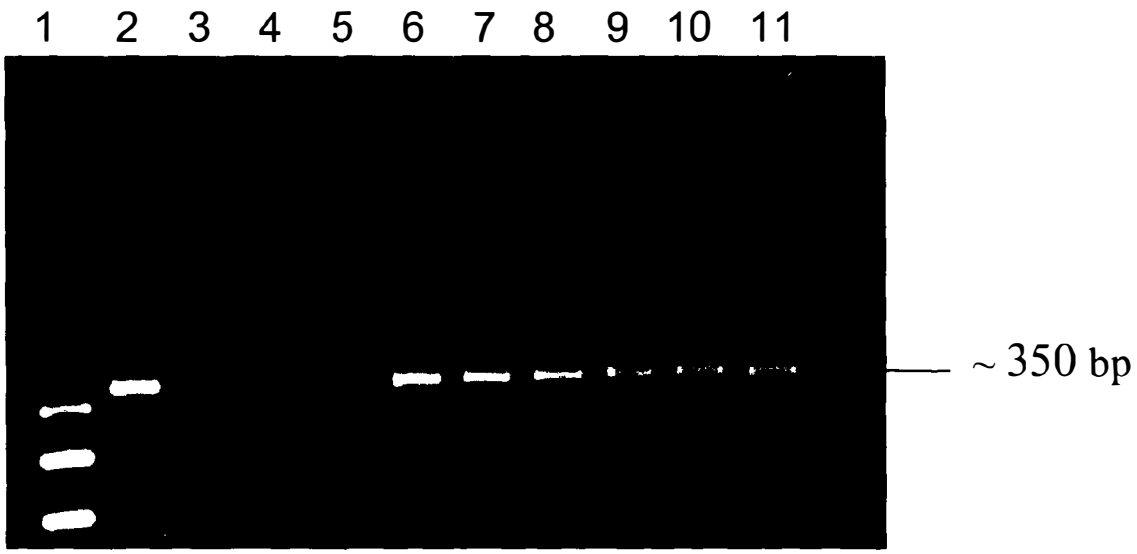

Figure 5.4: PCR products from leucocytes, lymphocytes, serum and whole blood amplified with primer pairs Np21+, Np6+. Lane 1 = 1 kb+ ladder, lanes 2 and 3 = positive and negative controls respectively, lanes 4 and 5 = serum samples, lanes 6 and 7 = leucocytes, lanes 8 and 9 = lymphocytes, lanes 10 and 11 = whole blood, lane 12 = an empty lane.

5.4 Discussion

The isolation of *N. caninum* DNA from the blood of naturally infected heifers by PCR is described here, to the authors' knowledge, for the first time. Only pregnant heifers were used in the present study because they were part of another study and thus available to provide blood samples for the amplification of *N. caninum* from blood. The PCR reactions and subsequent sequence analysis clearly demonstrate that the isolate from blood of these naturally infected heifers is *N. caninum.* The Nc-5 sequence amplified with the Np21+ and Np6+ primer pair was chosen for this study because it is a repetitive sequence (Kaufman *et al.* 1996; Muller *et al.* 1996; Yamage *et al.* 1996). This sequence is sensitive in real-time PCR (Collantes-Fernandez *et al.* 2002), quantitative-competitive PCR in mice (Liddell *et al.* 1999) and for diagnosis of *N. caninum* presence in bovine aborted foetuses (Baszler *et al.* 1999; Gottstein *et al.* 1998). The ITS 1 region of *N. caninum,* which was amplified with primer pair Tim 3 and Tim 11, was used as a confirmatory test in this work. This sequence has been successfully used to distinguish between closely related species and strains of *Neospora*, *Toxoplasma*, *Hammondia* and *Eimeria* (Ellis *et al.* 1998; Ellis *et al.* 1999; Mugridge *et al.* 2000; Dubey *et al.* 2001).

This study is in line with the findings of O'Handley *et al.* (2002), who isolated *Neospora* DNA from the blood of experimentally infected sheep, Ortega-Mora *et al.* (2003) who reported the isolation of *Neospora* DNA from the semen of naturally infected bulls, Burg *et al.* (1989) who isolated *Toxoplasma* DNA from leucocytes, and Hitt and Filice (1999) who detected *T. gondii* tachyzoites in the blood of infected rabbits. Detection of *N. caninum* DNA in blood, as in semen, brain, spinal cord and/or any body tissue presents *prima facie* evidence of infection. The detection of *N. caninum* in blood is expected because blood would appear to be the most likely transport medium for tachyzoites between body organs.

It appears that the fraction of blood that harbors *Neospora* DNA is leucocytes because the blood fractionation experiment carried out in this study revealed that *Neospora* DNA was amplified from whole blood, leucocytes and lymphocytes but not from serum, suggesting that the white blood cell fragment contains the *N. caninum* DNA. The reason for the absence of tachyzoite DNA in serum could not be explained, but in *T.*

gondii, tachyzoites were found intracellularly in leucocytes and not erythrocytes (Hitt and Filice 1999). It is hypothesised that *N. caninum* in the blood of infected cows circulates intracellularly in leucocytes. Intracellular transport would be protective for the organism because circulating antibodies could easily aid in killing tachyzoites in serum.

Authors' contributions to this experiment

N.B. Williamson helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the experiments and provided editorial advice.

C.M. Okeoma helped with animal inoculation, sample collection and clinical observations. She planned and conducted the experiments and wrote the manuscript.

Chapter 6

Quantification of *Neospora caninum* DNA present in the blood of naturally infected aborted and pregnant cows using real-time PCR[3]

[3] Published as: Okeoma CM, Stowell KM, Williamson NB, Pomroy WE. Experimental Parasitology

6.0 Abstract

This study quantified *N. caninum* DNA in the blood and brain of pregnant and aborted heifers by monitoring PCR product formation in real-time using SYBR Green 1, a double-stranded DNA-binding dye. Primers were designed to amplify a 188 bp product specific to *N. caninum* from the Nc-5 gene fragment of *N. caninum*. Similarly, a 71 bp product was amplified from the 28S rRNA gene of bovine genomic DNA that served as a control. Agarose gel electrophoresis and analysis of the melting curve for PCR products showed that both primer pairs were specific to their targets. Standard curves were generated for both bovine and *N. caninum* genomic DNA and were used to compute the relative concentration of parasite to bovine DNA in the test samples. The concentration of *N. caninum* DNA in 1 ng of bovine genomic DNA obtained from blood ranged between 0.097 ng at the 1st week of the observation to 0 ng at the 15th week in aborted cows. In pregnant cows, the values ranged between 0.080 ng at the 1st week to 0.155 ng at the 15th week of observation. There was a sustained decrease of DNA concentration in the aborted group after abortion and an increase in DNA concentration in the pregnant group. Comparison of parasite DNA in blood and brain of infected heifers showed a higher DNA concentration in brain than in blood. This study shows that *N. caninum* DNA can be quantified to obtain the relative concentration of parasite DNA to host genomic DNA in blood. This technique allows testing of a large number of samples at one time and can be done without the need for slaughter of tested animals.

6.1 Introduction

N. caninum is an apicomplexan protozoan parasite that causes neonatal neuromuscular disease in dogs and abortions in cattle (Lindsay *et al.* 2001). *N. caninum* induced-abortion was first reported in dairy cows in New Mexico in 1989 (Thilsted and Dubey 1989). Many factors may predispose cattle to neosporosis. Some of these factors include congenital transmission; presence of possible host animals on the farm e.g. the dog and domestic fowl; lactogenic transmission; immunosuppressive effect of bovine viral diarrhoea virus (BVDV) and pregnancy.

Pregnancy may trigger recrudescence of latent infection in a cow with chronic neosporosis. This may result in parasitaemia and foetal infection. In a naïve pregnant cow, infection with *N. caninum* is easily achieved because the immune regulation is suppressed (Quinn *et al.* 2002). It could be assumed that foetal infection follows a maternal parasitaemia although most infections occur in cattle already harbouring a persistent infection before pregnancy was established (Buxton *et al.* 2002). Infected cows may or may not abort their foetuses and most unaborted foetuses become congenitally infected and may or may not show clinical signs at birth (Dubey and Lindsay 1996). The above outcome of neosporosis during pregnancy may depend on all or a combination of the following: the gestational age/age of the foetus at the time of infection, the virulent nature of the particular *N. caninum* including the magnitude and time of parasitaemia (Innes *et al.* 2002; Williams *et al.* 2000). During pregnancy the body down-regulates the production of Th1 cytokines which may be incompatible with pregnancy (Quinn *et al.* 2002) since they promote foetal rejection. However, Th1 cells produce interleukin (IL)-2, tumour necrosis factor (TNF)- β and interferon (IFN)-γ and are the main effectors of phagocyte-mediated host defences (Mosmann and Coffman 1989; Romagnani 1991), which are highly protective against infections sustained by intracellular parasites. The down regulation of Th1 cytokine during pregnancy might lead to parasitaemia, when parasite tachyzoites can be found in several organs (Barr *et al.* 1994; Buxton *et al.* 1998) and body fluids (Okeoma *et al.* 2004a; Ortega-Mora *et al.* 2003).

Diagnosis of neosporosis is usually based on serology which detects parasite-specific antibodies in live animals (Björkman *et al.* 1999) or the visualization of characteristic

histopathological lesions in organs of dead animals and aborted materials. Foetal serology may also be used in diagnosis (Barr *et al.* 1995; Buxton *et al.* 1997; Paré *et al.* 1995). Most diagnostic analysis for neosporosis including some PCRs is done utilising materials from dead animals. We have shown that *N. caninum* DNA can be amplified in the blood of naturally infected cows allowing diagnosis of infection in live animals (Okeoma *et al.* 2004a).

In the present study, we quantified *N. caninum* tachyzoite DNA in the blood of naturally infected aborted and pregnant heifers by monitoring parasite DNA concentration in the animals. At the end of monitoring we compared parasite DNA concentration in brain with that obtained from blood of infected aborted and pregnant heifers. We also compared the sensitivity of real-time and block PCRs. The quantification of *N. caninum* DNA in the blood can complement serology in determining infection intensities in live animals and thus offers a tool to significantly improve research into the epidemiology and pathogenesis of *N. caninum* infections.

6.2 Materials and methods

6.2.1 Animals and sample collection

Six rising 2-year-old heifers (5 from a group of 20 heifers from one farm and 1 from a group of 4 heifers from two other farms) were in their 5th month of pregnancy when tested on 5th March 2003 or later. Pregnancy tests were repeated on all heifers on 16th April 2003. The latter test showed that of the 6 heifers used in this study, 3 were still pregnant and the other 3 had aborted. The exact time of abortion was not known for those aborting and no aborted material from them was recovered.

Heifers included in this study were selected from the initial 24 in the following way. Firstly, they were divided into 2 groups based on their initial serology i.e. a positive or negative IDEXX ELISA *N. caninum* antibody result. Then the positives were divided into pregnant and aborted groups. From these two groups, the DNA of one heifer in each group (that had the most obvious PCR band on an agarose gel) was cloned and sequenced; confirming that products were *N. caninum* sequences (Okeoma *et al.* 2004a).

These heifers (heifers 172 and 133) were selected. The other 4 heifers, two each from the aborted (35 and 95) and pregnant (64 and 86) groups were randomly selected. After abortions were noticed, blood samples were obtained from all heifers initially weekly for 7-weeks and then bi-weekly for 8 weeks. DNA was isolated from blood as described by Okeoma *et al.* (2004a). At calving, pre-colostral blood was obtained from calves of heifers 64, 86 and 133. The samples were tested for presence of *N. caninum* antibodies using indirect fluorescent antibody test (IFAT). The result (1:4000, 1:8000 and 1:2000 respectively for these calves showed that they were congenitally infected. In addition, brain samples were collected for analysis from all animals at slaughter. *N. caninum* tachyzoites were isolated from the brains of heifer 133 and her calf (Okeoma *et al.* 2004b). PCR analysis of the brains of all calves confirmed that they were congenitally infected.

Total DNA was isolated from the brains of heifers using a Qiagen DNAeasy blood kit according to manufacturer's instructions. Electrophoresis of each DNA sample on a 2% agarose gel in 1X TBE buffer was undertaken to check integrity. The DNA samples were then diluted 10 fold and quantified using a Hoefer DNA Quant 200 Fluorometer. A total of 2 μl of sample DNA containing 1 ng/μl of bovine brain genomic DNA was used in a reaction volume of 20 μl for the PCR assay.

6.2.2 Block-PCR assays

Following DNA isolation, samples were used for block-PCR amplification of *N. caninum* DNA using *N. caninum* specific primers Np21+ and Np6+ (Muller *et al.* 1996) as described by Okeoma *et al.* (2004a) in the polymerase chain reaction to detect *N. caninum* deoxyribonucleic acid in the blood of naturally infected cows. Amplified products were separated by 2% agarose gel electrophoresis. Separated products were stained with ethidium bromide and viewed under UV light.

6.2.3 Real Time PCR assays

6.2.3.1 Primers

A primer pair flanking 188 bp of Nc-5 gene (Yamage *et al.* 1996) of *N. caninum* was designed using LightCycler Probe Design Software version 1.0 from Roche Molecular Biochemicals. The primers thus called NeoF (5'-GTGAGAGGTGGGATACG-3') and NeoR (5'-GTCCGCTTGCTCCCTA) both had a GC content of 53.2%. For the quantification of bovine genomic DNA for use as a control, a specific fragment of 71 bp was amplified from the 28S rRNA gene (Collantes-Fernández *et al.* 2002). The sequence of the forward primer 28S-PF was 5'-TGCCATGGTAATCCTGCTCA-3' and that of the reverse primer 28S-PR was 5'-CCTCAGCCAAGCACATACACC-3'. Reaction conditions for the primers were optimised by titrating $MgCl_2$ concentration and varying annealing temperature and annealing time. The products were separated by 3% Nusieve 3:1 agarose gel electrophoresis to confirm size. Furthermore, melting curve analyses of the PCR products were performed. Standard curves were generated with both sets of primers using the optimised reaction conditions. Finally, the products amplified from *N. caninum* genomic DNA and bovine genomic DNA were purified, sequenced and subjected to BLAST using the nucleotide-nucleotide BLAST (blastn) at the National Centre for Biotechnology Information USA (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm its identity and specificity.

Standard curves were generated for both bovine and *N. caninum* genomic DNA and were used to calculate PCR efficiencies. The PCR efficiencies need to be the same in order to compare *Neospora* and bovine PCR products. PCR efficiency depends on one, or a combination, of the following; inhibition in the sample, how well primers are designed and how well the PCR conditions were optimised. The samples used were a 10-fold dilution series of purified genomic *N. caninum* and bovine DNA with known concentrations. Threshold cycles obtained with the real-time PCR of the standard samples were plotted against the log of the initial template concentration of the samples. The slopes of the lines were determined by linear regression and efficiency computed. Efficiency is derived from the idealized function for the amount of PCR product formed: $N = N_0 \times E^n$, where N is number (concentration) of amplified molecules, N_0

is the initial number (concentration) of molecules, n is the number of amplification cycles and E is the efficiency. The standard curves are derived from the function described above: $n = -(\frac{1}{\log E}) \times \log N_0 + (\frac{\log N}{\log E})$. Therefore, the slope of the line is $Slope = -(\frac{1}{\log E})$ and the efficiency can be calculated from the slope as $E = 10^{\{\frac{-1}{slope}\}}$.

6.2.3.2. The assay

Real-time PCR was carried out using the LightCycler software version 3.5 (Roche Molecular Biochemicals) and LightCycler-FastStart DNA Master SYBR Green I. Each assay was repeated 3-times. Briefly, DNA samples were quantified using a Hoefer DNA Quant 200 Fluorometer and a total of 2 µl of sample DNA containing 1 ng DNA/µl was used in a reaction volume of 20 µl. The PCR assay contained 1.6 µl of 3 mM magnesium chloride, 2 µl of 10 µM each primer, 2 µl of 1X SYBR Green master mix containing ready-to-use Hot Start reaction mix and 1 µl of uracil N-glycosylase (UNG) per reaction. Each reaction tube was layered with 20% dimethyl sulfoxide (DMSO). The reaction conditions for the assay comprised a sequence of denaturation, amplification, melting and cooling as follows: 1 segment of denaturation at 95°C for 10 min; 35 cycles of amplification at 3 segments of 95°C for 10 sec, 65°C for 5 sec and 72°C for 8 sec; 3 segments of melting at 95°C, 65°C for 15 sec and 95°C; and 1 segment of cooling at 40°C for 30 sec.

6.3 Results

6.3.1 PCR primers produced specific amplification products

When the amplified *Neospora* sequence result was subjected to a BLAST search, the best score was returned with *N. caninum* with the following accession numbers; AF190701-1, AY459289-1 and X84238-1 corresponding to *N. caninum* Nc-5 gene. Analysis of the melting curve for primer pairs *Neo*F, *Neo*R and 28S-PF, 28S-PR (Figures 6.1 and 6.2 respectively) shows that each primer pair amplified single targets.

This is shown by the presence of only one symmetrical peak in all samples analysed. The PCR products were further separated using electrophoresis with 3% NuSieve 3:1 agarose gel. Separated products were stained with ethidium bromide and viewed under UV light. Figures 6.3 and 6.4 show that the products obtained were of the expected sizes.

In order to compare *N. caninum* genomic DNA with bovine genomic DNA, the standard curves (Figures 6.5 and 6. 6) were used to calculate the efficiency (E) of the PCR from the given slopes in LightCycler software. The efficiency of a PCR cycle is calculated in the exponential phase using the equation $E = 10^{[-1/slope]}$. The standard curve was also used to estimate the concentration of the individual samples at the log or exponential phase. The results were expressed as concentration (ng/μl) of *Neospora* DNA in 1 ng /μl of bovine genomic DNA.

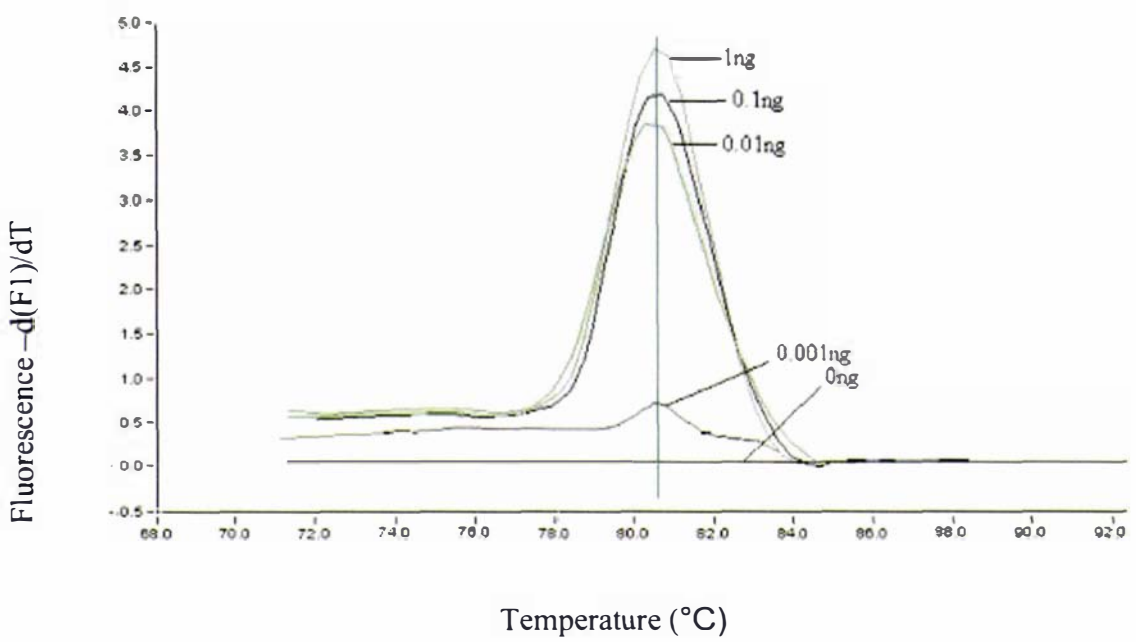

Figure 6.1: Melting curve analysis for primer pair *Neo*F and *Neo*R used to amplify *N. caninum* DNA in the blood and brain tissues of naturally infected pregnant (n = 3) and aborted (n = 3) heifers. Primer pair is specific to its target as revealed by one symmetric peak on the curve with no primer dimers, or unspecific binding.

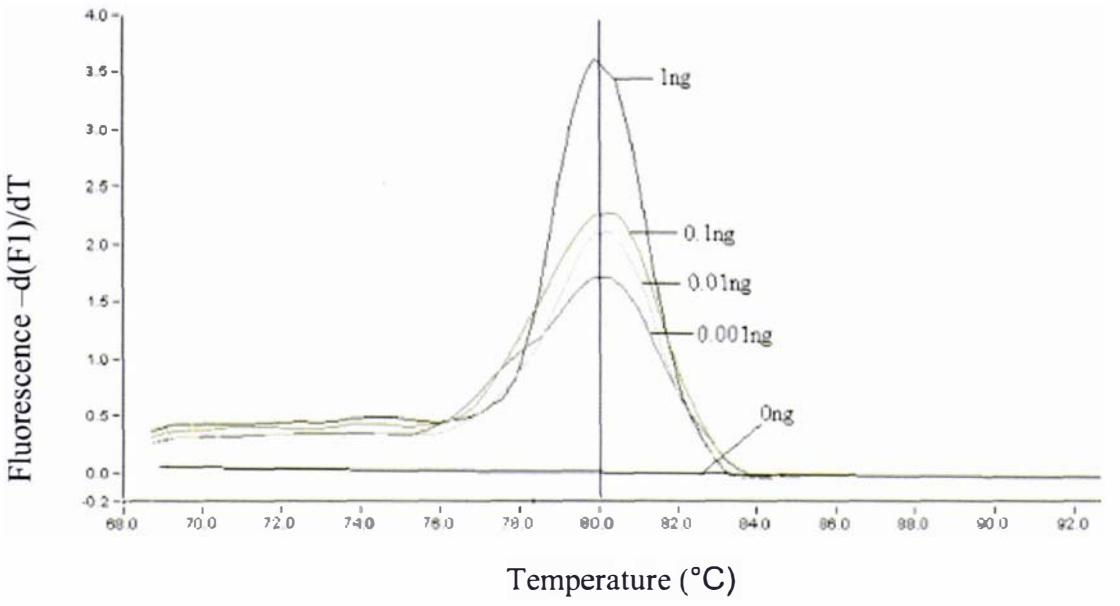

Figure 6.2: Melting curve analysis for primer pair 28S-PF and 28S-PR used as an internal control for the amplification of bovine genomic DNA in the blood and brain tissues of pregnant (n = 3) and aborted (n = 3) heifers naturally infected with *N. caninum*. Primer pair is specific to its target as revealed by one symmetric peak on the curve with no primer dimers, or unspecific binding.

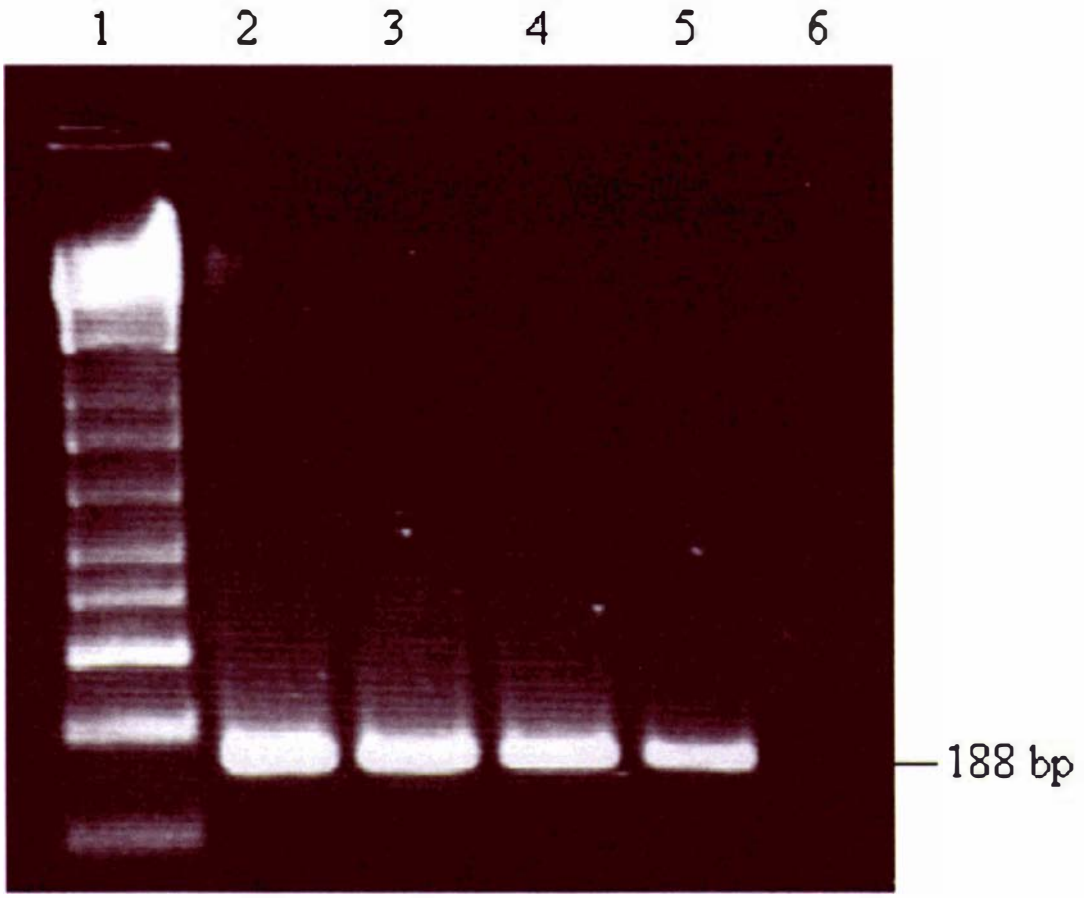

Figure 6.3: Agarose gel electrophoresis of PCR products from primer pair *Neo*F and *Neo*R indicating 188 bp size products whose band intensity decreases with a decrease in template concentration. Lane 1 is a 1 kb+ molecular weight marker. Lanes 2, 3, 4, 5 and 6 are the sample lanes with concentrations as follows 1, 0.1, 0.01, 0.001 and 0 ng respectively.

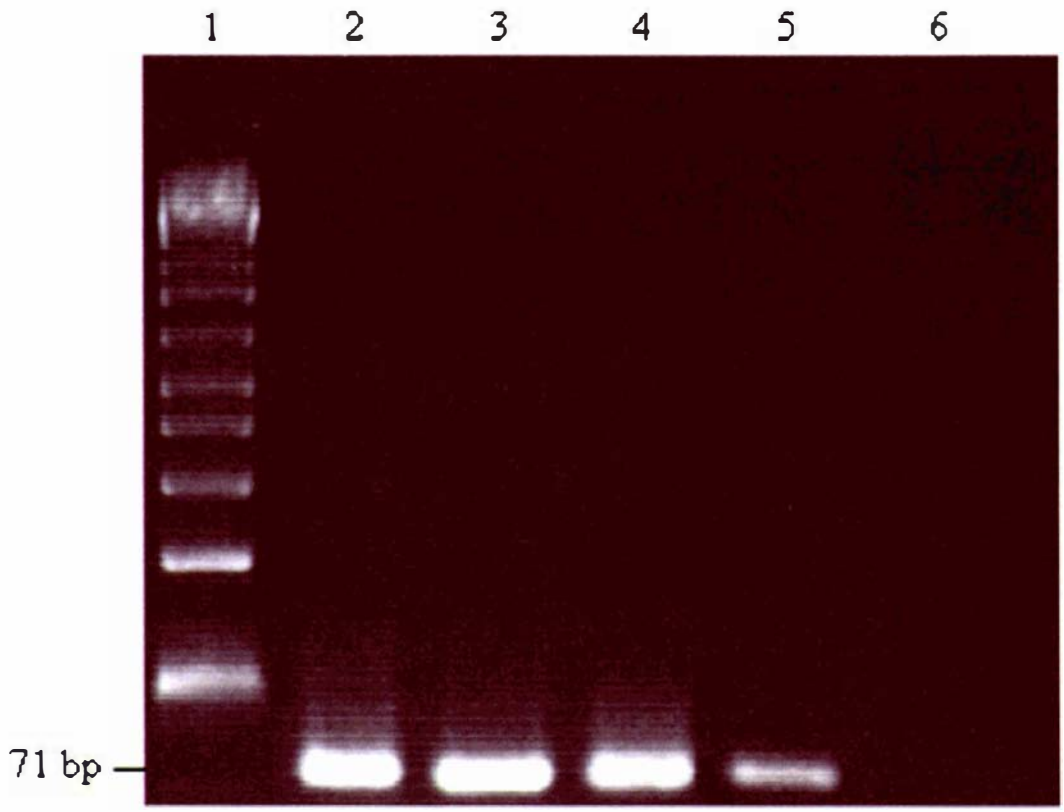

Figure 6.4: Agarose gel electrophoresis of PCR products from primer pair 28S-PF and 28S-PR indicating 71 bp size products whose band intensity decreases with a decrease in template concentration. Lane 1 is a 1 kb+ molecular weight marker. Lanes 2, 3, 4, 5 and 6 are the sample lanes with concentrations as follows 1, 0.1, 0.01, 0.001 and 0 ng respectively.

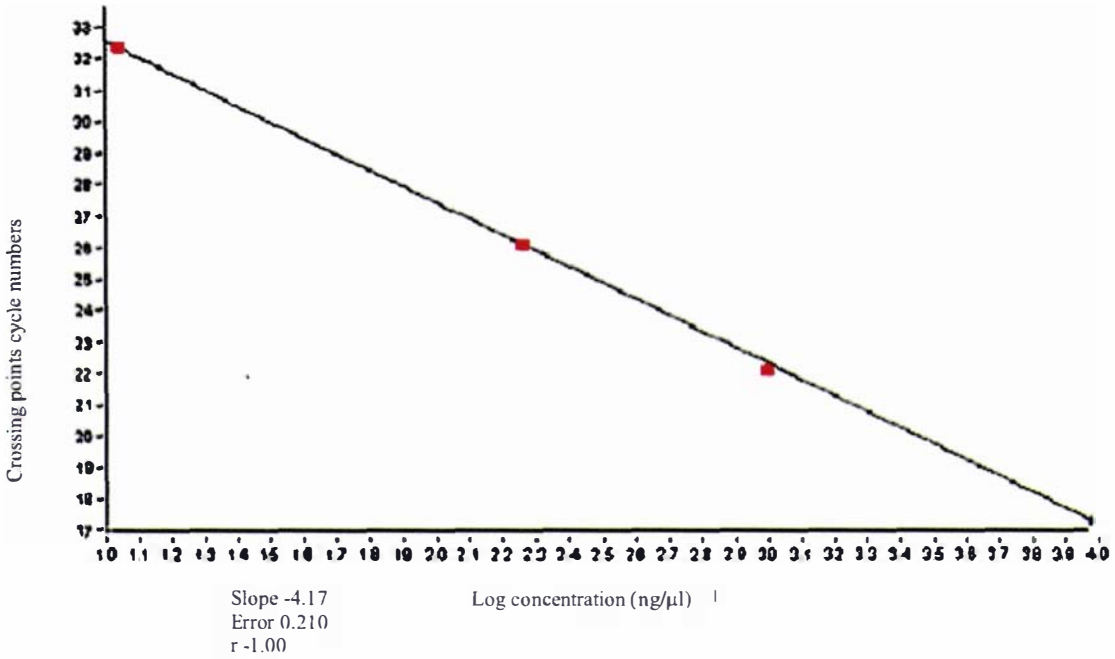

Figure 6.5: PCR amplification efficiency of primer pair *Neo*F and *Neo*R. The crossing point values plotted against the log of the initial template concentration (ng/µl) with efficiency (E) of 1.74.

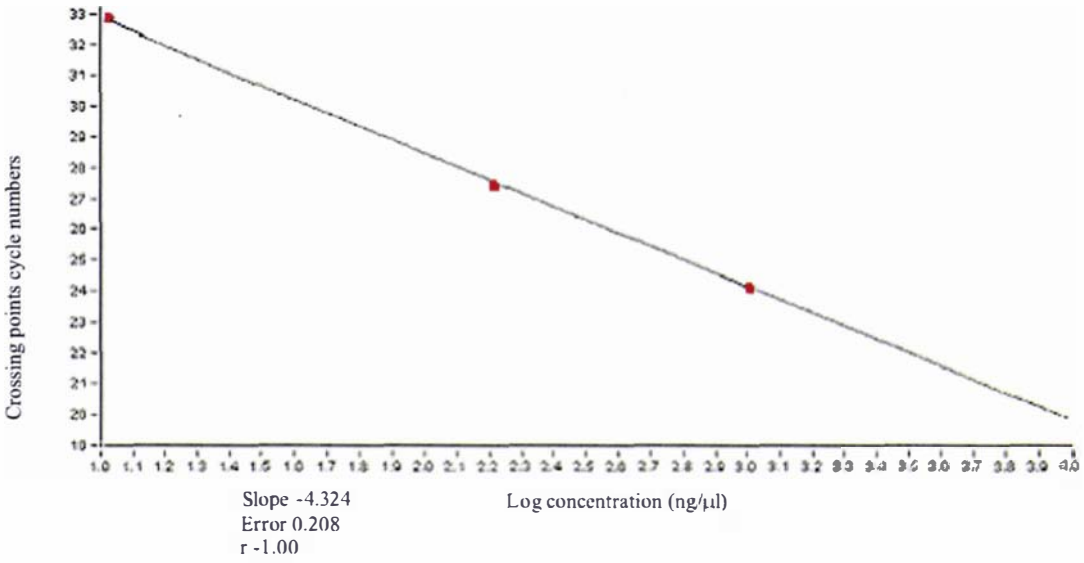

Figure 6.6: PCR amplification efficiency of primer pair 28S-PF and 28S-PR. The crossing point values plotted against the Log of the initial template concentration (ng/µl) with efficiency (E) of 1.70.

6.3.2 Quantification of *Neospora caninum* DNA in bovine genomic DNA from blood

The standard curve was used to calculate the amount of *Neospora* DNA in bovine blood at weekly intervals for 7-weeks and then biweekly for 8-weeks in both aborted and pregnant heifers. An increase in *Neospora* DNA concentration was observed in the aborting group for the first 2-weeks of the experiment and then there was a slight drop from the 3rd week. All aborted cows became negative from the 7th week with the exception of one. On the other hand, the pregnant group maintained their *N. caninum* status through to calving (15th week of the observation). Figure 6.7 is the graphic illustration of the observed variation in parasite load.

The starting concentration (ng/µl) of *N. caninum* DNA in 1 ng/µl of bovine genomic DNA obtained from blood ranged from 0.097 ± 0.0006 ng in the 1st week to 0 ng in the 15th week in aborted cows. This indicates a decrease in concentration of tachyzoites in the blood of this group. For heifers that remained pregnant, the values ranged between 0.080 ± 0.0009 ng on the 1st week to 0.154 ± 0.001 ng on the 15th week of the observation indicating an increase towards the end of gestation.

6.3.3. Comparison of *N. caninum* DNA in bovine genomic DNA from blood and brains of naturally infected cows

The concentrations (ng/µl) of *N. caninum* DNA present in 1 ng/µl of bovine genomic DNA from blood and brain of infected animals were computed by the LightCycler software using the standard curves shown in Figures 6.5 and 6.6. The concentration (ng/µl) of *N. caninum* DNA within 1 ng/µl of bovine brain genomic DNA ranged from 0.275 ± 0.0009 ng to 0.612 ± 0.001 ng per 1 ng/µl of bovine brain genomic DNA while in the blood, the concentration ranged from 0.080 ± 0.0009 ng to 0.154 ± 0.0006 ng per 1 ng/µl of bovine blood genomic DNA. Figure 6.8 compares the concentration (ng/µl) of *N. caninum* DNA present in blood and brains of infected heifers.

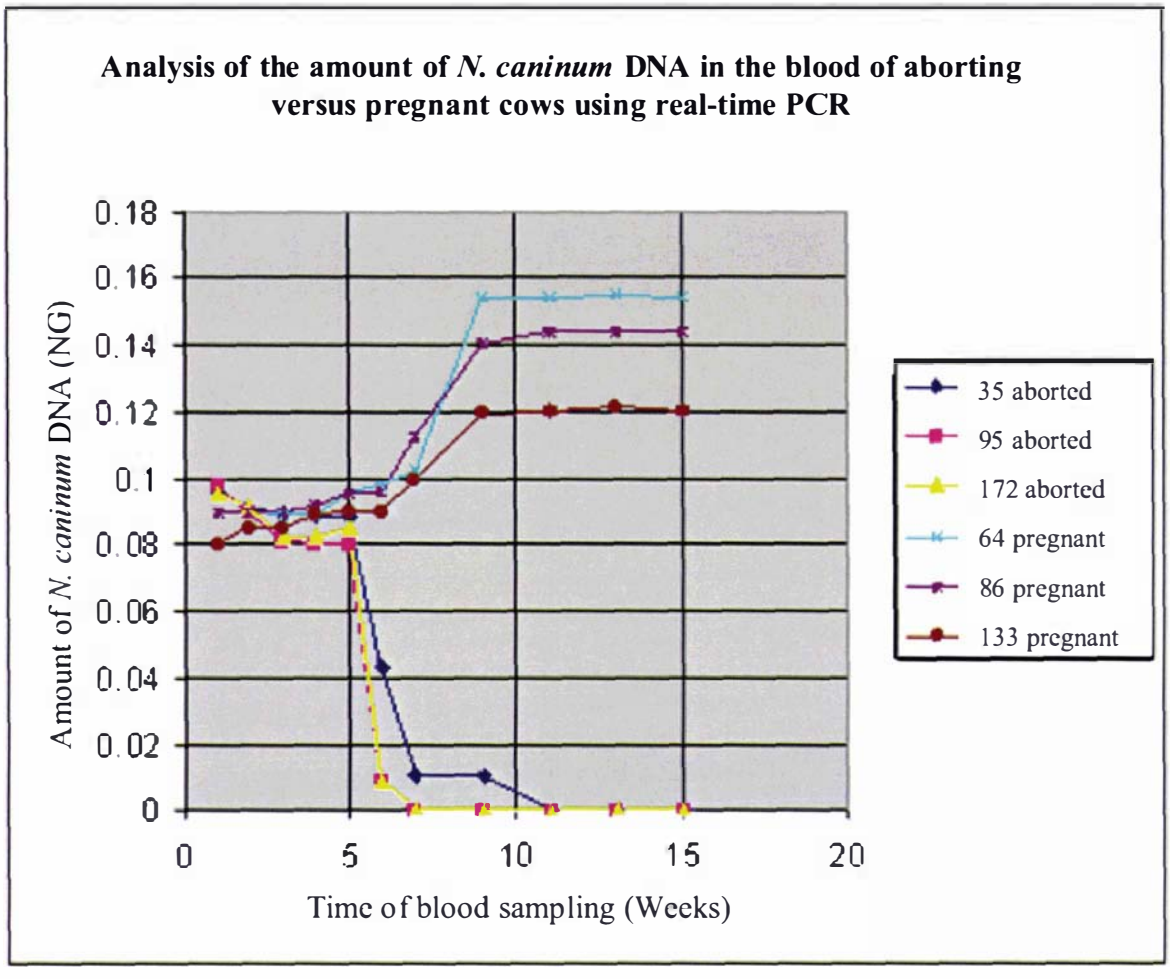

Figure 6.7: Quantification of the concentration (ng/µl) of *N. caninum* DNA present in ng/µl of total bovine genomic DNA from blood at different time points in aborting and pregnant cows using real-time PCR. All pregnant heifers (64, 86 and 133) were ~150 days pregnant, while abortion occurred in the group that aborted (35, 95 and 172) between days 100 to 150 of gestation. Sampling started around 150 days of gestation in the pregnant group and ~6 weeks after abortion in the aborting group.

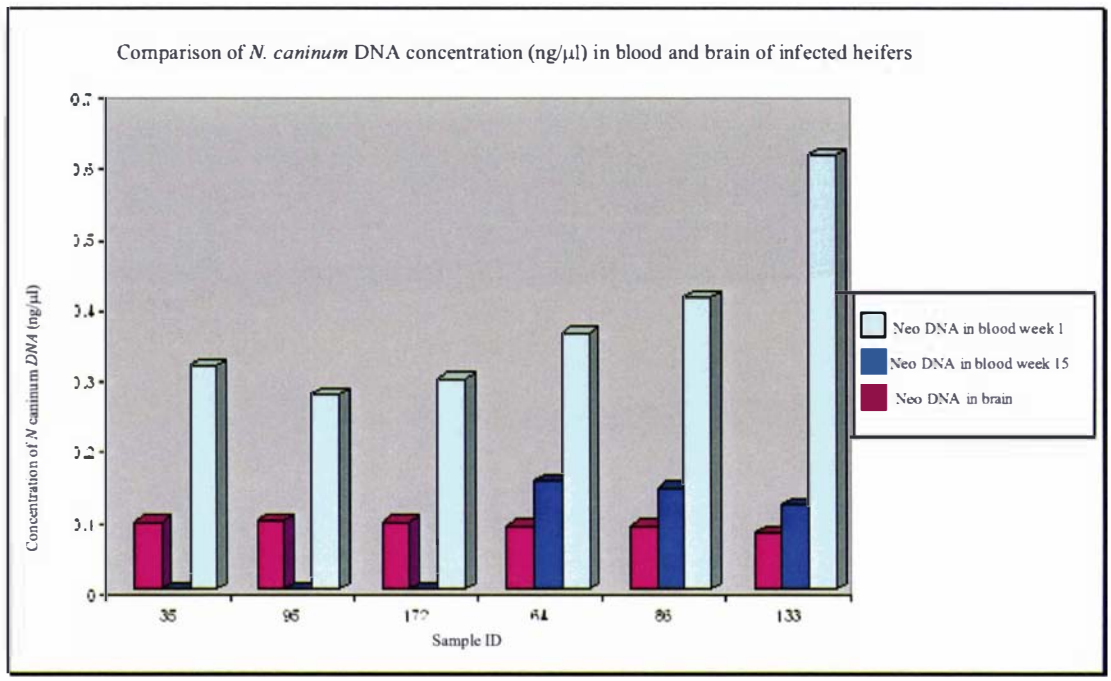

Figure 6.8: Comparison of *N. caninum* DNA concentration (ng/µl) in 1 ng/µl of genomic DNA from blood and brains of six aborting and pregnant cows naturally infected with *N. caninum* using real-time PCR. Bars represent animal blood at weeks 1 and 15 of observation and brains at slaughter. All pregnant heifers (64, 86 and 133) were ~150 days pregnant, while abortion occurred in the group that aborted (35, 95 and 172) between days 100 to 150 of gestation. Sampling started around 150 days of gestation in the pregnant group and ~6 weeks after abortion in the aborting group.

6.3.4 Real-time versus Block PCR assay

When real-time PCR was compared to block PCR using a kappa statistic, results were generally in agreement (kappa statistic = 0.87) but the real-time PCR was more sensitive (Table 6.1). Kappa is a measure of agreement between two variables. The result obtained was interpreted as follows:

Strength of agreement (Landis and Koch 1977)

< 0 = poor

0 – 0.20 = slight

0.21 – 0.40 = fair

0.41 – 0.60 = moderate

0.61 – 0.80 = substantial

0.81 – 1.0 = almost perfect

Table 6.1: Comparison of LightCycler and BlockCycler statistical results by quantifying the agreement between the two assays using Kappa statistic.

	Block PCR		
Real-time PCR	+	-	Total
+	49	3	52
-	0	14	14
Total	49	17	66
Kappa statistic	0.87		

The agreement between real-time and Block PCR experiments were compared in this table using kappa statistic. The result (0.87) indicates that both assays have comparable detection sensitivity. Note: + = positive sample and - = negative sample.

6.4 Discussion

This study describes a real-time PCR assay for the quantitative detection of *N. caninum* in the blood of naturally infected aborted and pregnant heifers. The primers used in this study were found to be specific for their targets by melting curve analysis, agarose gel electrophoresis and DNA sequencing of the PCR products. Our melting curves have one symmetrical peak in all samples analysed indicating that both primer pairs amplified single targets. Analysis of the PCR products for the melting curves by agarose gel electrophoresis further proved that the primers amplified single targets with no primer dimers.

The quantification by real-time PCR shows that at some point during pregnancy and around the time of abortion parenteral infection occurs and *N. caninum* DNA may be found in blood. This is in agreement with the qualitative detection of *N. caninum* in the blood of heifers (Okeoma *et al.* 2004a). In the present study, apparent parasitaemia cleared between 10 and 11 weeks after abortion in the aborting group, but persisted in the group that remained pregnant until calving. Of interest is the clearing of parasite DNA from the blood of the aborted group after the 7th week, yet parasite DNA was amplified from the brains of these animals when they were slaughtered after the 15th week of observations. The presence of parasite DNA in the brains of the heifers indicates that they were still infected with *N. caninum* although apparently it was not circulating. In the group that remained pregnant, there was a slight increase in parasite DNA concentration in the blood until calving. The brains of heifers in this group also demonstrated the presence of parasite DNA by PCR. Parasite tachyzoites were also isolated from the brains of a heifer and that of her calf (Okeoma *et al.* 2004b). All calves born to heifers in this study had positive brain PCR results and high IFAT titres indicating that they were infected *in utero* since precolostral blood was used.

Dubey *et al.* (1988) noted that bradyzoites are found in neural tissues; it appears that during pregnancy, the bradyzoites may be reactivated causing recrudescence of latent infection. When this happens, tachyzoites are produced which proliferate by endodyogeny (Hemphill *et al.* 1999), producing several hundred new parasites in a few days. Tachyzoites form a membrane-bound pseudocyst on proliferation and at a critical mass, host cell lysis releases tachyzooites, which then infect other cells/tissues

(Hemphill 1999). Thus, during tachyzoite movement from cell to cell and between tissues tachyzoites appear in blood. These events are more likely during pregnancy because of the down-regulation of the immune response (Quinn *et al.* 2002) to parasitic pathogens. In the present study, the heifers that remained pregnant maintained circulating *N. caninum* DNA. This may have resulted from the imbalance between cytokines secreted by T helper cells 1 and 2. As Th2 down-regulates the Th1 response during pregnancy, *N. caninum* survival in the host is favoured.

As in *T. gondii* and other intracellular parasitic infections, prevention and control of disease, particularly during pregnancy, is the role of cellular immunity and particularly the T cells. IFN-γ (Th1 cytokine) has a crucial role against parasitic infections (Dupouy-Camet 1998). *T. gondii* infected mice treated with anti-IFN-γ antibodies fully reactivated an asymptomatic infection, inducing massive necrotic areas in the brain with the appearance of free tachyzoites in blood and death of all animals within 2 weeks after treatment (Gazzinelli *et al.* 1992). During chronic *T. gondii* infection, parasite-specific T lymphocytes release high levels of IFN-γ, which prevent cyst reactivation (Denkers and Gazzinelli 1998). IFN-γ has also been found to be crucial to the prevention of *N. caninum* infection in mice. Nishikawa *et al.* (2001) stated that IFN-γ deficient mice died within 9 days of infection with *N. caninum*, whereas those treated with exogenous IFN-γ survived.

In contrast, circulating *N. caninum* DNA cleared from the group that aborted from the 7^{th} week of observations. The clearance of circulating *N. caninum* DNA could be as a result of the restoration of Th1/Th2 balance in this group of animals which may have occurred after abortion. Gazzinelli *et al.* (1992) stated that the production of IFN-γ (Th1) prevents the reactivation of chronic *T. gondii* infection in mice. Though the restoration of Th1/Th2 balance in animals (ruminants) has not been documented, researchers have reported a balance in Th1/Th2 from 3-months postpartum in humans (Russell *et al.* 1997; Ekerfelt *et al.* 1997; Matthiesen *et al.* 1998), while others have stated that the Th1/Th2 balance could take up to 1-year postpartum to re-establish (Watanase *et al.* 1997). In their work, Shimaoka *et al.* (2000) analysed changes in cytokine production by whole blood in humans and reported increases in Th1/Th2 cytokines in the postpartum period. Thus, the Th1/Th2 paradigm could be the reason

why circulating *N. caninum* DNA was maintained in the pregnant group but cleared in the aborted group.

To demonstrate the reproducibility and applicability of this study, we applied the methodology to both blood and brain samples in triplicate tests. Brain is the organ most consistently parasitized by *Neospora caninum* and has been recommended as the organ of choice for the diagnosis of neosporosis (Barr *et al.* 1990; Baszler *et al.* 1999; Hattel *et al.* 1998; Lindsay *et al.* 1995; Long *et al.* 1998). The ability to determine the infection status and intensity of *N. caninum* infection in cows using blood samples is an advance that may reduce or eliminate the need to slaughter animals in order to provide tissue samples intended for *N. caninum* diagnosis.

This study reports the observation of *N. caninum* induced parasitaemia in aborting and pregnant heifers using real-time PCR. The study shows that *N. caninum* tachyzoite DNA can be quantified to obtain the concentration of DNA relative to host genomic DNA in blood. In addition to testing a large number of samples at one time, quantification of *N. caninum* in blood samples of live animals can be done without the need for slaughter.

Authors' contributions to this experiment

N.B. Williamson helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the experiments as well as providing editorial advice.

C.M. Okeoma helped with animal inoculation, sample collection and clinical observations. She designed and conducted the experiments and she wrote the manuscript.

Chapter 7

Recognition patterns of *Neospora caninum* tachyzoite antigens by bovine IgG at different IFAT titres [4]

[4] Published as: Okeoma CM, Williamson NB, Pomroy WE, Stowell KM. *Parasite Immunology*, 26, 177-185

7.0 Abstract

This study describes qualitative and quantitative antibody response in cows naturally infected with *N. caninum*. The study was carried out with 269 serum samples obtained from 24 cows over a period of 15 weeks. Prior to sample collection, the cows were tested by ELISA. The 269 samples were screened with IFAT and categorised into seven IFAT titre groups (< 1:80, 1:80, 1:200, 1:600, 1:1000, 1:2000, > 1:2000). The samples were finally analysed by Western blotting.

Seven immunodominant antigens (~18, ~25, ~33, ~35-36, ~45-46, ~47 and ~60-62 kDa) and five minor antigens (~25, ~51, ~64, ~77 and ~116 kDa) were recognised by cow sera. The recognition of ~46 kDa antigen by cow sera was common to samples with IFAT titre 1:80 and above. Another common antigen was the ~18 kDa antigen which was recognised by samples with IFAT titre 1:200 and above. The most remarkable observation was the presence of the 45-46 kDa, the 77 kDa and absence of the 18 kDa antigenic bands in samples with IFAT titre 1:80. This observation was consistent even in the face of fluctuating antibody titre where serum antibody titres from an animal exceeded then failed to reach 1:80. Antibody fluctuation was observed across all cows (pregnant and aborted) with no discernible fluctuation pattern. However, the fluctuation in antibody titre observed appeared to be most remarkable in initially ELISA-negative pregnant cows, and to a lesser extent in ELISA-positive pregnant cows, and ELISA-positive aborted cows. Though there is fluctuation in antibody titre, the banding patterns of *N. caninum* tachyzoite antigens by cows within the same IFAT titre group remained similar.

7.1 Introduction

N. caninum is an apicomplexan parasite that is structurally similar to, but antigenically distinct from, *Toxoplasma gondii*, a close relative. *N. caninum* infection (neosporosis) can cause neuromuscular disorders, paralysis and death in dogs, abortion and neonatal morbidity in cattle and other ruminants and horses. The high prevalence of *N. caninum* in cattle has caused the recognition of neosporosis as an economically important disease with considerable impact on the livestock industry (Dubey and Lindsay 1996; Hemphill 1999). Diagnosis of *N. caninum* infection is mostly based on serology and the examination of the aborted foetus and the dam after abortion (Dubey and Lindsay 1996). In addition, serocpidemiological analyses in afflicted herds may demonstrate statistical association between seropositivity and abortion (Thurmond *et al.* 1997), as well as seropositivity and vertical transmission of infection.

Diagnostic methods for neosporosis could include one or a combination of the following: serology (enzyme linked immunosorbent assay and immunofluorescence antibody test) for the detection of antibodies in serum and/or body fluids; polymerase chain reaction (PCR) on specific tissues for the demonstration of parasite DNA; and histological examination for characteristic lesions. Furthermore, immunohistochemistry and Western blot are used to demonstrate parasite specific antigens.

A number of studies have been undertaken to identify and characterise, at the molecular level, specific antigenic components of *N. caninum* in order to improve serological diagnosis (Hemphill *et al.* 1999). About 20 immunodominant antigens ranging between ~16 and 80 kDa were identified by Western blot analysis (Barta and Dubey 1992) using polyclonal antibodies (pAbs). Similarly, 6 monoclonal antibodies (mAbs) raised to *N. caninum* immunostimulating complex (ISCOMs) were used in Western blot analysis in non-reduced and reduced conditions. These antibodies recognised antigens of 18, 30/32, 41 and 65 kDa (Björkman and Hemphill 1998). Antibodies directed against the 30/32 kDa doublet, the 18 kDa antigen and against the 41 kDa band, bound to the tachyzoite surface. Two other dense granule antigens of *N. caninum* tachyzoites are 33 kDa (Lally *et al.* 1997) and 36 kDa (Liddle *et al.* 1998). Immunoblot studies have also revealed 8 major and several minor *N. caninum* antigens ranging between 31 and 97.4 kDa using mAbs (Cole *et al.* 1994). Bjerkas *et al.* (1994) showed that immune sera

from a wide range of animal species exhibited a similar recognition pattern when visualised by immunoblotting, with five major (17, 27, 29, 30 and 46 kDa) and several minor *N. caninum* antigenic bands. The 17 kDa antigen was found to be localised in the rhoptries, while the 29 and 30 kDa antigens were distributed in the parasitophorous vacuole network (Bjerkas *et al.* 1994).

Antisera obtained from cows with confirmed *Neospora* induced abortions, were consistently observed to react with antigens of 25, 65 and 116 kDa on immunoblots (Baszler *et al.* 1996). Moreover, antisera from naturally and experimentally infected cattle, dogs and mice, identified immunodominant antigens of 29 and 35 kDa (Howe *et al.* 1998), while 4 immunodominant antigens (IDAs): 17-18, 34-35, 37 and 60-62 kDa, were reported as being commonly recognised by aborted foetuses and cows (Álvarez-García *et al.* 2002). Five antigens have been identified by immunoblot as predominant targets for the humoral immune response of *N. caninum*-infected animals. These antigens are 17, 29, 30 36 and 37 kDa (Barta and Dubey 1992; Bjerkas *et al.* 1994; Paré *et al.* 1995; Sonda *et al.* 1998).

The aim of this study was to evaluate the recognition pattern of *N. caninum* tachyzoite antigens by cows naturally infected with *N. caninum* using Western blot and to compare IFAT titres and Western blot antibody patterns in relation to observed IFAT antibody fluctuation in naturally infected cows.

7.2 Materials and methods

7.2.1 Serum samples and IFAT

Evaluation of tachyzoite antigen recognition at different IFAT titres was carried out with 269 serum samples obtained weekly for 7 weeks and biweekly for another 8 weeks from 24 cows used in this study. Twenty of these cows (12 were seronegative and 8 seropositive on an enzyme linked-immunosorbent assay, ELISA, of IDEXX Laboratories, Wörrstadt, Germany) were from a single herd. Out of the remaining 4 (all seropositive on ELISA), 3 were from one herd and 1 from a different herd. Blood sampling occurred from the last week of the 5th month of gestation through to the 2nd

week of the 9th month of gestation when calving started. Before the start of this study, 6 of the 24 cows aborted their foetuses but the exact dates of abortion were not known and foetuses were not recovered. These aborted cows were serologically positive on ELISA performed by serologists at ALPHA Scientific Laboratories Hamilton, New Zealand, using manufacturer's cut-off point for the S:P ratio of ≥0.5. Another 6 cows were also seropositive on ELISA but pregnant, while 12 cows were negative on ELISA and pregnant. Sera were obtained and stored until required for analysis. Serial serology on these cows was conducted using the IFAT at a dilution rate of 1:80. This cut-off point was used because at the initial cut-off of 1:200, parasite DNA was detected by PCR in the otherwise seronegative samples, thus the need to increase IFAT sensitivity by decreasing cut-off point. The technique for use of PCR in the detection of parasite DNA in blood samples was described by Okeoma *et al.* (2004a).

Analysis by IFAT was carried out by serologists at the Gribbles Pty Ltd Animal Health Laboratory (Palmerston North, New Zealand). The IFAT screening of the 269 samples revealed seven different titre groups of < 1:80, 1:80, 1:200, 1:600, 1:1000, 1:2000 and ≥ 1:2000. Based on these, Western blot analysis was carried out on all samples. All animal usage was approved by the Massey University Animal Ethics Committee, protocol number 02/29.

7.2.2 *In vitro* cultivation of *N. caninum* tachyzoites

N. caninum tachyzoites of the NcNZ 1 isolate (Okeoma *et al.* 2004b) were maintained by continuous passage in Vero cell cultures. Infected cultures were maintained with minimum essential medium (MEM) and supplemented with 10% foetal bovine serum (FBS), 10,000 units of penicillin, 10,000 μg of streptomycin, 25 μg of amphotericin B/ml. Once Vero cells were lysed, the tachyzoites were passed through a 5 μm filter and counted. The filtered *N. caninum* tachyzoites were washed twice in cold sterile phosphate-buffered saline (PBS), pH 7.2 and pelleted by centrifugation at 600 x *g* for 10 min. The pellets were re-suspended in PBS to a final concentration of approximately 2 x 10^6 tachyzoites per ml. The tachyzoites were cryo-preserved in liquid nitrogen in aliquots of 1 ml until used.

7.2.3 Antigen preparation

To obtain *N. caninum* antigens, 500 µl of 10 mM Tris–HCl containing 2 mM of phenylmethylsulfonyl fluoride (Sigma), was added to each vial containing 2 x 10^6 tachyzoites and disrupted by ultrasound treatment in an ice-bath. After sonication, the resulting antigens were centrifuged at 10,000 x *g* for 20 min at 4°C and the supernatant collected. The protein content of the supernatant was determined using the BioRad protein assay method. Antigens were stored at -20°C in 2 mg/ml aliquots until used.

7.2.4 SDS-PAGE

N. caninum antigens containing 2 mg/ml proteins were mixed with sample buffer containing 4% (v/v) β-mercaptoethanol and 2% (w/v) of sodium dodecyl sulphate in the ratio of 2 parts protein to 1 part sample buffer. Similarly antigens of *N. caninum* Liverpool isolate (Barber *et al.* 1993) and *T. gondii* containing tachyzoites of S48 of *T. gondii* (Toxovax AgVax Developments Limited,New Zealand) were used as positive and negative controls respectively and were diluted and treated as described above. Electrophoresis was performed using Bio Rad Tris-HCl Criterion Gel containing 12.5% Resolving Gel and 4% Stacking Gel (Cat #345-0014) in a Criterion cell (Cat #165 6001) for 55 minutes at 200 V. Molecular weight (MW) standard (Fermentas, Cat #SM0671) was loaded on each side of the gel to monitor antigen separation, electrophoretic transfer and estimation of the MWs of the different antigens recognised by the samples. *N. caninum* antigen was loaded in 10 wells while the control antigens were loaded in 1 well each. Each of the wells was loaded with 45 µl containing 2 mg/ml protein of either *N. caninum* or *T. gondii* antigen.

7.2.5 Western blot analysis

All incubations for Western blotting were conducted at room temperature for 1 hour under mild shaking unless otherwise stated. All washes were done 3-times for 10 minutes each with mild shaking. Following antigen separation, antigens were

electrophoretically transferred to 0.2 μm nitrocellulose membrane (Bio Rad) using Criterion Blotter (Bio Rad, Cat #170 4070) for 30 minutes at 100 V. Membranes were stained with Ponceau S and the different lanes marked for easy cutting. Membranes were then de-stained with water and non-specific binding sites blocked by incubation with a blocking buffer containing 5% non-fat milk powder diluted with phosphate-buffered saline (pH 7.4) containing 1% Tween-20 (PBS-T). After blocking, membranes were washed with PBS-T and then cut into strips. Each strip (including the controls) were incubated with a bovine serum sample (primary antibody) diluted with the blocking buffer at a dilution rate of 1:100. After incubation, membranes were washed with PBS-T and then exposed to a secondary antibody (sheep anti-bovine IgG: HRP, SERO TEC Cat #AA123P) at a dilution rate of 1:200. Following incubation, membranes were washed and amplified using Western blot amplification module (Bio Rad, Cat #170-8230). Following amplification, membranes were washed 4-times for 5 minutes each with 20% DMSO/PBS-T and finally washed twice for 5 minutes each with PBS-T. Membranes were then developed using opti-4CN^{TM} substrate kit (Bio-Rad Cat #170-8235), washed with water for 20 minutes, recorded, scanned and images saved as tiff files. Images were scanned into a Microsoft word document, exported to software called 'paint' and saved as tiff files. Annotation and/or labelling of images were done using paint. Blot images that were taken at the time of optimisation of the assays are presented in Appendix 4. These images were taken with a digital camera and were hand annotated. Unfortunately, no Coomassie stained gel images were taken during the course of these experiments. Though coomassie blue staining of protein gels is often used as a measure of loading consistency, this was not considered necessary as equivalent amounts of proteins were loaded into each lane of the SDS-PAGE gels. In addition, the Western blots (Appendix 5) show consistency in banding patterns of the various antigens detected at different IFAT titres. This in effect acts as an internal control for protein loading.

7.2.6 Data acquisition

The immunofluorescent antibody test (IFAT) was initially used to estimate the antibody levels in the serum samples used in this study. Seven IFAT titre levels were obtained as follows; ≤ 1:80 (54 samples), 1:80 (80 samples), 1:200 (18 samples), 1:600 (60

samples), 1:1000 (27 samples), 1:2000 (12 samples) and ≥ 1:2000 (18 samples). These IFAT titre levels constitute observational groups in this study. For each titre group, antigens of *N. caninum* tachyzoites were classified according to their occurrence and intensity of recognition by bovine sera in the Western blot analysis. Furthermore, intensity was categorised as low, medium or high and designated +, ++ and +++ respectively. Antigens with medium and high intensity (++ and +++) were designated as immunodominant antigens (IDAs) while those with low (+) intensity were designated as minor antigens.

7.3 Results

7.3.1 Patterns of *N. caninum* antigen recognition at different IFAT titres

Samples at IFAT titre of < 1:80 were considered to be seronegative to *N. caninum* by IFAT, while all other dilutions were regarded as seropositive to *N. caninum* by IFAT. All negative control samples (*Toxoplasma gondii, Toxo,* Figure 7.1) did not bind to any anti *N. caninum* antisera at any IFAT titre, but the positive control (*N. caninum* Liverpool isolate, NcLiv, Figure 7.1) had similar results to the positive samples. Table 7.1 and Figure 7.1 summarise the recognition patterns of *N. caninum* antigens based on the IFAT titres. Seronegative sera (IFAT <1: 80) did not bind to any *N. caninum* antigen (Figure 7.1, <80a), with the exception of one sample (Figure 7.1, <80b). Seven immunodominant antigens (IDAs) were recognised by bovine sera. These antigens (~18, ~25, ~33, ~35-36, ~45-46, ~47 and ~60-62 kDa) were termed IDAs because of the intensity of recognition. The recognition of a ~46 kDa band was common to all positive sera. Another common band was the ~18 kDa band which was recognised by all positive serum samples except for those at 1:80 IFAT titre (Figure 7.1, 80). At IFAT titre > 1:2000, ~25 kDa band was immunodominant because it was detected with a very high intensity (Figure 7.1, >2000).

Some minor antigens (~25, ~51, ~64, ~77 and ~116 kDa) were also observed to bind to anti-*N. caninum* antibodies (Figure 7.1). The minor antigen band of ~77 kDa was recognised across all positive IFAT titres (≥1:80) while the ~116 kDa band was recognised by all positive sera except for the 1:80 IFAT titre (Table 7.1, Figure 7.1).

Figures 7.2 and 7.3 show the recognition pattern of *N. caninum* antigen of different animals sampled at different time points at IFAT titres 1:80 and 1:600. These figures revealed that the banding pattern observed in this study is directly related to IFAT titre and that the antigen recognition pattern is consistent among different animals in the same IFAT titre group.

Table 7.1: Comparison of different IFAT titre with antigens recognised by anti-*N. caninum* antibodies from heifers infected with *N. caninum.*

IFAT titre	Immunodominant antigens							Minor antigens
	~ 18	~ 25	~ 33	~ 35 - 36	~45 - 46	~ 47	~ 60 - 62	
< 1: 80	-	-	-	-	+	-	-	One sample out of 54 recognised ~ 45 - 46 kDa antigenic bands
1: 80	-	-	-	-	+	-	-	~ 77 kDa, with 2 out of 80 samples having minor antigens higher than 77 kDa
1: 200	+++	-	-	-	++	-	-	~ 77 and 116 kDa, 2 samples had minor antigens between 77 and 116 kDa
1:600	+	-	-	-	+++	-	-	~ 77, 116 kDa, 5 samples had minor antigens between 77 and 116 kDa
1:1000	++	-	-	+++	+++	-	-	~ 25, ~ 77, and ~ 116 kDa
1:2000	+++	-	++	++	++	+++	++	~ 25, ~ 64, ~ 77, and ~ 116 kDa and other minor antigens between 77 and 116 kDa
>1:2000	+++	++	++	++	++	+++	++	~ 64, ~ 77, and ~ 116 kDa, and other minor antigens between 77 and 116 kDa

Note: number of + indicates degree of intensity of antigen recognition, while – indicates a negative result.

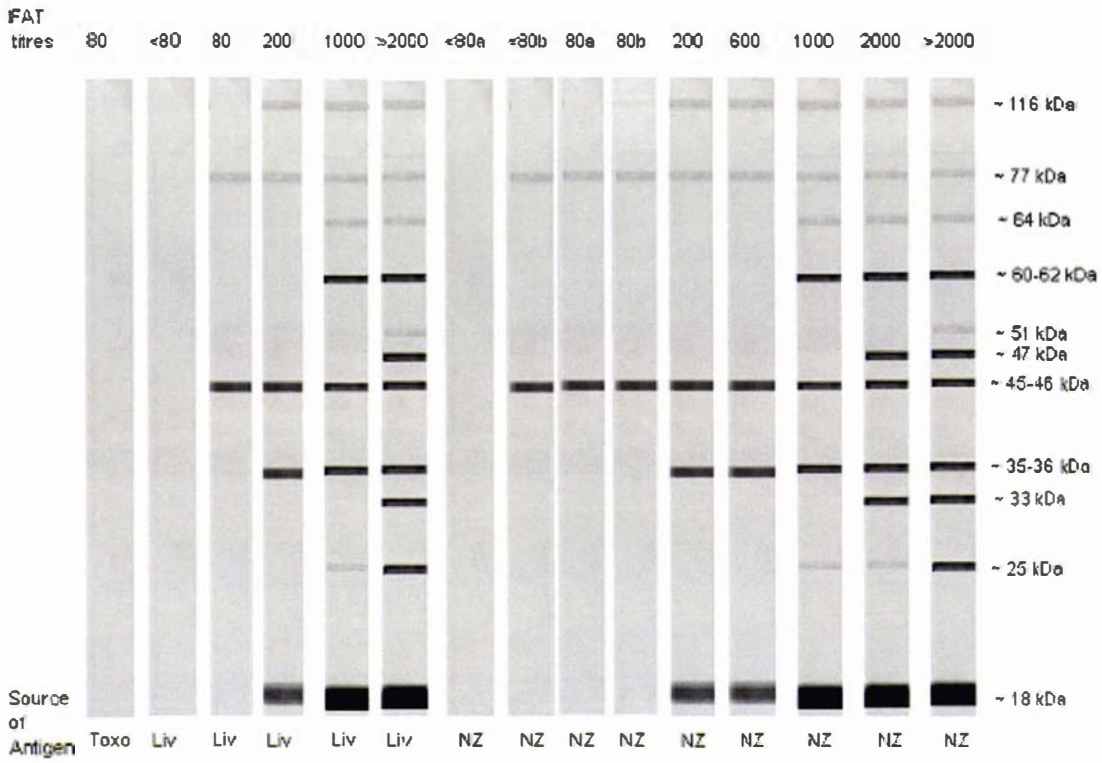

Figure 7.1: Banding pattern of *N. caninum* tachyzoite antigen (NcNZ 1) when reacted with anti-sera from naturally infected cows at different immunofluorescence antibody titres (IFAT). IFAT titre < 80 = negative IFAT result, a and b = same IFAT titre but different samples, *Toxo* = *Toxoplasma gondii* antigen, Liv = *Neospora caninum* Liverpool isolate (Nc Liv), NZ = *Neospora caninum* New Zealand isolate 1 (NcNZ 1).

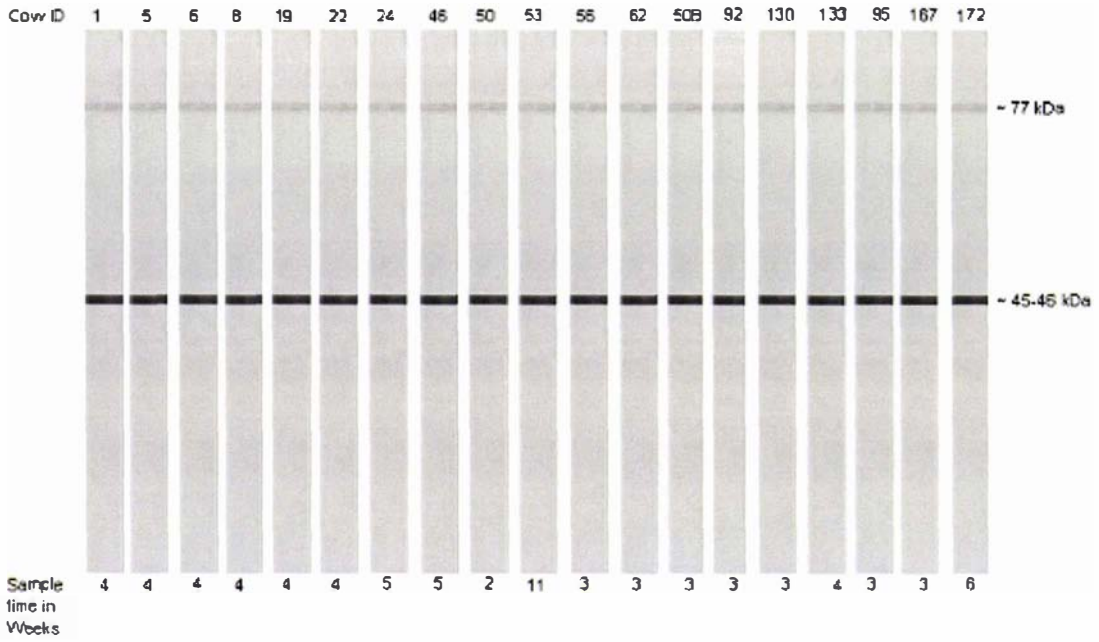

Figure 7.2: Analysis of banding pattern of *N. caninum* tachyzoite antigen with sera from 19 infected cows at an IFAT titre of 1:80. Cow ID = the identity of the cows and sample time = when serum sample was collected from the corresponding cow.

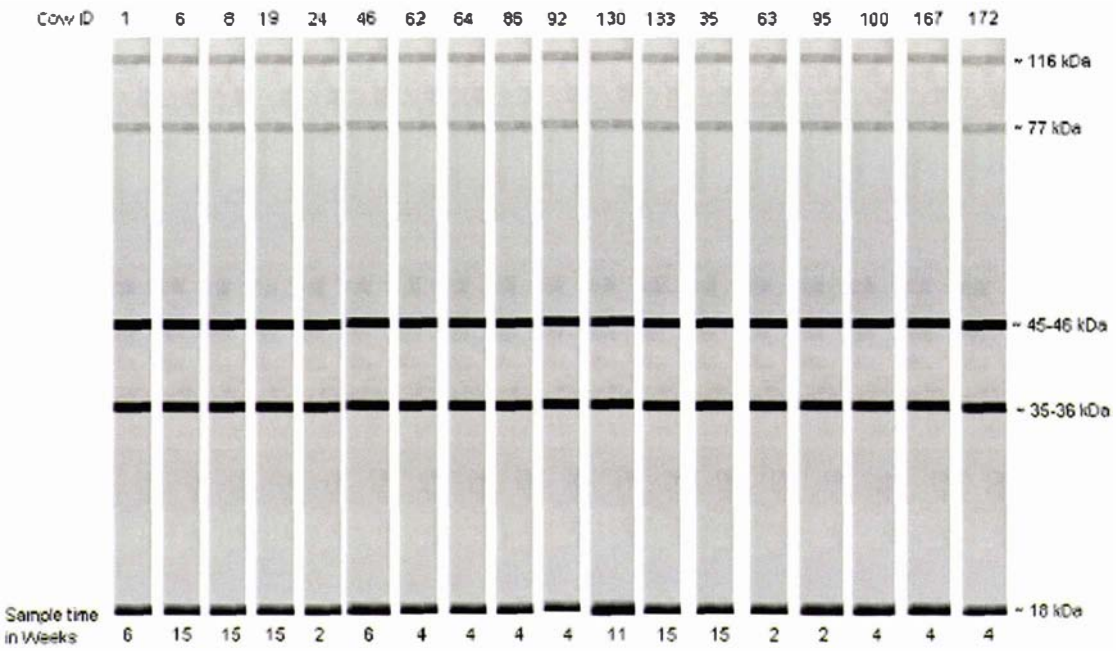

Figure 7.3: Analysis of banding pattern of *N. caninum* tachyzoite antigen with sera from 18 infected cows at an IFAT titre of 1:600. Cow ID = the identity of the cows and sample time = when serum sample was collected from the corresponding cow.

7.3.2 Comparison of the results obtained by IFAT and Western blot assays

Of the 269 serum samples analysed by both IFAT and Western blot, 54 samples had IFAT titres of < 1:80 (negative IFAT result). One sample out of the 54 reacted to *N. caninum* antigen showing 2 immunogenic antigen bands of ~45-46 and ~77 kDa (Figure 7.1, <80b), this sample was from cow 64 (Figure 7.4). Eighty serum samples had IFAT titre of 1:80 and 79 samples out of the 80 tested positive on Western blot. 78 samples reacted with the ~45-46 and 77 kDa antigenic bands (Figure 7.1, 80a), 1 sample in addition to the ~45-46 and 77 kDa antigenic bands reacted with a few other minor antigens (Figure 7.1, 80b), while the remaining 1 sample was negative on Western blot.

Eighteen serum samples had IFAT titres of 1:200 and all 18 reacted with *N. caninum* antigen (Figure 7.1, 200), while 60 serum samples had IFAT titres of 1:600 and all reacted with *N. caninum* antigen (Figure 7.1, 600). At an IFAT titre of 1:1000, 27 serum samples were tested and all 27 were also positive on Western blot assay (Figure 7.1, 1000). Similarly, 12 serum samples at IFAT 1:2000 were reactive with *N. caninum* antigen and 18 serum samples at IFAT > 1:2000 also reacted with *N. caninum* antigen (Figure 7.1, 2000 and > 2000 respectively).

7.3.3 IFAT, Western blot and antibody fluctuation

At IFAT < 1:80, all samples tested were negative on Western blot even when these samples were from animals with high IFAT titres (≥ 1:80 to > 2000) at other time points, except for one sample, which recognised two immunogenic bands of 45-46 and 77 kDa. This sample was from a pregnant animal (cow #64) with IFAT titres fluctuating between < 1:80 and 1:2000. The binding pattern of sera from this cow is shown in Figure 7.4. Similarly, the fluctuation pattern of a pregnant cow (cow #24) previously seronegative on ELISA is shown in Figure 7.5. Cow #172, a seropositive aborted cow, presented yet another example of antibody fluctuation (Figure 7.6) and the IFAT titre at different time points ranged from < 1:80 to 1:2000. A further illustration of antibody fluctuation pattern was from a seropositive pregnant animal (cow #92) with IFAT titres ranging from < 1:80 to 1:2000. Serum from this cow recognised more minor antigens at IFAT titre 1:80 (Figure 7.1, 80b) in addition to the ~45-46 and 77 kDa

antigenic bands commonly recognised by sera samples from this IFAT group. Antibody fluctuation was observed across all cows (pregnant and aborted) with no discernible fluctuation pattern.

The fluctuations in antibody response observed with IFAT across all animals were also observed by Western blot analysis. On Western blot, antigenic band recognition was associated with the IFAT titre and not on the animal's past serological record except for the few exceptions mentioned above, thus, serum from an animal with an IFAT titre of 1:80 recognised 2 *N. caninum* immunogenic bands (~45-46 and ~77 kDa), while serum from this same animal at an IFAT titre of 1:200 recognised 4 *N. caninum* immunogenic bands (~18, ~45-46, ~77 and 116 kDa).

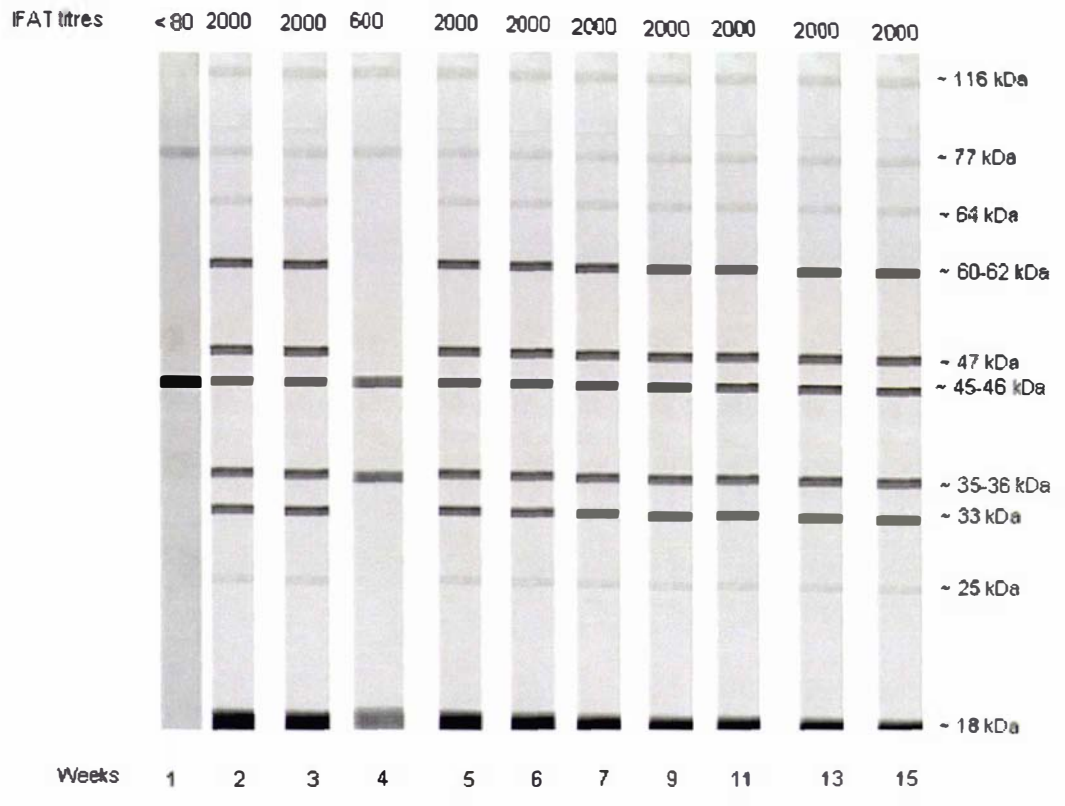

Figure 7.4: Binding pattern of *N. caninum* tachyzoite antigen (NcNZ 1) with anti-sera from a seropositive-pregnant cow (cow #64) showing antibody fluctuation. IFAT titre < 80 = a negative IFAT result, showing 2 antigenic bands (45-46 and 77 kDa) on Western blotting.

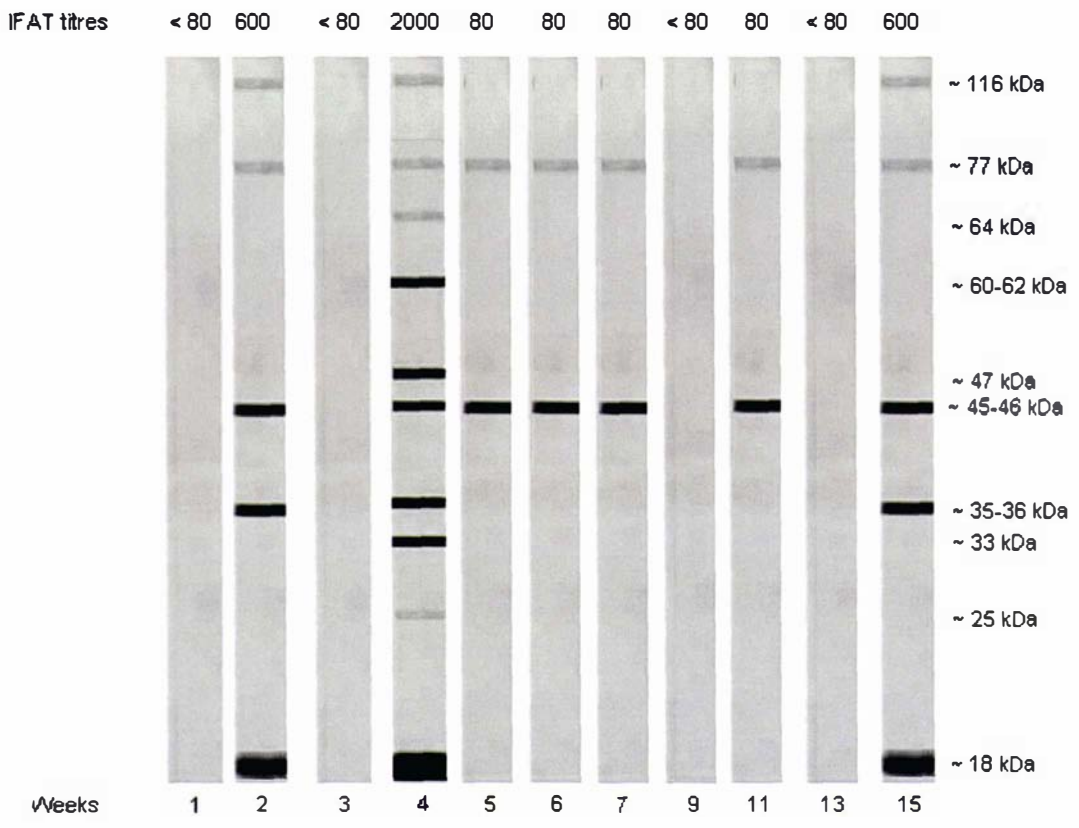

Figure 7.5: Binding pattern of *N. caninum* tachyzoite antigen (NcNZ 1) with anti-sera from a seronegative (IDEXX) - pregnant cow (cow #24) showing antibody fluctuation. IFAT titre < 80 = a negative IFAT result, showing no antigenic band on Western blotting.

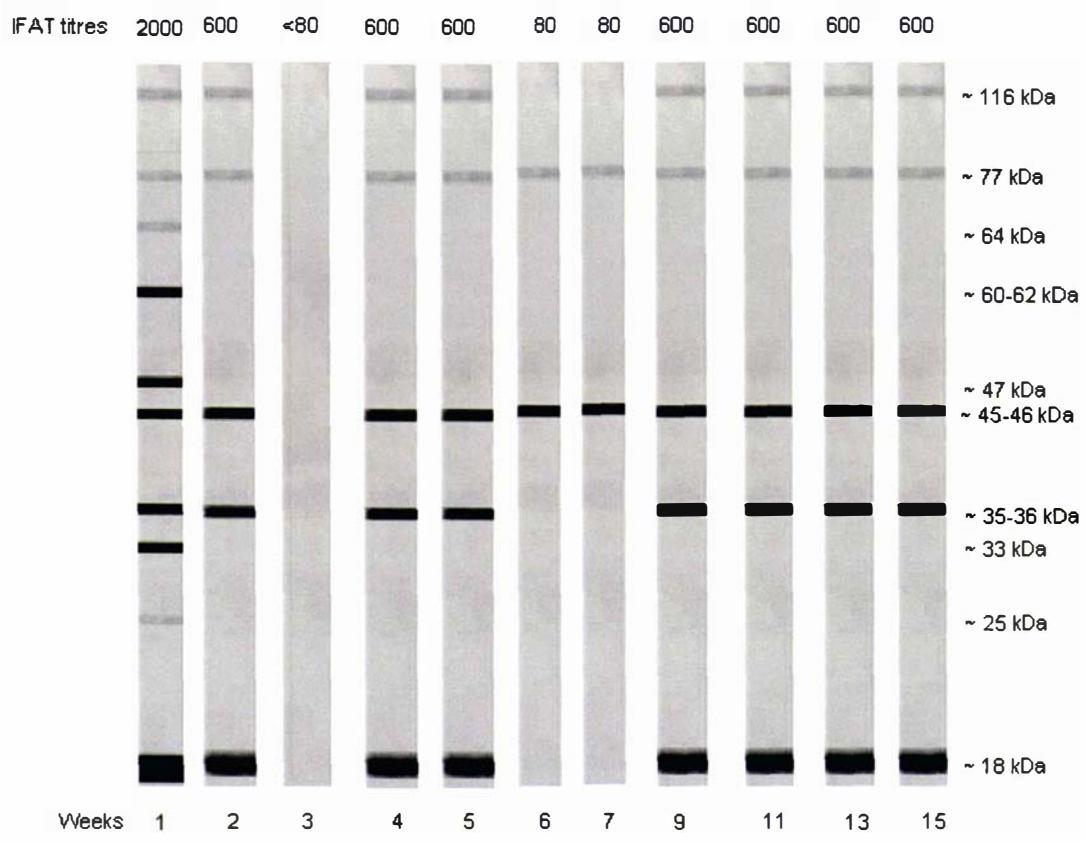

Figure 7.6: Binding pattern of *N. caninum* tachyzoite antigen (NcNZ 1) with anti-sera from a seropositive - aborted cow (cow #172) showing antibody fluctuation. IFAT titre < 80 = a negative IFAT result, showing no antigenic band on Western blotting.

7.4 Discussion

The recognition pattern of *N. caninum* tachyzoite antigen by anti *N. caninum* antibodies at different IFAT titres from cows naturally infected with *N. caninum* was investigated. Many authors' have reported the binding of *N. caninum* antigens by IgG from cattle and other animal species. In this study, the recognition of 7 *N. caninum* immunodominant antigens (18, 25, 33, 35-36, 45-46, 47 and 60-62 kDa) and 5 *N. caninum* minor antigens (25, 51, 64, 77 and 116 kDa) by anti-sera from naturally infected cows.

The 45-46 kDa antigen was recognised by all positive serum samples with IFAT titres ranging from 1:80 to >1:2000 dilutions. Schares *et al.* (1998) and Atkinson *et al.* (2000) reported the 46 kDa antigen as an *N. caninum* IDA for cattle. Similarly, the 18 kDa antigen localized in the rhoptries (Bjerkas *et al.* 1994) and tachyzoite membranes (Björkman *et al.* 1994; Schares *et al.* 1999) was recognised by all positive serum samples except for serum samples with IFAT titre 1:80. This finding accords with the works of Schares *et al.* (1998); Atkinson *et al.* (2000); Álvarez-García *et al.* (2002); Bjerkas *et al.* (1994); Björkman *et al.* (1994). However, the present study work differs from the findings of Álvarez-García *et al.* (2002), who stated that the 18 kDa antigen was recognised by sera with IFAT titres as low as 1:32 from infected foetuses. This is also the case with the findings of Bjerkas *et al.* (1994) who reported recognition of a 17 kDa antigen in some samples with low IFAT titres. The recognition of IDA 60-62 kDa by this work also supports the finding of Álvarez-García *et al.* (2002). This antigen was recognised by serum samples with 1: 2000 and >1: 2000 IFAT titres.

Baszler *et al.* (1996) observed that the 25 kDa antigen was recognised by all serum samples from *N. caninum* infected cattle. In our study, the 25 kDa antigen was only recognised by serum samples with IFAT titres 1:1000 and 1:2000 as a minor antigen and by serum samples with IFAT titre >1:2000 as an IDA. The variation in recognition intensity of 25 kDa could be attributed to the difference in IFAT titre. IDA 33 kDa, a dense granule antigen (Lally *et al.* 1997; Hemphill *et al.* 1998), was also recognised by sera with IFAT titres 1:2000 and >1:2000. This antigen was reported to be recognised by anti *N. caninum* sera (Lally *et al.* 1997). Another IDA identified in this study was 35-36 kDa. Hemphill and Gottstein (1996); Hemphill *et al.* (1996); Hemphill *et al.* (1997); Sonda *et al.* (1998) reported that the 36 kDa antigen is a major tachyzoite

surface protein. Tomioka *et al.* (2003) stated that in an early stage of infection, a 36-38 kDa antigen was clearly recognized by mouse anti-sera, but the signal weakened after day 48 post-inoculation.

While the 116 kDa antigen was reported as an IDA for *N. caninum* by Baszler *et al.* (1996), in our study it was recognised with low intensity by all serum samples with IFAT titre 1:200 and above; and thus was categorised as a minor antigen. Another minor antigen is the 77 kDa, which was recognised by all serum samples with IFAT titre ≥1:80. This finding agrees with the work of Álvarez-García *et al.* (2002) who also reported this antigen as minor. Other minor antigens identified in our study are; the 25 kDa (which is minor in IFAT titre 1:1000 and 1:2000 and major in IFAT titre > 1:2000), 51 kDa and 64 kDa antigens. These antigens have also been associated with *N. caninum* by other researchers. Álvarez-García *et al.* (2002) reported the recognition of minor antigens 24, 28, 30, 41, 51, 53, 55-57, 67 and 77 kDa from aborted cows when polyclonal secondary antibody was used. Bjerkas *et al.* (1994) reported that several minor *N. caninum* antigenic bands were recognised by immune sera from different animal species. Atkinson *et al.* (2000) and Schares *et al.* (1998) also reported the recognition of minor *N. caninum* antigenic bands by anti *N. caninum* sera.

Variation in the recognition pattern of *N. caninum* antigen by cow IgG was associated with differences in the IFAT titre. Thus, higher IFAT titres resulted in higher intensity and more immunogenic bands, the animal's serological records notwithstanding. This finding agrees with the works of Álvarez-García *et al.* (2002) and Ashburn *et al.* (1998), who reported changes in immunogenic binding based on different IFAT titres, but not with the work of Baszler *et al.* (1996) who stated that the variability in immunoblot binding patterns of *N. caninum* was not associated with the IFAT titre. In this study, the antigens first detected were the IDAs and later on, the minor antigens were detected suggesting that IDAs appear first before the minor antigens appear. This could also be associated with antibody titres because the minor antigens were detected sooner in the high IFAT titre groups than in the low titre groups. This observation was also made by Cazabonne *et al.* (1994), who stated that in *T. gondii*, the first IgG bound to the major antigens and minor antigens appeared later.

The pattern of *N. caninum* tachyzoite antigen recognition by sera from naturally infected cows was related to the level of seropositivity of serum samples at the time point, as defined by IFAT titres. The number of IDAs and minor antigens increased with higher IFAT titres. The intensity of IDA recognition also increased with IFAT titre. The most remarkable observation was the presence of the 45-46, the 77 kDa and absence of the 18 kDa antigenic bands in samples with 1: 80 IFAT titres. The absence of antigenic bands in all serum samples with IFAT titre <1:80 was an observation that held even in the face of antibody fluctuations where serum antibody titres from an animal exceeded then failed to reach 1:80.

Others have reported fluctuations in antibody response in pregnant cows infected with *N. caninum* (Quintanilla-Gozalo *et al.* 2000; Stenlund *et al.* 1999), but the reason for such fluctuations is yet to be elucidated. In this study, we observed antibody fluctuation in aborted and pregnant cows. The fluctuation in antibody titre observed appeared to be most remarkable in initially ELISA-negative pregnant cows and to a lesser extent in ELISA-positive pregnant cows and ELISA-positive aborted cows. However, it is not clear if the observed fluctuation depended on the physiologic (pregnant or aborted) condition of the cows. Despite fluctuation in antibody titre, the banding patterns in cows within the same titre group remained similar.

If the recognition of 3 or 4 immunodominant tachyzoite antigens (17, 29-30, 37 and 46 kDa) by sera from naturally infected cows should be considered as confirmation of *N. caninum* infection (Schares *et al.* 1998), then cows with fluctuating antibody titres need to be categorised differently since their antibody response changes. All the serum samples categorised as positive samples in this study have the IDA 45-46 and the 77 kDa minor antigens. If the detection of any of the IDA and minor antigenic bands reported in this work is a measure of infection or exposure to *N. caninum*, then serum samples with IFAT titre of 1:80 dilution should be considered as positive samples, especially since antibody fluctuation could mean that a cow with a high IFAT titre of 1:600 at one time could also have an IFAT titre of 1:80 at a different time. Conrad *et al.* (1993) and Dannatt (1997) stated that *N. caninum* antibodies fluctuate during pregnancy in cattle and that antibody titres may even drop below detection limits of some tests.

The use of serology for the diagnosis of infection is convenient when investigating abortion and experience indicates that setting high titres to assist with diagnosis of abortion and the examination of population prevalence in aborting and non-aborting animals is useful in reaching a diagnosis. However this technique is limited when attempting to define the infection status of individual animals, an essential step in defining the epidemiology of *N. caninum* infection. For this reason, we recommend further investigations into reason(s) for antibody titre fluctuations and a universal definition for infection with and exposure to *N. caninum* in cows. We further recommend the use of more definitive techniques, such as PCR on tissues and blood to define and document the infection status of animals when studying the epidemiology and pathogenesis of *N. caninum* abortion in cattle.

Authors' contributions to this experiment

N.B. Williamson helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the experiments as well as providing editorial advice.

C.M. Okeoma helped with animal inoculation, sample collection and clinical observations. She designed and conducted the experiments and she wrote the manuscript.

Chapter 8

Isolation and molecular characterization of *Neospora caninum* in cattle in New Zealand[5]

[5] Published as: Okeoma CM, Williamson NB, Pomroy WE, Stowell KM, Gillespie L. *New Zealand Veterinary Journal*, 52, 6 , 364-370

*It is worth mentioning that the isolation study reported in chapter 8 of this thesis was awarded the most commendable paper in 2004 by the Australian College of Veterinary Scientists

8.0 Abstract

The aim of this study was to isolate *N. caninum* from the brains of naturally infected cattle and use molecular techniques to characterise the isolate.

N. caninum tachyzoites were isolated in Vero cell culture from the brains of a cow and two calves. The isolates were characterised using the polymerase chain reaction (PCR), DNA sequencing, immunofluorescent antibody test (IFAT), transmission electron microscopy (TEM) and immunohistochemistry (IHC). The brains of the three cattle were subjected to histopathological examination. A pathogenicity study was conducted in 120 BALB/c mice.

N. caninum tachyzoites were first observed *in vitro* in all cases between 14 and 17 days post-inoculation. Parasites were sub-cultured and maintained in Vero cell culture for more than 6 months. The three isolates were PCR-positive using two different primers. Sequencing of the PCR products and a subsequent BLAST search identified the isolates as *N. caninum*. In addition, the isolates tested positive using the IFAT and IHC and were characteristic of *N. caninum* following TEM.

Histopathological examination revealed lesions characteristic of *N. caninum* in 1/3 brains. The mortality rate of the pathogenicity study in BALB/c mice was 3-7%. This was the first successful isolation of *N. caninum* in New Zealand confirmed by molecular characterisation tests.

8.1 Introduction

N. caninum is a protozoan parasite of the Apicomplexa phylum, which is a close relative of *T. gondii* (Lindsay and Dubey 1989). Infection with *N. caninum*, or neosporosis, occurs in cattle and several other species of ruminants, as well as dogs and horses. Neosporosis was first reported in dogs and caused neuromuscular disorders and mortality (Bjerkas *et al.* 1984; Dubey *et al.* 1988a). In horses, it caused myeloencephalitis (Marsh *et al.* 1996), while it is the major cause of abortion in cattle in many countries including New Zealand (Thornton *et al.* 1991). In calves infected *in utero*, neosporosis may cause congenital defects (Barr *et al.* 1990). At birth, such calves may have neurological signs, be underweight, unable to rise or have no clinical signs (Barr *et al.* 1993; Dubey and Lindsay 1996).

Bovine, ovine and canine neosporosis occur naturally, but there is no known major difference between isolates of *N. caninum* and thus all obtained from these species are considered to be *N. caninum* (Ellis *et al.* 1998; Wouda *et al.* 1999). Isolation of *N. caninum* has been documented in different countries and regions ranging from the United States of America (USA) through Europe to Japan and Australia. Several authors' reported some dissimilarity among isolates from different regions. An isolate designated Nc-LivB1 from the United Kingdom (UK) was found to be different from other bovine and canine isolates of *N. caninum* (Davison *et al.* 1999). Moreover, McGuire *et al.* (1997) showed that Nc-Liverpool (UK) produced more tissue cysts than the Nc-2 strain. In addition, Lindsay and Dubey (1990) reported differences in pathogenicity between Nc-1 and Nc-2, both isolates from the USA. They further stated that bradyzoites of the Nc-2 isolate were able to withstand treatment with pepsin-hydrochloride solution, unlike those of the Nc-1 isolate. Similarly, Magnino *et al.* (2000) reported minor differences when comparing an Nc-PV1 (Italy) sequence with two other available sequences of *N. caninum*. In addition, Atkinson *et al.* (1999) reported that a mouse model for infection of the central nervous system (CNS) demonstrated marked differences in pathogenicity between Nc-SweB1 (Sweden) and Nc-Liverpool (UK) isolates of *N. caninum*.

N. caninum can be isolated and cultivated *in vitro* or *in vivo*. *In vitro* isolation has been achieved in many cell lines, both primary and established (Dubey *et al.* 1988b; Cuddon

et al. 1992; Conrad *et al.* 1993; Stenlund *et al.* 1997; Davison *et al.* 1999; Dubey and Lindsay 2000; Kim *et al.* 2000; Sawada *et al.* 2000; Locatelli-Dittrich *et al.* 2003).

In vivo techniques using immunosuppressed, immunodeficient or knockout mice have also been used for the isolation of *N. caninum* and for the production of tissue cysts (McGuire *et al.* 1997; McGuire *et al.* 1999; Yamane 1997; Dubey *et al.* 1998; Peters *et al.* 2000; Sawada *et al.* 2000; Basso *et al.* 2001; Gondim *et al.* 2001; Koyama *et al.* 2001; Canada *et al.* 2002; Miller *et al.* 2002). *N. caninum* was the only recognised member of the genus *Neospora* before Marsh *et al.* (1998) isolated an apicomplexan parasite from the CNS of an adult equine. Although this isolate was ultrastructurally very similar to *N. caninum*, it differed from *N. caninum* of both bovine and canine origin, with respect to immunoreactive proteins. The sequence of the ITS-1 of the new isolate was seven-nucleotides different from that of *N. caninum.* The isolate was named *Neospora hughesi* (Marsh *et al.* 1998).

Bovine and canine neosporosis occur in New Zealand and the isolation of organisms from either or both is necessary for further study of the interaction between *N. caninum* and its hosts; its ability to establish infection, survive within its host, be transmitted to new hosts especially *in utero* and cause disease and/or abortion. We describe here the first confirmed isolation of *N. caninum* in New Zealand; its maintenance in Vero cell culture; and characterisation of the isolates using the PCR, IFAT, IHC and TEM.

8.2 Materials and methods

8.2.1 Animals used for isolation

Use of the animals in this study was approved (MUAEC 02/29 and 03/93) by the Massey University Animal Ethics Committee, Palmerston North, New Zealand. A 2-year-old Friesian cow from Waimana, Bay of Plenty, in the North Island of New Zealand had IFAT results ranging from 1:200 to 1:600, between May 2002 and immediately after calving in July 2003. The precolostral IFAT titre of her calf was 1:2,000. The IFAT titre of a stillborn calf was 1:8,000, while that of her dam (from Walton, North Island, New Zealand) ranged from ≤1:80 to 1:2,000, from May 2002 to

shortly after calving in August 2003. The dams were sampled weekly from approximately Day 150 of gestation for 7 weeks and then biweekly for 8 weeks.

The cow and calf were humanely slaughtered when the calf was 2 days old and their brains were taken immediately after slaughter. The brain was taken from the stillborn calf as soon as possible after it was born. The left hemispheres were used for isolation and PCR analysis. The right hemispheres were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin and cut at 4 μm and stained with haematoxylin and eosin, for histopathological examination.

8.2.2 Isolation of *N. caninum* tachyzoites

The brains were aseptically removed from the three animals and the left hemisphere separately homogenised in serum-free minimum essential medium (MEM) from Gibco (Invitrogen Corp NZ, Auckland, NZ) containing 10,000 units penicillin, 10,000 μg streptomycin (Gibco), 25 μg fungizone and antimycotic containing 0.25 μg amphotericin B (Gibco), per ml of MEM. The homogenates were trypsin-digested in 2% w/v phosphate buffered saline (PBS)-trypsin (Difco, Detroit, USA) by incubation at 37°C for 30 min, with shaking. The digests were washed three times with 1 x PBS pH 7.4 (Sigma-Aldrich, Milwaukee, USA) and centrifuged at 1,000 g for 10 min. The resulting pellets were re-suspended in 20 ml 1 x PBS pH 7.4 containing the same concentrations per ml of the same antibiotics and incubated for 48 h at 4°C, to control bacterial and fungal overgrowth. Each brain suspension was used to inoculate a 24 h Vero cell monolayer MEM containing 10% fetal bovine serum (FBS; Gibco) and the same antibodies at the same concentrations as previously. Three T^{75} tissue culture flasks were inoculated with each suspension at ratios of 1:2, 1:3 and 1:6 for Flasks 1, 2 and 3, respectively. The growth media were changed daily for 7 days after inoculation and thereafter at 3-day intervals for 17 days.

8.2.3 DNA isolation

Genomic DNA was isolated from parasites collected 21 days post-inoculation (p.i.), using a Qiagen DNeasy tissue kit (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia), according to the manufacturer's instructions. The integrity of each DNA sample was checked by electrophoresis on a 2% agarose gel in 1 x Tris-borate-EDTA (TBE) buffer. A 100 µl aliquot of total DNA was produced from each sample and stored at -20°C until required for PCR.

8.2.4 Genetic characterization by Polymerase Chain Reaction (PCR)

Genomic characterisation of *N. caninum* isolates was achieved by PCR, using two different primers. A *N. caninum* species-specific primer pair Np21+ (5'-CCAGTG-CGTCCAATCCTGTAAC-3') and Np6+ (5'-CTCGCCAGTCAACCTACGTCTTCT-3'), that anneals to the Nc-5 region (Müller *et al.* 1996) and the Tim 3 (5'-CGCTGC-AGAGGTGAACCTGCGG AAGGATC-3') and Tim 11 (5'-CACTGAAACAGA-CGTACC-3') primer pair (Payne and Ellis 1996), that targets the ITS-1 region, were used. PCR reactions for both primers were performed in 20 µl containing 1 µl 0.2 µg sample DNA, 0.4 µl 0.2 mM deoxynucleotide triphosphates (dNTPs) mix (Gibco), 1.4 µl 3.5 mM $MgCl_2$, 2 µl 1 X PCR Mg-free buffer, 0.12 µl 0.6 units platinum *Taq* polymerase (Gibco) and 0.4 µl 0.2 mM of each primer. PCR was performed in a Perkin Elmer GeneAmp PCR thermocycler System 2400 (Applied Biosystems, Foster City, California, USA). There was an initial denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, with a final extension of 72°C for 10 min. A negative control and positive control DNA from an Nc-Liverpool isolate (Barber *et al.* 1993) were included in each reaction. The amplified product of the Nc-5 fragment was separated by electrophoresis on a 2% Nusieve 3:1 agarose gel (Cambrex, Hallam, Victoria, Australia), while the product of the ITS-1 region was separated on a 2% agarose gel (Gibco). Separated products were stained with ethidium bromide at 4 µl/100 ml water for 15 min, destained in water and viewed under ultraviolet (UV) light.

8.2.5 Sequencing of PCR products

The PCR product of the ITS-1 region was sequenced from both ends using the BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Melbourne, Australia) and Tim 3 and Tim 11 as sequencing primers. The DNA sequence databases were searched using the nucleotide-nucleotide BLAST (blastn) at the National Centre for Biotechnology Information (USA) (http://www.ncbi.nlm.nih.gov/BLAST/) and comparison of the ITS-1 sequences of these isolates with previously described isolates was accomplished using the Align two sequences (bl2seq) (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html).

8.2.6 Characterisation of *N. caninum* by IFAT

Following isolation of *N. caninum* tachyzoites maintained by periodic passage in Vero cell cultures, heavily infected cultures were scraped with rubber pipettes and tachyzoites were purified by filtration through a 5 μm acrodisc syringe filter. Purified tachyzoites were washed twice in cold sterile PBS, pH 7.4 and pelleted by centrifugation at 1,000g for 10 min. The pellet was re-suspended in PBS to a final concentration of approximately 10^7 tachyzoites per ml, then aliquots frozen at -80°C until required for IFAT.

The procedure for demonstration of *N. caninum* by attachment of specific immunoglobulin G (IgG) antibodies was carried out using the purified tachyzoites obtained as described above. Briefly, 200 μl aliquots were dispensed onto SuperFrost Microscope Slides (Biolab Scientific NZ, Auckland, NZ). The slides were allowed to air dry at room temperature and then incubated at 37°C for 1 h. The prepared slides were processed for IFAT at the Gribbles Pty Ltd Animal Health Laboratory, Palmerston North, which conducts commercial testing of *Neospora*. Positive and negative controls were included on all runs according to normal practices of the laboratory. Reagents for *N. caninum* IFAT were commercially obtained from Veterinary Medical Research and Development (VMRD) Inc, Pullman, USA. Briefly, tachyzoites isolated from the three brain homogenates and grown on tissue culture were added to IFAT reagents and incubated with 50 μl anti-*N. caninum* rabbit antiserum (obtained from VMRD) diluted

1:5,000, plus sera from a cow and a dog naturally infected with *N. caninum*, at the concentration stated above. In each instance, 50 µl of a goat anti-rabbit fluorescein isothiocyanate (FITC) conjugated IgG antibody (Sigma, St Louis, USA) diluted at 1:160 in PBS was employed. These sera were samples submitted to the laboratory for diagnosis of neosporosis. Complete peripheral immunofluorescence of tachyzoites was considered a positive reaction specific to *N. caninum* (Barber *et al.* 1997). For the negative control, *T. gondii* tachyzoites were used and the slides were treated similar to the experimental and positive control slides.

8.2.7 Characterisation of *N. caninum* by TEM

N. caninum tachyzoites were maintained in culture to a concentration of approximately 10^7 tachyzoites per ml, as described above. Tachyzoites were pelleted and the supernatant discarded. The pellet was re-suspended in 0.25% trypsin in 0.1 M PBS pH 7.2 and incubated for 5 min at 37°C. The suspension was centrifuged and the pellet re-suspended in 30% Percoll in 0.1 M PBS pH 7.5. The suspension was centrifuged again and the pellet fixed in 3% glutaraldehyde in 0.1 M sodium dodecyl sulphate (SDS; BDH Laboratories Supplies, Poole, England) for 1 h at room temperature, then pelleted again and re-suspended in 20% bovine serum albumen (BSA; Sigma-Aldrich, Milwaukee, USA) and immediately pelleted. All centrifugations were at 2,000 g for 10 min, in a tabletop centrifuge. The BSA gel plug was extracted with the pellet and the pellet was cut into 1 mm slices and transferred into glass vials. The slices were fixed in 1% osmium tetroxide for 1 h and washed three times with 0.1 M PBS pH 7.5. The resulting slices were dehydrated for 10 min each in 30%, 60%, 90% and 100% ethanol, then twice for 15 min each in propylene oxide. Pellets containing parasites were embedded at 1:1 in resin and propylene oxide in a whirlybird mixer overnight and then for 4-6 h with fresh resin. Pellets were moulded and cured at 60°C for 48 h and thin sections (90 nm) stained with uranyl acetate and lead citrate prior to TEM. Appropriate sections of parasites were photographed for examination of structural features.

8.2.8 Characterisation of *N. caninum* by IHC

SuperFrost Microscope Slides were prepared as stated in the preparation of slides for IFAT. Slides were placed in an endogenous peroxidase wash (3% hydrogen peroxidase in 1 x PBS, pH 7.4) for 30 min and then equilibrated in 0.01 M PBS pH 7.4, for 1 min. Non-specific binding sites were blocked using 1% BSA in 1 x PBS, pH 7.4, for 1 min. Slides were then incubated in a caprine *N. caninum* antiserum (VMRD), diluted at 1:1,000 using 1% BSA in 1 x PBS, pH 7.4 and incubated in a humidified chamber at room temperature for 1 h. Slides were then washed in three changes of 1 X PBS, pH 7.4, for 1 min each and incubated in a biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) as the secondary antibody at a dilution of 1:200. Slides were washed as above and biotin-streptavidin-peroxidase preformed complex diluted at 1:200 was applied and incubated as above. Slides were also washed as above and peroxidase histochemistry was performed by applying 3, 3 diaminobenzidine (DAB) solution containing 4 mg DAB in 10 ml 1 x PBS, pH 7.4 and one drop of cobalt + nickel (heavy metal intensification system) in 10 µl 3% hydrogen peroxidase to the slides for 1.5 min. Immersing slides in three changes of PBS and rinsing in tap water stopped the reaction. Both positive and negative controls were included in all reactions. The positive control used was an Nc-Liverpool slide while the negative control was a *T. gondii* slide.

8.2.9 Pathogenesis in mice

N. caninum tachyzoites from the three isolates were maintained by periodic passage in Vero cell cultures. Heavily infected cells were left to lyse and tachyzoites pelleted by centrifugation at 1,000 g for 10 min and re-suspended in PBS, then titrated into 10^3, 10^4, 10^5 and 10^6 tachyzoites per ml. A 0.5 ml aliquot of each concentration of each isolate was inoculated intraperitoneally into 10 BALB/c mice (male and/or female, 20-30 g; total n = 120) and 10 mice inoculated intraperitoneally with 0.5 ml sterile saline acted as controls. The mice were only weighed at the start of the experiment, to ensure a minimum weight standard was met. They were observed daily for 60 days for survival, evidence of morbidity, mortality, or changes in appearance and behaviour. At the end of the 60-day period, all remaining mice were euthanised by anaesthetising with

isoflurane followed by cervical dislocation. Brains from all the mice were collected for parasite isolation.

8.3 Results

8.3.1 Isolation of *N. caninum* tachyzoites

Motile tachyzoites were observed in all flasks from the three animals between 14 and 17 days p.i. The isolates were passaged in Vero cell culture every 5-6 days and excess parasites cryopreserved with 1% dimethyl sulfoxide. The preserved parasites were subsequently thawed and used to infect Vero cells to assess viability and all were viable. The isolates were designated as NcNZ 1, NcNZ 2, NcNZ 3, for the cow, 2-day-old calf and stillborn calf, respectively.

8.3.2 Genetic characterisation by PCR

PCR with the primer pair Np21+ and Np6+ gave a product of 350 base pairs (bp) from all of the isolates (Figure 8.1). The bands co-migrated with the positive control. Similarly, PCR with the primer pair Tim 3 and Tim 11 gave a product of approximately 620 bp, which also co-migrated with the positive control (Figure 8.2). No band was amplified for the negative control in all PCR reactions. When the ITS-1 region sequence was subjected to a BLAST search, the best score was returned with *N. caninum* (AY463245, AF432123.1, NSPRGEBN, AF029702, AF038861, AF038860, NCU16160, NCU16159, AY259043.1, AY259037.1, AY259039.1, AY259038.1, AY259041.1, AY259040.1, AF249970, AF249969, AF249968, AF338411, AF038859, AY259042.1, AF249967; in listed order). The Genebank Accession Numbers for the tested sequences are AY601347, AY601349 and AY601348 for the NcNZ 1, NcNZ 2 and NcNZ 3 isolates, respectively.

8.3.3 Characterisation by IFAT

Tachyzoites from the three isolates reacted positively with anti-*N. caninum* antibodies in the IFAT (Figure 8.3), as did the positive control. No reaction was observed from the negative control.

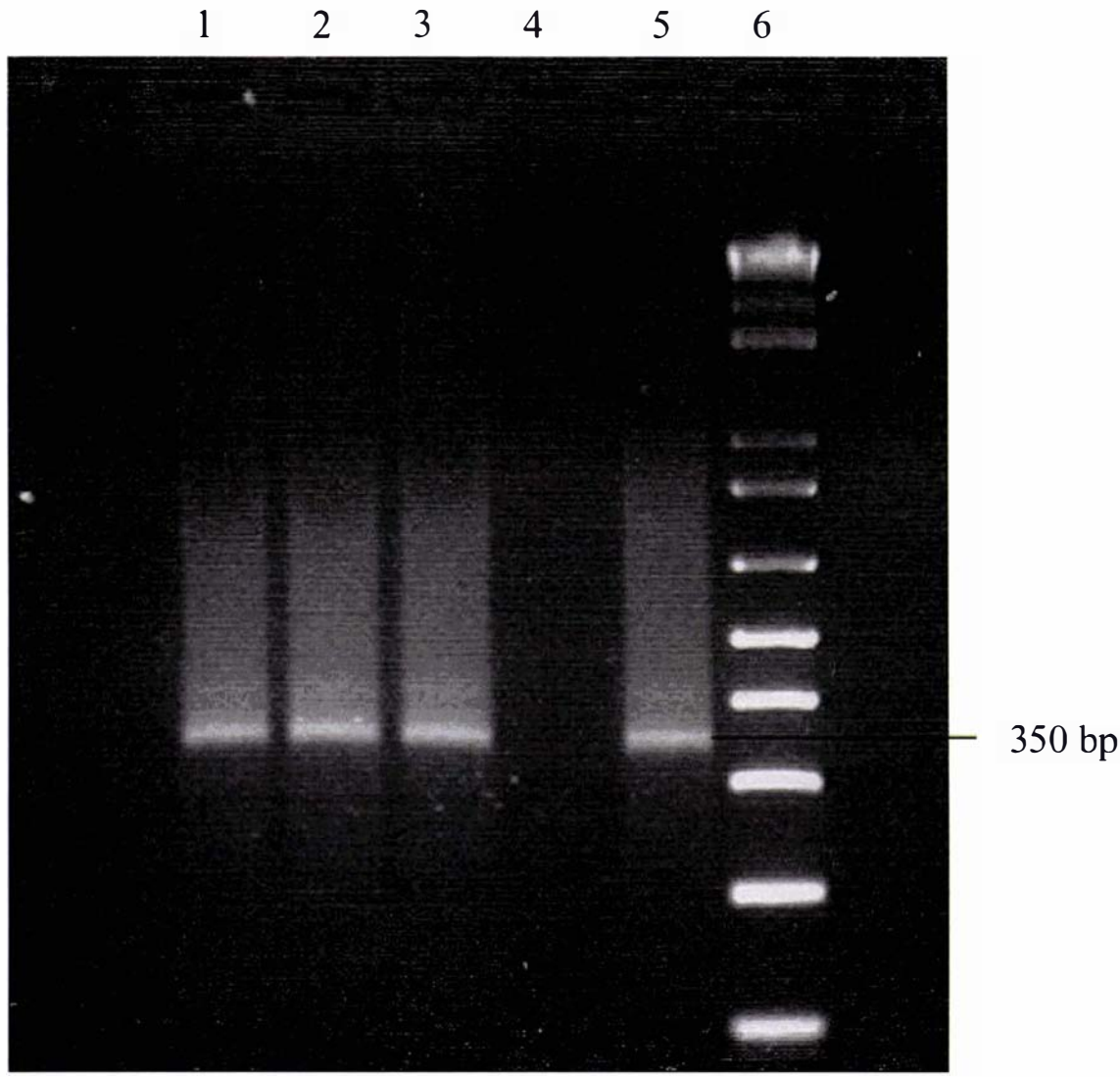

Figure 8.1: ~350 bp PCR products amplified with primer pair Np21+ and Np6+ from the brains of a cow and two calves. Lanes 1 to 3 are isolates NcNZ 1, 2 and 3 respectively. Lane 4 is a negative control, lane 5 is positive control (Nc Liverpool) and lane 6 is a 1 kb+ DNA ladder.

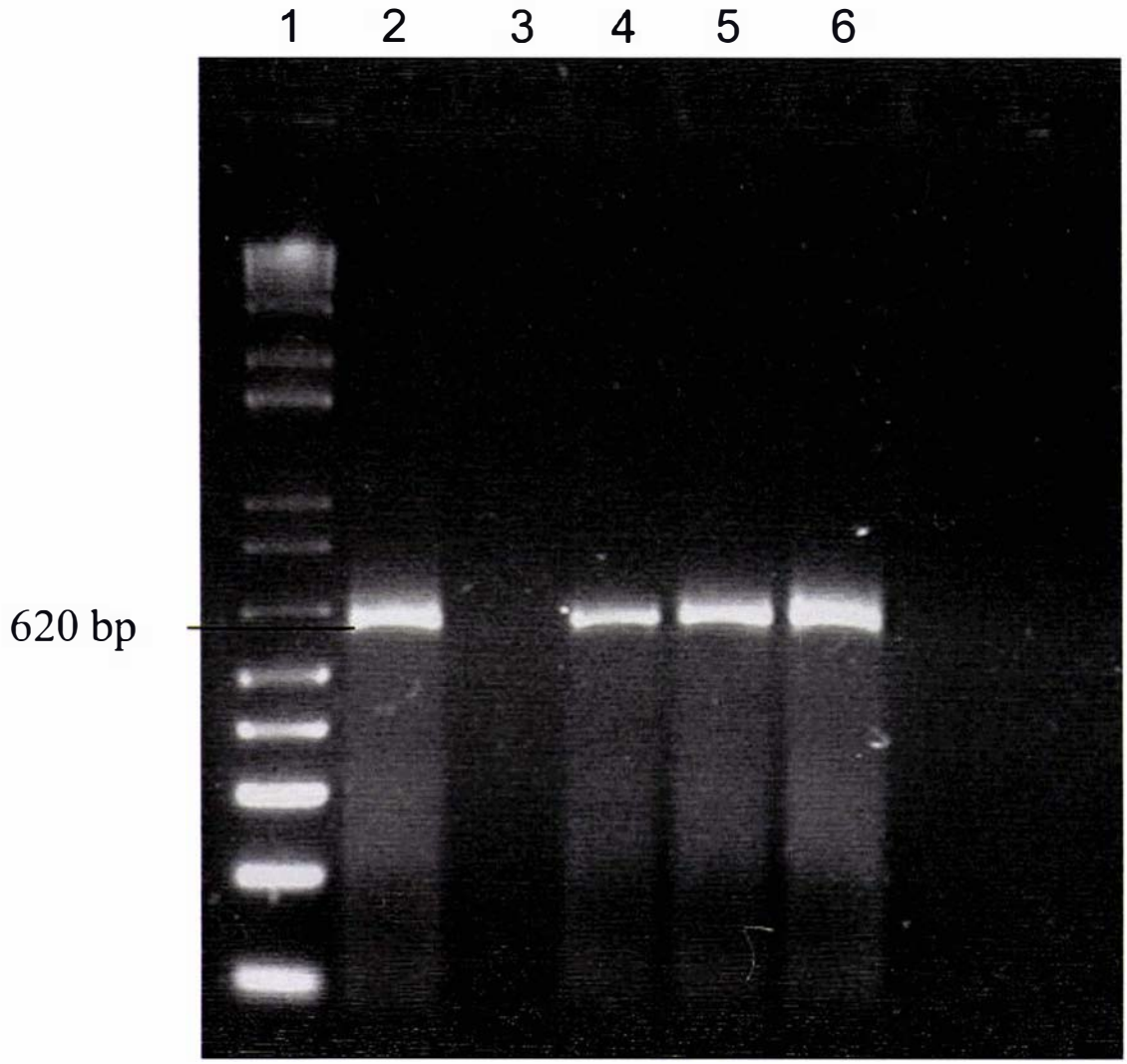

Figure 8.2: ~620 bp PCR products amplified with primer pair Tim 3 and Tim 11 from the brains of a cow and two calves. Lane 1 is a 1 kb+ DNA ladder, lane 2 is a positive control (Nc Liverpool), lane 3 is a negative control and lanes 4 to 6 are isolates NcNZ 1, 2 and 3 respectively.

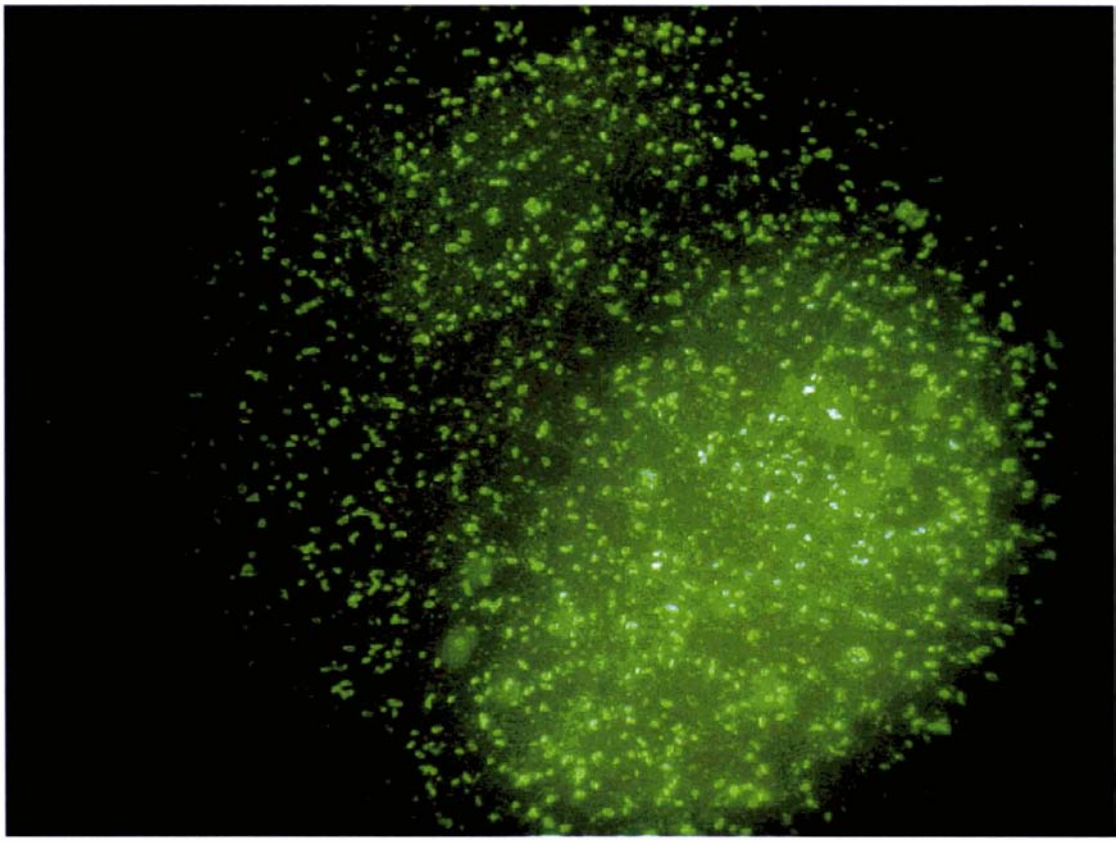

Figure 8.3: Representative IFAT slide for NcNZ 1, NcNZ 2 and NcNZ 3 isolated from the brains of a cow and two calves, showing fluorescence of *N. caninum* tachyzoites when reacted with anti *N. caninum* antibodies.

8.3.4 Characterisation by TEM

TEM revealed electron-dense rhoptries, numerous micronemes and dense granules (Figure 8.4), features consistent with those of *N. caninum* tachyzoites and were approximately 7.3 x 2 µm, unlike those of *T. gondii*, which measure approximately 6.8 x 1.5 µm; additionally, the rhoptries of *T. gondii* are labyrinthine or spongey (Lindsay *et al.* 1993).

8.3.5 Characterisation by IHC

Tachyzoites from the three isolates reacted positively with anti-*N. caninum* antibodies, as did the positive control. No reaction was observed from the negative control (figures not shown).

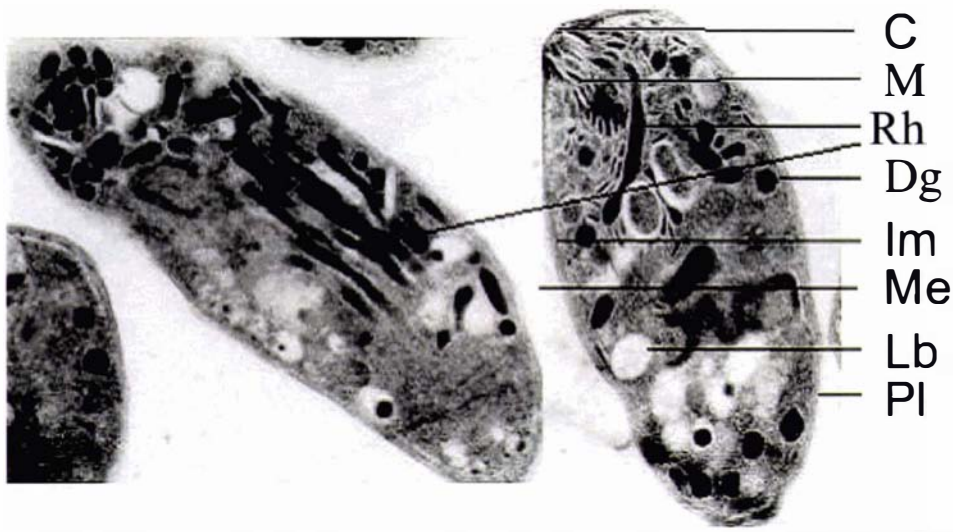

Figure 8.4: Representative transmission electron micrograph of NcNZ 1, NcNZ 2 and NcNZ 3 tachyzoites showing some of the ultra-structural features of *N. caninum*. Abbreviations: conoid (C), dense granule (Dg), inner membrane complex of pellicle (Im), lipid body (Lb), microneme (M), plasmalemma (Pl), growth medium (Me), rhoptries (Rh).

8.3.6 Pathogenesis in mice

Two of 30 (7%), 1/30 (3%) and 1/30 (3%) mice acutely infected with NcNZ 1, NcNZ 2 and NcNZ 3, respectively, died between 17 and 24 days p.i., whereas those chronically infected survived until the end of the 60-day trial and were euthanised (Table 8.1). Tachyzoites of *N. caninum* were isolated from all acute and chronic cases that were inoculated with 10^5 and 10^6 tachyzoites per ml. Brains of mice inoculated with 10^3 were pooled by isolate after euthanasia and tachyzoites of *N. caninum* were isolated from the pooled brains. Similarly, brains of mice inoculated with 10^4 tachyzoites per ml were treated as per 10^3 brains and tachyzoites were also isolated from the pool of brains. None of the control mice died and *N. caninum* was not isolated from the control mice.

8.3.7 Histopathology

Histopathological examination of the brains from the three cattle showed there were no inflammatory changes in the brain of the heifer or her calf, but moderate inflammation was observed in that of the stillborn calf, evidenced by perivascular lymphoid cuffing in the cerebellum and cerebrum.

Table 8.1: Result of a pathogenicity study in BALB/c mice (n = 130) inoculated with three isolates (NcNZ 1, NcNZ 2 and NcNZ 3) of a New Zealand strain of *N. caninum*.

Isolate	Inoculum Concentration	Number Inoculated	Number Dead/euthanase	Time of Death	Parasite Reisolated from
NcNZ 1	10^3	10	10[a]	Euthanase	Pooled Brain
	10^4	10	1/9[a]	24 days pi	Brain
	10^5	10	10[a]	Euthanase	Brain
	10^6	10	1/9[a]	22 days pi	Brain
NcNZ 2	10^3	10	1/9[a]	17 days pi	Pooled Brain
	10^4	10	10[a]	Euthanase	Brain
	10^5	10	10[a]	Euthanase	Brain
	10^6	10	10[a]	Euthanase	Brain
NcNZ 3	10^3	10	10[a]	Euthanase	Pooled Brain
	10^4	10	10[a]	Euthanase	Brain
	10^5	10	10[a]	Euthanase	Brain
	10^6	10	1/9[a]	20 days pi	Brain
Control	Sterile saline	10	None	N/A	N/A

a Euthanase at end of 60-day study; p.i. = post-inoculation; N/A = none dead or parasite not isolated

8.4 Discussion

N. caninum has been isolated from aborted foetuses, newborn calves (Conrad *et al.* 1993; Yamane *et al.* 1998; Davison *et al.* 1999; Magnino *et al.* 1999) and naturally infected cows (Sawada *et al.* 2000). In the study presented here, *N. caninum* was isolated from the brains of three animals and the isolates characterised by PCR, IFAT, IHC and TEM. Thus, this is the first report of successful isolation of *N. caninum* in New Zealand. The obtainment of these isolates represents a significant advance for *N. caninum* research in New Zealand since it now provides the opportunity to undertake further studies, including challenge studies and the possibility of vaccine development, that do not rely on the use of imported organisms. Ho *et al.* (1997) amplified *Neospora*-specific DNA fragments from the brains of naturally infected cows and PCR characterisation of their isolates was the same as that in our study. The results obtained by PCR were confirmed by comparing the ITS-1 sequence of each isolate against each other, as well as with that of related species. The ITS-1 region has been successfully used to distinguish between closely-related species of *Neospora*, *Hammondia*, *Toxoplasma* and other apicomplexan genera (Ellis *et al.* 1998; Ellis *et al.* 1999). Analyses of all available sequences of this region confirmed the isolates in our study were *N. caninum*.

The three isolates tested positive to *N. caninum* and reacted in a similar way to each other and to the positive control in the IFAT. This test normally detects antibodies directed to antigens present on the cell surface of tachyzoites. With parasites like *T. gondii* and *N. caninum*, such antigens are considered more specific than intracellular components (Hughes 1982; Björkman *et al.* 1994; Packham *et al.* 1998). Infection studies in different hosts have shown that the *N. caninum* IFAT shows very little cross-reactivity with other apicomplexan parasites (Trees *et al.* 1993; Dubey *et al.* 1996). We are confident that the IFAT used in the present study was specific for *Neospora* under the test conditions established by the laboratory, since we found no cross-reactivity with the negative control. The tachyzoites displayed complete peripheral immuno-fluorescence, which is characteristic of tachyzoites of *N. caninum* in an IFAT test (Figure 8.3).

In the IHC test, tachyzoites from the three isolates, like the control, tested positive to *N. caninum*. The isolates were also similar to each other and to the positive control. Furthermore, TEM showed that all three isolates were similar to each other and their ultrastructural features were characteristic of those described for *N. caninum* (Conrad *et al.* 1993; Lindsay *et al.* 1993).

Generally, immunocompetent mice are resistant to infection with *N. caninum* (Lindsay *et al.* 1995). Results of the pathogenicity study suggested that isolates of *N. caninum* in New Zealand were not very virulent for immunocompetent BALB/c mice, as the majority were resistant to infection. This mortality rate was low compared to the reports of Lindsay *et al.* (1995), who reported that 7/12 BALB/c mice died 26-70 days after subcutaneous inoculation with Nc-1 *N. caninum* tachyzoites. Similarly, female BALB/c mice immunised with 2.5 x 10^6 freshly harvested Nc-1 tachyzoites in 0.1 ml cell culture medium died within 28 days p.i. (Lundén *et al.* 2002). An isolate designated NC-LivB1 from the United Kingdom was found to be different from other bovine and canine isolates of *N. caninum* (Davison *et al.* 1999). Moreover, McGuire *et al.* (1997) showed that NC-Liverpool (UK) produced more tissue cysts than the NC-2 strain. In addition, Lindsay and Dubey (1990) reported differences in pathogenicity between NC-1 and NC-2 that are both United States of America isolates. They further stated that bradyzoites of the NC-2 isolate were able to withstand treatment with pepsin-hydrochloride solution unlike that of NC-1 isolate. Similarly, Magnino *et al.* (2000) reported minor differences when comparing an NC-PV1 (Italy) sequence with two other available sequences of *N. caninum*. In addition, Atkinson *et al.* (1999) reported that a mouse model for central nervous system (CNS) infection demonstrated marked differences in pathogenicity between isolates [NC-SweB1 (Sweden) and NC-Liverpool (UK)] of *N. caninum*.

Pathological differences observed in challenged mice between the present study and other similar studies could be as a result of; route of infection/inoculation for instance, oral, intranasal; intravenous; intraperitoneal, or subcutaneous infection. Differences could also be as a result of differences in the isolates used by different researchers or mouse strains used.

The New Zealand isolates of *N. caninum* reported in this study were used in a Western blot analysis of cows naturally infected with *N. caninum*, as described previously by Okeoma *et al.* (2004) Chapter 7. In that study, the recognition patterns of seven *N. caninum* tachyzoite immunodominant antigens (18, 25, 33, 35–36, 37, 45-46, 47 and 60-62 kDa) and five minor antigens (25, 30, 51, 64, 77 and 116 kDa) by bovine IgG at different IFAT titres were described. Those results agree variably with the results from studies by Bjerkas *et al.* (1994); Björkman *et al.* (1994); Schares *et al.* (1998); Schares *et al.* (1999b); Atkinson *et al.* (2000) and Álvarez-García *et al.* (2002).

DNA from each of the three isolates has been forwarded to the European *N. caninum* reference centre at the Moredun Institute in Edinburgh for characterisation and comparison with European strains. Information from this comparative study will prove valuable in determining if there is any difference between isolates from New Zealand and other countries. Any observed differences may explain differences in the epidemiology of *N. caninum* in New Zealand, such as lower vertical transmission rates (Schares *et al.* 1999a) and a higher seroprevalence in dogs (Antony and Williamson 2003), than are generally observed elsewhere.

Authors' contributions to this experiment

N.B. Williamson helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

W.E. Pomroy helped with animal inoculation, sample collection and clinical observations. He also provided editorial advice.

K.M. Stowell supervised the experiments as well as providing editorial advice.

C.M. Okeoma helped with animal inoculation, sample collection and clinical observations. She designed and conducted the experiments and she wrote the manuscript.

Chapter 9
General Discussion

9.0 General Discussion

N. caninum can be transmitted vertically (from cow to calf *in utero*) and horizontally (postnatal infection from the environment). Rates of vertical transmission of *N. caninum* have been estimated in many countries and vertical or congenital transmission has been found to be the major means of spreading *N. caninum* infection (Davison *et al.* 1999; Paré *et al.* 1996; Schares *et al.* 1998; Dubey and Lindsay 1996; Anderson *et al.* 1997; Bergeron *et al.* 2000; Bjorkman *et al.* 1996; Davison *et al.* 1999; Dubey *et al.* 1992; Liddel *et al.* 1998; Paré *et al.* 1996; Waldner *et al.* 1999; Romero and Frankena 2003; Okeoma *et al.* 2004b). In the present study, *N. caninum* DNA was detected in the brains of 14 out of 18 dam-daughter pairs, indicating a vertical transmission rate of 77.8%. Of these dam-daughter pairs, 38.8% (7/18) were consistently seropositive on IDEXX ELISA while 44.4% (8/18) were consistently seronegative on the same test. In this study, 7 out of the 18 dams were consistently seropositive and were also born to consistently seropositive dams. These dams all had calves that were infected *in utero*, indicating a possible 100% vertical transmission rate. This is in agreement with Dijkstra *et al.* (2003) who found that seropositive heifers had 80% congenitally infected offspring, while in older cows 66% of the offspring was congenitally infected.

An interesting observation in the transmission study was the rate (44.4%) of vertical transmission in consistently seronegative dams that were also born to dams that were consistently seronegative based on the ELISA. It is not known that seronegative dams transmit neosporosis to their calves *in utero*. However, it is plausible that the ELISA used in screening these animals may not have been sensitive enough to detect infection since PCR assay on the ELISA-seronegative dams and their calves showed *N. caninum* infection. This observation may also be true for the IFAT at 1:200 because the same dams tested negative at this IFAT dilution. However, when IFAT sensitivity was increased by reducing the cut-off titre to 1:80, 7/18 (38.8%) tested positive to *N. caninum* infection. Western blot analysis of sera from these animals corroborate the PCR and IFAT results since all 8 IDEXX-seronegative dams were positive on Western blot analysis. Reichel and Pfeiffer (2002) had compared IDEXX-ELISA with IFAT and recommended that the ELISA cut-off be reduced from the manufacturer's recommended S:P ratio value of 0.5 to 0.21 or 0.22 in order to increase sensitivity of the ELISA. In the present study, the manufacturer's recommended cut-off of 0.5 was used. Perhaps,

lowering the ELISA cut-off to 0.21 may have produced identical results as the IFAT (1:80), PCR and Western blot.

The ramifications of synergistic infection of *N. caninum* and BVDV are still not yet completely understood. However, BVDV is believed to potentially enhance diseases caused by other pathogens or precipitate illness by opportunistic pathogens (Bohac and Yates 1980; Malmquist 1985; Potgieter 1977). The present study seems to support this hypothesis since results show that *N. caninum* in cows that are also BVDV positive produced 44.4% (8/18) dam-calf pairs that were both BVDV and *N. caninum* seropositive. Bjorkman *et al.* (2000) had previously reported a significant association between BVDV and *N. caninum* infection in cattle. Though the dams and their calves in the present study had antibodies to BVDV, none of the calves tested positive on reverse transcription PCR indicating that there may be no viral RNA in their blood thus, they were not persistently infected.

The detection of *N. caninum* DNA in the blood has been elusive as cases of detection of *N. caninum* DNA prior to this study have mainly been in tissues (Yamage *et al.* 1996; Ho *et al.* 1997; and others), which almost always involves slaughtering animals. However, in this study, *N. caninum* DNA was amplified from the blood of 6 live *N. caninum* ELISA-seropositive aborted, 6 *N. caninum* ELISA-seropositive pregnant and 11 *N. caninum* ELISA-seronegative pregnant heifers. The presence of *N. caninum* DNA in the blood may have resulted from tachyzoites released when *N. caninum* tissue cysts rupture.

Other authors' have also reported the detection of *N. caninum* DNA from the blood of experimentally infected sheep (O'Handley *et al.* 2002); from bull semen (Ortega-Mora *et al.* 2003) naturally infected with neosporosis; semen and blood of bulls (Ferre *et al.* 2005) and from frozen extended semen (Caetano-da-Silva *et al.* 2004). However, the present study appears to be the only report on *N. caninum* DNA in the blood of animals previously ELISA-seronegative. The ability to detect *N. caninum* DNA in the blood of animals using PCR may complement serology in the diagnosis of *N. caninum* infection and may serve as a confirmatory test for neosporosis when results from ELISA and IFAT are inconclusive.

Furthermore, real-time PCR may be used in the quantification of *N. caninum* parasite load in the blood. The present study demonstrates the quantification of *N. caninum* DNA in the blood and brains of infected pregnant and aborted heifers. The presence of parasite DNA in blood is an indication of parasitaemia, while its presence in the brain indicates chronic infection. Unlike in brains where samples for DNA isolation for PCR involves slaughtering animals, the ability to isolate DNA from blood for PCR means that the circulating parasite load can be quantified in live animals. Thus the use of PCR offers valuable tools in farm management since the *N. caninum* status of animals may be determined and appropriate control strategies implemented.

Anti *N. caninum* antibodies can be detected in animals suffering from either chronic or active neosporosis. Detection of *N. caninum* antibodies is largely done by serology using different serological tests (IFAT, ELISA) with different cut-off points. In the present study, IFAT was used at a 1:80 dilution rate to detect *N. caninum* infection. This cut-off point was used in order to increase the sensitivity of the assay since it is known that antibody fluctuation in *N. caninum* infected cattle occurs (Conrad *et al.* 1993; Dubey *et al.* 1996; Dubey *et al.* 1997; Dannatt 1997; Hietala and Thurmond 1999). It is also documented that in cattle, *N. caninum* antibodies fluctuate during pregnancy and they may even drop below detection limits of some assays (Conrad *et al.* 1993; Dannatt 1997). In the present study, antibody titres rose and fell sometimes to levels below IFAT cut-off sensitivity, resulting in negative results in animals that had previously had positive tests. The fluctuations in antibody titres observed in this study could be as a result of a combination of the inherent characteristics of the cow and those of the parasite which could influence the immunological response by the host.

Another method of detecting *N. caninum* infection is by detecting antibodies to specific *N. caninum* antigens in animals. This may be tested by Western blot analysis of serum samples using *N. caninum* antigens. It has been documented that immune sera from a wide range of animal species show a similar antigen recognition pattern with five major (17, 27, 29, 30 and 46 kDa) and several minor response bands to *N. caninum* (Bjerkas *et al.* 1994). Antisera obtained from cows with confirmed *N. caninum* induced abortions, consistently reacted with antigens of 25, 65 and 116 kDa (Baszler *et al.* 1996). Antisera from naturally and experimentally infected cattle, dogs and mice reacted with antigens of 29 and 35 kDa (Howe *et al.* 1998). Four immunodominant antigens 17-18, 34-35, 37

and 60-62 kDa, were recognised by aborted foetuses and cows (Álvarez-García *et al.* 2002). In the present study, Western blot analysis using antigens of NcNZ 1 (Okeoma *et al.* 20004b), 7 immunodominant (~18, ~25, ~33, ~35-36, ~45-46, ~47, 60-62 kDa) and 5 minor (~30, 51, 64, 77 and 116 kDa) antigens were recognised by bovine sera.

The antigen provoking a 45-46 kDa antibody response was recognised across all positive sera including sera with IFAT titre as low as 1:80. This observation makes the 45-46 kDa antigen an important antigen which appears to be expressed in acute, chronic and even transient infections. It also appears that this antigen is a good candidate for detecting animals exposed to neosporosis. The 45 kDa antigen is an antigen provoking immunodominant antibody response in cattle (Schares *et al.* 1998; Atkinson *et al.* 2000). The ~18 kDa antigen provoked an antibody response on Western blot testing that was recognised in all positive sera except those with IFAT titre 1:80 or less. This antigen was also recognised by sera from cows (aborted or pregnant) and foetuses as was also observed by Álvarez-García *et al.* 2001.

Minor differences in the molecular weights of the antibodies to specific antigens reported in this study from those reported by other authors' could result from the different molecular weight size standards used, origin/variation in antigen and antibody used, or different methods of electrophoretic separation (native or denatured). For instance, the ~18 kDa antigen reported in the present study has been reported with a molecular weight ranging from 16-17 kDa (Schares *et al.* 1998; Atkinson *et al.* 2000), 17 kDa (Bjerkas *et al.* 1994), 17.8 kDa (Atkinson *et al.* 2001), 17-18 kDa (Quintanilla-Gozalo *et al.* 2000; Álvarez-García *et al.* 2001) and 18 kDa (Bjerkas *et al.* 1994; Okeoma *et al.* 2004). It is worth noting that the present study found that antibody to *N. caninum* has patterns for the appearance of protein band sizes that are the same for a specific IFAT titre regardless whether it is a rising or falling titre.

Isolating *N. caninum* has been an ongoing challenge at Massey University. A need to have a local isolate that can be compared with isolates from other parts of the world cannot be overemphasized. The study described in Chapter 8 of this thesis succeeded in obtaining 3 isolates of *N. caninum* that were named NcNZ 1, NcNZ 2 and NcNZ 3. The obtainment of these isolates represents a significant advance for *N. caninum* research in New Zealand since it provides the opportunity to undertake further studies that do not

rely on the use of imported organisms. It also makes possible vaccine development using one or more local isolates. The 3 local isolates of *N. caninum* exhibited features characteristic of *N. caninum* in line with other isolates of *N. caninum* from other countries. Study of the pathogenicity of the local isolates in BALB/c mice suggests that these isolates have low virulence for immunocompetent BALB/c mice. Differences in pathogenicity have been reported by various research workers (Lindsay *et al.* 1995; Lundén *et al.* 2002; McGuire *et al.* 1997; Dubey 1990; Magnino *et al.* 2000; Atkinson *et al.* 1999; Okeoma *et al.* 2004b) on isolates from different regions. A variation in pathological effects of different *N. caninum* isolates could depend on the route of infection/inoculation (oral, intranasal, intravenous, intraperitoneal, or subcutaneous). In the pathogenicity study in mice reported in Chapter 8, the route of infection was by intraperitoneal injection. This route of infection has previously been used by research workers and was reported to induce pathological effects (Ramamoorthy *et al.* 2005). Pathological differences observed could also be as a result of differences in the pathogenicity of isolates used by different researchers and/or differences in hosts' immune response to parasites of different pathogenicity.

DNA from each of the 3 isolates obtained as a result of the study has been forwarded to the European *N. caninum* reference centre at the Moredun Institute in Edinburgh for characterisation and comparison with other strains. Preliminary results indicate that NcNZ 1 and NcNZ 2 are more closely related but that these are distinct from NcNZ 3. This is not surprising since NcNZ 1 and NcNZ 2 were from the brains of a cow and her calf respectively. NcNZ 3 was from the brain of a stillborn calf from another cow that was from a different location. This comparative study should show any differences between New Zealand and other isolates.

Different isolates of *N. caninum* from different countries, regions and sources exist, so it is not surprising that there will be differences reported in antigenic reactivity; antibody responses of infected animals to specific antigens; serological reactivity on different assays; transmission routes and rates of transmission. The environment could also play a role in the differences observed for these factors.

9.1 Contributions of the research presented in this thesis to knowledge

The use of PCR in blood has provided a new and useful tool for studying *N. caninum* epidemiology and pathogenesis. PCR is an invaluable tool for the study of disease infection. PCR has been used to detect *N. caninum* from host's body fluids (Ortega-Mora *et al.* 2003; Okeoma *et al.* 2004; Caetano-da-Silva 2004; Ferre *et al.* 2005); cell culture (Cheah *et al.* 2004); and host's tissues (Pinitkiatisakul *et al.* 2005; Rodrigues *et al.* 2004; Maanen *et al.* 2004; Eleni 2004; Dubey *et al.* 2004; Ŝlapeta *et al.* 2002; Sager *et al.* 2001; Liddell *et al.* 1999). Prior to this research, there was no documented evidence of the use of PCR to amplify *N. caninum* DNA from the blood of naturally infected animals.

The ability to determine the infection status and intensity of *N. caninum* infection in cows using PCR on blood samples is an advancement that may reduce or eliminate the need to slaughter animals for tissue samples needed for *N. caninum* studies. Investigation of neosporosis in animals oftentimes involves assays like histology/immunohistochemistry and tissue PCR, all of which complement serology and serve as confirmatory tests for *N. caninum* infection. PCR on blood samples would no doubt complement serology as a tool for the study of neosporosis and may also serve as confirmatory test to serology with an added advantage of being performed in live animals. The fact that the levels of DNA in the serum in this study occurred in association with changes in the serological status of animals even in the short term confirm the usefulness of both types of tests in studying the pathogenesis of neosporosis when parasitaemia is occurring.

A qualitative PCR on blood may be used to differentiate between animals that are parasitaemic or not, while a quantitative PCR provides a measure of the intensity of the infection with *N. caninum* that will help to categorise animals based on the severity of their parasitaemia. Liddell *et al.* (1999); Ortega-Mora *et al.* (2003) and Collantes-Fernandez *et al.* (2002) have all applied quantitative PCR to the study of neosporosis in host tissues and fluids. The present study used quantitative PCR to study *N. caninum* parasitaemia in aborted and pregnant cows.

Furthermore, the isolation of *N. caninum* in New Zealand will allow further study of the pathogenesis of infection and abortion by challenge experiments in this country. Local isolates also allows local vaccine development efforts. The availability of a local isolate will also facilitate studies on host parasite interactions to improve our understanding of the intricacies of immunological interactions between the parasite and its host. This is especially true since different isolates have varying degrees of pathogenicity and virulence.

This study found that antibody to *N. caninum* has patterns for the appearance of protein band sizes that are the same for a specific IFAT titre regardless whether it is a rising or falling titre. *N. caninum* antibody fluctuation has been well documented (Conrad *et al.* 1993; Dubey *et al.* 1996; Dubey *et al.* 1997; Dannatt 1997; Hietala and Thurmond 1999). The current study revealed that in cows, antibody to *N. caninum* can rise and fall and sometimes can deteriorate below the detection limit of both IFAT and Western blot within a relatively brief period. It is also surprising that this was an observation which was made on cows labelled as seronegatives by long term monitoring using an ELISA test. This observation was true regardless of the pregnancy status of the cow. The observation was also supported on the whole by concurrent positive PCR responses demonstrating *N. caninum* DNA.

As a result of these observations, it is important to consider redefining what a *N. caninum* positive cow is including a need to critically examine and consider the cut-off points for different assays used in the study of neosporosis. Correct use of cut-off titres and their appropriate interpretation will help to reveal and identify animals that are suffering from exposure/transient infection, as well as, the chronically infected animals whose immunologic system is masking infection.

This thesis has made notable contributions to knowledge on *N. caninum* and neosporosis in general and to *N. caninum* and neosporosis in New Zealand in particular.

9.2 Limitations

The abortions that occurred in 6 of the 24 heifers destined to participate in the BVDV challenge trial reported in Chapter 4 reduced the major field experiment of this thesis to an observational study with inadequate statistical power. Despite this setback to the planned work, novel observations and findings were reported from this experiment.

The inability for biosecurity reasons to use another strain of *N. caninum,* for example, the Liverpool isolate in the study on mouse pathogenicity, as a positive control, was unfortunate. Its use in such a way would have allowed a valuable comparison of the ability of the locally isolated and well characterised strains of *N. caninum* to cause morbidity and death in mice. However, the lack of approval from MAF and the Massey University Animal Ethics Committee prevented us from introducing an isolate derived from overseas into mice and therefore conducting the pathogenicity study without the opportunity to use another *N. caninum* isolate as control.

A longitudinal study with blood sampling of heifers after parturition and prior to conception would have shown the antibody, antigen and DNA profiles at different physiological and gestational stages in the study heifers. Probably, no single study may capture all the possibilities and the present study is not an exception. Regardless of the limitations faced in this study, many novel findings were made as discussed above. The use of PCR in the detection of *N. caninum* DNA and quantification of parasite load is an advance that may eliminate the use of animal tissues in the study of neosporosis. Also this study provided practical data in support of the argument that cut-off point for the ELISA and IFAT should be re-evaluated. Western blot analysis provided the opportunity to evaluate fluctuation patterns of *N. caninum* antibodies in cows. The success in isolating a local strain of *N. caninum* in New Zealand opens a wide range of opportunities for research in neosporosis in New Zealand.

9.3 Future research

This study and its findings open new frontiers for future research. A logical advancement of knowledge would result from follow up studies. Serological data used in this study indicate that some cows may have seroconverted based on the ELISA. To better track the phenomenon of seroconversion, it would be useful to follow a set of seronegative and seropositive heifers with ELISA, IFAT, PCR and Western blot from preconception to post parturition. With all these assays in place, it would be possible to determine the true *N. caninum* status of a cow at all times. This would further tell us the point in time when a cow sero converts. Moreover, this would help in determining the specific period of parasitaemia in infected cows, the antibody response profile of cows and the differences in antibody response between infected pregnant and infected non-pregnant cows if any.

Western blot analysis of cow sera revealed an immunodominant antigen (45-46 kDA) that was seen across all seropositive samples. It may be helpful to characterise this antigen. By knocking down the gene that encodes this protein and studying its effect on *N. caninum* infection and the hosts, it may be possible to better understand the function(s) of this antigen (45-46 kDa).

In addition, it may be necessary to study the gene expression profiles of *N. caninum* seropositive-aborted, *N. caninum* seropositive-pregnant, *N. caninum* seronegative-aborted and *N. caninum* seronegative-pregnant cows using serial analysis of gene expression (SAGE). This would tell us about the genes of cows that are expressed (turned on) during infection with *N. caninum* at the different physiologic stages of the cow. If a gene is turned on, it may mean that this gene interacts with *N. caninum* and could be insightful in genetic manipulation of the parasite. A comparison of the gene expression profiles of *N. caninum* in naturally infected and experimentally infected heifers may help illustrate if there is any difference in gene expression profiles.

Finally, it may be necessary to analyse *N. caninum* genes involved with stage conversion/differentiation from tachyzoites to bradyzoites by examining gene expression patterns in bradyzoite and tachyzoite stages using microarrayed cDNAs. This study would help in better understanding the antigens of *N. caninum* that are

expressed during the different stages in its life cycle. If this is known, specific vaccines could be made targeting these life cycle stages to prevent infections during each infectious stage. Again, this would help in developing diagnostic tests to differentiate between chronic and acute infections of the host as the case may be.

In conclusion, the effort to study neosporosis in New Zealand cattle has led to the isolation of *N. caninum* in New Zealand and many other fascinating findings. Consequently, many opportunities for research have arisen from the need to extend the findings and overcome constraints in this study.

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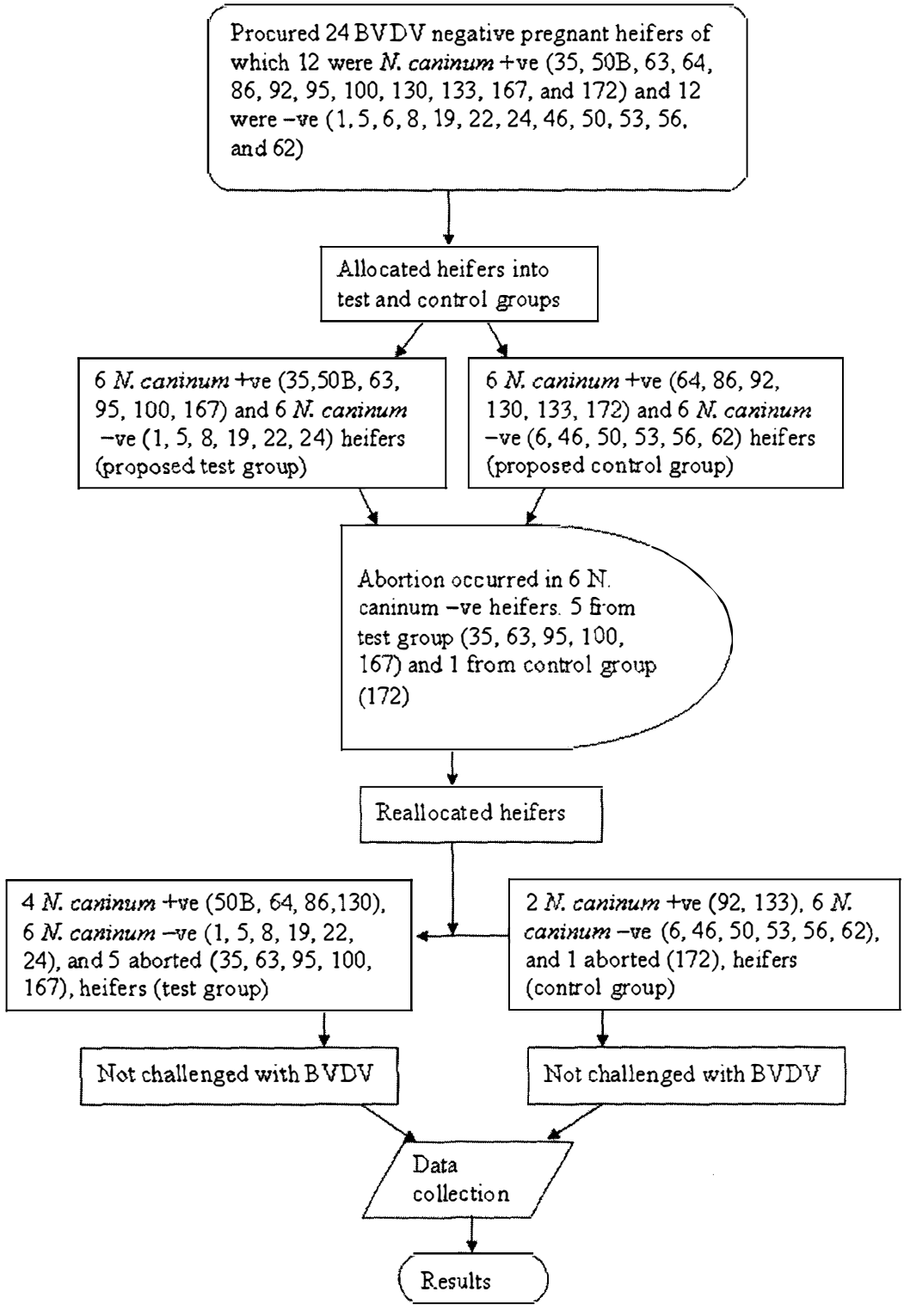
Appendices

Appendix 1: Results of BVDV antibody ELISA from arrival through to week 15 of experimental observation

Heifer ID	Physiologic status	BVDV antigen ELISA tested 3 weeks before arrival	Challenge Status	BVDV antibody ELISA												
				3 weeks before arrival	On arrival	Week 1	week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 9	Week 11	Week 13	Week 15
1	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	N/A
5	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Pos	Pos	Pos	Pos	N/A
6	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	N/A
8	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Sus	Sus	Pos	Pos	N/A
19	Pregnant	Neg	Challenged	Neg	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	N/A
22	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Pos	Pos	Pos	Pos
24	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos
46	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	N/A
50	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	N/A
53	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Neg	Neg	Neg	Pos	N/A
56	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg
62	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Sus	Neg	Sus	Neg	Neg	Neg	Neg	N/A
50B	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Pos	Sus	Neg	Pos
64	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Sus	Sus	Sus	Pos	N/A
92	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	N/A
86	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Sus	Neg	Pos	Pos	Pos
130	Pregnant	Neg	Challenged	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Sus	Sus	Pos	Pos	Pos	N/A
133	Pregnant	Neg	N/C	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Pos	N/A
35	Aborted	Neg	Challenged	Neg	Neg	Pos	Sus	Neg	Neg	Neg	Neg	Neg	Sus	Sus	Pos	Pos
63	Aborted	Neg	Challenged	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Sus	Pos	Pos	Pos
95	Aborted	Neg	Challenged	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Sus	Pos	Pos	Pos
100	Aborted	Neg	Challenged	Neg	Neg	Pos	Sus	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos
167	Aborted	Neg	Challenged	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Pos	Pos
172	Aborted	Neg	N/C	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Sus	Neg	Neg	Neg	Neg	Neg

Appendix 2: Flow chart of original experimental design before the occurrence of abortions and subsequent redesign of the experiment



Appendix 3: Result of *N. caninum* IFAT and Blood PCR assays on heifers (n = 24).

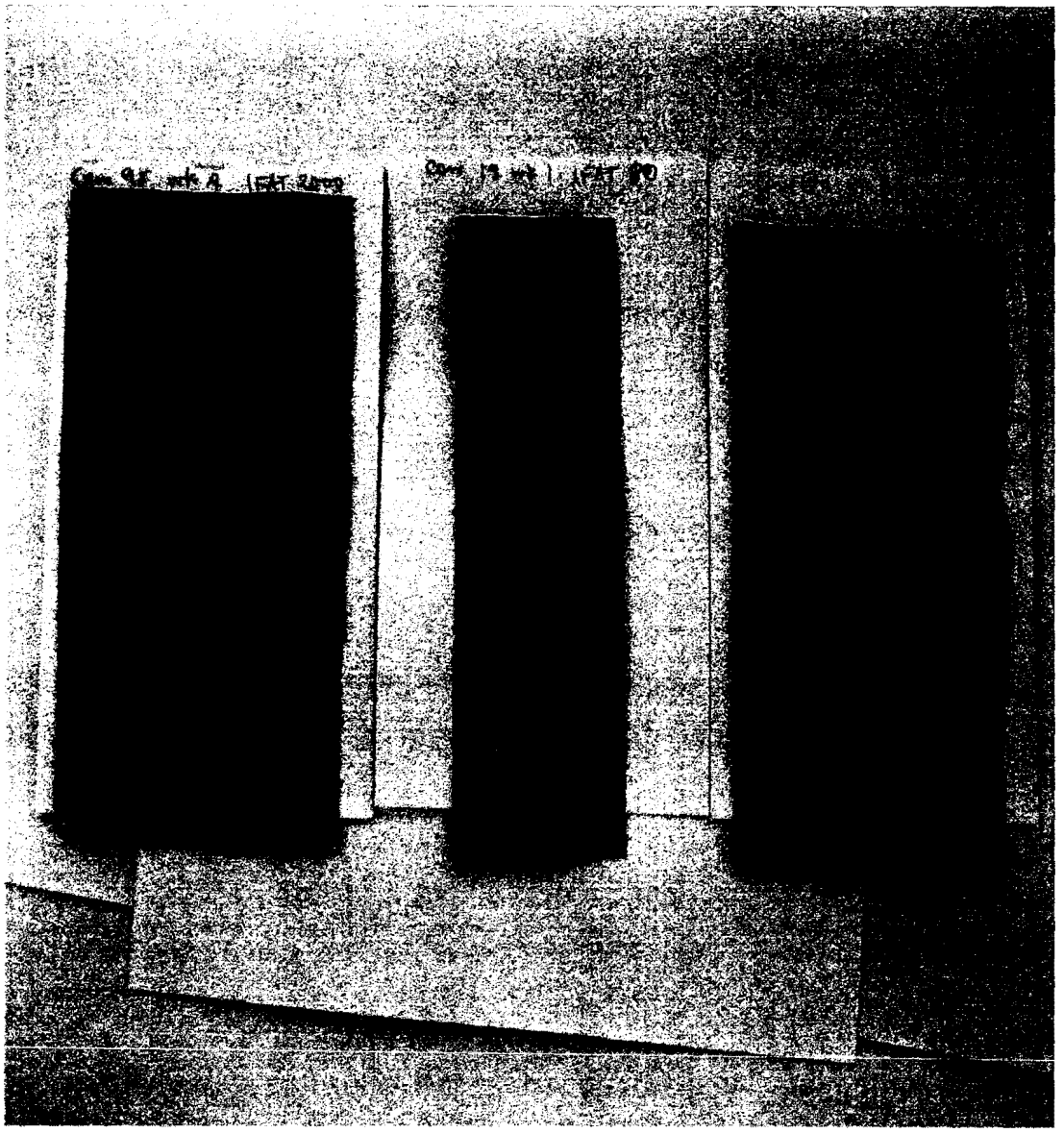
	PRE-EXPERIMENTAL STATUS		EXPERIMENTAL STATUS: POST-ABORTION AND PRE-PARTURITION									
			WEEK 1		WEEK 2		WEEK 3		WEEK 4		WEEK 5	
ID			IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR
1	NEG	PREG	2000	POS	6000	POS	80	POS	600	POS	200	POS
5	NEG	PREG	80	POS	80	POS	80	POS	80	POS	80	POS
6	NEG	PREG	0	NEG	2000	POS	80	POS	80	POS	0	NEG
8	NEG	PREG	80	NEG	2000	POS	80	POS	80	POS	80	NEG
19	NEG	PREG	80	NEG	80	NEG	80	NEG	80	NEG	0	NEG
22	NEG	PREG	0	NEG	1000	POS	80	POS	80	POS	0	NEG
24	NEG	PREG	0	NEG	600	NEG	0	NEG	2000	POS	80	NEG
46	NEG	PREG	0	NEG	80	NEG	0	NEG	0	NEG	80	NEG
50	NEG	PREG	0	NEG	80	NEG	0	NEG	0	NEG	0	NEG
53	NEG	PREG	0	NEG	200	NEG	0	NEG	N/A	N/A	0	NEG
56	NEG	PREG	0	NEG	80	NEG	80	NEG	2000	POS	0	NEG
62	NEG	PREG	80	NEG	1000	POS	80	POS	600	POS	80	POS
50B	POS	PREG	0	POS	1000	POS	80	POS	0	POS	80	POS
64	POS	PREG	0	POS	2000	POS	2000	POS	600	POS	2000	POS
86	POS	PREG	1000	POS	1000	POS	2000	POS	600	POS	2000	POS
92	POS	PREG	80	POS	0	POS	80	POS	600	POS	80	POS
130	POS	PREG	0	POS	80	POS	80	POS	80	POS	0	NEG
133	POS	PREG	200	POS	600	POS	600	POS	80	POS	200	POS
35	POS	ABOR	200	POS	600	POS	600	POS	600	POS	600	POS
63	POS	ABOR	200	POS	600	POS	600	POS	600	POS	1000	POS
95	POS	ABOR	200	POS	600	POS	80	POS	2000	POS	600	POS
100	POS	ABOR	200	POS	1000	POS	200	POS	600	POS	1000	POS
167	POS	ABOR	200	POS	600	POS	80	POS	600	POS	1000	POS
172	POS	ABOR	2000	POS	600	POS	0	POS	600	POS	600	POS

Appendix 3: Contd.

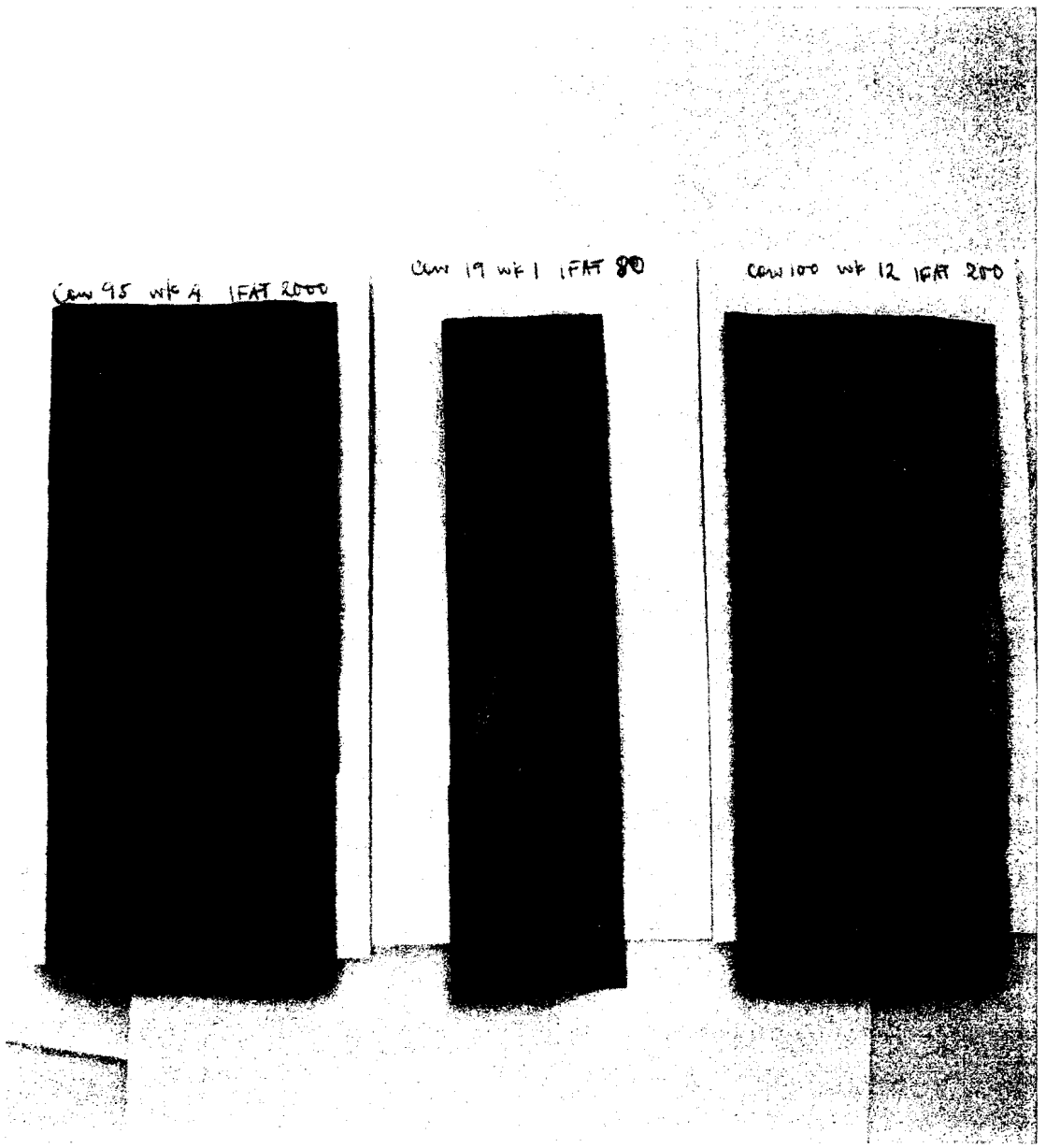
	PRE-EXPERIMENTAL STATUS		EXPERIMENTAL STATUS: POST-ABORTION AND PRE-PARTURITION									
			WEEK 6		WEEK 7		WEEK 9		WEEK 11		WEEK 13	
ID			IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR	IFAT, 1:80	PCR
1	NEG	PREG	600	NEG	80	NEG	0	NEG	0	POS	80	POS
5	NEG	PREG	80	POS	80	NEG	80	NEG	80?	NEG	200	POS
6	NEG	PREG	1000	NEG	80	POS	80	POS	0	POS	600	POS
8	NEG	PREG	0	NEG	0	POS	80	POS	0	POS	600	POS
19	NEG	PREG	80	NEG	0	NEG	0	NEG	0	NEG	600	POS
22	NEG	PREG	80	NEG	80	POS	0	POS	0	POS	80	POS
24	NEG	PREG	80	NEG	80	POS	0	POS	0	POS	600	POS
46	NEG	PREG	600	NEG	0	POS	80	POS	0	POS	0	POS
50	NEG	PREG	1000	NEG	80	NEG	0	NEG	0	NEG	80	NEG
53	NEG	PREG	200	NEG	0	POS	0	POS	0	POS	0	POS
56	NEG	PREG	2000	POS	0	NEG	0	POS	0	POS	80	POS
62	NEG	PREG	1000	POS	80	POS	80	POS	0	POS	80	POS
50B	POS	PREG	2000	POS	80	POS	80	POS	80	POS	80	POS
64	POS	PREG	2000	POS	2000	POS	2000	POS	2000	POS	2000	POS
86	POS	PREG	2000	POS	2000	NEG	2000	POS	2000	POS	2000	POS
92	POS	PREG	600	POS	0	NEG	80	NEG	0	POS	2000	POS
130	POS	PREG	0	NEG	0	NEG	80	NEG	0	POS	0	POS
133	POS	PREG	600	POS	200	NEG	600	NEG	200	POS	600	POS
35	POS	ABOR	600	POS	600	POS	600	POS	600	NEG	600	NEG
63	POS	ABOR	2000	POS	1000	NEG	600	POS	600	NEG	2000	NEG
95	POS	ABOR	600	POS	600	NEG	600	NEG	200	NEG	1000	NEG
100	POS	ABOR	1000	POS	1000	NEG	1000	POS	1000	NEG	600	NEG
167	POS	ABOR	600	POS	1000	NEG	600	NEG	600	NEG	600	NEG
172	POS	ABOR	80	POS	2000	NEG	600	NEG	600	NEG	600	NEG

	PRE-EXPERIMENTAL STATUS		EXPERIMENTAL STATUS: POST-ABORTION AND PRE-PARTURITION			
			WEEK 15		WEEK 16	
ID			IFAT, 1:80	PCR	IFAT, 1:80	PCR
1	NEG	PREG	80	POS	N/A	N/A
5	NEG	PREG	80	POS	N/A	N/A
6	NEG	PREG	80	POS	N/A	N/A
8	NEG	PREG	80	POS	N/A	N/A
19	NEG	PREG	80	NEG	N/A	N/A
22	NEG	PREG	200	POS	80	POS
24	NEG	PREG	80	POS	1000	POS
46	NEG	PREG	80	POS	N/A	N/A
50	NEG	PREG	N/A	N/A	N/A	N/A
53	NEG	PREG	N/A	N/A	N/A	N/A
56	NEG	PREG	200	POS	600	POS
62	NEG	PREG	80	POS	N/A	N/A
50B	POS	PREG	80	POS	600	POS
64	POS	PREG	1000	POS	N/A	N/A
86	POS	PREG	2000	POS	2000	POS
92	POS	PREG	0	NEG	N/A	N/A
130	POS	PREG	0	N/A	N/A	N/A
133	POS	PREG	N/A	N/A	N/A	N/A
35	POS	ABOR	600	NEG	600	NEG
63	POS	ABOR	1000	NEG	1000	NEG
95	POS	ABOR	600	NEG	1000	NEG
100	POS	ABOR	1000	NEG	1000	NEG
167	POS	ABOR	1000	NEG	600	NEG
172	POS	ABOR	600	NEG	600	NEG

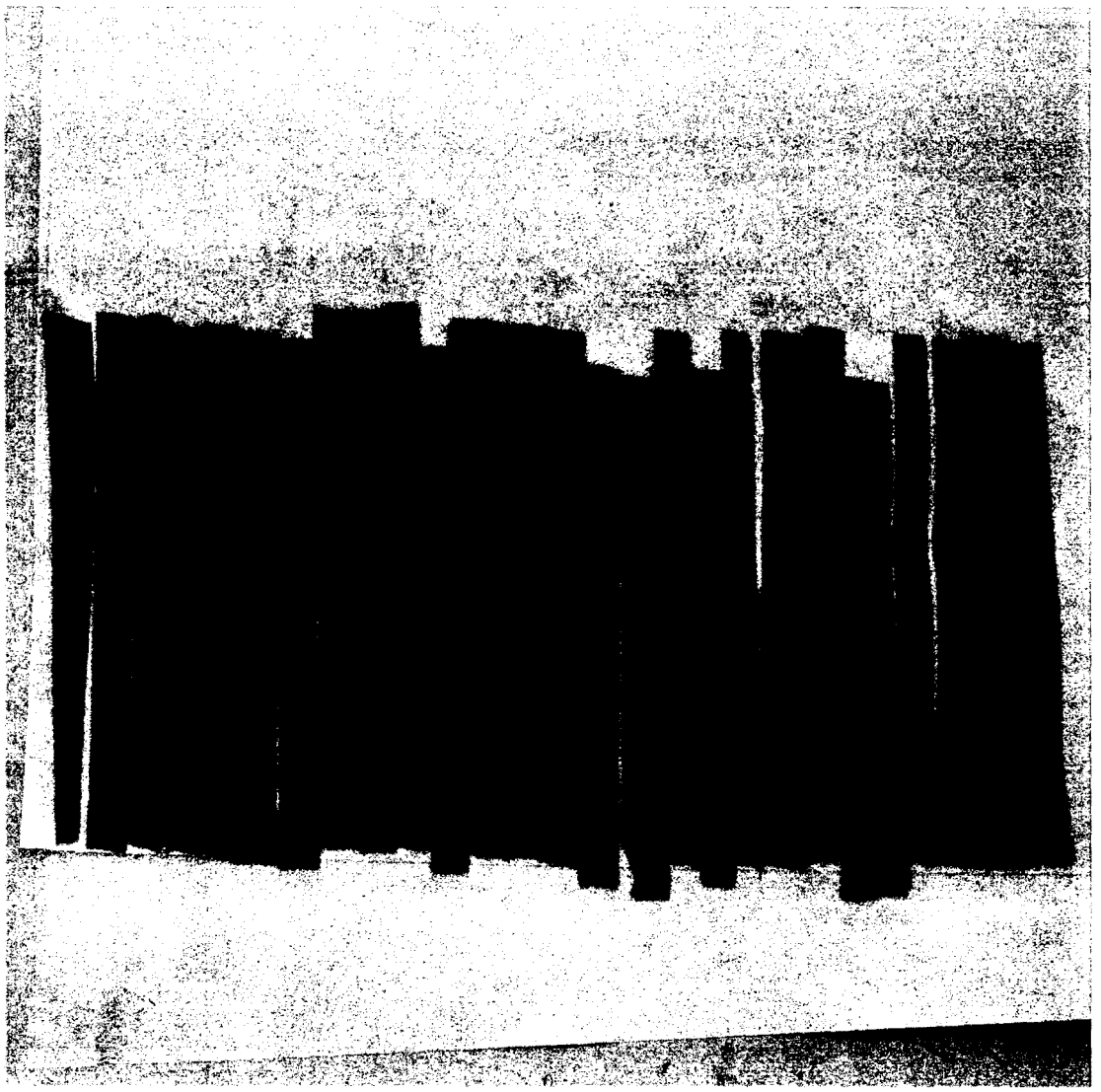
Appendix 4a: Initial Western blot images before assay optimisation. Photo taken with a digital camera after about 20 minutes exposure.



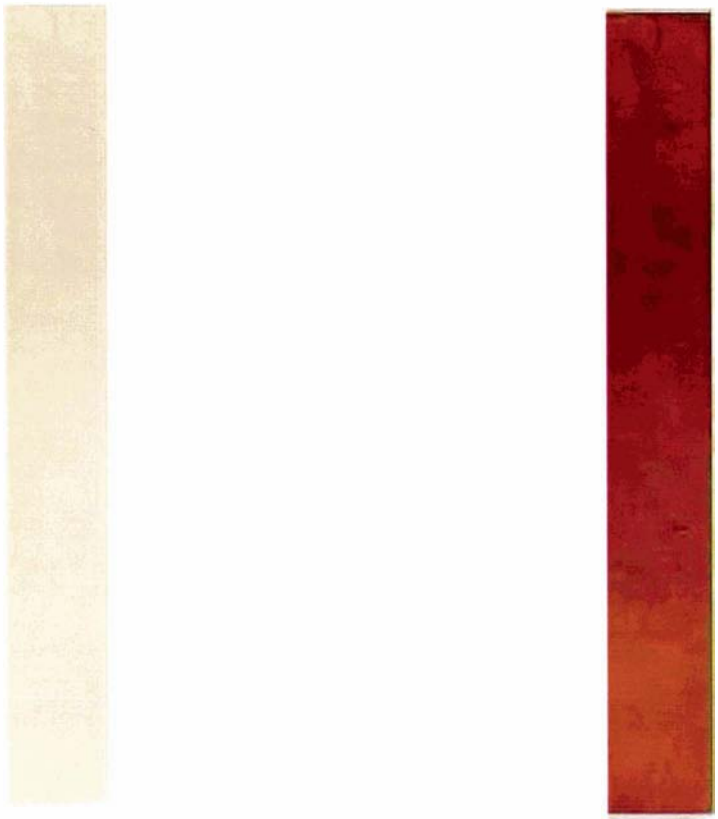
Appendix 4b: Initial Western blot images before assay optimisation. Photo taken with a digital camera after about 15 minutes exposure.



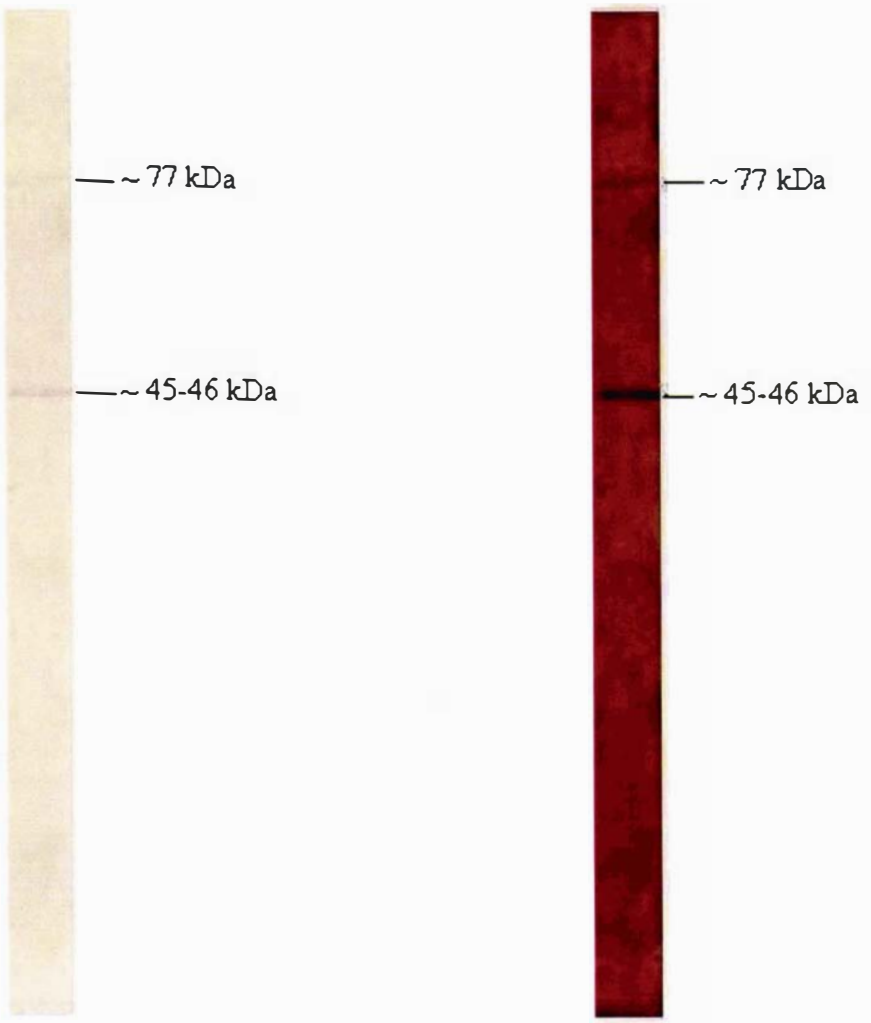
Appendix 4c: A sample of blot images cut in strips. Optimisation assay



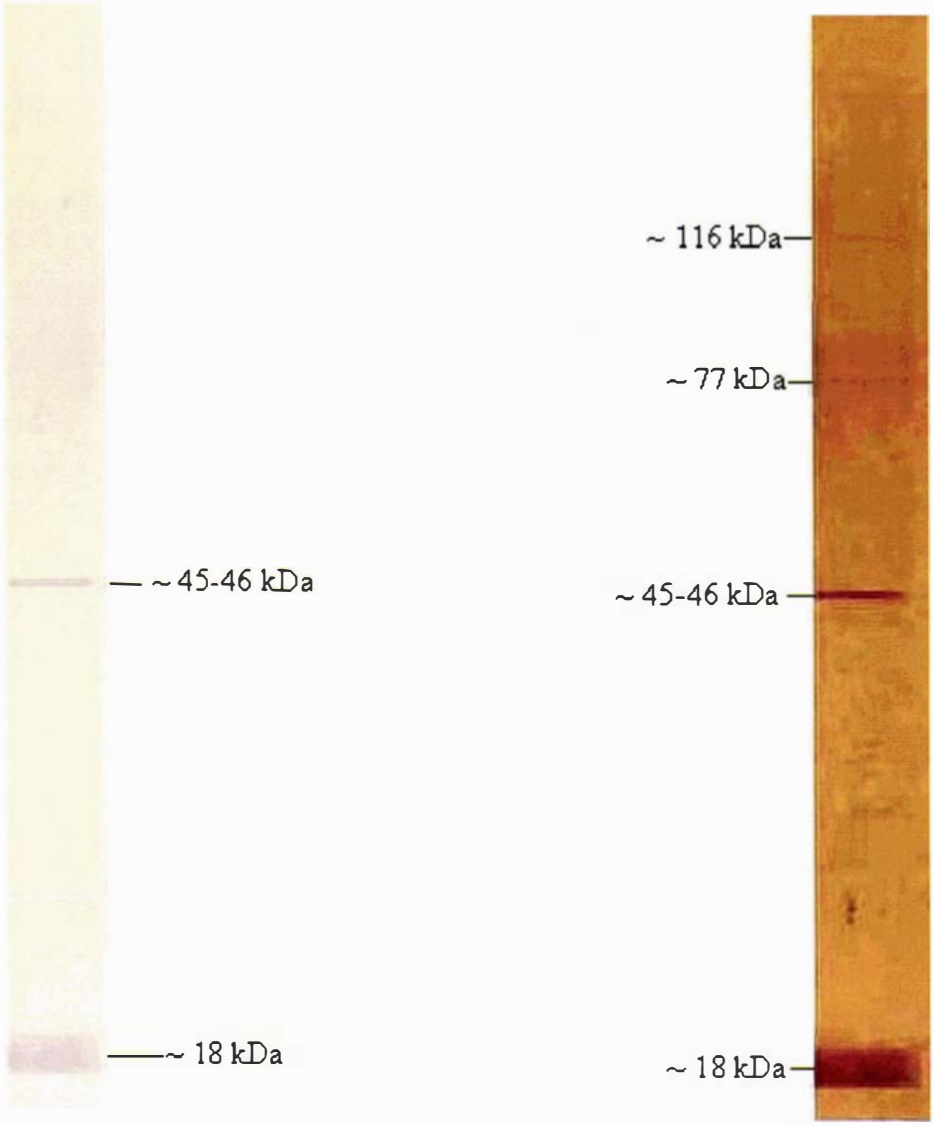
Appendix 5: Strips of Western blot membranes. Images on the left without "automatic balance". Images on the right are formatted with "automatic balance". Automatic balance is a feature in Microsoft photo editor.



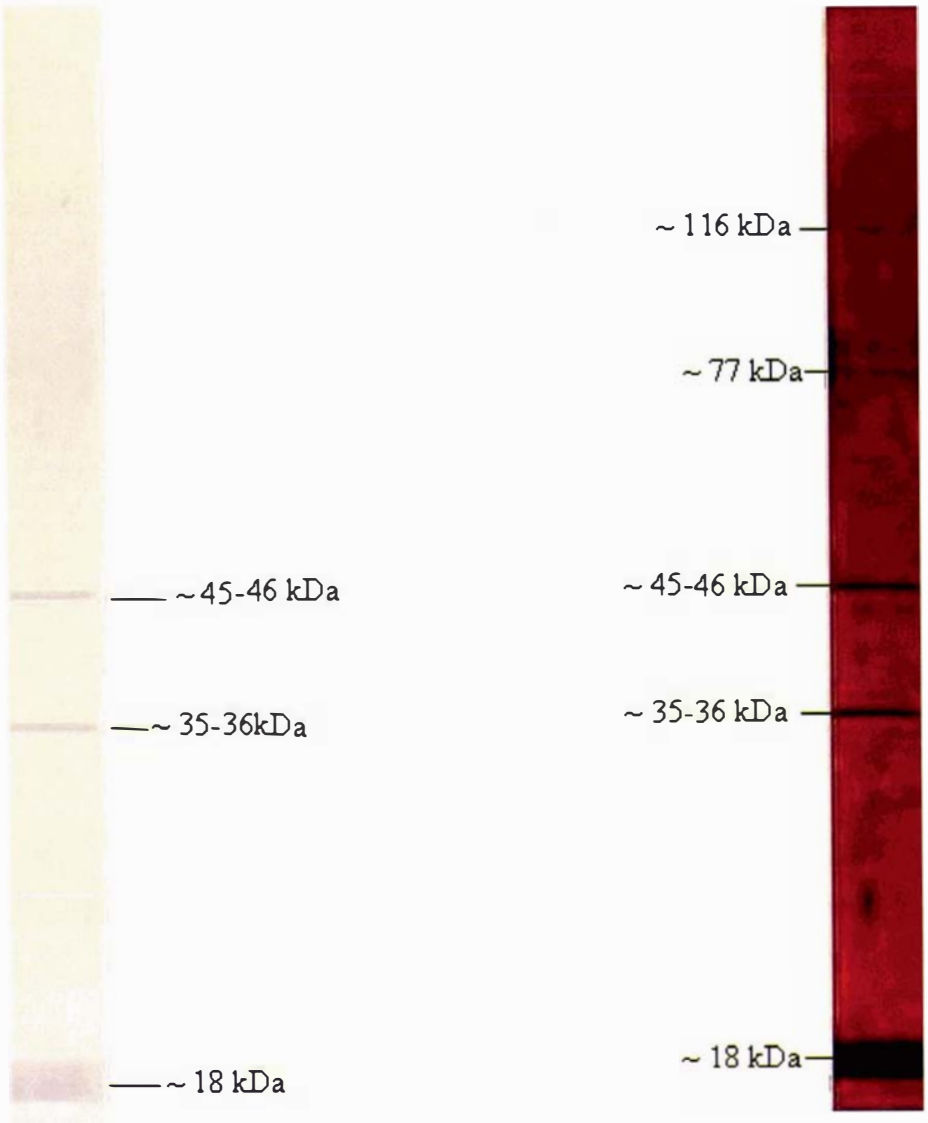
Recognition pattern of *N. caninum* antigen by cow IgG at IFAT <1:80



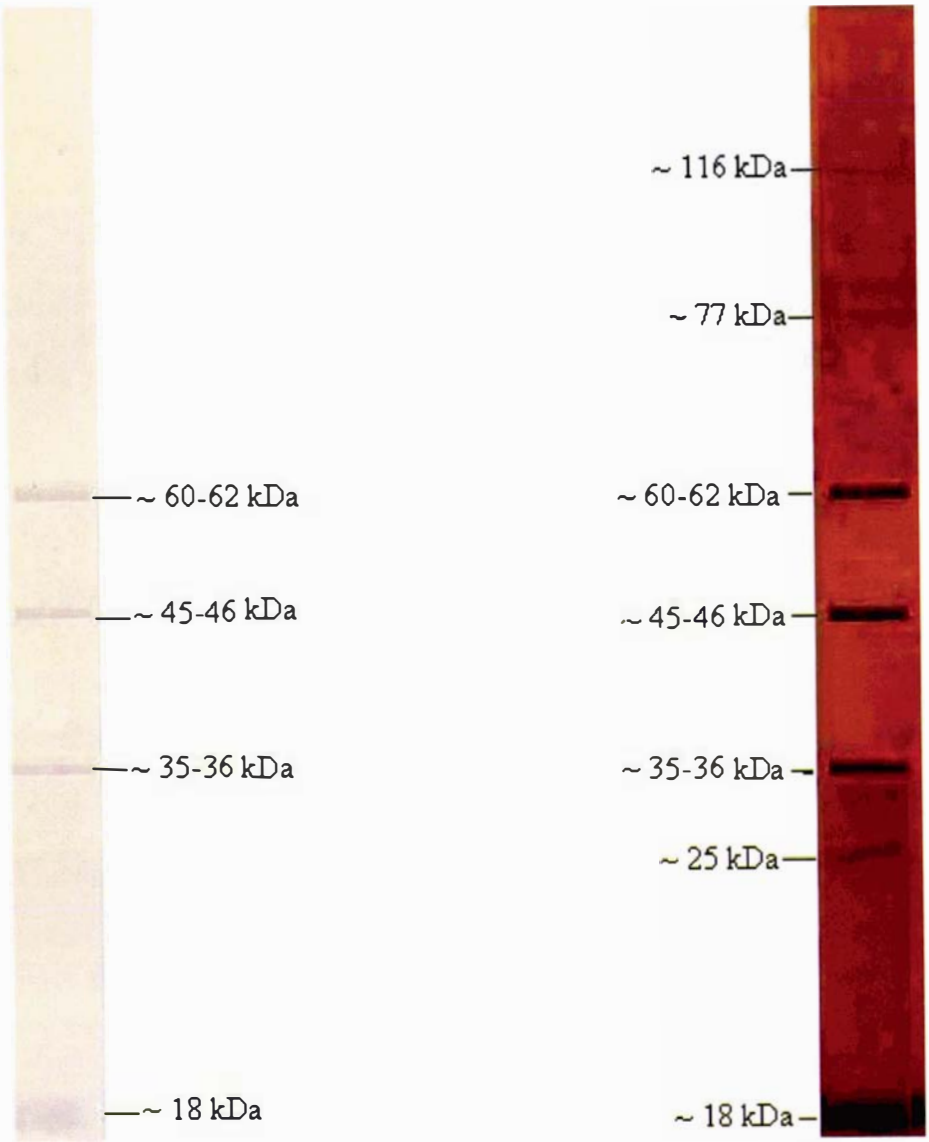
Recognition pattern of *N. caninum* antigen by cow IgG at IFAT 1:80



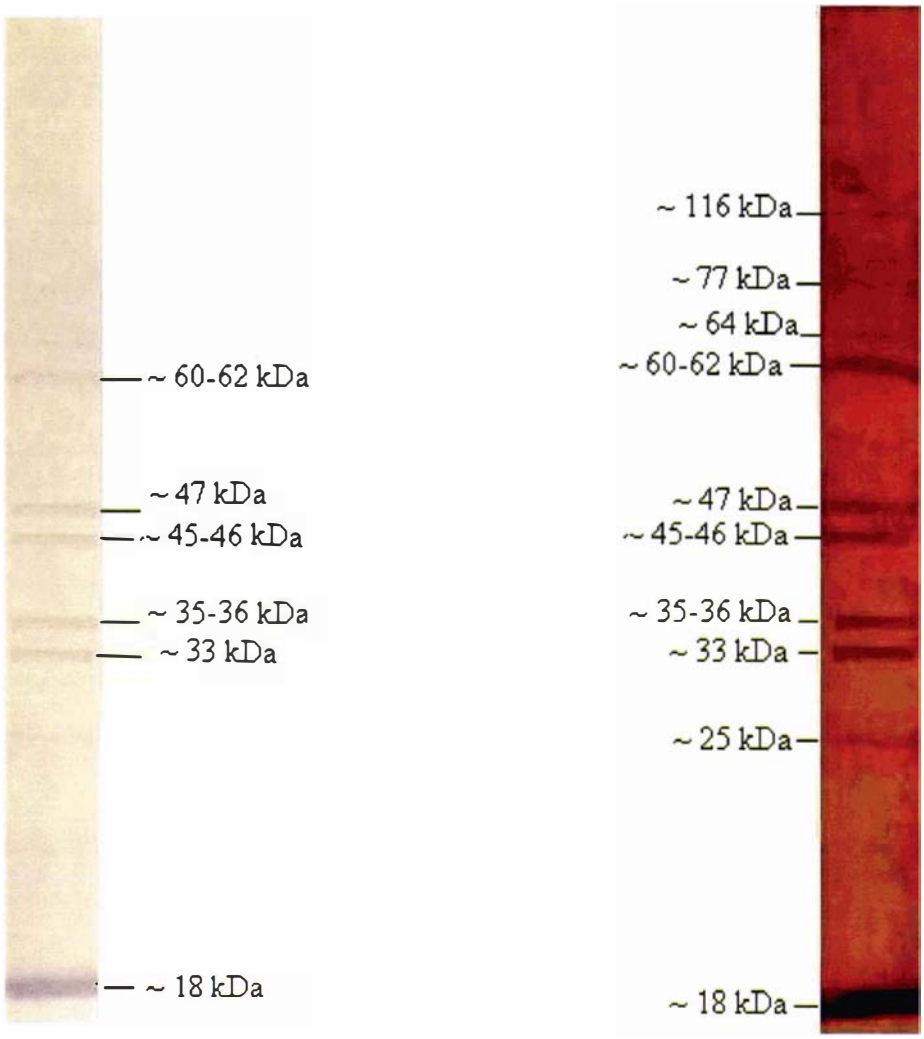
Recognition pattern of *N. caninum* antigen by cow IgG at IFAT 1:200



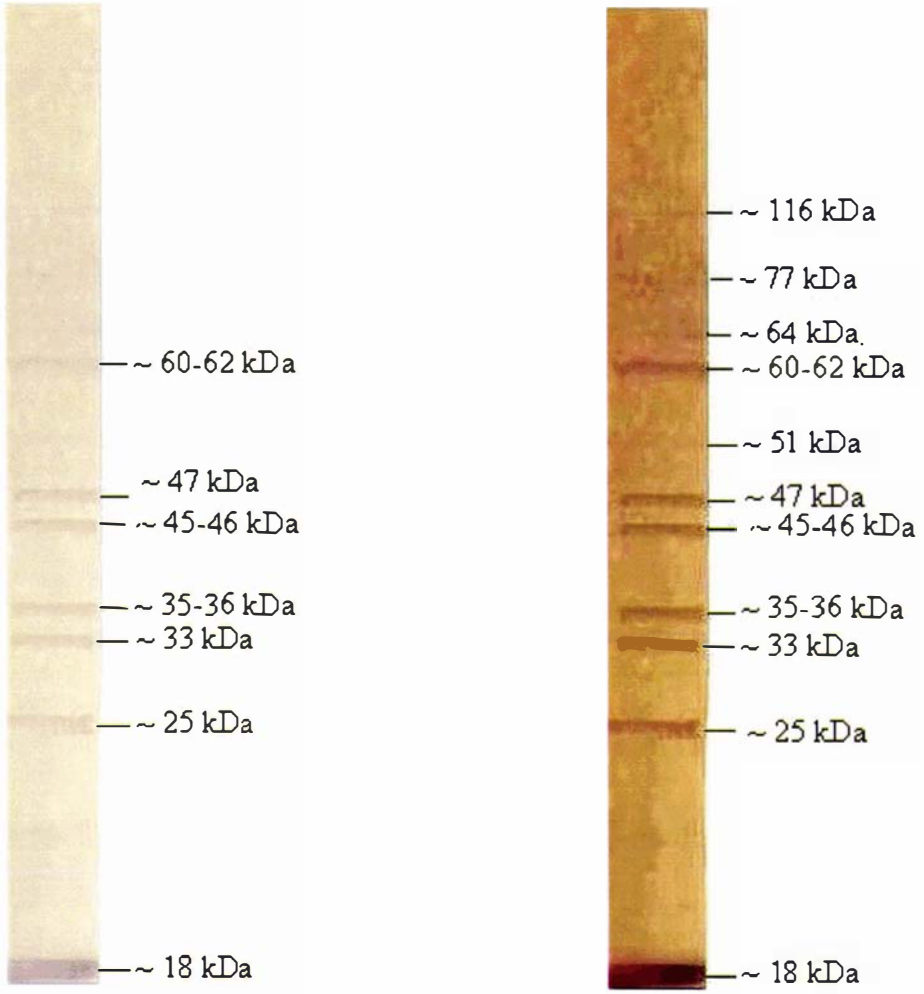
Recognition pattern of *N. caninum* antigen by cow IgG at IFAT 1:600



Recognition pattern of *N. caninum* antigen by cow IgG at IFAT 1:1000



Recognition pattern of *N. caninum* antigen by cow IgG at IFAT 1:2000



Recognition pattern of *N. caninum* antigen by cow IgG at IFAT >1:2000