Neospora caninum: Studies toward isolation in New Zealand

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Abstract

Background: *Neospora caninum* is a parasite that causes disease, largely in cattle and dogs. It is a disease of significant interest within New Zealand due to its association with bovine abortion. The economic impact of bovine abortion justifies the development of a bovine vaccine against *N. caninum*.

Aim: To develop and optimise diagnostic procedures for the detection of *Neospora* from a variety of blood and tissue samples and to isolate a New Zealand strain of *Neospora caninum.*

Methods: A local strain of *Toxoplasma gondii* and an imported *Neospora caninum* strain, Nc-Liverpool, were used to optimise tachyzoite growing conditions in bovine endothelial (BE) cells and Vero host cell cultures. A serum study using 112 tissue culture flasks was performed to determine whether foetal bovine serum or horse serum supplemented media provided the optimal growing conditions for Nc-Liverpool tachyzoites. Nc-Liverpool tachyzoites were also used to determine the optimal growth period between passage, and harvest for cryopreservation and cryopreservation conditions. Percoll gradients were also tested using Nc-Liverpool tachyzoites.

A known *Neospora* positive canine sample and murine tissues infected with *Toxoplasma,* were used during the development of the immunohistochemical diagnostic technique. Antibody concentrations and incubation temperatures were tested to reduce cross-reactivity and increase specific stain intensity. Immunohistochemistry was performed on sections of all tissue samples used for *N. caninum* isolation and experimentally infected murine tissue.

Several PCR techniques were developed, the final PCR used being a combination of the different techniques, which produced a 250kb band. PCR-3 used the NF6/GA1 primer combination for *Neospora* detection and TF6/GA1 for *Toxoplasma* detection, additional Mg^{2+} and an annealing temperature of 55°C were required. Whole tissue was processed via DNA elution whereas cell culture and Percoll purified tachyzoites were used following crude lysis techniques. All bovine and canine tissues used for parasite isolation as well as all experimentally infected mouse tissues were tested for *N. caninum* using PCR.

An immunoblot technique was developed for the detection of *N. caninum* antibodies in murine blood samples. Lysed Nc-Liverpool tachyzoites were used as antigen with varied results. The primary and secondary antibodies were commercially available and used at concentrations of 1:1,000 and 1:25,000 respectively.

BALB/c and CF1 mice were experimentally infected with *Toxoplasma gondii* and Nc-Liverpool. Forty female BALB/c and 40 female CF1 mice were used in 2 studies to determine the optimal Nc-Liverpool inoculation dose and immunosuppression requirements. Mice were immunosuppressed with 2.5mg of methylprednisolone acetate (MPA) and Nc-Liverpool inoculation ranged from $1.3x10^6$ to $5x10^3$ tachyzoites. Upon death, the brain and blood was harvested from the mouse carcases.

Attempts were made to isolate a New Zealand strain of *N. caninum* from bovine and canine central nervous system (CNS) tissue, and to maintain the parasites in cell culture and by small animal passage, in order to attenuate the parasite strain for use as a live large animal vaccine. Twenty one bovine tissue samples were used for *N. caninum* isolation attempts, 18 of which were positive for *Neospora* antibodies using a commercial IFAT. Isolation tissues were purified using a 30% Percoll

gradient and inoculated onto 8 cell culture flasks and into 8 immunosuppressed mice (BALB/c and CF1).

Results: Nc-Liverpool tachyzoites were found to be viable when grown at 37°C in antibiotic-MEM supplemented with either FBS or ES and grew optimally in FBS despite *Neospora* antibodies being detected using an IFAT. Passaging cultures at approx. 4 day intervals resulted in the greatest parasite growth. However, cryopreserved parasites should be harvested 2 days post inoculation (PI) for optimal viability. Viable parasites could be isolated using a 30% Percoll gradient and centrifuged at 2,700 x g (3,400 rpm) in a bucket centrifuge for 10 minutes.

Tissue cysts could be detected using immunohistochemistry but some degree of cross reaction remained despite optimisation. Cysts were not found in tissues used for isolation attempts or in mouse brains following inoculation with Nc-Liverpool, however cysts were commonly found in mice experimentally infected with *T. gondii* tachyzoites.

PCR-3 was successfully used to detect *N. caninum* and *T. gondii* infected tissue and tachyzoites from tissue culture. PCR-3 could detect *N. caninum* DNA in the brain tissue of 9/24 mice experimentally infected with Nc-Liverpool, even though most mice were culled within 1 week.

Although production of *N. caninum* antigen was only moderately successful, *N. caninum* antibody detection in mouse blood using one specific antigen batch was reliable and specific. The immunoblot could only detect *N. caninum* antibody approximately 14 days PI, but was sensitive enough to detect 100% of mice experimentally infected with Nc-Liverpool tachyzoites. PCR-3 strongly correlated with the immunoblot results from 14 days PI.

BALB/c mice were found to be far more sensitive to Nc-Liverpool than CF1 mice and developed severe disease at concentrations of approximately $1x10^6$ Nc-Liverpool tachyzoites. Neither BALB/c nor CF1 mice developed peritoneal exudate, irrespective of the parasite inoculation concentration.

Despite *Neospora* DNA being present in the brains of experimentally infected mice, re-isolation and continuous parasite passage from the brains could not be achieved. No mice experimentally infected with either Nc-Liverpool or isolation attempts were found to have brain cysts when tested using immunohistochemistry. Only 1 mouse inoculated with bovine isolation material was found to have a *Neospora* positive PCR.

Through the detection of DNA, antigens and antibodies, parasites were determined to have been present in 10 of the 18 IFAT positive bovine isolation samples, indicating that 55% of calves born to seropositive dams were infected with *N. caninum*.

However, despite numerous attempts to isolate *Neospora* parasites from naturally infected canine and bovine tissue and culturing using the optimised Nc-Liverpool technique, maintenance of a live culture of a New Zealand strain of *N. caninum* could not be established.

Conclusions: Findings from this study could be used to assist in the maintenance of *Neospora caninum* and *Toxoplasma gondii* parasite strains and for detection or diagnosis of these parasites in host tissues.

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Abbreviations

µMT	Antibody knock out
ABC	Avidin–biotin enzyme complex
ABPC	Avidin-biotin-peroxidase complex
AEC	3-amino-9-ethylcarbazole
ATV	Antibiotic trypsin-versene
BAG	Bradyzoite antigen
BE	Bovine endothelial
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
CMI	Cell-mediated immune
CNS	Central nervous system
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocytes
DAB	3-3'- diaminobenydine tetrahydrochloride
D-MEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribose nucleic acid
DPX	Distyrene plasticiser tyrene mounting medium
ELISA	Enzyme-linked immunosorbent assay
ES	Equine serum
FBS	Foetal bovine serum
gamma IFN-KO	Gamma interferon knock out
GST	Glutathione S-transferase
H&E	Haemotoxylin and eosin
HMAR	Heat mediated antigen retrieval
HMI	Heavy metal intensifier
HRP	Horseradish peroxidase
HS	Horse serum
ICC	Immunocytochemistry
ICT	Immunochromatographic test
IFN-γ	Interferon gamma
IgG1	Immunoglobulin G1
IHC	Immunohistochemistry
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
ITS	Internal transcribed spacer
IV	Intravenous
LFAT	Indirect fluorescent antibody test
LSU	Large subunit
MEM	Minimum essential medium
MPA	Methylprednisolone acetate
NAT	Neospora agglutination test
Nc	Neospora caninum
NC	Neospora caninum
NcSAG1	Neospora caninum surface antigen 1

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Post-inoculation
PVDF	Polyvynilidine difluoride
RIT	Rapid immunochromatographic test
rpm	Revolutions per minute
SC	Subcutaneous
SHP	Streptavidin-horseradish-peroxidase
TCNF – α	Tumour necrosis factor alpha
TEM	Transmission electron microscope
Th	T-helper
Th1	T-lymphocyte type 1
Th2	T-lymphocyte type 2
TNF - β	Tumour necrosis factor beta
UV	Ultraviolet
VMRA	Veterinary Medical Research and Development

Chapter 1.

1.0 Literature Review

1.1 The Veterinary Importance of *Neospora caninum*

Neospora caninum is an intracellular protozoan parasite of the family Sarcocystidae in the phylum Apicomplexa (Dubey et al., 1988a; Ellis et al., 1994). Structurally it closely resembles *Toxoplasma gondii,* but is antigenically different (Bjerkas and Presthus, 1988). *Neospora caninum* causes neosporosis, resulting in stillbirth and abortion in cattle and neuromuscular disease in dogs.

1.2 Historical background

Neospora caninum was first detected in Norway, where Bjerkas et al (1984) reported an unidentified cyst-forming sporozoan, which caused encephalomyelitis, myositis and lameness in a Boxer dog litter. Later, in 1987, *N. caninum* was identified in calves that were recumbent and unable to rise and were diagnosed with protozoan myeloencephalitis following necropsy (O'Toole and Jeffrey, 1987; Parish et al., 1987). A description of the new *Neospora* genus and species *N. caninum* was given following the first isolation in the USA and the parasite was named (Dubey et al., 1988a; Dubey et al., 1988b). Prior to this it is likely that neosporosis had been misdiagnosed as toxoplasmosis. Experimental infection of a litter of puppies with parasites from cell culture resulted in definitive identification of *N. caninum* as a causative agent of neurological disorder, paresis, paralysis and death. Diagnoses of neosporosis could be confirmed in 1989 when polyclonal antisera against *N. caninum* tachyzoites were raised in rabbits and were used for the immunohistochemical detection of parasite infected tissue sections (Lindsay and Dubey, 1989a). Around 1990 *N. caninum* was revealed to be associated with bovine abortion storms (Anderson et al., 1991; Barr et al., 1991a; Thilsted and Dubey, 1989) and became recognized globally as an economically important disease affecting the livestock industry (Dubey and Lindsay, 1993, 1996).

1.3 Discovery of *Neospora caninum* in dogs

In 1988 J.P Dubey reviewed case histories of 23 dogs diagnosed as having *T. gondii* and found that 10 had been infected with *Neospora caninum*. *N. caninum* was described to have infected host cell cytoplasm with a high incidence of infection in the brain and spinal cord. The parasites did not react with anti-*T. gondii* serum in immunoperoxidase tests. Based on this and differences in ultrastructure and cyst wall thickness, it was concluded that *N. caninum* was a different protozoan and it was named as a new genus.

Cummings et al. (1988) described a Labrador Retriever that whelped a litter of 8 puppies in which 5 had severe polyradiculoneuritis associated with a *Neospora*-like organism. Tissue isolates from subsequent litters of puppies, which had developed hind leg paresis shortly after birth, were used to inoculate cell cultures, mice and dogs. Antibodies to *N. caninum* were detected in sera of infected dogs and mice using an IFAT. *Neospora caninum* was successfully isolated and cultivated in cell culture within six months of its recognition as a new protozoan genus (Dubey et al., 1988a).

1.4 The biology of *Neospora caninum*

1.4.1 Morphology and Ultrastructure

Two asexual stages have been identified for *N. caninum*. These are the tachyzoite and the bradyzoite stages. The structures of the tachyzoites, bradyzoites and cysts are similar to those of *Toxoplasma gondii* and other closely related apicomplexan parasites (Speer et al., 1999). Within the host, tachyzoites and bradyzoites reside within a parasitophorous vacuolar membrane (PVM), which is originally derived from the surface membrane of the host cell, but is modified by the parasite following invasion (Hemphill et al., 1996). This membrane allows *N. caninum* infection to persist in the host animal for years.

Tachyzoites are rapidly dividing parasites that can penetrate host cells by active invasion (Hemphill et al., 1996). Tachyzoites are 3-7 x 1-5 μm in size and are ovoid, lunate or globular depending on the stage of division (Dubey and Lindsay, 1996). Tachyzoites derived from infected tissue and cell culture are almost identical ultrastructurally (Speer and Dubey, 1989). They possess a three-layered plasma membrane, a pellicle consisting of a plasmalemma and an inner membrane complex, 22 subpellicular microtubules, a conoid, anterior and posterior polar rings, 6 to 16 electron dense rhoptries, numerous micronemes, a single vesicular nucleus, tubular mitochondria, Golgi complexes, ribosomes, endoplasmic reticula, an inactive micropore, lipid bodies, a posterior pore, plastid, polysomes, amylopectin bodies and dense granules, which are secreted following host cell invasion. Tachyzoites are frequently located adjacent to the host cell nucleus enclosed in a parasitophorous vacuole that contains many intravacuolar tubules. Tachyzoites divide by endodyogeny, which produces two daughter cells (Lindsay et al., 1993; Speer et al., 1999).

Bradyzoites are approximately 8.1 x 2 μm in size (6.5-10 x 1.5-2.5μm) appearing more slender than tachyzoites. Bradyzoites have fewer rhoptries (approximately 6-12) and more amylopectin granules (periodic acid Schiff (PAS) positive granules) than tachyzoites, which are homogenously electron dense. They contain micronemes (>40), which are oriented perpendicular to the zoite pellicle. The nucleus is located approximately 1.5 μm anterior to the posterior tip of the zoite. Micronemes, dense granules, small dense granules, amylopectin granules, endoplasmic reticulum and occasionally mitochondria are located in the cytoplasm between the posterior tip and the nucleus. Small electron-dense granules (120-170 nm) arise from the forming face of the Golgi complex and can be seen scattered throughout the cytoplasm. Numerous vesicles, some of which contain several smaller vesicles or are multiple-membrane bound, arise from the

maturation face of the Golgi complex. Micropores are rare in *N. caninum* bradyzoites (Barr et al., 1991b; Speer et al., 1999).

Tissue cysts can be observed in the cytoplasm of neurons and other neural cells. Cysts are generally round to elliptical and on occasion ovoid and irregular containing 20-100 bradyzoites. Cysts contain dividing zoites that produce progeny by endodyogeny. Cysts measure approximately 17 μm long and 14.5 μm wide (ranging between 5.4-27.2 x 6.0-31.2 μm). Small cysts have thinner regular cyst walls that contain densely packed organisms with little ground substance. Large cysts have a thicker, convoluted wall with more dispersed bradyzoites and abundant ground substance (Speer and Dubey, 1989). The primary cyst wall consists of a thinner, electron-dense primary outer cyst wall called the parasitophorous vacuolar membrane (PVM) and a thick inner granular layer. Thickness of the combined cyst walls ranged from 0.5-4.3 μm (Speer et al., 1999). The granular layer contains electron-dense granules and vesicles embedded in a finely granular matrix. Vesicles appear to pinch off from indentations of the PVM. Host cell endoplasmic reticulum is usually situated immediately above the PVM and may protrude into the invaginations. Tubular structures measuring 75-85 or 30-40 μm in thickness and 300-950 μm in length extend from the inner cyst wall and may be in direct contact with the plasmalemmae of bradyzoites. The cyst wall ensures that the cyst is enclosed in a chemically and physiologically stable environment. Tissue cysts consisted of a fine granular matrix containing tubules and vesicles 30-40nm in diameter filled with moderately electron dense material (Jardine, 1996; Speer et al., 1999).

The sexual life cycle of *N. caninum* results in the production of oocysts gametes from bradyzoites. Oocysts go on to sporolate within a few days to contain two sporocysts, each consisting of 4 sporozoites (McAllister et al., 1998).

Oocysts are spherical to subspherical measuring approximately 11.7 x 11.3 μm (10.6-12.4 μm x 10.6-12.0 μm). The wall of the oocyst is smooth and colourless measuring 0.6-0.8 μm thick (Lindsay et al., 1999c).

Sporocysts are ellipsoidal and approximately 8.4 x 6.1 μm (7.4-9.4 μm x 5.6-6.4 μm) in size. The sporocyst wall is smooth, colourless and is 0.5-0.6 μm thick. A sporocyst residuum is spherical or subspherical in shape and consists of a cluster of small compact granules 4.3 x 3.9 μm in size (3.2-5.2 μm x 2.6-4.4 μm) or many larger 1μm diameter dispersed granules (Lindsay et al., 1999c).

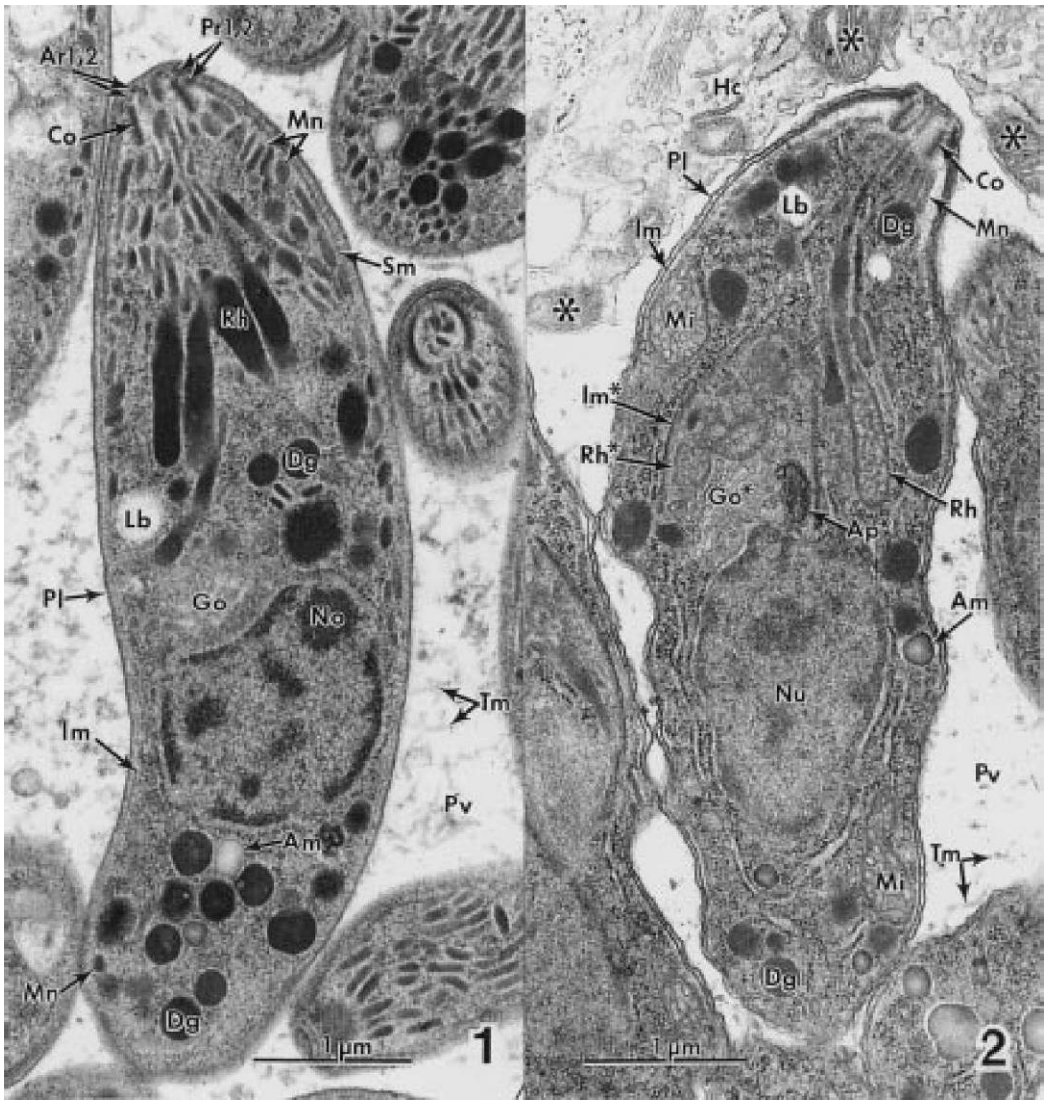
Sporozoites are elongate 6.5 x 2.0 μm (5.8-7.0 μm x 1.8-2.2 μm) in size and can appear slightly flattened on one side. The nucleus is located centrally or slightly posteriorly (Lindsay et al., 1999c).

Table 1.1: *Comparative ultrastructure of tachyzoites, tissue cysts, and bradyzoites of Neospora caninum and Toxoplasma gondii.*

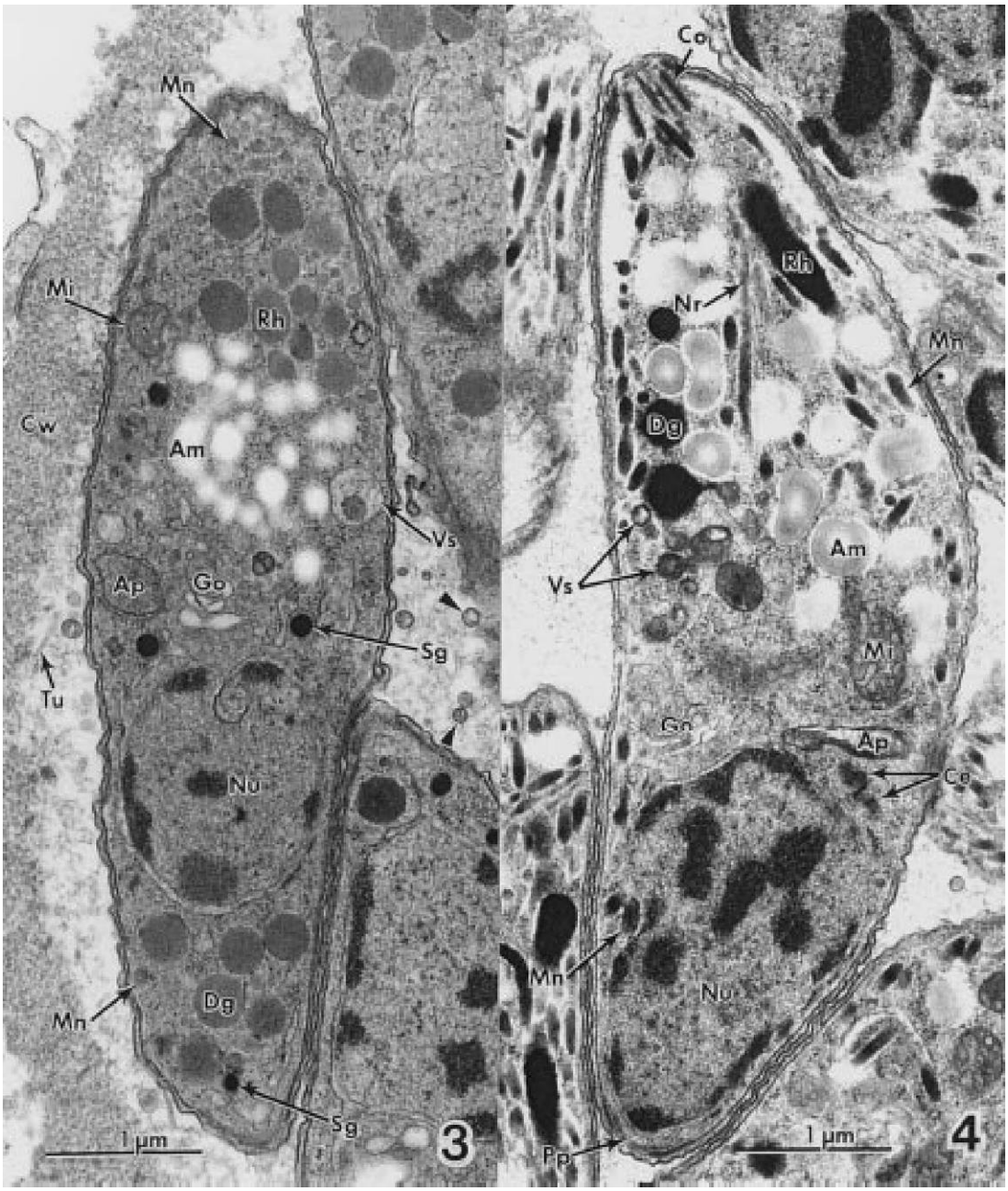
Table derived from (Speer et al., 1999)

Parasite stage/structure	*N. caninum*	*T. gondii*
Tachyzoite		
Anterior rhoptries[a]	Electron-dense, 6-16	Labyrinthine, 4-10
Posterior rhoptries[b]	Electron-dense	Rare
Looped-back rhoptries	Electron-dense, 1-2	Rare
Anterior micronemes	Many	Few
Posterior micronemes	Few	Rare
Anterior dense granules	Several	Several
Posterior dense granules	Several	Few
Micropores	Rare	Common
Tissue cyst wall		
Thickness	0.5-4 mm	≤0.5 mm
Contour	Irregular	Smooth
Bradyzoites		
Anterior rhoptries	Electron-dense, 6-12	Labyrinthine in young cysts, electron-dense in mature cysts, 6-8
Posterior rhoptries	Electron-dense, rare	None
Looped-back rhoptries	None	Electron-dense, 1-3
Anterior micronemes	Many	Many
Posterior micronemes	Few	Rare
Anterior dense granules	Several	Several
Posterior dense granules	Several	Rare
Small dense granules	4-8	None
Micropores	Rare	Common

[a]Rhoptries located anterior to the parasite nucleus.
[b]Rhoptries located posterior to the parasite nucleus.



Figures 1.1 & 1.2. *Transmission EM of tachyzoites of* <u>*N. caninum*</u> *and* <u>*T. gondii*</u>. Figures and Figure legend taken from (Speer et al., 1999). (Fig. 1) Tachyzoite of *N. caninum* NC-5 in a parasitophorous vacuole (PV) within a cultured M617 cell, showing various organelles and inclusion bodies. Some of the micronemes (Mn) are oriented perpendicularly to the zoite pellicle and the rhoptries are uniformly electron-dense, one of which (Rh) is oriented with its neck projecting posteriorly. Approximately 10 dense granules (Dg) are situated posterior and three anterior to the nucleus. (Fig. 2) Tachyzoite of *T. gondii* VEG strain in a parasitophorous vacuole (PV) within a mouse peritoneal macrophage, showing labyrinthine rhoptries (Rh) and an early stage of endodyogeny in which a progeny zoite, consisting of a plastid (Ap*), Golgi complex (Go*), inner membrane complex (Im*), and rhoptries (Rh*), is developing internally within the mother zoite. Several host-cell mitochondria (*) are situated close to the parasitophorous vacuole. Abbreviations: Am, amylopectin; Ar 1, 2, apical rings 1 and 2; Co, conoid; Go, Golgi complex; Hc, host cell cytoplasm; Im, inner membrane complex of pellicle; Lb, lipid body; Mi, mitochondrion; Mn, microneme; No, nucleolus; Nu, nucleus; Pl, plasmalemma; Pr 1, 2, polar rings 1 and 2; Sm, subpellicular microtubule; Tm, tubulovesicular membrane network.



Figures 1.3 & 1.4. *Transmission EMs of bradyzoites of N. caninum and T. gondii*. Figures and Figure legend taken from (Speer et al., 1999). (Fig. 3) *N. caninum* NC-Liverpool. The nucleus (Nu) is located subterminally and the cytoplasm behind the nucleus contains micronemes (Mn), six dense granules (Dg) and a small dense granule (Sg). Tubules (Tu) project from the inner surface of the cyst wall (Cw) and vesicles (arrowheads) are scattered amongst the bradyzoites. (Fig. 4) Bradyzoite of *T. gondii* VEG strain with its nucleus (Nu) occupying its posterior. One of the rhoptries (Rh) is looped-back with the base of its neck projecting posteriorly; from (Dubey et al., 1998c). Abbreviations: Am, amylopectin; Ap, plastid; Ce, centrioles; Co, conoid; Go, Golgi complex; Mi, mitochondrion; Nr, neck of rhoptry; Pp, posterior pore.

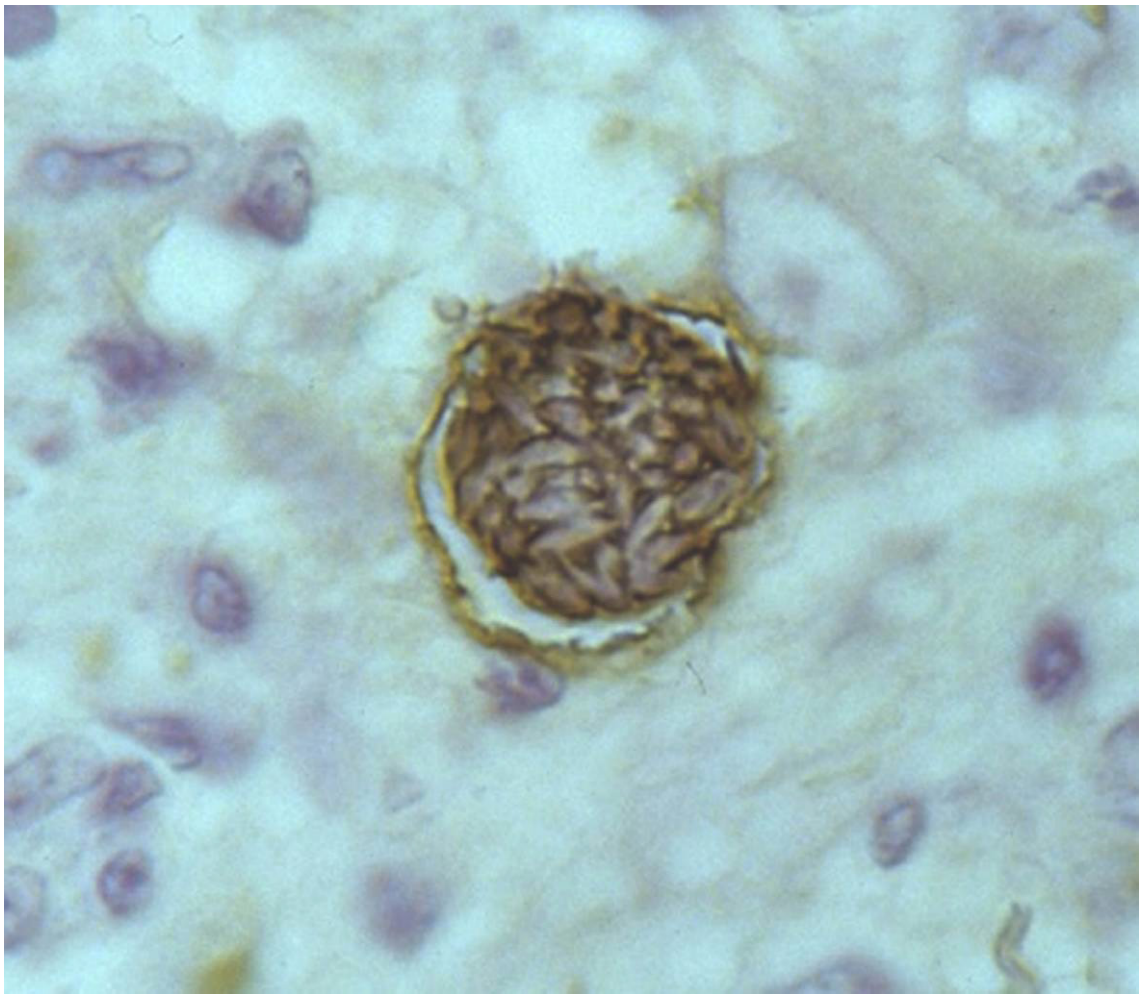
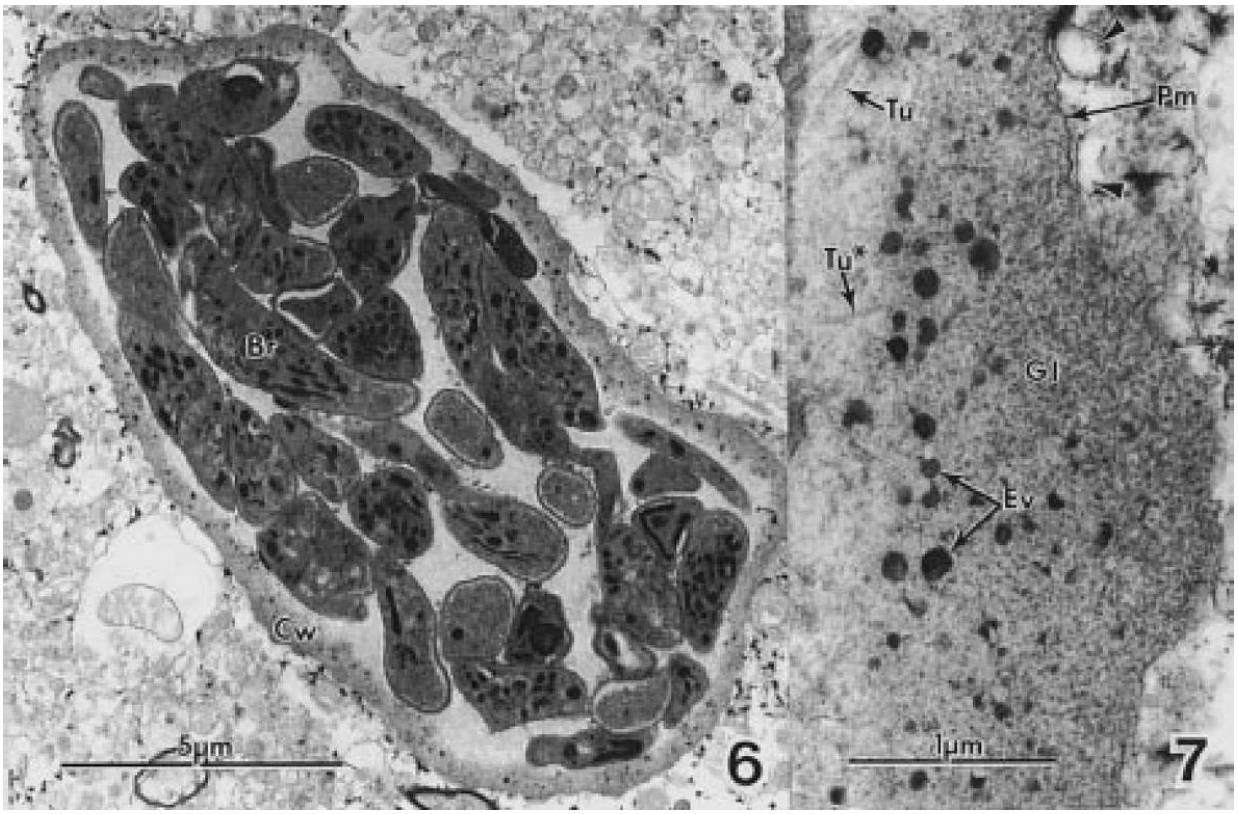


Figure 1.5. *Immunohistochemical staining of N. caninum tissue cyst.*



Figures 1.6 & 1.7. *Transmission EM of N. caninum tissue cysts.* Figures and Figure legend taken from (Speer et al., 1999). (Fig. 6) Tissue cyst of *N. caninum* NC-5 showing approximately 30 bradyzoites (Br) surrounded by an irregularly shaped cyst wall (Cw). (Fig. 7) High magnification of portion of cyst wall in Fig. 6, showing electron-dense vesicles (Ev) of various sizes embedded in the granular layer (Gl) and large (Tu) and small (Tu*) tubules projecting from the inner aspect of the cyst wall. Host cell endoplasmic reticulum with distended cisternae (arrowheads) is closely associated with the parasitophorous vacuolar membrane (Pm). From (Dubey et al., 1998b).

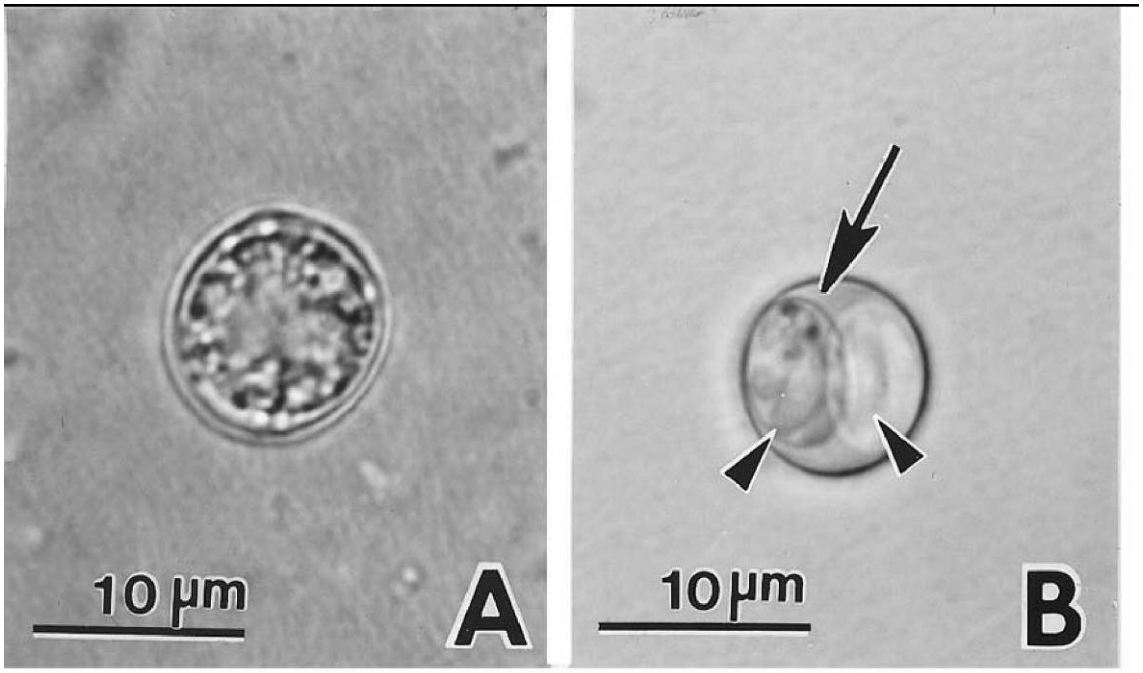


Figure 1.8. *Neospora caninum stages in dogs and mice.* Figure and Figure legend taken from (McAllister et al., 1998) [A] Unsporolated oocyst with a central mass[B] Sporolated oocyst with two sporocysts "arrow" and with sporozoites "arrowheads".

1.4.2 The Life Cycle

Neospora caninum has been shown to have a two-host life cycle in which dogs (McAllister et al., 1998) and coyotes (Gondim et al., 2004c) are the only recognised definitive hosts. A wide range of warm-blooded animals have been shown to act as intermediate hosts. Evidence of natural infections have been found in cattle, sheep (Dubey and Lindsay, 1990a), deer (Woods et al., 1994), water buffalo (Dubey, 1998), goats (Dubey et al., 1992), horses (Dubey and Porterfield, 1990) and a rhinoceros (Dubey, 2003; Williams et al., 2002). Experimental infections have been achieved in cats, mice, rats, dogs, foxes, goats, sheep, coyotes, pigs, gerbils, rabbits and cattle (Dubey and Lindsay, 1996; Hemphill, 1999).

N. caninum has three infectious stages; sporozoites, bradyzoites and tachyzoites. Tachyzoites and bradyzoites can be found in infected tissue of both intermediate and definitive hosts. However, sporozoites have only been found in oocysts produced by a definitive host.

Unsporolated oocysts are excreted in the faeces of dog and coyote definitive hosts and are produced by gamatogomy. Oocysts sporolate 3 days after excretion and contain two sporocysts. Each sporocyst has 4 sporozoites. Dogs have been shown to produce oocysts naturally (Basso et al., 2001a; McGarry et al., 2003) and experimentally (Dijkstra et al., 2001). Following experimental ingestion of infected tissue, dogs may begin shedding oocysts within 8 days (McAllister et al., 1998; Rodrigues et al., 2004) and may continue to shed for relatively long periods or re-shed at a later stage (Gondim et al., 2005; McGarry et al., 2003).

Tachyzoites are able to penetrate host cells by active invasion (Hemphill et al., 1996) and have been found in a variety of host tissues such as brain, spinal cord, heart, lung, liver, foetal membrane, muscle, placenta and skin (Dubey and Lindsay, 1996; Hemphill, 1999; Shivaprasad et al., 1989). *N. caninum* has been shown to infect many different nucleated cell types in vitro, including macrophages, hepatocytes, neural cells, fibroblasts, vascular endothelial cells, myocytes, placental tropoblasts and renal tubular epithelial cells (Dubey et al., 2002). Tachyzoites form a pseudocyst within the host cell, which lacks a cell wall. Tachyzoites continue to asexually replicate until host cell lysis occurs and the tachyzoites are freed into the host environment enabling them to infect neighboring cells (Hemphill, 1999).

In experimental inoculation studies, tachyzoites have been shown to be infectious by the subcutaneous (SC), intraperitoneal (IP), intramuscular (IM), intravenous (IV) and oral routes (Dubey and Lindsay, 1996). It is believed that the host immune response against tachyzoites is a likely trigger for the parasite to begin stage conversion to the slow growing bradyzoite stage (Buxton et al., 2002).

Bradyzoites are able to form intracellular tissue cysts, which are enclosed in a cyst wall. Bradyzoite cysts are found almost exclusively in central nervous tissue (Dubey, 2003). However, intramuscular tissue cysts have been reported in a dog (Peters et al., 2001) and in a foal (Lindsay et al., 1996). The bradyzoite slowly replicates within the cyst but can remain within the tissue undetected by the immune system for years without causing clinical manifestations (Dubey and Lindsay, 1996). Tissue cysts may vary in size depending on the number of bradyzoites they contain (Dubey et al., 2002). Bradyzoites may be released from tissue cysts and converted to the tachyzoite stage during recrudescence of *N. caninum* infection. Recrudescence is believed to occur due to suppression of the host immune system, which usually controls tachyzoite proliferation (Quinn et al., 2002a).

A definitive host can be infected by ingestion of fresh tissue containing tissue cysts. *N. caninum* tissue cysts can survive for at least 14 days at 4°C in tissue homogenate, or for a week within a carcass but are non-infective after being frozen for 1 day at -20°C (Lindsay et al., 1992).

1.5 Neosporosis in Dogs

Neosporosis was first reported in dogs by Bjerkas et al. (1984) in Norway with the discovery of *N. caninum*. However, earlier records of *N. caninum* misdiagnosis in dogs are present from 1957 (Dubey et al., 1990d). Studies from Japan, the Netherlands and Argentina have reported antibody prevalence in dogs. In Japan, 31% of dogs from dairy farms and 7% of urban dogs were positive to *N. caninum* antibodies (Sawada et al., 1998), whereas in the Netherlands dairy farm dogs were 23.6% positive compared to 5.5% of urban dogs (Wouda et al., 1999). Basso (2004) in Argentina reported 48% of dairy and 54.2% of beef farm dogs as seropositive compared to 22.2% of urban dogs (Basso et al., 2001b). However, the highest prevalence reported to date was from New Zealand, where 30.7% of urban dogs, 74.5% of dairy farm dogs and 96.8% of sheep/beef farm dogs were positive for *N. caninum* antibodies (Antony and Williamson, 2003). Variation in

seroprevalence for dogs is likely to vary with the country of origin and the IFAT cut off limit used by the laboratory testing the samples (Dubey et al., 2007).

Dogs have been accepted as the definitive host of *N. caninum* (McAllister et al., 1998). Congenitally infected dogs tend to display the most severe symptoms of neosporosis, such as hind leg paresis that may develop into progressive paresis, limbs may be locked in rigid hyperextension, neurologic signs may be present, dogs may have difficulty swallowing, paralysis of the jaw, muscle flaccidity, muscle atrophy and heart failure (Barber and Trees, 1996; Cuddon et al., 1992; Dubey and Lindsay, 1990b, 1993; Hay et al., 1990; Odin and Dubey, 1993; Trees et al., 1991). Older dogs may have dermatitis involving *N. caninum* tachyzoites or multi-organ infection that is frequently fatal (Dubey et al., 1988a; Hoskins et al., 1991; La Perle et al., 2001; McInnes et al., 2006a; Perl et al., 1998; Peters et al., 2000; Poli et al., 1998).

1.6 Neosporosis in Cattle

1.6.1 Transmission of infection

N. caninum can be transmitted horizontally and vertically through a herd of cattle. Horizontal transmission occurs when cattle ingest sporolated oocysts. Vertical transmission, also known as congenital transmission, is responsible for the spread of infection from a persistently infected dam to her calf *in utero* (Dubey et al., 1990a; Dubey et al., 1989; Landmann et al., 2002; O'Toole and Jeffrey, 1987; Parish et al., 1987; Shivaprasad et al., 1989; Thilsted and Dubey, 1989). Congenital transmission has also been shown in other animals, such as sheep, (Dubey et al., 1990b) dogs, (Dubey and Lindsay, 1990b; Uggla et al., 1989), horses (Dubey and Porterfield, 1990), goats (Barr et al., 1992; Dubey et al., 1992), rodents (Cole et al., 1995) and rhinoceros (Williams et al., 2002).

New evidence suggests that intrauterine infection from contaminated semen can lead to infection of the heifer but infection of the embryo has not yet been shown (Serrano et al., 2006). Evidence of intrauterine infection, taken with detection of *N. caninum* in bull semen provides a compelling argument for a new route of transmission between intermediate and possibly definitive hosts (Serrano-Martinez et al., 2007).

1.6.2 Vertical Transmission

Vertical transmission may occur via two proposed routes; exogenous transplacental transmission, which may occur following a primary infection of the pregnant dam following oocyst ingestion and endogenous transplacental transmission, occurring in persistently infected dams that experience recrudescence of the infection during pregnancy (Anderson et al., 1997; Dubey et al., 2006; Dubey and Lindsay, 1993).

Vertical transmission of infection during pregnancy may result in foetal abortion (Shivaprasad et al., 1989). Cattle infected with the parasite are three to seven times more likely to abort compared

to uninfected cattle (Thurmond et al., 1997). Considerable losses have been reported in cases where herds have suffered 30% loss of foetuses due to abortion storms, possibly caused by a primary herd infection (Anderson et al., 1991; Thilsted and Dubey, 1989; Thornton et al., 1991). In the majority of cases an infected dam will give birth to a healthy calf that may also be infected (Anderson et al., 1997; Davison et al., 1999b; Pare et al., 1996; Schares et al., 1998). Vertical transmission is the most important route of infection as it propagates the infection into successive generations of cattle supporting persistent *N. caninum* herd infection (Anderson et al., 1997; Bjorkman et al., 1996; Schares et al., 1998; Wouda et al., 1998b). Cows may continue to have a persistent *N. caninum* infection for life (Trees et al., 1999), which may be consistently or intermittently transmitted to offspring (Guy et al., 2001; Piergili Fioretti et al., 2003). There is evidence to indicate that the rate of congenital infection may decrease over time, suggesting that the dam does develop some immunity to prevent transmission (Andrew Thompson, 2000; Dijkstra et al., 2003; McAllister et al., 2000). It appears that cow to cow transmission does not occur, as animals housed in the same enclosure as infected animals do not seroconvert (Piergili Fioretti et al., 2003).

Neospora caninum may be excreted in milk, uterine discharge and semen. Ingested uterine fluids, foetal membranes or colostrum containing tachyzoites may result in infection (Davison et al., 2001; Schares et al., 1998; Uggla et al., 1998). *Neospora caninum* has been detected using PCR in the semen of bulls (Caetano-da-Silva et al., 2004; Ortega-Mora et al., 2003), however no evidence has been found to show that parasite transmission has occurred (Canada et al., 2006).

1.6.3 Horizontal Transmission

Mathematical modeling provides evidence that sustained herd infection is unlikely to occur without horizontal transmission, despite efficient vertical transmission routes (French et al., 1999). *Neospora caninum* abortion storms suggest a point-source exposure consistent with infection via horizontal transmission (McAllister et al., 2000; McAllister et al., 1996a; Sager et al., 2005; Yaeger et al., 1994), as does increasing seropositivity with age and unassociated seropositivity of calves and dams (Dyer et al., 2000; Mainar-Jaime et al., 1999; Thurmond et al., 1997).

The presence of dogs on farms is positively correlated with an increase in seropositivity to *N. caninum* and abortion in a cattle herd (Barber et al., 1997; Reichel, 1998; Sawada et al., 1998; Wouda et al., 1999). Cattle are likely to become infected from oocysts excreted in the faeces of dogs, which are also a likely source of re-infection. The number of dogs present on a farm is also related to abortion risk (Bartels et al., 1999; Pare et al., 1998; Sawada et al., 1998), however a lack of dogs does not exclude *N. caninum* seropositivity in a herd (Wouda et al., 2000). Herd infection may be via other wild canids such as coyotes (Gondim et al., 2004c) or foxes (Almeria et al., 2002; Wolfe et al., 2001).

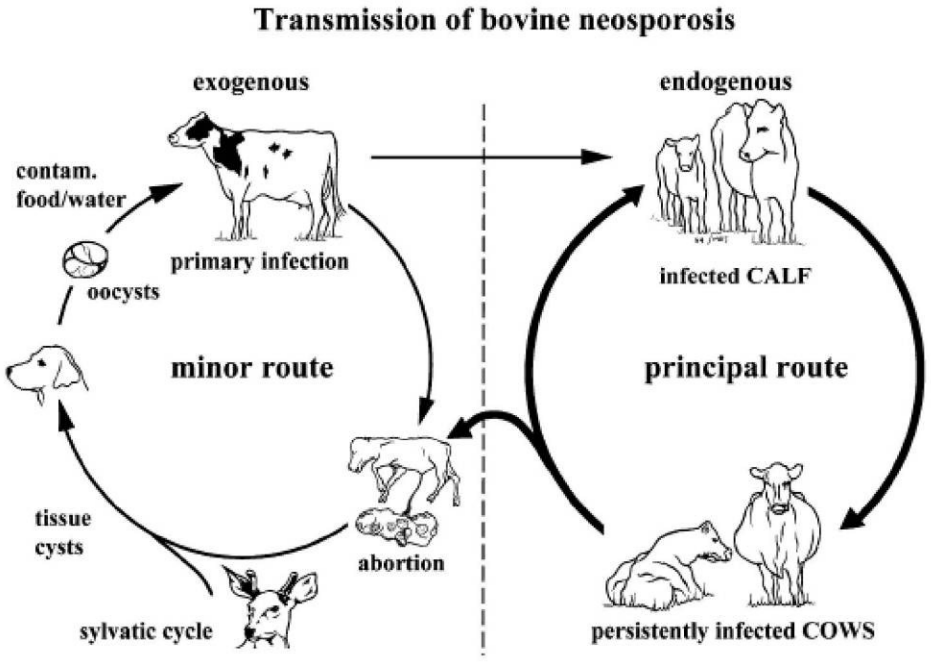


Figure 1.9. *Transmission of bovine neosporosis.*
Figure and Figure legend taken from (Dubey et al., 2006)

Oocysts are produced by the canine definitive host and their subsequent ingestion by a susceptible pregnant cow leads to infection of the foetus (exogenous transplacental transmission). Live born infected heifer calves would be expected to remain infected into adulthood when they, in turn, may pass infection to their fetus (endogenous transplacental transmission). Spread of *N. caninum* in this second way is the principal route whereby the parasite is propagated in a herd.

1.7 Beef and Dairy Farm Seropositivity

In a serological study in Spain, beef farms had a lower prevalence of *N. caninum* infection than dairy farms being 55% and 83% respectively. Specific antibody prevalence for individual cattle within the herds was also lower within beef (18%) than dairy herds (36%) (Quintanilla-Gozalo et al., 1999). Evidence has also been shown in New Zealand that beef cattle have a lower prevalence of *N. caninum* infection than dairy cattle (Tennent-Brown et al., 2000). However, dogs found on beef and dairy farms have a higher rate of *N. caninum* specific antibodies than urban dogs (Antony and Williamson, 2003; Malmasi et al., 2006).

1.8 Risk factors for Abortion

Several risk factors that may increase the likelihood of abortion storms and recrudescence of infection in persistently infected animals have been identified. High rainfall in the second trimester of gestation has been shown to be associated with decreased success of gestation

(Lopez-Gatius et al., 2005). Other risk factors include the presence of dogs, poultry, feeding of mouldy maize-silage and farm management practices. A significant association was found between the presence of dogs and seropositive cattle. Feeding of mouldy fodder is considered a risk factor as it can cause recrudescence of *N. caninum* infection due to mycotoxins causing immunosuppression (Bartels et al., 1999). Management practices such as a high prevalence of retained afterbirths, presentation of young stock at show grounds or fairs, grazing young stock on communal pastures (Bartels et al., 1999) and possibly contact with seropositive animals (Waldner et al., 1999), however this last point is considered to be debatable as other studies have found no evidence of cross-infection of this kind (Piergili Fioretti et al., 2003).

Bovine viral diarrhoea virus (BVDV) causes immunosuppression in the infected host and as such may also result in recrudescence of an existing *N. caninum* infection or susceptibility to a new infection (Bjorkman et al., 2000; Fray et al., 1999; Stahl et al., 2006).

1.9 Host-Parasite relationship

Clinical disease in non-pregnant animals is rarely observed since neosporosis is a disease that manifests during pregnancy.

1.9.1 Immunological responses to *N. caninum*

As *N. caninum* is an obligate intracellular pathogen, cell mediated immune (CMI) responses are likely to play an important role in protective immunity. Immunity to intracellular protozoa is generally controlled by a type-1 T lymphocyte (Th1) response. Interleukin-12 (IL-12) and interferon gamma (IFN-γ) mediate the development of antigen-specific T helper (Th) cells during initial intracellular infection. Th1 cells secrete effector cytokines IL-2, IFN-γ and tumour necrosis factor beta (TNF-β) (Baszler et al., 1999b). Resistance to *N. caninum* infection has been associated with high production of the Th1 cytokine IFN-γ and low production of the Th2 cytokine IL-4. IL-4 promotes the production of higher levels of IgG1, which is associated with disease susceptibility (Long et al., 1998).

Studies have shown that the treatment of *N. caninum* infected cells in vitro with ovine IFN-γ significantly inhibits intracellular multiplication of parasites within ovine fibroblast cells (Innes et al., 1995). Th-1 type cytokines were shown to play a key role in host defense mechanisms when tachyzoite multiplication was inhibited in bovine brain cell cultures when treated with IFN-γ and to a lesser extent with tumour necrosis factor alpha (TNF-α) (Yamane et al., 2000).

Neutralization of IL-12 and IFN-γ results in increased mortality in mice (Khan et al., 1997). Baszler et al (1999) showed that neutralization of IFN-γ results in increased mortality and severe morbidity during acute *N. caninum* infection, and additionally showed that that IL-12 resulted in decreased severity of disease at the early stages but did not alter long term prognosis. They suggested that IL-12 may be required for sustaining a Th1 response to *N. caninum* infection (Baszler et al., 1999b). IFN-γ knockout mice were found to be the most susceptible to *N. caninum*

infection when compared to other strains of mice and corticosteroid suppressed mice (Dubey et al., 1998b).

In addition, several authors have demonstrated IFN-γ production and antigen-specific cell proliferation in cattle (Andrianarivo et al., 2001; De Marez et al., 1999; Lunden et al., 1998; Marks et al., 1998; Williams et al., 2000). *N. caninum*-specific cytotoxic T lymphocytes (CTL) expanded from peripheral blood mononuclear cells (PBMC) have been used to demonstrate that CD4+ CTL killed autologous *N. caninum* infected cells and that killing was mediated through a perforin/granzyme pathway (Staska et al., 2003).

Administration of immunosuppressive corticosteroids can exacerbate neosporosis and cause recrudescence (Dubey and Lindsay, 1990b). Immunosuppression caused by feeding cattle mouldy fodder that harbors mycotoxins produced by fungi (Corrier, 1991) may also induce recrudescence of latent *N. caninum* infections (Bartels et al., 1999). Tissue cyst rupture has been shown in mice infected with *T. gondii* following immunosuppression (Venturini et al., 1996). Cyst rupture releases tachyzoites into the host tissue resulting in parasite multiplication and recrudescence of a latent infection.

1.9.2 Pregnancy and *N. caninum* infection

During pregnancy Th1-type inflammatory cytokines, such as IFN-γ and TNF-α, can exert detrimental effects on the placenta and are usually found in low concentrations. An immune response against pathogens that target the placenta may cause abortion or foetal damage (Entrican, 2002). The same immune responses that appear to be important in protecting against *N. caninum* parasite survival and multiplication during infection are modulated and suppressed during pregnancy (Innes et al., 2002; Quinn et al., 2002a).

A regulatory Th2-type cytokine environment, such as IL-10, TNF-β and IL-4, are favored in the placental environment and counteract the inflammatory responses induced by the Th-1 type cytokines (Entrican, 2002). Induction of an immune response against *N. caninum* during pregnancy via vaccination may result in foetal loss due to abortion; however, foetal infection via vertical transmission may occur in the absence of a Th-1 type immune response (Innes et al., 2005).

Production of the hormone progesterone by the pregnant dam directs the immune system towards a Th-2 type phenotype (Kalinski et al., 1997). Hence, IFN-γ is significantly down regulated and this may be a significant contributing factor to recrudescence of disease in persistently infected animals. Antibody titres and cell mediated immune responses of cattle infected with *N. caninum* decrease significantly around mid-gestation (Innes et al., 2005), which correlate to rising progesterone levels that steadily increase from early-mid gestation (Pope et al., 1969).

Foetal abortion will occur if either the foetus or the placenta are severely damaged and are no longer viable. In addition to maternal pro-inflammatory cytokines causing placental damage (Maley et al., 2006), primary infection with *N. caninum* tachyzoites may cause placental damage that can directly jeopardize foetal survival or may cause the release of maternal prostaglandins,

which causes contraction of the uterus resulting in foetal expulsion (Baetz et al., 1981). Foetal damage may occur due to insufficient transfer of oxygen or nutrients through the placenta following placental damage or may be caused by primary tissue damage due to active multiplication of tachyzoites (Dubey et al., 2006).

1.10 Pathological lesions

Tachyzoites are mainly found in brain and central nervous system tissues where they can rapidly multiply within the host cells, causing destruction of large numbers of neural cells (Barber et al., 1996) including those of cranial and spinal nerves and affecting the conductivity of the affected cells (Dubey and de Lahunta, 1993). Cell death can lead to areas of visible necrotic lesions within a few days. It can induce severe neuromuscular disease in host dogs and cattle. Encephalomyelitis and myositis have been associated with neosporosis since the discovery of the disease (Bjerkas et al., 1984). Live born calves may present with stunted growth, limb weakness and recumbence. Damage occurs to leg tendons, skull formation, with abomasitis, ulcerative oesophagitis (Barr et al., 1991b) and abnormal spinal cord development (Dubey et al., 1990a).

Severe lesions are usually found in the placenta and the brain of the foetus following experimental infection. During *N. caninum* invasion of the bovine uterus, parasites multiply in both the maternal and foetal tissues at the materno-fetal interface causing cell destruction and a non-suppurative inflammatory response (Macaldowie et al., 2004; Maley et al., 2003). Within 14 days following experimental infection, parasites can frequently be found in foetal placental villi associated with villous necrosis. Maternal inflammatory cells in the maternal septum have been shown to be CD4+, CD8+ and γδ T-cells, some of which are capable of producing IFN-γ (Innes et al., 2005). At 28 days following infection, breakdown of the placentome and separation of foetal cotyledons from maternal caruncles can be seen (Macaldowie et al., 2004).

At a later stage of foetal development the foetus is able to produce a better immune response and necrosis is usually confined to small foci and is surrounded by foetal inflammatory infiltrate, containing microglia, reactive astrocytes and monocyte and lymphoid cells (Wouda et al., 1997b). Lymphoid inflammation and foetal cell destruction have been noted in several tissues including the heart, skeletal muscle, lung, liver and brain (Anderson et al., 1991; Barr et al., 1991b; Wouda et al., 1997b). In a Californian study of 82 foetuses, encephalitis and myocarditis were seen in 100%, adrenalitis in 80%, myositis in 72%, nephritis in 66%, hepatitis in 62%, placentitis in 53%, pneumonia in 44% and protozoa were observed in 89% of the foetuses (Barr et al., 1990; Barr et al., 1991b). Severely infected foetuses may become mummified or autolysed in-utero (Dubey et al., 1998a)

1.11 Mouse models

Different strains of *N. caninum* appear to have quite varied pathogenicity *in vivo* (Dubey and Lindsay, 1993). It is possible that sex-related resistance may also be a factor in addition to mouse strain susceptibility to infection. Mice have been found to be susceptible to *N. caninum* induced encephalitis and congenital transmission in a dose dependent manner (Cole et al., 1995; Lindsay

et al., 1995a; Long and Baszler, 1996). Swiss Webster mice may develop tissue cysts but *N. caninum* is not highly infectious to the mouse.

Immunocompetent mice are generally resistant to infection with *N. caninum*, therefore immunosuppression with corticosteroids is commonly used to induce mouse susceptibility (Dubey et al., 1988b; Lindsay and Dubey, 1989c). Commonly 4 mg of methylprednisolone acetate is used to induce acute disease and polymyositis (Lindsay and Dubey, 1989c; Yamane et al., 1998). Inbred BALB/c mice are more susceptible to parasite infection and are commonly used for mouse modeling and isolation, however, corticosteroids must be used to suppress the immune system (Baszler et al., 1999b; Lindsay et al., 1995a). C57 BL/6 and BALB/c appear to be more susceptible to neosporosis than B10.D2 mice (Long et al., 1998). Other mouse strains used for isolation and passage are BALB/c J-nu (Peters et al., 2000), A/J (Khan et al., 1997), BALB/c nu/nu (Koyama et al., 2001), AKR mice (Peters et al., 2000), ICR (Koyama et al., 2001) and albino mice (Dubey et al., 2004). However, the most successful strain for promoting neosporosis is the gamma-interferon knockout mouse (gammaIFN-KO) (Dubey et al., 1998b). They have been shown to be highly susceptible to parental inoculation with tachyzoites and tissue cysts but are less susceptible to inoculation with oocysts. Mice with genetic/immunologic deficiencies, such as gammaIFN-KO or antibody knockout (μMT) provide a means in which to study the immune mediation to *N. caninum* and also provide a susceptible host that doesn't require immunosuppression (Eperon et al., 1999). IFN-γ has been shown to be important in host-mediated immune responses. The depletion of IFN-γ with antibodies renders mice susceptible to infection (Khan et al., 1997; Tanaka et al., 2000).

1.12 Mouse pathogenesis

Development of clinical neosporosis in mice depends on the mouse strain, the *N. caninum* strain and the dose of parasites as well as treatment of the mouse such as immunosuppression.

Mice suffering from neosporosis may present with tetraplegia, emaciation (Sawada et al., 1997), head-tilting, impaired movement and coat ruffling. Pneumonia, polymyositis, encephalitis, hepatitis and pancreatitis are the main lesions seen (Lindsay and Dubey, 1989c). Tachyzoites may been seen in inflammatory foci in peripheral nerves, skeletal muscles, central nervous system tissue (Sawada et al., 1997), muscle of the small intestine, skin, retina of the eye and tongue (Lindsay and Dubey, 1989c). Tissue cysts can be found in the brain several weeks following infection, particularly in the cerebrum (Lindsay and Dubey, 1989c).

Outbred Swiss Webster mice don't develop clinical neosporosis but cysts can form in the brain. Clinical neosporosis can be induced in these mice by varying the isolate of *N. caninum*, (for example NC-1 is more virulent than NC-2 or NC-3), or by varying the dose of parasites or the dose of corticosteroids (Dubey et al., 1988b; Lindsay and Dubey, 1989c). Nude mice develop severe neosporosis following inoculation with NC-1 tachyzoites (Sawada et al., 1997; Yamage et al., 1996). Depending on the isolate and dose of *N. caninum*, neosporosis can be induced in BALB/c mice. NC-Liverpool induces severe neosporosis whereas NC-SweB1 induces milder clinical signs. JPA1 produces no clinical signs (Atkinson et al., 1999). Inbred BALB/c mice are more susceptible than outbred BALB/c mice and may not require immunosuppression

particularly when inoculated with NC-1 (Lindsay et al., 1995a). Characteristic *N. caninum* tissue cysts in BALB/c mice are rare but tachyzoites are frequently found in tissue lesions (Dubey and Lindsay, 1996). ICR outbred mice do not develop clinical neosporosis but can be used to produce bradyzoite tissue cysts (Lindsay et al., 1995a) (McGuire et al., 1997b). Inbred A/J mice and B10.D2 mice can be used in studies of the immune response as they are resistant to *N. caninum* (Khan et al., 1997; Long et al., 1998). C57 BL/6 mice are sensitive to parasite dose. Severity of clinical neosporosis and brain lesions is increased with a higher dose (Eperon et al., 1999).

Transplacental transmission of *N. caninum* infection has been detected in BALB/c mice (Long and Baszler, 1996) and in outbred Swiss Webster mice (Cole et al., 1995).

Immunodeficient mice develop severe neosporosis frequently resulting in death (Dubey and Lindsay, 1996).

Athymic nude mice, which lack T cells but have a normal complement of bone marrow dependent B cells and elevated levels of macrophages and NK cells, are more susceptible to neosporosis with parasites detectable in the brain at a much earlier stage than wild type mice (Shibahara et al., 1999). B-cell and antibody (μMt) deficient mice will begin to die from neosporosis from 4 weeks following infection, whereas wild type mice show no clinical signs (Eperon et al., 1999). Neosporosis will kill SCID-beige mice, which lack functionally mature T cells, B cells and 50% NK cells, as they are highly susceptible to infection unlike BALB/c mice which are only mildly or not at all susceptible to *N. caninum* (Dreier et al., 1999). Swiss Webster mice can be treated with immunosuppressive drugs to induce susceptibility but IFN-γ knockout mice are highly susceptible and will die of overwhelming neosporosis (Dubey et al., 1998b).

1.13 Animal models

Dogs

Both naturally infected and experimentally infected dogs have been studied to show that dogs are the definitive hosts ascertained by oocyst shedding following infection (McAllister et al., 1998). It has been suggested that dogs shed more oocysts after ingesting infected bovine tissue (mean=160,700) than following ingestion of infected murine tissue (mean=5400) (Gondim et al., 2002). Dogs treated with a corticosteroid immunosuppressant were found to shed more than 100,000 oocysts after ingesting murine brains (Lindsay et al., 1999a; Lindsay et al., 2001). Puppies were also found to shed significantly more oocysts (mean=166,400) than adult dogs (mean=2900). The age, immunocompetence and infectious material consumed all appear to have a significant effect on oocyst production in dogs (Dubey et al., 2007).

Cattle

Ruminant models have been used but are costly and numbers are frequently limited (Harkins et al., 1998; Kritzner et al., 2002; Lunden et al., 1998).

Gerbils

Gerbils (*Meriones unguiculatus*) have frequently been used to isolate and passage parasites as they are susceptible to infection with tachyzoites and oocysts and do not require immunosuppression (Cuddon et al., 1992; Dubey and Lindsay, 2000; Gondim et al., 1999).

Sheep

Sheep models have been used as they provide a good alternative to study the typical features of bovine neosporosis but the cost is reduced. Natural *N. caninum* infection in sheep is rare but is observed (Dubey et al., 1990b; Koyama et al., 2001). Experimental infection of sheep can lead to abortion, birth of weak lambs or birth of infected lambs showing no clinical signs (McAllister et al., 1996b). Sheep are highly susceptible to experimental infection and produce high levels of *N. caninum* antibodies (Dubey and Lindsay, 1990a; Dubey et al., 1996a).

Goats

Naturally infected and experimentally infected goats have also been shown to behave in a similar fashion to natural and experimental models of neosporosis in sheep (Barr et al., 1992; Dubey et al., 1992; Dubey et al., 1996b; Lindsay et al., 1995b). A naturally occurring strain of *N. caninum* has not yet been isolated from goats (Koyama et al., 2001).

Cats

Cats have been used for experimental infection with *N. caninum*. Clinical neosporosis can be induced following infection (Dubey and Lindsay, 1989a). Congenital transmission of *N. caninum* infection has been shown in cats (Dubey and Lindsay, 1989b). Studies have established that wild and domestic cats are likely to be naturally infected with a high proportion of cats (25%) seropositive for antibodies against *N. caninum* (Bresciani et al., 2006; Ferroglio et al., 2005). Despite the prevalence of antibodies to *N. caninum* in cats, there is not yet a *N. caninum* isolate from cats.

Primates

At present there is no evidence of *N. caninum* infection in humans, however in a study on positive sera for *N. caninum* antibodies 6.7% of the samples had a low antibody titre against *N. caninum* when detected by indirect fluorescent antibody test (IFAT) (Tranas et al., 1999). In a similar study, sera from woman who had repeat abortions was tested by ELISA but none were found to be positive for *N. caninum* (Petersen et al., 1999). Rhesus macaques (*Macaca mulatta*) experimentally infected with *N. caninum* has been used to determine the zoonotic potential of *Neospora*. *Neospora* DNA was detected, using PCR, in the lung, brain and heart of monkeys (Ho et al., 1997b). Studies have also shown that nonhuman primates are susceptible to transplacental *Neospora* infection and it produces lesions similar to those induced by *Toxoplasma gondii* (Barr et al., 1994).

1.14 *Neospora caninum* Isolation

N. caninum was first isolated by Dubey et al (1988) from tissue of naturally infected dogs. Swiss white mice and beagle pups were used for an inoculation study in addition to cell cultures of bovine monocytes and cardiopulmonary arterial endothelial cells. *N. caninum* tachyzoites were seen in cell cultures from days 5-19. Experimentally inoculated dogs appeared clinically normal but parasites could be isolated from tissue and seen in cell culture after just 2 days. Tissue cysts were found in the brains of some of the mice inoculated with *N. caninum* infected tissue and all mice had positive IFA titers following inoculation (Dubey et al., 1988b).

The first bovine isolate came from the brains of two aborted foetuses. The parasites were cultured in Vero cells and antigenic and ultrastructural analysis confirmed that the parasites did belong to the *Neospora* genus (Conrad et al., 1993a).

Many isolations of *N. caninum* have been made since the characterization of NC-1 (Table 1.2). New strains have been isolated from a number of animal sources such as cattle (Barr et al., 1993; Canada et al., 2004b; Canada et al., 2002; Cheah et al., 2004; Conrad et al., 1993a; Davison et al., 1999a; Gondim et al., 2002; Hemphill and Gottstein, 2000; Hyun et al., 2003; Kim et al., 2000; Magnino et al., 1999; McAllister et al., 2000; Miller et al., 2002; Okeoma et al., 2004b; Piergili Fioretti et al., 2000; Sawada et al., 2000; Stenlund et al., 1997; Yamane et al., 1997), dogs (Barber et al., 1993; Basso et al., 2001a; Cuddon et al., 1992; Dubey et al., 1998b; Dubey et al., 1988b; Dubey et al., 2004; Gondim et al., 2001; Hay et al., 1990; Marsh et al., 1998; McInnes et al., 2006a; Peters et al., 2000), sheep (Koyama et al., 2001), deer (Vianna et al., 2005) and water buffalo (Rodrigues et al., 2004).

Successful isolation of *N. caninum* is difficult due to low numbers of parasites within host tissue (Wouda et al., 1997b) and low viability of parasites following tissue processing (Hemphill, 1999). In most cases, isolating viable parasites and maintaining them in an *in vitro* culture of passaging parasites have taken multiple attempts. Stenland et al., (1997) attempted isolation 10 times before an isolate was made, whereas Conrad et al., (1993a) made more than 100 attempts before succeeding at isolating them from bovine foetuses. More consistent success in isolation is achieved when using fresh neural tissue from infected newborn calves, stillborn calves and from congenitally infected dogs (Dubey and Lindsay, 1996). Success is likely to be based on the presence of an increased number of tissue cysts containing bradyzoites rather than the presence of tachyzoites. It is likely that bradyzoites are more resistant to the harsh treatment of tissue that is required in order to liberate parasites (Hemphill, 1999).

Observations of parasites in primary cultures can take from as little as 2 to more than 60 days after the culture is established (Davison et al., 1999a; Dubey et al., 1988b; Otter et al., 1995). A period of more than a month is common for parasite detection. There is also significant strain variation between in vitro growth characteristics and in vivo pathogenicity (Dubey and Lindsay, 1993) despite the fact that isolates obtained from cattle and dogs are considered to be the same species (Holmdahl et al., 1997). Isolates from horses are considered to be a separate species and are known as *Neospora hughesi* (Cheadle et al., 1999; Marsh et al., 1998; Marsh et al., 1999).

Parasites have been isolated from several tissue types. Commonly the brain of an infected animal is used, but also the spinal cord, muscle biopsy tissue, foetal tissue, skin lesions and canine faeces (Dubey and Lindsay, 1996).

Viable parasites can be isolated from whole tissue by physical disruption such as homogenization of tissue, whether with the use of a blender (Dubey et al., 1988b), a mortar and pestle, squashing through gauze (Yamane et al., 1998), passing through a syringe (Martinez-Moreno et al., 2006) or tissue squashes (Dubey et al., 1998b). In addition to physical disruption, chemical disruption such as treatment with trypsin solution may also be used to dislodge and disrupt tissue cysts (Dubey et al., 1998b). Tissue homogenates are incubated at 37°C in trypsin solution. A variety of trypsin solution concentrations and incubation times have been used (0.05% for 30-60 min, 0.25% for 20-45 min, 0.5% for 30 min and 2% for 30-60 min) (Barber et al., 1995; Conrad et al., 1993a; Dubey and Schares, 2006). Tissue may also be treated with acid pepsin solution to facilitate the release of bradyzoites (Dubey et al., 1998b). Lei et al (2005) have shown that trypsin treatment of *N. caninum* tachyzoites used to remove surface proteins does not increase cell invasion as it does for *T. gondii* (Lei et al., 2005).

As parasites are sparsely distributed within tissue, it is preferable to use as much tissue as possible in order to obtain cultures, particularly for brain tissue (Wouda et al., 1997b). Percoll® (Amersham Pharmacia Biotech, Uppsala, Sweden) can be used to reduce the amount of tissue required for inoculation into animal hosts and onto cell culture (McGuire et al., 1997a). Percoll solution provides a density gradient that can be used to separate the fraction containing parasites from others containing debris and fat. A 30% Percoll/phosphate-buffered saline (PBS) solution should be centrifuged at 3,000 g for 10 minutes, following which a pellet of parasites can be collected (Stenlund et al., 1997), or a 35% Percoll/PBS solution can be centrifuged at 4,400 g for 15 min (McGuire et al., 1997a). The brain in particular is densely populated with myelin, which is approximately 80% lipid and can easily be separated.

Parasites are isolated and cultured in the presence of antibiotics, generally 50-1000 U/ml penicillin and 50-100 ug/ml streptomycin, so as to avoid opportunistic microbial contamination in culture and a secondary infection occurring in an inoculated host (Dubey et al., 1998b).

Extracellular maintenance of tachyzoites in growth medium for longer than four hours results in loss of infectivity (Hemphill et al., 1996). Parasites that are disrupted from tissue should be inoculated onto host cells in culture and animal hosts promptly to ensure parasite viability.

Tissue homogenate inoculated onto cell culture should be removed 2-4 hours later to avoid damage to the cell monolayer, this is sufficient time for infection as reported by (Hemphill et al., 1996; Lei et al., 2005; Yamane et al., 1998). After the homogenate is removed, monolayers should be washed several times to remove tissue remnants before the addition of growth media.

Characterization of parasites following isolation is necessary for naming and family grouping. Isolates can been confirmed using PCR of known *Neospora* sequences (Ho et al., 1996), antigenic reactivity of tachyzoites using immunofluorescence (Barber et al., 1995; Kim et al., 2000) and study of ultrastructural features (Conrad et al., 1993a; Stenlund et al., 1997).

1.14.1 *In Vitro* Cultivation

Different cell types have been used for the intracellular proliferation of *N. caninum* tachyzoites. The first cell types to be used by Dubey et al (1988) were bovine monocytes and cardiopulmonary arterial endothelial cells for isolating *N. caninum* from dogs (Dubey et al., 1988b). Isolates of *N. caninum* have since been cultured in many cell lines, including Mandin-Darby bovine kidney, human foreskin fibroblasts, foetal mouse brain, Vero (Barber et al., 1993; Stenlund et al., 1997; Yamane et al., 1997), HS68 (Dubey et al., 1998b), COS- (Gondim et al., 2001) and CV1 cells (Dubey et al., 2004). Both bovine and non-bovine cell lines have been used in isolating *N. caninum* from cattle with equal success. Cells should always be cultured at 37°C with 5% CO_2.

Cultures of tachyzoites are commonly split 1-3 times per week (Stenlund et al., 1997; Yamane et al., 1998). Cell monolayers and tachyzoites can be disrupted with the use of a rubber scraper (Dubey et al., 1988b), or by trypsin treatment (0.25% in PBS) of the cells for 5 minutes (Stenlund et al., 1997). Trypsin treatment of cells involves lightly covering the cell layer with trypsin solution and incubating at 37°C until the cell layer comes away (usually 5-15min) (Hemphill et al., 2004).

Proliferation of tachyzoites appears to be strain dependent as some isolates multiply faster than others in cell culture and in animal models (Hemphill, 1999). Commonly, RPMI-1640 growth medium is used to culture tachyzoites but Dulbecco's modified Eagle's medium (D-MEM) (Koyama et al., 2001) and minimum essential media (MEM) (Yamane et al., 1998) can also be used. Growth medium is supplemented with serum, foetal bovine/calf serum (FBS) is considered to be the preferred serum for use. FBS can be used at 1% (Barber et al., 1995), 2% (Stenlund et al., 1997), 5% (Hemphill et al., 2004) and 10% (Dubey et al., 1988b) (Yamane et al., 1998). A 2% solution may be used if the growth rate of the host cell is preferentially minimized, which decreases the time required for sub-culturing but either may or may not provide optimal environmental conditions for the parasites (Barber et al., 1995; Stenlund et al., 1997). Barber et al., (1995) found that Nc-Liverpool tachyzoite growth was erratic when not supplemented with serum but could be grown in horse serum (HS) or 2% FBS. Increasing the concentration above 2% inhibited the growth of the parasites.

Commercially available batches of fetal bovine serum (FBS), used to propagate *N. caninum* tachyzoites in cell culture, have been found to contain antibodies directed against *N. caninum* (Torres and Ortega, 2006). Antibody contamination can lead to agglutination and death of parasites following cell lysis and release into the media (Hemphill, 1999). Studies have shown that *N. caninum* antibodies could be detected in 100% of FBS samples tested by western blotting and 88% of samples were shown to be positive to *N. caninum* by ELISA (Torres and Ortega, 2006). These results are perhaps unsurprising when compared to studies that show that up to 93% of calves born to infected dams are seropositive to *N. caninum* (Schares et al., 1998). Despite the presence of antibodies in *N. caninum* cell cultures, parasites have been shown to be infectious to mice following years of continuous passage in cell culture (Dubey and Lindsay, 1996). Horse serum can be used as an alternative to FBS, however cultures tend to be less prolific when supplemented with horse serum than with FBS (Hemphill, 1999).

Bradyzoites can be generated in vitro if tachyzoites are exposed to stress conditions, such as pH 8.1. Bradyzoite specific antibodies as well as ultrastructural changes can be detected following tachyzoite differentiation to bradyzoites. There is apparent evidence that low virulence strains of *N. caninum* have a higher spontaneous rate of cyst formation (Weiss et al., 1999). Another bradyzoite conversion technique is to treat tachyzoite cultures with 70μM sodium nitroprusside for 8 days. This treatment reduces parasite proliferation stimulating tachyzoite-to-bradyzoite stage conversion of *N. caninum* in vitro (Vonlaufen et al., 2002).

1.14.2 In Vivo isolation

Apart from isolating *N. caninum* in cell culture, isolation is also possible by inoculating infected tissue into mice. Several inbred, outbred and genetically defective strains of mice have been used to study and isolate *N. caninum*. Factors that can affect the susceptibility of the host to infection and neosporosis may include, mouse strain, parasite strain and life cycle stage, dose and route of inoculation and immunosuppressive drug treatment (Hemphill and Gottstein, 2000).

Inoculation of mice or gerbils with infected tissue homogenate has been shown to be an effective method for isolating parasites (Dubey et al., 1998b; Gondim et al., 2001). Mice are euthanized after several weeks and the brains are removed and can be checked for tissue cysts. A mouse brain homogenate can then be inoculated onto cell culture for tachyzoite passage (Dubey et al., 1988b; Dubey et al., 2004; McGuire et al., 1997a; Peters et al., 2000; Yamane et al., 1998).

Mouse strain or animal model selection for parasite isolation should be carefully made. It is often preferable to use more than one mouse strain or animal model as the virulence of the new parasite isolate may cause acute neosporosis in some animals or clinically undetectable disease in others (Dubey et al., 2004; Yamane et al., 1998).

Tissue homogenate or sediment is injected intraperitoneally (IP) into an animal. Injection IP allows for a large inoculation volume, up to 1ml of processed tissue (Canada et al., 2002; Dubey et al., 1998b). If tissue is separated over a density gradient, the sediment obtained can be the equivalent volume of injecting 1.5-8 grams of total homogenate (Yamane et al., 1998). In addition to using *N. caninum* brain cysts and other tissue infections such as lungs and liver as organism sources for isolation (Dubey et al., 1998b), tachyzoites may be found in the peritoneal exudate. The peritoneal cavity can be washed and inoculated onto cell culture (Canada et al., 2004b; Canada et al., 2002; Dubey et al., 1998b). A modified technique that allowed for the expansion of *N. caninum* tachyzoites in vivo by co-inoculating with mouse sarcoma cells and injecting these IP has been developed. This technique had previously been developed for *T. gondii* tachyzoites (Romand et al., 1998). Tachyzoites can subsequently be harvested from a peritoneal wash of the inoculated animals (Romand et al., 1998).

Table 1.2: *Neospora caninum Isolates*

Strain	Country	Source	Reference
NC-1	USA	Dog	(Dubey et al., 1988b)
NC-2	USA	Dog	(Hay et al., 1990)
NC-3	USA	Dog	(Cuddon et al., 1992)
NC-Liv	UK	Dog	(Barber et al., 1993) (Barber et al., 1995)
BPA-1	USA	Cattle	(Conrad et al., 1993a)
BPA-2	USA	Cattle	(Conrad et al., 1993a)
BPA-3	USA	Cattle	(Barr et al., 1993)
BPA-4	USA	Cattle	(Barr et al., 1993)
NC-SweB1	Sweden	Cattle	(Stenlund et al., 1997)
JPA-1	Japan	Cattle	(Yamane et al., 1997)
JPA-2	Japan	Cattle	(Yamane et al., 1998)
JPA-4	Japan	Cattle	(Yamane et al., 1998)
JPA-5	Japan	Cattle	(Yamane et al., 1998)
BT-2	Japan	Cattle	(Yamane et al., 1998)
CN-1	USA	Dog	(Marsh et al., 1998)
NC-4	USA	Dog	(Dubey et al., 1998b)
NC-5	USA	Dog	(Dubey et al., 1998b)
NC-LivB1	UK	Cattle	(Davison et al., 1999a)
NC-PVI	Italy	Cattle	(Magnino et al., 1999)
NC-GER1	Germany	Dog	(Peters et al., 2000)
NC-LivB2	UK	Cattle	(Hemphill and Gottstein, 2000)
NC-Beef	USA	Cattle	(McAllister et al., 2000)
KBA-1	Korea	Cattle	(Kim et al., 2000)
KBA-2	Korea	Cattle	(Kim et al., 2000)
BT-3	Japan	Cattle	(Sawada et al., 2000)
NC-PG1	Italy	Cattle	(Piergili Fioretti et al., 2000)
NC-Bahia	Brazil	Dog	(Gondim et al., 2001)
NC-6-Argentina	Argentina	Dog	(Basso et al., 2001a)
unknown	Japan	Sheep	(Koyama et al., 2001)
unknown	Brazil	Sheep	(Pena et al., 2007)
NC-Illinois	USA	Cattle	(Gondim et al., 2002)
NC-Porto1	Portugal	Cattle	(Canada et al., 2002)
NC-Nowra	Australia	Cattle	(Miller et al., 2002)
VMDL1	USA	Cattle	(Hyun et al., 2003)
Nc-MalB1	Malaysia	Cattle	(Cheah et al., 2004)
NC-SP-1	Spain	Cattle	(Canada et al., 2004b)
NC-6	USA	Dog	(Dubey et al., 2004)
NC-7	USA	Dog	(Dubey et al., 2004)
NC-8	USA	Dog	(Dubey et al., 2004)
NcBRBuf-1	Brazil	Water buffalo	(Rodrigues et al., 2004)
NcBRBuf-2	Brazil	Water buffalo	(Rodrigues et al., 2004)
NcBRBuf-3	Brazil	Water buffalo	(Rodrigues et al., 2004)
NcBRBuf-4	Brazil	Water buffalo	(Rodrigues et al., 2004)
NcBRBuf-5	Brazil	Water buffalo	(Rodrigues et al., 2004)
NcNZ-1	New Zealand	Cattle	(Okeoma et al., 2004b)
NcNZ-2	New Zealand	Cattle	(Okeoma et al., 2004b)
NcNZ-3	New Zealand	Cattle	(Okeoma et al., 2004b)
NC-WTDVA-1	USA	Deer	(Vianna et al., 2005)
NC-WTDVA-2	USA	Deer	(Vianna et al., 2005)
NC-WTDVA-3	USA	Deer	(Vianna et al., 2005)
WA-K9	Australia	Dog	(McInnes et al., 2006a)

1.15 Diagnosis of Neosporosis

Clinical signs of neosporosis in cattle other than abortion have been reported in newborn and young calves. Signs may include the neurological signs of ataxia, decreased patellar reflexes, loss of proprioception, hydrocephalus, exophthalmia, inability to rise, below average birth weight and limbs may be flexed or hyper extended (Barr et al., 1993; Barr et al., 1991b; De Meerschman et al., 2005; Dubey et al., 1998a; Dubey and de Lahunta, 1993; Dubey et al., 1990a).

Diagnosis of *N. caninum* in cattle showing no clinical signs of neosporosis is well established, especially for discriminating *N. caninum* from *T. gondii* and other similar parasites. Comprehensive diagnostic techniques utilizing serologic, electronmicroscopical, pathological examination, immunohistochemical, PCR, immunoblotting and other methods are commonly used to demonstrate chronic and acute infection both in infected cattle and in aborted foetuses (Dubey and Schares, 2006; Hemphill, 1999).

1.15.1 Serological studies

Serological studies may provide information on the stage of *N. caninum* infection. Following an initial infection with tachyzoites or oocysts, specific IgG_1 levels rise, followed by a slightly delayed rise in IgG_2 (Andrianarivo et al., 2001; De Marez et al., 1999; Williams et al., 2000). The avidity (functional affinity) of specific antibodies to *N. caninum* increases over time after a primary infection and can provide some information about the duration of infection (Bjorkman et al., 2005). A low avidity IgG response indicates a recent infection of approximately 2 months, whereas high avidity IgG is indicative of a chronic infection, usually infected for 6 months or more (Bjorkman et al., 2003).

1.15.1.1 Indirect Detection Techniques

These are techniques that can detect specific antibodies against *N. caninum* demonstrating exposure to the disease but cannot prove that an animal is currently acutely or chronically infected. However, these methods are useful for epidemiological studies to show exposure and risks of acquiring *N. caninum* and can sometimes lead to identification of animals which are more likely to be harbouring an active or recent infection as opposed to a chronic infection. All serological assays are based on tachyzoite antigens. There are not yet assays based on bradyzoite or sporozoite antigens.

1.15.1.2 Neospora Agglutination Test (NAT)

A *Neospora* agglutination test (NAT) can detect specific IgM and IgG antibodies that appear a few days after a primary infection. Serum IgG levels increase in the weeks following infection up to 3-6 months, while levels of IgM peak at 2 weeks following infection and drop below detection at 4 weeks post infection (Conrad et al., 1993b; De Marez et al., 1999). The NAT test

relies on whole fixed tachyzoites to detect specific antibodies in the serum to tachyzoite surface antigens (Packham et al., 1998; Romand et al., 1998).

1.15.1.3 Indirect Fluorescent Antibody Test (IFAT)

Cell culture derived *N. caninum* tachyzoites are fixed and used for the detection of specific antibodies in the serum. If antibodies are present they bind to the parasites and can be visualized using fluorescent reagents. The IFAT is the most widely used detection method and is very specific to *N. caninum* with little or no cross-reaction to *T. gondii* or other similar parasites (Dubey et al., 1996a; Hemphill, 1999). The first IFAT for *N. caninum* was created following the first isolation of *N. caninum* from dogs and was used to detect antibodies in dog and mouse serum (Dubey et al., 1988b).

The IFAT can be used to detect antibodies from peripheral blood, cerebrospinal fluid and from maternal and foetal sera (Barr et al., 1995; Hemphill, 1999). A positive sample is recognized by the entire surface of the tachyzoite fluorescing. Apical fluorescence may occur in uninfected animals is not considered a positive result as this is due to unspecific binding of serum component within the apical area (Dubey and Lindsay, 1996). False-positive results are likely to occur if tachyzoites have been grown in FBS as most batches of FBS contain antibodies to *N. caninum*. Tachyzoites can be grown in serum free media or IgG free horse serum (Hemphill, 1999; Torres and Ortega, 2006). IFAT titres decrease over a couple of months following abortion or in early pregnancy, which can lead to the missed diagnosis of a positive animal (Conrad et al., 1993b).

Results of an IFAT are not objective but rather are a subjective evaluation, therefore variation exists between different laboratories. Differences in buffers, incubation conditions, conjugates and the quality of the fluorescence microscope can influence detection limits. Additionally laboratories may vary the titre used to indicate a positive reaction (Hemphill, 1999; Wapenaar et al., 2006). An IFAT can identify exposure to *N. caninum* but cannot identify whether parasites are physically present in the animal.

1.15.1.4 Enzyme-Linked Immunosorbent Assay ELISA

Diagnosis of *N. caninum* using an ELISA is advantageous as it provides objective results due to automation of the procedure that measures changes in absorption. However, high background and non-specific reactions can be problematic dependent on the type of antigen tested (Dubey and Lindsay, 1996). The first ELISA used a crude lysate of *N. caninum* tachyzoites as antigen to detect positive bovine sera. The antigen is coated onto the surface of a multi-well plate. Serum samples are added to the wells, followed by a conjugate antibody and a substrate that causes an enzyme-substrate reaction producing a colour change that can be measured with a spectrophotometer. Comparison of the ELISA against an IFAT proved that the ELISA was at least as specific as the IFAT at a specific cutoff. Additionally, the outcome was not affected when either dog (NC-1) or bovine sourced (BPA-1) tachyzoites were used (Pare et al., 1995). Several ELISAs have since been developed to examine sera and utilize whole or fixed tachyzoites,

tachyzoite extracts and single native or recombinant tachyzoite antigens (Dubey and Schares, 2006). These different applications for antigen preparation have been developed in order to increase the sensitivity and specificity of the ELISA to serum antibodies.

Bjorkman et al. (1994) modified a procedure for using iscoms (immunostimulating complexes), which are used as adjuvants and carriers of immunogens or for the selection of surface membrane proteins, to incorporate parasite proteins. The iscom ELISA is highly sensitive and specific and has been used for detection of *N. caninum* antibodies in dogs, cattle and in the milk of cattle (Bjorkman et al., 1997; Bjorkman and Lunden, 1998; Bjorkman et al., 1994). The ability of the *N. caninum* iscom avidity ELISA to accurately assess the duration of infection has also been validated (Bjorkman et al., 2005).

A kinetic-ELISA that used water-soluble antigens to capture antibodies was developed and optimized (Pare et al., 1995). The ELISA was used on cattle sera and gave sensitive and specific results when compared to an IFAT. However, some cross-reaction did occur with sera infected with *Sarcocystis cruzi.*

Lally et al first reported the use of recombinant antigens in immunodiagnosis of *N. caninum* (Lally et al., 1996b). An *N. caninum* tachyzoite cDNA expression library was screened with sera from infected cattle. Two cDNA clones expressing antigens were identified and subcloned into a plasmid expression vector (pTrcHisB) and expressed in *E. coli.* Antibodies that bound to the recombinant antigens bound to a 33 and 36kDa protein localizing in the dense granules of *N. caninum* tachyzoites (Lally et al., 1997). An ELISA utilizing these recombinant antigens could discriminate between *N. caninum* and *T. gondii* and was very sensitive (Jenkins et al., 1997). However, the antigens could not be used for detection of foetal antibodies (Wouda et al., 1997a). Other recombinant antigens have also been produced using similar methods and have been shown to be more sensitive and at least as specific as whole tachyzoite lysate ELISA tests (Louie et al., 1997; Nishikawa et al., 2001a). Recombinant antigens can easily be produced in large quantities and can be standardised for the production of serological assays (Liao et al., 2005a). Care must be taken during purification of antigens as a bad preparation may lead to false-positive results (Jenkins et al., 2005).

1.15.1.5 Rapid Immunochromatographic test (RIT)

An immunochromatographic test (ICT) is a simple rapid method, which makes it suitable for clinical and field applications. Recombinant antigens are used for RITs as they require larger amounts of antibody than ELISAs. Liao et al have developed a RIT using the recombinant surface antigen 1 of *N. caninum* (NcSAG1) fused to glutathione S-transferase (GST-NcSAG1t). The method is sensitive and specific to *N. caninum* antibodies in cattle (Liao et al., 2005b).

1.15.1.6 Immunoblot

Immunoblot techniques can be used to detect antibodies found in sera, milk, or cerebrospinal fluid. Detection of antibodies in milk can be used as a less invasive test and has shown strong agreement with testing on sera (Ortega et al., 2006).

A western blot can be used to detect specific antigens as opposed to whole parasites. In a western blot, antigens migrate through an SDS-PAGE gel by electroporation and are separated depending on size and charge, therefore antigenic profiles can be revealed with western blots (Barber et al., 1995). Antigens are transferred to a membrane and exposed to serum, which binds to specific antibodies found on the membrane. Variations in antigenic profiles have been observed for *Neospora*-infected cattle serum and interspecies serum (Harkins et al., 1998; Pare et al., 1995). No major differences in antigenic profile have been found between different *Neospora caninum* isolates (Barber et al., 1995; Baszler et al., 1996; Hemphill et al., 1997; Hemphill and Gottstein, 1996; Lee et al., 2004). Stage specific antigens, such as tachyzoite versus bradyzoite, may vary in antigenic profile and in serum recognition of the antigens (Pare et al., 1995). The stage at which the blood or fluid sample was taken will also affect the antigens recognized (Tomioka et al., 2003). The source of examined fluid was also shown to be important. Peritoneal fluid was observed to be very sensitive and reliable for use (Sondgen et al., 2001) but a multitude of different fluids have been tested using immunoblots, including semen (Ortega-Mora et al., 2003). Immunoblotting is now accepted as a useful technique for serodiagnosing *N. caninum* infection (Hasler et al., 2006; Silva et al., 2007; Staubli et al., 2006).

A dot blot is a crude antigen detection method that utilizes a mixture of antigens and is directly applied to a membrane in a single dot rather than separating out specific antigens. A dot blot will give a positive/negative result whereas a western blot will detect the specific antigen the antibody is raised against (Bouillet et al., 1998).

Reduced and non-reduced parasite antigens in Western blots have been used to characterize the *N. caninum* antigens recognized by hyperimmune rabbit serum. They found antibodies recognised the non-reduced parasite antigen preparations more strongly than the reduced antigen preparations. Antigens were localised strongly to some organelles of *N. caninum*, in particular the dense granules, micronemes and the posterior portion of the rhoptries (Barta and Dubey, 1992; Pare et al., 1995). Bjerkas et al., (1994) found that sera from a wide range of animals recognised non-reduced immuno-dominant 17-, 29-, 30- and 27-kDa antigens.

Some antigens cross reacted with *T. gondii* but not the immuno-dominant antigens and this was also true for the *T. gondii* immuno-dominant antigens (Bjerkas et al., 1994). The strong interaction with the non-reduced antigens suggests that conformational epitopes are predominantly involved in *N. caninum*-specific antibody responses. Cross reaction with *T. gondii* and other similar parasites is less common when using a non-reduced antigen compared to a reduced antigen, which might be because conformational epitopes may be more species specific than linear epitopes (Atkinson et al., 2000; Baszler et al., 1996; Dubey and Schares, 2006).

1.15.2 Direct Detection

1.15.2.1 Pathological lesions

Pathological lesions are typically associated with neosporosis and are therefore an important diagnostic tool (Dubey et al., 2006), particularly as *N. caninum* tissue cysts have almost exclusively been found in the CNS tissue, as opposed to *T. gondii* cysts, which are generally found intramuscularly (Dubey and Lindsay, 1996). Inflammatory lesions produced by *N. caninum* tachyzoites are commonly found in the heart, liver and CNS tissue and occasionally the kidney and lung of foetuses and stillborn calves. Lesions in the placenta appear to be confined to the cotyledons. Lesions are less commonly found in cattle over the age of 2 months but have been noted in CNS, liver, kidney and heart (Dubey and Schares, 2006; Piergili Fioretti et al., 2000). The presence of lesions alone cannot be used to make a definitive diagnosis and antibody specific staining should be used to confirm a diagnosis as other parasites, such as *Sarcocystis,* may also cause similar lesions (Lindsay and Dubey, 1989a).

1.15.2.2 Transmission electron microscopy (TEM)

Parasites can be visualized using TEM to determine the number of rhoptries, the position of the micronemes and other morphological features. Viewing of slides is time consuming and requires screening a large number of samples as the tissue selected may not harbour parasites. Care must also be taken during fixation of the sample as morphological features can vary, particularly if fixation and processing is not standardised (Hemphill, 1999).

1.15.2.3 Hematoxylin and eosin (H&E) sections

Historically, tachyzoites are difficult to recognize in H&E sections as well preserved whole tachyzoites are rarely observed in preserved sectioned tissue. Tachyzoites can on occasion be identified by the vesicular nucleus, which distinguishes them from degenerating host cells (Dubey et al., 2006). Bradyzoites may stain red with periodic acid Schiff (PAS) as they have a terminally located nucleus (Dubey et al., 2002). H&E is more useful when used in combination with immunohistochemistry.

1.15.2.4 Immunohistochemistry (IHC)

Immunohistochemical methods can be used to detect *N. caninum* tissue cysts and distinguish them from *T. gondii* tissue cysts using anti-sera and the avidin-biotin peroxidase complex (ABPC) (Lindsay and Dubey, 1989a). Sections of tissue are stained with the primary anti-sera, followed by a biotinylated IgG. The ABP complex binds to the bound antibodies and is stained using a reactive chromogen such as 3,3'-diaminobenzidine tetrahydrochloride (DAB), which stains brown, or 3-amino-9-ethylcarbazole (AEC), which stains red.

Polyclonal antisera against *N. caninum* tachyzoites was raised in rabbits and used for immunohistochemical detection of *N. caninum* parasites in tissue sections following the discovery of the new species (Bjerkas and Presthus, 1988; Lindsay and Dubey, 1989a). Cole et al produced a murine monoclonal antibody that reacted positively with *N. caninum* tachyzoites and bradyzoites but did not react with *T. gondii* or 13 other protozoa tested (Cole et al., 1993). Both polyclonal and monoclonal anti-*Neospora* antibodies have been raised against *N. caninum* and are available commercially (Cole et al., 1994; Jenkins et al., 2002; Lindsay and Dubey, 1989a; Peters et al., 2001). It is difficult to distinguish between tachyzoites and tissue cysts unless a bradyzoite specific antibody is used (McAllister et al., 1996b).

Cross-reaction with *T. gondii* or *Sarcocystis* species may be encountered using IHC and may be associated with non-specific staining of the apical complex or dense granules (Sundermann et al., 1997). Specificity may vary between batches of serum, the animal the serum was raised in, the type of tissue treated, how the tissue has been processed and general cross reactivity due to non-specific binding (McAllister et al., 1996c; Sundermann et al., 1997). A murine monoclonal antibody is reported to be specific only to *N. caninum* and does not cross-react with *T. gondii.* It recognises both *N. caninum* tachyzoites and bradyzoites (Cole et al., 1993) and can be incorporated into a *Neospora* iscoms (Bjorkman et al., 1994).

Immunohistochemical detection of *N. caninum* is labour intensive and frequently few or no tachyzoites or cysts can be found due to the large volume of tissue and the relatively low numbers of parasites (Conrad et al., 1993a; Dubey, 1999; Dubey et al., 1998a). Tissue must be harvested quickly and fixed, particularly in the case of aborted foetuses as tachyzoites quickly die during host cell autolysis (Conrad et al., 1993a). However, IHC can sometimes be used more reliably than serological diagnostic tools for the detection of infection in foetuses as antibody synthesis in the foetus depends on the stage of gestation, level of exposure and the time between infection and abortion (Barr et al., 1995; Wouda et al., 1997a).

1.15.2.5 Polymerase Chain Reaction (PCR)

PCR provides a sensitive and specific test for *Neospora* DNA that may be present in body fluids and tissue samples. The sensitivity of PCR means that very small quantities of starting material are required.

A PCR procedure usually consists of three stages, DNA is denatured at high temperature (around 90°C), followed by a decrease in temperature for primer annealing (around 50°C) to complementary DNA sequences and then primer extension to create new strands of DNA (around 70°C). The three stages of PCR are known as a cycle, the cycles are repeated to amplify the DNA products.

PCR has been developed for the extraction of DNA from fresh, frozen and formalin-fixed or paraffin-embedded tissues (Baszler et al., 1999a). Several different types of PCR have been developed for use, such as standard PCR (Holmdahl and Mattsson, 1996; Kaufmann et al., 1996; Yamage et al., 1996), semi-quantitative PCR (Liddell et al., 1999b, 1999c), single tube nested

PCR (Ellis et al., 1999a; Lally et al., 1996a) or PCR followed by probe hybridization (Ho et al., 1997a; Muller et al., 1996).

The progression of PCR as a diagnostic tool for *N. caninum* was based on the assumption that canine and bovine neosporosis was caused by the same species (Ellis, 1998). Evidence did suggest that this was the case as sequences derived from both bovine and canine strains were identical (Ellis et al., 1998; Marsh et al., 1995; Stenlund et al., 1997).

Initially rDNA was used for the development of PCR due to a lack of knowledge relating to organism-specific genes. A comparison between *N. caninum* and *T. gondii* comparing the 18S rRNA genes showed they differed by only a small number of nucleotide positions (Ellis et al., 1994; Holmdahl et al., 1994). Sequence variation was determined by riboprints (Brindley et al., 1993), which clearly allowed the differentiation of *N. caninum* from *T. gondii* DNA and confirmed sequence variation of the two taxa in the 18S rDNA sequence. Sequence mismatch at the 3' end of the primer was found to increase specificity (Kwok et al., 1990), this is due to Taq polymerase lacking 3' exonuclease activity (Tindall and Kunkel, 1988). A mismatch 3-5 base pairs from the 3' end enabled specific amplification from *Neospora* and not *Toxoplasma* (Ellis, 1998). A more robust PCR method was developed (Ho et al., 1996) based on a conserved region of the ssrRNA gene. With this method, DNA from *N. caninum* could be detected in bovine brain, spinal cord, heart, lung, kidney, diaphragm, skeletal muscle, placenta and amniotic fluid. However, PCR based on rDNA may have issues with sensitivity as the sequence homology between *Toxoplasma* and *Neospora* is highly conserved (Ellis et al., 1994; Holmdahl et al., 1994).

Single tube nested PCR using internal transcribed spacer 1 (ITS) targeted primers was found to be robust in producing species-specific amplification but efficiency may be reduced if PCR products produced are large. The procedure amplifies using first external, followed by nested primers in a single tube (Holmdahl and Mattsson, 1996; Payne and Ellis, 1996). The nested single tube PCR can detect as little as 10fg of genomic DNA. The extremely high sensitivity of the method can occasionally lead to false positive results, particularly if aseptic technique is not strictly maintained (Ellis, 1998). The ITS 1 PCR detected *Neospora* DNA from experimentally infected mice in the cerebrospinal fluid, buffy coat cells, amniotic fluid, placenta, spinal cord, and in the heart and brain of experimentally infected ewes and foetuses (Hemphill, 1999).

Highly repeatable DNA sequences isolated from culture derived *N. caninum* tachyzoites were cloned into vectors and screened for cross-reaction with *T. gondii* DNA to identify sequences specific to *N. caninum* (Kaufmann et al., 1996). This type of PCR can be very sensitive, detecting as little as 100 pg of parasite DNA. Yamage et al., (1996) used a similar technique to screen oligonucleotides for *N. caninum* specificity. The resulting primer combinations could be used to detect as little as 10pg of genomic parasite DNA. The PCR was also able to detect a single parasite in a background of DNA derived from 2mg of brain tissue.

PCR internal controls can be used to assist in the development of quantitative procedures. The aim of quantitative PCR is to determine the quantity of target DNA sequences present in the specimen studies in order to correlate findings with parasite numbers (Collantes-Fernandez et al., 2002; Muller et al., 2002). Liddell et al., (1999b) developed the first quantitative-competitive PCR that could evaluate the levels of parasites in the brains and lungs of mouse pups following

vertical transmission. Competitive PCR effectively controls for tube-to-tube variation providing the ability to estimate the number of parasites in a background of host DNA. Incorporating a competitor molecule provides a control for PCR failure and identifies samples as true negatives. Quantitative PCR can be used to assess activity of new vaccines, therapies and drugs and for examining the pathogenesis of neosporosis (Cannas et al., 2003a; Cannas et al., 2003b; Esposito et al., 2005). All published quantitative PCR have been based on the pNc5 gene (Dubey and Schares, 2006). Real-time PCR is less labour intensive than artificial competitor techniques. A real-time PCR07 technique was developed, which was based on detection of PCR products by specific fluorescent probes (Muller et al., 2002). Another approach uses double-strand DNA-binding dye SYBR Green 1 to detect the number of tachyzoites in aborted foetus brain samples allowing for estimates to be made of parasite load in tissues at different stages of gestation (Collantes-Fernandez et al., 2006; Collantes-Fernandez et al., 2002).

PCR techniques have been developed for the detection of *Neospora* parasites in formalin fixed, paraffin-embedded sections of mouse, dog and cattle tissue (Baszler et al., 1999a; Ellis et al., 1999a; Loschenberger et al., 2004), for detecting oocysts in dog and coyote faeces (Basso et al., 2001a; Hill et al., 2001; McGarry et al., 2003; Slapeta et al., 2002), identifying *N. caninum* DNA in the blood of infected cattle (Ferre et al., 2005; Okeoma et al., 2004a), in the semen of bulls (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ortega-Mora et al., 2003) and in milk of lactating cows (Moskwa et al., 2006).

Most PCR methods developed for the diagnosis of *Neospora* were developed prior to the discovery of *N. hughesi* (Marsh et al., 1998). *Neospora hughesi* was isolated from the central nervous system of a horse. Differences in ultrastructural, antigenic and molecular data distinguished *N. hughesi* from *N. caninum,* supporting the recognition of a new species within the *Neospora* genus. Sequence information from the ITS1 and 28S rDNA of *N. hughesi* was compared to *N. caninum* PCR primer sequences and all differed by at least 1 base pair (Dubey and Schares, 2006). Selected primers from the pNc5 fragments, Np6 and Np21 also did not amplify *N. hughesi* DNA (Spencer et al., 2000), however not all primer pairs have been examined for *Neospora* species specificity. Despite the existence of many PCR primers available for the detection of *N. caninum*, no *N. hughesi*-specific PCR is currently available (Dubey and Schares, 2006).

Table 1.3: *Serological assays developed to detect antibodies in Neospora caninum-infected cattle.* Table derived from (Dubey and Schares, 2006)

Type of test	Test characteristics	Reference	Comment
Direct agglutination test	Whole fixed tachyzoite	(Packham et al., 1998)	Cell-culture-derived
		(Romand et al., 1998)	Mouse-derived
IFAT	Whole fixed tachyzoites	(Conrad et al., 1993b)	Air-dried
		(Buxton et al., 1997)	Formaldehyde fixed, air-dried
		(Schares et al., 1998)	Air-dried, acetone fixed
ELISA	Whole tachyzoite lysate antigen indirect ELISA (various lysate protocols)	(Pare et al., 1995)	Extracted with PBS, kinetic ELISA
		(Dubey et al., 1996a)	Extracted with PBS
		(Reichel and Drake, 1996)	Commercial antigen
		(Osawa et al., 1998)	Extracted with distilled water
		(Gottstein et al., 1998)	Extracted with PBS
		(Wouda et al., 1998a)	Extracted with PBS, 1% Triton X-100
		(Bae et al., 2000)	
	Fixed whole tachyzoite indirect ELISA	(Williams et al., 1997)	Formaldehyde fixed
	ISCOM antigen indirect ELISA	(Bjorkman et al., 1997)	Based on (Bjorkman et al., 1994)
	Recombinant antigen indirect ELISA	(Lally et al., 1996b)	rNcGRA6 and rNcGRA7
		(Louie et al., 1997)	rNcGRA7 and recombinant subtilisin-like serine protease
		(Nishikawa et al., 2001b)	rNcSRS2
		(Howe et al., 2002)	Truncated rNcSRS1
		(Chahan et al., 2003)	Truncated rNcSRS1
		(Ahn et al., 2003)	Truncated rNcSRS2
		(Jenkins et al., 2005)	rNcGRA6; HPLC purified
		(Gaturaga et al., 2005)	Truncated rNcSRS2
	Single native antigen indirect ELISA	(Schares et al., 2000)	NcSRS2
	Antigen-capture indirect ELISA	(Schares et al., 1999)	Polyclonal antiserum for antigen capture
	Antigen-capture competitive inhibition ELISA	(Dubey et al., 1997)	Monoclonal antibody for antigen capture
		(Baszler et al., 2001)	
	Competitive inhibition ELISA	(Baszler et al., 1996)	Based on a monoclonal antibody
		(Hemphill and Gottstein, 2000)	Based on a polyclonal antibody
		(McGarry et al., 2000)	
	Avidity ELISA	(Bjorkman et al., 1999)	ISCOM incorporated antigen
		(Maley et al., 2001)	Whole tachyzoite lysate
		(Schares et al., 2002)	NcSRS2
		(Sager et al., 2003)	Whole tachyzoite lysate
	Bulk-milk ELISA	(Bjorkman et al., 1997)	ISCOM incorporated antigen
		(Schares et al., 2003)	NcSRS2
		(Bartels et al., 2005)	Whole tachyzoite lysate
Immunoblot	Reduced whole tachyzoite antigen	(Bjerkas et al., 1994)	
	Non-reduced whole tachyzoite antigen	(Bjerkas et al., 1994)	
	Avidity Western blot	(Aguado-Martinez et al., 2005)	
RIT (rapid immunochromato-graphic test)	Recombinant antigen RIT	(Liao et al., 2005b)	Truncated rNcSRS1

Table 1.4: *Analytical sensitivity and specificity of polymerase chain reactions for the detection N. caninum DNA.*

Table derived from (Dubey and Schares, 2006).

Target DNA	Primer names	Type of PCR	Original description of analytical sensitivity	Parasites used to test analytical specificity	Reference
18S rDNA	COC-1, COC-2	One-step PCR + hybridisation	1 tachyzoite in medium, 5 tachyzoites in blood or amniotic fluid	*T. gondii, S. cruzi, S. tenella, S. capracanis, C. parvum, E. bovis*	(Ho et al., 1996)
18S rDNA	COC-1, COC-2	One-step PCR + restriction enzyme	ND	*T. gondii*	(Magnino et al., 1999)
18S rDNA	AP1, D SP4, A	Two-step nested PCR	ND	*T. gondii, S. cruzi*	(Ellis, 1998)
28S rDNA	GA1, NF6	One-step PCR	ND	*T. gondii, H. hammondi, B. besnoiti*	(Ellis et al., 1998)
ITS1	NS1, SR1	One-step PCR	ND	*T. gondii, S. cruzi*	(Payne and Ellis, 1996)
ITS1	PN1, PN2	One-step PCR	5 tachyzoites heated 2 min at 100°C in distilled water	*T. gondii, S. cruzi, S. fusiformis, S. gigantea, S. tenella*	(Holmdahl and Mattsson, 1996)
ITS1	NN1, NN2 NP1, NP2	Two-step nested PCR	ND	ND	(Buxton et al., 1998)
ITS1	TIM3, TIM11 NS1, SR1	Two-step nested PCR	ND	See (Payne and Ellis, 1996)	(Ellis, 1998)
ITS1	F6, 5.8B PN3, PN4	Two-step nested PCR	ND[a]	ND	(Uggla et al., 1998)
ITS1	NS2, NR1, NF1, SR1	One-step nested PCR	10–1 fg DNA (0.1–0.01 tachyzoites)	*T. gondii, S. cruzi*	(Ellis et al., 1999a; Ellis et al., 1999b)
pNc5 gene	Np1, Np 2	One-step PCR	100 pg genomic tachyzoite DNA	*T. gondii, S. cruzi, S. ovifelis, S. capracanis, S. moulei, S. miescheriana*	(Kaufmann et al., 1996)
pNc5 gene	Np6, Np21	One-step PCR	1 tachyzoite in 1 mg brain tissue	*T. gondii, H. hammondi, S. cruzi, S. tenella, S. capracanis, S. moulei, S. miescheriana, H. heydorni*[b], *Toxocara canis*[b]	(Yamage et al., 1996)
pNc5 gene	Np6plus, Np21plus	One-step PCR	DNA equivalent to 1–10 tachyzoite genomes	*T. gondii, H. hammondi, S. cruzi, S. tenella, S. capracanis, S. moulei, S. miescheriana*	(Muller et al., 1996)
pNc5 gene	Np6plus, Np21plus	One-step PCR + hybridisation	DNA equivalent to 1 tachyzoite genomes	*T. gondii, H. hammondi, S. cruzi, S. tenella, S. capracanis, S. moulei, S. miescheriana*	(Muller et al., 1996)
pNc5 gene	Np4, Np7	One-step PCR	1–2 tachyzoite equivalents per DNA sample (150 ng brain tissue DNA)	See (Yamage et al., 1996)	(Baszler et al., 1999a)
pNc5 gene	Np4, Np7 Np6, Np7	Two-step semi nested PCR	ND; sensitivity of semi nested PCR was not superior to one-step Np6–Np7 PCR	See (Yamage et al., 1996)	(Baszler et al., 1999a)

pNc5 gene	Np6plus, Np21plus	One-step quantitative PCR	9 fg DNA per 250 ng of mouse DNA	See (Muller et al., 1996)	(Liddell et al., 1999a; Liddell et al., 1999b)
pNc5 gene	Np6plus, Np21plus	Real-time PCR (probes)	DNA equivalent to 1 tachyzoite	See (Muller et al., 1996)	(Muller et al., 2001)
pNc5 gene	Np4B, Np21B	One-step PCR	ND	See (Yamage et al., 1996)	(Bergeron et al., 2001)
pNc5 gene	Nc5fwd, Nc5rev	Real-time PCR (Cyber green)	DNA equivalent to 0.1 tachyzoite genomes (10 fg) in 100 ng mouse brain DNA	*T. gondii*	(Collantes-Fernandez et al., 2002)
14–3–3 gene	Nc13F3, Nc13R2 Nc13F1, Nc13R4	Two-step nested PCR	25 tachyzoites in 5 mg brain tissue	*T. gondii, S. muris, S. tenella, S. cruzi*	(Lally et al., 1996a, 1996b)

ND, no data.
[a] Some information on analytical sensitivity in (Guy et al., 2001).
[b] Analysed by (Hill et al., 2001).

1.16 Neosporosis in New Zealand

In 1991 a paper recognizing bovine *Neospora* was published. Historical samples taken between 1987-1990 were re-examined and lesions caused by *N. caninum* were found in the brain, heart, and placental tissues (Thornton et al., 1991). *Neospora* was later recognized in dog tissues (Patitucci et al., 1997; Reichel et al., 1998).

Sero-epidemiological studies were performed to assess the extent of infection within bovine herds and to determine whether abortion and serostatus were associated. It was reported that the prevalence of anti-*Neospora* antibodies in dairy cattle that have aborted was around 40% (Reichel and Drake, 1996). A serological study found that although antibody levels were maximal within the weeks of an abortion they quickly returned to low levels and recrudescence of antibody titre did not lead to abortion in subsequent pregnancies (Cox et al., 1998). *Neospora* infection was reportedly responsible for 35% of abortions in New Zealand dairy cattle (Thobokwe and Heuer, 2004). The incidence of positive *Neospora* antibody titres in beef cattle was found to be much lower than dairy cattle with an overall prevalence of 2.8% (Tennent-Brown et al., 2000). A large serological study that looked at the prevalence of *N. caninum* in sheep/beef (n=154), dairy (n=161) and urban dogs (n=150) found that 30.7% of urban dogs, 74.5% of dairy and 96.8% of sheep/beef dogs were serologically positive for *N. caninum* antibodies using an IFAT (Antony and Williamson, 2003).

A New Zealand strain of *N. caninum* had not been isolated at the time of this study but has since been isolated. Three isolates have been generated, two from the brains of neonatal calves and one from the brain of an adult cow (Okeoma et al., 2004b).

1.17 Economic Impact of Neosporosis

The main economic loss associated with neosporosis is reproductive failure, milk yield, rebreeding, replacement of stock and professional help and expenses associated with diagnosis of

the disease are also significant concerns (Dubey et al., 2007). In 1998 the economic impact of neosporosis resulting in lost milk, meat and cattle through abortions was estimated to be NZ$17.8 million/annum (Pfeiffer et al., 1998) and a combined loss between New Zealand and Australia of $100 million Australian per annum (Reichel, 2000). In California it is estimated that losses reach about US$35 million per year (Barr et al., 1998).

The effect of *N. caninum* seropositivity on milk production is still to be settled. Studies in America have reported a decrease in milk production in seropositive cattle, approximately 1kg less milk in California (Thurmond and Hietala, 1997), or 3-4% decline in Florida equating to a loss of $128 per cow (Hernandez et al., 2001). In Costa Rica seronegative cows produced 84.7 litres more milk in 305 days (Romero et al., 2005). Yet in Canada they found no association between serostatus and milk production (Dubey et al., 2007). However, in New Zealand a positive association was found with *N. caninum* seropositivity and milk production (Pfeiffer et al., 2002).

Neospora antibody status has also been shown to be associated with calf weight gain, with a loss of approximately $16 per calf (Barling et al., 2000). Losses on beef farms have not accurately been determined due to difficulty of monitoring when small fetuses are expelled (Dubey et al., 2007).

In New Zealand and Australia, the control strategy of "no intervention" is optimal for herds with an 18 or 21% seroprevalence over a 1 or 5 year period but for herds with a higher seroprevalence, vaccination is the best option (Dubey et al., 2007; Reichel and Ellis, 2006).

1.18 Control Measures

Preventing the introduction of *N. caninum* to a naïve herd through biosecurity measures is the primary goal for most farms (Haddad et al., 2005). For herds that are infected with *N. caninum*, control programs to decrease vertical transmission and infection through oocyst ingestion are commonly used. Farm biosecurity measures such as quarantine and testing of replacement and purchased cattle, rodent control, prevention of waterborne transmission, prevention of transmission by definitive hosts and prevention of putative factors that may induce disease recrudescence should all be considered. Reproductive management to control infected embryo transfer and infection through artificial insemination have been proposed as sources of transmission. Infected cattle may need to be removed from the herd through testing and culling but this technique is only recommended for farms with predominantly endogenous vertical transmission of infection. Chemotherapy and vaccination may be considered but the long term effects and economic costs should be carefully considered (Dubey et al., 2007).

1.19 Vaccine development

An effective vaccine against *N. caninum* should protect against foetal loss and vertical transmission. Serological discrimination between vaccinated and infected animals would need to be available for infection control (Dubey et al., 2007). The age of the cattle appears to be

important for determining the nature of the immune response following initial infection and some in utero foetal immunotolerance to parasite development may exist (Innes et al., 2001b; Williams and Trees, 2006).

As *N. caninum* is an intracellular parasite, cell mediated immunity, in particular the cytokine interferon gamma and CD4 T cells should be considered as critical in combating infection (Innes et al., 2002; Omata et al., 2006). Abortion and vertical transmission occur during gestation when the immune system is suppressed to protect the foetus from the maternal antibodies, which is likely to affect the dams ability to control the infection particularly as recrudescence of infection is more likely during pregnancy (Innes et al., 2002). Identification of specific antibodies used by parasite-specific CTLs to kill *N. caninum* infected cells could be used to develop immunization strategies (Staska et al., 2003). Induction of pro-inflammatory responses to *N. caninum* during pregnancy may be detrimental to the foetus if maternal transfer of the parasite has occurred (Innes et al., 2002). Infection transmission to the fetus apparatus to be related to the gestational age and immunocompetence of the fetus (Collantes-Fernandez et al., 2006; Innes et al., 2002)

Animals vaccinated with inactivated *N. caninum* vaccines cannot accurately be evaluated using serological methods to determine infection status at a later stage as they develop antibody responses similar to those of naturally infected cattle (Moore et al., 2005). Vaccine markers detectable by serological tests may be required in order to distinguish between antibody responses to vaccination as opposed to antibody responses to naturally infected animals (Dubey and Schares, 2006).

A POLYGEN-adjuvanted killed *N. caninum* tachyzoite preparation was unable to protect against fetal infection following challenge of pregnant dams (Andrianarivo et al., 2000). In New Zealand, a HAVLOGEN-adjuvanted killed *N. caninum* tachyzoite vaccine (NeoGuard) gave protection to two of five herds studied with an efficacy of 5.2% to 54% (Heuer et al., 2004). The overall efficacy of the vaccine determined from trials in New Zealand and Costa Rica was 46% (Dubey et al., 2007).

Challenge with live tachyzoites during gestation has protected against foetal death whereas vaccination with whole-tachyzoite lysate did not protect despite strong antibody responses being seen in both the live and killed vaccination groups (Williams et al., 2007).

Challenge with *N. caninum* does not protect mice against *T. gondii* infection suggesting that *N. caninum* and *T. gondii* are distinct and cannot be used to infer protection from each other (Lindsay et al., 1990). However, *N. caninum* can give some protections particularly against a lethal *T. gondii* infection by inducing CD8+ T cells that are immunoreactive to both parasites (Kasper and Khan, 1998; Lindsay et al., 1998). Vertical transfer of *N. caninum* in mice can be protected against using crude lysate of *N. caninum* tachyzoites (Liddell et al., 1999a). Others have reported that vaccination of mice with a live temperature sensitive strain of *N. caninum* induces significant protection but killed tachyzoites did not (Lindsay et al., 1999b), similar studies have been performed in sheep using ToxoVax® (Innes et al., 2001a) and partial protection has been observed using killed tachyzoite vaccines (Jenkins et al., 2004; O'Handley et al., 2003). Studies directed at vaccination of the definitive host using recombinant canine herpes virus expressing *N. caninum* surface protein found that IgG antibodies against *N. caninum* were produced and clinical symptoms of neosporosis were avoided (Nishikawa et al., 2000a).

Recombinant viruses have also been used to show antibody production in host animals (Nishikawa et al., 2000b; Nishikawa et al., 2001c).

To date, no vaccine has shown protection from endogenous transplacental transmission. Recrudescence of a latent infection in naturally infected pregnant heifers immunized with killed tachyzoites or left untreated reportedly occurs regardless of immunization status and immunizing with killed vaccines does not protect against vertical transmission (Andrianarivo et al., 2005).

A highly conserved circular 35 kb DNA plastid has been identified from several genomes, including *Neospora* and *Toxoplasma*, within the Apicomplexa phylum (Gleeson and Johnson, 1999; Lang-Unnasch and Aiello, 1999; Zhu et al., 2000). The plastid is found in the apicoplast and is believed to be derived from either red or green algae. Inhibition of apicoplast DNA synthesis in *T. gondii* blocks replication of the parasite, suggesting a critical role in survival. This type of chloroplast-derived organelle is absent in humans and other mammals which means that the apicoplast genome or function could be a target for successful drug development against Apicomplexa (Zhu et al., 2000).

Chapter 2.

2.0 Cell Culturing Techniques for *Toxoplasma gondii* and *Neospora caninum* Tachyzoite Propagation

2.1 Introduction

Cell culturing of *N. caninum* parasites has been established as the most widely used and effective method for maintaining parasites long term and for increasing parasite numbers for the study of different aspects of neosporosis. These include ultrastructure description, antigen definition and elucidation of function, genetic manipulation, chemotherapeutic agent susceptibility, host adhesion and invasion mechanisms, immune modulation of experimentally infected animals and stage conversion induction studies (Hemphill et al., 2004). Parasites can be sustained *in vitro* for extended periods (Dubey and Lindsay, 1996; Lindsay and Dubey, 1989b) without losing infectivity to mice (Cannas et al., 2003b) or cattle (Kritzner et al., 2002).

At the time of this study, there were no available New Zealand strains of *N. caninum* as the parasites had not yet been isolated and successfully propagated within the country. However, a New Zealand strain of *Toxoplasma gondii* was available for use. The *T. gondii* strain had been isolated by Agvax Developments and had been used to produce a vaccine called ToxoVax®. ToxoVax® is made from live attenuated tachyzoites of the S48 strain of *Toxoplasma gondii*. The strain of *T. gondii* used for culturing was not attenuated and was expected to behave very similarly to *N. caninum* in culture (Hemphill, 1999; Sundermann and Estridge, 1999).

Both *N. caninum* and *T. gondii* have been shown to have a low degree of host cell specificity in vitro. In vitro culture of proliferating tachyzoites can be achieved in many different cell types. Primary and established cell lines have been used to culture *N. caninum* since it was first isolated. The cell lines first used were bovine monocytes and cardiopulmonary arterial endothelial cells (Dubey et al., 1988b). Cell lines that have since been used include Mandin-Darby bovine kidney, human foreskin fibroblasts, foetal mouse brain, Vero (Barber et al., 1993; Stenlund et al., 1997; Yamane et al., 1997), HS68 (Dubey et al., 1998b), COS- (Gondim et al., 2001) and CV1 cells (Dubey et al., 2004).

Parasites are inoculated onto a host cell monolayer (Lindsay and Dubey, 1989b). Seeding density of the monolayer determines the time required before splitting cultures is necessary. Cell lines

supporting parasite cultures are usually maintained at a higher cell density than cell monolayers in order to enable parasites time to invade and multiply within host culture cell prior to passaging on (Hemphill, 1996; Hemphill et al., 1996).

Initial attempts to subculture tachyzoites from culture supernatant were unreliable, which prompted subculturing from infected monolayers. Monolayers can be scraped with a rubber policeman to remove infected cells. Following harvest cells are ruptured by passing through a syringe with an attached 27-gauge needle (Lindsay and Dubey, 1989b). Another popular method of harvesting cells is by trypsin-treatment (0.1-0.25% trypsin). A 25 cm^2 flask of cells is covered with 2-3 ml of trypsin and incubated for 5-10 min at 37°C (Hemphill et al., 1996; Hemphill et al., 2004; Koyama et al., 2001; Stenlund et al., 1997; Uggla et al., 1998). Monolayers must be harvested prior to total destruction of the monolayer by the parasite. Passage onto an uninfected monolayer should occur when 70-90% of the cells become infected (Conrad et al., 1993a). Parasites should be harvested while still intracellular (Hemphill et al., 2004).

Intracellular tachyzoites begin replication by endodyogeny within as little as 6 hours post inoculation and can be seen in culture (Lindsay and Dubey, 1989b). Host cell invasion is an active process requiring metabolic energy from the parasite only, demonstrated by findings that *N. caninum* can infect formaldehyde-fixed host cells (Hemphill et al., 1996). Proliferating parasites cannot be notably observed in the initial phase of development following infection (1-3 days). However, after 3 days tachyzoite proliferation takes place more rapidly resulting in the formation of a pseudocyst (Muller et al., 2002). A pseudocyst can be identified by a parasitophorous vacuole membrane but a cyst wall will always be absent (Hemphill et al., 2004). Parasite induced cytopathic effect (CPE) of an infected monolayer may be seen around 3 days following inoculation. CPE is caused by parasites rupturing infected host cells and creating holes in the monolayer. Tachyzoites liberated from ruptured cells can be seen under a light microscope and are characteristically motile. Parasites display pivoting, gliding and flexing movements as they attempt to maneuver towards a new host cell. Tachyzoites are present in the supernatant of heavily infected cultures displaying a high level of CPE, cellular debris is increased and non-viable parasites are increasingly present (Lindsay and Dubey, 1989b). Unlike *T. gondii* tachyzoites, *N. caninum* tachyzoites are not well sustained outside of a host-cell as they are vulnerable to the effects of extracellular maintenance (Hemphill, 1999). However, a quantitative PCR-based assay has shown that only 50-80% of tachyzoites that adhere to the surface of a host cell eventually invade the target cell (Naguleswaran et al., 2003). Thus, a large number of parasites do not succeed in invasion and will eventually die in culture supernatant.

Cell culture procedures selected for parasite passage are frequently based on the type of host cell available. Media should be selected depending on which host cell is used as *N. caninum* tachyzoites have been shown to grow in a range of different media, such as RPMI-1640, Dulbecco's modified Eagle's medium (D-MEM) and minimum essential media (MEM) (Koyama et al., 2001; Lindsay and Dubey, 1989b; Yamane et al., 1998). Tachyzoite cultures are maintained in the presence of antibiotics, serum supplements and some cultures may also be supplemented with L-glutamine if it is not provided in the media (Hemphill et al., 2004; Lindsay and Dubey, 1989b; Stenlund et al., 1997).

Foetal bovine serum (FBS) has been found to harbour anti-*Neospora* antibodies. Depending on the detection technique performed, up to 100% of serum samples tested have been found to

contain varying *Neospora* antibody concentrations (Torres and Ortega, 2006). Parasites can be cultured in horse serum or equine serum (HS, ES) rather than FBS in order to avoid complications that may arise from antibodies (Hemphill, 1999). However, long term passage of *N. caninum* tachyzoites in vitro does not appear to be affected by the antibodies present in FBS as infectivity in mice and cultures is retained (Dubey and Lindsay, 1996).

Tachyzoites can be cryopreserved for long term storage (Barber et al., 1995). Cryopreservation is favourable as it ensures a back-up supply of parasites should anything happen to a primary passaged culture and also provides a standard culture for comparative studies following long term passage of parasites. Tissue cysts cannot be frozen when in host tissue as parasites become non-viable. Therefore, cysts must be separated, concentrated and enumerated prior to cryopreservation. McGuire et al., found a 65% viability of bradyzoites following cryopreservation of tissue cysts isolated from murine brains (McGuire et al., 1997a). Preserved tissue cysts may be used for oral inoculation trials of potential definitive host animals.

This study tested the growing conditions required for continuous passage of tachyzoites from the Nc-Liverpool strain of *N. caninum*. Infection of host cells and passage of tachyzoites in the presence of different types of serum was studied as well as the effect of parasite infection on the host cells in order to develop familiarity and good culturing techniques. Parasites were also checked for viability following exposure to a purification product and following cryopreservation for long term storage of parasites and host cell lines.

2.2 Materials and Methods

2.2.1 Host Cells Used for Cell Culture

The cell culture technique used was one developed by AgVax Developments Ltd for the continuous passage of *Toxoplasma gondii* for ToxoVax® production. Vero cells, originating from monkey kidney, were provided by AgResearch at Wallaceville, Upper Hutt and used as the primary cell line. Additionally, bovine endothelial cells were used as a cell line for cultures and were provided by the Institute of Veterinary, Animal and Biomedical Sciences (IVABS) at Massey University, Palmerston North.

2.2.2 Passage of Host Cells

A flask of host cells became 100% confluent once the cells had grown over the entire flask surface. Once cells become confluent they were passaged. Cell lines were passaged 1-2 times per week. Growth media was changes 2-3 times per week. Host cell lines were adherent cells and therefore required harvest from the flask surface, either mechanically using a rubber policeman or using enzymatic treatment with trypsin solution (Appendix 2.1 & Appendix 2.2).

At cell harvest, supernatant was tipped off and the cell layer was washed gently with two changes of PBS (Gibco) that was discarded. Cells were harvested by antibiotic-trypsin-versene (ATV) treatment for 5 min at 37°C, 1ml of ATV per 25 cm^2 flask surface area or enough to lightly cover the cell surface area. Following the initial 5 min incubation at 37°C, flasks were tapped to encourage cells to detach from their surface. ATV flasks were incubated for a further 1-5 min if cells had not properly detached. Cells were washed from 25 cm^2 flasks using 2 x 5 ml volumes of complete media (cMEM, made of 10% FBS in MEM plus antibiotics), which was retained. The ATV-cell suspension was centrifuged at 200 x g for 5 min. The supernatant was discarded and the cell suspension pipetted to break up cell clumps. Cells were washed again in cMEM before counting and dispensing aliquots into flasks with fresh growth media (cMEM).

Cells were counted using a haemocytometer following harvest. Cell lines were seeded at 50,000 cells per cm^2 of flask surface. Cell monolayers, used to culture tachyzoites, were seeded at 30,000 cells per cm^2 of flask surface. Cell seeding volume was calculated using the following formula: Volume = (required cell number (50,000 or 30,000) x flask surface area) / cell concentration.

2.2.3 Growth Media and Supplements Used for Culture

All cells were grown in Minimum Essential Medium (MEM) from Gibco (Invitrogen Corp NZ). Medium was stored at 4°C once opened. Growth medium was warmed to 37°C prior to addition to cultures. Cells were always washed with 2 changes of PBS prior to the addition of fresh media.

Growth media for cell lines and monolayers prior to inoculation were supplemented with 10% FBS (Gibco) or HS (Gibco). Cells were maintained in either FBS or HS but the sera were not used interchangeably. The primary serum used was FBS.

Following inoculation of a monolayer with parasites, the media was supplemented with only 2% serum, whether FBS or HS. Both *N. caninum* and *T. gondii* tachyzoite cultures were maintained in 2% serum supplemented MEM.

All cultures, cell lines, monolayers and inoculated monolayers were maintained in the presence of antibiotic-antimycotic (50ug/ml penicillin, 50 ug/ml streptomycin and 50 ug/ml amphotericin) from Gibco (Invitrogen Corp NZ) and 50 ug/ml of gentamycin (Serva- research grade, provided by AgResearch, Wallaceville).

Growth media or complete media (cMEM) consists of MEM supplemented with 2 or 10% serum and antibiotic-antimycotics.

2.2.4 Growth Conditions for Cells and Parasites

All cell lines, monolayers and infected monolayers were incubated at 37°C in 5% CO_2, 95% air.

2.2.5 Cytopathic Effect (CPE)

Cytopathic effect (CPE) could be identified by eye as a small clear dot in the cell monolayer. Parasitic movement around the area of CPE could be observed at 100x magnification. At 400x magnification parasites were readily distinguishable from surrounding debris and cells due to their characteristic shape and movement.

2.2.6 Passage of *Toxoplasma gondii* for Technique Development

Toxoplasma tachyzoites were initially used as a control parasite during the development of the cell culture techniques because a *N. caninum* strain was not available within New Zealand at that time.

Toxoplasma infected flasks were split when approximately 90% of the monolayer was infected with parasites. Parasites were inoculated onto new monolayers every 1-2 weeks or when CPE reached 75-90%. Media was changed 2-3 times per week. The procedure described for *N. caninum* passage was also used for *T. gondii.*

2.2.7 Passage of *Neospora caninum* Tachyzoites

Neospora caninum parasites were imported into New Zealand approximately six months following study commencement. Nc-Liverpool (isolated in England) was imported from Sydney, Australia. Dr John Ellis from the University of Technology, Sydney, kindly provided the *Neospora* parasites isolated by Prof. Trees at the University of Liverpool and the cooperation of both is acknowledged.

Parasites were primarily maintained in Vero cells supplemented with 2% FBS and antibiotics, which are the growing conditions used by Barber et al., (1993) following parasite isolation, with the exception of MEM being used instead of RPMI-1640. Parasites were also grown in a bovine endothelial host cell line which was maintained in the same manner as the Vero cell line.

Parasites were passaged onto a new monolayer every 1-2 weeks or when CPE reached 75-90%. Media was changed 2-3 times per week. Flasks were split using ATV as described for host cell lines. A split ratio of 1:3 to 1:5 was used to passage *N. caninum* (and *T. gondii*) infected cultures onto new monolayers. However, during culture trials parasites were counted and inoculated onto monolayers at known parasite densities.

2.2.8 FBS Evaluation

Foetal bovine serum (FBS) was tested for anti-*N .caninum* antibodies using a commercially available indirect fluorescent antibody test (IFAT) using a commercial test performed by AgriQuality, Palmerston North. All serum was heat inactivated at 56°C for 30 min to inactivate complement prior to testing. A minimum of 1 ml of serum was sent for each sample tested.

Several samples from the same bottle of FBS were also sent in some cases. Samples were submitted for serological testing on three separate occasions.

2.2.9 Serum Trial

Foetal bovine serum (FBS) and horse serum (HS) were used to study the growth rate of Vero cells and *N. caninum* parasites in order to evaluate whether anti-*Neospora* antibodies present in FBS affected *N. caninum* growth in culture. On day zero, 112 x 25 cm^2 monolayers were prepared from pooled Vero cells that had previously been grown in FBS. The flasks were seeded at 30,000 cells/cm^2, which totalled approximately 750,000 Vero cells per flask. On day 1, the 24-hour monolayers were divided into 4 groups. All monolayers were cultured in 10% serum MEM and maintained at this level throughout the trial. All monolayers were washed with PBS and cultured as follows: Group 1; Vero cells cultures in 10% FBS, Group 2; Vero cells cultured in 10% HS, Group 3; Vero cells inoculated with $2.5x10^6$ *N. caninum* tachyzoites and cultured in 10% FBS, Group 4; Vero cells inoculated with $2.5x10^6$ *N. caninum* tachyzoites and cultured in 10% HS. On days 4, 7, 10 and 13, three flasks from each group were rinsed twice with PBS, harvested using ATV, centrifuged at 200 x g for 10 minutes, resuspended into growth media and cell counts were performed for Vero cells and *N. caninum* tachyzoite. On the same days, all other remaining flasks were rinsed twice with PBS and the respective growth media were replaced. From day 7 onwards, the supernatant and wash media from all *N. caninum* infected cultures was also collected, centrifuged at 200 x g for 10 minutes, resuspended into growth media and either counted, for flasks that were harvested for analysis, or for all other flasks and supernatant captured tachyzoites were added back into the flask they were removed from for further culturing. Supernatant harvest was performed in order to maintain the correct parasite count for each flask throughout the trial.

2.2.10 Percoll Density Gradient Separation of Tachyzoites

A 30% Percoll-PBS solution was made and the pH tested using litmus paper. Nc-Liverpool tachyzoites were harvested from culture and observed under a microscope for signs of activity and viability. Tachyzoites were resuspended into the 30% Percoll-PBS solution and a sample of resuspended tachyzoites was observed under a microscope for signs of distress. The remaining parasites were centrifuged at 2,700 x g (3,400 rpm) in a bucket centrifuge for 10 minutes. The supernatant was removed with a pipette so as not to disturb the pellet and was examined microscopically for tachyzoite presence. The cell pellet was harvested, washed once and resuspended in growth media. A sample was taken from the resuspended solution and a microscope was used to identify tachyzoites. The remaining resuspended cell pellet was inoculated onto a 24-hour monolayer and incubated to validate parasite viability. The procedure used was based on that described by (McGuire et al., 1997a).

2.2.11 Cryopreservation of Tachyzoites

The cryopreservation procedure used for the *N. caninum* was based on the technique described by (Barber et al., 1995). Flasks were harvested for cryopreservation when many parasite clusters

were visible but little CPE could be seen, usually 2 days P.I. Cell layers were scraped from the flask surface using a rubber policeman (flasks were not treated with ATV). Cells were not disrupted by pipetting following harvest. The cell solution was centrifuged at 200 x g for 10 min. The sediment from each 25 cm^2 flask was resuspended into 2 ml of chilled cryopreservation mixture (5% serum (Gibco), 10% DMSO (Sigma), 85% MEM (Gibco)) and aliquoted into 2 x 1 ml cryotubes. Cryotubes were placed into an isopropyl alcohol cryo 1°C freezing container for a minimum of 3 hours or overnight before transfer to a liquid nitrogen Dewar. Tubes were placed onto cryo-straws and secured with a plastic sheath before placing into a liquid nitrogen Dewar.

Cultures were retrieved from cryopreservation by immersing the frozen vial in a 37°C water bath with mild agitation for 2-3 minutes (the lid of the vial was not immersed). The contents were removed from the tube, resuspended in PBS and centrifuged at 200 x g for 10 minutes. The cells were washed in PBS twice. Sedimented cells and parasites from each vial were resuspended in 2 mls per vial of 2% growth media and inoculated onto a 175 cm^2 monolayer (1 x cryopreserved vial: 175 cm^2 monolayer).

2.2.12 Cryopreservation Trial

A 25 cm^2 flask of *N. caninum* infected Vero cells was harvested 4 days after inoculation with *N caninum*. Small spots of CPE could be seen in the monolayer using a microscope but CPE could not be seen clearly by eye. Cells were harvested from the flask surface using a rubber policeman and resuspended into 2% FBS-MEM. Half of the cell solution was removed and plated onto a 24-hour monolayer. The other half of the cell solution was processed as described above and cryopreserved for 2 days. Following two days of storage at -196°C, the cells were retrieved from cryopreservation as described above and inoculated onto a 24 hour monolayer. The monolayer inoculated with parasites that had not been cryopreserved was harvested 7 days after seeding. The parasites were enumerated using a haemocytometer. The monolayer inoculated with cryopreserved parasites was also harvested 7 days after seeding and the parasites counted.

The same procedure was repeated for flasks of *N. caninum* infected Vero cells harvested 9 days after inoculation.

2.3 Results

2.3.1 Culturing of Host Cells and Tachyzoites

Cultures of Vero and endothelial cells underwent active growth and rapid expansion if maintained in fresh media (media changed every 2-3 days) and if regularly passaged at the appropriate seeding density. Cells that were seeded at a density that was too low did not grow well and took a long time to become confluent suggesting an extended lag phase following seeding. Cells that were not passaged and became overgrown, suffered from contact inhibition

resulting in a near cessation of growth. Overgrown cells appeared to suffer from decreased metabolic activity and slow growth in the subsequent passage. Cells required rapid passage in order to regain rapid growth rates.

ATV treated host cells occasionally appeared stressed following passage, exhibited by all or part of the adhered cell layer coming away from the flask surface. This may have been due to residual inactivated ATV present in the cultures or cellular damage caused by excessive ATV exposure. Adhered cell layers were initially ATV treated for 10 minutes at 37°C, however this time was reduced as it was found that a 5 minute incubation was sufficient for cells to be released into suspension and longer incubation appeared to damage host cells.

Toxoplasma parasites were found to be particularly hardy, even after digestion for up to 3 hours in a 1% trypsin solution at 37°C. No problems were experienced during the culturing of host Vero cells or *Toxoplasma gondii,* which was conducted at AgVax Developments. Parasites and Vero cells grew at an expected rate, no contaminants were discovered and all cells appeared healthy.

Parasites grew well in host Vero cells. The effect caused by *N. caninum* on the Vero cell monolayer was very similar to that seen with *Toxoplasma gondii*. *Neospora* tachyzoites required passage at a similar rate to *T. gondii* tachyzoites. Morphologically, cultures of *N. caninum* were indistinguishable from cultures of *T. gondii*. Parasites also grew easily in bovine endothelial cells.

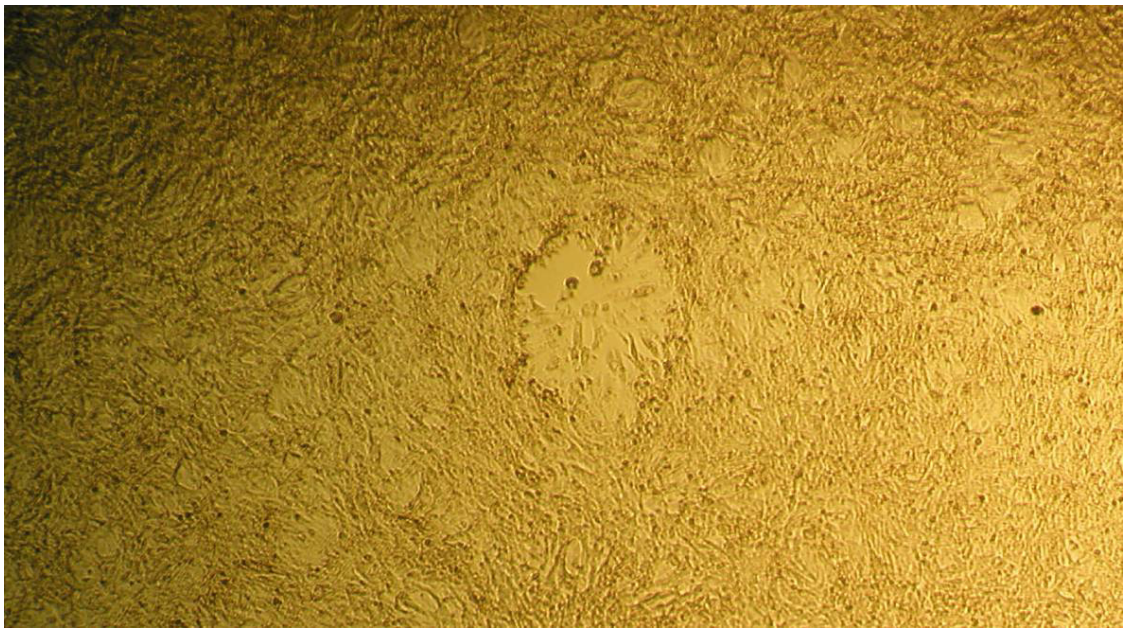


Figure 2.1. *CPE in a Vero cell monolayer infected with N. caninum tachyzoites.* CPE spots appear to have a darker edge, which is caused by a dense tachyzoite infection in the host cells and tachyzoites lysing out of the host cells (100x).

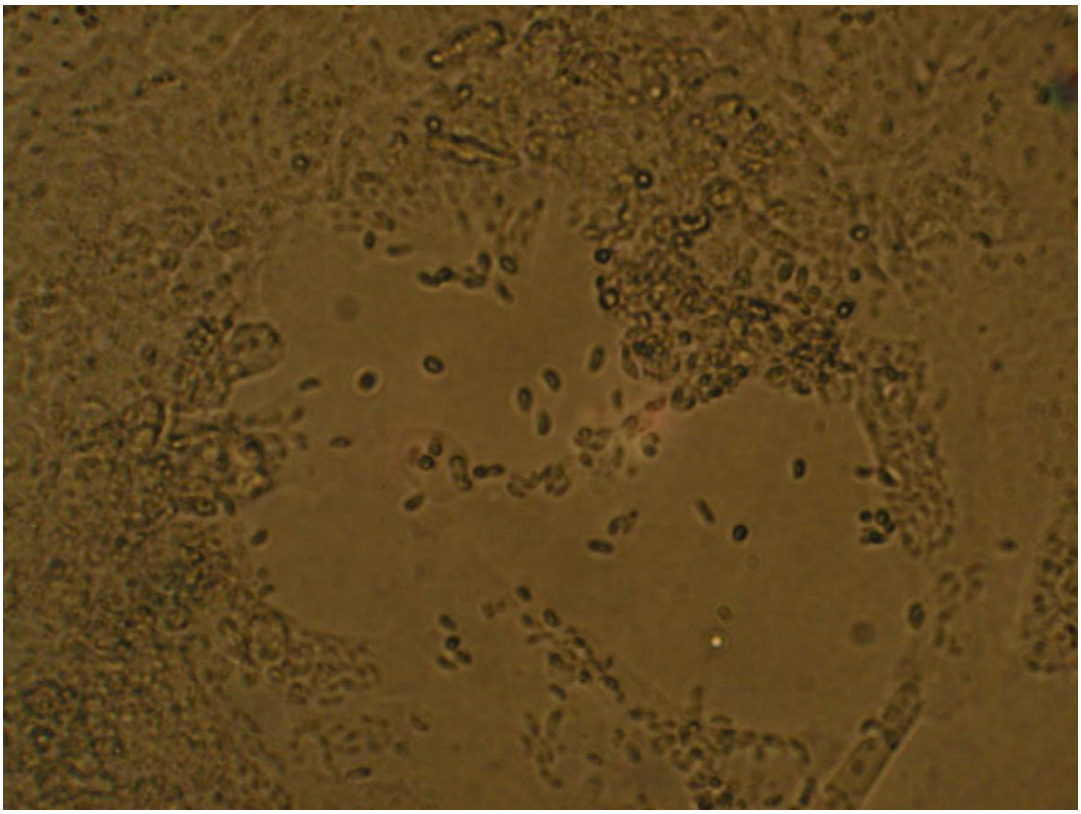
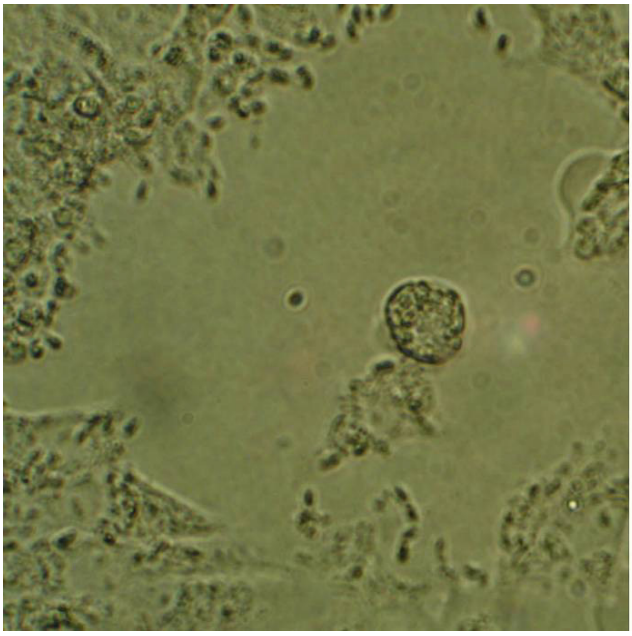
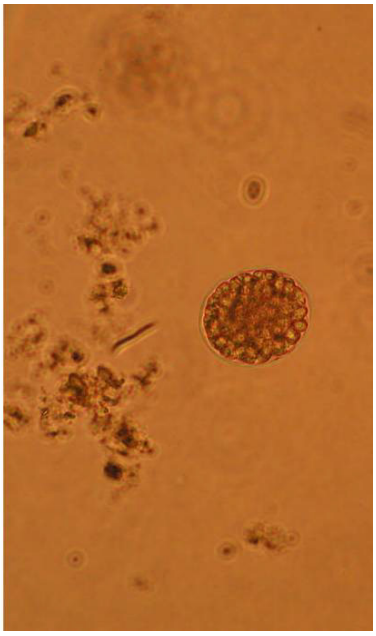


Figure 2.2. *Free N. caninum tachyzoites observed around areas of CPE.* Tachyzoites appear oval or banana shaped (400x).





Figures 2.3 & 2.4. *Heavily N. caninum infected host cells detaching from the monolayer during host cell lysis.* Detaching cells appear as dotted ball (400x).

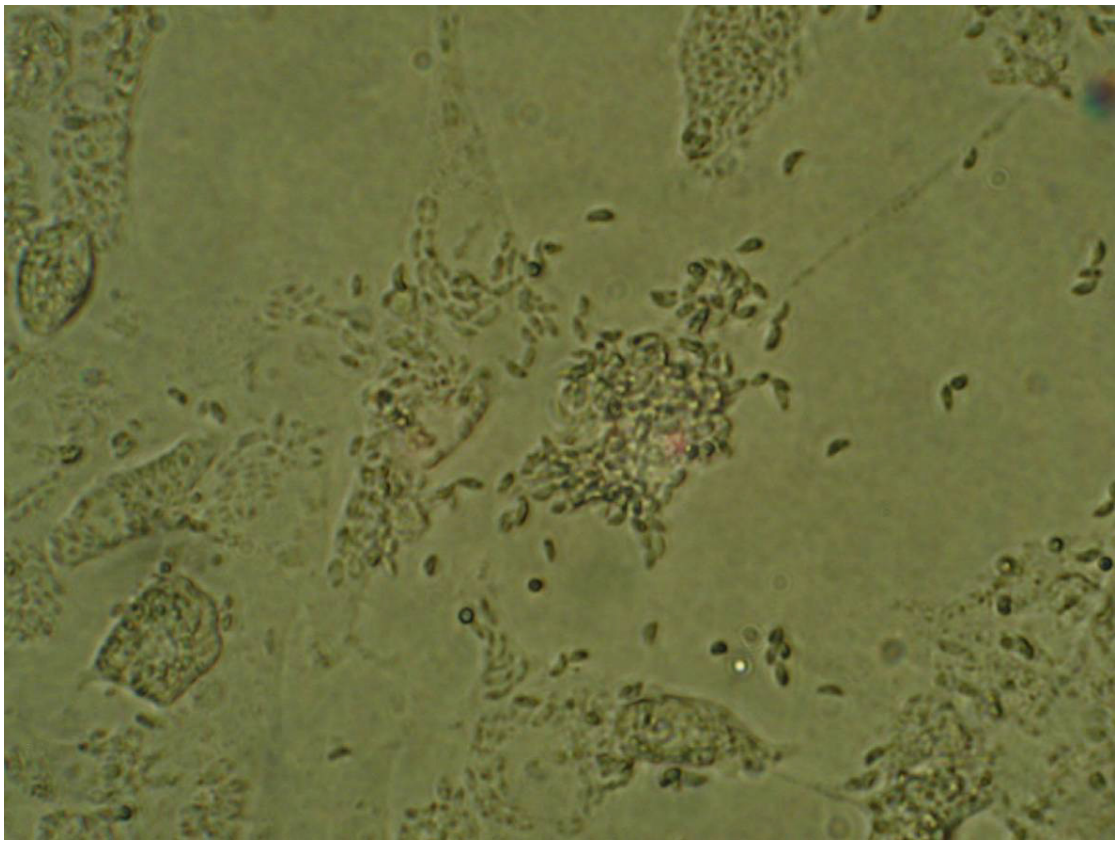


Figure 2.5. *Tachyzoites lysing a host cell and actively moving to search for a new host cell.* Tachyzoites can clearly be seen as small banana shaped cells (400x).

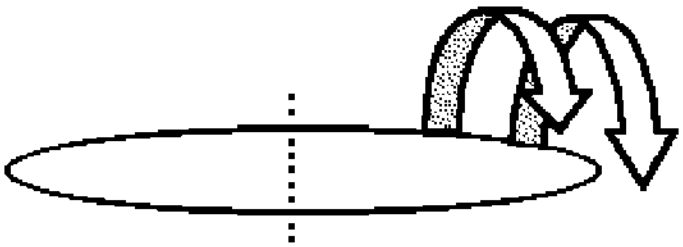


Figure 2.6. *Tachyzoite movement*. Parasites observed to twist half of the body in a circular movement.

2.3.2 Percoll Trial

The 30% Percoll-PBS solution was determined using litmus paper to have a pH between 7 and 8 as described by the Percoll protocol. Nc-Liverpool parasites resuspended in the 30% Percoll solution and when observed under a microscope appeared active without signs of distress. No

differences were noted when compared to Nc-Liverpool resuspended in growth media. The supernatant removed from the centrifuged Percoll density gradient did not contain *N. caninum* tachyzoites when examined using a microscope. Some cell debris was noted in the supernatant. Many parasites were observed in the resuspended cell pellet but parasite activity appeared to be reduced. Comparison with parasites that were freshly harvested showed that parasite activity was markedly reduced following Percoll processing relative to parasites that were freshly harvested. Parasite viability was not determined. Parasites inoculated onto monolayers from the density gradient pellet were able to infect the host cells and CPE was observed within 7 days.

2.3.3 Serological Status of Serum

Batches of FBS were tested on three separate occasions. A report was provided following testing which stated the IFAT titre and positive/negative sero-status. The cut off limit for a positive serum status was 1/200. The contractor performing the test was blinded as to the source of the samples and that aliquots may have been replicates of the same sample.

All FBS samples tested for the presence of anti-*Neospora* antibody were positive, including samples taken from different batches at different times and duplicates taken from the same batch of FBS. Replicate samples taken from the same batch of FBS often produced inconsistent results, however all results were consistently positive.

Table 2.1. *IFAT results for FBS samples*

Submission date	**FBS Sample**	**IFAT Titre**	**FBS Sero-status**
Sample 1	FBS	1/1000	Positive
Sample 2	FBS1 FBS2	1/600 1/600	Positive Positive
Sample 3	B1 B2 B3	1/1000 1/600 1/200	Positive Positive Positive

2.3.4 Serum Type Trial

Harvest of Vero cells supplemented with either FBS or HS

Vero cell cultures became overgrown from day 7 for all cultures. Vero cell growth was more prolific when cultured in FBS than in HS as can be seen by the higher number of Vero cells in the FBS and the ratio of FBS:HS propagated cells.

Table 2.2. *Serum trial Vero cell counts.*

Day	***Serum type***	***Confluence***	***Cell count per flask ($x10^6$)***	***Average cell count ($x10^6$)***	***Cell ratio FBS:HS***
4	FBS	90	12, 13, 17	14	2:1
	HS	90	8, 8, 5	7	-
7	FBS	100	26, 30, 30	29	2.2:1
	HS	100	19, 11, 9	13	-
10	FBS	100	100, 98, 96	98	4.1:1
	HS	100	21, 21, 31	24	-
13	FBS	100	59, 75, 44	59	1.5:1
	HS	100	46, 39, 33	40	-

Harvest of N. caninum (N.c) infected Vero cell cultures supplemented with either FBS or HS

For all cultures CPE was observed from day 4 using a microscope and from day 10 by the naked eye. Free-floating tachyzoites could be observed in the media from day 4 for all cultures. Tachyzoite numbers did not appear to be significantly different overall when grown in FBS as opposed to HS, whereas the number of Vero cells was reduced when grown in HS rather than FBS.

Table 2.3. *Serum trial Nc-Liverpool tachyzoite cell counts.*

Day	***Serum type***	***Confluence***	***Level floating cells***	***Flask Vero cell count ($x10^6$)***	***Avg Vero cell count ($x10^6$)***	***Flask Neo Count ($x10^6$)***	***Avg N.c count ($x10^6$)***	***Flask Super-natant N.c count ($x10^6$)***	***Avg Super-natant N.c Count ($x10^6$)***
4	FBS	100	Few	14 13 16	14	13 11 8	10	NA	NA
	HS	100	Mod	6 9 3	6	14 10 6	10	NA	NA
7	FBS	90	High	18 27 19	21	13 23 39	25	98 92 83	91
	HS	90	Med	2 6 4	4	28 23 28	27	84 19 42	48
10	FBS	50	High	8 9 14	10	82 38 49	57	150-250* 200-300* 200-300*	235
	HS	50	High	9 6 6	7	100 98 79	92	150-250* 200-250* 150-250*	210
13	FBS	5	Max	4 5 3	4	47 45 14	35	250-350* 200-300* 200-300*	365
	HS	5	Max	5 2 3	3	29 41 23	31	200-300* 200-300* 200-300*	250

NB. (All cell counts refer to cells per ml. All samples were resuspended to the same volume. Cell counts marked with an * were estimated, as too many cells were present for an accurate count to be made at the assigned volume)

By day 10 it was impossible to get an accurate tachyzoite count as the parasites were clumped together in large groups and cells could be seen lysing as parasites escaped. It was clear that cells were densely infected with parasites and were in different stages of parasite release. This was true for both the FBS and ES samples. The numbers of parasites could only be estimated. Estimates were considered conservative as large numbers could not be seen for counting due to clumping. By day 13 the clumps of parasites had mostly broken up, probably due to released parasites separating from each other following host cell lysis as they moved apart to search for new host cells.

2.3.5 Cryopreservation

Vero cell monolayers inoculated with cryopreserved tachyzoites and fresh parasites both appeared healthy and showed CPE from day 4. CPE covered approximately 70% of the monolayer by day 7. No obvious differences between the two cultures were noted. All flasks were harvested at day 7 following monolayer seeding to analyse tachyzoite growth. Tachyzoite harvest from each flask at day 7 revealed that the monolayer inoculated with tachyzoites that had been cryopreserved on day 4 contained 97% the number of tachyzoites observed in the culture inoculated with fresh tachyzoites.
Tachyzoites that were harvested and cryopreserved on day 9 were not as viable as those harvested on day 4. The same monolayer inoculation trial repeated with tachyzoites harvested at day 9 produced only 30% the number of tachyzoites in the cryopreserved flask relative to the freshly inoculated flask.

Table 2.4. *Cryopreservation retrieval*

Parasites harvested on day	Parasites cryopreserved	Cell count following harvest on day 7 *(x10^6)*	% of tachyzoites
4	No	63	100
4	Yes	61	97
9	No	44	100
9	Yes	13	30

2.4 Discussion

Tissue culture cell lines were found to grow faster and appeared healthier when passaged frequently. Optimal Vero cell growth appeared to be achieved if cells were passaged twice a week. Incubation time in antibiotic-trypsin-versene (ATV) solution should be kept to a minimum so as not to cause damage to cells. Serum deactivates trypsin activity. Serum should be added to the trypsin/cell supernatant following incubation with trypsin and cells should be further washed in 10% complete media to ensure trypsin is properly deactivated and removed. Properly deactivated trypsin does not show residual activity once cells are passaged. Cells were found to grow more vigorously following enzymatic cell harvest than mechanical harvest, possibly because cell damaged occurs during mechanical harvesting caused by scraping cells from the flask surface and from vigorous pipetting to break cell clumps down into a single cell suspension. It may also be possible that the trypsin activity stimulates active cell growth when used moderately.

Tachyzoites of *T. gondii* and *N. caninum* were shown to behave in a similar manner in Vero cell cultures. Bovine endothelial cells were later introduced for the passage of *N. caninum* but were not used for the passage of *T. gondii* tachyzoites. Using a bovine cell line when attempting to isolate and culture a new strain of *N. caninum* may be advantageous as the parasite would not have to adapt to cells from a new species. However, it has been shown that non-bovine cell lines

can successfully be used to isolate and maintain tachyzoite cultures. In particular, Vero cells have been reported as effective hosts for both *N. caninum* and *T. gondii* parasites during the isolation and maintenance of cultures (Dubey and Lindsay, 1996).

CPE could be seen by the naked eye after a minimum of 4 days for both *T. gondii* and *N. caninum*. Tachyzoite movement for both types of parasite was distinguishable from other Brownian motion movement in the flask as parasites appeared to move half of their body at a time in a twisting/circular motion. Parasites were particularly active immediately following harvest but activity decreased over time (Hemphill et al., 1996). Little *N. caninum* tachyzoite activity could be seen if parasites were harvested and left to stand for 1 hour, which is not unexpected as *N. caninum* tachyzoites do not survive extracellularly for long periods of time (Hemphill, 1999). Reportedly *T. gondii* tachyzoites survive for longer periods of time extracellularly than *N. caninum* parasites (Hemphill, 1999).

Percoll pH was appropriately within the required range. The optimal physiological pH is 7.4. Percoll appeared to provide the physiological requirements needed to sustain tachyzoites during processing. Using the specified conditions, it was found that free tachyzoites could be separated and pelleted, along with other cell types such as host cells. Parasites were not observed in the supernatant of the 30% Percoll-PBS solution following centrifugation, suggesting that all viable parasites had pelleted out when using the described conditions. Initial findings suggest that the described procedure may be appropriate for use during animal tissue isolations to reduce the volume of material to be inoculated onto cell monolayers. Parasite viability following separation on a Percoll gradient was undetermined, however a significant number of parasites must have survived the procedure as parasites were able to infect a host monolayer and cause CPE. However, it is expected that a large number of parasites may have lost viability, not due to toxicity from using Percoll but rather caused by a decrease in tachyzoite infectivity over the extended period taken to perform the Percoll procedure. A decrease in tachyzoite infectivity is extrapolated from the loss of active movement of tachyzoites observed following Percoll processing and the periods of extracellular tachyzoite maintenance required. Despite a likely loss of tachyzoite infectivity, Percoll density gradients have been successfully used by other researchers during tachyzoite isolation from tissue (McGuire et al., 1997a; Stenlund et al., 1997).

Foetal bovine serum (FBS) is commonly used as a component in media. FBS was used by AgVax developments for the development of ToxoVax and for *Toxoplasma* culture. The arrival of an *N. caninum* strain into New Zealand raised concern that the New Zealand FBS in use may contain antibodies against *N. caninum* (Dubey and Lindsay, 1996), as *N. caninum* is common amongst NZ dairy cows (Reichel, 1998). Batches of FBS tested using a commercial IFAT test showed that the New Zealand FBS in use contained *N. caninum* antibodies. IFAT test results of the FBS aliquots showed that the titre levels were not repeatable as samples from a single FBS bottle yielded 3 different titre levels. Despite the titre measurement variation between samples, all samples did return a consistent result in relation to positive or negative sero status. Test variation may have been due to operator inexperience, a faulty test kit or the subjective nature of the test. An IFAT test relies on personal evaluation rather than an objective assessment, which means that large variation between samples may be recorded (Hemphill, 1999).

The *N. caninum* strain, Nc-Liverpool, imported into New Zealand from Sydney, Australia, had been cultured in FBS. The serological status of the FBS used to culture the parasites in Australia

was unknown, therefore it was also not known whether the antibodies against *N. caninum* found in the New Zealand FBS would affect *N. caninum* tachyzoite viability. Antibodies to *N. caninum* have also been identified in equine serum (McDole and Gay, 2002). Additionally, it was unknown if the Vero cell line would grow well in media supplemented with anything other than FBS as the Vero cell line had previously only been supplemented with FBS.

The serum trial using FBS or horse serum (HS) to culture either Vero cells or *N. caninum* infected Vero cells produced interesting results. Vero cells did not proliferate as well when supplemented with HS as when supplemented with FBS. The decreased Vero cell numbers were observed when cells were or were not infected with *N. caninum* tachyzoites. Uninfected FBS supplemented cells grew twice as fast as HS supplemented cells until approximately day 10. From day 13 the ratio of cells grown in FBS to cells grown in HS began to even out. The ratio change around day 13 appears to be due to inhibition contact of cells grown in FBS and cell death as the number of cells decreased whereas in HS the number of cells was still increasing, suggesting that the HS supplemented cells suffered from a lower metabolic rate than those grown in FBS.

Not reported in this serum trial, but observed was the decreased growth and health of Vero cells grown in HS over long periods. Observations from the serum trial showed that cells grew slower than cells grown in FBS supplemented media. This was true also for cells passaged continuously in FBS for extended periods. The growth rate of the cells decreased further and the general health of the cells appeared to be adversely affected. Using HS to supplement Vero cell cultures would not provide the optimum growing conditions for this cell line. Vero cells should be grown in FBS to ensure a healthier culture. Alternatively however, it has been reported that *N. caninum* can be grown in human serum with no inhibitory effects (Omata et al., 2005).

Despite Vero cells being affected by the serum source, *N. caninum* tachyzoites appeared to be relatively unaffected by the serum change. Slightly higher numbers of tachyzoites were detected in the HS flasks following monolayer harvest, but this was balanced by slightly higher numbers of tachyzoites in the supernatant of the FBS supplemented flasks, resulting in comparable parasite counts between the two serum types. It appears that the serum type has little effect on the parasite numbers or tachyzoite growth rate but rather that the limiting factor, if there is one, is the growth rate and therefore availability of host Vero cells in the different sera. Calculations from the *N. caninum* infected Vero cells for both FBS and HS suggest that in each $25cm^2$ flask approximately 25,000,000 parasites were produced. This is an average of around 33 parasites produced per host cell (based on a host cell count of 750,000).

It would have been possible to have stained the cells with Trypan blue in order to assess whether the cells and parasites were alive or dead at the time of counting, but this procedure was not known to the investigator at the time of the study.

The serum trial suggests that the Nc-Liverpool strain of *N. caninum* could be successfully cultured in either FBS or HS. Given that Nc-Liverpool is able to grow in FBS known to contain antibodies to *N. caninum*, it is likely that a newly isolated New Zealand strain of *N. caninum* would also grow in sero-positive FBS.

Cryopreservation of *N. caninum* tachyzoites did not appear to effect viability to any significant degree. Almost complete retrieval of the tachyzoites was shown when compared to tachyzoites freshly harvested, based on subculturing of cryopreserved samples. Trypan blue exclusion was not used to assess cell viability. The stage of culturing for cryopreservation was found to be important. Free floating tachyzoites harvested from cultures that were almost completely destroyed by *N. caninum* did not survive cryopreservation as well as those that were still intracellular within the host cell. This may have been due to low viability or low infection ability of extracellular tachyzoites (Hemphill et al., 1996). The host cell may provide a degree of protection to the parasites during cryopreservation as well as both tachyzoites and host cells having greater health and viability at earlier stages of infection (Barber et al., 1995).

The cryopreservation procedure used was successful for freezing parasites and retrieving viable tachyzoites. Samples must be frozen in medium containing 5-15% of a cryopreservation agent such as dimethylsulfoxide (DMSO), otherwise the procedure is lethal to most eukaryotic cells (McGuire et al., 1997a). DMSO lowers the freezing point of the cells to protect their membranes against rupture. Cells will also be protected by using 5-25% serum during freezing. Increasing the serum content of the cryopreservation mixture to 20% and lowering the media volume to 70% would offer a more protective environment for the cells. A cell count should also have been performed to determine the optimal and maximum cell density for cryopreservation and the viability should also have been determined using trypan blue. Mechanical damage caused by ice crystals and dehydration can be decreased by freezing samples at 1-10°C per minute. Cell storage should be below -130°C to retard ice crystal formation. Cells must be thawed rapidly during retrieval as ice crystals may also form in the thawing cells above -50°C. Retrieval of cryopreserved samples should be done by adding complete media to the thawed sample in a drop wise manner. This decreases osmotic shock to the cells. Cryopreservation of tachyzoites can be used for the long-term storage of control strains of *N. caninum* and for storage of strains that may be newly isolated. By cryopreserving cells, their metabolic rate decreases and normal metabolic processes and destructive processes are inhibited. Therefore, cells are kept in a state of suspended animation that enables their long term storage and captures the cell's features at a specific point in time.

2.5 Conclusions

The described procedures used to culture host cell lines and tachyzoites of established *T. gondii* and *N. caninum* tachyzoite strains appear to be appropriate for long term propagation of the parasites. Parasites can also be harvested for long-term storage using the cryopreservation method described, which maintains tachyzoite viability upon retrieval.

The established Nc-Liverpool parasite strains appear to be unaffected by specific antibodies present in foetal bovine serum used to supplement media. Parasites can also be purified using a Percoll density with no apparent adverse effects.

Chapter 3.

3.0 Diagnostic techniques for the identification of *Neospora caninum* infection from tissue, cell culture and blood

3.1 Introduction

Serology is an indirect detection method that historically was the main diagnostic tool used for *N. caninum* diagnosis in cattle and dogs. Serological procedures, such as IFAT, ELISA and immunoblots, rely on the detection of antibodies in the blood and can be used to identify animals seropositive to *N. caninum* antibody caused either by infection or exposure to the parasite, but cannot definitively identify the presence of parasites in tissue. Serological titres can indicate the likelihood of a chronic versus acute phase infection but factors such as abortion or pregnancy, operator experience, sample handling and processing, the test used or laboratory practices can effect or give false results (Bjorkman et al., 2003; Conrad et al., 1993b; Hemphill, 1999). Direct detection methods such as immunohistochemistry (IHC) and PCR techniques complement serological testing by identifying parasites or parasite DNA present in tissue.

Immunohistochemistry has been used for the definitive diagnosis of neosporosis as the organism can be distinguished from other species using specific antibodies (Lindsay and Dubey, 1989a). Tachyzoites have been identified in many different tissue types. Bradyzoites had only been found in central nervous (CNS) tissue and the eye until recently where they have been discovered in skeletal muscle of 2 calves (Dubey et al., 2006; Dubey et al., 1990d; Dubey and Lindsay, 1993; Peters et al., 2001). Parasite density can vary markedly within the CNS tissue but parasites can be found most consistently in the cerebrum (Barber et al., 1996) and are also more commonly found in the grey matter in the brain and the white matter in the spinal cord (Jardine and Dubey, 1992). However, parasites have reportedly been seen distributed throughout the CNS, in the grey and white matter, within nerve roots and the spinal cord (Dubey et al., 1990c; Jardine and Dubey, 1992). Tachyzoites can often be identified in tissue due to host cell death and mononuclear cell infiltration in the affected areas causing encephalomyelitis (Bjerkas and Presthus, 1988). Tissue cysts however produce little or no host cell response (Dubey and Lindsay, 1993). Tissues such as placenta, which are high in peroxidase activity, should be treated with pepsin or trypsin (Dubey et al., 2001; Dubey and Schares, 2006) or by heat-mediated antigen retrieval (HMAR) (White et al., 1998) prior to immunohistochemical staining.

Antibodies specific for *N. caninum* bradyzoites and tachyzoites have been raised for the differentiation of the parasite stage in tissue. The bradyzoite antigen BAG 5 developed for *T.*

gondii cross reacts with *N. caninum* bradyzoites providing an effective mechanism for differentiating bradyzoites from tachyzoites but is unable to differentiate between *N. caninum* and *T. gondii* (McAllister et al., 1996c). Polyclonal antibodies against Nc-p43 (Hemphill and Gottstein, 1996) stains both tachyzoites and bradyzoites whereas Nc-p36 (Hemphill et al., 1997) stains only tachyzoites whilst neither antibody cross-reacts with *T. gondii* (Fuchs et al., 1998).

Cross-reaction with *T. gondii* antigens can be a problem when using IHC (Anfray et al., 2005; Dubey and Lindsay, 1996; Peters et al., 2000; Sundermann et al., 1997) although some researchers have also reported high specificity particularly when using monoclonal antibodies (Cole et al., 1993; Lindsay and Dubey, 1989a).

Given the low detection rate of parasites in tissues using IHC (Baszler et al., 1999a; Wouda et al., 1997b), researchers frequently use PCR to demonstrate *N. caninum* specific DNA isolated from fresh, frozen and formalin fixed tissues. PCR can provide a more sensitive and specific test when compared to IHC (Baszler et al., 1999a; Yamage et al., 1996). In addition to using PCR to detect bradyzoites and tachyzoites in tissue, PCR techniques have also been developed for the detection of oocysts in faeces (Hill et al., 2001), and tachyzoites in blood (Okeoma et al., 2004a), bull semen (Ortega-Mora et al., 2003) and in milk (Moskwa et al., 2006).

Several PCR's were developed based on rDNA sequences (Ellis et al., 1994; Ellis, 1998; Fazaeli et al., 2000; Ho et al., 1996; Holmdahl and Mattsson, 1996; Payne and Ellis, 1996). Ellis et al., (1998) developed a PCR that could distinguish *Neospora* from *Hammondia* and *Toxoplasma*. They compared the large subunit ribosomal DNA (lsu-rDNA) sequence of *N. caninum* and *T. gondii* and reported that the D2 expansion domain had the most variable nucleotide sequence (Ellis et al., 1998). However, using this PCR the *Toxoplasma* and *Hammondia* taxa could not be distinguished. Ellis' PCR technique was considered to be robust and was therefore used in this study. The advantage of this PCR is that rDNA is highly repeated in the coccidian genome providing a high copy target sequence for PCR detection.

A western blot test can be used to detect specific antigens by fractionating cell products using electrophoresis. In a western blot, antigens migrate through an SDS-PAGE gel by electroporation and are separated depending on size and charge. The separated antigens are then transferred onto either a PVDF or nitrocellulose membrane, the membrane is blocked and exposed to serum that may contain specific antibodies to the antigen (Pare et al., 1995). Antibodies bind to corresponding antigen bands and are stained with a secondary antibody, which couples to a detectable substrate. A dot-blot is a crude antigen detection method that utilizes a mixture of antigens immobilised onto a membrane in a single dot rather than fractionating specific antigens with electrophoresis. A dot-blot will give a positive/negative result whereas a western blot will detect the antigen the antibody is raised against. However, dot-blots have been shown to have a high comparative agreement with other diagnostic techniques, including western blotting (Bouillet et al., 1998; Mewis et al., 1999). Dot-blots are an easier and quicker method for accurately diagnosing disease than western blotting techniques and can be used as an alternative to plate ELISA (Folitse et al., 1998).

Dot-blots have been shown to be specific and sensitive for *Toxoplasma gondii* (Angel et al., 1997; Bouillet et al., 1998; Zhu et al., 2000), but no literature on the use of Dot-blots for *N. caninum* diagnosis could be found.

The aim of this study was to develop methods for diagnosing exposure to and infection with *N. caninum* in naturally and experimentally infected animals and for detecting parasites in tissue culture, cultured cells and blood products.

N. caninum infection in cattle and dogs could be assessed using a commercially available IFAT performed by AgriQuality in Palmerston North. Tests were not available for other species, such as mice, which were used for experimental inoculation. New diagnostic tests were required to screen tissue and blood products from experimental animals and cell culture samples for signs of *N. caninum* infection and exposure. Immunohistochemistry, PCR and immunoblot techniques were therefore developed for diagnostic purposes.

Immunohistochemistry was used for the direct detection of parasites in animal tissue and for screening experimental animals to test for *N. caninum* tissue cysts. Several PCR techniques were developed to screen DNA isolated from animal tissues and cells taken from tissue culture. Serum from experimentally infected animals was screened for the presence of anti-*Neospora* antibodies using immunoblots.

3.2 Immunohistochemistry (IHC)

3.2.1 General Description

Immunohistochemistry (IHC) is the study of cells and tissue using immunology and histology. Immunohistochemistry is frequently used interchangeably with Immunocytochemistry (ICC), which is the study of intracellular activity.

The streptavidin/avidin-biotin-enzyme complex (ABC) method described below relies on a specific antigen, formed by *Neospora caninum*, to be present within cells or tissue of sectioned samples. The sequence of reagent application is primary antibody (*N. caninum* or *T. gondii* antisera), biotinylated secondary antibody (biotinylated anti-goat secondary antibody), pre-formed streptavidin/avidin-biotin-enzyme complex, substrate solution, (DAB).

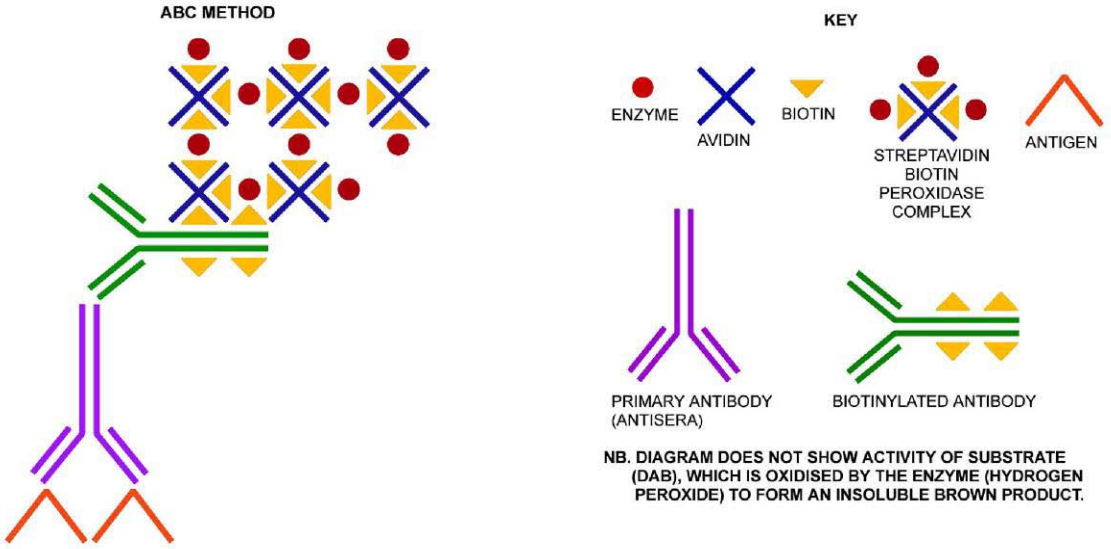


Figure 3.1. *Avidin-biotin enzyme complex (ABC) reaction with biotinylated secondary antibody.* Figure modified from (Boenisch et al., 1989)

3.2.2.1 Enzymology

SHP is a streptavidin-horseradish-peroxidase conjugated complex. Horseradish peroxidase is an enzyme, which in the presence of an electron donor, forms an enzyme-substrate complex and the oxidation of the electron donor. The electron donor is responsible for the continuing catalysis of H_2O_2. In the ABC method the substrate, 3,3'-diaminobenzidine tetrahydrochloride (DAB), becomes oxidised and produces an insoluble brown colour known as a chromogen. Heavy metal ions act as electrophilic (electron attracting) agents and are a requirement of many enzymes.

Steptavidin or avidin has four binding sites for biotin. Biotin covalently attached to an antibody is referred to as a biotinylated antibody, a biotinylated anti-goat secondary antibody was utilised in the described IHC procedure.

3.2.2 Materials and Methods

3.2.2.1 IHC Control Samples

A 1-year-old West Highland White Haired Terrier was diagnosed with hind limb paresis, which progressed to paralysis. Histological examination of the brain showed a large number of protozoan tissue cysts associated with lesions (Appendix 3.1). A paraffin embedded section of the dogs spinal cord had been held in archive at the Institute of Veterinary Animal and Biomedical Sciences (IVABS) at Massey University, Palmerston North, New Zealand, was used as an *N. caninum* positive control tissue sample. This tissue was identified as sample 11383.

Brain tissue from CF1 mice chronically infected with *Toxoplasma gondii* was used as the positive control tissue for *T. gondii*. Chronically infected mice and mouse tissue was supplied by AgVax Developments Limited.

3.2.2.2 Tissue processing for IHC

All tissue removed from animals experimentally or naturally infected with parasites was treated in the same manner. Animals were humanely destroyed and the spinal cord and/or brain was removed and placed into 10% buffered formalin solution for a minimum of 48 hours prior to tissue processing into formalin blocks. Formalin-fixed tissue was placed into a plastic cassette and cycled through a Leica, JUNG TP 1050 formalised tissue processor (Appendix 3.2). This required formalised tissue to be processed overnight in order to dehydrate the tissue with alcohol and infiltrate with wax. Once processed, tissues were embedded in paraffin wax using a Miles Scientific, Tissue-Tek embedder. Wax blocks were hardened overnight in a freezer before sectioning.

Sections were cut to a thickness of 5-7µm using a microtome, floated on a paraffin-section mounting bath and mounted onto a glass slide. Tissue sections were fixed securely to the slides by incubating at 56-60°C overnight before use in IHC.

3.2.2.3 IHC Primary and Secondary Antibodies

Specific antibodies to *T. gondii* were used in conjunction with *N. caninum*-specific antibodies to check for non-specific binding reactions between *N. caninum* and *T. gondii* parasites and cysts. (Refer to Appendix 3.3 for materials details.)

Neospora caninum primary antiserum: Anti-*Neospora caninum* polyclonal antiserum raised in goats (caprine) for use in immunohistochemistry and IFAT was purchased from Veterinary Medical Research and Development Inc (VMRD), Pullman, Washington.

Toxoplasma gondii primary antiserum: Anti-*Toxoplasma gondii* polyclonal antiserum raised in goats (caprine) for use in immunohistochemistry and IFAT was purchased from Veterinary Medical Research and Development Inc (VMRD), Pullman, Washington.

Secondary antiserum: Biotinylated anti-goat IgG (H+L) was purchased from DAKO Corporation.

3.2.2.4 Standard IHC staining procedure

The streptavidin/avidin-biotin-enzyme complex (ABC) method was used to detect parasites in sectioned tissue. Mounted sections were dewaxed in xylene and brought to water. Endogenous peroxide activity was decreased by incubation in a hydrogen peroxide wash. Slides were incubated in BSA solution to block non-specific binding sites. Primary antisera were used at the recommended titre. The antisera titre recommended by the supplier (VMRD) was 1:1000 for *N. caninum* and 1:500 for *T. gondii*. The secondary antibody was added and slides were incubated. A preformed streptavidin-biotin-enzyme complex (SHP) was incubated with each slide followed by a reaction mixture containing 3,3'-diaminobenzidine (DAB), heavy metal intensifier (HMI), and hydrogen peroxide. Once sections turned brown the reaction was stopped. Sections were counter-stained in Mayers-Haemalum and washed in tap water. Sections were dehydrated, mounted in DPX and sealed with a cover slip. Slides were left to dry before examined under a microscope (Appendix 3.4).

3.2.2.5 IHC Reaction Identification

Specific sites of immunoreactivity stained a chocolate-brown colour. Cysts containing bradyzoites or cells containing intracellular tachyzoites could be seen under high magnification (100-1000x).

3.2.2.6 Antibody Titre

Varying titres of primary antibodies were tested to determine whether stain colour consistency could be optimized. Only samples of *T. gondii* infected tissue were used in order to conserve limited *N. caninum* samples. *Neospora* primary antibody was used at a 1:1000 titre throughout (supplier recommendation). Primary antibody titres for *Toxoplasma* were used at 1:500 (supplier recommendation), 1:100 and 1:50. All other aspects of the described IHC protocol were followed as stated. The trial was repeated twice.

3.2.2.7 Antibody Incubation Experiment

Stain consistency optimization was also tested under different primary antibody incubation conditions. Tissue sections were incubated with a designated concentration of antibodies for either, 1 hour at room temperature (the standard application) or overnight at 4°C. Both *N. caninum* and *T. gondii* sections were stained with *N. caninum* and *T. gondii* antisera and incubated under the described conditions. *Neospora* primary antibody was used at a 1:1000 titre and primary antibody for *Toxoplasma* was used at a 1:500 titre.

An overnight incubation at 4°C was begun on the day before processing so that all aspects of staining after the primary antibody application would be the same for both the overnight incubation and the 1-hour incubation samples. The solution of primary antibody used for the overnight incubation was refrigerated and used the following day for the 1-hour incubation. All other aspects of the described IHC protocol were followed as stated. The trial was repeated twice.

3.2.2.8 Proteolytic Digestion of Sample Tissue

The incorporation of a proteolytic digestion step was used to optimize stain colour consistency. Tissue sections were incubated in a 0.1% Trypsin solution prior to incubation with the primary antibody to break down cross-linking that may interfere with the parasite antigen. Sections were deparaffinised, rehydrated and treated for endogenous peroxidase activity before incubation for 5-10 minutes in a humidity chamber at room temperature in 0.1% Trypsin solution/Tris buffer (pH 7.6). All other aspects of the described IHC protocol were followed as stated. The trial was repeated twice.

3.2.3 Results

3.2.3.1 Staining Observations

Most tissue cysts could be clearly identified following IHC staining. The background of Mayers haemalum increased cyst visibility within the tissue. Individual tachyzoites could be identified within the sections, particularly within areas of tissue lesion. Individual parasites were found mainly within parasitiphorous vacuoles of a host cell. The bulk of the parasites could be seen within tissue cysts. Tissue cysts frequently stained a rich chocolate brown colour. Bradyzoites within the cysts and areas of the cyst could be seen presenting stain. Obvious dark tissue cyst staining was almost always observed in tissue containing N. *caninum* tissue cysts when stained with *N. caninum* antibody for 1 hour at room temperature (See Appendix 3.5). However, positive tissue sections containing cysts or tachyzoites of either *N. caninum* or *T. gondii* parasites did not always immunoreact consistently throughout the same section or between sections from the same or different batches of samples. Cyst staining varied in brown-colour intensity, which is likely to

be caused by inconsistencies in immunoreaction across the section. The brown reaction colour was often pale and difficult to identify under low magnification.

Cross-reaction between *T. gondii* and *N. caninum* antibodies and positive control sections also occurred to varying degrees. The reaction colour observed due to cross-reaction staining was not as intense as that seen for specific staining. Cross-reaction staining did not typically occur the entire length of the parasite and not all cysts or free parasites stained within a section. Cross-reactive staining was relatively easily identified due to the paler and inconsistent nature of the immunoreaction compared to that observed in a true-positive section.

Non-specific staining of red blood cells was frequently observed but was easily distinguishable from specifically stained parasites. Cross-reaction with hemoglobin appeared as a pale brown/yellow colour and did not stain with the same brown intensity as cysts or tachyzoites.

3.2.3.2 Antibody Titres

3.2.3.2.1 *T. gondii* positive tissue sample incubation with various antibody titres

All *T. gondii* positive samples incubated with *T. gondii* antiserum at 1:50, 1:100 or 1:500 for 1 hour at room temperature immunoreacted (Figure 3.2). The staining intensity of cysts appeared to increase with increasing concentration of *T. gondii* antiserum. Cysts found in sections incubated with an antiserum titre of 1:50 appeared darker than cysts seen in sections incubated with either a 1:100 or 1:500 antiserum titre. Although cyst staining colour intensity increased, non-specific background staining also increased. Background staining appeared a darker brown in sections stained with a higher concentration of antiserum.

Individual parasites could be seen at magnifications between 100-1000x and appeared to stain over their entire length.

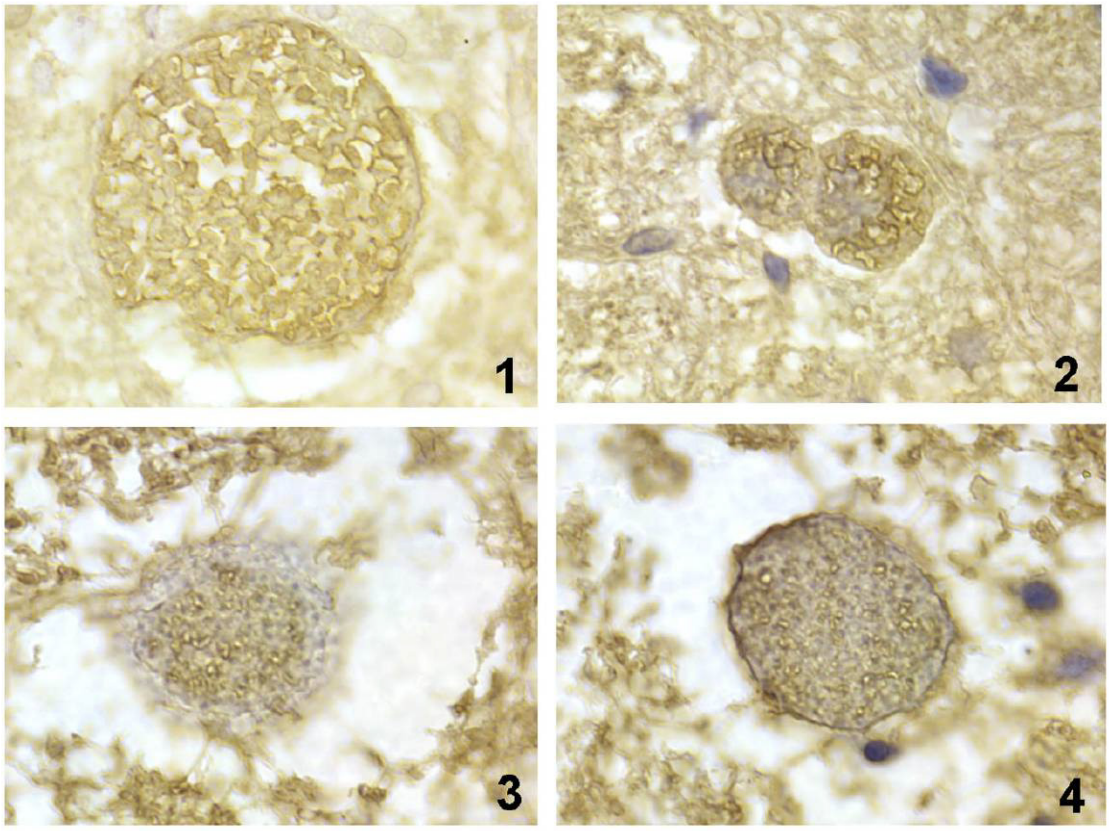


Figure 3.2. *T. gondii positive tissue sections (x1000) incubated with T. gondii antibody (1:1000) at room temperature for 1 hour. T. gondii* antibody used at 1:500 (1), *T. gondii* antibody used at 1:100 (2), *T. gondii* antibody used at 1:100, cyst beginning to degrade due to autolysis (3), *T. gondii* antibody used at 1:50 (4). Typical examples.

3.2.3.3 Incubation conditions

3.2.3.3.1 *N. caninum* tissue sections incubated with *T. gondii* antisera

Known-positive *N. caninum* tissue sections incubated with *T. gondii* antisera (1:500) for 1 hour at room temperature (Figure 3.3) or overnight at 4°C (Figure 3.4) immunoreacted with the *T. gondii* antibody resulting in stained tissue cysts. Cysts could be identified at 40x magnification. Cysts were stained a light brown colour but were distinct from the background tissue indicating a specific or cross-reactive immunoreaction had occurred. Cyst staining was not consistent throughout tissue sections. In some cases cysts did not immunoreact at all, while others were partially or strongly stained. Under high magnification individual bradyzoites and tachyzoites could be identified as having immunoreacted across the entire length of the organism, not just at the apical complex. At a magnification of 100-1000x the brown immunoreaction stain of the parasites was distinct from the colours of non-immunoreactive cells. All sections were counter

stained with Mayers-Haemalum (MH) enabling identification of lesions and individual neural cells. A clear difference in immunoreaction colour staining between sections incubated for 1-hour at room temperature or overnight at 4°C was not apparent.

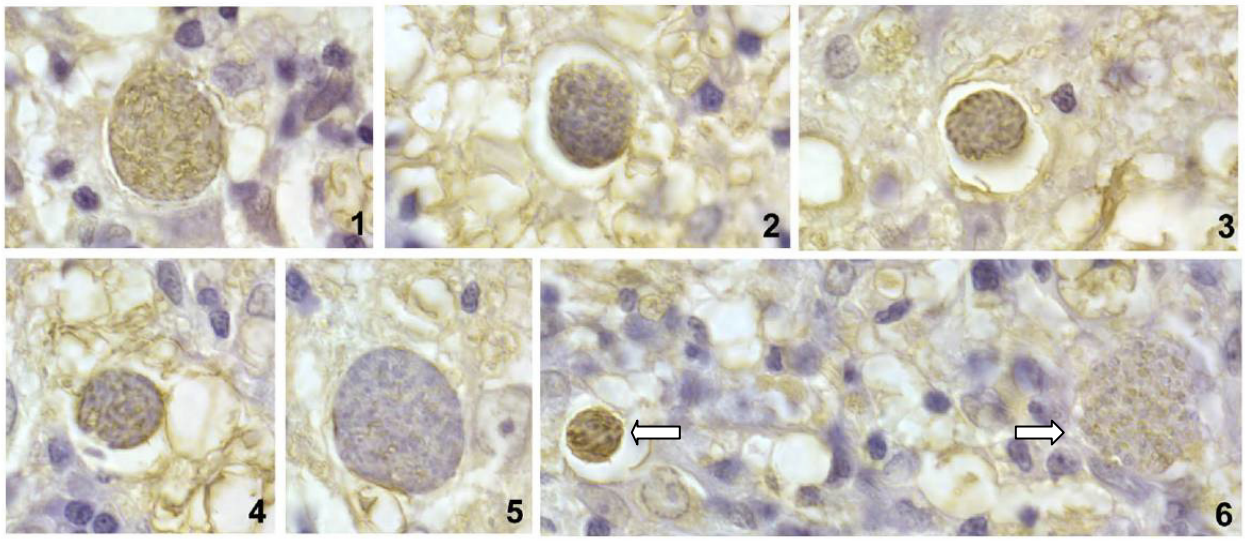


Figure 3.3. *N. caninum positive control sections (x1000) incubated with T. gondii antibody (1:500) for 1 hour at room temperature.* Cysts and parasites completely immunoreacted (1& 3), partially immunoreacted cysts (4 & 2), cyst that has not immunoreacted (5), two cysts located closely with a section, one of which has strongly immunoreacted and the other has not immunoreacted (6).

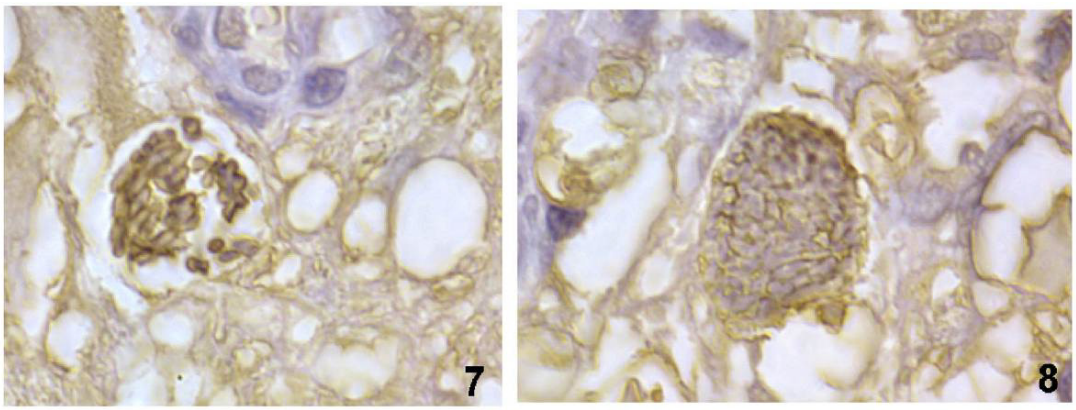


Figure 3.4. *Neospora caninum positive control sections (x1000) incubated with T. gondii antibody (1:500) at 4℃ overnight.* Tachyzoites or bradyzoites from a lysed cyst that have immunoreacted across their entire length (7). A cyst that has partially immunoreacted (8).

3.2.3.3.2 *N. caninum* tissue sections incubated with *N. caninum* antisera

Incubated for 1hour at room temperature

The *N. caninum* positive sections incubated with *N. caninum* antisera for 1 hour at room temperature usually stained a dark chocolate brown (Figure 3.5). Cysts were easily identified at 40x magnification due to their strong immunoreaction. Individual parasites could be identified within cysts at 100-1000x and tended to take up stain over their entire length. Cysts within a single cut section or from sections taken from the same sample did not always immunoreact consistently, since some cysts stained irregularly.

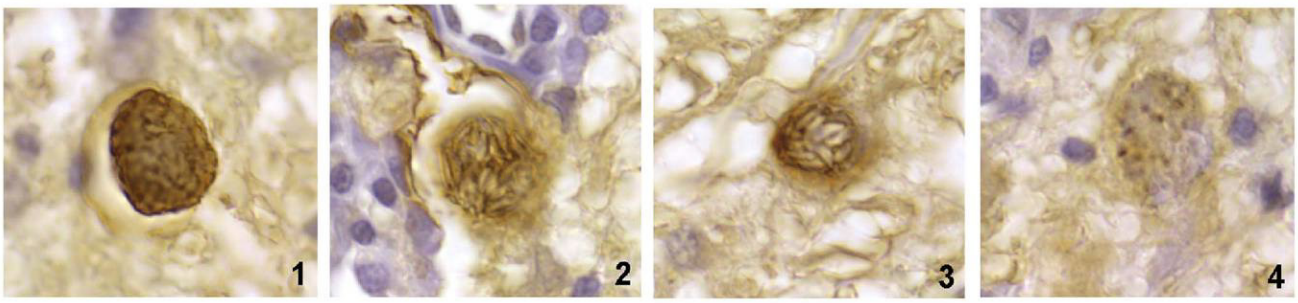


Figure 3.5. *Neospora caninum positive tissue sections (x1000) incubated with N. caninum antibody (1:1000) for 1 hour at room temperature.* Strongly immunoreacted tissue cysts staining a dark brown colour along the entire length of the parasites (1, 2 & 3). Tissue cyst that has stained intermittently (4).

Incubated overnight at 4℃

N. caninum positive sections that were incubated with the *N. caninum* antiserum overnight at 4°C, immunoreacted and were stained a brown colour (Figure 3.6). Individual parasites could be identified within cysts at magnifications between 100-1000x. At higher magnification (1000x), the cysts immunoreaction colour was accentuated in relation to that of background MH stained cells. Typically the entire length of the parasites immuno-reacted and stained brown.

A higher degree of non-specific background staining was observed in samples that were incubated overnight at 4°C than in samples incubated for 1 hour at room temperature. Staining intensity of cysts was not noticeably different between these 2 incubation conditions.

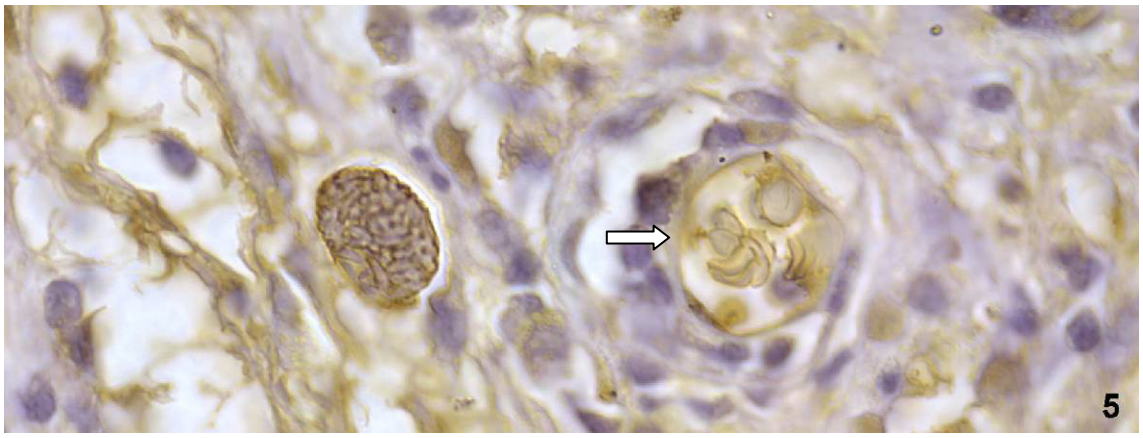


Figure 3.6. *Neospora caninum positive tissue sections (x1000) incubated with N. caninum antibody (1:1000) at 4℃ overnight.* A strongly immunoreacted *N. caninum* tissue cyst next to non-specifically stained red blood cells in a blood vessel (arrow) (5).

3.2.3.3.3 *T. gondii* tissue section incubated with *N. caninum* antisera

Incubated for 1hour at room temperature

Known *Toxoplasma gondii* positive tissue sections (*T. gondii* infected mouse brains), immunoreacted with *N. caninum* antisera following incubation for 1 hour at room temperature (Figure 3.7). Cysts and parasites stained a brown colour. Cysts from mouse tissue samples were large and clearly visible under 40 x magnification. Individual tachyzoites could be seen under magnification of 100-1000x and appeared to have immunoreacted and stained their entire length.

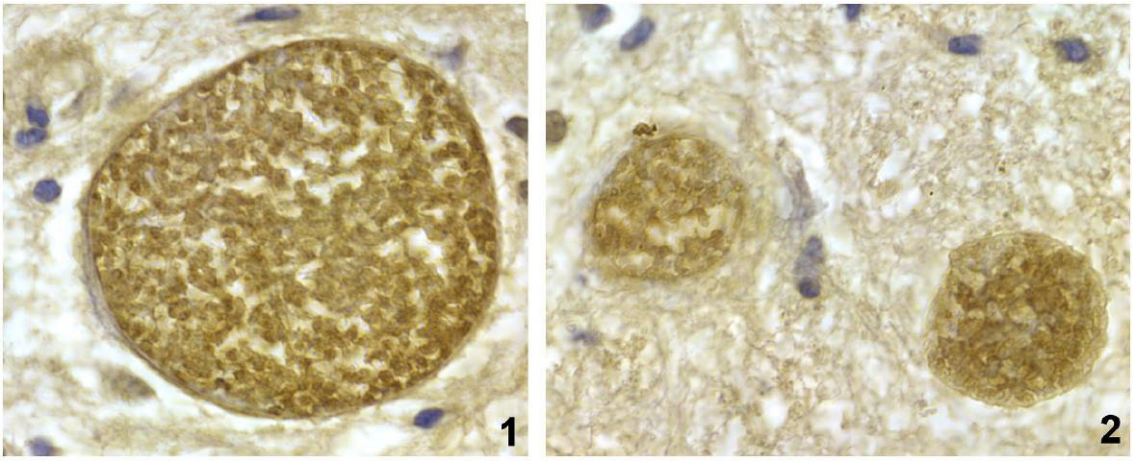


Figure 3.7. *Toxoplasma gondii positive tissue sections (x1000) incubated with N. caninum antibody (1:1000) for 1 hour at room temperature.* Examples of typically stained cysts (1 & 2)

Incubated overnight at 4℃

Toxoplasma gondii positive tissue sections incubated with *N. caninum* antisera at 4°C overnight immunoreacted and stained brown (Figure 3.8). Individual parasites could be identified when magnification from 100-1000x. There was a high degree of non-specific background staining in sections stained with *N. caninum* antibody. Cysts appeared to stain a lighter shade of brown than was seen in the *N. caninum* sections.

Sections were observed to stain relatively consistently. Most cysts were the same or similar in colour and most cysts did immunoreact with the antibody.

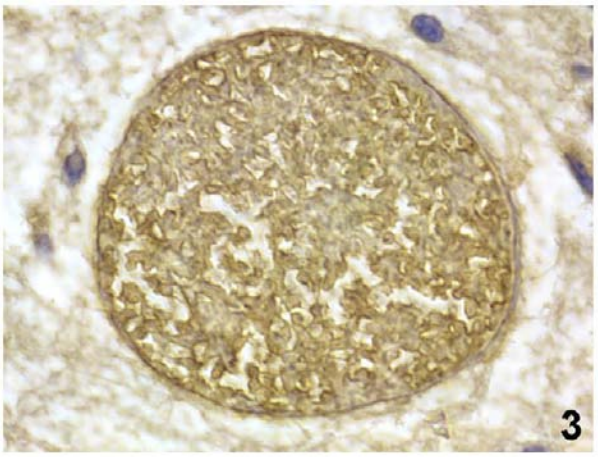


Figure 3.8. *Toxoplasma gondii positive tissue sections (x1000) incubated with N. caninum antibody (1:1000) at 4℃ overnight.* Typical example of stained cyst (3).

3.2.3.3.4 *T. gondii* tissue sections incubated with *T. gondii* antisera

Sections incubated for 1 hour at room temperature

T. gondii positive control sections stained with the *T. gondii* antisera for 1 hour at room temperature immunoreacted and stained brown (Figure 3.9). Cysts did not stain as darkly as the *Neospora* sections, but cysts were still distinguishable from the background staining as having immunoreacted. A high degree of non-specific background staining was observed. Individual tachyzoites within cysts appeared to stain over their entire length and were visible under magnification of 100-1000x.

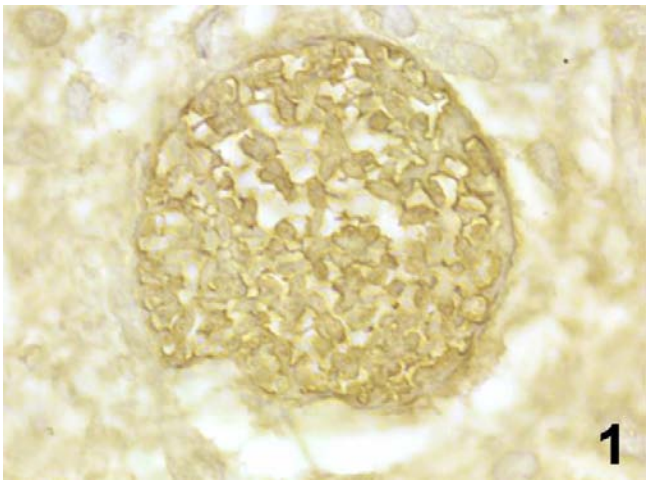


Figure 3.9. *T. gondii positive tissue sections (x1000) incubated with T. gondii antibody (1:500) for 1 hour at room temperature.* Typical example of a stained cyst (1).

Sections incubated at 4℃ overnight

T. gondii positive control sections immunoreacted with the *T. gondii* antisera when incubated at 4°C overnight (Figure 3.10). Individual tachyzoites were visible when magnified 100-1000x. Cysts were distinct from the non-specific background staining.

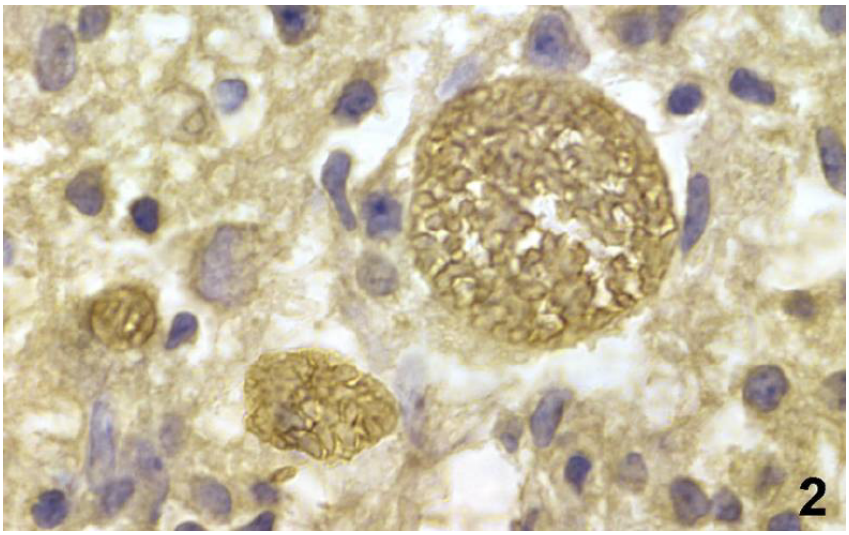


Figure 3.10. *Toxoplasma gondii positive tissue sections (x1000) incubated with T. gondii antibody (1:500) at 4℃ overnight.* Typical example of stained cyst (2).

3.2.3.4 Proteolytic digestion

Sections incubated in 0.1% Trypsin solution for 10 min at room temperature

Known *Neospora caninum* positive tissue sections (sample 11383) were incubated for 10 minutes in a 0.1% Trypsin solution at room temperature. The sections were then exposed to both *T. gondii* and *N. caninum* antisera and compared to sections that were not trypsin treated. Tissue sections immunoreacted with both *N. caninum* and *T. gondii* antisera. Cysts and parasites stained brown. Cysts that stained brown were clearly visible under 40x magnification. Individual tachyzoites could be seen under magnification of 100-1000x. A large variation in tachyzoite staining was observed. Tachyzoite staining ranged from little or no staining, partial staining, or dark staining where the entire length of the tachyzoite appeared to have stained.

Little difference was observed between sections that were treated with trypsin and those that were not. *Neospora* sections stained with *N. caninum* antisera did not consistently immunoreact either within a single cyst or between cysts found in the same section when treated (Figure 3.12) or untreated with trypsin (Figure 3.11). It did appear however that cross-reaction with *T. gondii* antiserum was increased following trypsin treatment (Figure 3.14), however in other experiments significant cross-reaction was noted without trypsin treatment (Figure 3.13).

The amount of background stain seen for the sections was similar for all slides, demonstrating that trypsin treatment had little effect on decreasing background staining within sections. Cysts that stained well were clearly visible within the cellular matrix irrespective of the amount of background staining. Therefore, only cyst staining intensity is likely to help improve detection.
Trypsin treatment was not found to be of any benefit when considering stain intensity, staining consistency or background staining. All cyst sections shown are typical examples of the immunoreactions observed for each treatment group.

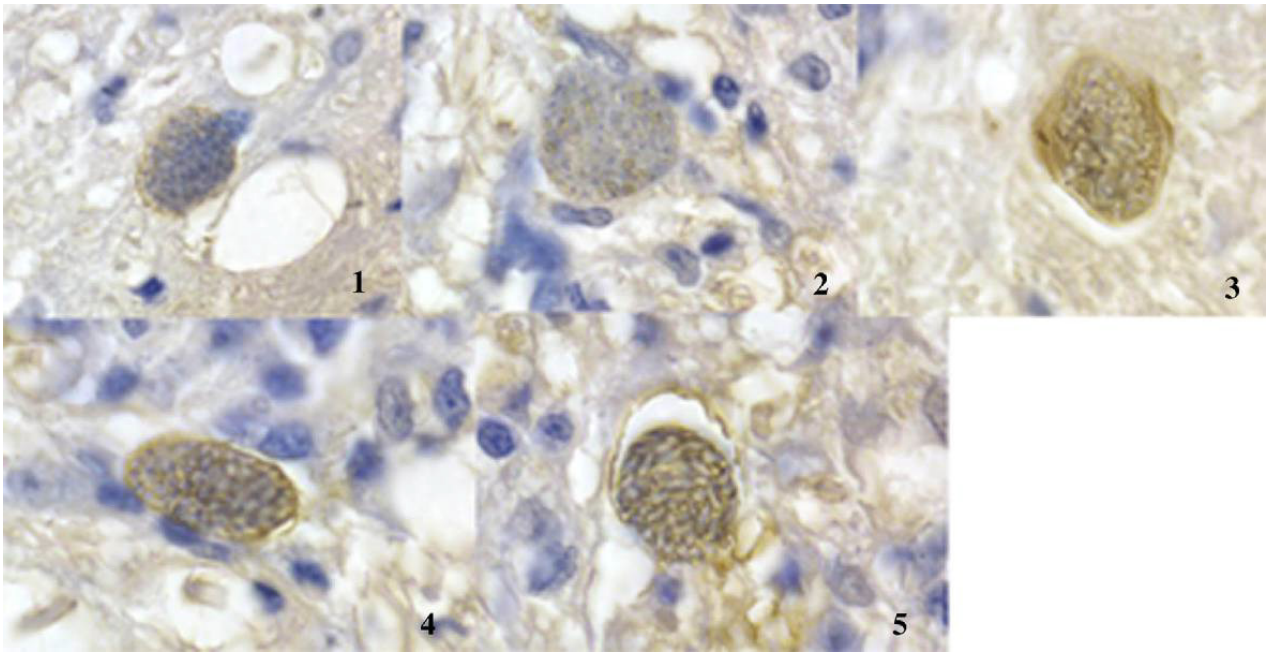


Figure 3.11. *Neospora caninum positive tissue sections (x1000) incubated with N. caninum antisera (1:1000) for 1 hour, no trypsin treatment.* Cyst immunoreactivity staining range showing intermittent to full staining (1 -5).

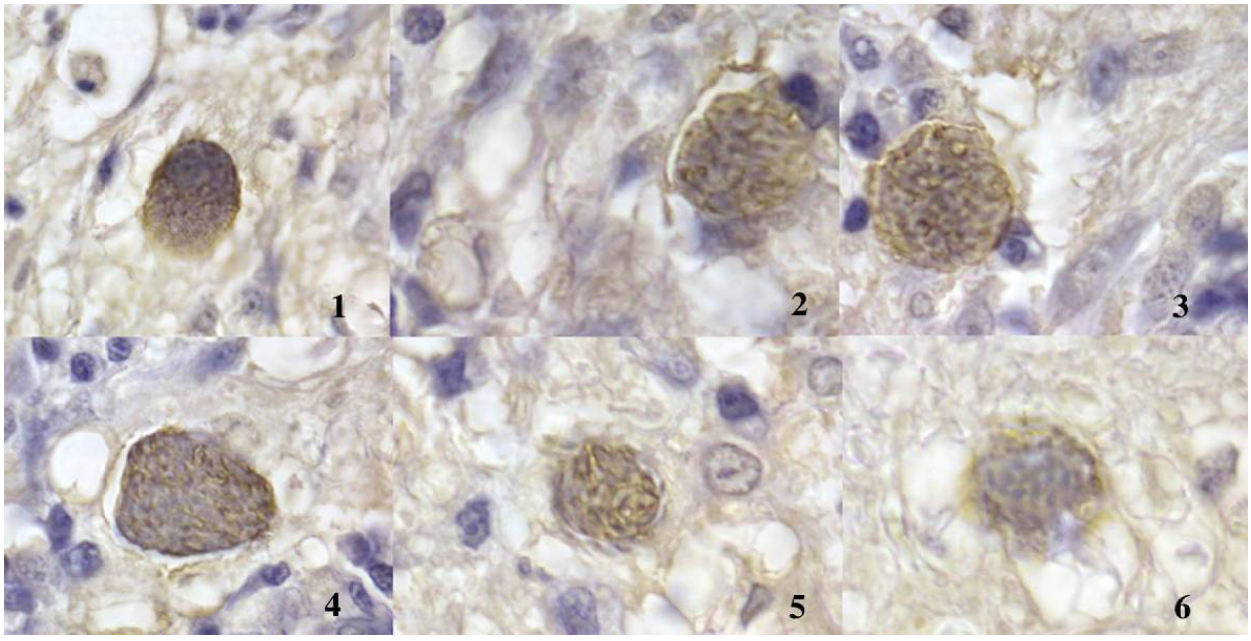


Figure 3.12. *Neospora caninum positive tissue sections (x1000) incubated with N. caninum antisera (1:1000) for 1 hour, following 1 hour of trypsin treatment.* Cyst immunoreactivity staining range showing intermittent to full staining (1-6).

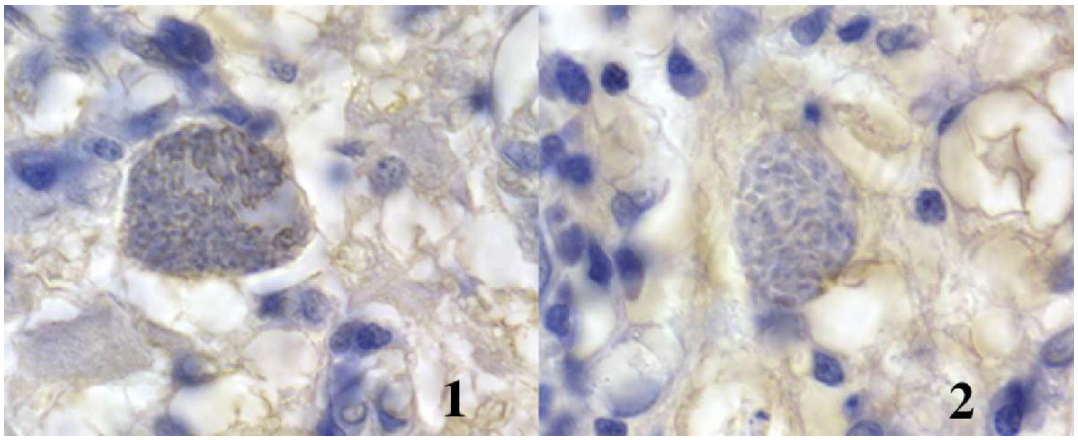


Figure 3.13. *Neospora caninum positive tissue sections (x1000) incubated with T. gondii antisera (1:500) for 1 hour, no trypsin treatment.* Cysts with little or no specific staining (1 & 2).

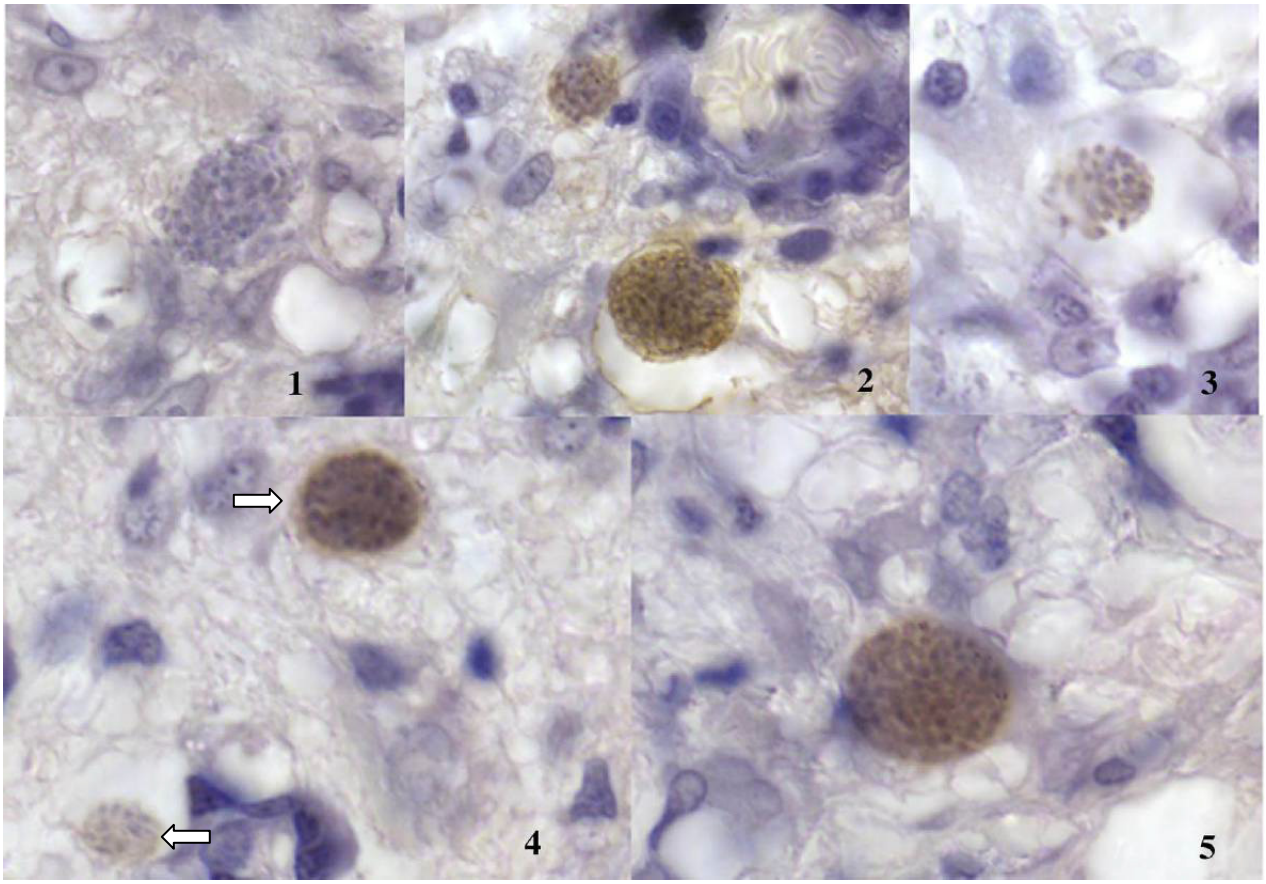


Figure 3.14. *Neospora caninum positive tissue sections (x1000) incubated with T. gondii antisera (1:500) for 1 hour, following 1 hour of trypsin treatment.* The range of immunoreactive staining of cysts shows no staining (1), intermittent (3) to full staining (2 & 5). Two cysts within a single section present different staining, one cyst is fully stained and the other is intermittently stained (4).

3.2.4 Discussion

Cysts seen in *Neospora caninum* and *Toxoplasma gondii* infected tissue sections reacted to antisera using the ABC protocol described. Under all conditions tested, some if not all cysts stained brown upon addition of DAB. In most cases parasites could be seen under high magnification as having stained along their entire length. The cyst wall usually stained which aided in identifying discreet cysts rather than intracellular tachyzoites. Staining of the cyst wall and in some cases the surrounding tissue is likely to be specific and is caused by antigen diffusion from the parasites to the surrounding tissue. However, this is not to be confused with background staining, which is caused by non-specific staining of tissue that does not directly surround parasites. Background staining may be caused by endogenous avidin, biotin or peroxidase activity or may be due to low affinity of the antibody.

Although immunohistochemistry proved to be a useful technique for the identification of tissue cysts, significant cross-reaction and inconsistent staining was observed. The *T. gondii* and *N. caninum* tissue sections both immunoreacted with *N. caninum* and *T. gondii* antisera resulting in cross-reactions between the antibodies and tissue cysts.

Non-specific background staining appeared darker in sections incubated with a higher concentration of antiserum, and in sections incubated for a longer period of time (incubated at 4°C overnight). Non-specific background staining also varied between sample types. Over exposure to DAB resulted in a darker brown colour intensity of cysts and background staining. Cysts were easier to identify in over exposed sections but a darker stain due to DAB overexposure did not increase the staining of individual parasites when inspected at high magnification.

Tissue sections can be incubated in dilute normal goat (Barr et al., 1991b; Lindsay and Dubey, 1989a; Peters et al., 2001), horse (Sundermann et al., 1997), chicken (Barber et al., 1996) or rabbit serum (Morales et al., 2001) to block non-specific binding of antibodies to tissue. Non-specific background antibodies were blocked using bovine serum albumin (BSA). Background staining was not an apparent problem during immunohistochemistry staining and analysis, therefore the BSA blocking step was considered to be effective in blocking non-specific antibodies in the sections. Had background staining been darker and resulted in high non-specific staining of tissue, an anti-goat blocking serum would have been used as the blocking serum and the link/secondary antibody should come from the same species for more effective blocking (Boenisch et al., 1989).

Endogenous peroxidase activity is common in hemoproteins such as hemoglobin (red blood) catalases (liver and kidney), myoglobin (muscle cells) and cytochrome (granulocytes and monocytes). Endogenous peroxidase activity results in the breakdown of hydrogen peroxide (H_2O_2) (Boenisch et al., 1989). Blood vessels and red blood cells did on occasion non-specifically react with the antibody due to endogenous peroxidase activity. Endogenous peroxidase activity in formalin fixed tissue can be suppressed by incubation in 3% H_2O_2 for 30min (Barber et al., 1996; Barr et al., 1991b; Lindsay and Dubey, 1989a; Morales et al., 2001; Peters et al., 2001; Sundermann et al., 1997). Some non-specific staining of blood cells occurred within the sections despite incubation in 5% hydrogen peroxide to eliminate internal peroxidase

activity and incubation with BSA to block non-specific binding. Sections that were not incubated in hydrogen peroxide showed more non-specific staining of blood and blood vessels than sections that were blocked for endogenous peroxidase activity.

Negative control sections that were not incubated with primary antibodies were seen to have very faint yellowing of blood cells and blood vessels. Blood cells and blood vessel staining appeared slightly darker in sections incubated with primary antibody, indicating that non-specific staining of blood and blood vessels was in part due to endogenous peroxidase activity that was not entirely eliminated, as well as some non-specific staining.

Neospora and *Toxoplasma* cysts present within a section, and in sections taken from the same tissue sample, frequently stained inconsistently. In some instances cysts did not stain at all. This was most often seen in sections where other cysts within the same section had stained. This implies that the technique and antibodies used were working appropriately. Cysts also varied significantly in the intensity of staining, with some cysts not staining and other cysts being stained a dark brown within the same section.

Attempts to increase staining consistency did not result in significant improvements. It was hypothesized that by increasing the primary antibody levels, cysts may stain a dark chocolate brown relative to a paler background colour. However, raising primary antibody titre did not result in darker staining of individual cysts or of individual parasites in comparison to background staining or other non-specific staining. Although a marginally darker cyst colour was observed when incubated with a higher antibody titre, background staining also increased. Therefore the cysts did not contrast any better against the background than they had previously. The recommended antibody titre levels of the manufacturers were used following antibody titre experiments, as no significant benefit could be observed by altering antibody titre.

Antibody titre and incubation time have an inverse relationship. The higher the antibody titre, the shorter the incubation time can be. Antibodies with a high affinity require incubation for shorter periods. Equilibrium is reached once the antigen is saturated with antibody. Prolonging primary antibody incubation time beyond saturation time, or the point of equilibrium, will not result in a better specific stain. Incubating samples overnight at 4°C was performed to determine whether the antibodies properly saturated the antigens. Saturation should result in increased stain intensity. Equilibrium is reached more rapidly at higher temperatures, but it was not known whether higher temperatures result in more specific antigen-antibody reactions without increasing non-specific background staining. Therefore, a temperature of 4°C was used, as the lower temperature was expected to decrease the level of background staining.

Increasing incubation time and decreasing incubation temperature had no significant positive effects on cyst staining. It appeared that antigen-antibody saturation occurred within the 1-hour incubation time at room temperature. Extended incubation periods tended to produce a slightly higher level of background staining than occurred in sections incubated for 1 hour at room temperature. Therefore, cyst stain intensity was not increased in relation to background staining. In addition to stain intensity not being improved by the increased incubation period and decreased temperature, cyst stain consistency was also not improved. Cysts within a section did not uniformly stain as some cysts stained darkly, others unevenly and some did not stain at all.

The altered incubation period did not appear to adversely affect stain consistency but no significant benefit was noted either. Following the incubation period experiments, all samples were incubated at room temperature for 1-hour.

Formalin fixes tissue by addition, which causes methylene-bridges and cross-linking between basic amino acids within the tissue. Cross-linking can result in low permeability of macromolecules such as antibodies (White et al., 1998). Proteolytic digestion will reverse the cross-linking effect of conformational changes resulting in formaldehyde reacting with amino acids adjacent to an epitope. Weak or non-specific staining often occurs due to over-fixation resulting in masking of antigenic determinants due to aldehyde cross-linking and increased hydrophobicity of tissues.

Some tissue antigens require a proteolytic digestion with trypsin prior to staining depending on the antigen, primary antibody and type and extent of fixing. Formalin requires 6-24 hours to permeate tissue properly. Antibody penetration is often reduced following over-fixation as tissue penetration is difficult for macromolecules.

Samples were incubated for 5-10 minutes in a 0.1% Trypsin solution during proteolytic digestion. Protease digestion appeared to have little or no effect in increasing staining consistency. Many cysts did not stain, nor was an increase in staining intensity observed. Proteolytic digestion can damage tissue morphology (D'Andrea, 2004). However, tissue damage was not observed following proteolytic digestion experiments. Heat-mediated antigen retrieval (HMAR) has been reported to be very effective for enhancing immunohistochemical detection. The procedure involves heating deparaffinised sections in the presence of an appropriate buffer solution. This technique will maintain normal cellular morphology better than proteolytic digestion. HMAR treatment resulted in superior staining for *N. caninum* than protease treatment of sections, which did not stain following treatment (White et al., 1998). During the course of this study HMAR was not utilized but *N. caninum* cysts stained with antibody regardless of proteolytic digestion, despite digestion having no significant benefit. As an increase in consistent staining potential was not observed in sections following proteolytic digestion, it was not considered a valuable step to add to the procedure and was therefore not used subsequently.

Polyclonal antibodies are immunochemically dissimilar antibodies produced by different cells that react with various epitopes on a given antigen. Cross-reactivity of an antibody may be the result of specific interaction with an identical epitope, which may be found on two or more different antigen molecules. Monoclonal antibodies react with a single epitope on an antigen, which decreases non-specific antibody staining, increases homogeny and decreases batch and lot variation of antibodies. Epitopes are the structural parts of the antigen, which react with an antibody. The *Neospora caninum* and *Toxoplasma gondii* antisera used were polyclonal antibodies, which increases the likelihood of cross-reaction.

Cysts could be identified in all known positive samples stained with primary antibodies. Cross-reaction occurred in all samples regardless of pre-treatment, incubation condition or primary antibody used. Known *Neospora*-positive sections cross-reacted with *T. gondii* antisera under all conditions tested. Incubation time and temperature appeared to have no effect on the occurrence of cross-reaction, neither did blocking with BSA, hydrogen peroxide treatment, proteolytic digestion or the titre of antisera. Similar cross-reaction occurred for *Toxoplasma*-positive sections

stained with *N. caninum* antisera. Cysts from both parasite types immunoreacted strongly with their own species-specific antisera, but cross-reaction with the other antisera could not be eliminated by the treatments used.

Cross-reaction appeared to be more marked in sections that were over-exposed to DAB. This is likely to be due to the increased brown background colour intensity seen across the entire section, suggesting that the amount of cyst staining relative to that of the background was much the same. Increased titres in antisera and increased incubation periods with primary antibody did not appear to increase non-specific staining of cysts but background staining was often increased. Despite the high level of cross-reaction, cysts did not immunoreact consistently. Not all cysts stained brown and the level of staining was often inconsistent or intermittent. Apical staining was observed in numerous cysts, which is associated with non-specific staining. Partial staining of the parasites' bodies may also be associated with non-specific staining of dense granules (Sundermann et al., 1997). Non-specific staining of apicomplexan parasites is frequently reported (McAllister et al., 1996c). Specificity may be increased by using alternative antibodies and by modifying antibody presentation (Cole et al., 1994; Bjorkman et al., 1994; Jenkins et al., 2002; Peters et al., 2001). Some parasites could be seen as having been stained over their entire length following cross-reaction despite other parasites and cysts only partially staining. In the case of the dog spinal sample (11383), it is possible that the tissue is infected with both *N. caninum* and *T. gondii* as this naturally infected sample may have been exposed to more than one pathogen. It is extremely unlikely, however, that experimentally infected mice had been infected with both parasites as they were housed in a containment unit and inoculated with a known *T. gondii* strain.

3.2.5 Conclusions

Improved identification of individual cysts at low magnification through producing a darker stain intensity could not be achieved, nor could cross-reaction between *N. caninum* and *T. gondii* antisera in cysts in tissue sections be eliminated.

It is recommended that antibodies be used at 1:1000 for *N. caninum* and 1:500 for *T. gondii* and that incubation in the primary antibody be for 1 hour at room temperature. Under these conditions background staining is minimized and cross-reacted cysts appear pale in comparison to cysts incubated with species-specific antibodies.

It is not recommended that IHC be used as a definitive test for either *N. caninum* or *T. gondii*. Although cysts can be identified using IHC, a clear distinction between *N. caninum* and *T. gondii* cannot be made due to cross-reaction.

3.3 Polymerase Chain Reaction (PCR)

3.3.1 General Description

A polymerase chain reaction (PCR) is a rapid molecular biology technique used for exponentially amplifying DNA via in-vitro enzymatic replication of a specific segment of DNA. PCR has many different applications, one of which is its use in detection and identification of disease agents.

PCR works by denaturing (separating) DNA strands and primers by heating, followed by annealing (binding) of the specific primers to the DNA strands at a lower temperature. Primers are chosen in order to flank each end of the DNA segment required for amplification. Annealing is followed by synthesis/extension/elongation, which requires deoxyribonucleoside triphosphates (dNTPs), DNA polymerase (Taq), buffers and salts. During elongation the DNA is copied to produce one new strand of DNA exactly the length between the primer ends. The new DNA strand will be bound to the old strand of DNA, thus amplifying the section of DNA two-fold. The denaturing, annealing and elongation phases are repeated approximately 20-30 times in order to amplify a small amount of DNA into a large detectable amount of DNA. Thus 30 cycles of amplification should result in 2^{28}-fold copies of the selected DNA fragment (270 million-fold).

3.3.1.1 PCR Primer Background

Ellis et al., (1998) found nucleotide differences between *Toxoplasma gondii* and *Neospora caninum* in the D2 expansion segment (or domain) (also called the C1/C1' region) of the large subunit (LSU) rDNA. These differences were incorporated into a primer that formed the basis of a species-specific polymerase chain reaction (PCR) for *N. caninum*. The D2 domain of the LSU rDNA represents a new genetic marker that can be used for the differentiation and identification of *Neospora* from other cyst-forming coccidia (Ellis et al., 1998).

PCR primers NF6 and TF6 were designed, based on the observed nucleotide differences between *Neospora* and *Toxoplasma* in the Tim15/GA1 sequence in an attempt to generate a species-specific PCR primer. The primer combination GA1/NF6, under PCR conditions that utilise a primer annealing temperature of 55°C, produce a unique species-specific PCR product of 250 bp from *N. caninum* DNA. No product was reportedly obtained using these two primers with *Toxoplasma*. The primer combination TF6 and GA1 generated no PCR product from *Neospora* using a primer annealing temperature of 55°C. However, products of approximately 250 bp were obtained from *T. gondii* and *H. hammondi* DNA respectively (Ellis et al., 1998).

Yamage et al., (1996) suggests that ratios of very large quantities of host material to low amounts of parasite material could yield false negative results during PCR reactions. Their results indicate that the primer pair Np21/Np6 can be used for PCR reactions to provide an efficient tool for the diagnosis of *Neospora* in central nervous tissues obtained at necropsy during large-scale

epidemiological studies. They found that the Np21/Np6 primer pair could detect a single tachyzoite in a background of DNA derived from 2 mg of brain tissue (Yamage et al., 1996).

3.3.2 Materials and Methods

3.3.2.1 Primers

Primers cloned by (Ellis et al., 1998).
GA1 (5'-AACCTCTCTCAGAGATCG)
NF6 (5'-GTCCCTCGTGGACCC)
TF6 (5'-GTTCCTTGTGGACCG)

Modified from primers (Np21/Np6) cloned by (Yamage et al., 1996).
*Neo*F (5'-GTGTGCGTCCAATCCTGTAAC)
*Neo*R (5'-GCCAGTCAACCTACGTCTTC)

3.3.2.2 Reconstitution and Quantification of Primers

All PCR primers were purchased from GibcoBRL Custom Primers.

Primers GA1, NF6 & TF6
Primer GA1: Reconstituted in sterile water to 100 μM solution
Primer NF6: Reconstituted in sterile water to 100 μM solution
Primer TF6: Reconstituted in sterile water to 100 μM solution

Primers NeoF & NeoR
Primer NeoF: Reconstituted in sterile water to 10 μM solution
Primer NeoR: Reconstituted in sterile water to 10 μM solution

3.3.2.3 PCR Reaction Mixtures and Running Procedures

Three PCR techniques were developed and used for the detection of *Neospora caninum* from cell culture samples, isolated tissue and isolated DNA.

3.3.2.3.1 PCR-1

PCR-1 Reaction Mixture

Buffer	2 µl
$MgCl_2$	0.6 µl
10 mM dNTP	0.4 µl
100 uM NF6 or TF6	0.1 µl
100 uM GA1	0.1 µl
Taq	0.1 µl
Template	*(1-5* µl*)*
Sterile Water	Add to make volume to 20 µl
Total mixture	20 µl volume

PCR-1 Running Protocol

For *N. caninum* specific primer combination GA1/NF6 and *T. gondii* primer combination GA1/TF6 the following conditions were used; initial denaturation at 94°C for 5 minutes, followed by 30 cycles of; denaturation at 95°C for 1 minute, primer annealing at 55°C for 30 seconds and primer extension at 72°C for 2 minutes. The PCR was terminated with a final primer extension reaction at 72°C for 5 minutes (Ellis et al., 1998).

3.3.2.3.2 PCR-2

PCR-2 Reaction Mixture

Buffer	1.25 *µl*
5 mM dNTPs	0.25 *µl*
10 uM Neo F	0.25 *µl*
10 uM Neo R	0.25 *µl*
Taq	0.1 *µl*
Template	*(1-5 µl)*
Sterile Water	Add to make volume to 12.5 µl
Total mixture	12.5 µl volume

PCR-2 Running Protocol

The PCR cycle was modified from that described by Yamage et al (1996). For the primer combination NeoF/NeoR the following conditions were used; initial denaturation at 94°C for 1.5 minutes, followed by 25 cycles of; denaturation at 94°C for 5 seconds, primer annealing at 60°C for 5 seconds and primer extension at 72°C for 15 seconds. Followed by a further 25 cycles of; denaturation at 94°C for 5 seconds, primer annealing at 60°C for 5 seconds and primer extension at 72°C for 45 seconds (Yamage et al., 1996).

3.3.2.3.3 PCR-3

PCR-3 Reaction Mixture

Buffer	1.25 µl
$MgCl_2$	0.6 µl
10 mM dNTP	0.25 µl
100 uM NF6 or TF6	0.25 µl
100 uM GA1	0.25 µl
Taq	0.1 µl
Template	*(1-5* µl*)*
Sterile Water	Add to make volume to 20 µl
Total mixture	20 µl volume

PCR-3 Running Protocol

PCR-3 was developed as a hybrid of PCR-1 and PCR-2. The GA1/NF6 and GA1/TF6 primers were amplified using a modified cycle of PCR-2. Initial denaturation at 94°C for 1.5 minutes, followed by 25 cycles of; denaturation at 94°C for 5 seconds, primer annealing at 55°C for 5 seconds and primer extension at 72°C for 15 seconds. This was followed by a further 25 cycles of denaturation at 94°C for 5 seconds, primer annealing at 55°C for 5 seconds and primer extension at 72°C for 45 seconds.

3.3.2.4 PCR Product Separation and Visualization

PCR products were mixed with 5 ul of loading dye, bromophenol blue (approx. 300 bp running speed) and/or xylene cyanol (approx. 4000-5000 bp running speed), prior to placing 10-20 ul into gel wells. Loading dyes were used to visualise the running distance of the samples within the gel. Xylene cyanol was the preferred running dye as it did not run at the same speed as the bands of interest. Marker running dyes can be seen under normal light.

A PCR ladder was used at a 1:5 dilution. Loading dye was mixed with 5 ul of ladder and loaded into a gel well for running alongside PCR products. DNA ladders of 50 bp and 100 bp were used, the later used most frequently. (See Appendix 3.6 for PCR Solutions and materials.)

PCR products were most frequently separated on 1% agarose gel that contained 7 ul of ethidium bromide per 100 ml of gel. Gels were run out in a large electrophoresis tank containing 1x TAE buffer and a further 24 ul of ethidium bromide. Gels were run at 100 volts for approximately 30-60 minutes.

DNA bands within agarose gels were visualized using ultra violet light (UV). In the presence of DNA ethidium bromide fluoresces under UV light. The gels were photographed with a digital camera and the images printed.

3.3.2.5 Crude Cell Samples

Crude whole tissue isolation samples, consisting of homogenised tissue, mostly from the brain or spinal cord, were initially used as PCR template.

Crude cell lysis was improved by separating and concentrating samples using a Percoll gradient, which increased the number of parasites in relation to the amount of host tissue. Tissue culture and mouse inoculum (Percoll purified tissue suspended in media) was chosen as a preferred crude sample for PCR.

3.3.2.5.1 Sample Water-bath Boiling

Boiling was used to prepare samples for PCR. A 30 ul aliquot of samples was placed into a micro-centrifuge tube and boiled in a water bath for 5 minutes. The samples were put directly on ice following boiling. Once chilled, either total cell lysate was used or samples were centrifuged for 2 minutes at 600 x g to pellet cell debris and the supernatant, containing DNA from lysed cells, was used for PCR.

3.3.2.5.2 Sample Microwave Boiling

Similarly to the sample boiling technique described above, PCR samples were also prepared by microwaving. Samples were microwaved on high for approximately 10-20 seconds each (or until just boiling). Samples were then placed directly on ice to chill. Following chilling, either total cell lysate was used or samples were centrifuged for 2 minutes at 14,000 rpm to pellet cell debris and the supernatant, containing DNA from lysed cells, was used in PCR.

3.3.2.6 DNeasy DNA Isolation

DNA isolation was performed using a QIAGEN DNeasy DNA extraction kit. The DNeasy kit was stored at room temperature for no longer than 1-year prior to use. The DNeasy kit was developed by QIAGEN for the purification of total cellular DNA and to completely remove contaminants and enzyme inhibitors such as proteins and divalent cations.

The procedures used for DNA isolation from animal tissue and cell culture samples were based on the technique described in the QIAGEN handbook, however some modifications were made. All samples were stored in buffer AE (supplied in kit) at -20°C.

Note: buffers AE, ATL AW1 and AL are all supplied with the DNeasy kit, no further information is provided with the DNeasy kit as to the components of the buffers.

3.3.2.6.1 DNeasy Protocol for Animal Tissues

In brief, up to 25 mg of tissue was homogenised in ATL buffer (supplied in kit) prior to the addition of Proteinase K for sample lysis and incubated overnight at 55°C. Samples were incubated for a further 10 min at 70°C with buffer AL (supplied in kit). Pure ethanol was added to the samples and the solution centrifuged using the mini columns provided. Column membranes were washed with buffer AW1 followed by buffer AW2 (supplied in kit) and the DNA eluted using buffer AE (supplied in kit) (Appendix 3.7).

3.3.2.6.2 DNeasy Protocol for Cultured Cells

A maximum of 5 x 10^6 cells were used per sample tube. Proteinase K and buffer AL (supplied in kit) were added to the samples and incubated for 10 min at 70°C. The technique continued as described in the DNeasy handbook for animal tissue DNA isolation with the addition of ethanol, wash buffers and DNA elution (Appendix 3.7).

3.3.2.7 DNA Purification

A High Pure™ PCR Product Purification Kit was used for DNA purification from agarose gel. Briefly, PCR products were separated on a 1% low melt agarose gel and stained with ethidium bromide. Bands of interest were excised and the DNA gel sample weight determined. 300 µl of Binding Buffer per 100 mg agarose gel was added to dissolve the agarose to release the DNA. The sample was mixed and incubated at 56°C for 10 minutes or until the gel had dissolved. 150 µl of isopropanol per 100 mg of agarose gel was added, mixed well and the solution placed into a High Pure™ Filter Tube in a Collection Tube. The sample was centrifuged at full speed and washed twice with wash buffer (as provided in the kit). Samples were eluted with elution buffer (as provided in the kit) and centrifuged at full speed (Appendix 3.8. Protocol derived from High Pure PCR Production Kit manual).

3.3.2.8 Ethanol/Glycogen DNA Precipitation

DNA precipitation was performed when DNA concentration was required. A 10:1 solution of DNA to sodium acetate (3M) solution was made, to which 1 µl of 20 mg/ml glycogen per ml of nucleic acid solution and 3 volumes of ethanol were added and the mixture incubated at -70°C for 20 min. Samples were centrifuged at 1500 rpm to pellet the DNA and washed with 70% ethanol. The pellet was air-dried and re-suspended in water, Tris buffer or TE buffer and stored at -20°C.

3.3.2.9 DNA Sequencing

DNA sequencing was used to analyse PCR products produced from two mouse blood samples, mouse #16 and mouse #4. The mouse blood products were compared to PCR products generated

from an *N. caninum* infected cell culture. DNA was isolated from a low melt agarose gel and the DNA samples were sequenced by the Massey University DNA Analysis Service. Nucleotide sequence results were generated for genetic analysis. DNA sequences were compared to sequences listed on the NCBI BLAST website.

3.3.2.10 PCR-1 Validation

The primers, reaction mixture and running protocol for PCR-1 were validated for sensitivity and specificity using both *Neospora* and *Toxoplasma* samples. ToxoVax® and harvested *T. gondii* and Nc-Liverpool tachyzoite cultures were used in combination with the NF6 (forward *Neospora* primer), TF6 (forward *Toxoplasma* primer) and the GA1 primer (reverse primer for both *Neospora* and *Toxoplasma*). Each sample was performed in duplicate. Samples were prepared by boiling for 5 minutes in a water bath then placing on ice, as described in 'sample boiling' above. PCR products were run on an agarose gel at 100 V for 45 minutes with a 100 bp ladder before being stained with ethidium bromide solution for 40 minutes and photographed.

3.3.2.11 PCR-2 Sensitivity Trial

DNA samples were prepared from Nc-Liverpool tachyzoite cultures using the DNeasy protocol for cultured cells. Samples DNeasy-1 and DNeasy-2 were made from $5x10^6$ and $5x10^5$ tachyzoites respectively (see Table 3.1). Each sample was diluted to extinction using a dilution series of 1:10, 1:100, 1:500, 1:1000, 1:5000 and 1:10,000. Samples were run according to PCR-2 as described above using the NeoF/NeoR primer pair. (Also see PCR-2 optimisation below.)

3.3.2.12 PCR-3 Development

PCR-3 was derived from both PCR-1 and PCR-2. The primers, reaction mixture and running temperatures from PCR-1 were used in combination with the running cycles and times described for PCR-2. Extra Mg++ was also added to PCR-3 to ensure that magnesium was not a limiting factor for this PCR.

3.3.2.13 DNeasy elution trial

The amounts of DNA collected in elutions 1 and 2 were compared. DNA from a *T. gondii* infected mouse brain sample (sample T15010208S) was isolated using the DNeasy protocol for tissues, as described. Elutions 1 and 2 were kept separate. The PCR-3 procedure was used to amplify the samples. A PCR mixture containing 1 μl of DNA template from elution 1 was made as well as PCR mixtures containing 1 μl, 2 μl and 5 μl of DNA template from elution 2.

3.3.2.14 PCR template preparation trial

Sample preparation techniques were compared using PCR. Three processing techniques were tested using Nc-Liverpool cultured tachyzoites to produce three different sample types: i) DNeasy protocol for cultured cells – purified DNA sample, ii) boiling by microwaving - whole cell lysate sample, iii) boiling by microwaving and centrifuging 2 min at 600 x g – supernatant sample.

All samples were processed from the same stock dilutions of $5x10^6$, $5x10^5$, $5x10^4$ or $5x10^3$ Nc-Liverpool tachyzoites per ml. For the purified DNA samples, the DNeasy elutions 1 and 2 were combined and the total volume made up to 1 ml. All boiled samples were made of 1 ml of the stock solution. For all samples, the $5x10^4$ and the $5x10^3$ tachyzoite concentrations were run in triplicate but the samples were prepared at different times, either on the day, the day before or 1 month prior to the day of PCR amplification.

Samples were prepared as described in (Table 3.1) below. Samples were run according to PCR-2, as described above.

Table 3.1. *PCR template preparation*

Sample ID	Nc-Liverpool Tachyzoite count	Preparation prior to PCR	Agarose gel lane (Figure 3.18)
DNeasy-1	$5x10^6$	Same day	1
DNeasy-2	$5x10^5$	Same day	2
DNeasy-3	$5x10^4$	Same day	3
DNeasy-4	$5x10^3$	Same day	4
DNeasy-5	$5x10^4$	Previous day	5
DNeasy-6	$5x10^3$	Previous day	6
DNeasy-7	$5x10^4$	1 month prior	7
DNeasy-8	$5x10^3$	1 month prior	8
Blank	Negative control	Same day	9
Lysate-1	$5x10^6$	Same day	10
Lysate-2	$5x10^5$	Same day	11
Lysate-3	$5x10^4$	Same day	12
Lysate-4	$5x10^3$	Same day	13
Lysate-5	$5x10^4$	Previous day	14
Lysate-6	$5x10^3$	Previous day	15
Lysate-7	$5x10^4$	1 month prior	16
Lysate-8	$5x10^3$	1 month prior	17
-	-	-	18
Blank	Negative control	Same day	19
100bp PCR ladder	-	Same day	20
Super-1	$5x10^6$	Same day	21
Super-2	$5x10^5$	Same day	22
Super-3	$5x10^4$	Same day	23
Super-4	$5x10^3$	Same day	24
Super-5	$5x10^4$	Previous day	25
Super-6	$5x10^3$	Previous day	26
Super-7	$5x10^4$	1 month prior	27
Super-8	$5x10^3$	1 month prior	28
-	-	-	29
Blank	Negative control	Same day	30
-	-	-	31
100bp PCR ladder	-	Same day	32

3.3.2.15 PCR-2 Optimisation

Problems with reproducibility of PCR-2 led to optimisation by adding a further 2.5 µl of Mg++ per sample and decreasing the volume of water by 2.5 µl. The NeoF/NeoR primer pair was run in parallel with the NF6/GA1 primer pair using the PCR running conditions described for PCR-2

and each primer pair was run with and without additional Mg++. Templates DNeasy-1, DNeasy-2, DNeasy-3 and DNeasy-4 (see Table 3.2) were used in each reaction mixture.

Table 3.2. *PCR templates and additives*

Sample ID	Nc-Liverpool Tachyzoite count	Primer Pair	Additive	Agarose gel lane (Figure 3.19)
DNeasy-1	$5x10^6$	NeoF/NeoR	None	1
DNeasy-2	$5x10^5$	NeoF/NeoR	None	2
DNeasy-3	$5x10^4$	NeoF/NeoR	None	3
DNeasy-4	$5x10^3$	NeoF/NeoR	None	4
DNeasy-1	$5x10^6$	NeoF/NeoR	Mg++	5
DNeasy-2	$5x10^5$	NeoF/NeoR	Mg++	6
DNeasy-3	$5x10^4$	NeoF/NeoR	Mg++	7
DNeasy-4	$5x10^3$	NeoF/NeoR	Mg++	8
100bp Ladder	-	-	-	9
DNeasy-1	$5x10^6$	NF6/GA1	None	10
DNeasy-2	$5x10^5$	NF6/GA1	None	11
DNeasy-3	$5x10^4$	NF6/GA1	None	12
DNeasy-4	$5x10^3$	NF6/GA1	None	13
DNeasy-1	$5x10^6$	NF6/GA1	Mg++	14
DNeasy-2	$5x10^5$	NF6/GA1	Mg++	15
DNeasy-3	$5x10^4$	NF6/GA1	Mg++	16
DNeasy-4	$5x10^3$	NF6/GA1	Mg++	17
100bp Ladder	-	-	-	18
Negative control	None	NeoF/NeoR	Mg++	19
Negative control		NF6/GA1	Mg++	20

3.3.3 Results

3.3.3.1 PCR-1 Validation Results

PCR with the primer pairs TF6 and GA1 resulted in positive bands of approximately 250 bp for all *Toxoplasma* positive control samples (ToxoVax® and *Toxoplasma* culture). The Nc-Liverpool positive control sample amplified with the NF6 primer pair also produced the expected 250 bp PCR products. Additionally neither the *Toxoplasma* nor *Neospora* positive control samples generated PCR products when amplified with PCR primers specific for the other genus. All replicate PCR product bands are of the same size and intensity and all mismatched sample and primer bands were absent, suggesting that this PCR is highly specific and repeatable.

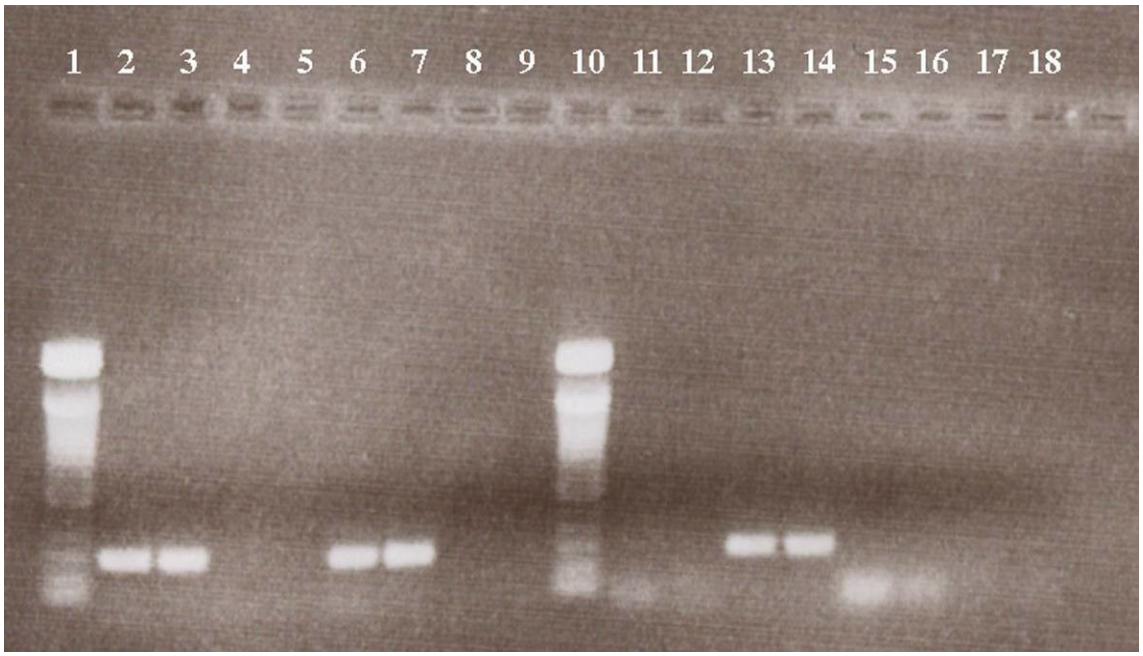


Figure 3.15. *PCR-1 validation agarose gel photograph.* 100bp PCR ladder (Lanes 1 & 10), ToxoVax® with TF6 (*Toxoplasma*) PCR primer (lanes 2-3), ToxoVax® with NF6 (*Neospora*) PCR primer (lanes 4-5), *Toxoplasma* culture with TF6 PCR primer (lanes 6-7), *Toxoplasma* culture with NF6 PCR primer (lanes 8-9), Nc-Liverpool culture with TF6 PCR primer (lanes 11-12), Nc-Liverpool culture with NF6 PCR primer (lanes 13-14), Blank (no sample) with TF6 PCR primer (lanes 15-16), Blank with NF6 PCR primer (lanes 17-18).

3.3.3.2 PCR-2 Sensitivity Validation Results

PCR-2 was performed using the primer pair NeoF/NeoR. The DNA amplification consisted of two running cycles each repeated 25 times. Sample DNeasy-1 could be detected to the lowest dilution of 1:10,000. The sample is made of DNA from $5x10^6$ tachyzoites in 1 ml of buffer from which 1 ul was removed for PCR template and diluted to the described volume, therefore 1 ul of undiluted sample should contain the DNA from 1000 tachyzoites. At the lowest dilution of 10,000 the PCR is detecting 0.1 tachyzoites (1000/10,000). Sample DNeasy-2 was made from $5x10^5$ tachyzoites and had a lower detection level of 1:500, which corresponds to a detection level of 1 tachyzoite. The bands observed for sample DNeasy-1, are not particularly uniform and do not decrease in intensity in a dose wise manner, unlike the bands seen for sample DNeasy-2. An error was made during the loading of this gel as a PCR ladder was not run.

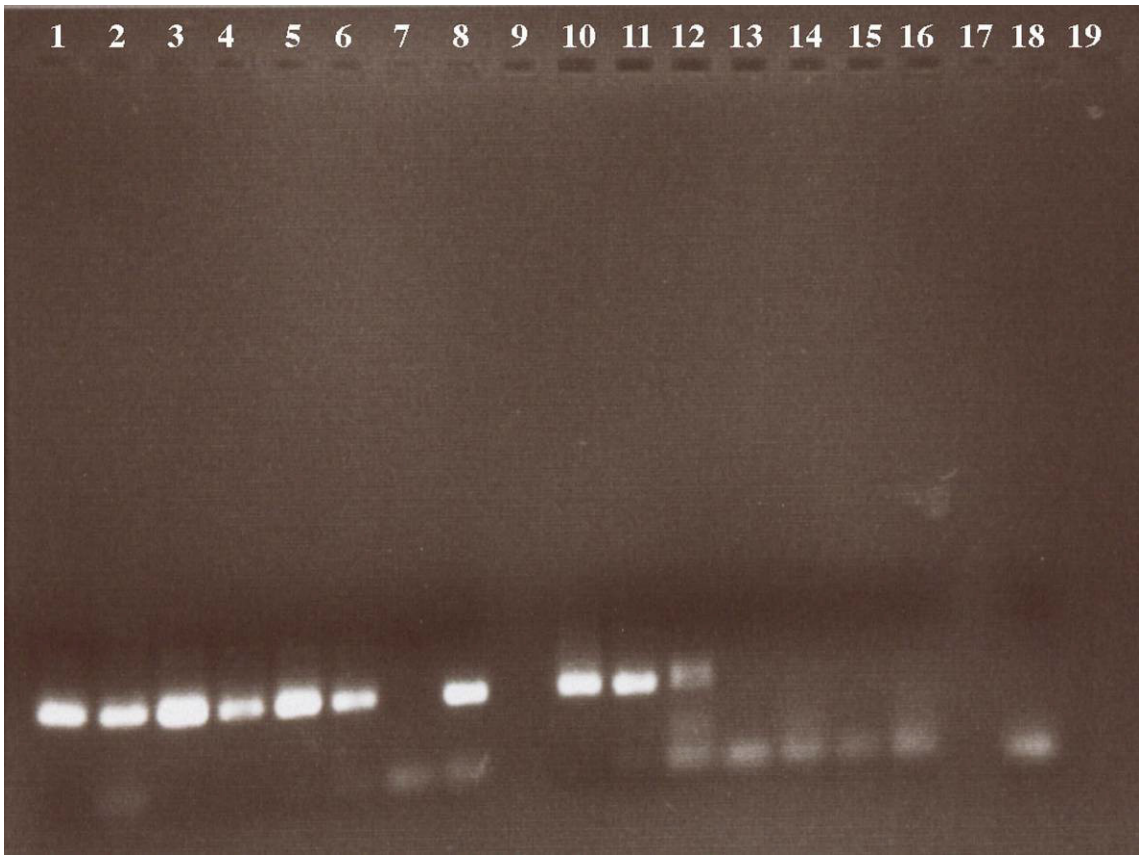


Figure 3.16. *PCR-2 sensitivity validation agarose gel photograph.* Sample DNeasy-1 diluted to 1:10, 1:100, 1:500, 1:1000, 1;5000, 1:10,000 respectively (Lanes 1-6), blank- no sample (Lanes 7 & 16), *N. caninum* positive control- undiluted (Lane 8), Sample DNeasy-2 diluted to 1:10, 1:100, 1:500, 1:1000, 1;5000, 1:10,000 respectively (Lanes 10-15), Ladder/error (Lane 16), empty wells (Lanes 9 & 17), ToxoVax® positive control sample (Lane 19).

3.3.3.3 DNeasy Elution Results

DNeasy processing of T. gondii infected mouse brain tissue J15010208S produced two elution samples that were tested using PCR. PCR product bands of the correct size (250 bp) for *T. gondii* samples were observed for both the first and second elutions. The PCR product from 1 µl of elution-1 DNA template in lane 2 produced a significant band that is comparable in intensity to the 2 µl of elution-2 band in lane 4. The band in lane 3 containing 1 µl of elution-2 appears to be approximately half the brightness of that seen in lane 2 for 1 µl of elution-1. It is likely that elution 2 contains approximately half the amount of DNA collected from elution 1. However, bands were seen for 1, 2 and 5 µl volumes of elution-2. The band for 5 µl of elution-2 is smudged which is likely to indicate the PCR was overloaded with template.

Figure 3.17. *DNeasy elutions PCR agarose gel photograph.* 100 bp PCR ladder (Lane 1), 1 ul of elution-1 DNA template (Lane 2), 1 ul of elution-2 DNA template (Lane 3), 2 ul of elution-2 DNA template (Lane 4), 5 ul of elution-2 DNA template (Lane 5).

3.3.3.4 PCR Template Preparation Results

All samples prepared using the DNeasy kit produced PCR products following amplification. However, only recently prepared samples produced PCR products for samples that were boiled, irrespective of whether the whole cell lysate or the supernatant was used. No bands were seen in lanes 16, 17, 27 or 28, which were all boiled samples prepared 1 month prior to PCR amplification, suggesting that the DNA in boiled samples degrades soon after boiling. It was not unexpected that the DNeasy sample integrity lasted longer than the boiled samples as the DNA is purified and stored in a DNA storage buffer (of unknown composition). However, Lanes 7 and 8 for the DNeasy samples, which contains DNA prepared 1 month prior to amplification, do not appear as bright as the recently prepared samples. The DNeasy samples are not as consistent as the samples seen for the boiling procedures. Lane 2 appears much fainter than samples of a lower concentration, suggesting that consistency and repeatability of the DNeasy purification technique may not be very reliable. Samples that were boiled and centrifuged and the supernatant used for PCR amplification appear to be effected in proportion to the number of tachyzoites used. This band intensity decrease can be seen for samples prepared on the same day and for samples prepared the previous day. Interestingly there also appears to be a slight decrease in band intensity for the samples prepared as little as 1 day prior to amplification. The same appears to be true for the whole lysate samples. When samples were boiled and the whole cell lysate used in the PCR the results appear to be most consistent. All bands for samples recently prepared appear bright with no apparent dose effect, indicating that little or no amplifiable material was lost

during processing, unlike what was seen for the DNeasy and the boil and spin samples, where it is likely that not all amplifiable material was retained during processing.

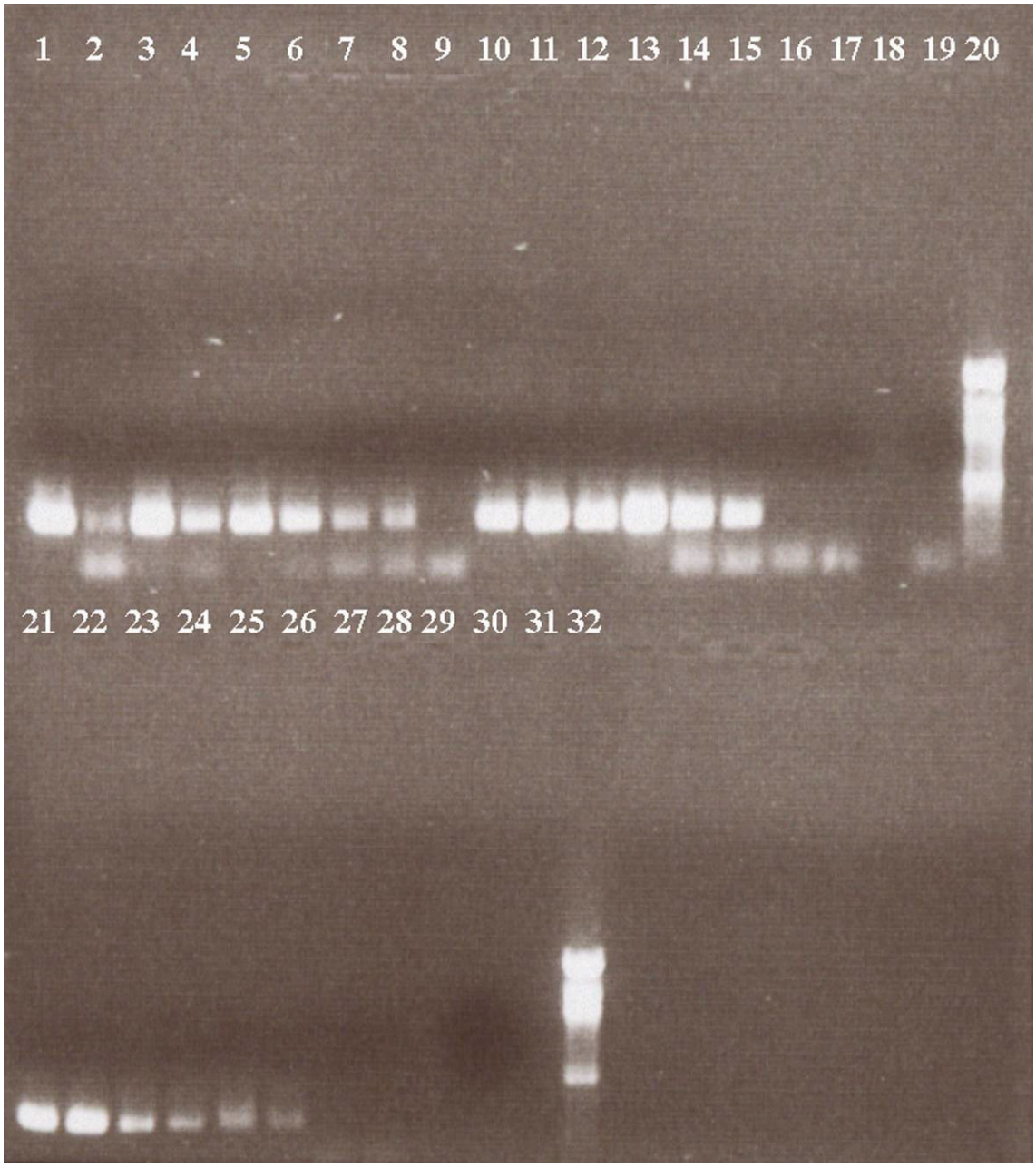


Figure 3.18. *PCR template preparation agarose gel photograph.* (Lanes as described in Table 3.1.)

3.3.3.5 PCR-2 Optimisation Results

The addition of extra Mg++ to the PCR reaction mixtures did appear to improve band consistency. The NeoF/NeoR primer pair bands without additional Mg++ do not appear consistently bright (Lanes 1-4), whereas the PCR product bands with additional Mg++ are consistently bright, except for sample DNeasy-4. Lanes 4, 8, 13 and 17 all contain PCR products amplified from DNeasy-4 sample. The lack of or faint banding in these lanes is likely to be due to sample degradation rather than a PCR fault, as all running conditions consistently showed poor banding.

Lanes 10-13 show moderate band intensity for the NF6/GA1 primer pair and a bright consistent band in the PCR reaction samples with additional Mg++, excepting band 16 which appears to have either been loaded badly or the template was not added correctly as the template band is much lighter than the others observed. The negative control samples for the NeoF/NeoR (lane 19) and the NF6/GA1 (lane 20) were both negative.

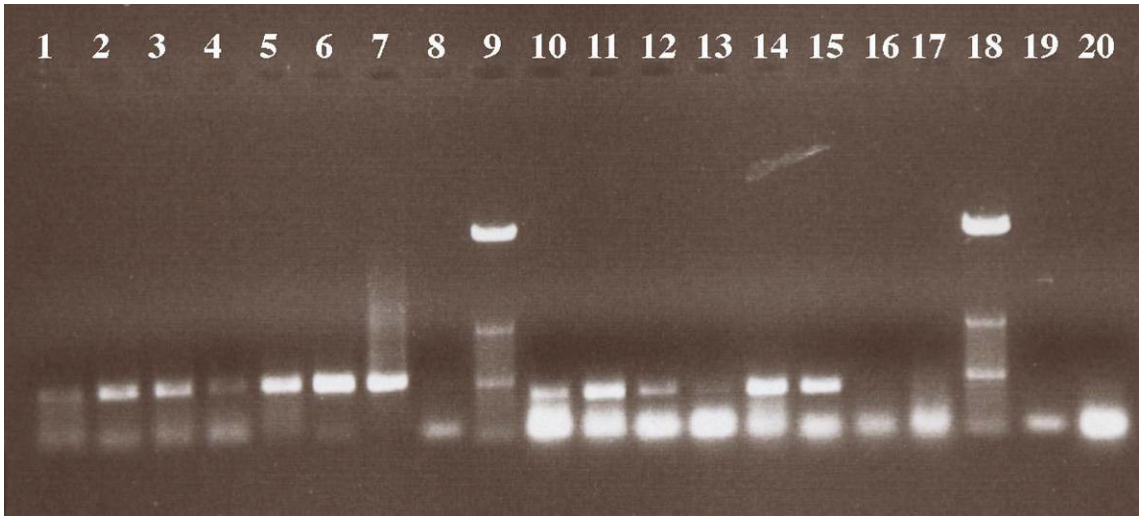


Figure 3.19. *PCR-2 optimisation agarose gel photograph.* (Lanes as described in Table 3.2.)

3.3.3.6 PCR-3 Development

PCR-3 was very successful as it had the specificity of PCR-1, the sensitivity of PCR-2 and did not produce false positive and negative bands. PCR-3 was the primary PCR used for analyzing experimentally and naturally infected animal tissues as described in the 'Small Animal Challenge' and '*Neospora* Isolation' chapters.

3.3.3.7 DNA Sequence Results

A BLAST search was performed on the generated DNA nucleotide sequences (Appendix 3.9). The *Neospora* control sample had positive sequence alignment with the *Neospora caninum*

strain, Australia 1, DNA detection probe (gi|10441733|gb|AF190701.1|AF190701). The entire sequence generated from the DNA of mouse blood sample #16 had a positive sequence alignment with an *N. caninum* species-specific DNA probe (gi|1085092|emb|X84238.1|NCSSDNAP). A more specific search, using only 44 bp resulted in the same sequence alignment identification. Other sequence homologies identified for mouse blood sample #16 were rat and human sequences. Mouse blood sample #4 did not show sequence alignment with any *N. caninum* sequences present in BLAST. Only mouse and human sequences were identified.

The NeoF and NeoR primer sequences developed for PCR-2 were screened in a BLAST search to check the species specificity of the primers in order to determine whether non-specific annealing was likely to occur. The NeoF and NeoR primers returned an *N. caninum* species-specific sequence alignment (E value = 0.004).

3.3.4 Discussion

It was reported that the D2 domain (C1/C1' region) of the large subunit (LSU) rDNA can be used as a genetic marker for the differentiation and identification of *Neospora* from other cyst forming coccidian (Ellis et al., 1998). Tachyzoites from the Nc-Liverpool, NC1 and NC-SweB1 isolates were used during the development of the PCR. Importantly, the sequence of the region amplified was shown to be identical for bovine (Nc-SweB1) and canine (NC1 and Nc-Liverpool) isolates (Stenlund et al., 1997). Nc-Liverpool was the only isolate used during the current study. The NF6 or TF6 GA1 primer pairs are able to amplify *Neospora*, *Toxoplasma* and *Hammondia* DNA. The TF6 /GA1 primer pair was proven not to be species-specific. Only *Neospora* and *Toxoplasma* were considered during this study, however as *Neospora* was the study organism and the NF6/GA1 primer pair is specific using the described PCR conditions, the PCR primers adopted were suitable for the purposes of identifying *Neospora*. Changes to the PCR protocol, such as lowering the annealing temperature, are likely to cause non-specific generation of PCR products as described by (Ellis et al., 1998). The rDNA is highly repeated in the coccidian genome which is advantageous as it provides a high copy target sequence for PCR detection, similarly described for the internal transcribed spacer (Holmdahl and Mattsson, 1996; Payne and Ellis, 1996)

Neospora detection in animal tissues was initially attempted using whole homogenised tissue as PCR template. This method was not effective as the tissue template would 'cook' in the PCR machine during the amplification cycles resulting in the loss of available DNA and a sticky intermittently solid PCR product that blocked the wells of the agarose gel. It was believed that detecting parasite DNA through the cellular debris was unlikely. Additionally, parasites are reportedly found in low numbers within whole tissue (Ellis et al., 1999a) and the chances of parasites being present in the 1 μl of template added to the sample decreased the likelihood of detection even further.

Yamage et al., (1996) showed that the DNA derived from 1 *Neospora* tachyzoite, mixed with 2 mg of brain tissue could be detected using the Np21/Np6 primer pair, which are the primer pair the NeoF and NeoR primers are based on (Yamage et al., 1996). However, *Neospora* DNA was

added to the sample, therefore the target DNA was known to be present, which it may not be the case in samples where tissue of unknown status is used. PCR-2, based on the primers described by Yamage et al, (1996) was developed in order to increase the level of detection from animal tissues. Purification of parasites or DNA would be necessary when testing animal tissue of unknown status using PCR.

Percoll density gradient tissue separation was used as the standard method for purifying parasites from large volumes of tissue. Small samples of purified tissue can be used directly in PCR or treated by boiling or DNeasy purification. Red blood cells were not found to adversely affect PCR amplification or running on agarose gels.

The DNeasy protocol for animal tissue was used for processing smaller volumes of animal tissue for PCR (McInnes et al., 2006a). Mechanical homogenization of tissues was described in the manual as being unnecessary, but it was found that homogenizing tissue with a pestle improved sample lysis and DNA yield. Overnight incubation with proteinase K was found to be the most effective process for digesting tissue.

The DNeasy protocol for cultured cells was adopted when samples were required for long-term use, such as for standards, as DNA did not degrade as quickly when placed into the DNeasy buffer. However, longer term storage, even at -20°C did result in some degradation of the DNA. Samples had to be aliquoted into smaller volumes so as to avoid freeze-thaw damage, which is also known to degrade DNA. Cell culture samples and purified tissue samples could be boiled or boiled and centrifuged if the sample was for immediate use and not to be stored. Heat treatment in a hot-water bath was initially used but sample boiling in a water bath often resulted in water contamination of the sample despite the use of tube cap locks and floatation devices, which prompted microwaving of samples to boil rather than using a water bath. Samples were microwaved only until they began to boil. Comparative PCR runs using PCR template that was boiled in a water bath compared to microwave boiled did not show any significant differences, therefore the latter procedure became the favoured technique. Samples that were boiled, whether the supernatant was removed or not, degraded quickly and could not be stored.

Many inconsistencies were observed for samples that were prepared using the DNeasy kit. Although DNA samples lasted longer and could be amplified using PCR after storage, the sample processing repeatability and consistency was low. Several serial dilutions were performed using the DNeasy kit but none produced even dose-wise band intensity decreases with decreasing numbers of parasites processed. Bands were frequently missing or were brighter at a lower concentration. The DNeasy dilution series was repeated three times.

Target DNA loss during DNeasy purification could have been caused by incomplete lysis or because the silica-gel membrane retained DNA during the elution steps. DNA elution can use either the AE buffer provided or water. It is recommended that DNA is eluted in AE buffer as DNA stored in water is subject to acid hydrolysis over time. It is suggested that 60-80% of DNA will elute out of the membrane after a single elution. Performing two elutions of 200 ul each is described in the manual to recover approximately 85% of the DNA. A third elution may increase DNA yield by a further 10-15%.

Although less DNA is present in the second DNeasy elution than the first, combining the two elutions would be a reasonable option for most samples provided that, an elution-1 sample produces a bright band and that dilution of the sample by approximately 25% would not dampen the band brightness to the point where it could not be detected. It is not known exactly how much DNA was yielded from the first and second elutions. Attempts were made to quantify the amount of DNA using a mass ladder, however due to the age and low remaining quantity of the sample, quantification was not possible. It was not deemed necessary to purchase more DNA mass ladder to repeat the experiment. Combining the first and second elutions would provide more DNA template overall, which could be particularly useful for control samples. If necessary, combined elution samples can be concentrated using ethanol/glycogen DNA precipitation to produce a sample of a higher concentration. DNeasy yields should also be quantified using real-time PCR (Collantes-Fernandez et al., 2002; Muller et al., 2002; Okeoma et al., 2005).

PCR-1 was a successful PCR and proved to be highly specific. However, it was believed that PCR-1 may not have been as sensitive as required. PCR-1 was a reliable PCR for positively confirming suspected positive and negative samples when significant target DNA was available. Samples that contained a high volume of host DNA were difficult to diagnose as PCR products were often found to be smeared or inhibited in some way (Yamage et al., 1996), which lead to the development of PCR-2.

PCR-2 was expected to be highly sensitive as it was based on the PCR described by (Yamage et al., 1996). PCR-2 sensitivity was tested on many occasions. The DNeasy-1 sample could be detected to the lowest dilution of 1:10,000, suggesting that PCR-2 can detect as little as 0.1 tachyzoites. Whereas, the DNeasy-2 sample made from $5x10^5$ tachyzoites, had a lowest level of detection of 1:500, which corresponds to a detection rate of 1 tachyzoite. The difference in detection levels is more likely to be due to different quantities of DNA isolated using the DNeasy kit. It is possible that some of the DNA was lost during isolation of the $5x10^5$ tachyzoites sample. The PCR detection limit of less than 1 tachyzoite is likely to reflect the high copy number of the target DNA.

Despite the promising results observed during the sensitivity trial, use of PCR-2 produced inconsistencies and problems. There appeared to be significant cross-reaction issues when analysing samples isolated from animal tissues that resulted in non-specific bands of a similar size to the target PCR products. PCR-2 was run 20-30 times in an effort to standardise the procedure. PCR-2 was developed to be a more sensitive detection system than PCR-1, but it appeared to be highly inconsistent and unreliable. Additionally, on multiple occasions known positive samples did not produce a PCR product band on an agarose gel but the same sample when run later or earlier would sometimes produce a clear PCR product band. False negative PCR results may have been caused either by inhibition of the PCR reaction, or a truly negative result (Ellis, 1998). Inhibition may also be caused by very large amounts of host DNA in relation to low amounts of parasite DNA and hence yield a negative result. This may also be the case for whole unpurified animal tissue (Yamage et al., 1996).

The PCR primers, based on the Np21/Np6 primers were run in a PCR cycle that differed to that originally described (Yamage et al., 1996). It is not known whether the higher annealing temperature (60°C rather than 50°C) and slightly lower extension temperature (72°C rather than

74°C) effected the specificity of the primers or whether it was the difference of running two shorter cycles of 25 amplifications each. Several attempts were made to improve the reproducibility of the PCR, including changing the volume and concentration of primers and template and increasing the amount of Mg++, and these did appear to stabilise the PCR to some degree.

PCR-2 optimisation trials led to the design of PCR-3, which was a hybrid of PCR-1 and PCR-2 with additional Mg++. PCR-3 became the most successful PCR as it was both specific and sensitive. Following the development of PCR-3, PCR-2 was abandoned as the non-specific banding issues and false positive and negative results could not be resolved. PCR-3 however was used for all final diagnosis of culture and animal tissue samples.

DNA contamination of the primers was identified on one occasion. In response to this contamination, all primers and reagents were discarded and replaced with fresh stock and the PCR workspace and equipment was thoroughly cleaned with DNA-away and Vircon. All PCR's were re-run to ensure that they were negative prior to continuing with testing of samples.

A sample of FBS used as PCR template in PCR-3 produced a faint band when run out on an agarose gel. The band was of approximately 350 bp and may have been due to non-specific amplification of a product found in the FBS or contamination. The expected band size should be approximately 250 bp. However, using the GA1 and Tim15 primer set described by Ellis et al., (1998) a 350 bp product is produced. It is unlikely that non-specific binding occurred in order to specifically amplify a 350 bp *N. caninum* product as the Tim15 and NF6 primers are very different (Ellis et al., 1998). The temperatures used during the running procedure for this PCR are the same as those used for PCR-1, however the procedure is broken into two steps of 25 cycles each and the denaturation, annealing and extension times are different to those described by Ellis et al. (1998) for the running of these primers. It is likely that either the extra cycles or the different running temperature times adversely affected this PCR and resulted in non-specific bands being produced. It is possible that the FBS did contain *Neospora* DNA. *Neospora* DNA has since been identified in the blood of naturally infected cattle (Okeoma et al., 2004a) using a PCR that produced a 350 kb product based on the Np21 and Np6 primers (Yamage et al., 1996).

The production of a highly specific sequence for the *N. caninum* positive control DNA sample proves that the sequencing procedure used worked as anticipated. The identification of a *N. caninum* sequence alignment with *N. caninum* sequences on BLAST, suggests that DNA sequencing of mouse blood #16 may have been specific, although sequence homology was lower than that seen for the positive control. However, it is unlikely that mouse blood #4 contained *N. caninum* DNA as no sequence alignment with *N. caninum* was seen following a BLAST search, indicating that PCR bands observed for this sample were likely to be non-specific PCR products of a similar size to those expected for an *N. caninum* product.

Although detection of *N. caninum* in blood using PCR was not adequately shown, the possibility remains that *N. caninum* DNA was present. Claims have previously been made that a sporadic blood-born dissemination of *Neospora* during neosporosis may occur, as is demonstrated in AIDS patients with active toxoplasmosis and in immunocompetent patients with toxoplasmic lymphadenopathy (Ellis, 1998; Guy and Joynson, 1995). The use of PCR to detect *Neospora* in the blood of infected cattle and dogs has since been established (Ho et al., 1996; McInnes et al.,

2006b; Okeoma et al., 2004a; Serrano-Martinez et al., 2007). However, it is likely that PCR detection would only be possible during neosporosis when an active infection is disseminating throughout the body (McInnes et al., 2006a).

A PCR that could differentiate between tachyzoites and bradyzoites would have been very useful for determining the life cycle stage of parasites isolated from tissue and culture (Risco-Castillo et al., 2007).

3.3.5 Conclusions

The QIAGEN DNeasy tissue kit for purification of DNA from animal tissue and cultured cells is effective at isolating small volumes of parasite DNA from tissues and cultures, although reproducibility is not optimal. An *N. caninum* tachyzoite count of approximately $5x10^4$ is recommended for preparing positive PCR template standards. Isolated DNA should be aliquoted into small volumes and stored at or below -20°C. Cell culture samples required for immediate use can be prepare for PCR by microwaving for approximately 10-20 seconds, however these samples should not be stored for long-term use as a PCR template.

PCR-3 utilizing the NF6/GA1 primer pair combination was the most sensitive and specific PCR and was able to detect small amounts of *N. caninum* DNA amongst large volumes of host DNA. This PCR was not quantified, but quantification of the PCR is recommended for future studies. The NF6/GA1 primers were observed to be sensitive to changes in annealing temperature. An annealing temperature of 55°C should be used for this PCR. Ensuring sufficient Mg++ availability was shown to increase the sensitivity of PCR-3.

PCR-1, also using the NF6/GA1 primer pair, is a very reliable PCR when a large amount of target template is available. The only significant difference between PCR-1 and PCR-3 is the number of amplification cycles and the addition of Mg++. PCR-1 primers should be used as the base primers for future PCR developments.

PCR-2 proved to be consistently unreliable. Sensitivity was sporadic and false positive and negative bands were almost always observed as well as non-specific banding. All attempts to improve this PCR failed, resulting in the abandonment of PCR-2.

Given that a PCR for detecting *N. caninum* in blood was established, no further work in this area should be necessary. However, detection windows have yet to be established.

3.4 Immunoblotting

3.4.1 General Description

N. caninum serological testing for mice is unavailable commercially in New Zealand. It became necessary to develop a serological test to check the sero-status of mice following inoculation in parasite isolation attempts and challenge with Nc-Liverpool, as mice did not show signs of illness and little was known about infection rates and capabilities.

Western blotting is used to identify and measure the size of proteins/antigens that react with specific antibodies. Proteins are separated with electrophoresis through SDS-polyacrylamide gel and transferred onto a nitrocellulose, polyvinylidene difluoride (PVDF) or cationic nylon membrane. The non-specific reaction sites are blocked and the membrane exposed to polyclonal or monoclonal proteins to form an antigen-antibody complex that can be located by radiographic, chromogenic or chemiluminescent reactions.

The dot-blot technique is used for detecting, analysing and identifying proteins. It is similar to the Western blot technique except that proteins are not separated electrophoretically but are spotted through circular templates directly onto the membrane. Protein concentrations in crude preparations can be estimated semiquantitatively using the dot blot method if both purified protein and specific antibody against it are available.

3.4.2 Materials and Methods

3.4.2.1 Antigens

- Antigen-1 (A1). Whole lysed *N. caninum* (cultured Nc-Liverpool tachyzoites), sample provided by John Ellis' group in UTS, Sydney. Sample was of unknown concentration.
- Antigen-2 (A2). Whole lysed *N. caninum* cultured tachyzoites, prepared at Massey University on the 12.11.02 (see Antigen Production below).

3.4.2.2 Primary Antibodies

- *N. caninum* -Negative control serum , Canine origin (VMRD Inc, Cat #: 211-N-NC-CAN)
  Description: Canine sera diluted in PBS, 1% BSA, 0.09% sodium azide.
  Intended use: As negative control in detection of antibody to *N. caninum* by indirect fluorescent antibody technique (IFAT). This serum should be used undiluted to demonstrate non-specific fluorescence, if any.

- *N. caninum* –Positive control serum, Canine origin (VMRD Inc, Cat #: 211-P-NC-CAN).
  Description: Canine sera diluted in PBS, 1% BSA, 0.09% sodium azide.
  Intended use: As control serum in detection of antibody to *N. caninum* by IFAT. This control should be used undiluted to demonstrate positive fluorescence.

3.4.2.3 Secondary Antibody

- Peroxidase Anti-mouse IgG (Vector, Cat# PI-2000)
- Peroxidase Anti-dog IgG (Sigma, Cat# A6792)

(Refer to Appendix 3.10 for further information on Materials and Solutions)

3.4.2.4 Western Blot Gels

Gels were prepared as described in Appendix 3.10. The TEMED and ammonium persulfate were added last to set the gel. If necessary additional ammonium persulfate was added to aid gel setting. Gloves were worn at all times and all gel pouring equipment, including glass, spacers and sheets were thoroughly cleaned using methanol. The resolving gel was poured ¾ of the way up the gel apparatus and covered with a small volume of methanol or water during setting. Water or methanol was removed, the gel rinsed with water and the stacking gel poured onto the resolving gel and the combs placed. Once set, gels were transferred to a gel electrophoresis tank. Running buffer was added to the inner and outer reservoirs ensuring that the top and bottom of the gel was immersed in chilled buffer. The combs were removed to make the gel ready for loading.

3.4.2.5 Antigen Manufacture

A fresh 25 cm^2 flask of *N. caninum* tachyzoite culture was harvested by cell scraping. Cell debris was centrifuged at 2000 rpm for 10 minutes to pellet parasites. Parasites were frozen by dunking into liquid nitrogen then thawed by placing into a 37°C incubator. The freeze-thaw process was repeated several times. The parasite culture was then sonicated on ice for 20-40 seconds to break up the cells, sonication was repeated several times.

3.4.2.6 Antigen Preparation

Western Blot Antigen Preparation

Antigen samples were prepared in 1x SDS-gel loading buffer and heated at 100°C for 3 min to denature proteins then immediately placed on ice. Antigen was aliquoted into usable volumes before storage between -20°C and -70°C.

Immunoblot Antigen Preparation

Antigen was heat treated for 3 min at 100°C as described above but no loading buffer was added.

3.4.2.7 Running Western Blot Gel

Between 5-10 µl of antigen was added to each well. A standard protein of known molecular weight (BSA 1mg/mL) was also added. Unused wells were loaded with loading buffer only.

Gels were run at 90V for approximately 45 minutes or until the Bromophenyl blue neared the bottom of the gel. Then the gel was removed from the electrophoresis equipment, separated from the glass plates and placed directly into fixative solution in preparation for silver staining.

3.4.2.8 Silver Staining of Western Blot Gel

To check antigen preparations, gels were stained with silver stain to determine the band sizes before continuing with the western blot. Silver staining was performed using Silver nitrate solution (Silver Stain Plus, Biorad). Refer to Appendix 3.11.

Gels were stained, placed into a plastic bag and photocopied so that the bands could be visualised.

Gels were destained in 1% hydrogen peroxide, washed and used for western blotting.

3.4.2.9 Western blot Gel to Membrane Transfer

Gel transfer onto a membrane was performed using electrophoresis.
3M Whatman paper was cut into quarters and soaked in transfer buffer for 15 minutes. A PVDF membrane was cut to size (slightly larger than the gel), wet with methanol and rinsed with 3-4 changes of deionised distilled water before placing into transfer buffer.

The saturated 3M paper was placed on the anode side of the gel and the PVDF membrane on the other side of the gel. Transfer of the bands from the gel to the membrane occurs when using a current running perpendicular to the direction that the gel was run in. The apparatus was run for 20-40 min at 15V.

3.4.2.10 Ponceau S Stain

Gel transfer was checked using Ponceau S stain (Ponceau Red). Ponceau S stain was diluted 1:10 with distilled water and the membrane soaked for 5 minutes. Transferred protein bands appear pink. Membranes were destained by washing several times in deionised distilled water and shaking.

3.4.2.11 Dot-Blot Procedure

A PVDF membrane was wet by passing through methanol and washing well in milliQ water while nitrocellulose membranes were wet with water directly. The dot-blot apparatus was set up as shown in Figure 3.20. All air bubbles were removed from under the membrane once placed. The apparatus was screwed together. Wash buffer was placed in each well to wet the membrane followed by 10 µl of antigen (lysed tachyzoites). Antigen was diluted in Blocking Solution where necessary. Antigen was drawn onto the membrane and each well rinsed with washing buffer. To each designated well, 10 µl of primary antibody diluted in Blocking Solution was added, the apparatus was covered and incubated overnight at 4°C with gentle agitation.

The following day, the membrane was removed from the apparatus and treated as described in the Immunoblot and Development Procedure below.

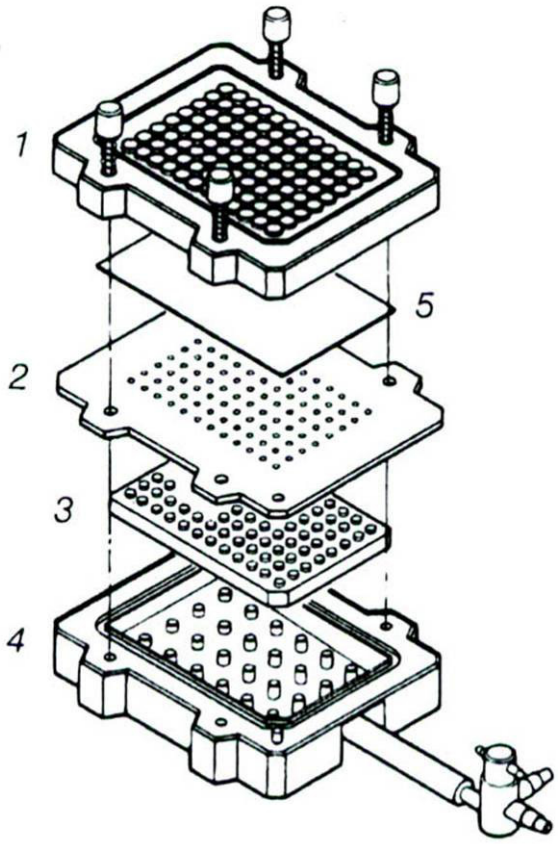


Figure 3.20. *Assembly of Dot-Blot Apparatus.*
Sample Template (1), gasket (2), gasket support plate (3), vacuum manifold base (4), membrane (5).

3.4.2.12 Immunoblot and Development Procedure

The PVDF membrane was washed in PBS-TWEEN 0.5%, blocked with Blocking Solution (5% skim milk powder/PBS-TWEEN) for 1 hour, washed with PBS-TWEEN and incubated with primary antibody. Primary control antibodies were diluted 1:1,000 in Blocking Solution. Membranes were covered and incubated overnight at 4°C on a shaker.

Membranes were washed thoroughly in PBS-TWEEN and incubated in secondary antibody at room temperature, covered on a shaker, for 1 hour. Secondary antibody was diluted 1:25,000 in Blocking Solution.

Membranes were thoroughly rinsed and placed into a plastic sleeve. A 200 µl mixture of SuperSignal West Femto was added to the enclosed membrane and smeared to ensure an even coverage and to remove all air bubbles. The sleeve was heat sealed and taped to a radiographic film cassette.

In a dark room, a Kodak Biomax film was exposed to the membrane and developed through an X-ray developer.

3.4.2.13 Membrane Stripping

Membranes were washed for 30 minutes at 50°C in Western Blot Stripping Solution (Appendix 3.10). Then they were washed well with deionised distilled water, left in wash buffer overnight and reused from the membrane blocking step of the immunoblot procedure (described above). Membranes can be stripped up to 5 times.

3.4.2.14 Western Blot Antigen Validation

Antigen-1 and Antigen-2 batches were tested by western blot. BSA was also used as a positive control antigen. Antigens were either heat treated by boiling for 3 min or were used without heat treatment and run on a western blot. The western blot gel was silver stained and the gel photocopied to visualise antigen banding (Figure 3.21). The gel was washed and then transferred to a PVDF membrane and the membrane exposed to canine positive control primary sera (1:1,000) (VMRD) and peroxidase anti-dog IgG secondary antibody (1:25,000) (Sigma). The membrane was developed using SuperSignal West Femto (200 µl substrate A:200 µl Substrate B) and an X-ray film was taken (Figure 3.22).

3.4.2.15 BSA Standard

The optimal amount of BSA for use in western blots was determined. BSA was prepared using antigen heat treatment at 1%, 2% and 3% concentrations and run on a western blot gel along with Antigen-1 and Antigen-3 (Figure 3.23).

3.4.2.16 Dot-Blot Antibody Optimisation

A 96 well dot-blot apparatus was set up and each well coated with Antigen-1, diluted to 1:100 in Blocking Solution. Mouse sample #34 was chosen for use as the primary antibody positive control (this sample had previously been shown to be positive for *N. caninum* by western blot and PCR analysis as is reported below in Chapter 4). The membrane was incubated in the assigned primary antibody concentrations (Table 3.3) to determine the optimal dilutions for the primary and secondary antibodies. The membrane was cut into 4 strips based on the concentration requirements of the secondary antibody. The strips were blocked in Blocking Solution and incubated in the assigned secondary antibody dilution. Strips were otherwise all treated in the same manner for development.

Table 3.3. *Dot-Blot antibody optimisation.* 96 well dot-blot PVDF membrane with bound antigen used at 1:100. Primary antibody (mouse #34) used at 1:250, 1:500 or 1:1,000. Secondary positive control antibody used at 1:10,000, 1:30,000 or 1:50,000. Secondary negative control antibody used at 1:10,000.

	1:10,000 Positive Control			1:30,000 Positive Control			1:50,000 Positive Control			1:10,000 Negative Control			2° antibody dilution
	1:250	1:500	1:1,000	1:250	1:500	1:1,000	1:250	1:500	1:1,000	1:250	1:500	1:1,000	1° antibody dilution
Well	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	
A	●	●	●	●	●	●	●	●	●	●	●	●	
B	●	●	●	●	●	●	●	●	●	●	●	●	
C	●	●	●	●	●	●	●	●	●	●	●	●	
D	●	●	●	●	●	●	●	●	●	●	●	●	
E	●	●	●	●	●	●	●	●	●	●	●	●	
F	●	●	●	●	●	●	●	●	●	●	●	●	
G	●	●	●	●	●	●	●	●	●	●	●	●	
H	●	●	●	●	●	●	●	●	●	●	●	●	

3.4.3 Results

3.4.3.1 Western Blot Antigen Treatment

All antigen samples were observed to produce bands following electrophoresis and staining with silver stain. The bands of the ladder could not be seen following silver staining. Both *N. caninum* Antigen-1 and Antigen-2 produced at least two bands, one of which was a similar size to that seen for BSA (60-70 kDa). The antigen bands for the heat-treated samples were not significantly darker than the bands observed for the untreated antigen. The *N. caninum* antigen samples that were boiled (lanes 3 and 5) appear to have produced a slightly darker smudge throughout the lane in comparison to the untreated samples. Boiling BSA samples did not appear to increase the band size, but increasing the concentration of BSA (to 8 mg) did produce a slightly larger band (lanes 4 and 9). However, both the 4 mg/ml and 8 mg/ml BSA samples have overloaded the gel as the bands are too large and are not properly separated (Figure 3.21). The gel should have been run for longer. Future gels were run longer.

Antigen bands observed on the western blot membrane following development were in agreement with the bands observed on the silver stained gel. Two significant bands were observed for the boiled *N. caninum* antigen samples, which correlated with the sizes seen using the silver stain. A bright, slightly smaller third band was observed for the unboiled Antigen-1 sample (lane 2) and was also faintly seen for the heat-treated Antigen-1 sample (lane 3). The boiled Antigen-2 sample produced the two expected bands (lane 5) but the unboiled sample lacked the second band (lane 8). None of the BSA samples produced bands following exposure to the *N. caninum* antibodies. Molecular weight ladder bands can be seen following incubation with the *N. caninum* antibody. Both Antigen-1 and Antigen-2 have been shown to be capable of producing *N. caninum* antigen bands.

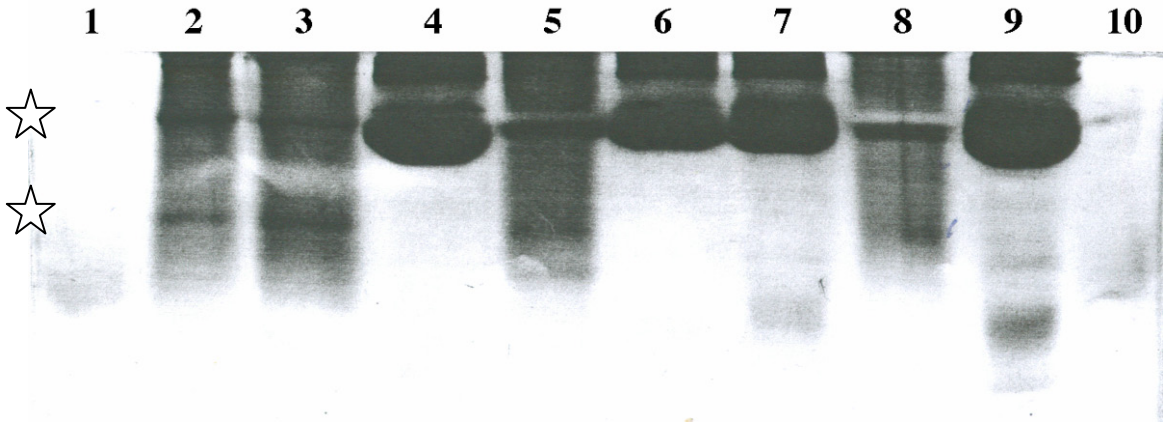


Figure 3.21. *Silver stained western blot photocopy.* SDS-Page MW Standard (Lane 1 & 10), Antigen-1 (Lane 2), Antigen-1 boiled (Lane 3), BSA 8 mg (Lane 4), Antigen-2 boiled (Lane 5), BSA 4 mg (Lane 6), BSA boiled 4 mg (Lane 7), Antigen-2 (Lane 8), BSA boiled 8 mg (Lane 9). Stars indicate positions of two *N. caninum* specific bands.

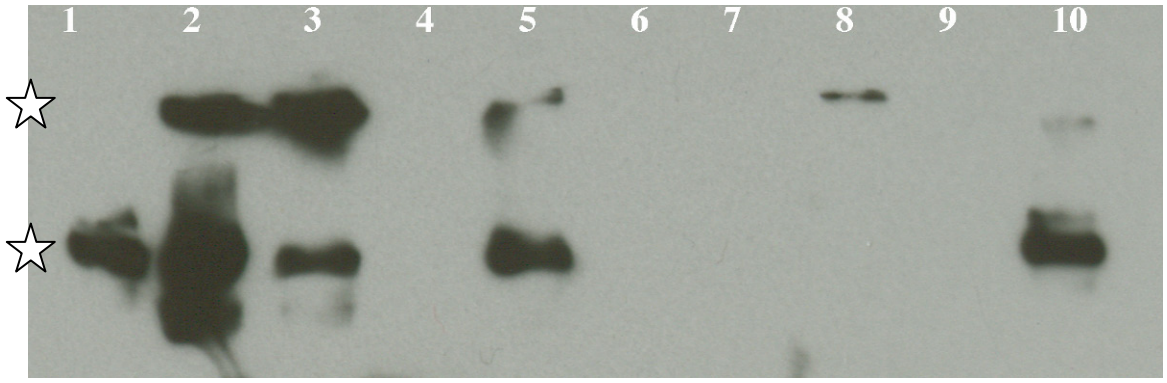


Figure 3.22. *Western blot Biomax film image.* SDS-Page MW Standard (Lane 1 & 10), Antigen-1 (Lane 2), Antigen-1 boiled (Lane 3), BSA 8 mg (Lane 4), Antigen-2 boiled (Lane 5), BSA 4 mg (Lane 6), BSA boiled 4 mg (Lane 7), Antigen-2 (Lane 8), BSA boiled 8 mg (Lane 9). Stars indicate positions of two *N. caninum* specific bands.

3.4.3.2 BSA Standard Results

The most appropriate BSA standard appears to be 1% BSA or 1 mg/ml BSA. All BSA standards have separated into multiple distinct bands. The first large band is too wide using 2% and 3% BSA samples. Only the 1% BSA sample bands appear well separated and distinct. The *Neospora* antigens, Antigen-1 and Antigen-2, did not produce dark bands with Silver staining. The first, largest, band can be seen for all *Neospora* antigens but the second band can only faintly be seen for Antigen-2. These bands are only used for checking antigen electrophoresis, therefore overloading with antigen would not be beneficial.

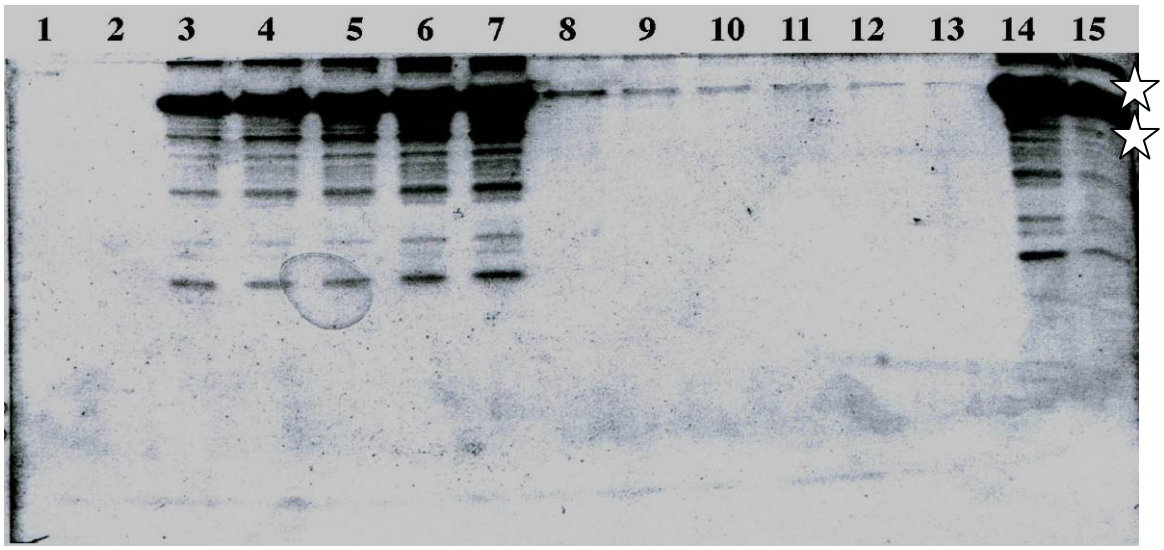


Figure 3.23. *Silver stain of BSA Western blot gel.* SDS-Page MW Standard (Lane 1 & 2), 1% BSA (Lane 3-5), 2% BSA (Lane 6 & 7), Antigen-2 (Lane 8-10), Antigen-1 (Lane 11-13), BSA 3% (Lane 14 & 15). Stars indicate positions of two *N. caninum* specific bands.

3.4.3.3 Dot-Blot Antibody Optimisation

The dot-blot membrane has consistently reacted with the chemiluminescent substrate in a dose wise manner dependent on the amounts of primary and secondary antibody. At a primary antibody concentration of 1:250 and a secondary antibody concentration of 1:10,000 excessive chemiluminescence was produced which resulted in exposure of the X-ray film surrounding the dots as well as the dots themselves. The primary antibody concentration of 1:100 in combination with a low secondary antibody concentration of 1:50,000 produced dots that were fainter and less even in appearance. All of the negative control dots were negative. Some leakage or over exposure to the chemiluminescent substrate appears around the periphery of the membrane.

This membrane was initially exposed to the chemiluminescent substrate at a concentration of 100µl:100µl of substrates A:B and the volume made up to 600 µl with sterile water. The membrane reaction was restricted for lane 9, where the concentrations of primary and secondary antibodies were low (Figure 3.25). The membrane was exposed to X-ray film, then washed in sterile water and re-exposed to the chemiluminescnt substrate at a concentration of 300 µl:300 µl of substrate A:B (Figure 3.24).

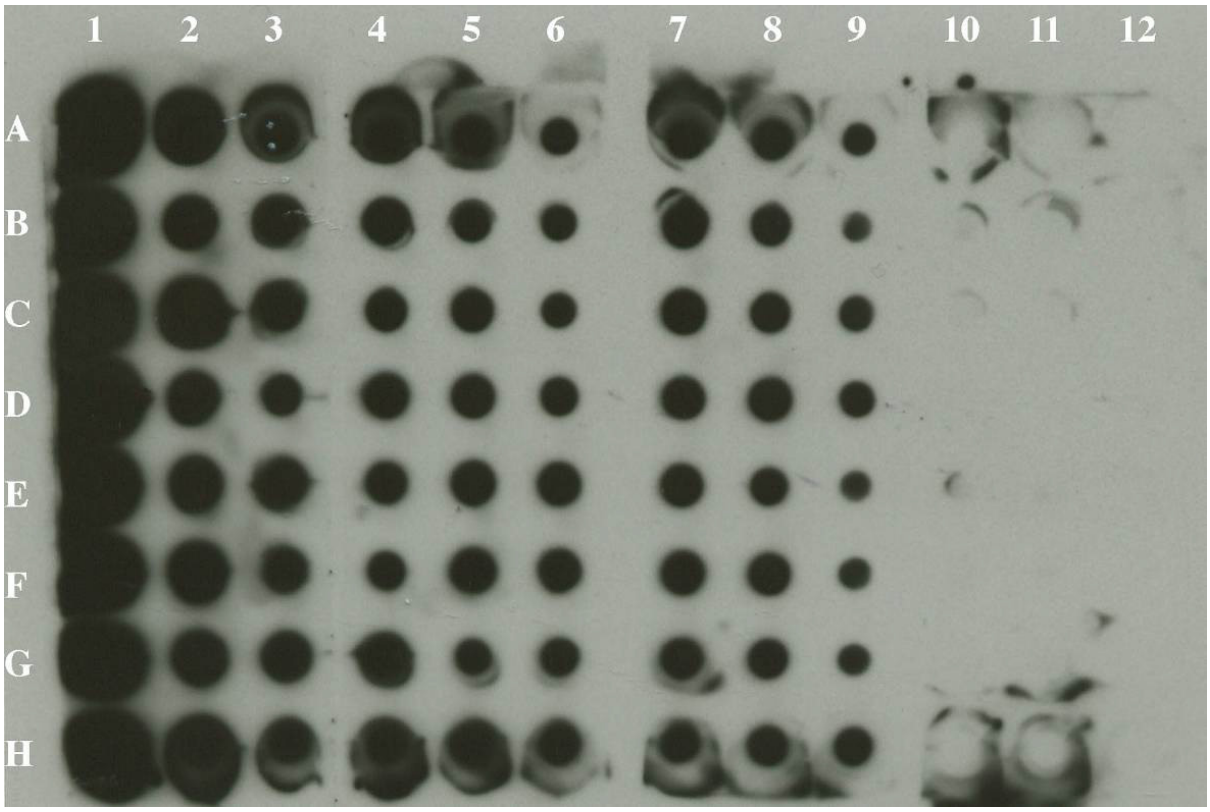


Figure 3.24. *Dot blot of 1° and 2° antibody dilution series.* 1° antibody 1:250 (lanes 1, 4, 7 & 10), 1° antibody 1:500 (lanes 2, 5, 8 &11), 1° antibody 1:1,000 (lanes 3, 6, 9 & 12), 2° antibody 1:10,000 (lanes 1-3), 2° antibody 1:30,000 (lanes 4-6), 2°antibody 1:50,000 (lanes 7-9). Membrane reacted with 600 ul SuperSignal West Femto undiluted. X-ray film exposed to membrane for 1 minute.

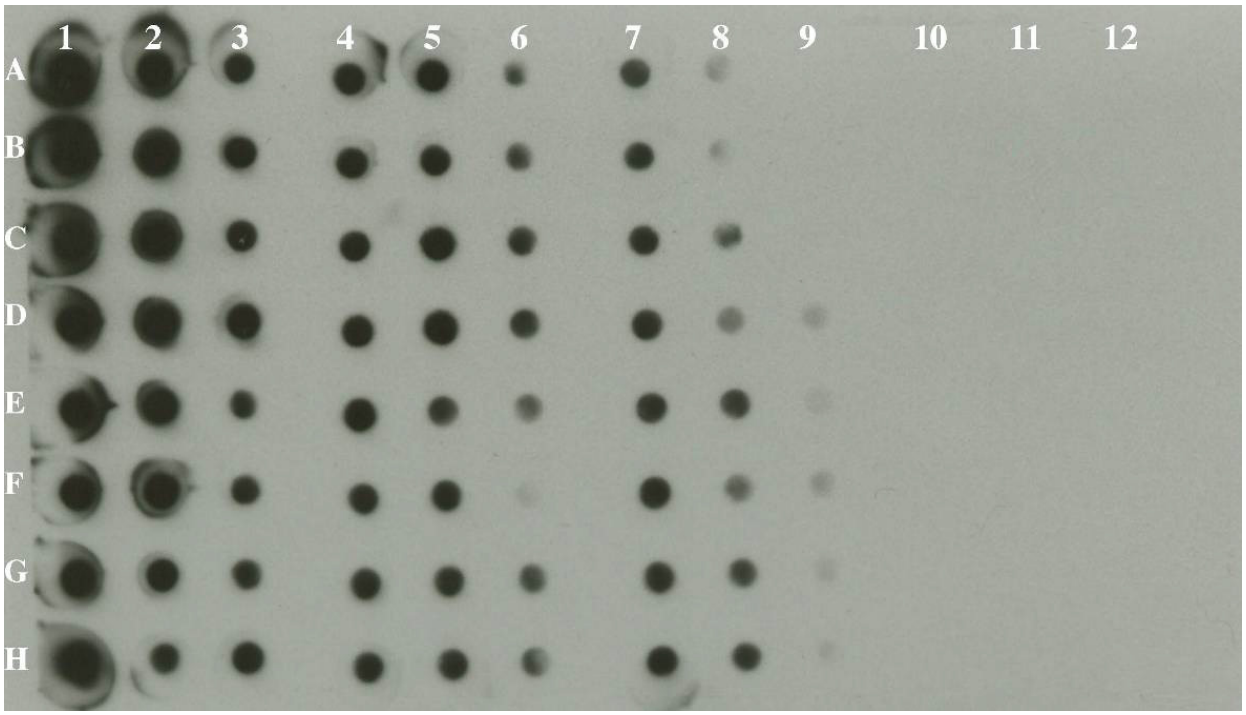


Figure 3.25. *Dot blot of 1° and 2° antibody dilution series.* (Lanes as described for Figure 3.24) Membrane exposed to 200 ul SuperSignal West Femto diluted 1:2. X-ray film exposed to membrane for 1 minute.

3.4.4 Discussion

Serological diagnostic procedures for murine samples were not commercially available, which prompted the development of the western and immunoblots. An ELISA had been used by several research groups for the detection of immune responses in mice (Nishikawa et al., 2001a; Quinn et al., 2002b). Otherwise mouse infection is often diagnosed with immunohistochemistry, PCR (Gottstein et al., 2001; Liddell et al., 1999a; Pinitkiatisakul et al., 2005) or in some cases western blotting (Atkinson et al., 1999; Atkinson et al., 2001).

Despite indications from the silver stained western blot gel that boiling samples did not affect the size or position of the antigen bands, the necessity to heat treat samples prior to use became clear during analysis of the western blot membrane, following exposure to specific antibodies. Specific bands were observed and bands were more clearly defined following heat treatment of antigen. Heat treatment of antigens was used as standard practice for immunoblots after this observation. Both the Australian (Antigen-1) and New Zealand (Antigen-2) made antigens produced *N. caninum* specific protein bands of the same sizes, suggesting that the protocol used for antigen production in New Zealand was adequate (Atkinson et al., 1999).

The western blot was not tested for cross-reaction with *T. gondii*. Evidence suggests that detection using specific immunodominant antigens such as the 17, and 29-32 kDa proteins should

be selected as markers in serological assays (Silva et al., 2007). During the course of this study, clear separation of antigens was not observed and this may have been due to impure antigen. The molecular weight markers did not separate clearly either, which may be due to running conditions and overloading. Further work would be required to ensure that the antigens formed clear bands.

The western blot technique was abandoned for a quicker dot-blot procedure as the dot-blot allowed for many times more sample to be tested in each run and the entire procedure was significantly faster and easier to perform. Dot-blots are commonly used for *T. gondii* but evidence for use with *N. caninum* has not been found (Angel et al., 1997; Bouillet et al., 1998; Zhu et al., 2000).

The concentrations of the primary and secondary antibodies should be determined by titration when running dot-blots. It may be sensible to use excess primary antibody and limit the concentration of secondary antibody to produce more consistent dots. Where both the primary and secondary antibodies were limited, dot formation became more inconsistent, however when only the secondary antibody was limited, dots were still observed to be consistent in shape and intensity. Therefore the limiting factor for a dot blot should be the secondary antibody rather than the primary antibody concentrations.

Additionally it was found that the amount of chemiluminescent substrate used had a significant effect on the detection of the dots (Nelson and Carlson, 2005). The volume and concentration of chemiluminescent substrate should be considered and determined carefully for dot-blot processes. The West Femto substrates saturate above a certain level, causing non-linear relationship between antibody concentration and chemiluminescent intensity (Nelson and Carlson, 2005). The most appropriate approach is to expose the membrane to a lower concentration of substrate first and if necessary wash the membrane and re-expose to a higher volume/concentration of substrate. This will prevent the horse radish peroxidase (HRP) reaction from burning out due to overexposure prior to determining the correct volume of chemiluminescent required. At the correct concentrations the chemiluminescence should be detectable for at least 6-8 hours. It is equally as important to ensure an even coverage of the membrane as uneven distribution will result in marking of the membrane and uneven staining of the dots. Multiple experiments were conducted to optimise the uniform exposure of the membrane to the chemiluminescent substrate to ensure an even membrane reaction. These included, dunking into substrate solutions, adding substrate to a sealed bag or dripping substrate onto the membrane (results not shown). Adding substrate to a sealed bag and evenly distributing across the membrane appeared to be the most effective method. However, if the membrane had been cut into pieces for exposure to different secondary antibody concentrations, then the dunking procedure was adopted. If membranes are dunked into substrate it is important to consider that the concentration will change between the first and last membrane as wet membranes will dilute the substrate.

Significant issues arose during the development of the dot-blot technique. Antigen production was not as successful as anticipated. Although antigen manufacture from *N. caninum* tachyzoites was shown by western blot to produce *N. caninum* bands that bound specific antibodies, use of the antigen in the dot blot apparatus produced inconsistent results. Dot antigen coating of membranes was not always uniform and some dots appeared lighter or darker than others. The Australian antigen (Antigen-1) was found to produce more consistent results and was used as a positive control against the New Zealand antigens (multiple antigen batches were produced and

tested). Immunostimulation complexes (iscoms) could be adopted for use during antigen preparation in an attempt to improve antigen reliability, as has been shown for ELISAs (Bjorkman and Lunden, 1998). BSA would be useful as a negative control antigen for immunoblots and should be used at 1 mg/ml.

Antigen application onto membranes was performed using several methods, including dot-blot and slot-blot apparatuses as well as dotting known volumes of antigen directly onto strips of membrane with a pipette. Using the dot-blot apparatus was found to be the most successful technique. Strips of membrane resulted in additional issues such as uneven distribution of chemiluminescent substrate (Nelson and Carlson, 2005).

Tachyzoites were harvested at different culturing stages, such as early development of CPE, heavy infection and moderate CPE or late stage infection when almost the entire cell layer was destroyed. Early or mid culture harvests were found to yield better antigens. Treatment of parasites was also important as the amount of cell lysis and the solution the parasites were lysed into effected the durability of the antigen. Cells lysed well in water but water based antigen cultures did not remain active for long periods, which resulted in inconsistent spotting on membranes. SDS-page buffer was used as standard practice (Atkinson et al., 1999).

Dark patches that appear as leakage or over exposure to the chemiluminescent substrate were often observed around the periphery of membranes or in patches across the membrane. The dark patches are probably non-specific fluorescence as it also occurs around the negative control samples and may have resulted from insufficient blocking, excess primary or secondary antibody, insufficient washing or too much HRP in the system. Changes of blocking buffer, such as using different brands and batches of skim milk powder, had little effect. Additional wash steps and incubation times in blocking buffer did not markedly improve the background staining either. A BSA blocking solution as opposed to a Skim milk blocking solution may have produced better results (Nishikawa et al., 2000b). Decreasing antibody concentrations helped decrease background staining. A different detection method may be worth considering, as the West Femto SuperSignal was very sensitive to peroxidase activity (Nelson and Carlson, 2005). Internal peroxidase activity found in red blood cells was considered to possibly be interfering with tests. Whole blood was not used but mouse blood samples were frequently quite red due to red blood cell lysis caused during blood harvest.

Background staining must not be confused with ‘halos’, which appear as rings immediately around all or part of the dot. Halos are most likely caused by leakage from the well onto the membrane surrounding the well and could possibly be caused by damage to the membrane, an uneven membrane surface or the apparatus not being placed tightly enough over the membrane. Some samples with specifically stained halos were considered to be positive samples results.

Dot-blotting was found to be very specific and sensitive, which is in agreement with published literature which suggests that dot-blot techniques are equally as good as other ELISA and IFA tests (Bouillet et al., 1998; Noya, 1998). Dot-blot DNA-hybridisation could also be considered for *N. caninum* screening (Angel et al., 1992; Toso and Omoto, 2007). DNA-hybridisation dot-blots can detect as little as 80 pg of purified DNA (Blanco et al., 1992).

3.4.5 Conclusions

The western blot and immunoblot techniques were developed for screening and diagnosing *N. caninum* serostatus of dogs and mice following experimental infection. Dot-blotting has not commonly been used for diagnosis of *N. caninum*. However, given a reliable antigen and further technique development, dot-blotting would be a useful indicator of parasite exposure. Once sero-positive animals were identified, a western blot could be used to positively identify the specific antigens detected. The dot-blot technique is very sensitive and requires only small volumes of sample, which makes it ideal for testing mouse serum samples. Additional benefits are, that a large number of samples can be tested at any one time, the procedure is fast and easy and once antigen is membrane bound it can be re-used several times.

Chapter 4.

4.0 Small Animal Challenge

4.1 Introduction

Animal models of infection, such as mouse models, have been used to study the biology, immunology and susceptibility to chemotherapy of *N. caninum.* Mice are also used for isolation of the parasite from tissue. Susceptibility to neosporosis is dependent on the mouse strain, immunosuppressants given and the strain and number of *N. caninum* parasites used (Dubey and Lindsay, 1996).

In chronic neosporosis of mice, *N. caninum* is usually found only in the brain and spinal cord. In generalised neosporosis, tachyzoites can be found in several tissues. Generalised neosporosis can be induced in immunocompetent mice by treating them with 2 x 4 mg of methylprednisolone acetate (MPA), which commonly results in death following inoculation with $1x10^5$ tachyzoites. Lower doses (2 mg) of MPA result in milder neosporosis and frequently the development of neurologic signs (Dubey and Lindsay, 1996). Interestingly, IgG antibody titres in mice given 3 treatments of 4 mg MPA were only slightly (1-2 dilutions) higher than those mice given 3 treatments of 2 mg MPA or no MPA (Lindsay and Dubey, 1989c).

Immunodeficient mouse strains such as athymic nude, SCID Bg and INFγ-knockout mice, develop severe neosporosis (Dreier et al., 1999; Lindsay et al., 1995a; Yamage et al., 1996) in addition to immunosuppressed mice. In vivo treatment with antibodies against IFN-γ or IL-12 result in severe neosporosis and death (Khan et al., 1997).

Differences in virulence have been observed for *N. caninum* isolates. The NC-1 isolate is widely accepted as being highly virulent and frequently causes death in immunocompromised mice, whereas strains such as NC-2, NC-3 and Nc-Liverpool cause less sever neosporosis and may result in chronic infection in mice leading to brain cysts (Lindsay et al., 1995a; McGuire et al., 1997b). Small differences in the internal transcribed spacer 1 (ITS-1) region have been reported among *N. caninum* strains, in particular between strains from different continents or regions, indicating that some genetic variability exists between isolates (Gondim et al., 2004a).

BALB/c models for *N. caninum* infection have been well established (Liddell et al., 1999c; Lindsay et al., 1995a; Long and Baszler, 1996; Tanaka et al., 2000). BALB/c mice are usually treated with immunosuppressants and inoculated by interperitoneal injection of parasites.

However, CF1 mice have not reportedly been used for *N. caninum* infection. The CF1 mouse strain is commonly used for *T. gondii* infection where parasites can be harvested from peritoneal

fluid (Harper et al., 2006; Sibley et al., 1989). The CF1 mouse strain was used by AgVax Developments for the attenuation of the *T. gondii* strain 48.

Infected mice may develop clinical disease consisting of encephalitis, radiculoneuritis, polymyositis, and myonecrosis. Neosporosis in mice is often characterised by coat roughening, body hunching, weight loss, neurological signs such as head tilting, impaired movement and death in some mice (Baszler et al., 2000; Lindsay and Dubey, 1989c; Sawada et al., 1997).

4.2 Materials and Methods

4.2.1 Mouse Strains

CF1 Outbred mouse strain. Albino. (No additional susceptibility information provided)

BALB/c Inbred mouse strain. Albino. Susceptible to *N. caninum* following subcutaneous inoculation with tachyzoites of the NC-1 strain (Lindsay et al., 1995a)

(Information gathered from mouse genome informatics website http://www.informatics.jax.org)

4.2.2 Mouse Immunosuppression

Mice were immunosuppressed with 2.5 mg of methylprednisolone acetate (MPA) (Vetacortyl) injected subcutaneously (s.c) into the neck scruff. MPA treatment occurred on the day of or 3 days prior to inoculation with *N. caninum* parasites

4.2.3 Experimental Inoculation

Mice were inoculated with an assigned volume of purified tissue sample or cultured tachyzoites. Typically, both BALB/c and CF1 mice were injected intraperitoneally (i.p) with 0.2-0.3 ml of sample.

For cultured tachyzoite inoculations, Nc-Liverpool was grown in vitro, parasites were enumerated and a specific number of tachyzoites used for inoculation (from 5 $x10^3$ to 1.3$x10^6$).

Mice were checked regularly for signs of peritoneal exudate and disease. Any mice that became severely ill within 72 hours were culled on humane grounds since their illness was likely to be due to bacterial infection and these were not considered to have neosporosis.

Procedures were conducted in accordance with the guidelines of the Ministry of Agriculture and Fisheries (MAF).

4.2.4 Humane Destruction of Mice

Mice were placed into a plastic housing box and the top of the box was covered with a piece of perspex that had five holes in it, one on each corner and a larger hole in the centre for the CO_2 tube. Mice were gassed with CO_2 for approximately 5 min or until they had stopped breathing and were dead. Mice were then removed from the box, placed into a clear bag and taken to the laboratory for dissection. Stress to the mice was minimised as much as possible by keeping them in a box containing nesting material while they were gassed.

4.2.5 Mouse Post-Mortem Dissection

Dead, mice were taken to the laboratory to harvest tissues and blood. Dissection was performed in a laminar flow hood. Mice were placed on their backs and a small amount of ethanol was used to wet the abdominal/chest area to stop fur contaminating the sample. The skin along the chest was pinched and cut to expose the rib area. Using small sterile dissection scissors the ribs were cut on either side close to the axillae. Ribs were lifted and pulled back exposing the lungs and heart. Blood was removed from the heart with a syringe. If blood could not easily be removed, the heart was cut and the blood drawn up by syringe. Approximately 0.5 ml of blood was collected from each mouse.

Mice were bled as soon after death as possible. Following death, mice were kept warm to stop blood from clotting. Blood was always removed before the organs.

To remove the brain, the mouse was placed prone. The neck scruff was tented and cut with sterile scissors to expose the neck muscles and skull. The muscles at the base of the skull were cut and the scissors were inserted up into the lateral base of the skull. The skull was carefully cut along each edge and flipped up to expose the brain. Using a small metal spatula the brain was removed. The brain was bisected longitudinally and one half placed into 10% formalin buffer for immunohistochemistry (IHC), while the other half was placed into antibiotic PBS solution for isolation of tissue and DNA.

Mouse carcasses were destroyed by incineration following sample harvest.

4.2.6 Mouse Blood Processing

Blood samples were stored at 4°C for 24 hours then spun at 200 x g for 10 min. The serum was removed, frozen and stored for subsequent use in immunoblots.

4.2.7 Mouse Tissue Processing

Mouse brains were processed in a similar method to processing used for dog and cattle tissue (see Isolations chapter 5). Brains were stored in antibiotic PBS at 4°C until processed. Processing was usually performed within 24 hours of tissue harvest and not longer than 3 days. Up to 30 samples

were processed per day. Each mouse brain was kept separate during processing. Briefly, brains were homogenized in a 1% Trypsin digest mixture and incubated for 1 hour at 37°C with agitation. The sample was washed with PBS, filtered and resuspended into a 30% Percoll solution. Samples were centrifuged at 2,700 x g and the pellet collected for diagnostic and cell culture inoculation procedures.

4.2.8 Mouse Brain Smears

A small sample of fresh brain tissue, approximately the size of a pinhead, was taken from some samples for a brain smear. The tissue was put onto a microscope slide and a cover slip placed over the tissue. By applying a small amount of digital pressure to the cover slip, the neural tissue was squashed to produce a thin layer that could be viewed under a microscope for tissue cyst screening.

4.2.9 Mouse Brain Histology

Formalin fixed mouse brains were embedded into paraffin blocks and sectioned for immunohistochemical (IHC) analysis (see Diagnostics chapter for IHC procedures). Sections were stained using the protocol described in Appendix 3.4. (See Appendix 3.3 for materials.) *Toxoplasma gondii* infected mouse tissue was used as a positive control using *T. gondii* specific antibodies.

4.2.10 Nc-Liverpool Trial-1

This trial was conducted to investigate whether CF1 and BALB/c mice could be infected with Nc-Liverpool and to determine the dose required to induce neosporosis.

Twenty female CF1 mice and 20 female BALB/c mice were divided into 5 groups of 4 mice each and used for experimental inoculations with Nc-Liverpool and treatment with methylprednisolone acetate (MPA) a corticosteroid immunosuppressant.

Tachyzoites were harvested once they had destroyed approximately 90-95% of the cell culture monolayer. Most tachyzoites were free floating at this stage and could be easily quantified using a haemocytometer.

Mice from groups 2, 3, and 4 were injected with 2.5 mg of MPA s.c in the scruff of the neck. Group 3 received one dose of MPA on day –3. Groups 2 and 4 received MPA on day 0. Groups 1 and 5 did not receive MPA. Groups 1, 2, and 3 were inoculated with 0.2 ml of culture containing $6.5x10^6$ Nc-Liverpool tachyzoites i.p on day 0, giving a dose of $1.3x10^6$ tachyzoites per mouse. Groups 4 and 5 did not receive tachyzoites (Table 4.1).

Table 4.1. *Treatment groups for Nc-Liverpool Trial-1.*

Group number*	Treatment
1	*N. caninum* Day 0, §
2	*N. caninum* Day 0 + MPA Day 0, § ¥
3	*N. caninum* Day 0 + MPA Days –3, § ¥
4	MPA Day 0 (No *N. caninum* given), ¥
5	Control (No *N. caninum* or MPA).

* Each group contained 4 mice per mouse strain (BALB/c and CF1 mice)

§ Mice inoculated with 1.3 x 10^6 Nc-Liverpool parasites.

¥ Mice inoculated with 2.5 mg MPA.

Mice were assessed daily for signs of disease. Mice exhibiting severe signs of disease were culled immediately. Each day mouse condition was checked according to the set mouse health chart, which recorded mouse activity, coat ruffling, body hunching, paralysis, weekly weight gain and survival (refer to Appendix 4.1).

From groups with surviving mice, one mouse per group was killed each week for examination. After death, the brain was removed and heart blood obtained. Brain smears were performed on brains from mice killed at 28 days PI.

Harvested brain tissue was dissected longitudinally. One half was placed into 10% formalin buffer for IHC analysis while the other was frozen for use in DNA isolation. DNA was isolated from the mouse brains using the DNeasy kit (QIAGEN). Samples were left to digest overnight. Mouse blood and Nc-Liverpool DNA for sequencing was isolated using the Roche High Pure Viral NA kit. Mouse blood was centrifuged at 200 x g for 10 min and the serum was harvested and stored for immunoblot analysis (see chapter 3 - Diagnostics).

Serum was tested using a dot-blot immunoblot apparatus. Antigen-1 (Australian antigen) (1:1,000 dilution in wash buffer, 10 µl loaded into each well) was bound to a PVDF membrane and the mouse serum was diluted in blocking solution and used as the primary antibody at 1:250 (side A) or 1:10,000 (side B of Figure 4.1). The secondary antibody was in blocking solution and used at 1:50,000 dilution. The membrane was incubated with 600 µl (300 µl substrate A:300 µl substrate B) of WestFemto SuperSignal chemiluminescent substrate and exposed to an X-ray film.

Isolated DNA was tested using PCR-1 (not shown), PCR-2 (Figure A4.3.1-A4.3.3 and Table A4.3 in Appendix 4.3) and PCR-3 (Figures 4.2-4.3).

2.11 Nc-Liverpool Trial-2

Twenty mice, (10 x CF1 and 10 x BALB/c) were inoculated with relatively low doses of Nc-Liverpool to examine long-term survival up to 55 days and chronic infection. Both the CF1 and BALB/c mice were divided into two groups of five and infected with either $5x10^4$ or $5x10^3$ Nc-Liverpool tachyzoites.

No MPA was given to any of the mice in order to increase the chances of long-term survival.

4.2.12 Nc-Liverpool Infection and Re-Isolation Trial

Four BALB/c and 4 CF1 mice from the Nc-Liverpool Trial-2 were culled 55 days after inoculation (PI) and the blood and brain were removed for research. Mice had been inoculated with $5x10^3$ or $5x10^4$ Nc-Liverpool tachyzoites (Table 4.2).

Table 4.2. *Nc-Liverpool tachyzoite inoculation of mice.*

Nc-Liverpool Parasite count	No. BALB/c mice	No. CF1 mice
5×10^3	2	2
5×10^4	2	2

One mouse brain from each group was used for diagnostic purposes and the other brain was used for attempted re-isolation into tissue culture.

Infection Diagnosis

One brain from each mouse inoculation group was used purely for diagnostic purposes. The brain was dissected longitudinally. Half of the brain was frozen for DNeasy DNA isolation (Appendix 3.7) for use in PCR. The other half brain was placed in 10% formalin buffer for use in IHC (see Diagnostics Chapter 3.1 and 3.2 – IHC and PCR).

Re-Isolation into Tissue Culture

One brain from each mouse inoculation group was homogenized and the homogenate split into two equal volumes. Half of the brain homogenate was purified using a 30% Percoll solution and the other half was used for crude lysis isolation, as described in Appendix 4.2.

Cell cultures were checked for signs of infection and passaged each week as described in Chapter 2 – cell culture. Samples were removed at each passage for PCR analysis.

Cell samples removed at passage 1, 2 and 3 as well as the supernatant retained following flask inoculation were tested for *N. caninum* using PCR-2. Samples were prepared using the sample boiling technique (as described in the Diagnostics Chapter 3.2 – PCR).

4.3 Results

4.3.1 Nc-Liverpool Trial-1 Results

In most cases, inoculated BALB/c mice developed severe signs of neosporosis within 7 days PI. On day 5 of the study, 1 mouse in BALB/c-2 had developed coat ruffling, by the sixth day 2 mice displayed hunched bodies and subsequently died overnight. The 2 remaining mice in the group were culled at the end of week 1 in view of severe coat ruffling, body hunching and 1 mouse displayed signs of paresis. Due to the severity of neosporosis observed for the BALB/c-2 mice, all Nc-Liverpool BALB/c mice were killed at the end of week 1 as all were showing signs of neosporosis. All mice in BALB/c-1 were moderately active and developing coat ruffling by the end of week 1, only 1 mouse from this group had developed some coat ruffling on day 5. By the end of week 1 all mice in BALB/c-3 had become inactive and displayed coat ruffling and hunched bodies, only 1 mouse had displayed signs of coat ruffling from day 5. No mice from the control groups 4 and 5 developed signs of neosporosis.

One mouse in treatment group CF1-1, two mice in CF1-2, and 1 mouse in CF1-3 began to develop significant coat ruffling by the end of week 1 and were all subsequently chosen for euthanasia. One mouse in CF1-3 developed coat ruffling by the end of week 2 and was selected for euthanasia. No other mice in the CF1 groups showed signs of neosporosis, however 1 mouse from each group was killed for examination each week for the duration of the study.

Brain smears were done on two mouse samples, one CF1mouse from each of groups 1 and 3 taken on week 4. No cysts were seen in either of these mice. The slides were subsequently destroyed after examination.

Samples were unavailable for mouse 20 and only a small sample of brain could be retrieved from mouse 19. Both of these mice died overnight and tissues were not retrievable.

Table 4.3. *Nc-Liverpool Trial 1 mouse cull table.*

Mouse strain group and treatment	Week 1 (7 days PI)	Week 2 (14 days PI)	Week 3 (21 days PI)	Week 4 (28 days PI)
BALB/c-1 (Nc-Liv D0)	4 culled	-	-	-
BALB/c-2 (MPA + Nc-Liv D0)	2 dead, 2 culled	-	-	-
BALB/c-3 (MPA D-3 + Nc-Liv D0)	4 culled	-	-	-
BALB/c-4 MPA D0 + No Nc-Liv	1 culled	1 culled	1 culled	1 culled
BALB/c-5 (No MPA+ No Nc-Liv)	1 culled	1 culled	1 culled	1 culled
CF1-1 (Nc-Liv D0)	1 culled	1 culled	1 culled	1 culled
CF1-2 (MPA + Nc-Liv D0)	2 culled	1 culled	1 culled	-
CF1-3 (MPA D3 + Nc-Liv D0)	1 culled	1 culled	1 culled	1 culled
CF1-4 (MPA D0 + No Nc-Liv)	1 culled	1 culled	1 culled	1 culled
CF1-5 (No MPA+ No Nc-Liv)	1 culled	1 culled	1 culled	1 culled

(-) No tissue.

Table 4.4. *Nc-Liverpool Trial 1 summary table of results.* Dot-blot X-ray film (Figure 4.1), PCR-3 gel photographs (Figures 4.2. – 4.3.) PCR-2 gel photographs (Appendix 4.3). PCR-1 gel photographs (not shown). Positive (+), strongly positive (++), negative (-). No sample available (NS). Text in bold (Nc-Liverpool inoculated mice)

Sample No.	Mouse strain	Cull day PI	MPA given	Parasite inoculate	PCR-1 results	PCR-2 results	PCR-3 results	Blot results	IHC result
1	**CF1**	**7**	**Day 0**	**Nc-Liv**	**-**	**+**	**-**	**-**	**-**
2	**CF1**	**7**	**Day 0**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
3	**CF1**	**7**	**None**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
4	**BALB/c**	**7**	**Day -3**	**Nc-Liv**	**+**	**+**	**-**	**-**	**-**
5	**BALB/c**	**7**	**Day -3**	**Nc-Liv**	**-**	**+**	**+**	**-**	**-**
6	**BALB/c**	**7**	**Day -3**	**Nc-Liv**	**-**	**+**	**+**	**-**	**-**
7	**BALB/c**	**7**	**Day -3**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
8	BALB/c	7	None	None	-	-	-	-	-
9	BALB/c	7	Day 0	None	-	-	-	-	-
10	**CF1**	**7**	**Day -3**	**Nc-Liv**	**-**	**-**	**+**	**-**	**-**
11	**BALB/c**	**7**	**Day 0**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
12	**BALB/c**	**7**	**Day 0**	**Nc-Liv**	**+**	**-**	**++**	**-**	**-**
13	**BALB/c**	**7**	**None**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
14	**BALB/c**	**7**	**None**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
15	**BALB/c**	**7**	**None**	**Nc-Liv**	**-**	**-**	**-**	**-**	**-**
16	**BALB/c**	**7**	**None**	**Nc-Liv**	**+**	**+**	**+**	**-**	**-**

17	CF1	7	Day 0	None	-	-	-	-	-
18	CF1	7	None	None	-	-	-	-	-
19	**BALB/c**	**7**	**Day 0**	**Nc-Liv**	**-**	**-**	**++**	NS	**-**
20	**BALB/c**	**7**	**Day 0**	**Nc-Liv**	NS	NS	NS	NS	**-**
21	CF1	14	None	None	-	-	-	-	-
22	**CF1**	**14**	**None**	**Nc-Liv**	**+**	**-**	**+**	**+**	**-**
23	CF1	14	Day 0	None	-	-	-	-	-
24	**CF1**	**14**	**Day 0**	**Nc-Liv**	**+**	**+**	**++**	**++**	**-**
25	**CF1**	**14**	**Day -3**	**Nc-Liv**	**-**	**-**	**-**	**+**	**-**
26	BALB/c	14	None	None	-	-	-	-	-
27	BALB/c	14	Day 0	None	-	-	-	-	-
28	BALB/c	21	None	None	-	-	-	-	-
29	BALB/c	21	Day 0	None	-	-	-	-	-
30	CF1	21	None	None	-	-	-	-	-
31	CF1	21	Day 0	None	-	-	-	-	-
32	**CF1**	**21**	**None**	**Nc-Liv**	**-**	**-**	**-**	**+**	**-**
33	**CF1**	**21**	**Day 0**	**Nc-Liv**	**+**	**+**	**++**	**++**	**-**
34	**CF1**	**21**	**Day -3**	**Nc-Liv**	**-**	**-**	**-**	**+**	**-**
35	CF1	28	None	None	-	-	-	-	-
36	BALB/c	28	None	None	-	-	-	-	-
37	BALB/c	28	Day 0	None	-	-	-	-	-
38	CF1	28	Day 0	None	-	-	-	-	-
39	**CF1**	**28**	**None**	**Nc-Liv**	**-**	**-**	**-**	**+**	**-**
40	**CF1**	**28**	**Day -3**	**Nc-Liv**	**-**	**-**	**-**	**+**	**-**

(-) Negative, (+) Positive, (++) Strongly positive.

4.3.1.1 Nc-Liverpool Trial-1: Immunoblotting Results

Positive dot-blot samples were detected for all mice inoculated with Nc-Liverpool that were culled 14 days or more PI (Table 4.5). No mice culled within 1 week of inoculation produced immune responses nor did any of the negative control mice that did not receive Nc-Liverpool. Treating mice with MPA did not inhibit the immune response. Only inoculated CF1 mice survived for more than 7 days PI. All BALB/c mice inoculated with Nc-Liverpool were culled at the end of week 1 due to severe neosporosis.

Table 4.5. *Nc-Liverpool Trial-1 summary table of positive immunoblot results*

Sample No.	Mouse strain	Cull day PI	MPA given	Inoculate day 0	Blot results	Group blot detection (total)	Detection from 14 days PI
24	**CF1**	**14**	**Day 0**	**Nc-Liv**	++	2/4	2/2
33	**CF1**	**21**	**Day 0**	**Nc-Liv**	++		
22	CF1	14	None	Nc-Liv	+	3/4	3/3
32	CF1	21	None	Nc-Liv	+		
39	CF1	28	None	Nc-Liv	+		
25	**CF1**	**14**	**Day -3**	**Nc-Liv**	+	3/4	3/3
34	**CF1**	**21**	**Day -3**	**Nc-Liv**	+		
40	**CF1**	**28**	**Day -3**	**Nc-Liv**	+		

(+) Positive, (++) Strongly positive.

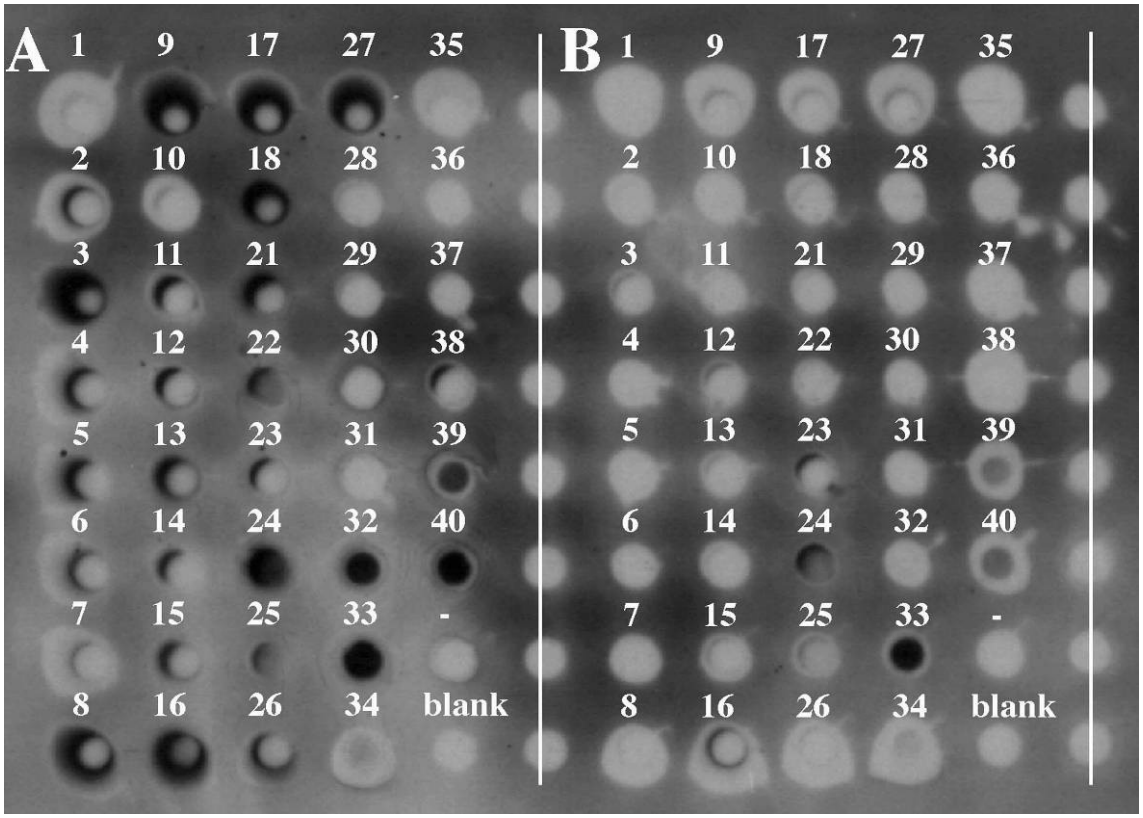


Figure 4.1. *Nc-Liverpool Trial-1 dot-blot of mouse serum.* Mouse serum primary antibody used at 1:250 (side A), and 1:10,000 (side B). Individual dots are as described in Table 4.4.

4.3.1.2 Nc-Liverpool Trial-1: Immunohistochemistry Results

No tissue cysts were observed in any of the mice. Occasionally tachyzoites may have been observed but could not be positively identified, therefore the sections were considered to be negative.

4.3.1.3 Nc-Liverpool Trail-1: PCR Results

At least one sample was taken from each brain and processed through the DNeasy DNA isolation kit. The resulting DNA was used as template in all three PCR techniques (described in diagnostics Chapter 3- PCR). PCR-3 was considered to be the best PCR to use since it was found to be the most sensitive (high detection rate) and specific (few non-specific bands). (Refer to Figures 4.2.-4.3 for PCR-3 gel photographs of brain samples.)

No *N. caninum* specific bands were observed for any negative control mice. All mice from group 2 had a high rate of detection of *N. caninum* DNA using PCR for both mouse strains. The PCR detection summary (Table 4.6.) refers to the combined detection from all PCR procedures performed. (Refer to Table 4.4. for individual sample results.)

Table 4.6. *Nc-Liverpool Trial-1 summary table of PCR detection results*

Mouse strain group and treatment	PCR detection for group
BALB/c-1 (Nc-Liv day 0)	1/4
BALB/c-2 (MPA + Nc-Liv day 0)	2/3
BALB/c-3 (MPA day-3 + Nc-Liv day 0)	3/4
BALB/c-4 (MPA day 0 + No Nc-Liv)	0/4
BALB/c-5 (No MPA+ No Nc-Liv, Control)	0/4
CF1-1 (Nc-Liv day 0)	1/4
CF1-2 (MPA + Nc-Liv day 0)	3/4
CF1-3 (MPA day-3 + Nc-Liv day 0)	1/4
CF1-4 (MPA day 0 + No Nc-Liv)	0/4
CF1-5 (No MPA+ No Nc-Liv, Control)	0/4

Blood samples from mice #16 and #4 were tested using PCR and the PCR product was sequenced. (Refer to Chapter 3- PCR for further details.)

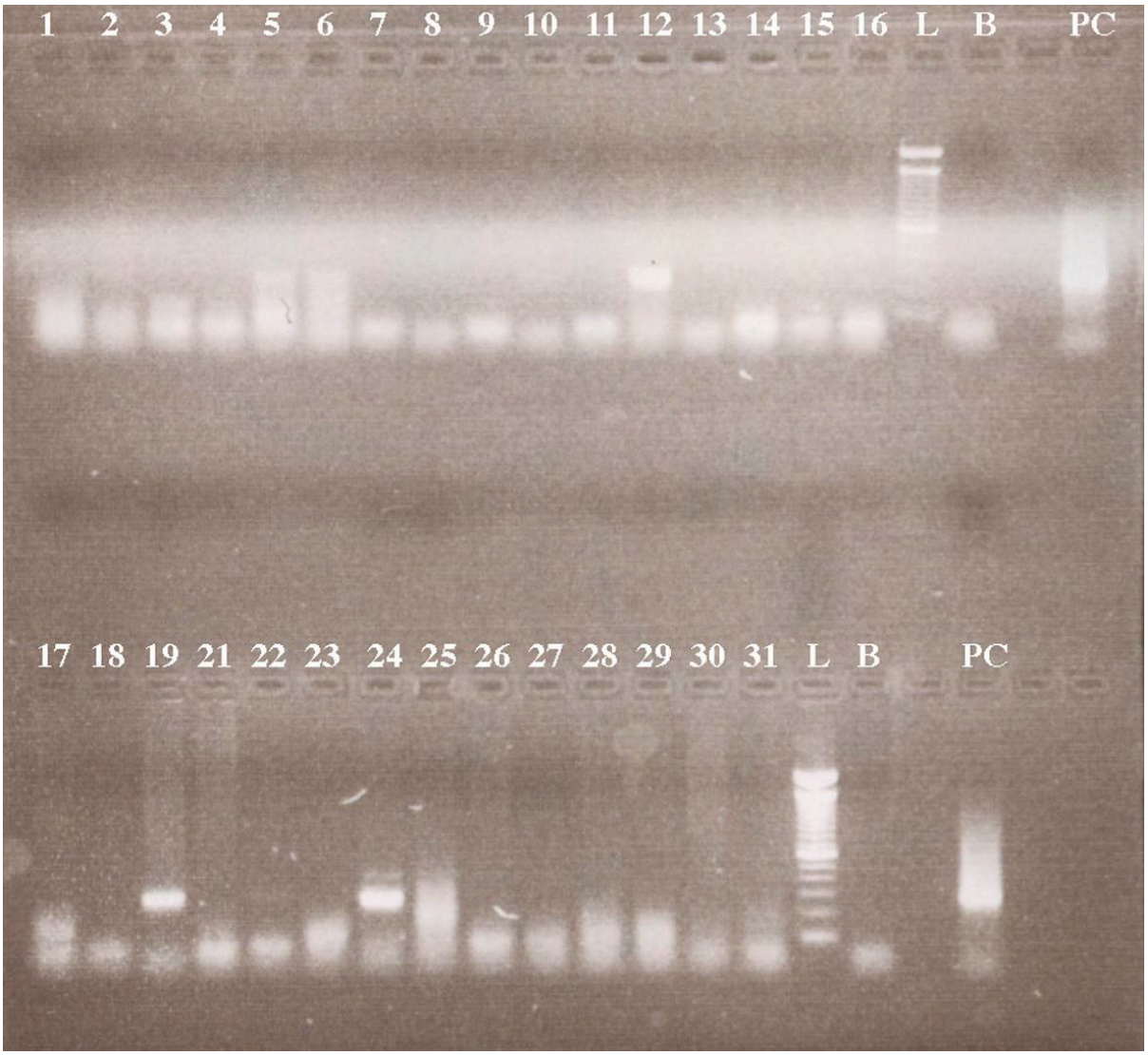


Figure 4.2. *PCR-3 gel photograph of Nc-Liverpool Trial-1 mouse brain samples (gel 1).* Lane numbers correspond to samples 1-31 as described in Table 4.4. Ladder (L), Blank (B), Positive Nc-Liverpool control sample (PC).

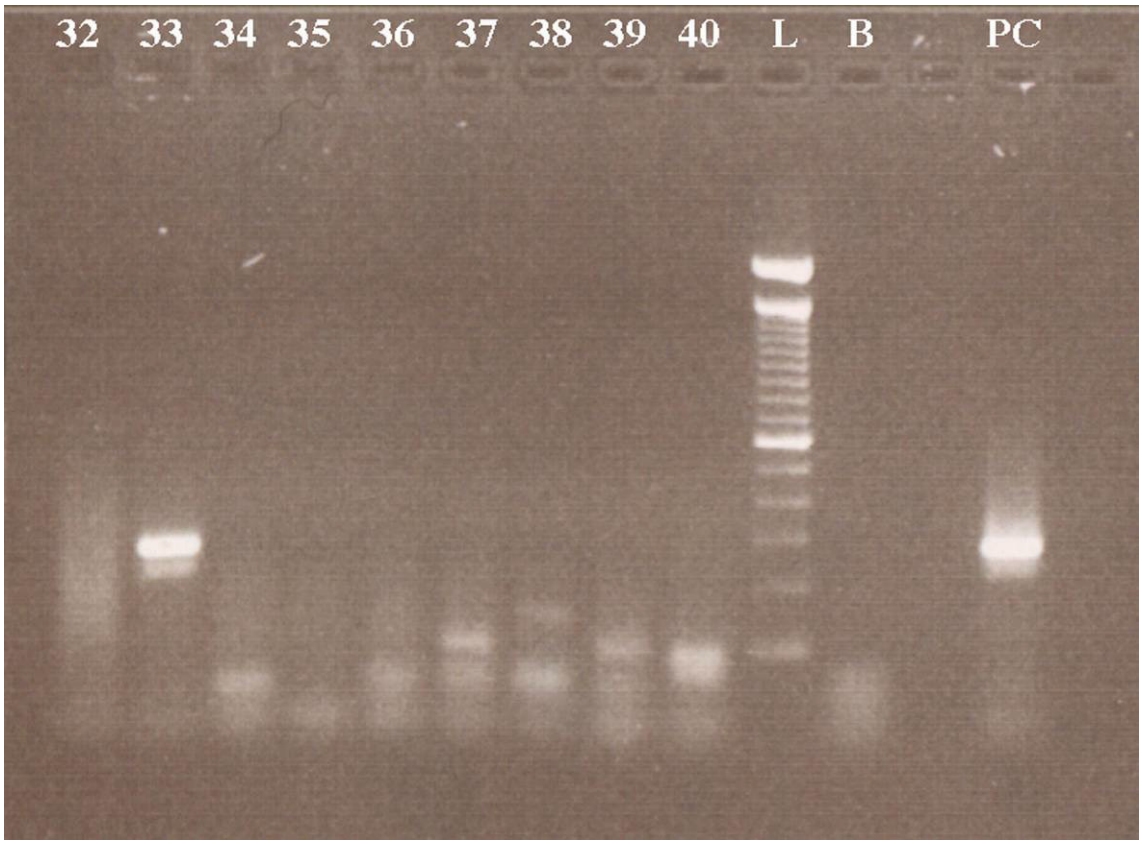


Figure 4.3. *PCR-3 gel photograph of Nc-Liverpool Trial-1 mouse brain samples (gel 2).* Lane numbers correspond to samples 32-40 as described in Table 4.4. Ladder (L), Blank (B), Positive Nc-Liverpool control sample (PC).

4.3.2 Inoculation and Re-Isolation Results

Just one of the four mice that had brain tissue frozen and the DNA isolated using the DNeasy kit was found to be positive for *N. caninum* using PCR (Figure 4.4). The CF1 mouse inoculated with $5x10^3$ tachyzoites produced a strong band (lane 4) consistent with the Nc-Liverpool positive control band (lane 7). Although bands can clearly be seen for the BALB/c mouse inoculated with $5x10^4$ tachyzoites and faintly for the other two mice, none of the bands are of the correct size for an *N. caninum* specific product.

No tissue cysts were identified in any of the formalin fixed brains following sectioning and immunohistochemical staining (Table 4.8).

Cell culture monolayers that were inoculated either with crude brain homogenate or with brains that were homogenized and purified using a Percoll density gradient, did not show signs of parasitic colonisation at any stage. Cultures were checked using an inverted microscope for signs of parasite growth. No CPE was observed.

Supernatant for both Percoll treated and crude lysate brain homogenate that was retained following the 3 hour monolayer inoculation incubation was not found to be positive for *N. caninum* using PCR for any of the mouse samples (PCR gel not shown). However, *N. caninum* was identified using PCR (Figure 4.5) in samples taken from passage 1 of the inoculated monolayers for the BALB/c mouse inoculated with $5x10^3$ tachyzoites and purified using a Percoll gradient (lane 4), as well as for both the Percoll purified (lane 6) and crude brain (lane 5) homogenates from the CF1 mouse inoculated with $5x10^4$ tachyzoites. By passage 2, only the BALB/c mouse culture showed signs of *N. caninum* infection (lane 14) and by passage 3 (Figure 4.3) none of the cultures appeared to be positive for *N. caninum* Table 4.7.

Table 4.7. *PCR results table for re-isolation cultures.*

Mouse strain, mouse inoculation dose, tissue treatment	**PCR Sample**			
	Supernatant	**Passage 1**	**Passage 2**	**Passage 3**
BALB/c, 5 x 10^4, crude	-	-	-	-
BALB/c, 5 x 10^4, Percoll	-	-	-	-
BALB/c, 5 x 10^3, crude	-	-	-	-
BALB/c, 5 x 10^3, Percoll	-	+	+	-
CF1, 5 x 10^4, crude	-	+	-	-
CF1, 5 x 10^4, Percoll	-	+	-	-
CF1, 5 x 10^3, crude	-	-	-	-
CF1, 5 x 10^3, Percoll	-	-	-	-

(-) Negative, (+) Positive.

Table 4.8. *Summary of results from inoculation and re-isolation trial.*

Mouse strain	**Tachyzoite inoculation dose**	**IHC results**	**DNA purification results**	**Cell culture results**
BALB/c	$5x10^4$	-	-	-
BALB/c	$5x10^3$	-	-	+
CF1	$5x10^4$	-	-	+
CF1	$5x10^3$	-	+	+

(-) Negative, (+) Positive.

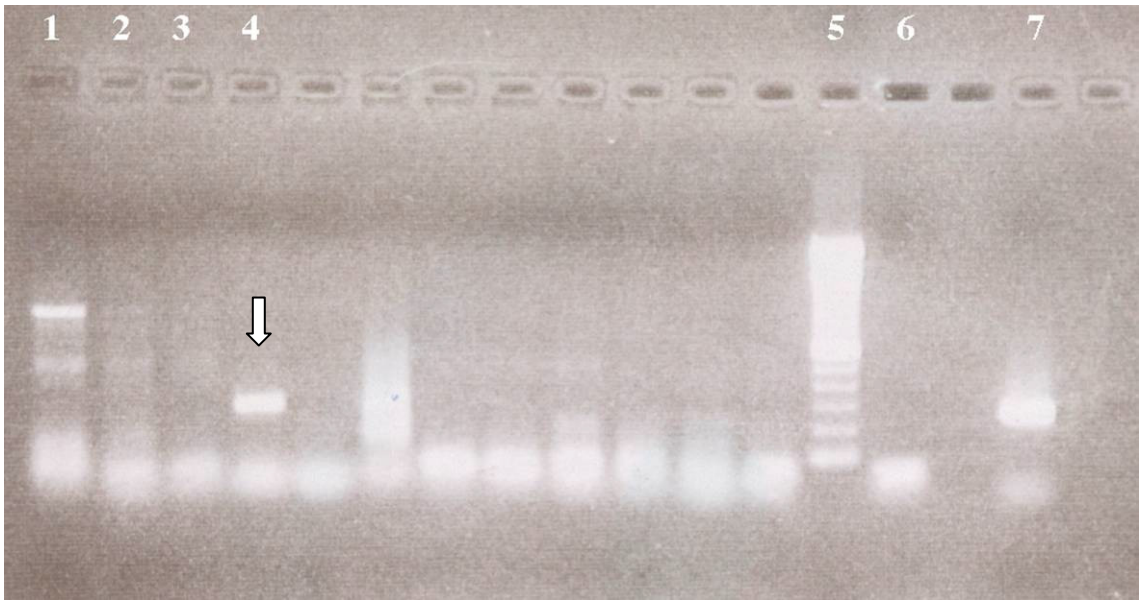


Figure 4.4. *PCR gel photograph of mouse brain DNA isolations.* BALB/c mouse $5x10^4$ and $5x10^3$ tachyzoite inoculation (lanes 1 & 2 respectively), CF1 mouse $5x10^4$ and $5x10^3$ tachyzoite inoculation (lanes 3 & 4 respectively), 100bp PCR ladder (lane 5), negative control (lane 6), positive control (lane 7). (Arrow indicates positive sample band) Un-numbered lanes contain samples from a separate study.

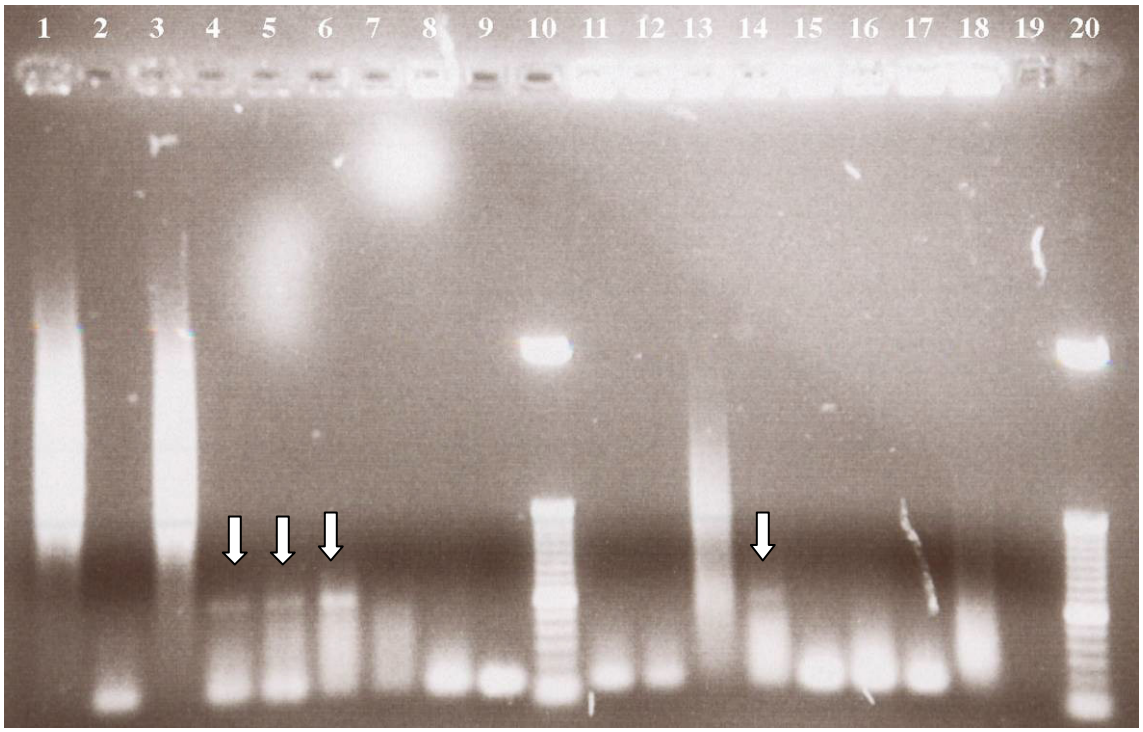


Figure 4.5. *PCR gel photograph of re-isolation cell culture passage 1 & 2 samples.* Passage 1 samples (lanes 1-8), Passage 2 samples (lanes 11-18) BALB/c mouse $5x10^4$ crude isolate (lanes 1

& 11) and $5x10^4$ Percoll isolate (lanes 2 & 12), BALB/c mouse $5x10^3$ crude isolate (lanes 3 & 13) and $5x10^3$ Percoll isolate (lanes 4 & 14), CF1 mouse $5x10^4$ crude isolate (lanes 5 & 15) and $5x10^4$ Percoll isolate (lane 6 & 16), CF1 mouse $5x10^3$ crude isolate (lanes 7 & 17) and $5x10^3$ Percoll isolate (lane 8 & 18), 50 bp PCR ladder (lanes 10 & 20), negative control (lane 9), positive control (lane 18 Figure 4.6), blank (lane 19). (Arrow indicates positive sample band)

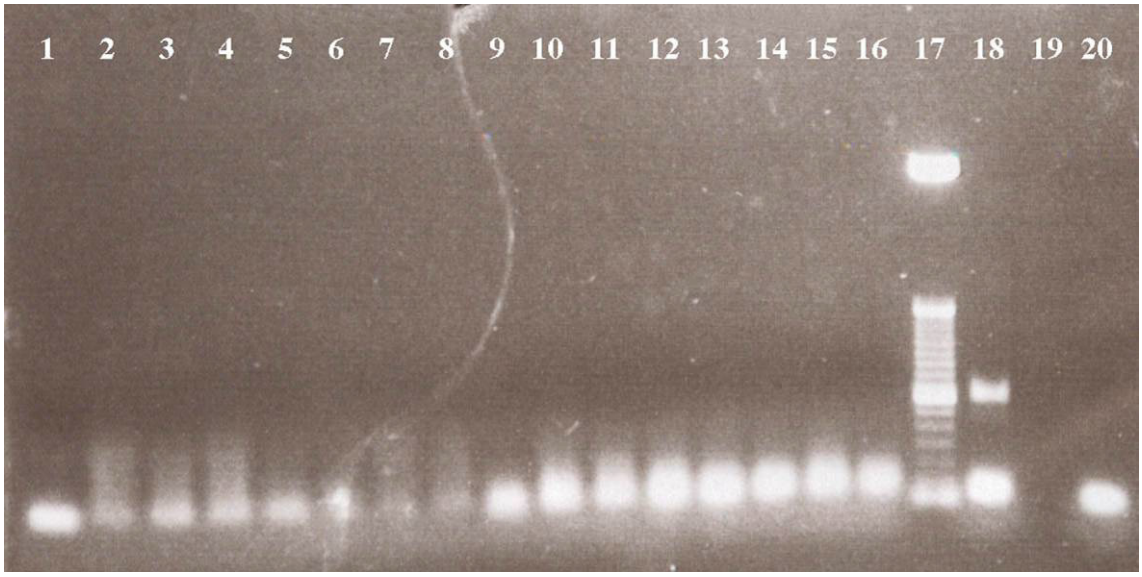


Figure 4.6. *PCR gel photograph of re-isolation cell culture passage 3 samples.* 1 µl sample template (lanes 1-8), 5 µl sample template (lanes 9-16) BALB/c mouse $5x10^4$ crude isolate (lanes 1 & 9) and $5x10^4$ Percoll isolate (lanes 2 & 10), BALB/c mouse $5x10^3$ crude isolate (lanes 3 & 11) and $5x10^3$ Percoll isolate (lanes 4 & 12), CF1 mouse $5x10^4$ crude isolate (lanes 5 & 13) and $5x10^4$ Percoll isolate (lane 6 & 14), CF1 mouse $5x10^3$ crude isolate (lanes 7 & 15) and $5x10^3$ Percoll isolate (lane 8 & 16), 50 bp PCR ladder (lane 17), positive control (lane 18), blank (lane 19), negative control (lane 20).

4.4 Discussion

The Nc-Liverpool Trial-1 proved that both BALB/c and CF1 mice could be infected with Nc-Liverpool by intraperitoneal inoculation. Infection of BALB/c mice with Nc-Liverpool has been shown previously (McGuire et al., 1997b), but evidence for infection of CF1 mice has not been reported. Mice were inoculated with $1.3x10^6$ Nc-Liverpool tachyzoites. However the number of live tachyzoites was undetermined. It is likely that the inoculation dose consisted of a mixture of live and dead tachyzoites as they were harvested from late stage cell cultures where most of the monolayer had been destroyed. Trypan blue elimination could have been used to determine live:dead ratios but the technique was not known of at the time of the evaluation. The parasite dose inoculated into the mice was too high, as severe neosporosis was induced within a short time. Although dose has been shown to be important for inducing acute and chronic neosporosis

(Atkinson et al., 1999; Dubey and Lindsay, 1996; Eperon et al., 1999; McGuire et al., 1997b), a dosage of 10-1000 fold fewer parasites may have been more appropriate, especially as immunosuppressants were also used.

BALB/c mice all displayed signs of neosporosis following inoculation with Nc-Liverpool tachyzoites. Signs included inactivity, coat ruffling, body hunching, paresis and death. Mice that were immunosuppressed (groups 2 and 3) showed more severe neosporosis than those that were not immunosuppressed (group 1). Mice injected with immunosuppressants on the day of inoculation developed the most severe signs of neosporosis, resulting in death for two of the mice. All BALB/c mice inoculated with parasites had to be euthanised by day 7 due to severe disease. Immunosuppression with MPA to overcome natural resistance in mice has been well established (Lindsay and Dubey, 1989c; McGuire et al., 1997b). Clinical disease severity has been shown to vary from acute to chronic depending on the amount of MPA administered (Lindsay and Dubey, 1989c, 1990), however BALB/c mice have been shown to develop neosporosis when infected with the NC-1 strain of *N. caninum* without immunosuppressive treatment (Lindsay et al., 1995a).

CF1 mice were not affected as severely from neosporosis as the BALB/c mice. Only one mouse developed body hunching and most exhibited moderate coat ruffling. Five of 12 CF1 mice inoculated developed signs of neosporosis, whereas 12/12 BALB/c mice did. CF1 mice appeared to have more abdominal distension than the BALB/c mice and were checked for peritoneal exudate, however no parasites were identified in peritoneal fluid. CF1 mice may just appear to have larger abdomens as a characteristic of the breed, or in comparison to the BALB/c mice, which were unwell and unlikely to be eating. CF1 mice treated with MPA displayed more severe signs of neosporosis. One mouse that was not treated with MPA developed moderate coat ruffling.

The signs of neosporosis observed were similar to those described by other groups (Atkinson et al., 1999; Lindsay and Dubey, 1989c; McGuire et al., 1997b; Sawada et al., 1997; Yamage et al., 1996). Coat ruffling was observed from day 5, which is consistent with some reports (Lindsay et al., 1995a) while others have observed signs in BALB/c mice commencing from day 10 PI, even when injected with $1x10^7$ parasites (Atkinson et al., 1999). These authors reported that i.p. injection of Nc-Liverpool into BALB/c mice failed to yield clinical signs of disease, nor were brain lesions or cysts found, but s.c. inoculation resulted in classic neosporosis signs (Atkinson et al., 1999).

Immunoblot results were consistent with mouse infection and the number of days PI. No mice developed antibodies by the first cull date at 7 days PI. This result is not unexpected as it is likely to be due to the fact that the immunoblot detects IgG antibodies, which take approximately 10 days to develop to detectable levels in vivo. Using an immunoblot, antibodies against Nc-Liverpool have been detected in the serum of BALB/c mice from day 9 (Atkinson et al., 1999). From day 14 PI, all remaining mice that had been challenged with Nc-Liverpool produced a positive dot-blot result. Mice that had not been exposed to Nc-Liverpool were all negative using the dot-blot. From day 14 there was a 100% correlation between exposure to Nc-Liverpool and a positive dot blot result. Blood samples were tested at 1:1,000, the secondary antibody was used at a concentration of 1:50,000. Therefore, the dot-blot procedure can be used as a sensitive and

reliable test for detecting specific *N. caninum* antibodies in mouse blood following parasite challenge.

A correlation between PCR-3 and the immunoblot results was observed. From 14 days PI, all mouse brain samples that were positive using PCR-3 also produced positive results using the immunoblot detection system. However not all samples that were positive using immunoblotting were found to be positive using PCR. Samples 24 and 33 were strongly positive using PCR and were also strongly positive using the dot-blot. The correlation between PCR and immunoblot detection was surprising as it was anticipated that a strong immune response may result in clearance of the disease. It appears that a strong immune response is likely to be associated with a high neural parasite load.

Despite indications by PCR that parasites were present in the mouse brains, they could not be positively identified using immunohistochemistry. Reports suggest that small tissue cysts in the brain can only be observed from 21 days PI (Lindsay and Dubey, 1989c) and 28 days PI in BALB/c mice (McGuire et al., 1997b). However, a complete lack of brain tissue cysts in BALB/c has also been reported, even after 140 days, despite brain lesions frequently being observed (Lindsay et al., 1995a). Given the large volume of brain tissue and the small sample size taken during sectioning, it is possible that developing tissue cysts in mice culled in weeks 3 or 4 may have been missed during screening despite multiple sections from different parts of the brain having been examined. Reportedly brain cysts in BALB/c mice are rare and are infrequently seen in most mice (Dubey and Lindsay, 1996; Lindsay and Dubey, 1989c; McGuire et al., 1997b). The strain of *N. caninum* used for infection has a significant effect on tissue cyst formation in the brain (Atkinson et al., 1999). Given a longer period of chronic infection, cysts may have been identifiable in this study.

Interestingly, the number of positive brain samples detected using PCR for both the BALB/c and CF1 mouse groups challenged with *N. caninum* were similar. From the CF1 mouse groups 5/12 mice were positive using PCR whereas 6/11 BALB/c mice were positive using PCR. PCR has been shown to be an effective detection tool, even in the presence of high background DNA (Ellis et al., 1999a; Yamage et al., 1996). Most CF1 mouse brain samples could only be detected from 14 days PI, whereas all PCR positive BALB/c mouse brain samples were detectable by 7 days PI. Had the BALB/c mice survived for longer it is possible that more samples may have demonstrated detectable parasites.

Dose of tachyzoites and tissue cyst numbers are reportedly unrelated but dose and time of MPA treatment does increase cyst production. Mice receiving 2.5 mg of MPA on day 0 PI were reported to produce the largest number of tissue cysts (McGuire et al., 1997b). However, 21% of BALB/c mice infected with Nc-Liverpool were not observed to develop tissue cysts at any stage following inoculation. It is suggested that BALB/c mice should not be used for tissue cyst production models due to early mortality (McGuire et al., 1997b).

The Nc-Liverpool Trial-2 mice were inoculated with $5x10^3$ and $5x10^4$ tachyzoites following insights gained in the Nc-Liverpool Trial-1. To support long-term survival and chronic infection, mice were not treated with immunosuppressants (Lindsay et al., 1995a). No mice from this trial died prematurely from neosporosis, nor did mice develop clinical signs of neosporosis. Only Nc-Liverpool Trial-2 mice used for the Inoculation and Re-isolation Trial will be discussed further.

PCR diagnostic tests performed on the wash supernatant removed from the cell culture monolayer following inoculation with crude lysis or Percoll purified brain samples did not produce positive *N. caninum* bands, suggesting that either parasites were not present to begin with or that they had infected the new host cells. A positive PCR sample would have indicated that either the parasites were dead prior to addition into cell culture or that parasites were unable to infect the new monolayer cells. One week after monolayer inoculation a cell sample was removed during the first passage of the re-isolation cultures and tested using PCR-2. Three of the eight cultures showed positive tests for *N. caninum* as *Neospora* specific bands were observed on the PCR gel. By the second passage, at week 2, only 1 culture appeared to have a positive band and by passage 3, week 3, no detectable *Neospora* was present. Interestingly, both the Percoll and crude lysis samples infected the cultures and could be detected at passage 1. There appeared to be no correlation with isolation ability and mouse strain or inoculation dose, which is consistent with reports (McGuire et al., 1997b). A larger study would be required to determine which mouse strain is better for re-isolating parasites from tissue, but it is likely that re-isolation would be dependent on the number of cysts present to start with.

Again, none of the mouse brains were determined to be positive when screened using IHC. The entire brain was not screened. It would be necessary to check the entire brain in order to gain an accurate assessment of parasitic infection as cysts are usually rare within tissues (Dubey and Lindsay, 1996).

One mouse was found to be positive when a sample of brain was tested using the DNeasy DNA isolation kit. Again, only a sample of brain was tested which is likely to result in missed diagnosis due to processing of tissue that does not hold the tissue cyst. However, it is clear that both CF1 and BALB/c mice can become infected with *Neospora* given an inoculation dose as low as 5000 tachyzoites without immunosuppression. Despite having supposedly functional immune systems, the mice were still susceptible to low numbers of parasites, which resulted in chronic infection. The results observed from this trial cannot be considered conclusive, as the sample size is small. Indications of dose, breed and isolation requirements are suggested as a result of these studies but further work is required before firm conclusions can be drawn.

The results produced appear to indicate that *N. caninum* parasites can be re-isolated from murine brain tissue and introduced to cell cultures. It is unclear whether the parasites infect the cells. Parasites could not be detected in the supernatant, which contained the original inoculum but could be detected in a cell suspension sample at the first passage. It appears that the parasites had infected the host cells as the monolayer had been washed six times with PBS and the media had been changed at least once prior to passage. Presumably following this treatment, any parasites that were not intracellular would have been removed.

It is not understood why the parasites disappear during the following two passages. It is possible that the process of cell passage is causing parasite loss. This may be due to toxicity caused by the ATV, or perhaps host cells infected with parasites are not adhering back onto the flask and are washed away and discarded during subsequent washes and media changes. It is not possible using the PCR to determine whether the *N. caninum* parasites are present in their tachyzoite or bradyzoite form. If the parasites are not stimulated to convert from bradyzoites to tachyzoites, they may not subsequently be replicating within the host cells and may die within a short period

of time. Parasite death following cell infection could explain why parasites are seen for only the first 1-2 passages but were absent in later passages. Cell culture conditions may not be appropriate for parasite growth at early infection stage. It has previously been suggested that *N. caninum* antibodies identified in FBS may affect the parasites. Studies showed that this was not the case for the established Nc-Liverpool strain of *N. caninum* that is in the tachyzoite life stage, but the results could differ for parasites that are unaccustomed to culture conditions or exposure to antibodies.

4.5 Conclusions

Cultured Nc-Liverpool tachyzoites can infect both BALB/c and CF1 mice. BALB/c mice are more susceptible to severe neosporosis following parasite challenge than CF1 mice. Immunosuppression of both mouse strains resulted in increased neosporosis signs, with MPA given on the day of inoculation producing the most significant disease for both mouse strains.

An immunoblot is a reliable indicator of *N. caninum* parasite exposure, but detection is only possible if blood is harvested at approximately 10 or more days PI. The production of *N. caninum* specific antibodies does not result in clearance of disease. A positive relationship between a strong immune response and high disease burden may exist.

A correlation was observed between PCR-3 and immunoblot results but only for samples taken from 14 days PI (IgG production dependent). Despite parasites clearly being present in neural tissue, cysts could not be identified in brains from infected mice within a 4 week period from challenge.

BALB/c mice developed more severe neosporosis than CF1 mice that were also clearly infected with *N. caninum* parasites. It is recommended that both CF1 and BALB/c mice are utilized for isolation studies and that mice are treated with 2.5 mg of MPA on the day of inoculation in order to increase the likelihood of infection.

Isolation of *N. caninum* can be done using Percoll purification or a crude lysis technique. Percoll isolation may provide a better isolation option as it concentrates the number of parasites. However, parasites do not subsequently survive in culture for any length of time. Nor do the parasites appear to increase in number in culture. This indicates that the parasites may initially be invading cells but are not surviving and replicating within them.

Mouse infection following inoculation with Nc-Liverpool appears to be unpredictable. Infection does not necessarily occur after exposure to the parasite and the concentration of parasites used to inoculate mice appears to have little effect on infection rate or cyst development.

Chapter 5.

5.0 Attempts to Isolate *Neospora caninum* from Naturally Infected Animals

5.1 Introduction

Since *Neospora caninum* was first isolated in 1988 it has been recognised in many different countries and found to infect a range of animals (Dubey et al., 1988). At least 60 strains of *N. caninum* have been isolated around the world from tissues of naturally infected dogs, cattle, deer, water buffalo and sheep (Dubey et al., 2007). However, viable *N. caninum* is recognised as difficult to isolate, normally requiring multiple attempts (Conrad et al., 1993; Stenlund et al., 1997) as parasites are usually found in low numbers within host tissue (Wouda et al., 1997) and processing can result in low parasite viability (Hemphill, 1999). Additionally, not all isolates can be adapted to cell culture (Vianna et al., 2005). Biological diversity between strains has been proven in both *in vitro* and *in vivo* studies. Recently significant genetic diversity has been shown between isolates, which also revealed no association between genetic similarities and host or geographic origin (Regidor-Cerrillo et al., 2006).

Host infection in large animals is usually detected with serological tests such as IFATs, and ELISAs. Serological tests can provide evidence that an animal has been exposed to the parasite but do not represent the infection or clinical status of the host. None of the serological tests have been validated based on viable *N. caninum* parasite recovery from host tissue (Dubey et al., 2007).

Most *N. caninum* isolates have come from clinically infected animals and have been achieved in a variety of cell cultures and animal challenge bioassays in immunosuppressed mice, gerbils and dogs (Dubey and Schares, 2006). Establishing a new culture may take anything from a week to several months (Davison et al., 1999; Dubey et al., 1988) with a period of approximately one month being a common period of time before parasite detection. Significant strain variation exists for *in vitro* growth characteristics and *in vivo* pathogenicity (Dubey and Lindsay, 1993).

Parasites in tissue cysts can remain viable for up to 2 weeks if refrigerated (4°C) but will die within a day if frozen (Lindsay et al., 1992). Tachyzoite infectivity is also significantly affected by extracellular maintenance in growth medium for more than 4 hours (Hemphill, 1999). In vitro culture media for tachyzoites is usually supplemented with foetal bovine serum (FBS), however FBS has been shown to contain *Neospora* antibodies (Hemphill, 1999; Torres and Ortega, 2006). Despite the presence of antibodies, the tachyzoites can be maintained in culture and appear to be unaffected by them (Dubey and Lindsay, 1996). In fact, parasites have been shown to grow erratically without serum supplementation (Barber et al., 1995).

Animal challenges have been performed where dogs, mice and gerbils have been inoculated with or fed cyst-infected tissue or harvested oocysts to induce host infection that leads to tissue cysts or oocyst production in dogs (Dubey et al., 1988). However, the most common method used for inoculation of experimental animals is via intraperitoneal or subcutaneous injection (Lindsay and Dubey, 1990).

The purpose of this study was to isolate a New Zealand strain of *N. caninum* using inoculation into cell cultures and mice and then to passage any isolated strain in cell culture. In addition to isolation of a new strain, confirmation of the presence of *N. caninum* in the original tissues and in the biological models was also attempted.

5.2 Materials and Methods

5.2.1 Bovine Tissue Selection

The Institute of Veterinary Animal and Biomedical Sciences (IVABS) at Massey University, Palmerston North, New Zealand, had conducted a *N. caninum* serological study on a large South Waikato dairy herd since 1997. The serological profile results from the study were used to select cattle that were likely to be infected as a source of samples for the isolation of *N. caninum* from naturally infected hosts.

Cows were identified that were consistently serologically positive. Calves from these cows were selected at birth for tissue isolation. The calves were transported to Massey University a few days after birth. Blood samples were taken and sent for *N. caninum* antibody testing using an IFAT at the AgriQuality Laboratory in Palmerston North.

Additionally, cattle from other regional New Zealand farms that were suspected of being infected with *Neospora* due to a high *Neospora* IFAT level or calves suspected of suffering from neosporosis were also occasionally selected to obtain tissue for isolation attempts.

5.2.2 Foetal Tissue Selection

On two occasions, bovine aborted foetal tissues were used in isolation attempts. One such sample was the placenta from a strongly IFAT positive cow that had aborted. The second sample was a foetal calf almost at full term. The foetal tissues were transported in bins with ice packs to remain chilled and were received within 48 hours.

Twenty-one attempts at isolation from bovine sources were performed. (Refer to Table 5.1 for further details).

5.2.3 Dog Tissue Selection

Few dog samples were used for isolation attempts. Two dogs from a trial at Massey University, which was performed to observe the sero-status of dogs after feeding CNS and muscle tissue from a known serologically positive cow, were used for isolation attempts after sero-conversion was observed. (Refer to Table 5.2 for further details.)

A naturally infected boxer dog that presented with neosporosis at the Massey University veterinary clinic and was subsequently euthanased due to severe illness (possibly related) was donated to this trial (see Boxer case study 5.2.13).

5.2.4 Bovine Processing and Tissue Removal

Adult cattle were housed in isolation at a Massey University research farm until culled. Calves were housed in stalls on site at IVABS for a maximum of 7 days (usually 24-48 hours) and were euthanased with approximately 20 ml pentabarbatone intravenously (i.v). Following euthanasia the animals were taken to the post mortem (PM) room. A stethoscope was used to check for signs of a heartbeat. In the absence of a heartbeat the throat was cut to exsanguinate the animal before dissection.

The head was removed from the body, skinned and excess muscle was removed. The head was secured in a vice and a hacksaw was used to saw the top of the skull off. Once the brain was exposed the head was removed from the vice and held over a beaker of sterile antibiotic-PBS. The brain was prized out of the skull into the PBS with sterile forceps and scalpel and the container was sealed.

The limbs and muscles were removed to expose the vertebral column before being separated from the carcass. The vertebral column was cut using a band saw. The cut was made into the vertebral canal attempting to keep to one side of the spinal cord, exposing it for removal. The spinal cord was removed using sterile forceps and a scalpel and it was placed in a beaker of sterile antibiotic PBS.

All equipment and surfaces were thoroughly cleaned with Vircon and hot water and sterilized with 70% ethanol to reduce bacterial contamination of the tissues.

Once the spinal cord and brain were removed from the carcass they were taken to a PC2 laboratory where they were processed. The carcass was destroyed.

Initially calves were culled on the same day as tissue processing. However, it was discovered that tissues could be maintained in antibiotic PBS for several days at 4°C without parasite destruction (Lindsay et al., 1992). This allowed up to three calves to be killed per day and tissue processing performed during the subsequent days. One sample per day was usually processed in the laboratory. The standard procedure was to kill two calves on a day and process the calf tissue in the laboratory over the following two days.

5.2.5 Foetal Tissue Processing

Aborted foetal tissues were examined for signs of neosporosis prior to tissue processing. The cotyledons were removed from the placenta. The cleanest and freshest looking cotyledons were used for isolation, using approximately 3 cotyledons. These were homogenized and treated as described for CNS tissues.

An aborted foetus was not used for isolation if it was found that its brain had already undergone severe autolysis and isolation was not worth pursuing. Samples of brain tissue were removed for PCR analysis to confirm a *Neospora* diagnosis.

5.2.6 Dog Processing and Tissue Removal

Experimental dogs were euthanased and the brains removed for tissue isolation. The spinal cord from dogs was not removed, as the procedure would have been too difficult due to their smaller size. The procedure developed for calf tissue isolation was also used for dog samples.

5.2.7 Central Nervous System (CNS) Tissue Processing

All tissues were processed in a biohazard containment hood once removed from the animal in order to reduce the incidence of contamination of the samples. To further decrease contamination, tissues were washed several times in antibiotic-PBS and pieces of bone, blood vessels and membranes were removed from the tissue and discarded where possible.

A section of spinal cord and/or brain was removed from each sample and stored in 10% buffered formalin for immunohistochemical detection. All remaining tissue was used for parasite isolation. The brain and spinal cord from each animal were kept separate during processing.

Processing of tissues (including brains, spinal cords and cotyledons) was performed by first homogenising the tissue in a 1% Trypsin digest mixture at a ratio of approximately 1:5, tissue to digestion mixture, then incubating at 37°C with agitation for 1 hour. Following digestion, samples were washed several times with sterile PBS, filtered through sterile muslin cloth and re-suspended into a 30% Percoll solution. Samples were centrifuged at 2,700 x g to separate the myelin (lipid cells) from the denser parasites, which formed a layer at the top of the solution and a pellet at the bottom of the tube respectively. Once purified, the parasite fraction of the sample could be used to inoculate cell cultures and mice and a sample was removed for diagnostic purposes.

Using this isolation method, a 200-300 g sample could be reduced to a mass of 1-3 g. Following tissue processing the reduced mass was most frequently resuspended to a volume of 7 ml in growth media (antibiotic / 2% serum / MEM). The resuspended sample was then divided into three; a 4 ml volume for cell culture inoculation, 2.5 ml for mouse inoculation and 0.5 ml for PCR diagnostic testing.

(Refer to Appendix 5.1 for further details on tissue processing, Chapter 2 - Cell Culture for details on culturing techniques and Chapter 3 - Diagnostics for details on PCR and IHC)

5.2.8 Inoculation of Cell Cultures

The final method adopted for inoculation of cell cultures involved using Vero and Bovine Endothelial (BE) cell lines. Commonly, eight 25 cm^2 monolayers were used per host cell line. For each cell line, four of the monolayers were trypsinised and resuspended prior to inoculation and the other four monolayers were inoculated directly, therefore providing adhered or suspended cells for parasite infection.

Each 24 hour monolayer was inoculated with 250 ul of purified tissue isolate and incubated at 37°C. Adhered monolayers were incubated with inoculum for no more than 3 hours (Hemphill, 1999; Hemphill et al., 1996; Hemphill et al., 2004). Resuspended monolayers were incubated with inoculum for 8-12 hours, after which time the media was removed, centrifuged, and the pellet kept for PCR analysis. Fresh growth media was added to the monolayer.

The growth media on each culture was changed daily for at least 3 days after inoculation to ensure that antibiotic levels were maintained and toxins did not build up. Media changes were extended to every 2-3 days following this period.

Experimental cultures were first passaged at 1-2 weeks following setup or once the monolayer became confluent and fresh cells were added if required. Experimental cultures were maintained for a minimum of three months and samples were removed during cell passage for *N. caninum* diagnosis using PCR.

(Refer to Chapter 2 – Cell culture, for further details on culturing procedures and materials.)

5.2.9 Mouse Inoculation with Tissue for Isolation Attempts

Mice were immunosuppressed with 2.5 mg of methylprednisolone acetate (MPA) (Vetacortyl) given subcutaneously (s.c) into the neck scruff on the day of inoculation. (Refer to Chapter 4 – Small Animal Challenge, for further details on mouse models.)

Mice were inoculated with the retained volume of purified tissue sample. Mice were usually inoculated on the day after tissue isolation as mice were located at a site 2 hours away from the isolation site. Typically, both BALB/c and CF1 mice were inoculated with 0.3 ml of sample intraperitoneally (i.p). Four mice from each strain were inoculated per spinal sample and/or four mice per brain sample for canine and bovine isolates.

Mouse brains suspected of being infected with *N. caninum* of bovine or canine origin were occasionally purified and inoculated into other mice. Each mouse brain sample passaged back into mice was split between no more than 6 mice.

Mice were kept for a minimum of two months and checked regularly for signs of peritoneal exudate and disease. Mice that became severely ill within 72 hours were culled and were not considered to have neosporosis.

Of the mice inoculated with early study isolates, at least one mouse from each group was culled and the brain checked using IHC for signs of *N. caninum* infection. Half of the brain was used for IHC and PCR and the other half was used for inoculation into cell culture.

All mice were culled near the completion of the study. The brain and blood was removed. The brain was processed and Percoll purified. A sample of Percoll purified brain was taken for PCR-3 diagnosis and the remaining brain sample used to inoculate three 24-hour Vero monolayers in a 25 cm^2 culture flask.

PCR positive brain samples were also used to inoculate 3 immunosuppressed BALB/c mice.

Serum was separated and used in an immunoblot test.

5.2.10 Bovine Isolation Samples

All tissue samples were processed and used to inoculate 24-hour tissue culture monolayers and mice (Table 5.1).
Samples 1-5 were only inoculated onto Vero host cells and into CF1 mice, whereas samples 6-21 were inoculated onto Vero and BE cells as well as into CF1 and BALB/c mice.

Samples 1-3 were inoculated directly onto host cell lines as crude homogenate, whereas samples 4-21 were all purified using a Percoll purification gradient.

Samples 1-5 were tested using only PCR-1 as no viable sample remained for further testing at the conclusion of this study. Whole crude lysate was used as PCR template for samples 1-3. Percoll purified sample tissue was used as PCR template for samples 4-21. Samples 6-21 were tested using PCR-3.

5.2.11 Immunohistochemical Detection

Samples were taken from all bovine tissues used in isolation attempts and stored in 10% buffered formalin for IHC detection. IHC was performed as described in the Diagnostics Chapter 3- IHC.

Brain tissue from mice inoculated with early tissue isolates (samples 1-5), were placed into 10% buffered formalin, processed and tested using IHC.

5.2.12 Case Study 1: Bobby Calf Isolation Attempt

Sample Selection

Bobby calf 99-167 from a farm outside of the serological study group was identified as being serologically positive for *N. caninum* with an IFAT of 1/1000. The cow that gave birth to the calf had a serological IFAT of 1/2000.

Calf Treatment and Cull

The calf was treated with immunosuppressant prior to destruction in an attempt to induce recrudescence. The calf received 20 mg/kg (18.6 ml) of 0.5% Opticortenol via an intramuscular injection across several sites 2 weeks prior to euthanasia.

Post-mortem Dissection and Tissue Processing

The Bobby calf was euthanased with pentabarbatone administered intravenously in the post mortem room at Massey University. The brain and spinal cord were dissected, removed and placed into sterile plastic bags (refer to Bovine Processing and Tissue Removal technique described above). The bagged tissues were taken to a clean laboratory where they were dissected further. Tissues were not processed in a biohazard containment unit. Sections of the brain and spinal cord were removed and kept for use in PCR and IHC. Samples for use in PCR were stored in sterile plastic bags at -20°C. Samples for IHC were placed in 10% buffered formalin and kept at room temperature until processed.

A sample of the pellet was removed after the Percoll centrifugation and viewed under 400 x magnification for parasites and flasks were viewed following inoculation for signs of parasites infecting cells.

Cell Culture Inoculation

Calf 99-167 was the first sample for which Percoll purification was used.

Variations to the described Tissue processing protocol (Appendix 5.1) are as below.

Three 25 cm^2 Vero monolayer flasks were inoculated with 1 ml of suspension for brain and spinal cord samples. The media for two flasks was supplemented with 2% FBS and one with 2% ES for each sample. Media was topped up to 35 ml with either the FBS or the ES supplemented media and incubated at 37°C for 3 hours.

Three hours post-inoculation, cell culture flasks were washed twice with sterile PBS and the appropriate media replaced to a volume of 40 ml and returned to the incubator at 37°C.

Mouse Inoculation

The day following processing tissue to attempt isolation, the remainder of the brain and spine samples was taken to the small animal unit at Wallaceville for inoculation into female CF1 mice. Eight mice were inoculated, four each for the brain and spinal samples. Each mouse was treated with 2.5 mg of MPA subcutaneously into the scruff of the neck and 0.5 ml of the isolate sample i.p.

5.2.13 Case Study 2: Boxer Dog

Diagnosis and Treatment

A 6-year old female Boxer dog treated at the Massey University veterinary clinic presented with chronic diarrhoea lasting more than three weeks, severe lethargy, intolerance for exercise, vomiting, weight loss/anorexia, face and forelimb swelling, inappetence, a corneal ulcer, anaemia, hypoadrenocorticism, hyposthenuria, polyphagia, proteinuria, panhypoproteinaemia, mild azotaemia and had a history of chronic skin disease. She was diagnosed with protein losing nephropathy, inflammatory bowel disease /small and large bowel diarrhoea and chronic skin allergies. She was non- responsive to treatment with Benzepril and Augmentin.

A $ZnSO_4$ faecal floatation was performed which revealed oocysts of either *Hammondia* or *Neospora* species. Oocysts were sporulated and tested using a PCR.

The dog was euthanased and a post-mortem examination conducted. The brain and spinal cord were removed and used in a parasite isolation attempt (as described for CNS Tissue Processing above).

Boxer Isolation Cell Culture

Following tissue processing of the brain, bovine endothelial (BE cells) and Vero cell cultures were inoculated with the Percoll purified tissue sample. Three Vero monolayers were inoculated with purified brain tissue sample, as was 1 bovine endothelial monolayer. Two Vero monolayers and 1 bovine endothelial monolayer were inoculated with the purified spinal tissue sample.

Following a 3-hour incubation of inoculum on the monolayers, the supernatant was removed and combined with other host cell line sample groups for analysis with PCR.

Monolayers were treated as described for Experimental Cell Culture Inoculation above.

Mouse Inoculation with Boxer Tissue

Four BALB/c mice were inoculated with 0.3 ml, 3 mice with 0.5 ml and 3 mice with 1 ml of purified tissue homogenate of the Boxer tissue and all mice were treated with 2.5 mg of MPA on the day of inoculation.

Mice were culled 2 months PI. Their brains were removed and stored for PCR analysis and re-isolation into cell culture.

Boxer Diagnostic Procedures

A blood sample was drawn from the Boxer during the post-mortem examination and sent to Gribbles Veterinary Pathology (AgriQuality) for *Neospora* and *Toxoplasma* testing using the IFAT method.

A small sample of purified brain and spine tissue inoculum was retained for PCR analysis. The samples were run in triplicate using PCR-3 along with Nc-Liverpool positive control samples.

The retained monolayer inoculum samples and samples of cell culture taken at the first culture passage were tested using PCR-3. PCR-3 was repeated for the Vero cell culture samples taken during passage-1 and for samples taken at passage-2 (chosen based on results observed from the first PCR attempt) and faecal floatation samples.

Mice inoculated with Boxer samples were euthanased after 2 months and their brains removed for passage in cell culture and for PCR diagnosis. Half of the brain was processed and inoculated onto 24-hour Vero monolayers and the other half was frozen for PCR. DNA was isolated using the DNeasy isolation kit and used as template in PCR-3.

5.3 Results

5.3.1 Bovine Isolations

Twenty-one bovine samples were used to attempt *N. caninum* isolation. One sample was a placenta (sample 6), one an aborted foetus near full term (sample 8), one sample was received from a diagnostic laboratory but was derived from Farm 1 (sample 1), one calf was still-born (sample 11), two were adult cows (samples 2 and 3) and the remaining animals were all live calves born to serologically positive dams. Most animals were from Farm 1, which was the subject of a long-term serological study, but some calves were received from regional farms that suspected *N. caninum* involvement (samples 4 and 5 from Farm 2, and samples 10 and 11 from Farm 3).

Three of the bovine samples were found to come from seronegative cattle (samples 8, 11 and19). None of the other diagnostic procedures performed produced a positive *N. caninum* result, which led to cultures made from these samples being discarded.

Ten of the 21 samples tested produced a positive PCR product. Sample 4 was only tested using PCR-1. Refer to bobby calf case study for further details on sample 4. All other positive samples were detected using PCR-3. Sample 15 produced several non-specific bands using PCR-3 but a band of the correct product size for *N. caninum* was also observed, this sample will be considered as positive but a degree of uncertainty remains.

Detection of *N. caninum* in the brain indicates that *N. caninum* DNA was present in the samples used to inoculate cell cultures and mice. Cell cultures tested using PCR were not found to be positive for *N. caninum* following subsequent passages, except for cultures from animal 99-167 described in the bobby calf case study (Figure 5.4).

All remaining mice inoculated with bovine samples were euthanased near the conclusion of this study. Their brains were inoculated onto cell culture and were also tested using PCR. Only one mouse brain from a BALB/c mouse inoculated with a Percoll purified brain sample from Calf 062 (sample 13) was found to be positive using PCR-3 (Figure 5.2). The mouse brain was inoculated onto cell culture and into more BALB/c mice, however neither the cultures nor the mice were subsequently found to be positive for *N. caninum*.

All bovine samples were tested using IHC. None of the samples had detectable *N. caninum* parasites or cysts. Mice inoculated with samples 1-5 were culled 6-18 months following inoculation and brain tissue was checked using IHC for signs of *N. caninum* infection. No tissue cysts or *N. caninum* parasites were identified in the mouse brain tissue.

Mouse blood harvested from inoculated mice was processed and the serum was removed for immunoblot analysis. Although mouse serum was tested using dot-blot, slot-blot and direct application methods, the results were too varied for conclusions to be drawn. Positive samples were observed, however as one negative control sample also produced a positive spot, the reliability of the results gained from the immunoblotting technique is questionable. The procedure was repeated on many occasions using antigen-2 (see Appendix 5.2 for X-ray images of immunoblots). Most tests produced negative results across the immunoblot (results not shown). Due to the inconsistent nature of the immunoblot, the immunoblot results are not presented and were not considered when determining which samples were truly positive for *N. caninum*.

Table 5.1. *Bovine isolates summary of results table.* Samples in italics refer to samples that are shown in Figure 5.1. Text in bold refer to positive samples. (PCR gel photographs for earlier samples (1-5) have not been shown.)

Sample Number	**Animal ID**	**Source**	***N. caninum* IFAT**	**PCR results**	**Inoculated Mice PCR**
1	155	Farm 1	1/2000	-ve	-ve
2	008	Farm 1	1/2000	-ve	-ve
3	118	Farm 1	1/2000	-ve	-ve
4	**99167**	**Farm 2**	**1/1000**	**+ve spinal cord**	**-ve**
5	99210	Farm 2	1/600	-ve	-ve
6	*199 placenta*	*Farm 1*	*Dam +ve*	*-ve*	-ve
7	***661***	***Farm 1***	***1/2000***	***+ve brain*** ***+ve spinal cord***	**-ve**
8	*7-8 mth foetus*	*Farm 1*	-ve	*-ve*	-ve
9	***148***	***Farm 1***	***1/2000***	***+ve brain***	**-ve**
10	***014***	***Farm 3***	***1/600***	***+ve spinal cord***	**-ve**
11	*168*	*Farm 3*	*-ve*	*-ve*	-ve
12	***538***	***Farm 1***	***1/2000***	***+ve brain***	**-ve**
13	***062***	***Farm 1***	***1/1000***	***+ve brain*** ***+ve spinal cord***	***1 mouse +ve***
14	***188***	***Farm 1***	***1/2000***	***Brain +ve***	**-ve**
15	***210***	***Farm 1***	***1/2000***	***Possible brain +ve***	**-ve**
16	*186*	*Farm 1*	*1/2000*	*-ve*	-ve
17	*218*	*Farm 1*	*1/1000*	*-ve*	-ve
18	*150*	*Farm 1*	*1/1000*	*-ve*	-ve
19	*003*	*Farm 1*	*-ve*	*-ve*	-ve
20	***038***	***Farm 1***	***1/2000***	***+ve brain*** ***+ve spinal cord***	**-ve**
21	***125***	***Farm 1***	***1/2000***	***+ve brain***	**-ve**

Farm 1 = serological study farm
Farm 2 = outsourced farm 1
Farm 3 = outsourced farm 2

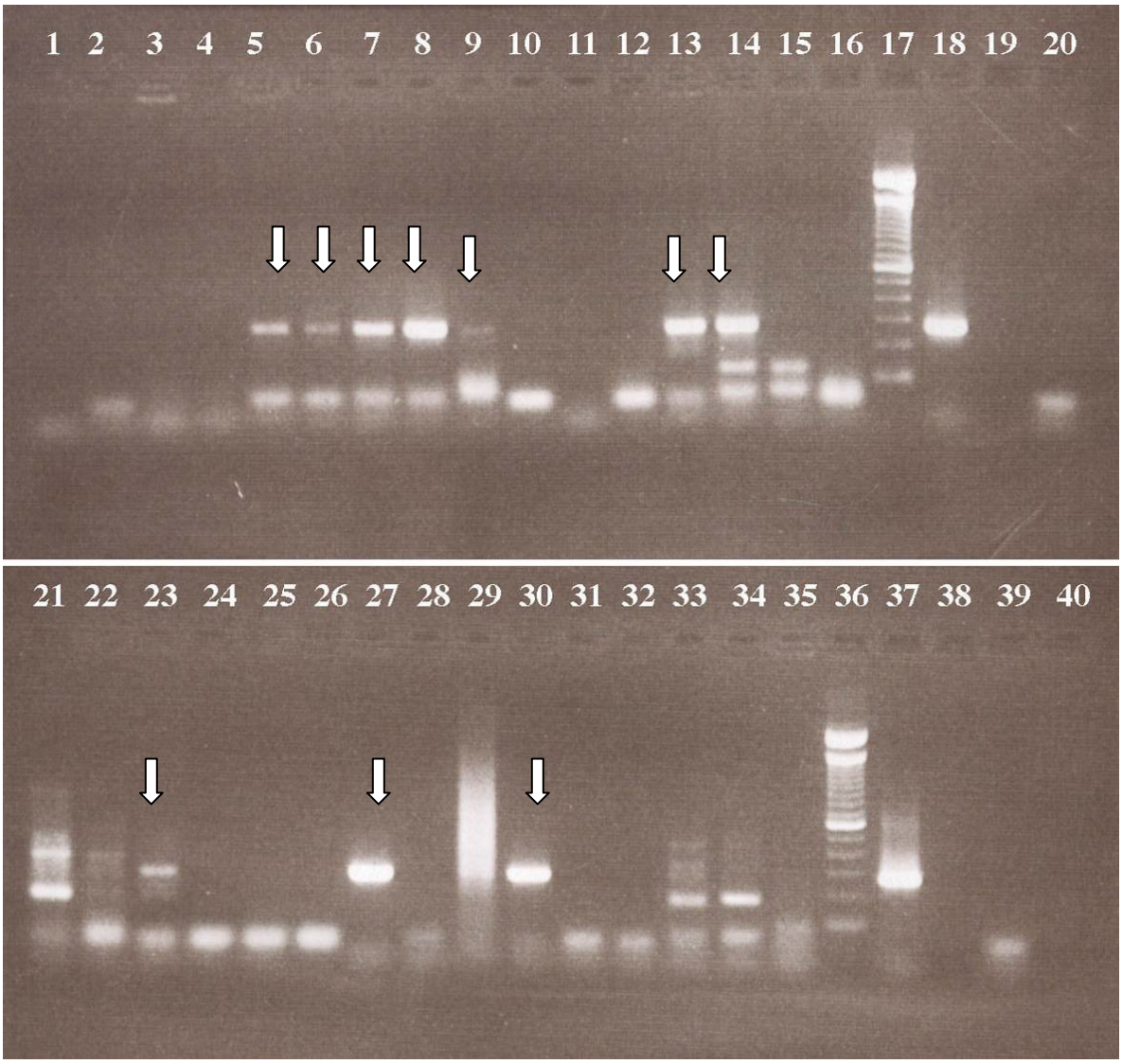


Figure 5.1. *PCR-3 gel photograph of bovine isolate samples.* Brain 186 (lane 1), spine 186 (lane 2), brain 210 (lane 3), spine 210 (lane 4), **brain 661 (lane 5), spine 661 (lane 6), brain 62 (lane 7), spine 62 (lane 8), brain 538 (lane 9)**, spine 538 (lane 10), brain 218 (lane 11), spine 218 (lane 12), **brain 38 (lane 13), spine 38 (lane 14),** brain 3 (lane 15), spine 3 (lane 16), 100 bp ladder (lanes 17 & 36), Nc-Liverpool positive control (lane 18 & 37), no sample (lanes 19, 38 & 40), negative control (lane 20 & 39) brain 150 (lane 21), spine 150 (lane 22), **brain 125 (lane 23),** spine 125 (lane 24), brain 188 (lane 25), spine 188 (lane 26), **brain 148 (lane 27),** spine 148 (lane 28), brain 014 (lane 29), **spine 014 (lane 30)**, brain 078 (lane 31), spine 078 (lane 32), **brain 210 (lane 33),** spine 10 (lane 34), placenta (lane 35). (Positive samples in bold text.)

All mice inoculated with material to attempt isolation were culled, DNA purified from samples of their brains was tested using PCR-3 (Figure 5.2). All but one mouse sample was negative for *N. caninum* (negative PCR gels not shown). The brain from a BALB/c mouse inoculated with brain sample from calf 62 produced a strong *N. caninum* specific positive band (lane 10).

Three cell culture monolayers (2x Vero and 1x BE) were inoculated with the mouse brain infected from calf 62. Cultures were passaged 8 days PI and split into 6 cultures. Samples from all of the cultures were taken during passage and tested using PCR-3. No flasks produced an *N. caninum* positive PCR band (not shown). Cultures were maintained for at least 3 months before being discarded due to absence of *N. caninum*.

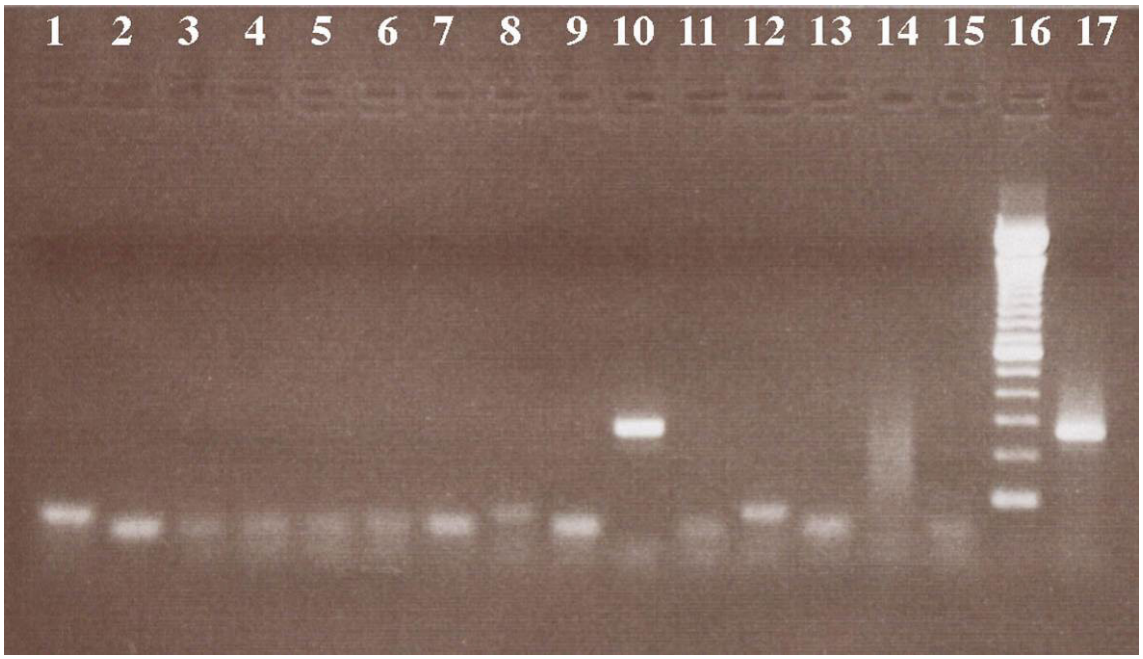


Figure 5.2. *PCR-3 gel photograph of brains from BALB/c mice inoculated with bovine isolates.* Spine 661 (lane 1), brain 661 (lane 2), brain 661 (lane 3), foetal calf brain (lane 4), foetal calf spine (lane 5), spine 62 (lane 6), spine 62 (lane 7), spine 62 (lane 8), brain 62 (lane 9), **brain 62 (lane 10),** brain 118 (lane 11), spine 118 (lane 12), brain 150 (lane 13), spine 150 (lane 14), negative control (lane 15), 100 bp ladder (lane 16), Nc-Liverpool positive control (lane 17).

5.3.2 Experimental Inoculation: Pups 2 and 5

Samples of brain and spinal tissue from puppies (Pups) 2 and 5 were homogenised and inoculated directly onto 24-hour monolayers and tested using PCR-1. A faint band was observed in lane 4 (the band was observed more clearly on the original gel), which contained the inoculated brain culture-1 sample from Pup 2 amplified with *Neospora* primers (Figure 5.3). Lane 17 contained a band for the *Neospora* primer amplified sample of the brain culture-2 from Pup 5. Both the ToxoVax® positive control amplified with *Toxoplasma* specific primers and the Nc-Liverpool positive control amplified with *Neospora* specific primers produced parasite specific bands but did not produce bands when primers of the other species were used for PCR amplification. Both of the bands from the experimental sample observed at passage 1 were lost in future passages. Parasites could not be detected in brain tissue using IHC.

Table 5.2. *Canine isolates summary of results table.*

Dog	Source	IFAT serology	PCR result
Pup 2	Fed Cow 8	+ve	+ve
Pup 5	Fed cow 8	+ve	+ve

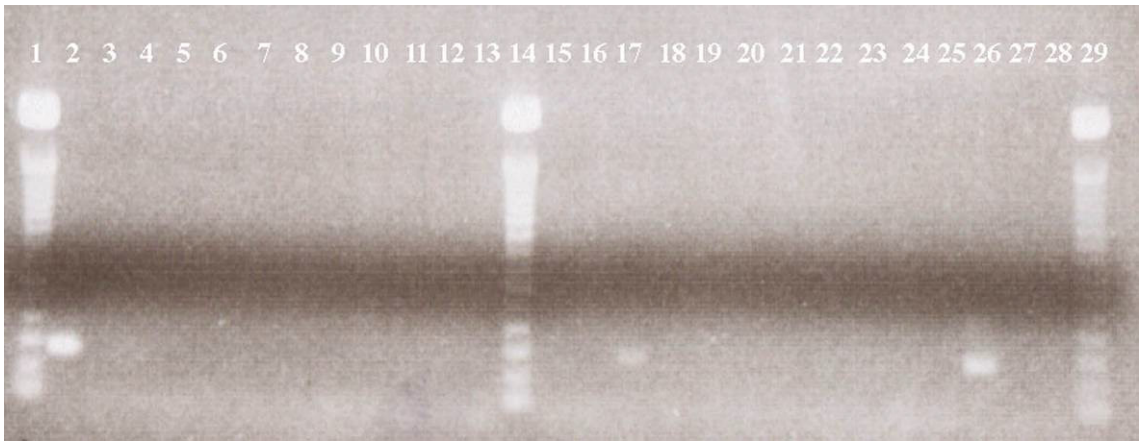


Figure 5.3. *PCR-1 gel photograph of Pup 2 & 5 samples amplified with Neospora or Toxoplasma specific primers.* 100 bp ladder (lanes 1, 14 & 29), Nc-Liv + Neo (lane 2), Nc-Liv + Toxo (lane 3), **Pup-2 brain-1 + Neo (lane 4)**, Pup-2 brain-1 + Toxo (lane 5), Pup-2 brain-2 + Neo (lane 6), Pup-2 brain-2 + Toxo (lane 7), Pup-2 brain-3 + Neo (lane 8), Pup-2 brain-3 + Toxo (lane 9), Pup-2 spinal-1 + Neo (lane 10), Pup-2 spinal-1 + Toxo (lane 11), Pup-2 spinal-2 + Neo (lane 12), Pup-2 spinal-2 + Toxo (lane 13), Pup-5 brain-1 + Neo (lane 15), Pup-5 brain-1 + Toxo (lane 16), **Pup-5 brain-2 + Neo (lane 17),** Pup-5 brain-2 + Toxo (lane 18), Pup-5 brain-3 + Neo (lane 19), Pup-5 brain-3 + Toxo (lane 20), Pup-5 spinal-1 + Neo (lane 21), Pup-5 spinal-1 + Toxo (lane 22), Pup-5 spinal-2 + Neo (lane 23), Pup-5 spinal-2 + Toxo (lane 24), ToxoVax + Neo (lane 25), ToxoVax + Toxo (lane 26). (Positive samples in bold text.)

5.3.3 Case study 1: Bobby Calf Isolation

Two of the eight CF1 mice injected with brain from calf 167 died within a few days PI due to secondary bacterial infections. One mouse had a lesion on its abdomen where the sample had been injected. One mouse from each of the brain and spinal inoculation groups died.

Two days following the inoculation of Percoll purified tissue from Calf 167 onto Vero cell cultures, five of the six cultures appeared to be destroyed by a contaminating infection. Samples from the flasks were sent for microbial identification. Three flasks were found to be infected with *Enterobacter cloacae* and two with *Staphylococcus epidermidis*, both of which were gentamycin resistant. Gentamycin was the only antibiotic used at the time, a fault was found with the

culturing procedure in that it was discovered that gentamycin was being used at half the recommended strength.

An attempt was made to clear the bacterial infection from the flasks by washing the monolayers with sterile PBS and replacing the media with 3x the normal dose of gentamycin and double the dose of Penicillin/Streptomycin/Amphotericin (antibiotic-antimycotic), which was purchased and used to control the infections. The increased dose of gentamycin did not appear to limit the infections, however once Pen/Strep/Amphotericin was introduced, further growth of the contaminants appeared to stop. Although the infection did not progress during treatment, the Vero cells did not recover following the infection. The five infected flasks were eventually discarded.

The remaining cell culture flask also appeared to contain the secondary infection but it was controlled with the use of antibiotics. The cultures were treated with high doses of antibiotic-antimycotic. During the first week the flask was washed daily with sterile antibiotic PBS and fresh growth media was replaced. Cell washing and media changes were decreased to every 2-3 days once the infection was controlled.

The remaining flask, which was inoculated with the spinal tissue, was passaged for 7 days PI and a sample removed for PCR analysis. Passage 2 was performed at 26 days PI and passage 3 on day 36 PI. A cell sample was collected at each passage and tested using PCR-1.

Parasite DNA was clearly detected using PCR-1 in the Percoll purified spinal culture inoculum sample (lane 5), very faintly in a sample of cell culture from passage 1 of the remaining spinal cell culture flask (lane 4) and in a sample from passage 2 (lane 12) of the same flask. The PCR band was not detectable at passage 3, 36 days PI (Figure 5.4).

Two mice inoculated with calf 99-167 tissue were suspected of having neosporosis. One mouse was particularly small and the other was beginning to go blind. Both mice were culled and their brains were removed, processed and used to inoculate 24-hour Vero cell monolayers. PCR analysis of the mouse brain samples produced *N. caninum* positive bands for the blind mouse (lanes 4-6) and more faintly for samples from the small mouse (Figure 5.5).

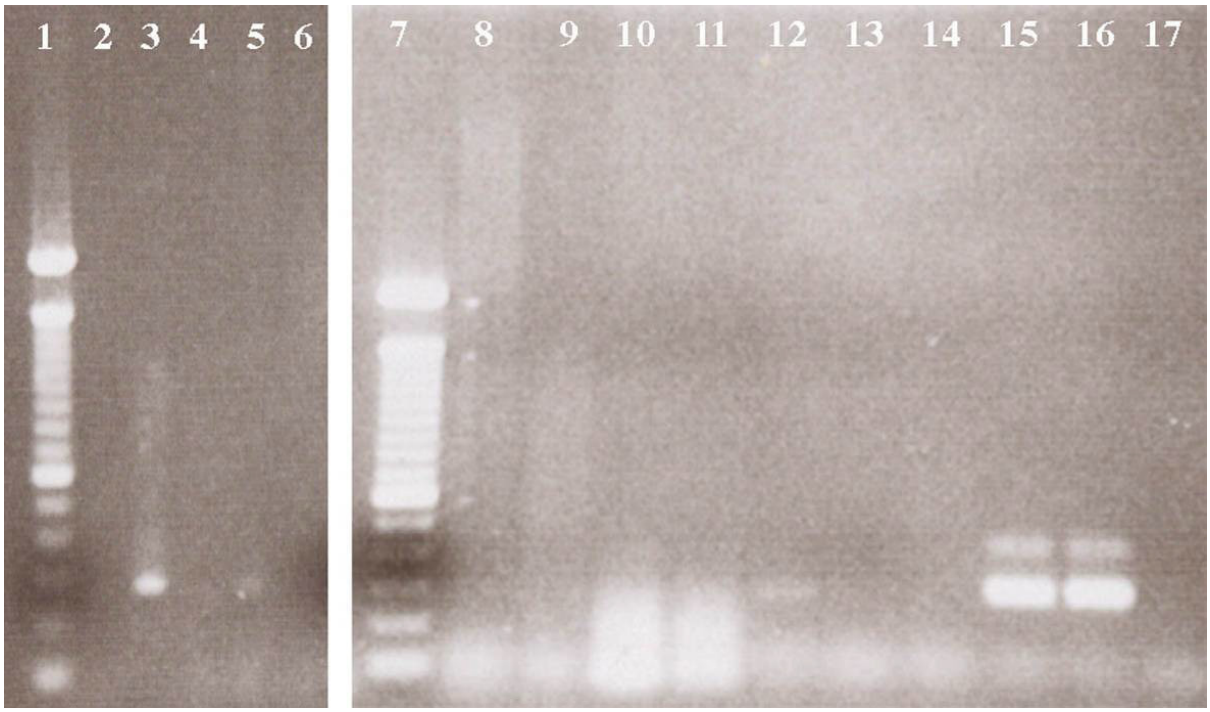


Figure 5.4. *PCR gel photograph of Bobby calf 99-167 purified tissue and cell culture samples.* 100 bp ladder (lanes 1 & 7), spinal cell culture passage-1 (lane 4), purified spine inoculum (lane 5) purified brain inoculum (lane 6), unrelated samples (lanes 8-11), Spinal cell culture passage-2 (lane 12-14), Nc-Liverpool positive control (lanes 3, 15 & 16), negative control (lanes 2 & 17).

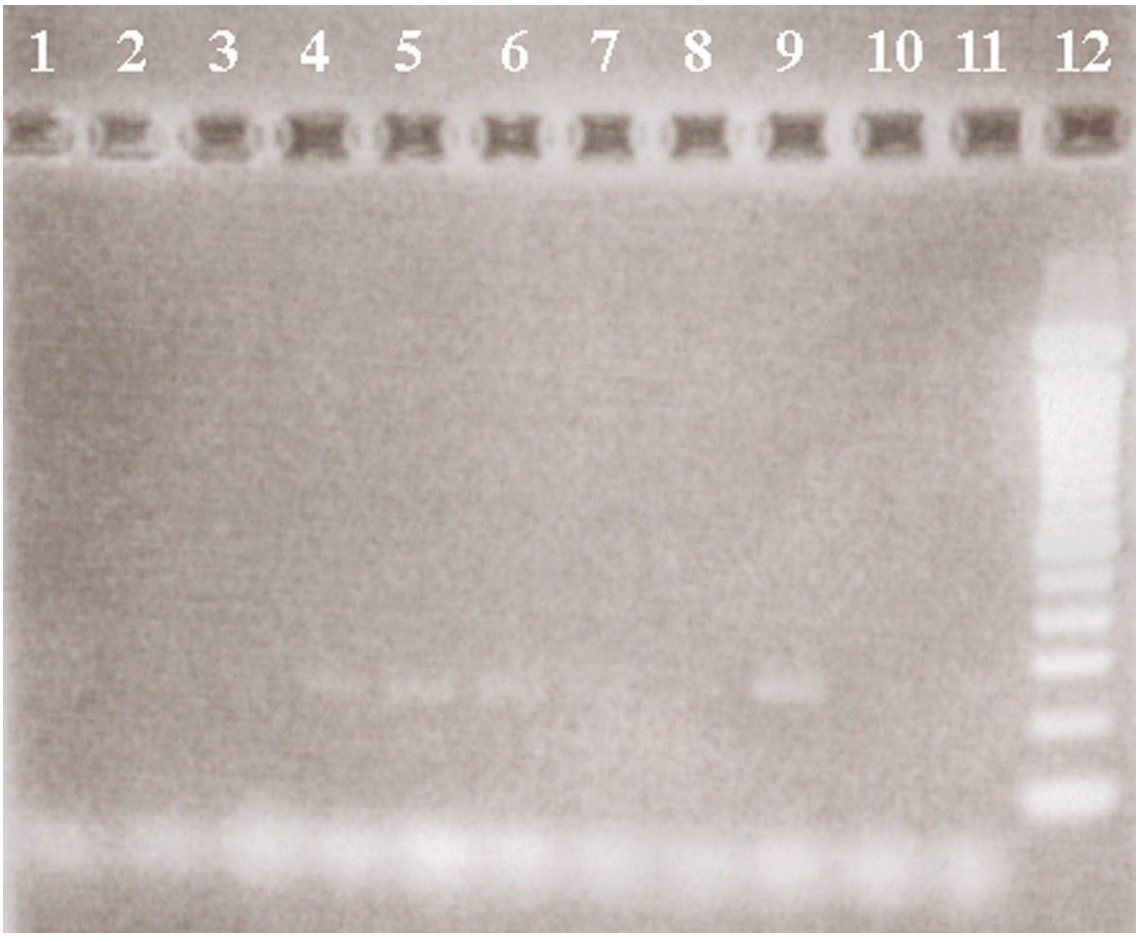


Figure 5.5. *PCR gel photograph of mouse brains following Bobby calf 167 tissue inoculation.* Spinal cell culture passage-3 (lanes 1-3), blind mouse (lanes 4-6), small mouse (lanes 7, 8 & 11), Nc-Liverpool positive control (lane 9), negative control (lane 10), 100 bp ladder (lane 12).

5.3.4 Case Study 2: Boxer Dog Isolation

Blood sent for *N. caninum* serological testing using IFAT was positive for *N. caninum* at a dilution of 1/2000 and the blood sample was negative for *Toxoplasma* antibodies.

Both the brain and the spinal cord samples of the purified tissue were clearly positive for *N. caninum* using PCR-3. The brain samples did not produce as strong a band as the spinal samples, which are very similar in intensity to the Nc-Liverpool control samples (Figure 5.6).

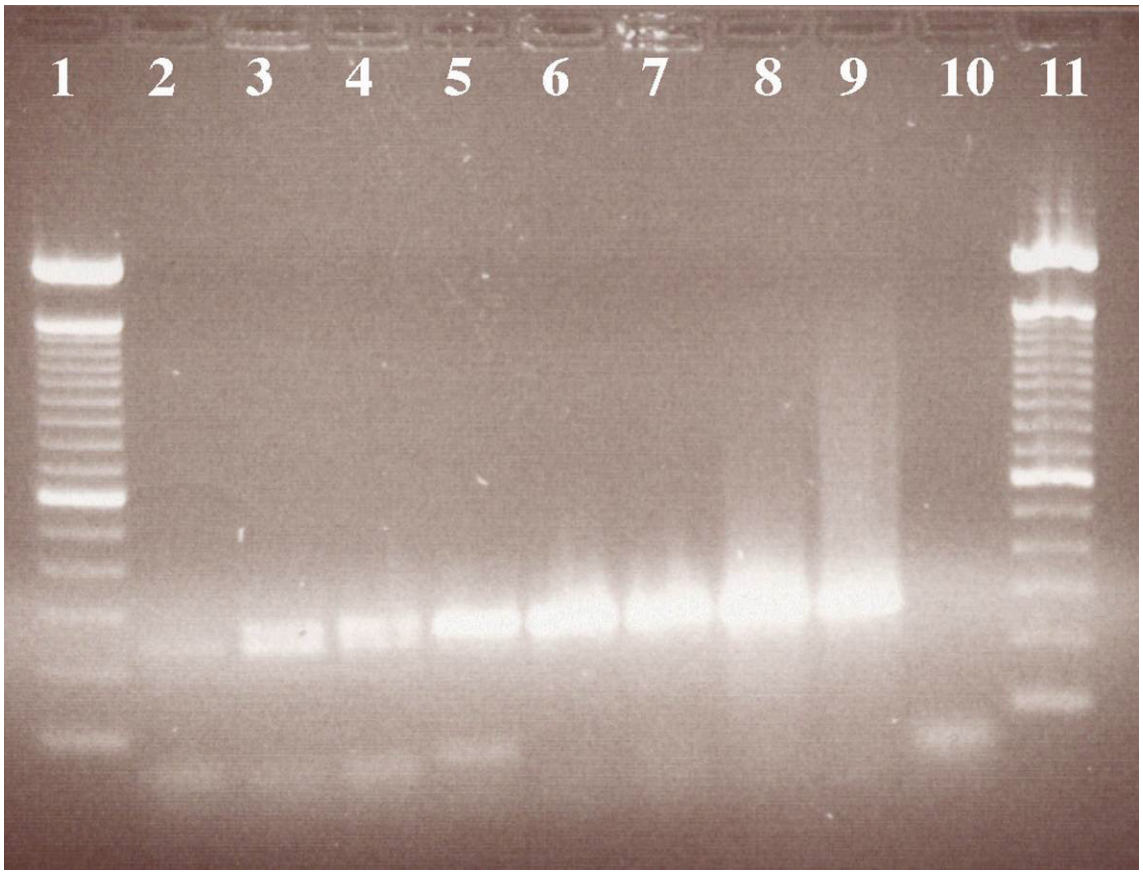


Figure 5.6. *Boxer dog purified tissue inoculum PCR gel photograph.* 100 bp ladder (lanes 1 & 11), purified brain inoculum (lanes 2-4), purified spine inoculum (lanes 5-7), Nc-Liverpool positive control (lanes 8 & 9), negative control (lane 10).

PCR revealed that parasite DNA was present in the post-inoculation culture supernatant that was removed from the cell cultures inoculated with brain homogenate for both the BE cells (lanes 3 & 4) and the Vero cells (lanes 7 & 8) as well as showing a fainter band for one of the duplicates of the Vero cell cultures inoculated with spinal tissue homogenate (lane 6). Faint bands were also observed in lanes 9, 12 and 20 (Figure 5.7) (refer to Appendix 5.3 for the supplementary gel photograph with high exposure showing bands in lanes 9 and 12). The bands in lanes 9 (Vero Brain culture flask 1) and 20 (Vero Spinal culture flask 1) are not of the correct molecular size for *N. caninum* specific products. Lane 12 (Vero Brain culture flask 2) has an *N. caninum* specific band, although it could only be observed under high light exposure (Figure A5.3).

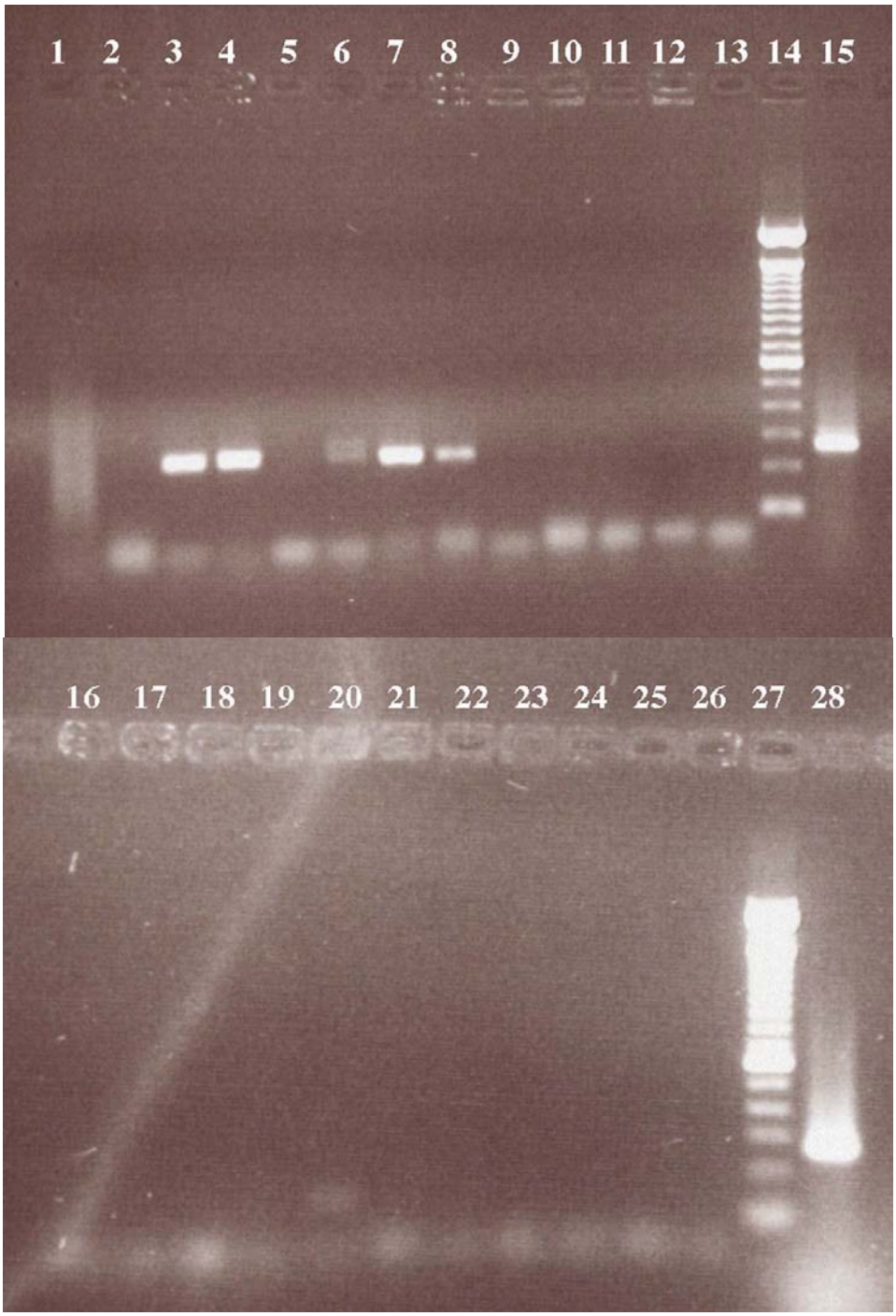


Figure 5.7. *Boxer dog monolayer post-inoculum and passage-1 samples PCR gel 1.* BE cell PI spine sample (lanes 1 & 2), BE cell PI brain sample (lanes 3 & 4), Vero cell PI spine sample (lanes 5 & 6), Vero cell PI brain sample (lanes 7 & 8), Vero brain culture 1 (lanes 9 & 10) Vero brain culture 2 (lanes 11 & 12), negative control (lane 13), 100 bp ladder (lane 14), Nc-Liverpool positive control (lane 15). Vero brain culture 3 (lanes 16 & 17), Vero spine culture 1 (lanes 18 & 19), Vero spine culture 1 (lanes 20 & 21) BE brain culture 3 (lanes 22 & 23), BE spine culture 1 (lanes 24 & 25), negative control (lane 26), 100 bp ladder (lane 27), Nc-Liverpool positive control (lane 28).

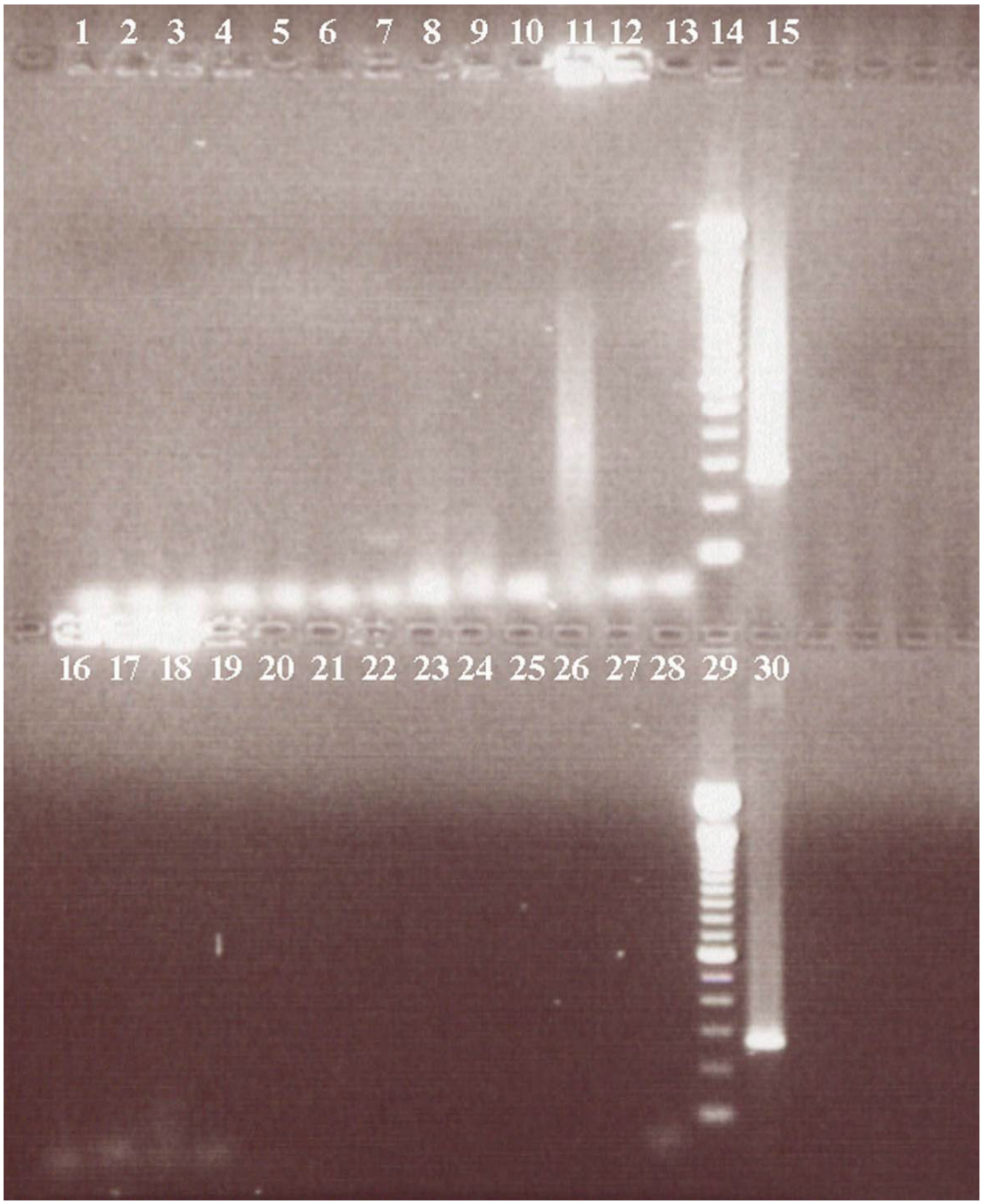


Figure 5.8. *Boxer dog Vero cell culture passage-1 and passage-2 and faecal floatation samples PCR gel photograph.* Vero brain culture-1 P2 (lanes 1 & 2) Vero brain culture-2 P2 (lanes 3 & 4), Vero brain culture-3 P2 (lanes 5 & 6) Vero spine culture-1 P2 (lanes 7 & 8), Vero spine culture-2 P2 (lanes 9 & 10), Vero brain culture-1 P1 (lanes 11 & 12) Vero brain culture-2 P1 (lanes 16 & 17), Vero brain culture-3 P1 (lanes 18 & 19), Faecal floatation sample (lanes 20-27), negative control (lanes 13 & 28), 100 bp ladder (lanes 14 & 29), Nc-Liverpool positive control (lanes 15 & 30).

Following the detection of bands from samples taken from passage-1 of the Vero cell culture flasks, all passage-1 samples of Vero cell cultures were re-tested using PCR-3 along with samples of Vero cell cultures taken during passage-2 (Figure 5.8.). A faint band was observed in lane 7 (Vero spine culture flask 1 passage-2) as it was observed in lane 20 of Figure 5.7 for the same sample, however the band is not of the correct size for *N. caninum*. A band similar to that seen in lane 7 may also be present in lane 18 (Vero brain culture flask 3 passage-1) but again it is not an *N. caninum* specific band. Significant streaking has occurred in lane 11 (Vero brain culture flask 1 passage-1), but no clear band can be observed, therefore the lane was considered negative. None of the oocyst faecal floatation samples produced a band of any kind. Additionally, genomic DNA bands cannot be seen indicating that very little, if any DNA was loaded as template for this sample, which is likely caused by the difficulty in separating oocysts during faecal flotation.

BALB/c mice that were inoculated with various volumes of purified Boxer brain tissue were examined for *N. caninum* DNA in the brain. No clear *N. caninum* positive bands were observed following PCR, however, a faint band of the correct size appeared to be present in lane 3 (0.3 ml inoculum) (Figure 5.9.).

Mouse brain samples that were inoculated onto cell culture did not result in positive cultures, no CPE or evidence of parasite infection was observed and PCR analysis of the cell cultures was negative (PCR gels not shown).

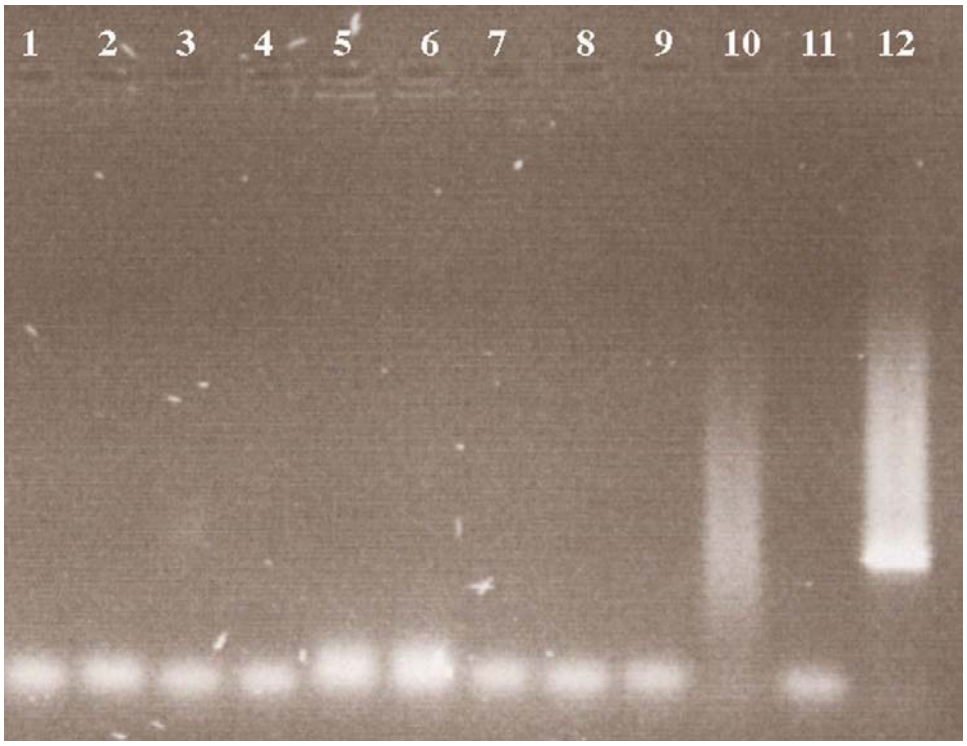


Figure 5.9. *Mouse brain PCR gel photograph following inoculation with Boxer dog samples.* Mice inoculated with 0.3 ml boxer brain (lanes 1-4), mice inoculated with 0.5 ml boxer brain (lanes 5-7), mice inoculated with 1 ml boxer brain (lanes 8-10), negative control (lane 11), Nc-Liverpool positive control (lane 12).

5.4 Discussion

Isolation and passage of a New Zealand strain of *N. caninum* was not successful. The parasite could be concentrated from tissue using the Percoll density gradient technique and parasite DNA could be detected but live tachyzoites could not be grown and passaged in culture.

Serological testing of cattle only shows exposure to the parasite and is not a guaranteed indicator that parasites are present in the tissue (Kyaw et al., 2005). Three bovine samples were discarded as serological testing and PCR were all negative. The animals in these cases were selected from seropositive dams or there was reason to suspect that *N. caninum* may have caused birth defects such as abnormal limb development. Samples were processed for culture before results from serology were available for these samples. Of the remaining 18 bovine samples arising from cows that were positive using the IFAT, 8 of the samples were negative using PCR and 10 samples produced PCR specific bands for *N. caninum* DNA. If these findings are correct, then it suggests that approximately 55% of seropositive dams produce seropositive calves that harbour *N. caninum* parasites in their CNS tissue. A relationship between rising antibody titres and a higher calf infection rate has been described and not all calves born from serologically positive dams were infected. The success of transplacental transmission was influenced by the gestational stage when the dam became infected (Gondim et al., 2004). Others have reported that histological examination for lesions is the most reliable diagnostic tool, as serological and PCR analysis produced much lower diagnostic capabilities and there was little agreement among results (Pereira-Bueno et al., 2003). Multiple diagnostic procedures should be used to determine the true infection status of animals (Innes et al., 2000).

Eight of ten bovine samples tested showed *N. caninum* parasite DNA in the brain, 5 in the spine and on three occasions in both the brain and the spine. Parasites are most frequently isolated from the brain of infected animals (Dubey et al., 2007). This is possibly because the brain is easier to access than the spinal cord, sterility is easier to maintain and the tissue is large and easy to process.

Animal tissues were originally processed on a work bench but the process was soon modified to use a biohazard cabinet following many contaminating infections in cell cultures and mice. Problems were also experienced with a laminar flow cabinet used for cell line maintenance and monolayer production. An air vent located in the room was found to be blowing fungi and other microbes directly into the hood, which contaminated and infected cell lines. Additionally, a bottle of FBS became contaminated. Contamination of the FBS solution was suspected to have arisen from the water bath used to heat-treat the FBS before use.

Although the FBS was heat treated, antibodies to *N. caninum* were still detected in the serum. Due to antibody detection in serum, isolation attempts were made using both FBS and equine serum as additives to culture media. FBS was shown not to affect Nc-Liverpool tachyzoites, but this may not be the case for bradyzoites newly isolated from tissues. Bradyzoites may have been more sensitive to the antibodies, which could have caused parasite death. Only the FBS was tested for specific antibodies, however horses can be infected with both *Neospora caninum* and *Neospora hughesi.* Antibodies against either or both *N. hughesi* and *N. caninum* are likely to be found in ES and could act against the parasites (Dubey and Porterfield, 1990; Dubey et al., 1999;

Pitel et al., 2003). Serum-free culturing techniques have been developed for use in *N. caninum* passage (De Meerschman et al., 2002).

Despite the detection of *N. caninum* DNA in 10 of the bovine tissues, the parasites could not be isolated and passaged in cell cultures. On one occasion parasites appeared to infect the host cells, but cultures were lost during their subsequent passage. Cultures were frequently checked for signs of parasitic growth and CPE development but no clear parasitic infections were observed. It was suspected that parasites were not converting from the bradyzoite to the tachyzoite stage. Isolated bradyzoites can be treated with a pepsin/HCl solution to induce stage conversion (McGuire et al., 1997). It is possible that the trypsin digestion may be inducing bradyzoite-tachyzoite stage conversion, but if it is not, then bradyzoites may not be infecting host cells or may not be actively growing in host cells.

Cell cultures were inoculated for a maximum of 3 hours. It was observed that leaving inoculum on cell cultures for periods longer than three hours frequently damaged the monolayers. Monolayers had to be washed thoroughly following inoculation and the media changed at least once within the first 24 hours. The periphery of the flask was often observed to have an absence of cells following monolayer inoculation, seen as a clear ring approximately 0.5-1 cm around the periphery of the flask. Cells growing at the corners/joints of the flasks or in the centre did not appear to be affected. Cell death occurred for up to 24 hours post-inoculation of the monolayer, despite the inoculum having been removed and the monolayer washed several times with PBS and fresh media added. The ringing effect may have been caused by toxins remaining from the isolation procedure or may have been caused by toxins released from the isolated cells, particularly if cell death was occurring. Lymphocytes were observed in purified tissue inoculum but the cause of the toxic effect was not ascertained. The ringing effect was of particular concern as in Nc-Liverpool infected flasks, CPE and parasite infection was first observed around the periphery of the flasks, in the same areas that the ringing effect was observed.

Capillary effect is the likely cause of the denser parasite population around the periphery of the Nc-Liverpool flasks. The same effect may have been occurring in experimental flasks and cells within that area were the most likely to be infected and are also the most likely to be lost due to ringing. This would greatly reduce the likelihood of maintaining the parasites in culture. Monolayer ringing was the reason that re-suspended monolayers were used during isolation attempts. The ringing effect was occasionally observed in resuspended monolayers but usually monolayers that had been resuspended and replated immediately prior to inoculation appeared healthier directly after inoculation. The number of cells that settled after re-suspension and inoculation appeared to be lower than the number originally disrupted. This could indicate that during the re-suspension, unhealthy and dead cells are lost and do not resettle or it could indicate that some cells are lost during the re-suspension due to toxicity or another unidentified cause. Whether parasites are able to infect cells in suspension is unknown. An inoculation study with an *N. caninum* isolate would need to be performed to determine this.

Inoculation of host cells should occur within a 3-hour window. Studies have shown that tachyzoites do not survive outside of cells for long and rapidly lose infectivity after 3 hours. Parasites have been shown to infect most cells within 1 hour (Hemphill, 1999; Hemphill et al., 1996; Hemphill et al., 2004). The loss of infectivity to cells during extracellular maintenance may have been a major factor in the inability to establish parasites in cell culture and maintain

them by passaging. Tissue processing was a lengthy process, frequently taking as long as 6 hours. Parasites may have been extracellular for as long as 3 hours before inoculation onto cell culture, by which time infectivity is significantly decreased and parasites may have been unable to convert from the bradyzoite life stage to the tachyzoite life stage.

Three samples from 28 white tailed deer that were serologically positive for *N. caninum* produced infection of interferon-gamma gene knockout mice, and of the three mouse isolates, only 1 could be passaged in cell culture. The average duration between passages was 100 days (Vianna et al., 2005). These findings suggest that not all isolates can be readily adapted to grow in cell culture (Dubey et al., 2007). During the current study, bovine samples came from three different farms. It is possible that one *N. caninum* strain was responsible for infecting the entire herd on each farm, therefore limiting the available strains to just three. On the occasion that parasites did appear to infect the host monolayer, the bovine sample was not from the serologically studied farm, which made up 17 of the 21 bovine samples tested. Unfortunately, the culture that was established was lost due to contaminating infection. Isolation in cell culture can only be achieved if other microbes do not contaminate the materials (Dubey et al., 2007). Infection of cultures could usually be avoided or mitigated during isolation attempts through the use of sterile technique in a biohazard containment hood and by using antibiotics and antimycotics.

Care was always taken to ensure that equipment used during isolations was thoroughly cleaned and where possible sterilised. A specific band saw blade was kept for preparing samples for this study. The tissues removed for isolation did not contact surfaces or tools that were not previously sterilised. Care was taken to make as few incisions as possible into the brain and spinal cord while in the PM room, as these may have resulted in unwanted infections. Antibiotics were utilized as early as possible. The tissues were placed directly into antibiotic PBS upon removal from the body. The antibiotics were used at double strength for transport and storage of tissues prior to entering the laboratory. Tissues were kept in the antibiotic/PBS solution until they were processed in a biohazard containment unit. After sample inoculation of the cells, the antibiotics were kept fresh within the media so as to kill any contamination. The dose of antibiotics within the media was not increased compared to normal levels for routine cell passage as it was not known whether increasing antibiotic levels would adversely affect parasites, particularly parasites which were not accustomed to growth with antibiotics in a cell culture environment.

Aborted foetal placenta is not recommended as a source of *N. caninum* for isolation. Not only are the parasites reportedly rare within the tissue but the likelihood of contamination is greatly increased (Piergili Fioretti et al., 2000). The tissue is prone to bacterial and fungal invasion, which is then transferred to cell culture. No cell culture flasks containing cotyledon isolations survived for more than a week before contaminant infection destroyed the cells.

Cells were encouraged to grow actively in the first 3 weeks following inoculation as parasites were expected to multiply more rapidly in healthier active cells. *Toxoplasma* has been found to grow best under active conditions (AgVax). If parasites could not be seen after 3 weeks, host cell growth activity was decreased by allowing the monolayer to grow denser and the media was changed less frequently.

Mice were monitored over two weeks PI for signs of illness and peritoneal exudate. Peritoneal exudate was not identified in any of the mouse samples. *N. caninum* does not produce peritoneal exudate in the same manner as *T. gondii* (Canada et al., 2002; Holmberg et al., 2006).

Approximately 2-5% of the mice died or were culled due to severe illness within the first 2-3 days after inoculation due to iatrogenic bacterial infection. Almost all other mice survived and showed no clinical signs of disease. Mice were kept for a minimum of two months, many for as long as nine months before being humanely killed with CO_2 gas. Of all the mice inoculated, only one mouse was found to be infected with *N. caninum*. Neosporosis was never identified in the mouse, only *N. caninum* DNA in the brain indicated the infection. Significant attempts to re-isolate the parasites from the mouse tissues failed to produce a viable culture. Parasites were not found in the culture after the first passage, suggesting that they were unable to infect the host cells. The same was true for mice inoculated with the dog samples that appeared to be positive for *N. caninum*, since parasites could not be recovered from the tissues and grown in culture. An interferon-gamma gene knockout mouse strain would have been the preferred mouse strain for use had it been available. Interferon gamma (IFN-γ) has been shown to inhibit intracellular multiplication of *N. caninum* (Innes et al., 1995), depleting IFN-γ renders mice susceptible to infection (Baszler et al., 1999; Dubey and Lindsay, 1996; Khan et al., 1997).

Two mice injected with the bobby calf sample died within a few days PI due to bacterial infections. One mouse appeared to have a lesion on its abdomen where the sample had been injected. *N. caninum* does not kill mice within such a short period indicating that mouse death was not due to neosporosis. One mouse from each of the brain and spinal inoculation groups died. The mice were young and small at the time of inoculation, which may have reduced their ability to survive an infection.

It was not possible to determine the source of contamination in the bobby calf isolate cell cultures. It is possible that the tissue forming the inoculum became contaminated during the dissection or tissue isolation steps. However, is it also possible that the Vero cell monolayer was contaminated with the gentamycin resistant bacterial strains as the secondary contamination also appeared within Nc-Liverpool and Vero cell cultures. The cell line may have become infected at some stage, possibly through contaminated serum and the infection undetected long enough for it to become gentamycin resistant.

The Boxer dog samples became available near the end of this study. Although parasites were clearly shown within the dogs tissue and could be purified with Percoll, the parasites were not able to infect the host cell lines. Parasite DNA was shown in the pre-inoculum sample and the post-inoculum sample but not in the cell culture samples. Mice inoculated with dog tissue did not develop signs of neosporosis. The reason for the inability to isolate the parasites is not known. Even if the animal had been dead for a longer period of time prior to isolation, viable parasites should still have been retrievable as others have retrieved parasites from animals that have been dead for some time (Vianna et al., 2005). The dog was being treated for the infection and it is possible that parasite viability was lost due to treatment prior to the isolation attempts.

Although oocysts were harvested from the faeces of the Boxer, confirmation of species was not established as the oocyst DNA did not amplify using PCR. Attempts to treat the oocysts to induce

them to sporolate were not effective and it was expected that the PCR technique was unable to penetrate the oocysts to access the DNA.

Others have shown that the detection of parasite DNA in the brain does not indicate that viable parasites are present. Gerbils and KO mice were inoculated with feral rat or mouse brain tissue. Although parasite DNA was detected in as many as 23% of mice and 70% of rat brains used for the inoculation, experimentally inoculated animals had a complete absence of clinical neosporosis or serological titres. It is suggested that the parasites in the brain tissue of the feral rodents was not viable but that intact DNA was present and that perhaps the time between animal death and isolation was too long, resulting in the loss of viable tissue cysts (Jenkins et al., 2007). These suggestions are reasonable explanations for the inability to isolate viable parasites from the dog and mouse tissues, as neither of these tissues could be processed immediately. The viability of the tissue cysts is likely to be dependent on the temperature of the tissues they are in prior to separation (Lindsay et al., 1992).

Serological screening of mouse serum for antibodies against *N. caninum* was unsuccessful. It is expected that the antigen was to blame as antigen production was not well developed and the antigen made on site did not produce consistent results. Time restraints restricted the development of this procedure further. The secondary antibodies were tested to determine whether they were at fault, but both the mouse and dog secondary antibodies were active. The primary antibody could not be altered as it was the subject of the study.

5.5 Conclusions

A viable New Zealand strain of *N. caninum* tachyzoites could not be isolated and passaged in cell culture. Parasite DNA was recovered from the tissues of naturally infected animals on 10 occasions, as shown by PCR but parasites could not be maintained in culture.

Only 1 mouse from approximately 300 mice inoculated with isolation attempts had detectable *N. caninum* DNA in the brain. Re-isolation from the tissue of this mouse was unsuccessful.

Low parasite viability following isolation is likely to be the cause of this inability to culture the parasite. It is likely that parasites were either unable to infect host cells or were dying within the culture following host cell penetration. The culturing procedure was shown to work well for Nc-Liverpool, which is a strain adapted to grow in cell culture but a new isolate may not survive and grow in the same culturing conditions.

PCR appears to be a reliable diagnostic tool for detecting parasite DNA. Immunohistochemistry was not a reliable technique for detecting tissue cysts or parasites in the brain but may have been useful for detecting parasites in lesions. Normal histological examination using H & E would probably suffice for detecting lesions but not parasite presence. The immunoblotting technique has previously been shown to be useful for detecting antibody exposure in mice but it proved to be inaccurate during the isolation inoculation studies. Further work is required to develop the immunoblotting technique, including the production of a reliable antigen.

Chapter 6.

6.0 General Discussion

At the time this study was conducted, *Neospora caninum* had not been isolated and cultured sustainably in New Zealand. This study represents the first concerted attempt in New Zealand to isolate *N. caninum* from naturally infected hosts in order to passage viable tachyzoites in cell culture or *in vivo*. *Neospora* infection in New Zealand cattle and dogs has been well established as being widespread, endemic and of considerable economic significance (Antony and Williamson, 2003; Reichel, 2000; Reichel and Drake, 1996; Thornton et al., 1991).

The study of *Neospora caninum* within New Zealand had been limited by the lack of a local isolate. Although a British isolate was imported to allow development of skills and to assist with laboratory studies, the strain could not be studied in large animal models or in any non-PC2 containment facility. The ultimate goal of this study was to propagate the organism and to develop an attenuated strain of *N. caninum* that could be used as a vaccine in the same manner as ToxoVax® is used for control of toxoplasmosis in sheep.

Characterisation of a New Zealand strain would also help to identify similarities and differences between strains. As New Zealand is an isolated island, differences in antigenicity, virulence and culturing requirements may have been observed between a local strain and other international strains. The broad host range and worldwide distribution of *N. caninum*, as well as its ability to reproduce both sexually and asexually, cause expectations that it has a large biological diversity and large genetic polymorphism (Regidor-Cerrillo et al., 2006). Murine challenges have identified differences in virulence of individual strains of *N. caninum* (Atkinson et al., 1999) as well as biological diversity that is recognised in different growth rates *in vitro* (Perez-Zaballos et al., 2005; Schock et al., 2001). Genetic differences between strains have been observed by randomly amplifying polymorphic DNA (Atkinson et al., 1999; Davison et al., 1999; Schock et al., 2001; Spencer et al., 2000), sequence analysis of rDNA internal transcribed spacer (ITS1) regions (Gondim et al., 2004) and multilocus microsatellite analysis (Regidor-Cerrillo et al., 2006). Multilocus analysis showed that the geographic origin, host and genetic similarity were not associated with the unique profiles observed for 9 different strains of *N. caninum* (Regidor-Cerrillo et al., 2006).

N. caninum has been reportedly maintained in culture for many years without losing virulence in mice (Dubey and Lindsay, 1996). The NC-1, NC-SweB1 and Nc-Liverpool strains all had increased fitness after 250 passages, indicating that *N. caninum* can adapt to new environments without sexual recombination and supporting the idea that the parasite has the capacity for clonal propagation in nature (Perez-Zaballos et al., 2005). Contradicting this study was another one suggesting that long-term passage of these parasites in tissue culture can lead to attenuation of virulence. Neosporosis was more severe in BALB/c mice inoculated with low-passage parasites and that high-passage parasites initially multiplied more rapidly but mouse survival time was

extended, suggesting that high-passage parasites had become more adapted to tissue culture resulting in a possible attenuation of virulence (Bartley et al., 2006). Attenuation of parasites is an important technique for establishing a new vaccine. ToxoVax® was developed by passaging *T. gondii* in mice over 3000 times until the parasite lost its ability to form tissue cysts (Bartley et al., 2006).

Isolation and diagnostic trends identified in this study were consistent with those reported for *N. caninum* by many other groups. Inability to passage parasites in culture is not uncommon. Many groups have processed as many as a 100 samples and have been able to maintain only 1 or 2 isolates (Conrad et al., 1993; Stenlund et al., 1997). It has been shown that not all isolates are able to adapt to cell culture (Vianna et al., 2005). *Neospora* is considered difficult to isolate for many reasons. Parasites are known to be scarce within the host tissue (Wouda et al., 1997), have low viability (Hemphill, 1999), be sensitive to temperature (Lindsay et al., 1992), lose infectivity within a short time (Hemphill, 1999), take a long time to establish in culture (Davison et al., 1999; Dubey et al., 1988b) and have variable virulence and infection rates in animal models (Dubey and Schares, 2006).

During the study reported here, sample tissues for isolation attempts were selected based on serological findings in animals from which the samples were obtained. Serological tests are unable to accurately diagnose infection but are able to indicate exposure to parasites (Dubey et al., 2007). Comparisons of serological procedures have highlighted some disagreement between different techniques and many can vary significantly depending on the operator, the cut off values, the standards applied and laboratory conditions (Hemphill, 1999; Wapenaar et al., 2007).

Although through PCR diagnosis, 55% of the seropositive samples chosen during this study were found to have detectable *N. caninum* DNA, no parasite DNA could be detected in almost half of the samples. Lack of detection using PCR does not conclusively exclude samples from being positive, as only a sample of the isolation product is tested. A negative finding merely indicates that the infection could not be detected at that time. Therefore it is conceivable that more samples may have been infected but it is also quite likely that around 50% of strongly seropositive cattle harbour parasite DNA (Canada et al., 2004; Garcia-Vazquez et al., 2005). Of 18 seropositive samples tested, 15 were from one herd and the remaining three from two separate herds. Eight of the 10 PCR positive samples were from the same herd and 1 each from the other two farms was also PCR positive. The bobby calf 99167 sample that was the most successful in culture did not come from the main study herd. Unfortunately however this culture was lost due to a contaminating infection of the cultures that most likely occurred during inoculation. This overwhelming secondary infection prompted future isolation attempts to be performed in a biohazard containment cabinet and extra care was taken to ensure sterile materials and technique was used, as well as high levels of antibiotic-antimycotic solution. Although the culture was not viable, parasites could be detected until at least 26 days PI. Secondary contamination was a significant problem during this study causing the loss of at least 1 early stage isolate. The main source of contamination was found to be a faulty ventilation system, which once tested was shown to be blowing microbes directly into the cell culturing hood causing cell line infection.

Several techniques were adopted to overcome the inability to passage parasites in culture. Following the identification of antibodies in foetal bovine serum (FBS), equine serum (ES, also known as horse serum (HS)) was used for experimental isolation techniques and for Nc-

Liverpool maintenance in culture (Hemphill, 1999). The equine serum was never tested for specific antibodies, therefore the presence of *N. caninum* antibodies in the serum cannot be ruled out. An extensive serum trial was performed to determine whether serum antibodies adversely effected Nc-Liverpool parasites. It was found that Nc-Liverpool was unaffected by FBS and in fact grew better in FBS than in ES. Whether or not this finding is applicable to a newly isolated strain of *N. caninum* that is not adapted to growing in culture is uncertain. Strains that have adapted to grow in cell culture are less likely to be affected by variations to or adverse culturing condition than new isolates, as not all new isolates can be adapted to grow in culture (Vianna et al., 2005). In addition to FBS and ES being used for cultures inoculated with tissues for isolation attempts, a Vero and a bovine endothelial cell line were also used (Dubey, 1999). The bovine endothelial cell line was introduced as it may have more closely resembled the cell types that the parasites were accustomed to growing in within the animals that they infected. Previous studies reported that when bovine endothelial cells were used for *Neospora* culturing a rapid proliferation of parasites and host cell lysis resulted (Hemphill, 1999). Numerous cell lines have been used to grow *N. caninum* in culture. Vero cells are most commonly used but *N. caninum* does not have a recognised cell line preference (Dubey and Schares, 2006; Lei et al., 2005).

As a result of observed damage to monolayers following inoculation, monolayers from both cell lines were also treated in two ways, half of the monolayers were left as adherent monolayers and inoculated directly and the other half were resuspended immediately prior to inoculation. Monolayers were inoculated for a maximum of 3-hours after which parasites lose their ability to infect cells. Parasites should have infected host cells within the first hour (Hemphill et al., 1996; Hemphill et al., 2004). The cause of the monolayer damage observed was never determined but additional wash steps did not eliminate the problem. Attempts were made to identify the causative agent. A significant number of lymphocytes were observed in the inoculation solution, however nothing could be concluded.

The culturing techniques adopted were considered appropriate for the culture and maintenance of *N. caninum* parasites as Nc-Liverpool was readily passaged under all described conditions. In addition to culturing conditions, the time between passages was also carefully considered. It was recommended by staff experienced in culturing *Toxoplasma gondii* tachyzoites at AgVax Developments Ltd that parasites should be rapidly passaged in order to establish cultures. To maintain healthy host cells, media was changed at least once within the first 24 hours PI, then again every 2-3 days. Cultures were passaged for the first time at approximately 5-7 days following culture inoculation. Cultures had their media changed and were passaged more frequently in the first 3-4 weeks following inoculation. After this time, media was changed less frequently and cultures were passaged every 7-10 days. Cultures were maintained for a minimum of 2 months and in many cases up to 4 months, as recommended when attempting isolations (Dubey and Schares, 2006). A limiting factor for culture passage was the number of flasks generated from each subsequent passage, therefore rapid passage could only be maintained for 3-4 weeks.

Once the number of cultures being maintained became unmanageable, cultures were harvested and cryopreserved according to the procedure established using Nc-Liverpool tachyzoites. Nc-Liverpool could successfully be cryopreserved and retrieved back into viable culture. Although it was not known of at the time of conducting this study, Trypan blue exclusion would have been a very useful technique to use during cell culturing and isolation procedures to determine parasite

viability. Using the trypan blue exclusion method, an optimal cryopreservation cell concentration should be determined for future studies.

It was shown during this study that Percoll and the purification conditions used for tissues were appropriate for retrieving parasites from both tissue and cell culture. Viable parasites could not be discriminated from non-viable parasites but Nc-Liverpool parasites inoculated back into cell culture following Percoll purification were able to infect the monolayer and cause CPE in a similar manner to parasites that had not been separated over a Percoll gradient. Therefore the tissue purification process was considered to be adequate for retrieving viable parasites.

Although Nc-Liverpool was not successfully re-isolated from mouse brain tissue and passaged in cell culture using the technique outlined in this study, *T. gondii* was re-isolated and passaged in culture. The inability to re-isolate Nc-Liverpool in culture was more likely due to few or no cysts being present in the mouse tissue used, rather than the procedure itself, as tissue cysts could not be found in the brains of mice inoculated with Nc-Liverpool using IHC. However, concerns were always held that *N. caninum* parasites were either not converting to tachyzoites in culture or that perhaps trypsin treatment was too harsh and was causing parasites to become non-viable even though trypsin is commonly used during parasite isolation (Hemphill et al., 2004; Stenlund et al., 1997).

T. gondii tachyzoites and bradyzoites can survive in 1% Trypsin for more than 2 hours although infectivity decreases after 1 hour. There are no quantitative reports of *N. caninum* survival in trypsin or pepsin solutions but *N. caninum* is considered to be more delicate than *T. gondii*. Reports by other researchers now suggest that trypsin should be used for no longer than 60 minutes at a concentration not exceeding 0.25% (Dubey and Schares, 2006). The 1% trypsin concentration used in this study was recommended by AgVax Development Ltd, as this is the concentration commonly used for *T. gondii* isolation.

During this study *T. gondii* could be reisolated from tissue using a 1% trypsin solution as found by Bjerkas et al., (1984) but *N. caninum* could not be. Tachyzoites are unable to survive in pepsin whereas bradyzoites can. If the cysts lyse early in the tissue isolation procedure and release bradyzoites that quickly convert to tachyzoites, then the tachyzoites may not survive in the trypsin treatment solution (Dubey and Schares, 2006). Whether or not the trypsin treatment caused loss of parasite viability during processing is purely speculation. Further work would be required to determine the optimal and lethal trypsin treatment times and concentrations. It is also possible that tissue cysts were not lysed and that whole cysts were inoculated onto cell culture, but tissue cysts are not infective to cell cultures (Dubey and Schares, 2006). Even so, it is recommended that for future attempts at isolating *N. caninum*, a lower concentration of trypsin should be used as well as a shorter incubation period.

Toxoplasma inoculated mice that had tissue cysts in the brain were used as IHC procedural positive controls and for cross-contamination studies. *Toxoplasma* cysts could be identified in brain tissue, validating the IHC procedure used and confirming that tissue cysts are present in the mouse tissue providing evidence that *T. gondii* inoculated mouse tissue was appropriate for use in other procedural validations that required the identification of parasites from whole tissue, such as tissue isolation and PCR. IHC failed to identify *N. caninum* in any tissue sample other than an historical dog sample that had previously been identified as positive for *N. caninum* using IHC

(Patitucci et al., 1997). All bovine isolation samples were checked using IHC as were many mouse brain samples, particularly those of Nc-Liverpool inoculated mice. It is believed that parasites persist as bradyzoites in cysts found in the tissue of adult cattle, although cysts have not yet been identified in histological sections of tissue from naturally infected adult cattle (Dubey et al., 2006). *N. caninum* has however been isolated from the brain of adult cows (Okeoma et al., 2004; Sawada et al., 2000).

Many researchers report that lesions are observed in the CNS and other tissues of *N. caninum* infected animals (Dubey et al., 1988a; Lindsay and Dubey, 1990; Okeoma et al., 2004). During this study sections were not screened for lesions, due to time and training limitations. The identification of lesions should be considered during histological examination of tissues suspected of being infected with *N. caninum*. Parasite cysts are usually rare within tissues and individual tachyzoites are very difficult to positively identify, therefore identification of lesions is a sensible indicator of *N. caninum* infection. Specific staining with antibodies would not be required for normal histological examination, which would significantly decrease the slide preparation time although positive identification of parasites can only be assured with IHC (Boger and Hattel, 2003; McAllister et al., 1996).

As almost none of the cultures inoculated with tissue in isolation attempts had detectable parasite DNA after the first passage, it is reasonable to assume that parasites were either unable to infect the host cells at the time of inoculation or were not surviving and multiplying within the host cell following inoculation, which may also have led to the death of the host cell. The case study of the Boxer dog highlights that significant parasite DNA was present in the tissue used to inoculate the cultures. The DNA was most likely derived from whole parasites but the inoculation material removed from the cultures after 3 hours still contained the parasite DNA, which presumably consisted of non-viable parasites. It is possible that the parasites were not viable prior to inoculation as the detection of parasites in cysts or by PCR is not an indication of viability. None of the mice inoculated with samples from the Boxer dog developed signs of neosporosis.

Out of approximately 300 mice inoculated with tissue samples, just 1 mouse had detectable *N. caninum* DNA using PCR. Only 1 bovine tissue inoculated into mice resulted in detectable *N. caninum* DNA in the brain of the mouse. The calf came from the main serological study herd. None of the mice inoculated with experimental isolations developed any signs of neosporosis, unlike the mice inoculated with Nc-Liverpool tachyzoites. The mice inoculated with the Nc-Liverpool isolate developed clinical signs of disease similar to that noted by other groups (Baszler et al., 2000; Lindsay and Dubey, 1989; Sawada et al., 1997).

In this study, clinical signs were only observed at high doses of approximately 1 million tachyzoites. At lower doses of $5x10^3$ or $5x10^4$ tachyzoites, clinical signs of neosporosis were not observed. BALB/c mice were significantly more susceptible to overwhelming neosporosis than CF1 mice. All BALB/c mice inoculated with a high concentration of tachyzoites either died or had to be humanely destroyed within 1 week of inoculation (PI), whereas very few of the CF1 mice developed severe neosporosis, many didn't develop any signs of infection and were able to survive until the final cull date. Despite being highly susceptible to high doses of *N. caninum* tachyzoites, BALB/c mice inoculated with low doses ($5x10^3$ or $5x10^4$) did not show any signs of disease. It is clear that the number of parasites used to inoculate mice has a significant impact on the severity of neosporosis as well as the strain of mice used. Susceptibility to neosporosis based

on inoculation dose and the mouse strains used have been well reported by other groups and the findings of this study are consistent with those previously described (Dubey and Lindsay, 1996).
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Appendix 3.3 – IHC Materials

PBS
Disodium Hydrogen Phosphate
Scientific Supplies Ltd
Cat # SSL
Sodium Chloride
Univar- AJAX Chemicals
5 kg
Cat# 7647-14-5

Na_2HPO_4 (anhyd)	4.54 g
KH_2PO_4	1.09 g
NaCl	36.00 g

Wax Pen
PAP PEN
Daido Songyo.co.ltd.
Japan
Xylene
Mobil Oil Ltd
ASCC – Australasia solvents and chemicals company
100% xylene
17 kg
Secondary Antibody
Anti-goat antibody
Goat immunoglobulins- Biotin
DAKO corporation
1 ml
Cat# E0466
DPX
DPX mountant for microscopy
BDH
500 ml
Cat# 360294H
Primary Antisera/Antibodies
VMRD Inc
Goat origin
1ml
Neospora caninum

Potassium Dihydrogen Orthophosphate
Unilab- AJAX Chemicals
500 g
Cat# G85
H.M.I
Heavy Metal Intensifier
1% Cobalt chloride
Cobaltous Chloride
BDH
500 g
Cat# 27790
1% Nickel Chloride
BDH
Mixture 1:1
Peroxidase Anti-mouse IgG
Vector
1 mg
Cat# PI-2000
DAB
3,3'-Diaminobenzidine
Sigma
1 g
Cat# D5637

SHP
Streptavidin- biotinylated- horseradish peroxidase complex
Amersham Life Science
2 ml
Cat# RPN1051
BSA
Bovine Serum Albumin
Sigma
50 g
Cat# A4503
Alcohol
Chemical Bulk store supply Massey University
Absolute ethanol.

Cat# 210-70-NC
Toxoplasma gondii
Cat# 210-70-TOXO

Appendix 3.4 – Immunohistochemistry Protocol

Using biotin-streptavidin detection system

1. Dewax sections and bring to water (2 x 6 min xylene through alcohol to water ~1-2 min each).
2. Place slides in an endogenous peroxidase wash for 30 min (3% hydrogen peroxide in PBS).
3. Equilibrate in phosphate buffered saline (PBS) 0.01M, pH 7.2 for 1min. Repeat.
4. Dry slide around section with paper tissue, be careful not to touch tissue section.
5. Encircle section with PAP pen to create a fluid barrier.
6. Block non-specific binding sites using 1% BSA in PBS for 5 min (200 mg of BSA in 20 ml PBS, do not swirl), ~5 drops per slide.
7. Drain BSA drops, NB do not drain BSA from negative control slide.
8. Sparingly apply primary antisera to the positive section. Antisera is diluted 2:1000 in 1 % BSA/PBS (2 ul in 1 ml). Incubate in a humidity chamber at room temperature for 1 hour.
9. Drain both sections and wash in 3 changes of PBS for 1 min each.
10. Thoroughly drain and dry slides around section but ensure the section does not dry out.
11. Apply 4-5 drops of 1:200 diluted biotinylated antigoat IgG to sample slides (5 ul in 1 ml BSA), except the negative control slides. Incubate in a humidity chamber at room temperature for 30 min.
12. Drain sections and wash in 3 changes of PBS 1 min each.
13. Thoroughly drain and dry slides around section.
14. Apply the biotin-streptavidin-peroxidase preformed complex (SHP), diluted to 1:200 (5 ul in 1 ml BSA). Incubate in a humidity chamber at room temperature for 15 min.
15. Drain sections and wash in 3 changes of PBS 1 min each.
16. Peroxidase histochemistry. React sections in 3,3 diaminobenzidine (DAB) solution at room temperature for ~1 ½ - 2 min, (4 mg DAB in 10 ml PBS). A heavy metal intensification (HMI) system is also included (1 drop of HMI (cobolt + nickel) in 10 ul hydrogen peroxide). NB wear gloves, DAB is carcinogenic.
17. Halt reaction by immersing slides in PBS, 3 changes of 1 min each.
18. Rinse in tapwater.
19. Counterstain step may be inserted here, for a nuclei stain, immerse for 30 sec in Mayers haemalum. Rinse in tapwater
20. Dehydrate in 70 % alcohol, 2 changes of absolute alcohol, and 2 changes of xylene (4-5 dunks).
21. Drain slides and dry carefully. Mount in DPX.

NB 1, at no stage should the samples be allowed to dry out completely.
2, add household bleach to the left over DAB solution, this will inactivate it.
3, specific sites of immunoreactivity will appear brown.

Appendix 3.5 – IHC Staining of *Neospora caninum* Tissue Cysts

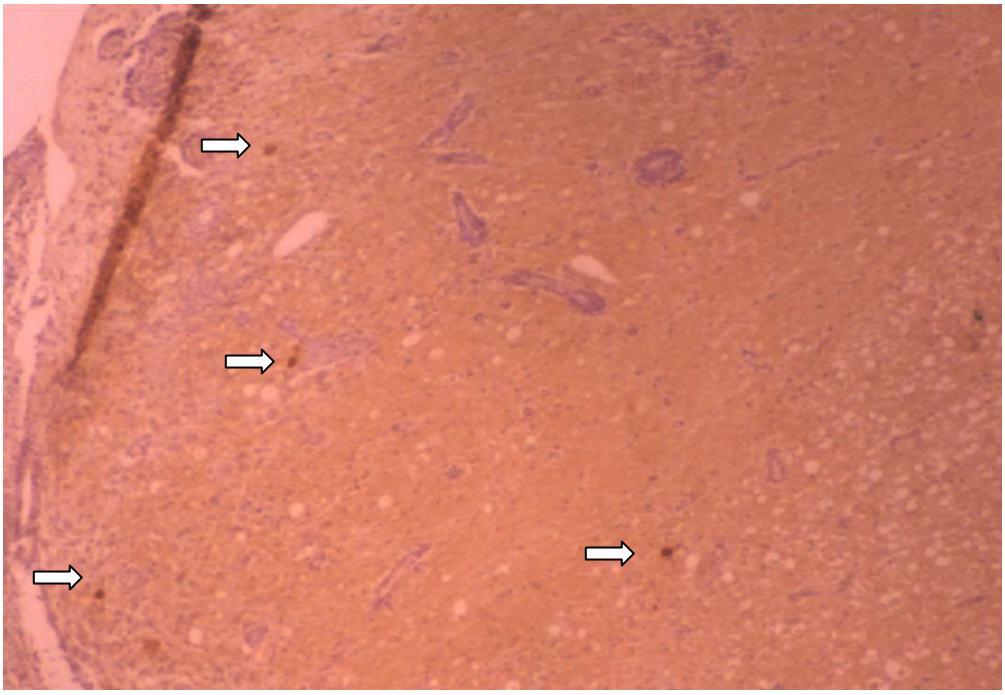


Figure A3.5.1. *N. caninum tissue section stained with N. caninum antibody for IHC.* Stained cysts appear as dark brown spots in the tissue section (arrows).

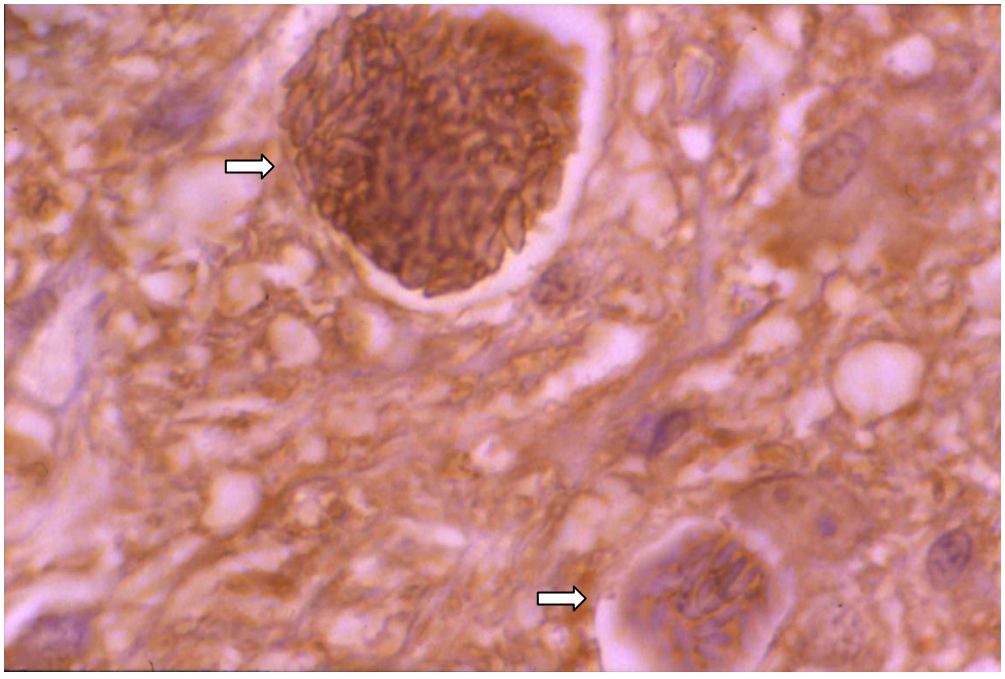


Figure A3.5.2. *N. caninum tissue section stained with N. caninum antibody for IHC.* Two stained cysts, individual tachyzoites can be observed within the cysts (1000x).

Appendix 3.6 - PCR Materials & Solutions

1% Agarose gel
1g agarose per 100 ml TAE buffer
High pure PCR Product Purification Kit
Boehringer Mannheim
Cat# 1732668
- for purification of PCR reaction products

50bp DNA ladder
GibcoBRL- LifeTechnologies
Cat# 10416-014
EDTA
GibcoBRL- LifeTechnologies
Cat# 15576028
Proteinase K
GibcoBRL- LifeTechnologies
Cat# 25530015
Chloroform
Sigma
Cat# C2432
PCR nucleotide mix
Promega
Cat# C114G
PCR Buffer
GibcoBRL- LifeTechnologies
Cat# 18067-017
Agarose gel
GibcoBRL- LifeTechnologies
Cat# 15510019
TBE buffer (10x)
108 g Tris base (890mM)
55 g Boric acid (890mM)
40 ml 0.5M EDTA, pH 8.0 (20nM)
Make up to 1000 ml with milli-Q water
Tris-acetate (TAE) buffer
(1x) = pH 8.0
0.04M Tris (Trisma) Acetate (acetic acid)
0.01M EDTA
(50x) = pH 8.0
242 g Tris base (2.0M)
57.1 ml glacial acetic acid
100ml 0.5M EDTA pH 8.0 (NaOH used for pH)

- make up to 800ml with milli-Q water,
- autoclave and store at 4°C.

Custom Primers
GibcoBRL- LifeTechnologies
DNeasy Tissue Kit
QIAGEN
Cat# 69504
- for DNA isolation from tissues, rodent tails, and cultured cells
100bp DNA ladder
Invitrogen
Cat# 15628-019
Tris-HCL
GibcoBRL- LifeTechnologies
Cat# 15504012
Phenol
Sigma
Cat# p4557
Taq DNA Polymerase
GibcoBRL- LifeTechnologies
Cat# 18038-018
Ethidium Bromide
BDH
Cat# 44392 2U
High DNA Mass Ladder
GibcoBRL- LifeTechnologies
Cat# 10496-016
DNAzole Reagent
GibcoBRL- LifeTechnologies
Cat# 598-84-0
TE buffer
(10x)
10mM Tris-Cl, pH 7.4, 7.5 or 8.0
1mM EDTA, pH 8.0

PCR loading dye
30% dH_2O
70% glycerol
0.1% w/v Bromophenol Blue (dark blue)
0.25% w/v xylene cyanol (optional) (light blue)
- Make 10-15 mls, aliquote out 500 ml samples into 1.7 ml microtubes for bench use.
- Store at 4°C or for long-term storage keep at -20°C

Appendix 3.7 – DNeasy Isolation Techniques

Procedure protocols described by QIAGEN DNeasy tissue kit handbook.

NB. The protocol described by QIAGEN was modified slightly for best results. Refer to the Handbook for the technique described by QIAGEN.

* refers to modified steps.

DNeasy Protocol for Animal Tissues

1. Up to 25 mg of tissue was homogenised in 180 µl of buffer ATL using a pestle in a 1.5 ml centrifuge tube*.
2. 20 µl of Proteinase K was added to the tube and mixed by vortexing 5-10 seconds. The tissue homogenate was incubated overnight at 55°C in a water bath to ensure complete lysis*.
   NB. Following incubation the lysate should appeared viscous with little tissue debris present.
3. Samples were vortexed for 15 seconds and 200 µl of Buffer AL was added to each sample. Samples were mixed thoroughly by vortexing and incubated at 70°C for 10 minutes.
4. 200 µl of absolute ethanol (96-100%) was added to each sample and the samples mixed thoroughly by vortexing.
5. The mixture from step 4 was pipetted into a DNeasy mini column placed into a 2 ml collection tube (provided with kit). Columns were centrifuged at 6000 x g (8000 rpm) for 1 minutes. The flow-through and collection tube were discarded.
6. The DNeasy mini column was placed into a new 2 ml collection tube (provided in kit) and 500 µl of buffer AW1 was added. Columns were centrifuged for 1 minute at 6000 x g (8000 rpm). The flow-through and collection tube were discarded.
7. The DNeasy mini column was placed into a 2 ml collection tube (provided in kit) and 500 µl of buffer AW2 was added. Columns were centrifuged for 3 minutes at full speed to dry the DNeasy membrane. The flow-through and collection tube were discarded.
8. The DNeasy mini columns were placed into a clean 1.5 ml microcentrifuge tube, and 200 µl of buffer AE was pipetted onto the DNeasy membrane. Columns were incubated at room temperature for 1 minute, then centrifuge for 1 minute at 6000 x g (8000 rpm) to elute the DNA (elution 1).
9. Elution was repeated again into a second 1.5 ml microcentrifuge tube (elution 2).
10. Elutions 1 & 2 were either combined or kept separate depending on the volume and DNA concentration required.

DNeasy protocol for Cultured Animal cells

1. A maximum of 5 x 10^6 cells per sample were centrifuged for 5 minutes at 2700 x g (3500 rpm). Cell pellets were resuspended in 200 ul PBS.
2. 20 µl of Proteinase K and 200 µl of Buffer AL were added to each sample. Samples were mixed thoroughly by vortexing and incubated at 70°C for 10 minutes.
3. 200 µl of absolute ethanol (96-100%) was added to each sample and mixed thoroughly by vortexing.
4. The mixture from step 3 (including any precipitate) was pipetted into a DNeasy spin column sitting in a 2 ml collection tube (provided in DNeasy Kit). Columns were centrifuged at 6000 x g (8000 rpm) for 1 minute. The flow-through and collection tube were discarded.
5. The DNeasy spin column was placed into a new 2 ml collection tube (provided in kit), and 500 µl of buffer AW1 was added. Columns were centrifuged for 1 minute at 6000 x g (8000 rpm). The flow-through and collection tube were discarded.
6. The DNeasy spin column was placed into a new 2 ml collection tube (provided in kit), and 500 µl of buffer AW2 was added. Columns were centrifuged for 3 minutes at full speed to dry the DNeasy membrane. The flow through and collection tube were discarded.
7. The DNeasy spin column was placed into a clean 1.5 ml microcentrifuge tube, and 200 µl of Buffer AE was added directly onto the DNeasy membrane. Columns were incubated at room temperature for 1 minute, then centrifuged for 1 minute at 6000 x g (8000 rpm) to elute the DNA (elution 1).
9. Elution was repeated again into a second 1.5 ml microcentrifuge tube (elution 2).
10. Elutions 1 & 2 were either combined or kept separate depending on the volume and DNA concentration required.

Appendix 3.8 – DNA Purification

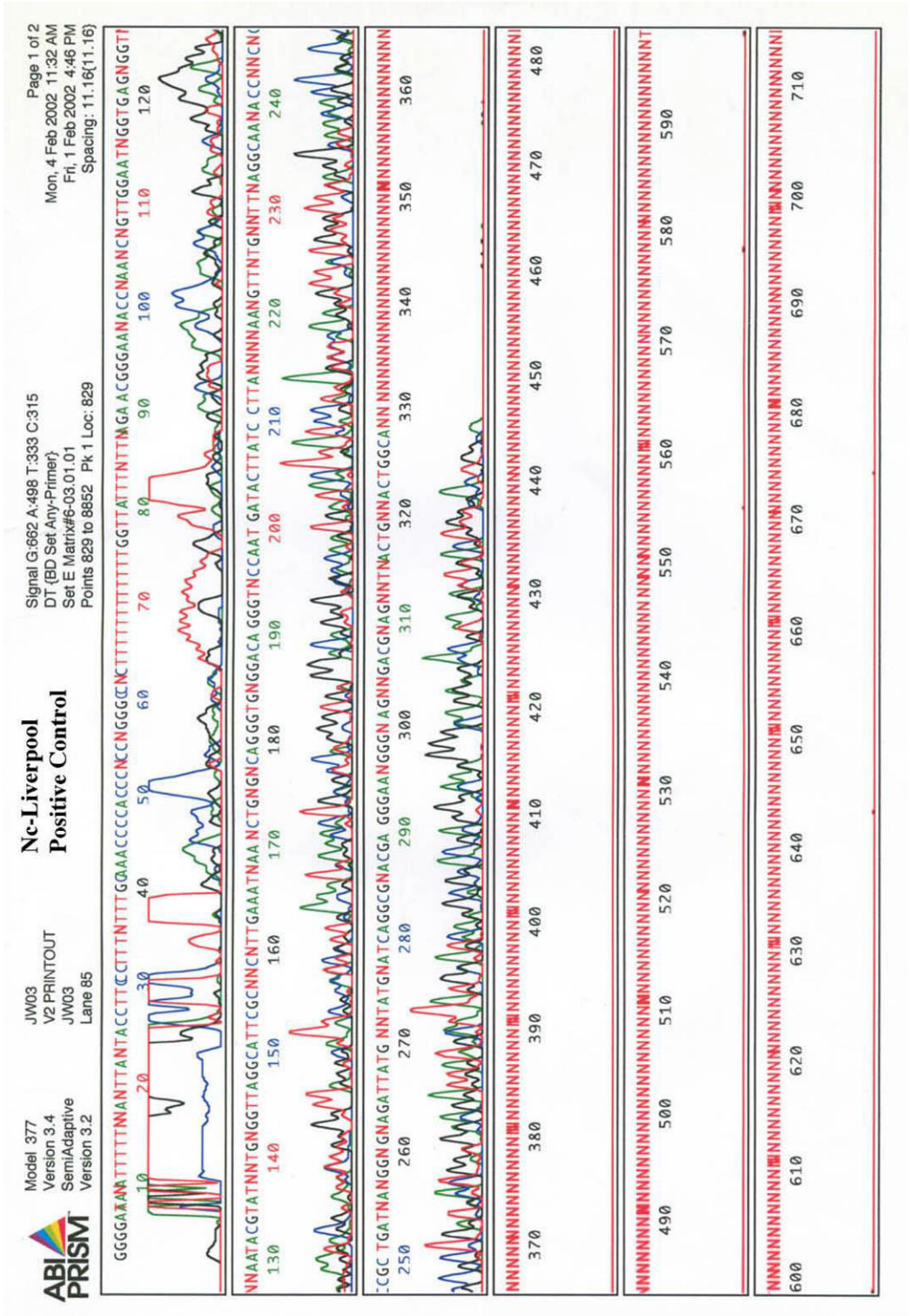
DNA Purification from Low Melt Agarose Gel Electrophoresis

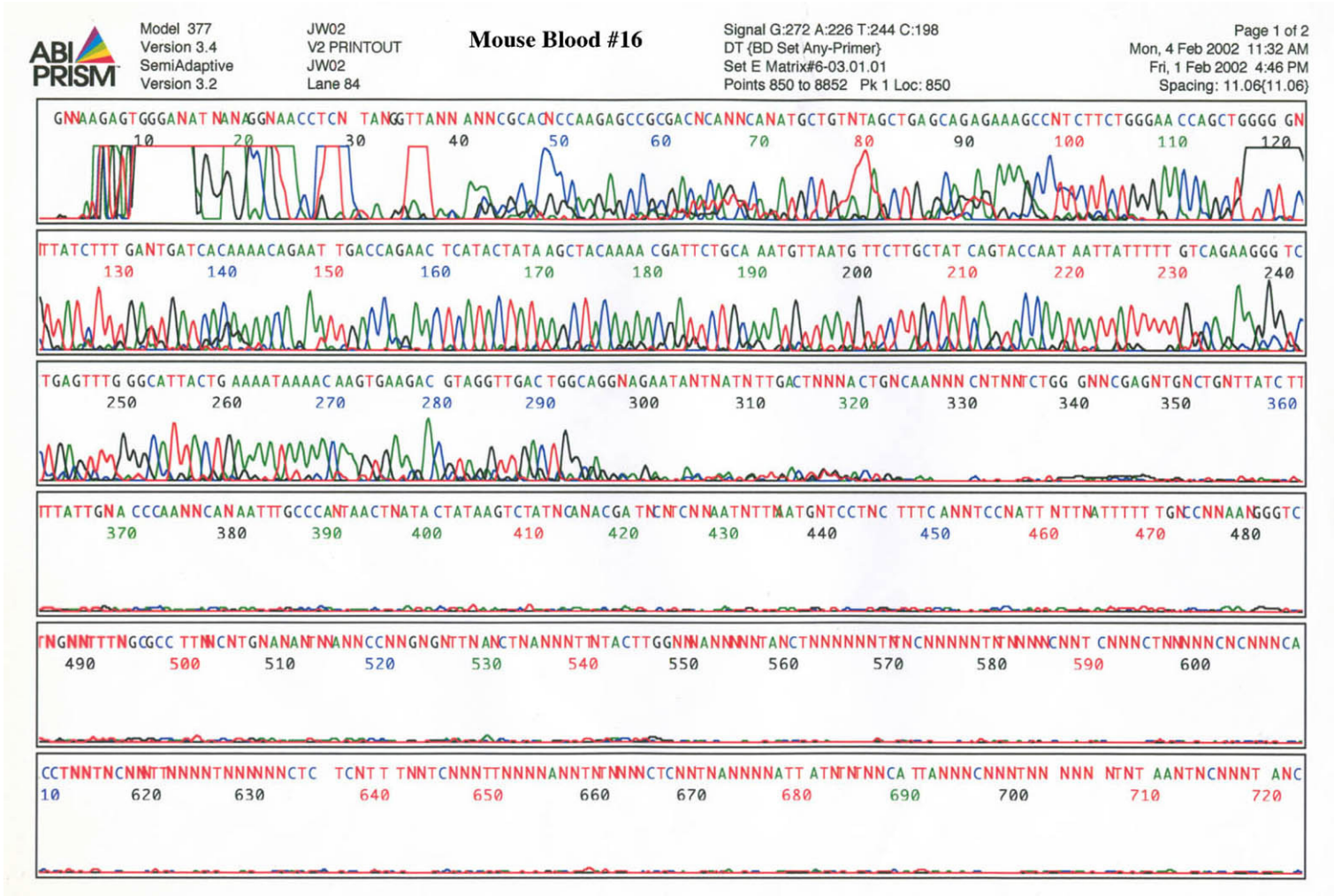
Purifying DNA from a 100 mg slice of agarose gel

1. A 1.0% low melt agarose gel made with Boehringer Mannheim agarose (MP, LE, MS, or LM-MP) was prepared and loaded with the DNA, a 1 kb plus DNA ladder and mass ladder.
2. The gel was run in 1 x (chilled) TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0) or 1 x TBE (45mM Tris-borate, 1mM EDTA, ph 8.0) running buffer at 90-100V for 40-60 min or until bands were clearly separated. Following gel electrophoresis, stain the gel with ethidium bromide.
3. DNA bands were cut from the gel with a sharp scalpel or razor blade. The smallest possible gel slice was removed to ensure high DNA to gel proportions.
4. The agarose gel slice was placed into a sterile 1.5 ml microfuge tube of known weight, then the tube reweigh to determine the weight of the agarose gel.
5. 300 µl Binding Buffer for every 100mg agarose gel was added to the microcentrifuge tube.
6. To dissolve the agarose gel slice and release the DNA: the microfuge tube was vortexed to resuspend the gel slice in the Binding Buffer and incubated for 10 min at 56°C, vortexing every 2-3 min during incubation.
7. Once the gel was completely dissolved: 150 µl of isopropanol for every100 mg of agarose gel was added to the tube and the tube vortexed thoroughly.
8. A High Pure™ Filter Tube was inserted into a Collection Tube (High Pure™ PCR Product Purification Kit. Roche- Cat # 1732 668) and the entire contents of the microcentrifuge tube was pipetted into the upper reservoir of the Filter Tube.
9. The entire High Pure™ Tube assembly was inserted into a standard tabletop microcentrifuge, and centrifuged for 30 sec at full speed (approx. 13,000 x g).
10. The Filter Tube from the Collection Tube was removed and the flow-through liquid in the Collection Tube was discarded.
11. The Filter Tube was re-inserted, 500 µl of Wash Buffer was added to the upper reservoir and the tube centrifuged for 30 sec at full speed.
12. The flow-through and collection tubes were discarded and the Filter Tube placed into a clean, sterile 1.5 ml microcentrifuge tube.
13. DNA was eluted from the Filter Tube by adding 50-100µl of Elution Buffer or distilled water (pH 8.0-8.5) to the upper reservoir and the tube centrifuged at full speed for 30 sec.
14. DNA contained in the microcentrifuge can be used in applications such as cloning or sequencing, stored at 4°C for later analysis or alternatively DNA can be concentrated by Ethanol/Glycogen precipitation (refer Chapter 3, PCR).

Protocol derived from the Boehringer Mennheim Nucleic High Pure™ PCR Purification Kit manual.

Appendix 3.9 – DNA Sequences

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Version 3.4
SemiAdaptive
Version 3.2
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V2 PRINTOUT
JW02
Lane 84
Mouse Blood #16
Signal G:272 A:226 T:244 C:198
DT {BD Set Any-Primer}
Set E Matrix#6-03.01.01
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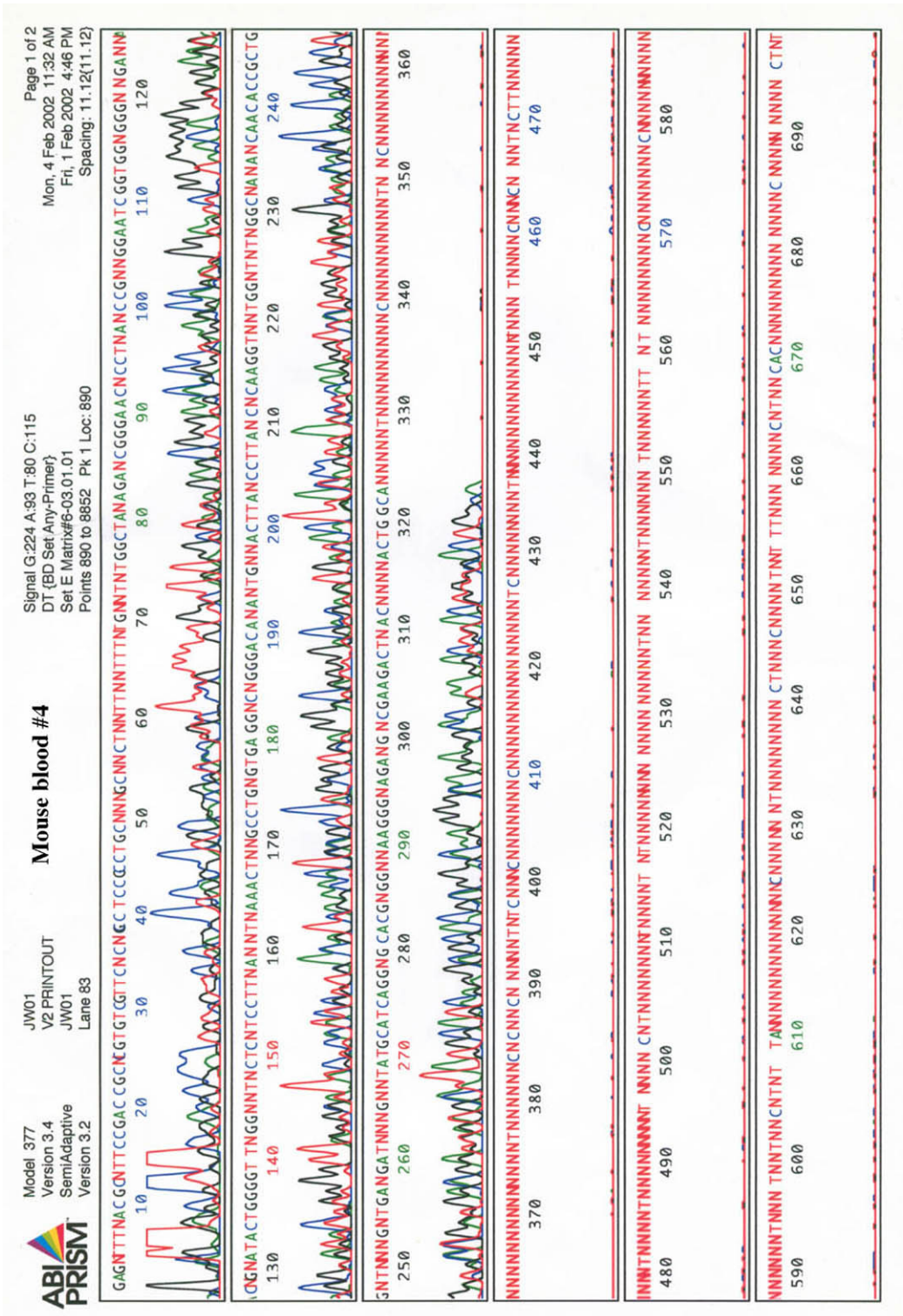
Page 1 of 2
Mon, 4 Feb 2002 11:32 AM
Fri, 1 Feb 2002 4:46 PM
Spacing: 11.06{11.06}


ABI PRISM
Model 377
Version 3.4
SemiAdaptive
Version 3.2
JW01
V2 PRINTOUT
JW01
Lane 83
Mouse blood #4
Signal G:224 A:93 T:80 C:115
DT (BD Set Any-Primer)
Set E Matrix#6-03.01.01
Points 890 to 8852 Pk 1 Loc: 890
Page 1 of 2
Mon, 4 Feb 2002 11:32 AM
Fri, 1 Feb 2002 4:46 PM
Spacing: 11.12(11.12)

Appendix 3.10 - Immunoblot Materials & Solutions

PVDF membrane
Biorad
10 sheets
Cat# 162-0175

Hybond-C pure Nitrocellulose membrane
Amersham
20 cm x 3 m roll
Cat# RPN203W

SuperSignal WestFemto
Pierce
20 ml kit
Cat# 34094

Peroxidase Anti-dog IgG
Sigma Biotech
1 ml
Cat# A6792

MagicMark Western Protein Standard
Invitrogen
250 µl
Cat# LC5600

2-mercaptoethanol (2ME)
Merck
50 ml
Cat# 1120060050

Peroxidase Anti-mouse IgG
Vector
1 mg
Cat# PI-2000

Guanidine HCL
Sigma
25 g
Cat# G3272

CL-Xposure film
Pierce
Cat# 34090

Kodak Biomax Film
Radiographic Supplies
Cat# 8701302

Lauryl Sulfate (SDS)
Sigma
100 g
Cat# L4509

Gel Dryer filter paper
Biorad
25 sheets
Cat# 165-0962

Tween20
Biorad
1 L
Cat# 161-0781

SDS Page MW standard
Biorad
200 µl
Cat# 161-0304

N. caninum -Negative control serum
Canine origin
VMRD Inc
1 ml
Cat# 211-N-NC-CAN

N. caninum –Positive control serum
Canine origin
VMRD Inc
1 ml
Cat# 211-P-NC-CAN

0.5M Tris-HCL (pH 6.8)
60.55 g Tris base
800 ml MilliQ water
- adjust pH then make to 1 L

1.5M Tris-HCL (pH 8.8)
181.65 g Tris base
800 ml MilliQ water
- adjust pH and make to 1 L

Silver Stain Plus
Biorad
Cat# 161-0049

Skim Milk Powder
Pam's Supermarket brand

10% SDS
10 g SDS
- make up to 100 ml with MilliQ water

Ponceau S stain (Red)
2 g Ponceau S
30 g TCA
dH_2O to 100 mls

1x SDS gel-loading buffer (20ml)
10% glycerol (2 ml of 100% stock)
2% SDS (4 ml of 10% stock)
0.1% bromophenol blue (200 µl 100% stock)
50 mM Tris-HCL pH 6.8 (2 ml of 0.5M stock)
50 mM DTT (1ml of 1M stock)
10.8 mls water

5x Tris Glycine Gel electrophoresis buffer (pH 8.3)
15.1 g Tris base
94 g glycine
900 ml deionised water
50 ml 10% SDS stock
- adjust pH and make to 1 L

10% Ammonium persulfate
Biorad
Cat# 161-0700
10 g Ammonium persulfate
- make up to 100 ml with MilliQ water

Transfer Buffer
24 mM Tris Base (5.82g Tris)
192 mM glycine (2.93g glycine)
20% methanol (200 mLs)
- adjust to pH 9-9.3. store at 4°C.

Stacking Gel (2ml)
1.208 mls water
0.25 mls 40% acrylamide
0.5 mls 0.5M Tris pH 6.8
20 µl 10% SDS
20 µl 10% Ammonium persulfate
2 µl TEMED
- Add TEMED and ammonium persulfate last to set gel

Resolving Gel (5ml)
1.773 ml water
1.875 mls 40% acrylamide
1.25 ml 1.5M Tris
50 µl 10% SDS
50 µl 10% ammonium persulfate
2 µl TEMED
- Add TEMED and ammonium persulfate last to set gel

0.1M glycine pH 3.0
0.7507 g Glycine
80 ml dH_2O
- adjust to pH 3.0, add dH_2O to make 100 ml

Western Blot Stripping Solution
62.5 mM Tris pH 6.7
2% SDS
100 mM B-Mercaptoethanol

PBS-TWEEN 0.5%
5 ml TWEEN
995 ml PBS

Blocking Solution
5 g Skim milk powder
Make up to 100 ml with PBS-TWEEN 0.5% solution

Appendix 3.11 – Silver Staining

Silver staining protocol derived from the Bio-Rad “Silver Stain Plus Kit”, Cat# 161-0049.

Silver staining containers were thoroughly cleaned with detergent and rinsed with 50% nitric acid and then deionised water. Gloves were worn at all times when handling gel or equipment and gels were not touched with metal objects. The gel was completely submerged at all times in the silver stain to ensure even development

The Development Accelerator solution was either made fresh or stored for short periods at 4°C (< 3 months). If the solution is not fresh the gel will go black.

1. Following gel electrophoresis, gels were placed into Fixative Enhancer Solution and gently agitated for 20 minutes.

2. The Fixative Enhancer Solution was decanted from the staining vessel and the gel rinsed in 400 ml of deionized distilled water for 10 min with gentle agitation. The water was decanted and the gel washed for a further 10 min in fresh water.

3. The Staining Solution was prepared within 5 minutes of use. 35 ml of deionised distilled water was laced into a large beaker and stirred with a plastic coated flea. 5ml of Silver Complex Solution, 5 ml of Reduction Moderator Solution and 5 ml of Image development Reagent were mixed in. Immediately before use, 50 ml of room temperature Development Accelerator Solution was added, mixed well and the mixture added to the gel in the staining vessel with gentle agitation.

4. Gels were stained for approximately 20 min.

5. Once the desired staining was reached, gels were placed into 5% acetic acid for 15 min to stop the reaction. Gels were then washed in deionised distilled water for 5 minutes and placed into a plastic bag for photocopying.

Appendix 4.1 – Mouse Health Chart (Example)

	Mouse	Mouse active	Coat ruffled	Hunched body	Hind-limb paralysis	Death	Weight gain (Weekly)	Comments	Signed
Day 1	1								
	2								
	3								
	4								
Day 2	1								
	2								
	3								
	4								
Day 3	1								
	2								
	3								
	4								
Day 4	1								
	2								
	3								
	4								
Day 5	1								
	2								
	3								
	4								
Day 6	1								
	2								
	3								
	4								
Day 7	1								
	2								
	3								
	4								

Appendix 4.2 – Re-isolation of Parasites from Mouse Tissue

Each mouse brain was treated separately using the following procedure.

Digestion buffer = 5g Trypsin 1:250 in 500ml sterile PBS

1. Isolation work was performed in a biohazard cabinet.
2. Brains were homogenized in digestion buffer using a pestle.
3. Homogenized brain the solution was poured into a sterile 15ml tube and digestion buffer added to a volume of 14 ml.
4. Tubes were mixed well and incubated for 1 hour at 37°C on a shaker.
5. Tubes were removed from incubation and centrifuged at 2700 x g (3400 rpm) for 10 min.
6. Supernatant was discarded and the pellet re-suspended into 7 ml of antibiotic PBS and mixed well.
7. A double layer of muslin cloth was used to filter the samples into a clean 15ml tube. The sample tube was rinsed with a further 3ml of antibiotic-PBS and put through the muslin filter. The muslin was discarded between samples and the funnel thoroughly cleaned.
8. Sample tubes were re-suspended to a volume of 14 ml and vortexed.
9. Samples were divided into 2x 7 ml samples (samples A and B), re-suspended to 10ml with antibiotic-PBS and centrifuged at 2700 x g (3400 rpm) for 10 min.
10. The supernatant was discarded and the pellet re-suspended in a further 14 ml of antibiotic-PBS.
11. Samples were mixed well and centrifuged at 2700 x g (3400 rpm) for 10 min.
14. The supernatant was discarded from all samples.

Sample A:

I. The pellet for sample A was re-suspended into antibiotic-2%FBS-MEM to a volume of 1 ml.
II. Sample A was used to inoculate 1x 75 cm^2 Vero monolayer flask and the volume topped up with 2% FBS-MEM to 35 ml.
III. The sample was left on the cell monolayer for 3 hours before being removed (and stored) and the monolayer washed several times with antibiotic PBS before having the antibiotic 2% FBS-MEM replaced to a volume of 35-40 ml.

Sample B:

I. The pellet for sample B was re-suspended in 30% Percoll in antibiotic PBS.
II. Sample B was mixed well and centrifuged at 2700 x g (3400 rpm) for 10 min.
III. The top layer of the density gradient (fats) was removed and discarded using a disposable 3ml pipette. The Percoll solution was carefully removed using a new pipette ensuring that the pellet was not disturbed.
IV. The pellet was then re-suspended to a volume of 1ml using antibiotic-2%FBS-MEM.
V. The sample was used to inoculate 1x 75^2 cm Vero monolayer flask and antibiotic 2% FBS-MEM was added to a volume of 35-40 ml.
VI. The sample was left on the cell monolayer for 3 hours before removal (the removed supernatant was stored at 4°C for diagnostic purposes.) The monolayer was washed several times with antibiotic PBS and the antibiotic 2% FBS-MEM was replaced to a volume of 35-40 ml.

Appendix 4.3 – PCR-2 results from Nc-Liverpool Trial-1

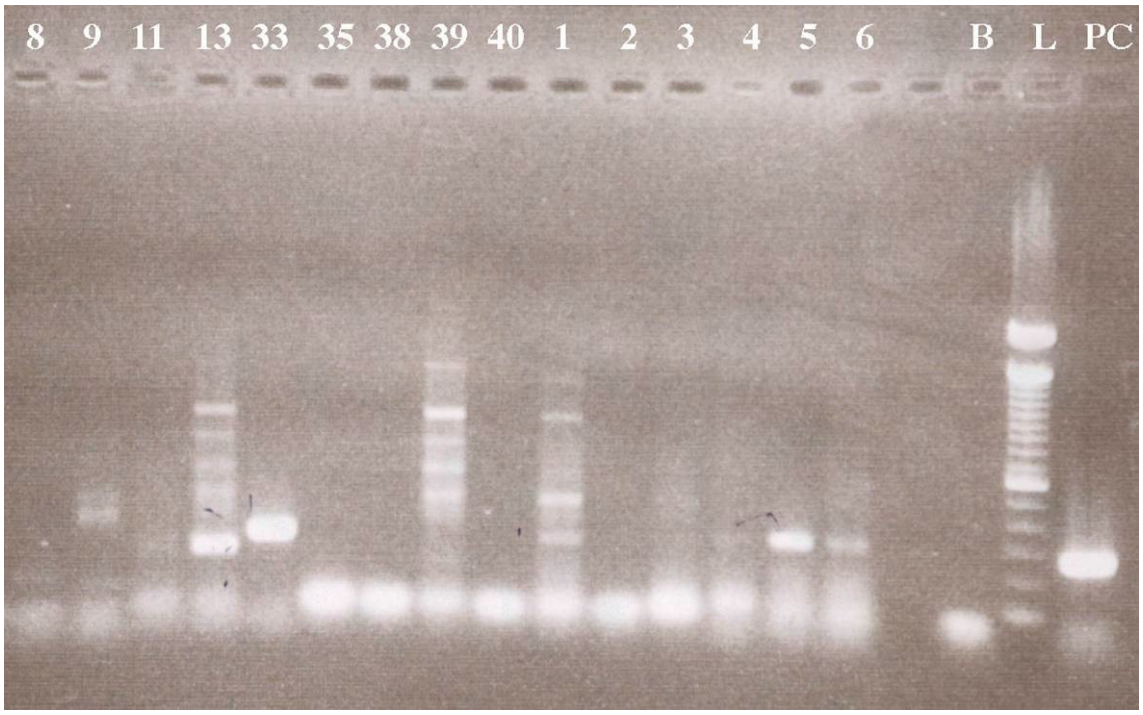


Figure A4.3.1. *PCR-2 gel of Nc-Liverpool Trial 1 mouse brain samples (gel 1)*

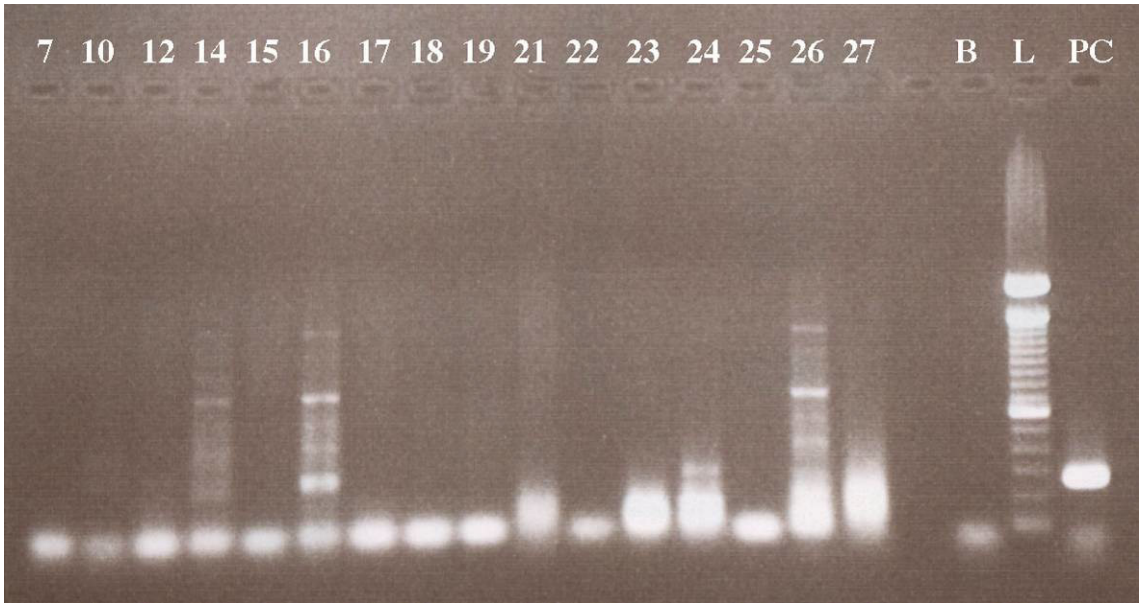


Figure A4.3.2. *PCR-2 gel of Nc-Liverpool Trial 1 mouse brain samples (gel 2)*

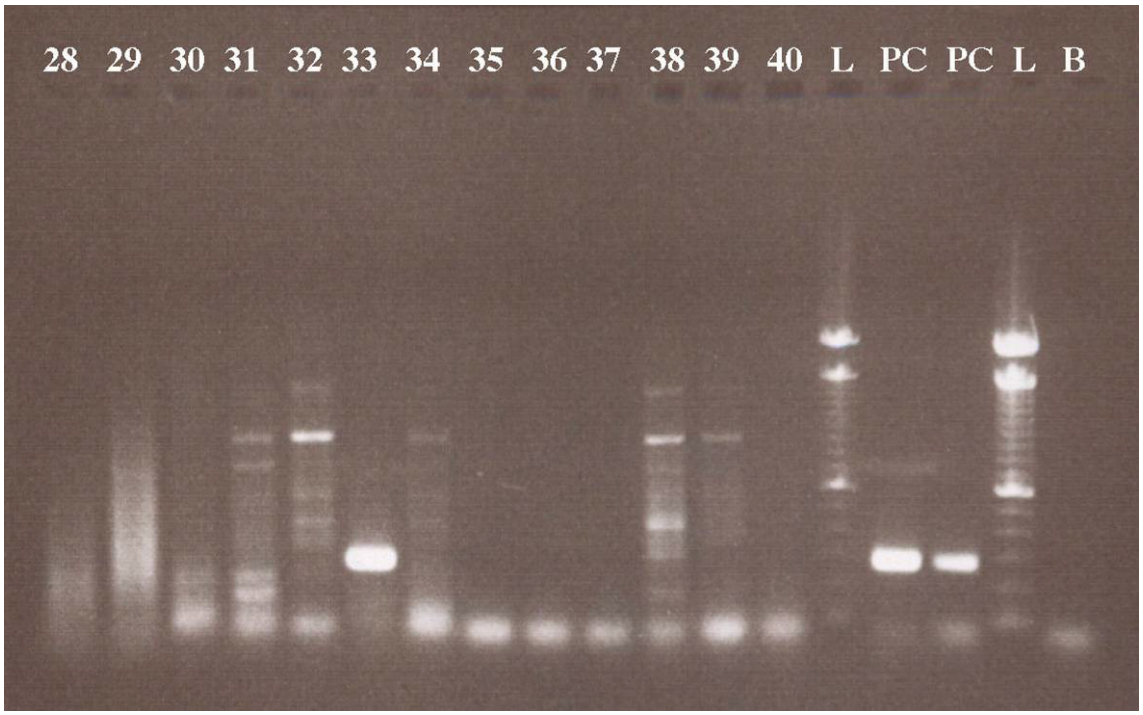


Figure A4.3.3. *PCR-2 gel of Nc-Liverpool Trial 1 mouse brain samples (gel 3)*

Table A4.3. *Sample and N. caninum detection results table*. (ns = no sample)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	ns
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

Appendix 5.1 – CNS Tissue Processing

Reagents:

a. 1% Trypsin Digest Mixture - 5 g Trypsin (1:250) in 500 mls sterile PBS. Digestion mixture should be warmed to 37°C to prevent shock to organisms.
b. Antibiotic wash solution - 5 ml antibiotic-antimycotic + 5 ml Gentamycin in 500 mls sterile PBS.

1. Tissues were processed in a biohazard hood to decrease secondary infection.
2. Tissues were rinsed 2-3 times in sterile antibiotic PBS.
3. Bone debris was cleaned away from the tissue. Blood vessels and membranes were removed from the tissue where possible and discarded.
4. Sections of the spinal cord and brain were removed and placed in 10% buffered formalin for use in IHC.
5. The brain and spinal cord were kept separate during processing.
6. The remaining brain was homogenised in a food processor with digestion mixture (1% trypsin in sterile PBS). Digestion mixture was added until the solution was fluid enough to be easily poured, approximately 200-300 ml for a calf brain. Tissues were homogenise until no chunks remained.
7. The homogenate was divided into 50 ml tubes, approximately 15-20 ml per tube and topped up to 50 ml with digestion mixture in order to reach a ratio of approximately 1:5 of brain to digestion mix.
8. The tubes were mixed well and left on a shaker in a 37°C incubation room for 1 hour.
9. Tubes were removed from incubation at 37°C and spun in a bucket centrifuge for 10 min at 2,700 x g (3,400 rpm).
10. The tubes were returned to the biohazard hood and the supernatant was tipped off. The samples were re-suspended in 20-25 ml of antibiotic PBS and vortexed.
11. The samples were filtered through a double layer of sterile muslin cloth. Two sample were combined and the tubes were topped up to 50 ml with antibiotic PBS. Producing half the number of original tubes.
12. The tubes were re-spun at 2,700 x g for 10 min.
13. In the biohazard hood, the supernatant was tipped off and the samples were re-suspended again in PBS to a volume of 50 ml and vortexed.
14. The samples were spun at 2,700 x g for 10 min.
15. Samples were returned to the Biohazard hood and the supernatant tipped off.
16. Samples were re-suspended in 30% Percoll solution in antibiotic PBS to a volume of 40 ml and vortex well.
17. The samples were spun again at 2,700 x g for 10 min.
18. The samples were returned to the Biohazard hood and a sterile syringe was used to remove the white layer of lipid cells present at the top of the tube, the remaining supernatant was carefully sucked off as close to the pellet as possible without disturbing it.
19. The pellet from all the tubes was re-suspend in antibiotic-MEM with either 2% or 10% FBS and pooled.

20. The pooled samples were spun at 2,700 x g for 10 min and the supernatant removed by gentle pipetting.
21. The pellet was re-suspended in 7 ml of antibiotic-MEM with either 2% or 10% serum supplement.
22. A 4 ml volume was removed so that a 0.25 ml volume of re-suspended sample could be used to inoculate each 25 cm^2 flask of 24 hour monolayer cells. Cell monolayers were prepared the previous day.
23. Eight Vero cell monolayers and eight bovine endothelial cell monolayers were used for inoculation. Four monolayers from each cell line were treated with ATV, cells were re-suspended and put back into their respective flasks.
24. The 4 monolayers and 4 re-suspended monolayers for each cell line were inoculated with 0.25 ml of isolation material.
25. The sample on the monolayers (not re-suspended monolayers) was left on the cell lines for 2-3 hours before being washed twice with antibiotic-PBS and fresh 2% serum supplemented MEM (growth media) added. Half of the flasks were resuspended into 2% foetal bovine serum (FBS) supplemented MEM and half into 2% equine or horse serum (HS or ES)
26. The re-suspended monolayers were left for 8-12 hours before being washed twice with antibiotic-PBS and fresh growth media added. Half supplemented with HS and half with FBS as described above.
27. All monolayers were washed twice and the media changed each day following infection for 3-5 days, beginning 12 hours PI. After which time the washing and media change was reduced to every 2-3 days.
28. Approximately 1 week post inoculation the monolayers were harvested using ATV and the cells put back into 25 cm^2 or 75 cm^2 flasks depending on the density of cells. If the cell layer was dense no extra cells were added. For monolayers where cells were not as dense fresh cells were added.
29. On the day of tissue processing, 2.5ml of the remaining isolated sample was used for inoculation in mice. BALB/c and CF1 mice were used. 0.3ml of sample was inoculated into each mouse via i.p injection. The mice were also given 2.5 mg (0.05ml) of MPA (Vetacortyl) to suppress their immune system. Four BALB/c and four CF1 mice were given brain sample and the same number was given spinal sample (if available). Therefore, 16 mice were inoculated from each isolation attempt.
30. The remaining 0.5 ml of sample was used for diagnostic purposes. A PCR was usually started on the same day as the tissue processing. The samples were left to cycle overnight in the PCR machine and run out on a 1-1.5% agarose gel the following day.

Appendix 5.2 – Isolations Mouse Immunoblot

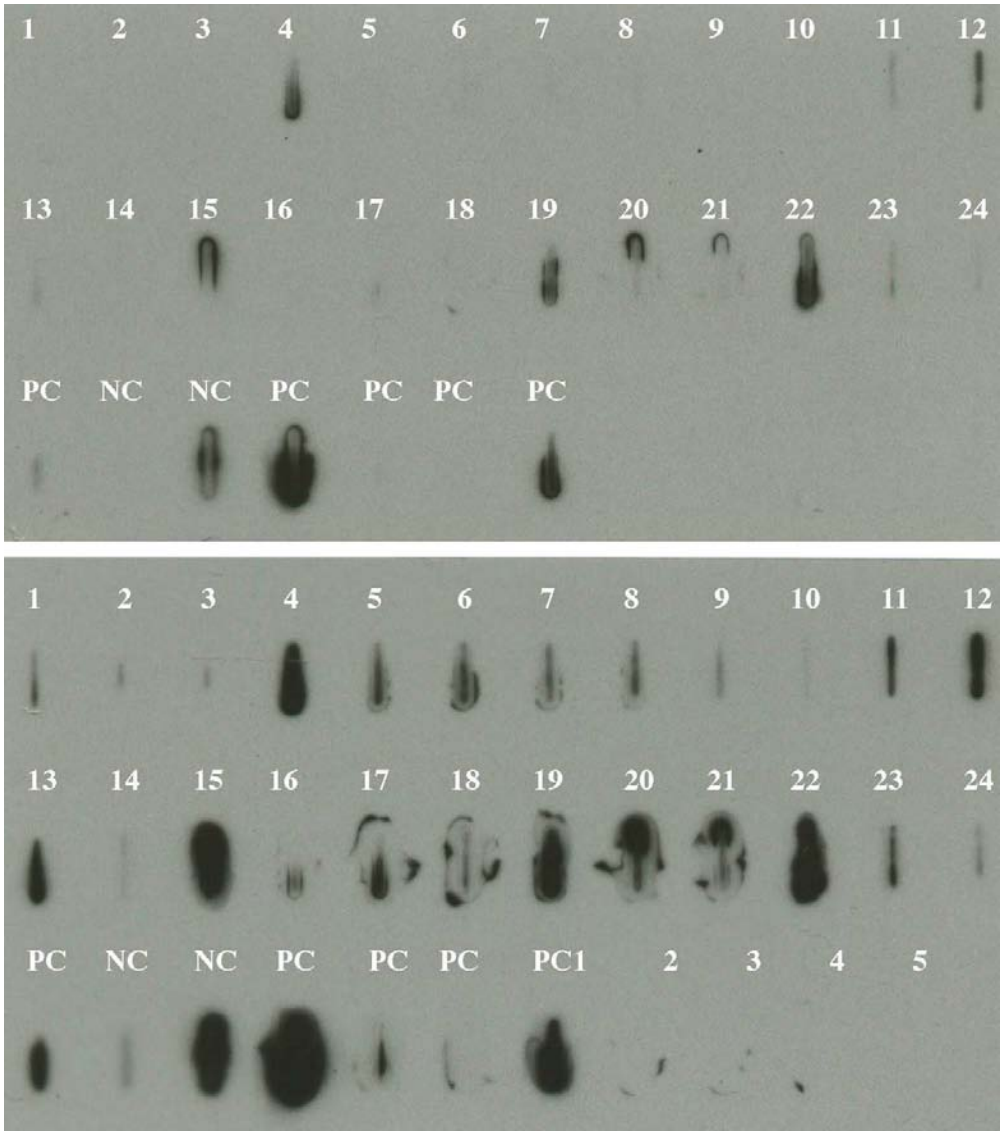


Figure A5.2. *Immunoblot X-ray of serum from mice experimentally inoculated with bovine isolate samples.* Immunoblots shown are the same except for exposure time to x-ray film. Blot 1 was exposed for 1 minute (top blot), blot 2 was exposed for 10 minutes (bottom blot). CF1- brain S7 (slot 1), CF1- brain S8 (slot 2), CF1- spine S8 (slot 3), CF1- spine S7 (slot 4), BALB/c- brain S7 (slot 5), BALB/c- brain S8 (slot 6), BALB/c- spine S7 (slot 7), BALB/c- spine S7 (slot 8), BALB/c- brain S13 (slot 9), CF1- brain S13 (slot 10), BALB/c- brain S12 (slot 11), CF1- brain S12 (slot 12), BALB/c- brain S14 (slot 13), CF1- brain S14 (slot 14), BALB/c- brain S10 (slot 15), CF1- brain S10 (slot 16), BALB/c- brain S9 (slot 17), CF1- brain S9 (slot 18), BALB/c- brain S19 (slot 19), BALB/c- brain 20 (slot 20), BALB/c- brain S21 (slot 21), BALB/c- brain S17 (slot 22), CF1- brain S17 (slot 23), BALB/c- brain S18 (slot 24), PC = positive control, NC = negative control. (Mouse strain – inoculum sample type, S refers to the bovine sample, followed by the sample number.)

Appendix 5.3 – Boxer Case study Supplementary PCR Gel

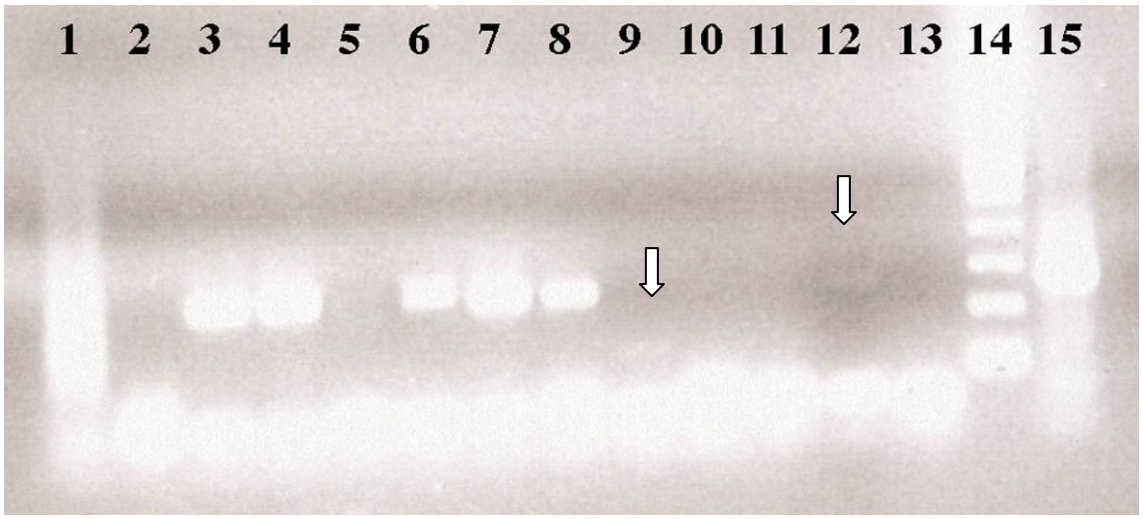


Figure A5.3. *Boxer dog monolayer post-inoculum and passage-1 samples PCR gel 1 high exposure supplementary image.* BE cell PI spine sample (lanes 1 & 2), BE cell PI brain sample (lanes 3 & 4), Vero cell PI spine sample (lanes 5 & 6), Vero cell PI brain sample (lanes 7 & 8), Vero brain culture 1 (lanes 9 & 10) Vero brain culture 2 (lanes 11 & 12), negative control/blank (lane 13), 100 bp ladder (lane 14), Nc-Liverpool positive control (lane 15).

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