# *Neospora caninum*: Studies toward isolation in New Zealand

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

Master
of
Veterinary Studies

At Massey University, Palmerston North,
New Zealand.

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**2008**

# 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.

# Acknowledgements

I would like to acknowledge and thank my supervisors, Prof. Norman Williamson and Dr William Pomroy for their guidance, assistance and unfailing support and patience.

I would also like to acknowledge and thank AgVax Developments and their staff for providing technical training and support throughout this study and for providing study materials and procedural assistance.

Furthermore I would like to thank Darelle Thomson and Chris DuPont for their assistance in molecular techniques, Sarah Hodges, Emily Smith and Renae Carr for assistance in the laboratory, Barbara Adlington for parasitology laboratory assistance, Mike Hogan for assisting with pathology, Mahmud Fathalla for his help with large animals, Jason Isaac and Barry Cooper for their help with small animal inoculations, the late Mervyn Birtles for immunohistochemical support and Andrea Coleman for computer assistance.

Thank you to the many individuals who have given so much of their time and knowledge to assist on this project throughout the years, your efforts were sincerely appreciated.

I wish to acknowledge the financial support for this thesis, which was provided by the New Zealand Foundation for Research Science and Technology, AgVax Developments and the New Zealand Dairy Board.

Finally, I would like to thank my brothers Paul and Mark, my partner Simon Jones, my friends, particularly Alison Ineson, and I would especially like to thank my parents, Dennis and Wendy for their assistance, guidance and their infinite patience and support.

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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 |