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**INVESTIGATIONS INTO THE CONTROL OF
NEOSPOROSIS IN CATTLE**

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

**Doctor of Philosophy
In
Veterinary Clinical Sciences**

**Massey University, Palmerston North
New Zealand**

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“It is a riddle,
wrapped in a mystery,
inside an enigma;
but perhaps there is a key.”

Winston Churchill
October, 1939

General Abstract

The research presented in this thesis was undertaken to further understanding of the control of neosporosis in cattle. A prospective cohort study of primiparous heifers on a farm with a history of *Neospora*-associated abortion found a 0.65 risk of abortion among seropositive heifers, suggesting that identification and culling of seropositive heifer replacements may be cost-effective.

A clinical trial of a registered *Neospora caninum* vaccine utilising 2,246 cattle from five farms with endemic *N. caninum* infection was assessed for efficacy in preventing abortion and vertical transmission. Overall vaccine efficacy was 0.25 ($p=0.12$) and vaccination increased the risk of vertical transmission. Histopathological and serological results from 148 cases of abortion from this trial were compiled to establish aetiological diagnoses. Nine of 34 cases where the fetus was examined had histopathological evidence of *N. caninum* infection. Histopathology revealed dual infectious aetiologies in 2 cases and serology suggested that, in another 17 cases, there had been recent exposure to a second infectious agent capable of causing abortion in conjunction with *N. caninum* lesions in the fetus or fetal bacteraemia.

As a prelude to cattle challenge trials, a challenge study conducted on pregnant sheep revealed a strong dose-response for abortion and that indirect fluorescent antibody test results did not correlate well with infection status or pregnancy outcome. A novel challenge method of applying tachyzoites to an abraded oral mucosa was undertaken in pregnant heifers to establish whether oral lesions could facilitate the direct horizontal transmission of *N. caninum* between cattle. One of eight heifers seroconverted, her calf and one other were seropositive when sampled within 12 hours of birth, and three other heifer-calf pairs had at least one positive polymerase chain reaction result at parturition. This method of transmission between cattle may be responsible for only a small proportion of infections but is a major new finding in the epidemiology of *N. caninum* infection and warrants further investigation.

Finally, inoculation with mouse-passaged *N. caninum* tachyzoites prior to mating did not prevent abortion when heifers were challenged again on Day 70 of gestation, suggesting that live inoculation may not be a suitable control option.

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Abbreviations

AI	Artificial insemination
BLAST	Basic local alignment search tool
BVDV	Bovine viral diarrhoea virus
CD	Cluster of differentiation system
CI	Confidence interval
CMI	Cell-mediated immunity
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAT	Direct agglutination test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleotide 5'-triphosphate
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
IB	Immunoblot
IBR	Infectious bovine rhinotracheitis
ICT	Immunochromatographic test
IFAT	Indirect fluorescent antibody test
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ISCOM	Immunostimulating complex
ITS	Internal transcribed spacer
IVABS	Institute of Veterinary, Animal and Biomedical Sciences (Massey University)
kDa	Kilodalton
<i>k/o</i>	<i>knock out</i>
MAT	Microscopic agglutination test
MEM	Minimum essential media

MLST	Multilocus sequence typing
MUAEC	Massey University Animal Ethics Committee
<i>N. caninum</i>	<i>Neospora caninum</i>
Nc-NZ	<i>N. caninum</i> New Zealand
NcSAG1	<i>N. caninum</i> surface antigen 1
NcSRS2	<i>N. caninum</i> surface antigen-1 related sequence 2
NK	Natural Killer
NPV	Negative predictive value
OD	Optical density
PAG	Pregnancy-associated glycoprotein
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Persistently infected
PP	Percent positivity
PPV	Positive predictive value
PSM	Planned start of mating
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RR	Relative risk
RT-PCR	Reverse transcription polymerase chain reaction
SAG	Surface antigen
SAS	Statistical analysis system
SD	Standard deviation
SDS	Sodium dodecyl sulphate
S/P ratio	Sample to positive ratio
TEM	Transmission electron microscopy
<i>T. gondii</i>	<i>Toxoplasma gondii</i>
Th	T cell helper
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VMRD	Veterinary Medical Research and Development
VNT	Virus neutralisation test

Preface

This thesis is presented as a series of papers which have been published (or submitted for publication). Consequently, there is some repetition, particularly in the Material & Methods sections. Different formats between journals mean that the format of chapters within this thesis will also vary. The references for each chapter have been collated at the end of this thesis in the Bibliography.

The literature review (Chapter Two) provides basic details of the life cycle, epidemiology, clinical consequences and immune response to *Neospora caninum* infection in cattle with a detailed review of the diagnosis of neosporosis in cattle and current recommendations for its control.

Chapter Three details the risk of abortion in primiparous Friesian-cross dairy heifers according to their serostatus for *N. caninum*. The cattle on the study farm had been sampled quarterly for the previous six years to measure antibody to *N. caninum* following an abortion outbreak in 1997. The relative risk for abortion among ELISA-positive heifers was 23.6 compared to the seronegative heifers and eleven of seventeen seropositive heifers aborted. These findings suggested that identification and culling of infected heifer replacements may be a cost-effective control option on farms with a history of abortion due to *N. caninum* infection.

In the same year as the prospective cohort study was being carried out, a killed vaccine containing *N. caninum* tachyzoites was licensed for use in New Zealand and several other countries. A large-scale clinical trial of this product was conducted over five commercial dairy farms in four regions of New Zealand that had a history of abortion associated with *N. caninum* infection (Chapter Four). A total of 2,246 cattle were enrolled in this trial of the first, commercially available vaccine with a claim to reduce the risk of abortion in cattle. The vaccine was found to have a significant effect on reducing abortion in one of five farms in this study suggesting that farm-specific effects need to be elucidated to identify farms which may benefit from such a product. Vaccination increased the risk of vertical transmission.

A thorough investigation was conducted on all cases of abortion from cows and heifers from the farms that were enrolled in the clinical trial. This consisted of histopathological examination of fetus and placenta as well as serological testing of the dam for antibody to *N. caninum* and bovine viral diarrhoea virus and compared to antibody status the time of at enrolment in the trial. A diagnosis of abortion due to *N. caninum* infection was made in nine of 34 cases of abortion where fetal histopathology was undertaken and on one of the five farms, there was no evidence that any of the 33 abortions were due to *N. caninum* infection. Although *N. caninum* was the most common cause of abortion on these farms, there were at least as many abortions that were associated with other infectious causes, or in which there was evidence of co-infection with other organisms. It is likely that the importance of *N. caninum* as a cause of abortion is over-estimated on some farms following the confirmation of its involvement in cases of abortion. Ongoing diagnostic efforts should be made to identify all causes of abortion on an affected farm as some may be able to be controlled and the removal of other immunological stressors may decrease the incidence of abortion in cows that are infected with *N. caninum*.

Pregnant sheep were used as a model for challenge prior to undertaking such studies in cattle due to their earlier onset of puberty, shorter gestation length and the lower cost. New Zealand isolates of *Neospora caninum* that had been kept in Vero cell culture at Massey University were used to produce the tachyzoite challenge in all subsequent studies. A strong dose-response relationship was observed with five of ten sheep aborting when challenged with 5×10^3 tachyzoites intravenously at 70-90 days gestation and all sheep aborting at higher doses. Additionally, it was found that there was little agreement between two different serological methods used to detect antibody to *N. caninum* in these animals which called into question the use of the indirect fluorescent antibody test on sheep sera.

A challenge study involving the inoculation of pregnant cattle with *Neospora caninum* tachyzoites on Day 70 of gestation was then conducted. Intravenous inoculation with 2×10^8 tachyzoites resulted in seroconversion in all eight heifers but only one heifer subsequently aborted, suggesting that the New Zealand isolates were of low virulence. A novel challenge model involving the application of tachyzoites to an abraded oral mucosa resulted in seroconversion in one of eight heifers, two calves from these heifers were seropositive and *N. caninum* DNA was found in samples from three other heifer-calf pairs in this group. These results were consistent with the hypothesis that aborting and

parturient cattle may be a source of infection for other cattle that become infected by direct horizontal transmission if tachyzoites gain entry to the bloodstream via lesions in the oral mucosa.

The final study involved a two-by-two trial design in which cattle were inoculated with either *N. caninum* tachyzoites that had been passaged through mice or with serum-free tissue culture media prior to mating. Pregnant heifers within each group were then randomly allocated to inoculation with either *N. caninum* tachyzoites or serum-free tissue culture media on Day 70 of gestation. Other authors had reported that pre-mating inoculation with *N. caninum* tachyzoites was protective against abortion when cattle were subsequently challenged. A challenge dose of 2×10^9 tachyzoites intravenously was chosen to increase the risk of abortion. Two of six heifers aborted that had previously been inoculated with *N. caninum* tachyzoites aborted when subsequently challenged as opposed to five of six heifers aborting that received a *N. caninum* tachyzoite challenge on Day 70 of gestation and had previously been inoculated with serum-free tissue culture media. This study casts doubt on the efficacy of pre-mating inoculation with live tachyzoites to prevent abortion due to *N. caninum*.

For each chapter my input was greatest. The preliminary ideas for some of the studies were a collaboration of several of the authors but I devised the details of the research, undertook the fieldwork, analysed the data and wrote the manuscripts. I was, however, assisted by my co-authors and a declaration of authors' contribution to each paper is included at the conclusion of each chapter that reports a published paper. Further assistance such as technical work and editorial input is reported in the Acknowledgements section of each chapter.

All experiments documented in this body of work were approved by the Massey University Animal Ethics Committee as documented within each chapter.