

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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.

Julie K. Walton
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 1.3×10^6 to 5×10^3 tachyzoites. Upon death, the brain and blood was harvested from the mouse carcasses.

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 1×10^6 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.

Table of Contents

ABSTRACT	I
ACKNOWLEDGEMENTS.....	V
TABLE OF CONTENTS.....	VI
LIST OF TABLES	XIII
LIST OF FIGURES	XIV
ABBREVIATIONS	XVII
 CHAPTER 1.	 1
1.0 Literature Review.....	1
1.1 The Veterinary Importance of <i>Neospora caninum</i>	1
1.2 Historical background	1
1.3 Discovery of <i>Neospora caninum</i> in dogs.....	1
1.4 The biology of <i>Neospora caninum</i>	2
1.4.1 Morphology and Ultrastructure	2
1.4.2 The Life Cycle	9
1.5 Neosporosis in Dogs	10
1.6 Neosporosis in Cattle	11
1.6.1 Transmission of infection	11
1.6.2 Vertical Transmission.....	11
1.6.3 Horizontal Transmission.....	12
1.7 Beef and Dairy Farm Seropositivity	13
1.8 Risk factors for Abortion	13
1.9 Host-Parasite relationship	14
1.9.1 Immunological responses to <i>N. caninum</i>	14
1.9.2 Pregnancy and <i>N. caninum</i> infection.....	15
1.10 Pathological lesions.....	16
1.11 Mouse models	16
1.12 Mouse pathogenesis	17
1.13 Animal models	18
1.14 <i>Neospora caninum</i> Isolation	20
1.14.1 In Vitro Cultivation	22

1.14.2 In Vivo isolation	23
1.15 Diagnosis of Neosporosis.....	25
1.15.1 Serological studies	25
1.15.1.1 Indirect Detection Techniques	25
1.15.1.2 <i>Neospora</i> Agglutination Test (NAT)	25
1.15.1.3 Indirect Fluorescent Antibody Test (IFAT)	26
1.15.1.4 Enzyme-Linked Immunosorbent Assay ELISA.....	26
1.15.1.5 Rapid Immunochromatographic test (RIT)	27
1.15.1.6 Immunoblot	28
1.15.2 Direct Detection.....	29
1.15.2.1 Pathological lesions.....	29
1.15.2.2 Transmission electron microscopy (TEM)	29
1.15.2.3 Hematoxylin and eosin (H&E) sections.....	29
1.15.2.4 Immunohistochemistry (IHC)	29
1.15.2.5 Polymerase Chain Reaction (PCR)	30
1.16 Neosporosis in New Zealand	35
1.17 Economic Impact of Neosporosis	35
1.18 Control Measures	36
1.19 Vaccine development	36
CHAPTER 2.	39
2.0 Cell Culturing Techniques for <i>Toxoplasma gondii</i> and <i>Neospora caninum</i> Tachyzoite Propagation.....	39
2.1 Introduction.....	39
2.2 Materials and Methods.....	41
2.2.1 Host Cells Used for Cell Culture.....	41
2.2.2 Passage of Host Cells	41
2.2.3 Growth Media and Supplements Used for Culture	42
2.2.4 Growth Conditions for Cells and Parasites.....	42
2.2.5 Cytopathic Effect (CPE).....	43
2.2.6 Passage of <i>Toxoplasma gondii</i> for Technique Development	43
2.2.7 Passage of <i>Neospora caninum</i> Tachyzoites.....	43
2.2.8 FBS Evaluation.....	43
2.2.9 Serum Trial	44
2.2.10 Percoll Density Gradient Separation of Tachyzoites.....	44
2.2.11 Cryopreservation of Tachyzoites.....	44
2.2.12 Cryopreservation Trial.....	45
2.3 Results.....	45
2.3.1 Culturing of Host Cells and Tachyzoites.....	45

2.3.2 Percoll Trial	48
2.3.3 Serological Status of Serum	49
2.3.4 Serum Type Trial.....	50
2.3.5 Cryopreservation	52
2.4 Discussion	52
2.5 Conclusions.....	55
CHAPTER 3.	56
3.0 Diagnostic techniques for the identification of <i>Neospora caninum</i> infection from tissue, cell culture and blood	56
3.1 Introduction.....	56
3.2 Immunohistochemistry (IHC)	59
3.2.1 General Description.....	59
3.2.2.1 Enzymology	59
3.2.2 Materials and Methods	60
3.2.2.1 IHC Control Samples	60
3.2.2.2 Tissue processing for IHC.....	60
3.2.2.3 IHC Primary and Secondary Antibodies.....	60
3.2.2.4 Standard IHC staining procedure	61
3.2.2.5 IHC Reaction Identification	61
3.2.2.6 Antibody Titre	61
3.2.2.7 Antibody Incubation Experiment.....	62
3.2.2.8 Proteolytic Digestion of Sample Tissue.....	62
3.2.3 Results	62
3.2.3.1 Staining Observations	62
3.2.3.2 Antibody Titres	63
3.2.3.2.1 <i>T. gondii</i> positive tissue sample incubation with various antibody titres	63
3.2.3.3 Incubation conditions.....	64
3.2.3.3.1 <i>N. caninum</i> tissue sections incubated with <i>T. gondii</i> antisera	64
3.2.3.3.2 <i>N. caninum</i> tissue sections incubated with <i>N. caninum</i> antisera	66
3.2.3.3.3 <i>T. gondii</i> tissue section incubated with <i>N. caninum</i> antisera.....	67
3.2.3.3.4 <i>T. gondii</i> tissue sections incubated with <i>T. gondii</i> antisera	68
3.2.3.4 Proteolytic digestion	70
3.2.4 Discussion.....	73
3.2.5 Conclusions	76
3.3 Polymerase Chain Reaction (PCR)	77
3.3.1 General Description	77
3.3.1.1 PCR Primer Background.....	77
3.3.2 Materials and Methods	78

3.3.2.1	Primers	78
3.3.2.2	Reconstitution and Quantification of Primers	78
3.3.2.3	PCR Reaction Mixtures and Running Procedures	78
3.3.2.3.1	PCR-1	79
3.3.2.3.2	PCR-2	79
3.3.2.3.3	PCR-3	80
3.3.2.4	PCR Product Separation and Visualization	80
3.3.2.5	Crude Cell Samples	81
3.3.2.5.1	Sample Water-bath Boiling	81
3.3.2.5.2	Sample Microwave Boiling	81
3.3.2.6	DNeasy DNA Isolation	81
3.3.2.6.1	DNeasy Protocol for Animal Tissues	82
3.3.2.6.2	DNeasy Protocol for Cultured Cells	82
3.3.2.7	DNA Purification	82
3.3.2.8	Ethanol/Glycogen DNA Precipitation	82
3.3.2.9	DNA Sequencing	82
3.3.2.10	PCR-1 Validation	83
3.3.2.11	PCR-2 Sensitivity Trial	83
3.3.2.12	PCR-3 Development	83
3.3.2.13	DNeasy elution trial	83
3.3.2.14	PCR template preparation trial	84
3.3.2.15	PCR-2 Optimisation	85
3.3.3	Results	86
3.3.3.1	PCR-1 Validation Results	86
3.3.3.2	PCR-2 Sensitivity Validation Results	87
3.3.3.3	DNeasy Elution Results	88
3.3.3.4	PCR Template Preparation Results	89
3.3.3.5	PCR-2 Optimisation Results	91
3.3.3.6	PCR-3 Development	91
3.3.3.7	DNA Sequence Results	91
3.3.4	Discussion	92
3.3.5	Conclusions	96
3.4	Immunoblotting	97
3.4.1	General Description	97
3.4.2	Materials and Methods	97
3.4.2.1	Antigens	97
3.4.2.2	Primary Antibodies	97
3.4.2.3	Secondary Antibody	98
3.4.2.4	Western Blot Gels	98
3.4.2.5	Antigen Manufacture	98

3.4.2.6	Antigen Preparation	98
3.4.2.7	Running Western Blot Gel	99
3.4.2.8	Silver Staining of Western Blot Gel.....	99
3.4.2.9	Western blot Gel to Membrane Transfer	99
3.4.2.10	Ponceau S Stain.....	99
3.4.2.11	Dot-Blot Procedure	100
3.4.2.12	Immunoblot and Development Procedure	101
3.4.2.13	Membrane Stripping.....	101
3.4.2.14	Western Blot Antigen Validation.....	101
3.4.2.15	BSA Standard.....	101
3.4.2.16	Dot-Blot Antibody Optimisation.....	102
3.4.3	Results	103
3.4.3.1	Western Blot Antigen Treatment	103
3.4.3.2	BSA Standard Results	104
3.4.3.3	Dot-Blot Antibody Optimisation.....	105
3.4.4	Discussion.....	107
3.4.5	Conclusions	110
CHAPTER 4.		111
4.0	Small Animal Challenge	111
4.1	Introduction	111
4.2	Materials and Methods.....	112
4.2.1	Mouse Strains	112
4.2.2	Mouse Immunosuppression.....	112
4.2.3	Experimental Inoculation	112
4.2.4	Humane Destruction of Mice	113
4.2.5	Mouse Post-Mortem Dissection	113
4.2.6	Mouse Blood Processing	113
4.2.7	Mouse Tissue Processing.....	113
4.2.8	Mouse Brain Smears.....	114
4.2.9	Mouse Brain Histology.....	114
4.2.10	Nc-Liverpool Trial-1	114
4.2.11	Nc-Liverpool Trial-2	116
4.2.12	Nc-Liverpool Infection and Re-Isolation Trial.....	116
4.3	Results	117
4.3.1	Nc-Liverpool Trial-1 Results.....	117
4.3.1.1	Nc-Liverpool Trial-1: Immunoblotting Results.....	119
4.3.1.2	Nc-Liverpool Trial-1: Immunohistochemistry Results.....	121
4.3.1.3	Nc-Liverpool Trail-1: PCR Results	121

4.3.2	Inoculation and Re-Isolation Results.....	123
4.4	Discussion	126
4.5	Conclusions	130
CHAPTER 5.		131
5.0	Attempts to Isolate <i>Neospora caninum</i> from Naturally Infected Animals.....	131
5.1	Introduction.....	131
5.2	Materials and Methods.....	132
5.2.1	Bovine Tissue Selection	132
5.2.2	Foetal Tissue Selection.....	132
5.2.3	Dog Tissue Selection	133
5.2.4	Bovine Processing and Tissue Removal.....	133
5.2.5	Foetal Tissue Processing	134
5.2.6	Dog Processing and Tissue Removal	134
5.2.7	Central Nervous System (CNS) Tissue Processing.....	134
5.2.8	Inoculation of Cell Cultures	135
5.2.9	Mouse Inoculation with Tissue for Isolation Attempts	135
5.2.10	Bovine Isolation Samples	136
5.2.11	Immunohistochemical Detection.....	136
5.2.12	Case Study 1: Bobby Calf Isolation Attempt	137
5.2.13	Case Study 2: Boxer Dog	138
5.3	Results.....	139
5.3.1	Bovine Isolations	139
5.3.2	Experimental Inoculation: Pups 2 and 5.....	143
5.3.3	Case study 1: Bobby Calf Isolation	144
5.3.4	Case Study 2: Boxer Dog Isolation	147
5.4	Discussion	152
5.5	Conclusions.....	156
CHAPTER 6.		157
6.0	General Discussion.....	157

Appendices

Appendix 2.1 - Disposables & General Use Materials	Error! Bookmark not defined.
Appendix 2.2 - Cell Culture/Isolations Materials.....	Error! Bookmark not defined.
Appendix 3.1 – IHC Positive Control Sample 11383	Error! Bookmark not defined.
Appendix 3.2 - Leica, JUNG TP 1050 Formalised Tissue Processor.....	Error! Bookmark not defined.
Appendix 3.3 – IHC Materials	162
Appendix 3.4 – Immunohistochemistry Protocol	163
Appendix 3.5 – IHC Staining of <i>Neospora caninum</i> Tissue Cysts	164
Appendix 3.6 - PCR Materials & Solutions	165
Appendix 3.7 – DNeasy Isolation Techniques.....	166
Appendix 3.8 – DNA Purification	168
Appendix 3.9 – DNA Sequences	169
Appendix 3.10 - Immunoblot Materials & Solutions	172
Appendix 3.11 – Silver Staining	174
Appendix 4.1 – Mouse Health Chart (Example)	175
Appendix 4.2 – Re-isolation of Parasites from Mouse Tissue	176
Appendix 4.3 – PCR-2 results from Nc-Liverpool Trial-1	177
Appendix 5.1 – CNS Tissue Processing	179
Appendix 5.2 – Isolations Mouse Immunoblot	181
Appendix 5.3 – Boxer Case study Supplementary PCR Gel	182
REFERENCES	183

List of Tables

CHAPTER 1.

Table 1.1: Comparative ultrastructure of tachyzoites, tissue cysts, and bradyzoites of <i>Neospora caninum</i> and <i>Toxoplasma gondii</i>	4
Table 1.2: <i>Neospora caninum</i> Isolates	24
Table 1.3: Serological assays developed to detect antibodies in <i>Neospora caninum</i> -infected cattle.	33
Table 1.4: Analytical sensitivity and specificity of polymerase chain reactions for the detection <i>N. caninum</i> DNA.	34

CHAPTER 2.

Table 2.1. IFAT results for FBS samples	49
Table 2.2. Serum trial Vero cell counts	50
Table 2.3. Serum trial Nc-Liverpool tachyzoite cell counts.....	51
Table 2.4. Cryopreservation retrieval	52

CHAPTER 3.

Table 3.1. PCR template preparation	85
Table 3.2. PCR templates and additives	86
Table 3.3. Dot-Blot antibody optimisation.....	102

CHAPTER 4.

Table 4.1. Treatment groups for Nc-Liverpool Trial-1.	115
Table 4.2. Nc-Liverpool tachyzoite inoculation of mice.....	116
Table 4.3. Nc-Liverpool trial 1 mouse cull table.....	118
Table 4.4. Nc-Liverpool Trial-1 summary table of results.....	118
Table 4.5. Nc-Liverpool Trial-1 summary table of immunoblot results	120
Table 4.6. Nc-Liverpool Trial-1 summary table of PCR detection results	121
Table 4.7. PCR results table for re-isolation cultures.....	124
Table 4.8. Summary of results from inoculation and re-isolation trial.	124

CHAPTER 5.

Table 5.1. Bovine isolates summary of results table.	141
Table 5.2. Canine isolates summary of results table.....	144

List of Figures

CHAPTER 1.

Figures 1.1 & 1.2. Transmission EM of tachyzoites of <i>N. caninum</i> and <i>T. gondii</i>	5
Figures 1.3 & 1.4. Transmission EMs of bradyzoites of <i>N. caninum</i> and <i>T. gondii</i>	6
Figure 1.5. Immunohistochemical staining of <i>N. caninum</i> tissue cyst.....	7
Figures 1.6 & 1.7. Transmission EM of <i>N. caninum</i> tissue cysts.	8
Figure 1.8. <i>Neospora caninum</i> stages in dogs and mice.	9
Figure 1.9. Transmission of bovine neosporosis.....	13

CHAPTER 2.

Figure 2.1. CPE in a Vero cell monolayer infected with <i>N. caninum</i> tachyzoites.....	46
Figure 2.2. Free <i>N. caninum</i> tachyzoites observed around areas of CPE.	47
Figures 2.3 & 2.4. Heavily <i>N. caninum</i> infected host cells detaching from the monolayer during host cell lysis.....	47
Figure 2.5. Tachyzoites lysing a host cell and actively moving to search for a new host cell.	48
Figure 2.6. Tachyzoite movement.....	48

CHAPTER 3.

Figure 3.1. Avidin-biotin enzyme complex (ABC) reaction with biotinylated secondary antibody.....	59
Figure 3.2. <i>T. gondii</i> positive tissue sections (x1000) incubated with <i>T. gondii</i> antibody (1:1000) at room temperature for 1 hour.....	64
Figure 3.3. <i>N. caninum</i> positive control sections (x1000) incubated with <i>T. gondii</i> antibody (1:500) for 1 hour at room temperature.....	65
Figure 3.4. <i>Neospora caninum</i> positive control sections (x1000) incubated with <i>T. gondii</i> antibody (1:500) at 4°C overnight.....	65
Figure 3.5. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antibody (1:1000) for 1 hour at room temperature.....	66
Figure 3.6. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antibody (1:1000) at 4°C overnight.....	67
Figure 3.7. <i>Toxoplasma gondii</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antibody (1:1000) for 1 hour at room temperature.....	67
Figure 3.8. <i>Toxoplasma gondii</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antibody (1:1000) at 4°C overnight.....	68
Figure 3.9. <i>T. gondii</i> positive tissue sections (x1000) incubated with <i>T. gondii</i> antibody (1:500) for 1 hour at room temperature.....	69
Figure 3.10. <i>Toxoplasma gondii</i> positive tissue sections (x1000) incubated with <i>T. gondii</i> antibody (1:500) at 4°C overnight.....	69

Figure 3.11. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antisera (1:1000) for 1 hour, no trypsin treatment.	71
Figure 3.12. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>N. caninum</i> antisera (1:1000) for 1 hour, following 1 hour of trypsin treatment.	71
Figure 3.13. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>T. gondii</i> antisera (1:500) for 1 hour, no trypsin treatment.	72
Figure 3.14. <i>Neospora caninum</i> positive tissue sections (x1000) incubated with <i>T. gondii</i> antisera (1:500) for 1 hour, following 1 hour of trypsin treatment.	72
Figure 3.15. PCR-1 validation agarose gel photograph.	87
Figure 3.16. PCR-2 sensitivity validation agarose gel photograph.	88
Figure 3.17. DNeasy elutions PCR agarose gel photograph.	89
Figure 3.18. PCR template preparation agarose gel photograph.	90
Figure 3.19. PCR-2 optimisation agarose gel photograph.	91
Figure 3.20. Assembly of Dot-Blot Apparatus.	100
Figure 3.21. Silver stained western blot photocopy.	103
Figure 3.22. Western blot Biomax film image.	104
Figure 3.23. Silver stain of BSA Western blot gel.	105
Figure 3.24. Dot blot of 1° and 2° antibody dilution series.	106
Figure 3.25. Dot blot of 1° and 2° antibody dilution series.	107

CHAPTER 4.

Figure 4.1. Nc-Liverpool Trial-1 dot-blot of mouse serum.	120
Figure 4.2. PCR-3 gel photograph of Nc-Liverpool Trial-1 mouse brain samples (gel 1). .	122
Figure 4.3. PCR-3 gel photograph of Nc-Liverpool Trial-1 mouse brain samples (gel 2). .	123
Figure 4.4. PCR gel photograph of mouse brain DNA isolations.	125
Figure 4.5. PCR gel photograph of re-isolation cell culture passage 1 & 2 samples.	125
Figure 4.6. PCR gel photograph of re-isolation cell culture passage 3 samples.	126

CHAPTER 5.

Figure 5.1. PCR-3 gel photograph of bovine isolate samples.	142
Figure 5.2. PCR-3 gel photograph of brains from BALB/c mice inoculated with bovine isolates.	143
Figure 5.3. PCR-1 gel photograph of Pup 2 & 5 samples amplified with <i>Neospora</i> or <i>Toxoplasma</i> specific primers.	144
Figure 5.4. PCR gel photograph of Bobby calf 99-167 purified tissue and cell culture samples.	146
Figure 5.5. PCR gel photograph of mouse brains following Bobby calf 167 tissue inoculation.	147
Figure 5.6. Boxer dog purified tissue inoculum PCR gel photograph.	148
Figure 5.7. Boxer dog monolayer post-inoculum and passage-1 samples PCR gel 1.	149
Figure 5.8. Boxer dog Vero cell culture passage-1 and passage-2 and faecal floatation samples PCR gel photograph.	150

Figure 5.9. Mouse brain PCR gel photograph following inoculation with Boxer dog samples.	
.....	151

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'-diaminobenzidine 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	Polyvinylidene 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