

**Approaches towards vaccine development against  
*Neospora caninum***

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute  
and State University in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy  
In  
Biomedical and Veterinary Sciences

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June 5, 2006  
Blacksburg, Virginia

Keywords: *Neospora caninum*, *Brucella abortus* strain RB51, recombinant vaccine,  
mouse model, cell-mediated immunity, vertical transmission

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## **ABSTRACT**

*Neospora caninum* is an apicomplexan parasite that causes neuromuscular paralysis in dogs and abortions in cattle. *N. caninum* is responsible for losses of several million dollars to the dairy and beef industries in several parts of the world. The key players in the host immune response to *N. caninum* include CD4<sup>+</sup> T cells, the Th1 cytokines IL-12, Interferon gamma and IgG2a isotype antibodies. There are currently no chemotherapeutic agents that are effective against adult cattle neosporosis. A commercially available, inactivated vaccine induces the undesirable Th2 type of immunity against *N. caninum*. Therefore, two approaches towards vaccine development against *N. caninum* that were designed to induce potent cell mediated immunity have been explored in this dissertation. The first approach consisted of the development of a bivalent recombinant vaccine for both brucellosis and neosporosis, while the second approach involved gamma irradiation of *N. caninum* tachyzoites for use as an attenuated vaccine against *N. caninum*.

Since *N. caninum* research has been conducted with several strains of mice and the different strains of mice vary in their susceptibility to infection with *N. caninum*, there is a need to develop a standard lab animal model for *N. caninum*. A gerbil and a C57BL/6 mouse model for *N. caninum* vaccine testing have been developed. It was found that the LD<sub>50</sub> of *N. caninum* tachyzoites in gerbils was  $9.3 \times 10^5$  tachyzoites per gerbil delivered intra-peritoneally, (i.p) while for C57BL/6 mice the LD<sub>50</sub> was  $1.5 \times 10^7$  tachyzoites per mouse delivered i.p. Vertical transmission rates in C57BL/6 mice infected with *N. caninum* tachyzoites during mid-gestation were determined and found to be in the range of 96-100%.

Putative protective antigens of *N. caninum* that included MIC1, MIC3, GRA2, GRA6 and SRS2 were expressed in *B. abortus* strain RB51 to create recombinant vaccine strains. C57BL/6 mice were vaccinated with either the recombinant strains or the irradiated tachyzoites. Antigen specific IgG2a and IgG1 responses and high levels of interferon gamma and IL-10 were induced by vaccination. Mice vaccinated with irradiated tachyzoites, RB51-MIC1 and RB51-GRA6 were completely protected against lethal challenge, while the mice vaccinated with RB51-SRS2, RB51-GRA2 and RB51-MIC3 were partially protected.

To determine the efficacy of the vaccines in preventing vertical transmission of *N. caninum*, mice were vaccinated and bred after administration of a booster dose four weeks after the primary vaccination. Antigen specific IgG1 and IgG2a and significant levels of IFN- $\gamma$  and IL-10 were detected in vaccinated, pregnant mice. Pregnant mice were challenged with  $5 \times 10^6$  *N. caninum* tachyzoites between days 11-13 of pregnancy. Brain tissue was collected from pups three weeks after birth and examined for the presence of *N. caninum* by a semi-nested PCR. Protection against vertical transmission elicited by the RB51-GRA6, RB51-MIC3, irradiated tachyzoite, RB51-GRA2, RB51-MIC1 and RB51-SRS2 vaccinated groups were 43%, 38%, 34%, 34%, 18%, and 7% respectively. Since not all the antigens that were highly protective against acute disease were not very effective in preventing vertical transmission, the role of the selected antigens in preventing acute disease and vertical transmission appear to differ. Only GRA6 was found to be effective in protecting against an acute lethal challenge as well as preventing vertical transmission 43% of the time.

In summary, two animal models for the testing of *N. caninum* vaccines were developed. *N. caninum* protective antigens were successfully expressed in *B. abortus* strain RB51. The irradiated tachyzoite and recombinant RB51-*Neospora* vaccines were highly effective in protecting against acute neosporosis and partially protective against vertical transmission. Therefore, both these approaches show great promise as practical and effective means to achieve the goal of successful prophylaxis against *N. caninum* induced abortions and reduce the chances of vertical transmission.

## ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge the support and guidance of my advisor Dr. Sriranganthan. I am immensely grateful to him for providing me with an environment that fostered a spirit of scientific enquiry and for always encouraging the exploration of new ideas. I would also like to thank him for his approachability and being as much a friend as a mentor.

I would also like to extend my grateful thanks to Dr. David Lindsay for his encouragement and support in my development as a scientist. I would like to express my deep appreciation for his generosity, his guidance in manuscript preparation and for helping to broaden my perspective about my chosen field of work.

This research project would not have been possible without the valuable contribution of our collaborators, Dr. Vemulapalli and his team. I would also like to thank Dr. Vemullapalli for his suggestions that have guided the course of my research. I would like to extend my warm appreciation to Dr. Boyle for his guidance and advice regarding my research, technical writing and career. I am indebted to my other committee members Dr. Schurig, Dr. Lathigra and Dr. Suzuki for their periodic suggestions and constructive criticism that have helped me to keep the big picture in perspective as my research evolved during the course of my study. My thanks are also due to Dr. Bob Duncan for carrying out histological analysis of samples generated during the study.

My special thanks are due to Kay Carlson for ensuring the timely availability of lab supplies and for being a friend. I would like to thank Mohammed Seleem for making my stint at CMMID memorable by the long technical and non-technical debates and for being a good friend and colleague. I would like to thank Abey Bandara for his guidance and help with experimental work and my other lab-mates Rajiv Prasad, Moemen Mohammed, Alexa Rosypal, Sheila Mitchell, Dave Goodwin and Ashraf Omar for their company.

The National Research Initiative of the USDA Cooperative State Research, Education and Extension Service supported this project through grant number 2002-35204-12337. I would like to thank the college of veterinary medicine for financial support during the last two years of my study.

I would like to thank my friends Sharad Gupta, Ramya Ramanath, Narinder Kaur, Anbumani Subramanian, Krishnaraj Varma and Vidya Rajagopalan for the wonderful times we have shared in Blacksburg, listening and for being there in my times of need. Last but certainly not the least; I would like to thank my parents, brothers and their families for their love and unwavering support and acceptance of my aspirations. They have been the source of my strength and inspiration.

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## LIST OF ABBREVIATIONS

AVMA	American Veterinary Medical Association
Bp	Base-pairs
BSL-3	Bio-safety Level 3
CMI	Cell Mediated Immunity
CpG	Cytosine Guanine dinucleotide
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
GRA2	Dense Granule Protein 2
GRA6	Dense Granule Protein 6
HSP	Heat Shock Protein
IFN- $\gamma$	Interferon gamma
IgG	ImmunoGlobulin
IL-4	Interleukin 4
IL-10	Interleukin 10
IL-12	Interleukin 12
i.p	Intraperitoneal
Kd	Kilo Daltons
LD <sub>50</sub>	Lethal Dose <sub>50</sub>
LPS	Lipo Polysaccharide
MHC -I	Major Histo-compatibility Complex I
MHC-II	Major Histo-compatibility Complex II
MIC1	Microneme 1
MIC3	Microneme 3
NC-1	<i>Neospora caninum</i> strain1
OIE	Office des epizootics
PBS	Phosphate Buffer Saline
RPM	Revolutions per Minute
SCID	Severe Combined Immunodeficiency Disease

SEM	Standard Error of the Mean
SRS2	Surface Antigen Gene Related sequence 2
Th1	T Helper cell 1
Th2	T Helper cell 2
TMB	Tetramethylbenzidine
TNF- $\alpha$	Tumor Necrosis Factor $\alpha$
USDA	United States Department of Agriculture

## GENERAL INTRODUCTION

*Neospora caninum* is an Apicomplexan parasite that affects cattle and dogs. It causes mid to late term abortions in cattle and neuromuscular paralysis in dogs. Incidence of the disease has been recorded in several parts of the world and the major impact of *N. caninum* is due to the severe economic losses it causes to the dairy and beef industries (31, 34). The dog has been identified as the definitive host of the parasite. Cattle acquire the infection trans-placentally from infected dams, thus maintaining the disease in the herd. Protective immune responses to this disease in cattle and mice involve CD4+T cells and secretion of interferon gamma (69, 82). Antibodies are considered to have a secondary role in parasitic clearance.

There are no available chemotherapeutic agents that are effective against cattle neosporosis. Due to the severe economic impact of the disease, there is an urgent need to develop an effective vaccine for this disease. The only available vaccine for this disease is an inactivated tachyzoite preparation that induces antibody responses. Although it is reported to reduce the rates of abortion by about 35%, its efficacy in preventing vertical transmission has not been studied (22).

*Brucella abortus* is a gram negative, intracellular bacterium that is also a cause of bovine abortions. *B. abortus* strain RB51 is the official vaccine for bovine brucellosis in the U.S. Strain RB51 is an excellent vector for heterologous protein expression and vaccination with recombinant strains of RB51 expressing heterologous proteins induces strong Th1 type immunity against the expressed protein (119).

The primary focus of this dissertation is the development of an effective vaccine for *N. caninum*. Two approaches that were followed consisted of a) over-expression of *N. caninum* protective antigens in *B. abortus* strain RB51 to create a bivalent vaccine against both the diseases and b) the use of gamma-irradiated whole tachyzoites as an attenuated vaccine. Both the approaches were designed to stimulate a Th1 type of immune response that is indispensable for protection against *N. caninum* induced abortions and vertical transmission of the parasite.

The main objectives of this study were to a) develop a robust animal model for *N. caninum* vaccine testing, b) develop the recombinant RB51-*Neospora* vaccines and

irradiated vaccine, test the ability of both vaccines to induce Th1 type immune responses and protection against acute challenge, c) determine the effect of pregnancy upon immune responses to vaccination and d) determine the efficacy of both vaccines in preventing vertical transmission. The development of laboratory animal models for vaccine testing, characterization of the immune responses elicited by vaccination and determination of the efficacy of these vaccines in protecting against acute challenge and vertical transmission in vaccinated mice are described herein.