UNIVERSIDADE FEDERAL DE PELOTAS

Programa de Pós-Graduação em Biotecnologia

Tese

DESENVOLVIMENTO DE MÉTODOS IMUNOQUÍMICOS E MOLECULARES PARA O DIAGNÓSTICO DA NEOSPOROSE

Gizele Lima de Sá

Pelotas, 2016

Gizele Lima de Sá

DESENVOLVIMENTO DE MÉTODOS IMUNOQUÍMICOS E MOLECULARES PARA O DIAGNÓSTICO DA NEOSPOROSE

Tese apresentada ao Programa de Pós-Graduação em Biotecnologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área do Conhecimento: Biotecnologia).

Orientador: Sibele Borsuk

Pelotas, 2016

Dados de catalogação na fonte:
Maria Beatriz Vaghetti Vieira – CRB-10/1032
Biblioteca de Ciência & Tecnologia - UFPel

S111d Sá, Gizele Lima de
Desenvolvimento de métodos imunoquímicos e moleculares para o diagnóstico da neosporose / Gizele Lima de Sá. – 102f. : il. – Tese (Doutorado). Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas. Centro de Desenvolvimento Tecnológico. Pelotas, 2016. – Orientador Sibele Borsuk.

1.Biotecnologia. 2. *Neospora caninum*. 3. Nc-p43. 4. Nc-p29. 5. Imunodiagnóstico. I.Borsuk, Sibele. II. Título.

CDD: 615.37

BANCA EXAMINADORA

Prof. Drª. Sibele Borsuk (Universidade Federal de Pelotas, CDTec)

Prof. Drª. Fabiane Seixas (Universidade Federal de Pelotas, CDTec)

Prof. Drª. Maria Elisabeth Aires Berne (Universidade Federal de Pelotas, IB)

Dr. Sergio Jorge (Universidade Federal de Pelotas, CDTec)

Para minha família e companheiro, com carinho e gratidão.

Dedico.

Agradecimentos

Eu gostaria de agradecer as minhas irmãs e meus pais. A vida pode ser uma escola bastante puxada, e sem apoio e amor de uma família a existência se torna bem mais difícil.

A Gabriel Guerreiro meu parceiro, sem seu enorme apoio nos níveis espiritual, mental e emocional durante este período, certamente eu não seria hoje a pessoa que sou.

Aos integrantes do laboratório de imunodiagnóstico e CDTec. Pelo companheirismo e apoio durante o doutorado, para mim foi um privilégio conhecê-los, e estou certa de que nossa amizade continuará muito além do curto período da pós-graduação.

As minhas orientadoras Cláudia Hartleben e Sibele Borsuk pelo aprendizado profissional e principalmente pessoal que obtive. As palavras não podem expressar apropriadamente minha gratidão.

E enfim agradeço a Deus por permitir-me viver a experiência da pós-graduação com essas pessoas.

Resumo

SÁ, Gizele Lima de. **DESENVOLVIMENTO DE MÉTODOS IMUNOQUÍMICOS E MOLECULARES PARA O DIAGNÓSTICO DA NEOSPOROSE.** 2016. 102f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O *Neospora caninum* é um protozoário filogenéticamente relacionado a vários coccídeos de importância médico veterinária, além de ser um parasito intracelular obrigatório capaz de causar abortamentos em bovinos e paralisia neoromuscular em cães. A interação ligantes/receptores são requisitos que permitem que o parasito explore sua capacidade invasiva, sendo o contato inicial mediado por proteínas de superfície imunodominantes como Nc-p43 e Nc-p29. O estudo de antígenos e proteínas recombinantes de *N. caninum* gerou uma série de informações para aprimorar o diagnóstico e diferenciação deste protozoário e demais agentes a ele relacionados, principalmente com relação ao *Toxoplasma gondii*. No presente trabalho foi realizada uma revisão bibliográfica das proteínas envolvidas na interação hospedeiro-parasito utilizadas como alvos vacinais e na aplicação em testes diagnósticos devido a sua especificidade. Dentre as proteínas descritas, os antígenos de superfície Nc-p43 e Nc-p29 são amplamente estudados devido a sua especificidade e capacidade de induzer uma resposta imune protetiva, além de não apresentar reação cruzada com outros parasitos Apicomplexas quando utilizadas em ensaios diagnósticos. Frente a isso, propomos a expressão das proteínas específicas de *N. caninum*, Nc-p43 e Nc-p29 em sistema procarioto, para avaliar sua antigenicidade frente a soros de animais naturalmente infectados por *N. caninum* e especificidade quando testados frente a soros de animais infectados por *T. gondii*. A proteína rNc-p43 foi utilizada na produção de um anticorpo policlonal, que foi purificado, conjugado a peroxidase (HPR) e isotiocianato de fluoresceína (FITC) afim de detectar as proteínas recombinantes e nativas Nc-p43, respectivamente. pAb e pAb/HRP foram capazes de reconhecer rNcp-43, enquanto pAb/FITC se mostrou eficiente na imunomarcação do complexo apical de taquizoítos. Um ensaio imunoenzimático de bloqueio (b-ELISA) foi realizado para avaliar a performance do pAb/HRP como ferramenta de diagnóstico. A porcentagem de inibição média para as amostras de soros positivos e negativos de bovinos com neosporose obteve diferença estatística ($P < 0,0001$). Estes resultados sugerem que o pAb pode ligar-se aos mesmos epítopos da Ncp-43 que os anticorpos anti-*N. caninum* de amostras positivas testadas. O b-ELISA utilizando o pAb/HRP representa uma opção interessante aos testes de diagnóstico disponíveis para a neosporose, uma vez que menos passos estão envolvidos na sua realização e seu formato evita a reatividade cruzada com anticorpos anti-espécie específicos. Em resumo, este trabalho descreve a clonagem e expressão das proteínas Nc-p43 e Nc-p29 de *N. caninum* em sistema procarioto, a produção de um anticorpo policlonal monoespecífico contra a proteína recombinante Nc-p43 e a avaliação de sua aplicabilidade como ferramenta no diagnóstico para neosporose.

Palavras-chave: *Neospora caninum*, Nc-p43, Nc-p29, Imunodiagnóstico.

Abstract

SÁ, Gizele Lima de. **DEVELOPMENT OF IMMUNOCHEMICAL AND MOLECULAR METHODS FOR THE DIAGNOSIS OF NEOSPOROSIS.** 2016. 102f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Neospora caninum is a protozoan phylogenetically related to several coccidia with importance in veterinary medicine. This obligate intracellular parasite causes abortions in cattle and neoromuscular paralysis in dogs. Interaction ligand/receiver are required that allow the parasite explore your ability invasive, being the initial contact mediated for immunodominant surface proteins as Nc-p43 and Nc-p29. The study of antigens and recombinant proteins of *N. caninum* have generated a serie of informations aiming the improvement of diagnosis and differentiation of this protozoan and other agents related to it, especially *Toxoplasma gondii*.In the present work was carried out a literature review focusing in the proteins involved in host-parasite interaction used as vaccine targets and application in diagnostic tests due to its specificity. Among the described proteins, antigens of surface Nc-p43 and Nc-p29 are widely studied due to their specifity and ability to induzer a protective immunity, and not reported cross reaction with other Apicomplexa parasite when used in trials diagnostics. Furthermore, the expression of specific proteins of *N. caninum*, Nc-p43 and Nc-p29, in prokaryote system, are here described, followed by their evaluating concerning their antigenicity, using sera from animals naturally infected with *N. caninum;* and their specificity through reaction with sera from animals infected with *T. gondii*. The rNc-p43 protein was used for polyclonal antibody production in rabbit, purified and conjugated to peroxidase (HPR) and fluorescein isothiocyanate (FITC), in order to detect the recombinant and native Nc-p43, respectivelly. pAb and pAb/HRP were able to recognize rNc-p43, which was evaluated by Dot blot and ELISA assays, while pAb/FITC was able to mark the apical complex of tachyzoites. A blocking enzyme-linked immunosorbent assay (b-ELISA) was performed to evaluate the performance of pAb/HRP as diagnostic tool. The average percentage of inhibition for the positive sera pool and the negative sera pool of cattle neosporosis was significantly different ($p < 0.0001$). These results suggest that pAb may bind to the same epitopes of Nc-p43 and anti-*N. caninum* antibodies positive samples tested. b-ELISA using pAb/HRP is an interesting option for diagnostic tests that are available for neosporosis since fewer steps are involved in their implementation and their shape prevents cross-reactivity with anti-species-specific antibodies. In short, this work describes the cloning and expression of Nc-p43 and Nc-p29 proteins *N. caninum* in prokaryotic system, to produce a monnoespecific polyclonal antibody against the recombinant protein Nc-p43 and evaluation of its suitability as a tool in diagnosis of neosporosis.

Keywords: *Neospora caninum*, Ncp-43, Ncp-29, immunodiagnostic.

Lista de Abreviaturas

µg – Microgram (Microgramas)

µL – Microliter (Microlitro)

µm – Micrometre (Micrômetro)

2-DE – Two-dimensional electrophhoresis techniques (eletroforese bi dimensioal)

b-ELISA - Blocking enzyme-linked immunosorbent assay (Ensaio imunoenzimático de bloqueio)

CONCEA – Conselho Nacional de Controle de Experimentação Animal

DNA – Deoxyribonucleic acid (Ácido desoxirribonucleico)

ELISA – Enzyme-linked immunosorbent assay (Ensaio imunoenzimático)

Fig – Figura

FITC – Fluorescein isothiocyanate (Isotiocianato de fluoresceina)

g – Força de gravidade

GRA – Dense granule protein (Proteínas de grânulos densos)

H – Hora

H_2O_2 – Peróxido de hidrogênio

HRP - Horseradish Peroxidase (Peroxidase)

HSP – Heat shock protein (Proteína de choque)

IF – Immunofluorescence (Imunofluorescência)

IFD – Direct immunofluorescence [DIF] (Imunofluorescência direta)

IFI – Indirect immunofluorescence test [IIFT] (Imunofluorescência indireta)

IgG – Imunoglobulina G

IHC – Immunohistochemistry test (Imunohistoquimica)

IMAC – Immobilized metal íon affinity chromatograph (Íons metálicos imobilizados)

IPTG – Isopropilα-D-thiogalactoside

KDa – Quilodalton

mAbs – Monoclonal antibodies (Anticorpos monoclonais)

MIC – Microneme protein (Proteìna de micronemas)

mM – milimolar

M – Molar

MS – Mass spectrometry (espectrometria de massa)

NcGRA – *Neospora caninum* dense granule protein (Proteína de grânulos densos de *Neospora caninum*)

NcMIC – *Neospora caninum* microneme protein (Proteìna de micronemas de *Neospora caninum*)

Nc – *Neospora caninum*

Nc-p29 – Proteína 29 de *Neospora caninum*

Nc-p43 - Proteína 43 de *Neospora caninum*

NcRON - *Neospora caninum* roptry neck protein (Proteína do pescoço de roptrias de *Neospora caninum*)

NcROP – *Neospora caninum* roptry body protein (Proteína do corpo de roptrias de *Neospora caninum*)

NcSAG1 – Sequência codificadora para antígeno de superfície de *Neospora caninum*

NcSRS2 – Sequência codificadora para antígeno de superfície de *Neospora caninum*

nm - Nanômetro

OD – Optical density (Densidade óptica)

pAb/FITC – Fluorescein isothiocyanate conjugated polyclonal antibody (Anticorpo policlonal conjugado a isotiocianato de fluoresceína)

pAb/HRP – Horseradish Peroxidase conjugated polyclonal antibody (Anticorpo policlonal conjugado a peroxidase)

pAbs – Polyclonal antibody (Anticorpo policlonal)

PAGE – Polyacrylamide gel electrophoresi (Gel de poliacrilamida)

PBS - Phosphate-buffered saline (Tampão fosfato salino)

PBS-T – Phosphate-buffered saline with 0,05% Tween-20 (Tampão fosfato salino com 0,05%de Tween 20)

PCR – Polynerase chain reaction (Reação em cadeia da polimerase)

rNc-p29 – Proteína 29 de *Neospora caninum* na forma recombinante

rNc-p43 - Proteína 43 de *Neospora caninum* na forma recombinante

RON - Rhoptry neck protein (Proteína do pescoço de roptrias)

ROP – Roptry body protein (Proteína do corpo de roptrias)

SAG – Surface antigens (Antígenos de superfície)

SDS – Sodium dodecyl sulfate (Dodecil sulfato de sódio)

SRS – Surface antigens related sequences (Sequências relatadas de superfície)

VERO – African Green monkey kidney cell line (Células de rim de macaco africano)

VP – Vacúolo parasitóforo

WB – Western blotting

SUMÁRIO

1 INTRODUÇÃO GERAL

A neosporose é uma doença causada por um protozoário intracelular obrigatório denominado *Neospora caninum* (Dubey & Schares, 2011), que foi erroneamente diagnosticado como *Toxoplasma gondii* devido à grande similaridade morfológica, molecular e antigênica até o ano de 1988 (Dubey et al., 1988). A infecção por *N. caninum* esta associada a abortos e mortalidade neonatal em bovinos, ovinos, caprinos, eqüinos e cervídeos, seus hospedeiros intermediários (Dubey, 2003) e desordens neuromusculares severas em canídeos, seus hospedeiros definitivos (McAllister, Dubey et al., 1998; King et al., 2010).

Ao longo do ciclo o parasito se apresenta nos estágios de taquizoítos e bradizoítos, encontrados nos hospedeiros definitivos e intermediários, e em oocistos que são exclusivos de hospedeiros definitivos e eliminados juntamente com as fezes desses animais (McAllister et al., 1998). A resposta imune do hospedeiro e outros fatores fisiológicos estimulam diferenciação e reconversão de taquizoítos em bradizoítos (Peters et al., 2001) o que permite a permanência do parasito durante toda a vida do hospedeiro sem grandes manifestações clínicas (Goodswen et al., 2013). Em situações de imunossupressão, como durante a prenhez, pode causar infecção do feto acarretando em aborto, natimorto, terneiro malformado ou clinicamente saudável, porém cronicamente infectado (Andrianarivo et al., 2005; Sengupta et al., 2012). Devido a esses fatores, a neosporose, representa um prejuízo de milhões de dolares ao Brasil (Reichel et al., 2013).

O diagnóstico da neosporose é baseado na identificação de lesões compatíveis com a doença e ensaios que identificam diretamente a presença do parasito nos tecidos, como a reação em cadeia da polimerase (PCR) e a imuno-histoquímica (Benavides et al., 2012). Contudo, os ensaios sorológicos apresentam vantagens sobre o diagnóstico direto, pois podem ser aplicados *antemortem* (Dubey & Schares, 2006) sendo a imunofluorescência indireta (IFI) considerada como padrão ao avaliar novos métodos (DUBEY et al., 1988). Entretanto, a IFI é um teste laborioso e dispendioso, pois necessita do cultivo de taquizoítos em células de mamíferos (Dubey & Schares, 2011).

Como alternativa ao teste de IFI, outros formatos de testes foram avaliados como o *Enzime-Linked Immunosorbent Assay* (ELISA) (Ahn et al., 2003; Byrem et

al., 2012) e teste de aglutinação (*Neospora Agglutination Test*) (Romand et al., 1998). Porém, estes testes baseiam-se na utilização de antígenos solúveis totais do parasito, incluindo antígenos intracelulares, o que diminui a sensibilidade e especificidade destes ensaios, enquanto que na IFI prevalece os antígenos de superfície dos parasitos (Bjorkman & Uggla, 1999).

Neste contexto, ensaios diagnósticos que utilizam antígenos recombinantes, espécie-especificos de *N. caninum* para a detecção de anticorpos nos soros animais são uma alternativa para aumentar a acurácia do diagnóstico, pois apresentaram maior sensibilidade e especificidade quando comparados à técnica padrão (Dong et al., 2012; Moraveji et al., 2012; Selahi et al., 2013). Além disso, representam uma alternativa importante, devido à possibilidade de produção rápida, facilitando a geração de insumos e a padronização de ensaios sorológicos (Khow & Suntrarachun, 2012).

Estudos da antigenicidade do *N. caninum* sugeriu que os antígenos imunodominantes específicos encontram-se principalmente na superfície celular (Bjorkman & Uggla, 1999; Jung et al., 2004). Testes sorológicos que possuem como alvo antígenos de superfície ou insumos produzidos contra estes antígenos apresentam menor percentual de reações cruzadas com parasitos do mesmo filo, tal como os antígenos protéicos imunodominante de taquizoítos, Nc-p43 e Nc-p29 (Howe et al., 1998; Howe et al., 2002). Ainda, a expressão aumentada da proteína Nc-p29 foi associada a virulência de cepas de campo do parasito (Regidor-Cerrillo et al., 2012) e uma resposta imune protetiva foi alcançada quando utilizado como vacina de DNA (Cannas et al., 2003). ELISAs indiretos foram relatados para o diagnóstico da neosporose, utilizando a proteína recombinante Nc-p43 (Schares et al., 2005; Andreotti et al., 2009; Borsuk et al., 2011) apresentando uma sensibilidade e especificidade variando em torno de 93% a 96%. Anticorpos policlonais monoespecíficos desenvolvidos contra este antígeno apresentaram potencial para detecção do parasito (Sa et al., 2014).

Assim, o trabalho aqui proposto visa a expressão das proteínas recombinantes Nc-p43 e Nc-p29, a produção de anticorpo policlonal contra a proteína rNc-p43 e o desenvolvimento de ensaios imunoquímicos e moleculares para o diagnóstico da neosporose.

2 REVISÃO BIBLIOGRÁFICA

2.1 Neosporose

O agente infeccioso causador da neosporose é um protozoário intracelular obrigatório pertencente ao filo Apicomplexa, classe Sporoasida, sub-classe Coccidiasina, ordem Eucoccidiorida, família Sarcocystidae, sub-família Toxoplasmatinae, gênero *Neospora* e espécie *Neospora caninum* (Dubey et al., 1988). Entretanto até 1988 foi erroneamente classificado como *Toxoplasma gondii* devido à grande similaridade (Dubey et al., 1988). A neosporose tem sido confirmada como uma das principais causas de abortos e mortalidade neonatal em diversas espécies de mamíferos (Dubey, 2003). Os canídeos infectados apresentam desordens neuromusculares severas, principalmente aqueles com infecção congênita (Dubey et al., 1998).

A importância econômica dessa enfermidade na bovinocultura está diretamente relacionada às falhas reprodutivas e descarte precoce de fêmeas (Hasler et al., 2008). Em muitos casos ocorre o nascimento de animais clinicamente saudáveis, no entanto cronicamente infectados, os quais são de suma importância na manutenção do ciclo do parasito em rebanhos (Dubey, 2003).

A neosporose afeta tanto o gado de corte como o de leite, tendo sido registrada em todos os continentes, logo, grande parte dos rebanhos vem sendo constantemente exposto ao agente (Dubey & Schares, 2011; Reichel et al., 2013; Reichel et al., 2014). Estimativas indicam que no Brasil os prejuízos relacionados à neosporose variam de 51,3 a 101 milhões de dólares em rebanhos de leite e corte (Reichel et al., 2013).

2.2 Ciclo Biológico

Os canídeos são os hospedeiros definitivos do *N. caninum*, e a eliminação de oocistos desse parasito, foi confirmada, até o momento, em cães (*Canis lupus familiaris*) (McAllister et al., 1998), coiotes (*C. latrans*) (Gondim et al., 2004), dingos (*C. lupus dingo*) (King et al., 2010), e lobos cinzentos (*C. lupus lupus*) (Dubey et al., 2011). Uma ampla gama de outros mamíferos é suscetível a este parasito assumindo importância como hospedeiros intermediários.

Vários aspectos do ciclo biológico deste parasito não estão completamente esclarecidos, principalmente com relação à fase sexuada que ocorre no intestino do hospedeiro definitivo, no entanto, pode-se afirmar que o período pré-patente é de cinco a oito dias após a ingestão dos cistos teciduais pelos canídeos (Lindsay & Dubey, 2000). Ao longo do ciclo o parasito se apresenta nos estágios de taquizoítos e bradizoítos, fases intracelulares encontradas nos hospedeiros definitivos e intermediários, e em oocistos, que são exclusivos de hospedeiros definitivos e eliminados juntamente com as fezes desses animais (McAllister et al., 1998). A esporulação dos oocistos ocorre no ambiente, sendo esta a forma infectante para hospedeiros intermediários (Dubey, 2003). Há relato da presença de taquizoítos de *N. caninum* em diversos tipos de tecidos e células como macrófagos, fibroblastos, células endoteliais, miócitos, células epiteliais dos túbulos renais e hepatócitos, no entanto o parasito demonstra predileção pelo sistema nervoso quando no hospedeiro definitivo (Hemphill, 1999; Dubey & Schares, 2011).

A resposta imune do hospedeiro intermediário e outros fatores fisiológicos estimulam a diferenciação de taquizoítos em bradizoítos, os quais são menores, apresentando metabolismo e multiplicação mais lentos, formando cistos teciduais (Peters et al., 2001), comumente encontrados no encéfalo, medula, nervos e retina, podendo permanecer durante toda a vida do hospedeiro sem grandes manifestações clínicas (Goodswen et al., 2013).

2.3 Transmissão

A primeira observação de transmissão vertical em canídeos ocorreu na Noruega em três ninhadas sucessivas, que apresentaram problemas neurológicos similares a sintomas observados em animais infectados por *T. gondii (*Bjerkas et al., 1984). A porta de entrada para o *N. caninum* é o trato digestivo do hospedeiro definitivo, pois a infecção ocorre pela ingestão de tecidos de animais infectados contendo cistos, ou pela ingestão de oocistos esporulados presentes em água ou alimentos contaminados (Barr et al., 1993). A transmissão horizontal é mais freqüente entre cães do que a vertical, justificando as elevadas taxas de soroprevalência encontradas nesta espécie (Barber & Trees, 1998). A presença de oocistos em fezes de cães foi confirmada após a ingestão de cistos teciduais presentes em bovinos (Dijkstra et al., 2002), bubalinos (Rodrigues et al., 2004), ovinos (Pena et al., 2007) e cervídeos (Vianna et al., 2005).

Em hospedeiros intermediários a transmissão pode ser horizontal, através da ingestão de oocistos esporulados presentes nas pastagens ou água, ou vertical, através da infecção transplacentária por taquizoítos. Em bovinos a principal via de transmissão é a transplacentária e dependendo do estágio gestacional pode ocorrer aborto, natimorto ou ainda o nascimento de um bezerro clinicamente saudável, porém, cronicamente infectado (Sengupta et al., 2012). A transmissão lactogênica foi possível em condições laboratoriais, no entanto, não há confirmação da ocorrência desse tipo de transmissão naturalmente (Davison et al., 2001).

A taxa de infecção transplacentária em bovinos pode chegar a uma frequência de 95% dos casos, sendo a rota de transmissão responsável pela manutenção e expansão da infecção no rebanho devido às sucessivas passagens de geração em geração (Davison et al., 2001). A transmissão transplacentária exógena ocorre quando a fêmea sofre a primo-infecção durante a gestação, através da ingestão de oocistos, o que culmina com a infecção do feto. Já a transmissão transplacentária endógena ocorre quando uma fêmea cronicamente infectada, possivelmente de forma congênita, sofre reativação dos cistos durante a gestação, ocasionando a reversão de bradizoítos em taquizoítos que atingem o feto (Miller et al., 2002).

2.4 Epidemiologia

A associação do parasito *N. caninum* a abortos bovinos foi comprovada em estudos soroepidemiológicos realizados em diferentes países, os quais relatam taxas de prevalência variando de 10 a 60% (Reichel et al., 2013; Reichel et al., 2014). A neosporose afeta tanto o gado de corte como o gado de leite e tem sido registrada em todos os continentes (Cardoso et al., 2012; Reichel et al., 2013). Estimativas indicam que na Califórnia, Estados Unidos, os prejuízos causados a bovinocultura giram em torno de 35 milhões de dólares à indústria do leite, por ano (Chi et al., 2002), já no Brasil este prejuízo varia de 51 a 101 milhões de dólares anuais (Reichel et al., 2013).

Existem evidências que sugerem que bovinos leiteiro são mais susceptíveis à infecção por *N. caninum* que bovinos de corte, talvez em função do sistema de criação mais intensivo (Almería et al., 2009), que favorece uma maior exposição, devido a existência de vários mecanismos que possibilitam a disseminação da doença. Por exemplo, a utilização de *pool* de colostro, o que pode propiciar uma

possivel trasmissão do *Neospora caninum* pela via lactogênica (Corbellini et al., 2006). Outro fator de risco para neosporose é a presença de fetos abortados ou restos placentários que constituem fontes de infecçaõ do agente, principalmente aos hospedeiros definitivos, que passam a contaminar o ambiente com oocistos nas fezes. Dessa forma, ocorre a trasmissão horizontal aos hospedeiros intermediários, podendo causar o aborto. Nesse sentido, segundo Williams et al. (2009), uma vaca infectada com N. caninum tem de três a sete mais probabilidade de abortar que uma não infectada.

O hospedeiro definitivo representa um papel de destaque na cadeia epidemiológica da neosporose devido à grande frequência de infecção na população de canídeos combinada à ausência de sinais clínicos na maioria dos casos (Reichel et al., 2007; Al-Qassab et al., 2010).

Além disso, o risco de infecção aumenta quando diferentes espécies que participam de uma mesma cadeia epidemiológica são criadas no mesmo ambiente, em contato direto ou indireto. Em estudo realizado por Vanleeuwen e colaboradores (2010), em rebanhos bovinos, os fatores de riscos estavam associados à presença de cães, principalmente em propriedades rurais onde foi relatada ocorrência de sinais clínicos da neosporose. Nesse mesmo estudo foi verificado que, independente de os cães se alimentarem ou não de restos de placentas ou de fetos abortados, sua presença constitui um fator de risco à ocorrência de neosporose. Isso corrobora com os resultados de Corbellini et al. (2006), que verificaram que vacas de fazendas menores apresentavam maiores chances de serem soropositivas para o agente, pois os cães têm maior contato com as vacas e, assim, maiores chances de se infectar e, consequentemente, contaminar o ambiente com oocistos eliminados nas fezes, aumentando o risco de transmissão horizontal.

Deste modo, é importante considerar os fatores de riscos para neosporose em estudos de prevalência, uma vez que tais fatores influenciam diretamente na prevalência da neosporose nos rebanhos de corte e de leite. Essa influência pode ser constada em estudos soroepidemiológicos, onde rebanhos bovinos de propriedades leiteiras apresentaram uma prevalência maior quando comparada com as propriedades de rebanhos de corte (Kamga-Waladjo et al., 2010; Nasir et al., 2011; Ghalmi et al., 2012).

No Brasil, os dados desta parasitose em rebanhos bovinos de corte e de leite foram relatados em estudos de prevalência realizados nos estados do Mato

Grosso do Sul, Paraná, Goiás, Rio de Janeiro, Rondônia, Rio Grande do Sul, Santa Catarina e São Paulo, com taxas de prevalência que variaram de 6 a 58% (Locatelli-Dittrich et al., 2001; RAGOZO et al., 2003; Melo et al., 2006; Munhoz et al., 2006; Benetti et al., 2009; Andreotti et al., 2010; MOURA et al., 2011; Piagentini et al., 2012). Em levantamento soroepidemiológico de cães da região sul do Rio Grande do Sul a prevalência da neosporose foi de 15,62%, sendo os cães da zona rural os que apresentaram maior percentual de soropositividade (Da Cunha Filho et al., 2008).

2.5 Resposta imune

Existem vários fatores não esclarecidos acerca da resposta imune induzida frente à infecção por *N. caninum*, porém, mecanismos de reconhecimento e estímulo de hospedeiros infectados por outros Apicomplexas intimamente relacionados, como *T. gondii*, podem elucidar a interação parasito hospedeiro na neosporose.

Em uma primo infecção o mecanismo de defesa frente o neospora requer uma resposta imune inata e adaptativa, envolvendo altos níveis de interleucina-12 e INF-γ e uma razão aumentada de IgG2:IgG1, indicando uma predominância da resposta Th1 (Almeria et al., 2012; Ellis et al., 2012; Orozco et al., 2013). Macrófagos ativados via INF-γ produzem espécies ativas de oxigênio, como óxido nítrico que auxilia no controle da fase aguda da infecção (Staska et al., 2003; Boysen et al., 2006; Williams & Trees, 2006). Esses fatores provavelmente são responsáveis por induzir a diferenciação de taquizoítos em bradizoítos, levando à formação de cistos teciduais (Williams et al., 2009). Esta conversão desempenha um papel fundamental no estabelecimento da fase crônica da infecção (Lyons et al., 2002).

O papel crucial do INF-γ e da IL-12 foi confirmado em experimentos nos quais camundongos tratados com anticorpos anti-INF-γ e anti-IL12 foram infectados por *N. caninum,* e apresentaram maior suscetibilidade ao parasito (Khan et al., 1997). Além disso, animais deficientes para estas citocinas apresentaram elevadas cargas parasitárias devido à multiplicação exacerbada de taquizoítos em diversos órgãos resultando em altas taxas de mortalidade. A importância das células T foi confirmada experimentalmente quando camundongos atímicos infectados por *N. caninum* apresentaram 100% de mortalidade (Shibahara et al., 1999).

Anticorpos resultantes da infecção desempenham um papel auxiliar em infecções posteriores participando da neutralização e destruição de taquizoítos extracelulares (Innes et al., 2002). Estudos sugerem que a imunidade protetora pode ser alcançada devido a uma infecção anterior, e pode ser mantida através de mecanismos não elucidados, pois animais com evidências de exposição prévias têm menor probabilidade de aborto do que animais com infecção primária (McAllister et al., 2000).

Os bovinos, de forma geral, apresentam soroconversão rápida com altos títulos de anticorpos IgG2 e tendem a manter esses títulos durante um longo período (Dijkstra et al., 2003; Andreotti et al., 2010).

2.6 Proteína Nc-p43

A identificação de diferenças gênicas entre *T. gondii* e *N. caninum* possibilita a distinção entre essas duas espécies, e contribui na compreensão de sutis diferenças fenotípicas (Hemphill et al., 1999). Dentre as sequências gênicas descritas até o momento a maioria codifica antígenos que se encontram na superfície do parasito, como *NcSRS2* que codifica o antígeno de membrana Nc-p43 e apresenta 44% de homologia com *TgSRS2* em *T. gondii* (Howe et al., 1998). No entanto, ensaios imunológicos que utilizam esta proteína como alvo não apresentam reações cruzadas com *T. gondii* (Borsuk et al., 2011; Dong et al., 2012).

O antígeno Nc-p43 foi a primeira proteína de superfície de *N. caninum* a ser clonada (Hemphill & Gottstein, 1996), cujo os epítopos são majoritariamente de natureza proteica . Estudos *in vitro* demonstram que o bloqueio desta proteína limita a capacidade de adesão e penetração de células hospedeiras pelo parasito salientando a importância desta proteína nestes processos (Lekutis et al., 2001; Nishikawa et al., 2002; Dubey & Schares, 2011). Além disso, estudos *in vivo* também demonstraram que a proteína recombinante Nc-p43 (rNc-p43) tem efeitos protetores contra a encefalite e a transmissão transplacentária (Nishikawa et al., 2002; Haldorson et al., 2005), enquanto vacinas que utilizam antígenos totais do parasito demonstram eficácia abaixo de 25% (Weston et al., 2012). Estes resultados corroboram com estudos onde a proteína Nc-p43 foi capaz de gerar uma forte resposta imune em animais infectados, sendo, portanto, um candidato interessante para diagnóstico e antígeno vacinal (Jung et al., 2004).

2.7 Proteína Nc-p29

Hemphill e colaboradores (1997) caracterizaram a glicoproteína Nc-p29 de 36 KDa localizada tanto na superfície do parasito quanto no interior dos grânulos densos, e em 1998 Howe et al. clonaram uma proteína idêntica de 29 KDa.

O antígeno de superfície imunodominante Nc-p29 codificado pelo gene *SAG1* de *N. caninum* representa uma boa alternativa de relevância vacinal e para o diagnóstico da neosporose (Moraveji et al., 2012; Ibrahim, 2013; Takashima et al., 2013). Este antígeno apresenta uma homologia de 30% com a proteína 29 de *T. gondii* (Howe et al., 1998). Entretanto, estudos utilizando a proteína Nc-p29 na sua forma recombinante (rNc-p29) como antígeno em ELISA (Chahan et al., 2003), ensaio imunocromatográfico (Liao et al., 2005), imprint (Wilkowsky et al., 2011) e aglutinação em latex (Moraveji et al., 2012) apresentaram sensibilidade e especificidade satisfatórias quando comparados a IFI. Além disso, Hiasa e colaboradores (2012) descreveram a capacidade de se utilizar a anticorpos gerados contra a proteína Nc-p29 como marcadores de infecção aguda em bovinos infectados experimentalmente, já que níveis identificáveis desta molécula foram constatados somente até 30 dias pós aborto.

No entanto, a utilização direta da proteína rNc-p29 como antígeno vacinal apresentou um baixo efeito protetivo contra a infecção cerebral em camundongos infectados experimentalmente, quando comparado com uma combinação de vacina de DNA e imunização conjunta de Nc-p29 (Cannas et al., 2003).

2.8 Diagnóstico

O diagnóstico da neosporose pode ser realizado com a identificação de lesões microscópicas compatíveis a patologia, e ensaios que identificam a presença do parasito nos tecidos, como a reação em cadeia da polimerase (PCR) e a imuno-histoquímica utilizando soros hiperimunes contra o antígeno bruto (Dubey & Schares, 2011). A confirmação da neosporose como causa do aborto não deve ser baseada somente no método sorológico (Sager et al., 2001; Pereira-Bueno et al., 2003). O resultado sorológico positivo em um animal com histórico de aborto indica somente que ocorreu exposição do animal ao parasito, sendo necessário demonstrar o protozoário no feto e, se possível, incluir os dados histopatológicos

para confirmar o diagnóstico (Pereira-Bueno et al., 2003), tais como: encefalite multifocal não supurativa, necrose focal do miocárdio, miocardite não supurativa, com posterior identificação do agente por imunoistoquímica (Dubey & Schares, 2006; Pescador et al., 2007). Se o exame do soro materno, fluídos corporais fetais ou tecidos fetais for positivo para *N. caninum*, o aborto pode estar associado à neosporose, porém é importante relacionar outras causas potenciais e/ou a detecção direta do parasito em lesões cerebrais e cardíacas (Dubey & Schares, 2006). No entanto, ensaios sorológicos são essenciais em levantamentos epidemiológicos. Dentre os testes que detectam anticorpos, a imunofluorescência indireta (IFI) é considerada o teste de referência (Dubey et al., 1988; Ghalmi et al., 2014).

Outros formatos de testes foram descritos para identificar anticorpos específicos, tais como o ensaio imunoenzimático (ELISA) (Ahn et al., 2003; Byren et al., 2012) e teste de aglutinação (*Neospora* Agglutination Test) (Romand et al., 1998). Contudo, os ensaios de ELISA desenvolvidos apresentaram menor especificidade quando comparados a IFI, por se basearem na utilização de antígenos solúveis totais do parasito, incluindo antígenos intracelulares, enquanto a IFI utiliza taquizoítos fixados intactos, prevalecendo os antígenos de superfície do parasito (Bjorkman & Uggla, 1999).

Ensaios que utilizam antígenos espécie-especificos de *N. caninum* apresentam maior sensibilidade e especificidade quando comparados à técnica padrão (Borsuk et al., 2011; Dong et al., 2012; Moraveji et al., 2012; Selahi et al., 2013). Anticorpos policlonais e monoclonais contra antígenos nativos específicos de *N. caninum* foram desenvolvidos com o objetivo de aperfeiçoar o diagnóstico (Latif & Jakubek, 2008; Uchida et al., 2013). No ano de 2011 Sohn e colaboradores relataram um painel de anticorpos monoclonais (mAbs) contra proteínas nativas de *N. caninum,* onde os mAbs gerados reconheceram uma variedade de proteínas presentes em micronemas, roptrias, grânulos densos, mitocôndrias, apicoplasto, complexo membrana interna, e superfície do parasito.

A obtenção da proteína nativa é laboriosa devido à necessidade de grande quantidade de taquizoítos obtidos através de cultivo celular, portanto, antígenos recombinantes representam uma alternativa importante, devido à possibilidade de

produção em quantidades significativas, facilitando a geração de insumos e padronização de ensaios sorológicos (Dubey & Schares, 2006). A identificação e escolha de alvos antígenicos específicos baseiam-se em estudos do perfil proteômico do parasito (Ferreirinha et al., 2014). Dentre os antígenos protéicos descritos até momento, os mais estudados encontram-se na superfície (SAG) ou associados à superfície (SRS) (Weston et al., 2012; Ibrahim, 2013), como as proteínas Nc-p29 e Nc-p43 respectivamente.

Na ultima década alguns ensaios ELISAs para o diagnóstico da neosporose foram relatados utilizando as proteínas recombinantes Nc-p43 (Borsuk et al., 2011; Sinnott et al., 2015) e Nc-p29 (Ibrahim, 2013), além de outros formatos de testes diagnósticos com a rNc-p29 como aglutinação em látex (Moraveji et al., 2012), imprint (Wilkowsky et al., 2011) e anticorpos policlonais monoespecíficos desenvolvidos contra o antígeno rNcp-43, os quais apresentaram potencial para detecção do parasito (Sa et al., 2014).

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

As proteínas recombinantes Nc-p29 e Nc-p43 são antigênicas e reconhecidas por anticorpos presentes em soros de animais infectados. Anticorpo específico anti-rNc-p43 é capaz de reconhecer taquizoítos e bradizoítos de *N. caninum*, podendo ser aplicado em ensaios diagnósticos.

3.2 Objetivo Geral

Desenvolver insumos biológicos e padronizar ensaios de diagnóstico da neosporose.

3.3 Objetivos Específicos

- Obter as proteínas recombinantes Nc-p29 e Nc-p43 de *N. caninum*;
- Avaliar a reação das proteínas produzidas com anticorpos de soros imunes de animais naturalmente infectados com *N. caninum;*
- Produzir um anticorpo policlonal (pAb) anti-rNcp43 ;
- Avaliar a reação do pAb produzido através de ELISA, *Western blotting* e Imunofluorescência com as proteínas recombinantes e nativas de taquizoítos;
- Avaliar a utilização do pAb como insumo diagnóstico quando conjugados a enzima e fluoróforo em ELISA direto e Imunofluorescência direta, utilizando taquizoítos como antígeno alvo;
- Desenvolver, padronizar e avaliar um teste de ELISA, para identificação de anticorpos anti Neospora em soros bovinos e de outras espécies acometidas, baseados nas proteínas recombinantes.

4 CAPÍTULOS

4.1.1 Artigo 1 – Proteins of the *Neospora caninum* apical complex as targets of new immunotherapeutic tools

Artigo submetido na revista Journal of Veterinary Diagnostic Investigation

Proteins of the *Neospora caninum* apical complex as targets of new immunotherapeutic tools

Gizele L. Sá[1*], Magda V. Benavides[2], Camila B. Bender[1] and Sibele Borsuk[3]

[1]Laboratório de Imunodiagnóstico and [3]Laboratório de Pesquisa em Doenças Infecciosas, Núcleo de Biotecnologia-CDTec, Universidade Federal de Pelotas, Corredor da Embrapa s/n, CEP 96160-000, Capão do Leão, RS, Brasil.

[2]Embrapa Pesca e Aquicultura, Av. LO 1, Quadra 104 Sul, Conjunto 4, CEP: 77020-020, Palmas, TO, Brasil.

[*]Corresponding author: gezelha@hotmail.com Phone: + 55 (53) 32757350

Running Title: Proteins of the *Neospora caninum* as potential vaccine targets

Abstract

Abortion caused by *Neospora caninum* infection is a major cause of economic losses to the cattle industry worldwide. To prevent infection and/or abortion, considerable research funds have been spent over the years to develop an effective vaccine, highly specific and sensitive diagnostic tests, and other control measures. Though a commercial vaccine is available in some countries, it is of unknown efficacy. The vaccine is based on inactive *N. caninum* tachyzoites, though other approaches based on specific antigens or attenuated strains may also be feasible. A number of studies have been undertaken to identify and characterize species-specific proteins from Apicomplexan parasites to improve vaccines and serological diagnostic control strategies. Proteins involved in adhesion, invasion and attachment of the parasite to the host cells may also confer protection against *N. caninum* infection. Such proteins represent important targets for the development of control strategies against bovine neosporosis. In this review we address the expression patterns of *N. caninum* proteins during the host cell invasion process, as well as the relevance and applications of these proteins to the development of new immunotherapeutic tools for the control of neosporosis.

Key Words: Apicomplexa, *Neospora caninum*, diagnostic tests, vaccine

INTRODUCTION

The phylum Apicomplexa includes many parasites of importance to human and animal health. Plasmodium, the causative agent of malaria, resulted in 584 000 deaths in the year 2013.[131] Cryptosporidium, a parasite that causes cryptosporidiosis, results in serious morbidity and high mortality in immunocompromised individuals.[83,101,132] *Toxoplasma gondii* causes severe congenital toxoplasmosis and death in immunocompromised individuals[65] and *Neospora caninum*, which to date shows no records of infection in immunocompetent humans, causes abortions in cattle, and represents costs of millions of dollars annually to global cattle farming.[20,24,104,107]

The economic importance of neosporosis has become the driving force in developing strategies to prevent or control the disease.[33,107] Control programs including early diagnosis, slaughter of infected animals, chemotherapy, and vaccination have been proposed as measures to reduce the transmission of the parasite, and have contributed to the reduction in cases.[107,105] However, the available commercial vaccine based on inactivated tachyzoites [Bovilis® Neoguard, Intervet] show conflicting results,[66,123] and gives only partial immunity.[114] Non-commercial vaccines using isolated live non-virulent tachyzoites has conferred protective immunity with a 90% reduction in vertical transmission for mice in pregnancy,[43] 50% protection against *Neospora* induced abortion in cattle,[133] and 100% protection against vertical transmission and bovine fetal death, when administered as an intravenous immunization.[112] However, these vaccines are economically untenable due to the various logistical problems arising in production, handling, quality control, shelf-life (limited), and risk of reversion to their virulent phenotype.[133]

Diagnosis of neosporosis can be accomplished by identifying microscopic lesions consistent with the disease pathology, and by assays that identify the presence of the parasite in tissues,

such as the polymerase chain reaction (PCR) and immunohistochemistry using hyperimmune sera that react with *N. caninum* surface antigens.[40] Serological tests are essential in sero-epidemiological surveys. Among the tests that detect antibodies, indirect fluorescence antibody test (IFAT) is considered the golden test.[38,47] Other test formats have been described to identify specific antibodies, such as enzyme-linked immunosorbent assay (ELISA),[3] and agglutination test (Neospora Agglutination Test).[113] However, the ELISAs developed, since they are based on the use of soluble parasite antigens, (including intracellular antigens) have shown a lower specificity as compared to IFAT which uses intact fixed tachyzoites, the parasite´s surface antigens thus prevailing.[14]

In vaccine development, the use of specific antigenic proteins with relevance to *N. caninum*'s maintenance cycle may represent an advantage when inducing a protective immune response.[43] Assays using species-specific proteins show higher sensitivity and specificity when compared to standard techniques.[16,34,92,117] Therefore, identification of specific *N. caninum* proteins is an important step in defining vaccine, drug, and diagnostic targets.[57,85] The functions and expression patterns of such proteins are increasingly used to define the parasite's developmental stages, and to morphologically differentiate similar Apicomplexan species [50] such as *N. caninum* and *T. gondii*.[76]

Considering that neosporosis remains a problem for livestock, and that many of its proteins (being used for diagnoses) have already presented initial results,[40] the present review summarizes its transmission, the host-parasite interaction and the potential for some of its antigens in developing vaccines and diagnostic tests.

BIOLOGICAL ASPECTS OF *N. CANINUM*

N. caninum is an obligate intracellular parasite protozoan belonging to the phylum Apicomplexa, the family Sarcocystidae, sub-family Toxoplasmatinae, genus *Neospora*, and

species *Neospora caninum*.[38] *N. caninum* infections cause neosporosis, resulting in neuromuscular disorders (in definitive hosts), and neonatal morbidity and abortions (in intermediate hosts).[40] *N. caninum* has canines as its definitive hosts, yet a wide range of mammals are susceptible to the parasite, as intermediate hosts. [26,48] However, due to its high incidence in cattle, neosporosis is usually characterized as a disease of relevance to animal health, and of economic importance to cattle industry.[10,53]

All Apicomplexan species have a number of phylum-specific organelles in their apical region called the apical complex and responsible for active penetration into the host cell.[75] Further, Apicomplexan parasites are capable of modulating the host's cellular environment via protein export to the surface of the parasitized cell.[103] This permits development of immune system escape mechanisms and maintenance of the parasite's biological cycle. Because of this cellular panorama, identification and location of specific antigenic Apicomplexan parasite proteins becomes important. Identification of these proteins allows insights into their roles in the metabolic routes, and in the host-parasite interaction.[135]

Several aspects of the parasite biological cycle are not fully understood, particularly the intestinal phase in the definitive host, known as the sexual stage.[80] Throughout its cycle, the parasite has three different forms: tachyzoites and bradyzoites, (intracellular forms found in definitive hosts and intermediaries), and the oocysts containing sporozoites eliminated in the feces of the definitive hosts.[87,88] After sporulation, which takes place in the environment, these oocysts become infective to intermediate hosts.[106] Tachyzoites are highly infectious and capable transplacental infection in any host.[39]

Intermediate host transmission can be horizontal through oocyst intake, or vertical via transplacental infection with tachyzoites.[134] In cattle, the main route of transmission is trans-placental, and depending on the gestational stage, abortion, stillborn birth, or the birth of a

clinically healthy calf (though chronically infected) may occur.[118] Exogenous transplacental transmission occurs when the female contracts a primary infection during pregnancy, through oocyst ingestion, which culminates with the infection of the fetus.[134] Endogenous transplacental transmission occurs when a chronically infected female, possibly congenitally, reactivates cysts during pregnancy, resulting in the conversion of bradyzoites into tachyzoites that reach the fetus.[91] Lactogenic transmission in cattle has been demonstrated under laboratory conditions; however, there is no confirmation of the occurrence of this type of transmission in nature.[29]

N. caninum tachyzoites are found in many types of host tissues and various cell types such as nerve cells, macrophages, fibroblasts, endothelial cells, myocytes, renal tubular epithelial cells, and hepatocytes.[57] This suggests that the parasite exhibits low specificity, and is capable of invading a wide variety of nucleated cells. In contrast, bradyzoites are found within tissue cysts, predominantly in the central nervous system,[18,49] that features an escape mechanism from the host immune system.

The host immune response and other physiological factors stimulate tachyzoite cell penetration, and conversion into bradyzoites which are very similar to tachyzoites, however smaller and with slower metabolisms. This allows the formation of tissue cysts surrounded by a solid cyst wall,[97] and is responsible for chronic infections that may continue yet without clinical manifestation for many years.[49] However, reactivation of the disease may occur during episodes of immunosuppression, such as in pregnancy, which often leads to bradyzoite conversion into metabolically active tachyzoites, which are responsible for the acute infection phase.[39] The point of interaction between the tachyzoite and the host cell is the protein profile expressed by the apical complex organelles.

HOST-PARASITE INTERACTION

To identify new and specific antigens with potential for vaccine development, and to improve diagnostics; analyzing *N. caninum's* proteomic profile becomes important, and consequently, an understanding of the various aspects of its biological life cycle, such as parasite adhesion, fixation, penetration, development, progression, and pathogenicity is required.[45]

The initial recognition of the host cell, adhesion, and then invasion are mechanisms mediated by surface antigens.[93] An arsenal of proteins is secreted, being orchestrated by specialized secretory organelles, present at the apical complex called micronemes, rhoptries, and dense granules.[8,78,100] Yet, the host-parasite interaction is heavily dependent on the host immune response, which directly influences the proteome expressed by the parasite, the infection outcome, and ultimately, the elimination or survival of the pathogen.[59]

In the past, the antigens identified and characterized during the host-parasite interaction were discovered on the cell surface (SAG), or related to surface antigens (SRS), through the direct contacts established between the parasite and the host cell. These contacts are mediated through cell receptors,[58,63,64] and via rhoptry (ROP), microneme (MIC) or dense-granule (GRA) activity (Table 1).[57]

Advances in two-dimensional electrophoresis techniques (2-DE) and mass spectrometry (MS), together with bioinformatics have enabled mapping and identification of new *N. caninum* stage-specific proteins.[85,86] These include GRA14,[81] MIC11,[100] and MIC13[27] proteins; respectively present in the dense granules and micronemes. In addition, progress has been made on the molecular composition of these organelles, obtained using the molecular interaction model of Apicomplexa host-parasite *T. gondii*, which shares remarkable similarities with the *N. caninum* morphology and antigenic profile.[5,31,59,56,90] This was possible because the basic mechanisms of the host-parasite interaction are conserved among the parasites of this phylum.[21]

THE STUDIED ANTIGENS

Surface Antigens

The invasion process starts with recognition of the host cell and low affinity tachyzoite adhesion to the surface of the plasma membrane; followed by an adhesion process which provides a stable association between the tachyzoite and the cell surface.[59] Ligand/receptor interactions, which allow tachyzoites to begin their invasion, are required. In 2013, Uzeda et al.[128] emphasized the importance of surface antigens, since the use of a monoclonal antibody, generated against a ≈38 kDa surface protein improved sensitivity for tachyzoite detection in immunohistochemistry (IHC) test.

At the molecular level, the recognition and the initial low affinity contacts are mediated, at least in part through *N. caninum* tachyzoite immunodominant surface antigens such as NcSAG1 and NcSRS2 (Fig. 1), both already characterized and anchored to plasma membrane phospholipids.[63,116] Polyclonal and monoclonal antibodies directed against these antigens inhibit both adhesion and consequent invasion of the host cell.[52] Monoclonal antibodies produced against a *N. caninum* surface protein ≈73 kDa inhibited the invasion process,[127] implicating its involvement. A monoclonal antibody generated against another *N. caninum* ≈65 kDa glycosylated surface antigen revealed potential to diagnose bovine neosporosis.[12] These studies demonstrate the importance of surface proteins in the initial stage of the invasion process.

The immunodominant protein NcSRS2 has been widely used in vaccine trials, with satisfactory results in a murine model; lowering both the blood[99] and brain parasite loads[98] when used in recombinant form as an immunogen, or expressed in a *Brucella abortus* strain vaccine in combination with other antigens.[102] Gerbils immunized with NcSRS2 antigen, associated with *N. caninum* dense granule protein 1 (GRA1) also showed reductions in

clinical signs when compared to the control group.[25] However, these results do not occur when the NcSAG1 protein is used directly in a recombinant form as an antigen vaccine. The best results occur when used in a DNA vaccine format.[19] Another surface protein which may be of relevance to future vaccine studies is the transmembrane protein Ncp40. The use of this recombinant protein in ELISA tests revealed a sensitivity and specificity of approximately 98% in naturally infected bovine sera.[54]

Microneme antigens

For parasites belonging to the Apicomplexa phylum, the invasion process is active, depending on motility and resulting in the formation of a parasitophorous vacuole.[42] The micronemes are the first organelles to be secreted by the tachyzoite in the initial stage of invasion. These micronemes then secrete proteins with structural domain adhesives that can interact with surface receptors of target cells[115] allowing fixation.

It is believed that *N. caninum* microneme proteins (NcMICs), (by analogy with *T. gondii*) are involved in the formation of complexes promoting the development of a bridge between the parasite and host cell (Fig. 1).[22,36,37] Yet, it is also known that these proteins are related to host specificity. Since, in spite of *N. caninum* and *T. gondii* MIC13 proteins being orthologous, the binding capacity of Nc MIC13 is narrower, preventing neosporosis in humans.[27]

Of the MICs described in the literature, four have been characterized in *N. caninum*. The soluble proteins, NcMIC1,[72] NcMIC2[82] and NcMIC4,[73] which undergo proteolytic processing for activation of their functions, and NcMIC3 which shows no evidence of this processing and is expressed in the organelle membrane.[94] The mechanism of action of these proteins is still unknown, however there is evidence in *T. gondii* that MICs 1/2/3/4 act by forming complexes with each other,[71,109] which represent virulence factors.[46] Mice vaccination with *T. gondii* strain attenuated by deletion of the MIC1 and MIC3 genes, induced a mild form of the

disease, and also protected against chronic and congenital toxoplasmosis.[67] Further, in subsequent work, cross-immunity, against lethal *N. caninum* infection using the same attenuated vaccine was obtained.[96]

Studies have revealed the importance of the MIC protein complex. Strains of *T. gondii* with the *MIC1* gene deleted showed invasion reductions as compared to wild strains.[23] Anti-NcMIC2 and anti-NcMIC3 used in combination with antibodies generated against rhoptry protein-2 (ROP2) reduced the invasive capacity of tachyzoites by 75% when compared to the use of anti-ROP2 antibodies alone at 50 %.[30] The combination of these protein complexes varies according to genus of the Apicomplexa and distinguishes the host target cell.[27]

Another microneme protein MIC10 (NcMIC10), is potentially immunodominant and specific to *N. caninum.* Antibodies generated against this recombinant protein were able to recognize the native protein present in tachyzoites, and showed no cross-reactivity with *T. gondii* MIC10.[137] The anti-TgMIC10 antibodies also displayed no cross-reactions with *N. caninum* MIC10.[28] Further, direct application of NcMIC10 for diagnosis of neosporosis may be relevant to the detection of current infections, because studies by Yin and collaborators[137] revealed the presence (with peaks) of MIC10 in pregnant goats experimentally infected with *N. caninum* until the occurrence of abortion, in Capture ELISA using a standard recombinant NcMIC10 curve, has indicating its potential for use in diagnostic tests.

Studies recently aimed at identifying the proteins expressed during cell invasion have noted the overexpression of the NcMIC11 protein.[100,111] However, there is no indication of immunologic reactivity, and therefore future studies are required to determine its function and relevance to the *Neospora caninum* life cycle.

Rhoptry antigens

The rhoptries are organelles that integrate the apical complex of Apicomplexan parasites, they exhaust their contents after tachyzoite fixing to the host cell surface, following microneme exocytosis, thereby contributing to the formation of the parasitophorous vacuole.[41] Evidence suggests that the rhoptry neck proteins such as RON2, RON4, and RON5 are conserved in parasites of the phylum,[7,6] and RON8 is common to both *N. caninum* and *T. gondii* (Fig. 1).[13]

The identified RONs all present proteolytic processing for activation of their functions.[5,13] Rhoptry neck proteins are exported to the host cell membrane early in the target cell invasion, RON2 is an integral membrane protein that interacts with the parasite surface proteins and synergistically with RONs 4/5/8 anchored on the cytosolic face of the plasma membrane.[13] At the end of the invasion process such proteins help comprise the parasitophorous vacuole membrane.

Debache and collaborators[32] showed the importance of another rhoptry protein named ROP2, localized in the organelle bulb with a molecular weight ≈43 kDa. This protein conferred protection against infection by *N. caninum*, associated with antibodies anti-NcROP2 IgG1 and IgG2; being used as an immunogen.[32,31] In addition, recombinant antibodies generated against ROP2 used in an inhibition assay of host cell invasion, were able to significantly reduce the invasive capacity of *N. caninum* tachyzoites.[30] Rhoptry antigens present suitable targets for the development of preventive vaccination strategies and early diagnosis.

Dense granule antigens

Exocytosis of dense granules occurs during the process of host cell invasion and afterwards. It is believed that dense granule (GRA) proteins are involved in both maturation,[17] and maintenance of the parasitophorous vacuole towards survival and replication of the parasite (Fig. 1).[95] Previous studies have determined the specificity of certain *N. caninum* dense granule proteins (NcGRA); such as NcGRA1, NcGRA2, and NcGRA7,[77,76] since it does not

present cross-reactions with other parasites of the same phylum, even when sharing a certain low GRAs homology with *T. gondii*.[119]

A vaccine trial conducted by Ellis et al.[43] using recombinant NcGRA1 and NcGRA2 antigens demonstrated immunogenicity and a small capacity to reduce transplacental infection in experimentally infected mice when compared to the control group vaccinated with live tachyzoites of avirulent strain. There is still a gap in the evaluation of these antigens in diagnostic assays against neosporosis. However when these antigens were tested in ELISA assays, to identify anti-GRA1 and anti-GRA2 *T. gondii* antibodies, they were highly sensitive.[35,120,62] In addition, a DNA vaccine using the coding sequence of the *T. gondii* GRA1 gene was able to prolong survival in experimentally infected mice.[136]

In turn, the GRA7 protein of *N. caninum* is widely applied in vaccine studies and in diagnosis of neosporosis,[60,68,69,70] due to its high specificity, lack of cross-reactivity with related parasites,[128] and correlation with virulence.[70] Also, anti-NcGRA7 antibodies were detected in experimentally infected dogs, yet mostly in the acute phase of the disease,[61] which sets up its potential for use to distinguish current infections. However, Jimenez-Ruiz and collaborators[69] found a fail in protection against cerebral neosporosis and vertical transmission in pregnant mice immunized with recombinant GRA7 protein from *N. caninum* when challenged with a virulent strain, probably due to an imbalance in Th1/Th2 immune response modulation.

Recently a new dense granule protein called GRA14 was identified in *N. caninum* tachyzoites.[81] Future studies characterizing and investigating its antigenicity and specificity should be conducted to reveal its applicability in vaccines or for diagnostics.

Bradyzoite antigens

Although some antigens such as NcGRA7[2] and SRS2[121] are common to both tachyzoite and bradizoite stages, variations in metabolism and parasite mitochondrial function should be

taken into consideration because they are correlated directly with the proteomic profile of the stage, and immune response.[15]

Accordingly, Marugan-Hernandes et al.[2] describe the super-expression of ROP9 and GRA9 proteins as possible *N. caninum* chronic infection markers, due to their location (after invasion) on the parasitophorous vacuole membrane, and for being bradyzoite stage-specific. Another antigen has been described as a possible marker of chronic infection, with 87% homology and similar weight to a heat shock protein (HSP) specific for *T. gondii* bradyzoites (HSP21).[130] The surface antigens of *N. caninum* SAG4, BSR4, and SRS9 exhibit respectively 69%, 66%, and 59% homology with the proteins SAG4, and BSR4, SRS9 present in *T. gondii*.[44,110] These might also be used as chronic infection markers due to the absence of these proteins in tachyzoites. However, future studies are needed to characterize them as to their role in the conversion and reconversion processes of tachyzoites / bradyzoites.

It is noteworthy that some of the proteins identified in bradyzoites have been studied as therapeutic targets for diseases caused by parasites of the Apicomplexa phylum; such as blockage of the metabolic pathway of phospholipids in *Plasmodium*,[9] topoisomerase I inhibitors,[84] and HSP blockers that affect parasite invasion and conversion.[11]

With regard to vaccine development and improvement, antigens which are important to the survival of the parasite, such as ROP9 or GRA9 being likely involved in the formation of the parasitophorous vacuole (VP),[108] and tubular network which delimits the membrane VP[1] respectively, are potential candidates. Although proteomic studies of *N. caninum* bradyzoites still pose challenges, due to the difficulties involved in obtaining a sufficient quantity of antigen to be applied for trials,[89,129] vaccination studies have begun to be developed. For example, work using a gestacional murine model with primary infection, and recombinant surface proteins NcSAG4, NcBSR4, and NcSRS9,[69] which failed to protect against cerebral

neosporosis or vertical transmission, did however produce a slight reduction in mortality and in maternal parasite load. In contrast, non-pregnant mice vaccinated with recombinant antigens from bradyzoites, and then challenged with a virulent strain showed a significantly smaller cerebral parasitic load when immunized with NcBAG1, NcMAG1, and NcSAG4 proteins as compared to control animals, or when immunized with recombinant antigen NcBSR4.[126]

Further, it is known that Apicomplexa phylum parasites share proteins.[81] Studies have identified several antigens that can be identified in both *N. caninum* and *T. gondii*. *These include* protein disulfide isomerase, heat shock proteins 60 and 70, ribosomal protein 1,[76,79] rhoptry protein of ≈42 kDa,[4] and actin 1,[77] which are all equally important in parasite maintenance and proliferation.[139] An important microneme protein preserved in the Apicomplexa phylum is AMA1. Blocking its action prevents host cell penetration by *Plasmodium* sp.,[124] *Babesia* sp.,[122,125] *T. gondii* [55] and *N. caninum* [138]. Therefore, because of *in vivo* cross-protection, conferred by antigenic relationships between these Apicomplexan parasites, such antigens should be taken into account for development of broad-spectrum vaccines.

CONCLUSIONS

The authors believe that the proteins involved in the processes of adhesion, attachment and penetration of the host cell by the parasite represent targets for vaccination against *N. caninum* infection and for the development of diagnostic tests. We assume that a vaccine could be obtained through the use of multiple and specific antigenic proteins. However, a rigorous and comprehensive assessment of the available data is necessary in order to define the best type of vaccine and the best biotechnology strategy for its production.

Studies indicate that certain proteins involved in the physical parasite-host interaction reveal the potential to mediate protective immunity. However, the life cycle of the parasite is complex and not yet fully understood, making it necessary to incorporate new vaccine antigens into the existing arsenal. Research that seeks to elucidate the cycle and characterization of new cycle maintaining relevant antigens is needed.

Finally, due to the fact that *N. caninum* survival, multiplication, and life cycle completion is host dependent; invasion and intracellular development processes are of crucial importance. The proteins thus involved, represent potential targets for both vaccines and chemotherapy, in addition to specific and sensitive diagnoses.

Reference List

1. Adjogble KD et al. GRA9, a new *Toxoplasma gondii* dense granule protein associated with the intravacuolar network of tubular membranes. Int J Parasitol 2004; 34: 1255-1264.

2. Aguado-Martinez A et al. Characterisation of NcGRA7 and NcSAG4 proteins: immunolocalisation and their role in the host cell invasion by *Neospora caninum* tachyzoites. Acta Parasitologica 2010; 55: 304-312.

3. Ahn HJ et al. ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. Korean J Parasitol 2003; 41: 175-177.

4. Ahn HJ et al. Protease activity and host cell binding of the 42-kDa rhoptry protein from *Toxoplasma gondii* after secretion. Biochem Biophys Res Commun 2001; 287: 630-635.

5. Alaeddine F et al. Molecular cloning and characterization of NcROP2Fam-1, a member of the ROP2 family of rhoptry proteins in *Neospora caninum* that is targeted by antibodies neutralizing host cell invasion in vitro. Parasitology 2013; 140: 1033-1050.

6. Alexander DL et al. *Plasmodium falciparum* AMA1 binds a rhoptry neck protein homologous to TgRON4, a component of the moving junction in *Toxoplasma gondii*. Eukaryot Cell 2006; 5: 1169-1173.

7. Alexander DL et al. Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. PLoS Pathog 2005; 1: 137-149.

8. Alvarez-Garcia G et al. Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Vet Parasitol 2002; 107: 15-27.

9. Ancelin ML et al. Potent inhibitors of Plasmodium phospholipid metabolism with a broad spectrum of in vitro antimalarial activities. Antimicrob Agents Chemother 2003; 47: 2590-2597.

10. Andreotti R et al. Association between seropositivity for *Neospora caninum* and reproductive performance of beef heifers in the Pantanal of Mato Grosso do Sul, Brazil. Rev Bras Parasitol Vet 2010; 19: 119-123.

11. Angel SO et al. A review of recent patents on the protozoan parasite HSP90 as a drug target. Recent Pat Biotechnol 2013; 7: 2-8.

12. Baszler TV et al. Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. J Clin Microbiol 2001; 39: 3851-3857.

13. Besteiro S et al. Export of a *Toxoplasma gondii* rhoptry neck protein complex at the host cell membrane to form the moving junction during invasion. PLoS Pathog 2009; 5: e1000309-

14. Bjorkman C and Uggla A Serological diagnosis of *Neospora caninum* infection. Int J Parasitol 1999; 29: 1497-1507.

15. Bohne W et al. Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. Infect Immun 1994; 62: 1761-1767.

16. Borsuk S et al. Development of an indirect ELISA-NcSRS2 for detection of *Neospora caninum* antibodies in cattle. Vet Parasitol 2011; 177: 33-38.

17. Breinich MS et al. A dynamin is required for the biogenesis of secretory organelles in *Toxoplasma gondii*. Curr Biol 2009; 19: 277-286.

18. Buxton D et al. The comparative pathogenesis of neosporosis. Trends Parasitol 2002; 18: 546-552.

19. Cannas A et al. Vaccination of mice against experimental *Neospora caninum* infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology 2003; 126: 303-312.

20. Cardoso JM et al. A longitudinal study of *Neospora caninum* infection on three dairy farms in Brazil. Vet Parasitol 2012; 187: 553-557.

21. Carruthers V and Boothroyd JC Pulling together: an integrated model of Toxoplasma cell invasion. Curr Opin Microbiol 2007; 10: 83-89.

22. Carruthers VB and Tomley FM Microneme proteins in apicomplexans. Subcell Biochem 2008; 47: 33-45.

23. Cerede O et al. Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence. J Exp Med 2005; 201: 453-463.

24. Chi J et al. Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, Mycobacterium avium subspecies paratuberculosis, and *Neospora caninum*. Prev Vet Med 2002; 55: 137-153.

25. Cho JH et al. Protective efficacy of vaccination with *Neospora caninum* multiple recombinant antigens against experimental *Neospora caninum* infection. Korean J Parasitol 2005; 43: 19-25.

26. Costa KS et al. Chickens (*Gallus domesticus*) are natural intermediate hosts of *Neospora caninum*. Int J Parasitol 2008; 38: 157-159.

27. Cowper B et al. The molecular basis for the distinct host and tissue tropisms of coccidian parasites. Mol Biochem Parasitol 2012; 186: 1-10.

28. Dautu G et al. *Toxoplasma gondii*: detection of MIC10 antigen in sera of experimentally infected mice. Exp Parasitol 2008; 118: 362-371.

29. Davison HC et al. Experimental studies on the transmission of *Neospora caninum* between cattle. Res Vet Sci 2001; 70: 163-168.

30. Debache K et al. Vaccination with recombinant NcROP2 combined with recombinant NcMIC1 and NcMIC3 reduces cerebral infection and vertical transmission in mice experimentally infected with *Neospora caninum* tachyzoites. Int J Parasitol 2009; 39: 1373-1384.

31. Debache K et al. Intraperitoneal and intra-nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in differential effects with regard to protection against experimental challenge with *Neospora caninum* tachyzoites. Parasitology 2010; 137: 229-240.

32. Debache K et al. Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites. Int J Parasitol 2008; 38: 1455-1463.

33. Debache K et al. Vaccination of mice with chitosan nanogel-associated recombinant NcPDI against challenge infection with *Neospora caninum* tachyzoites. Parasite Immunol 2011; 33: 81-94.

34. Dong J et al. Development of a diagnostic method for neosporosis in cattle using recombinant *Neospora caninum* proteins. BMC Biotechnol 2012; 12: 19-

35. Doskaya M et al. Diagnostic value of a Rec-ELISA using *Toxoplasma gondii* recombinant SporoSAG, BAG1, and GRA1 proteins in murine models infected orally with tissue cysts and oocysts. PLoS One 2014; 9: e108329-

36. Dowse T and Soldati D Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis. Curr Opin Microbiol 2004; 7: 388-396.

37. Dowse TJ and Soldati D Rhomboid-like proteins in Apicomplexa: phylogeny and nomenclature. Trends Parasitol 2005; 21: 254-258.

38. Dubey JP et al. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med Assoc 1988; 193: 1259-1263.

39. Dubey JP et al. Congenital transmission of *Neospora caninum* in white-tailed deer (*Odocoileus virginianus*). Vet Parasitol 2013; 196: 519-522.

40. Dubey JP and Schares G Neosporosis in animals--the last five years. Vet Parasitol 2011; 180: 90-108.

41. Dubremetz JF Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. Cell Microbiol 2007; 9: 841-848.

42. Dubremetz JF et al. Apical organelles and host-cell invasion by Apicomplexa. Int J Parasitol 1998; 28: 1007-1013.

43. Ellis J et al. Evaluation of recombinant proteins of *Neospora caninum* as vaccine candidates (in a mouse model). Vaccine 2008; 26: 5989-5996.

44. Fernandez-Garcia A et al. Identification and molecular cloning of the *Neospora caninum* SAG4 gene specifically expressed at bradyzoite stage. Mol Biochem Parasitol 2006; 146: 89-97.

45. Ferreirinha P et al. Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract. Immunology 2014; 141: 256-267.

46. Friedrich N et al. Members of a novel protein family containing microneme adhesive repeat domains act as sialic acid-binding lectins during host cell invasion by apicomplexan parasites. J Biol Chem 2010; 285: 2064-2076.

47. Ghalmi F et al. Comparison of different serological methods to detect antibodies specific to *Neospora caninum* in bovine and canine sera. J Vet Diagn Invest 2014; 26: 136-140.

48. Gondim LS et al. *Toxoplasma gondii* and *Neospora caninum* in sparrows (Passer domesticus) in the Northeast of Brazil. Vet Parasitol 2010; 168: 121-124.

49. Goodswen SJ et al. A review of the infection, genetics, and evolution of *Neospora caninum*: from the past to the present. Infect Genet Evol 2013; 13: 133-150.

50. Graewe S et al. Hostile takeover by Plasmodium: reorganization of parasite and host cell membranes during liver stage egress. PLoS Pathog 2011; 7: e1002224-

51. Guionaud C et al. Molecular characterization of *Neospora caninum* MAG1, a dense granule protein secreted into the parasitophorous vacuole, and associated with the cyst wall and the cyst matrix. Parasitology 2010; 137: 1605-1619.

52. Haldorson GJ et al. *Neospora caninum*: antibodies directed against tachyzoite surface protein NcSRS2 inhibit parasite attachment and invasion of placental trophoblasts in vitro. Exp Parasitol 2006; 112: 172-178.

53. Hasler B et al. Epidemiological and financial considerations for the control of *Neospora caninum* on Swiss dairy farms. Schweiz Arch Tierheilkd 2008; 150: 273-280.

54. He P et al. *Neospora caninum* surface antigen (p40) is a potential diagnostic marker for cattle neosporosis. Parasitol Res 2013; 112: 2117-2120.

55. Hehl AB et al. *Toxoplasma gondii* homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. Infect Immun 2000; 68: 7078-7086.

56. Hemphill A et al. Proteins mediating the *Neospora caninum*-host cell interaction as targets for vaccination. Front Biosci (Elite Ed) 2013; 5: 23-36.

57. Hemphill A et al. The antigenic composition of *Neospora caninum*. Int J Parasitol 1999; 29: 1175-1188.

58. Hemphill A and Gottstein B Identification of a major surface protein on *Neospora caninum* tachyzoites. Parasitol Res 1996; 82: 497-504.

59. Hemphill A et al. Cellular and immunological basis of the host-parasite relationship during infection with *Neospora caninum*. Parasitology 2006; 133: 261-278.

60. Hiasa J et al. ELISAs based on rNcGRA7 and rNcSAG1 antigens as an indicator of *Neospora caninum* activation. Vet Parasitol 2012; 187: 379-385.

61. Hiasa J et al. Enzyme-linked immunosorbent assays based on *Neospora caninum* dense granule protein 7 and profilin for estimating the stage of neosporosis. Clin Vaccine Immunol 2012; 19: 411-417.

62. Holec-Gasior L et al. The optimal mixture of *Toxoplasma gondii* recombinant antigens (GRA1, P22, ROP1) for diagnosis of ovine toxoplasmosis. Vet Parasitol 2014; 206: 146-152.

63. Howe DK et al. The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infect Immun 1998; 66: 5322-5328.

64. Howe DK and Sibley LD Comparison of the major antigens of *Neospora caninum* and *Toxoplasma gondii*. Int J Parasitol 1999; 29: 1489-1496.

65. Innes EA A brief history and overview of *Toxoplasma gondii*. Zoonoses Public Health 2010; 57: 1-7.

66. Innes EA and Vermeulen AN Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. Parasitology 2006; 133 Suppl: 145-168.

67. Ismael AB et al. Mic1-3 knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice. J Infect Dis 2006; 194: 1176-1183.

68. Jia LJ et al. Generation and immunity testing of a recombinant adenovirus expressing NcSRS2-NcGRA7 fusion protein of bovine *Neospora caninum*. Korean J Parasitol 2013; 51: 247-253.

69. Jimenez-Ruiz E et al. Low efficacy of NcGRA7, NcSAG4, NcBSR4 and NcSRS9 formulated in poly-epsilon-caprolactone against *Neospora caninum* infection in mice. Vaccine 2012; 30: 4983-4992.

70. Jimenez-Ruiz E et al. Specific antibody responses against *Neospora caninum* recombinant rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 proteins are correlated with virulence in mice. Parasitology 2013; 140: 569-579.

71. Kaasch AJ and Joiner KA Protein-targeting determinants in the secretory pathway of apicomplexan parasites. Curr Opin Microbiol 2000; 3: 422-428.

72. Keller N et al. Identification of a *Neospora caninum* microneme protein (NcMIC1) which interacts with sulfated host cell surface glycosaminoglycans. Infect Immun 2002; 70: 3187-3198.

73. Keller N et al. Identification and characterization of a *Neospora caninum* microneme-associated protein (NcMIC4) that exhibits unique lactose-binding properties. Infect Immun 2004; 72: 4791-4800.

74. Kobayashi T et al. Gene cloning and characterization of the protein encoded by the *Neospora caninum* bradyzoite-specific antigen gene BAG1. J Parasitol 2013; 99: 453-458.

75. Kohler S et al. A plastid of probable green algal origin in Apicomplexan parasites. Science 1997; 275: 1485-1489.

76. Lee EG et al. Application of proteomics for comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites. J Chromatogr B Analyt Technol Biomed Life Sci 2005; 815: 305-314.

77. Lee EG et al. Establishment of a two-dimensional electrophoresis map for *Neospora caninum* tachyzoites by proteomics. Proteomics 2003; 3: 2339-2350.

78. Lekutis C et al. Surface antigens of *Toxoplasma gondii*: variations on a theme. Int J Parasitol 2001; 31: 1285-1292.

79. Liao M et al. Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. Parasitology 2005; 130: 481-488.

80. Lindsay DS and Dubey JP Determination of the activity of diclazuril against *Sarcocystis neurona* and *Sarcocystis falcatula* in cell cultures. J Parasitol 2000; 86: 164-166.

81. Liu G et al. GRA 14, a novel dense granule protein from *Neospora caninum*. Acta Biochim Biophys Sin (Shanghai) 2013; 45: 607-609.

82. Lovett JL et al. Molecular characterization of a thrombospondin-related anonymous protein homologue in *Neospora caninum*. Mol Biochem Parasitol 2000; 107: 33-43.

83. Mac Kenzie WR et al. A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. N Engl J Med 1994; 331: 161-167.

84. Malik M and Nitiss JL DNA repair functions that control sensitivity to topoisomerase-targeting drugs. Eukaryot Cell 2004; 3: 82-90.

85. Marugan-Hernandez V et al. Identification of *Neospora caninum* proteins regulated during the differentiation process from tachyzoite to bradyzoite stage by DIGE. Proteomics 2010; 10: 1740-1750.

86. Marugan-Hernandez V et al. Identification of novel rhoptry proteins in *Neospora caninum* by LC/MS-MS analysis of subcellular fractions. J Proteomics 2011; 74: 629-642.

87. McAllister D and Latham S *Neospora* 2001. Trends Parasitol 2002; 18: 4-5.

88. McCann CM et al. *Neospora caninum* in cattle: experimental infection with oocysts can result in exogenous transplacental infection, but not endogenous transplacental infection in the subsequent pregnancy. Int J Parasitol 2007; 37: 1631-1639.

89. McGuire AM et al. A protocol for the production of *Neospora caninum* tissue cysts in mice. J Parasitol 1997; 83: 647-651.

90. Mercier C et al. Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? Int J Parasitol 2005; 35: 829-849.

91. Miller CM et al. Characterisation of the first Australian isolate of *Neospora caninum* from cattle. Aust Vet J 2002; 80: 620-625.

92. Moraveji M et al. Development of latex agglutination test with recombinant NcSAG1 for the rapid detection of antibodies to *Neospora caninum* in cattle. Vet Parasitol 2012; 189: 211-217.

93. Naguleswaran A et al. *Neospora caninum* protein disulfide isomerase is involved in tachyzoite-host cell interaction. Int J Parasitol 2005; 35: 1459-1472.

94. Naguleswaran A et al. Vero cell surface proteoglycan interaction with the microneme protein NcMIC(3) mediates adhesion of *Neospora caninum* tachyzoites to host cells unlike that in *Toxoplasma gondii*. Int J Parasitol 2002; 32: 695-704.

95. Nam HW GRA proteins of *Toxoplasma gondii*: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. Korean J Parasitol 2009; 47 Suppl: S29-S37.

96. Penarete-Vargas DM et al. Protection against lethal *Neospora caninum* infection in mice induced by heterologous vaccination with a mic1 mic3 knockout *Toxoplasma gondii* strain. Infect Immun 2010; 78: 651-660.

97. Peters M et al. Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. Int J Parasitol 2001; 31: 1144-1148.

98. Pinitkiatisakul S et al. Immunogenicity and protective effect against murine cerebral neosporosis of recombinant NcSRS2 in different iscom formulations. Vaccine 2007; 25: 3658-3668.

99. Pinitkiatisakul S et al. Quantitative analysis of parasite DNA in the blood of immunized and naive mice after infection with *Neospora caninum*. Parasitology 2008; 135: 175-182.

100. Pollo-Oliveira L et al. Unravelling the *Neospora caninum* secretome through the secreted fraction (ESA) and quantification of the discharged tachyzoite using high-resolution mass spectrometry-based proteomics. Parasit Vectors 2013; 6: 335.

101. Proctor ME et al. Surveillance data for waterborne illness detection: an assessment following a massive waterborne outbreak of *Cryptosporidium* infection. Epidemiol Infect 1998; 120: 43-54.

102. Ramamoorthy S et al. Prevention of lethal experimental infection of C57BL/6 mice by vaccination with *Brucella abortus* strain RB51 expressing *Neospora caninum* antigens. Int J Parasitol 2007; 37: 1521-1529.

103. Ravindran S and Boothroyd JC Secretion of proteins into host cells by Apicomplexan parasites. Traffic 2008; 9: 647-656.

104. Reichel MP et al. What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. Int J Parasitol 2013; 43: 133-142.

105. Reichel MP and Ellis JT *Neospora caninum*--how close are we to development of an efficacious vaccine that prevents abortion in cattle? Int J Parasitol 2009; 39: 1173-1187.

106. Reichel MP et al. Neosporosis and hammondiosis in dogs. J Small Anim Pract 2007; 48: 308-312.

107. Reichel MP et al. Control options for *Neospora caninum* - is there anything new or are we going backwards? Parasitology 2014; 141: 1455-1470.

108. Reichmann G et al. Characterization of TgROP9 (p36), a novel rhoptry protein of *Toxoplasma gondii* tachyzoites identified by T cell clone. Mol Biochem Parasitol 2002; 119: 43-54.

109. Reiss M et al. Identification and characterization of an escorter for two secretory adhesins in *Toxoplasma gondii*. J Cell Biol 2001; 152: 563-578.

110. Risco-Castillo V et al. Molecular characterisation of BSR4, a novel bradyzoite-specific gene from *Neospora caninum*. Int J Parasitol 2007; 37: 887-896.

111. Rocchi MS et al. Selection of *Neospora caninum* antigens stimulating bovine CD4+ve T cell responses through immuno-potency screening and proteomic approaches. Vet Res 2011; 42: 91-

112. Rojo-Montejo S et al. Effect of vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. Vet Res 2013; 44: 106-

113. Romand S et al. Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitol Res 1998; 84: 50-53.

114. Romero JJ et al. Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions. Vet Parasitol 2004; 123: 149-159.

115. Santos JM and Soldati-Favre D Invasion factors are coupled to key signalling events leading to the establishment of infection in apicomplexan parasites. Cell Microbiol 2011; 13: 787-796.

116. Schares G et al. Structural analysis of free and protein-bound glycosyl-phosphatidylinositols of *Neospora caninum*. Mol Biochem Parasitol 2000; 105: 155-161.

117. Selahi F et al. Development of a disperse dye immunoassay technique for detection of antibodies against *Neospora caninum* in cattle. Korean J Parasitol 2013; 51: 129-132.

118. Sengupta PP et al. Prevalence of *Neospora caninum* antibodies in dairy cattle and water buffaloes and associated abortions in the plateau of Southern Peninsular India. Trop Anim Health Prod 2012; 45: 205-210.

119. Shin YS et al. Comparison of proteome and antigenic proteome between two *Neospora caninum* isolates. Vet Parasitol 2005; 134: 41-52.

120. Song KJ et al. A rapid diagnostic test for toxoplasmosis using recombinant antigenic N-terminal Half of SAG1 linked with intrinsically unstructured domain of gra2 protein. Korean J Parasitol 2013; 51: 503-510.

121. Staska LM et al. Identification of vaccine candidate peptides in the NcSRS2 surface protein of *Neospora caninum* by using CD4+ cytotoxic T lymphocytes and gamma interferon-secreting T lymphocytes of infected holstein cattle. Infect Immun 2005; 73: 1321-1329.

122. Tonkin ML et al. Babesia divergens and *Neospora caninum* apical membrane antigen 1 structures reveal selectivity and plasticity in apicomplexan parasite host cell invasion. Protein Sci 2013; 22: 114-127.

123. Trees AJ and Williams DJ Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. Trends Parasitol 2005; 21: 558-561.

124. Triglia T et al. Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. Mol Microbiol 2000; 38: 706-718.

125. Tyler JS et al. Focus on the ringleader: the role of AMA1 in apicomplexan invasion and replication. Trends Parasitol 2011; 27: 410-420.

126. Uchida M et al. Comparative study of protective activities of *Neospora caninum* bradyzoite antigens, NcBAG1, NcBSR4, NcMAG1, and NcSAG4, in a mouse model of acute parasitic infection. Parasitol Res 2013; 112: 655-663.

127. Uchida Y et al. Monoclonal antibodies preventing invasion of *Neospora caninum* tachyzoites into host cells. J Vet Med Sci 2004; 66: 1355-1358.

128. Uzeda RS et al. Combination of monoclonal antibodies improves immunohistochemical diagnosis of *Neospora caninum*. Vet Parasitol 2013; 197: 477-486.

129. Vonlaufen N et al. In vitro induction of *Neospora caninum* bradyzoites in vero cells reveals differential antigen expression, localization, and host-cell recognition of tachyzoites and bradyzoites. Infect Immun 2004; 72: 576-583.

130. Weiss LM et al. Bradyzoite development in *Toxoplasma gondii* and the hsp70 stress response. Infect Immun 1998; 66: 3295-3302.

131. WHO WHO procedures for certification of malaria elimination. Wkly Epidemiol Rec 2014; 89: 321-325.

132. Widerstrom M et al. Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. Emerg Infect Dis 2014; 20: 581-589.

133. Williams DJ et al. Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. Infect Immun 2007; 75: 1343-1348.

134. Williams DJ et al. Endogenous and exogenous transplacental transmission of *Neospora caninum* - how the route of transmission impacts on epidemiology and control of disease. Parasitology 2009; 136: 1895-1900.

135. Woodcroft BJ et al. Determination of protein subcellular localization in apicomplexan parasites. Trends Parasitol 2012; 28: 546-554.

136. Wu XN et al. Multicomponent DNA vaccine-encoding *Toxoplasma gondii* GRA1 and SAG1 primes: anti-Toxoplasma immune response in mice. Parasitol Res 2012; 111: 2001-2009.

137. Yin J et al. Characterization of *Neospora caninum* microneme protein 10 (NcMIC10) and its potential use as a diagnostic marker for neosporosis. Vet Parasitol 2012; 187: 28-35.

138. Zhang H et al. Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii*, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites. Mol Biochem Parasitol 2007; 151: 205-212.

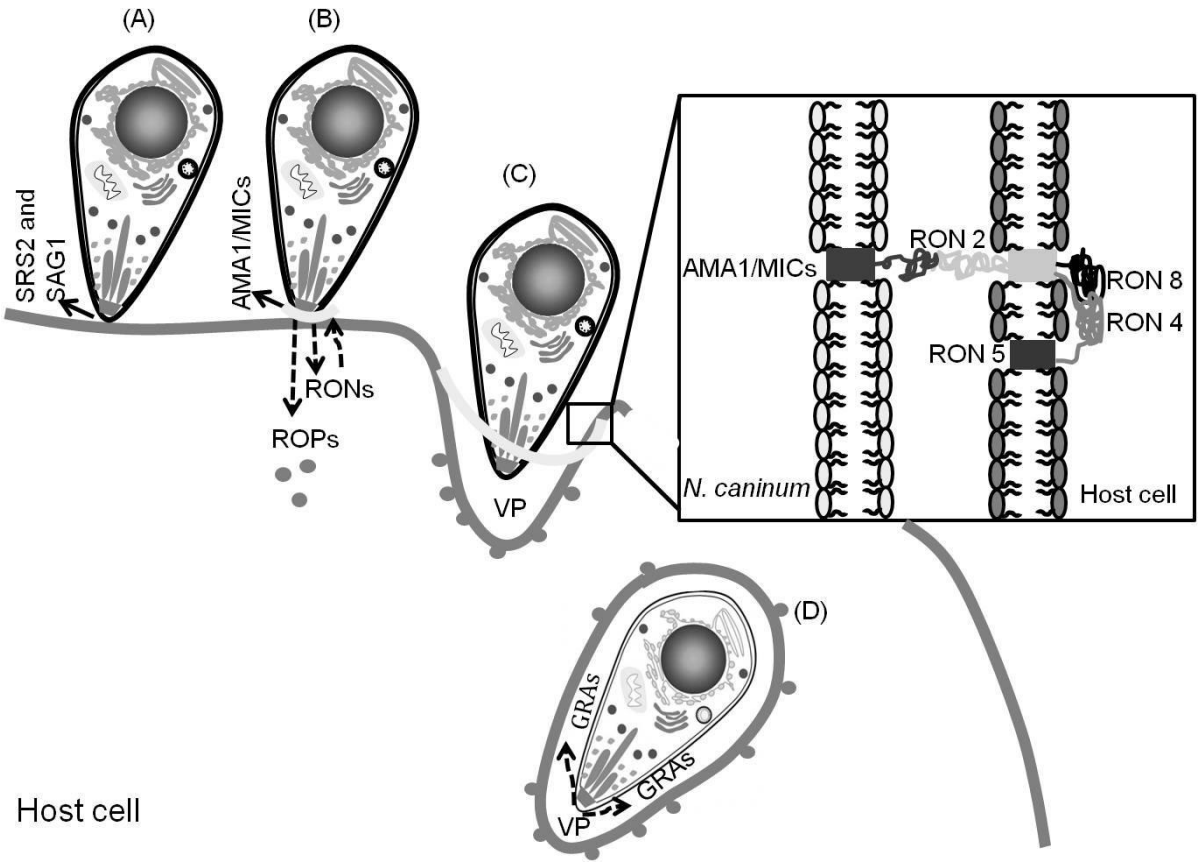
139. Zhang H et al. Identification of the cross-reactive and species-specific antigens between *Neospora caninum* and *Toxoplasma gondii* tachyzoites by a proteomics approach. Parasitol Res 2011; 109: 899-911.

Figure 1: Schematic representation of the host-parasite interaction during the *N. caninum* host cell invasion process. The apical complex organelles are responsible for the process of active penetration into the host cell tachyzoite. (A) The tachyzoite attaches to the host cell through interaction of SRS2 and SAG1 surface proteins expressed on its membrane. (B) the micronemes express their AMA1 and MICs proteins, which are directed to the surface of tachyzoite to mediate adhesion of the parasite complex to the host cell, initiating the invasion through expression of ROP and RON rhoptry proteins (C) ROP proteins are directed at the target cell membrane, while the RON 2/4/5/8 proteins are directed towards the cell membrane of the parasite to interact with AMA1/ MICs allowing the formation of the parasitophorous vacuole [VP] (D) the VP membrane is extensively modified during the invasion of the rhoptry proteins through anchoring and host protein deletions. VP maturation occurs through the expression of dense granules proteins.

Table 1: *N. caninum* antigens characterized by SDS / PAGE under reducing conditions

Recommended designation	Molecular Weight (≈KDa)	Location	Stage	Reference
SRS2	35	Cellular Membrane	Tachyzoite/ Bradyzoite	Howe et al., 1998
SRS9	-	Cellular Membrane	Bradyzoite	Risco-Castillo et al., 2001
SAG1	29	Cellular Membrane	Tachyzoite	Howe et al., 1998
SAG4	21	Cellular Membrane	Bradyzoite	Fernandez-Gracia et al., 2006
BSR4	51	Membrane Cellular	Bradyzoite	Risco-Castillo et al., 2007
Ncp40	40	Cellular Membrane	Tachyzoite	He et al., 2013
-	38	Cellular Membrane	Tachyzoite	Uzeda et al., 2013
-	73	Cellular Membrane	Tachyzoite	Uchida et al., 2004
-	65	Cellular Membrane	Tachyzoite	Baszler et al., 2001
MIC1	50	Micronemes	Tachyzoite	Keller et al., 2002
MIC2	80	Micronemes	Tachyzoite	Lovett et al., 2000
MIC3	38	Micronemes	Tachyzoite	Naguleswaran et al., 2002
MIC4	55	Micronemes	Tachyzoite	Keller et al., 2004
MIC10	18	Micronemes	Tachyzoite	Yin et al., 2012
MIC11	16	Micronemes	-	Rocchi et al., 2011
MIC13	56	Micronemes	Tachyzoites	Cowper et al., 2012
RON2	155	Rhoptries	Tachyzoite	Besteiro et al., 2009
RON4	120	Rhoptries	Tachyzoite	Besteiro et al., 2009
RON5	179	Rhoptries	Tachyzoite	Besteiro et al., 2009
RON8	329	Rhoptries	Tachyzoite	Besteiro et al., 2009
ROP2	43	Rhoptries	Tachyzoite	Debache et al., 2008
ROP9	36	Rhoptries	Bradyzoite	Reichmann et al., 2002
GRA1	29	Dense Granules	Tachyzoite	Lee et al., 2003
GRA2	28	Dense Granules	Tachyzoite	Lee et al., 2003
GRA7	33	Dense Granules	Tachyzoite/ Bradyzoite	Lee et al., 2005
GRA9	41	Dense Granules	Bradyzoite	Adjogble et al., 2004
GRA 14	44	Dense Granules	Tachyzoite	Liu et al., 2013
MAG1	-	Dense Granules	Bradyzoite	Guionaud et al., 2010
BAG1	30	-	Bradyzoite	Kobayashi et al., 2013

Figure 1


(A)
(B)
(C)
(D)
SRS2 and SAG1
AMA1/MICs
RONs
ROPs
VP
AMA1/MICs
RON 2
RON 8
RON 4
RON 5
N. caninum
Host cell
GRAs
GRAs
VP
Host cell

4.1.2 Artigo 2 – Diagnostic Potential of Anti-rNcp-43 Polyclonal Antibodies for the Detection of *Neospora caninum*

Artigo aceito na revista Current Microbiology.

DIAGNOSTIC POTENTIAL OF ANTI-rNcp-43 POLYCLONAL ANTIBODIES FOR THE DETECTION OF *Neospora caninum*

Gizele Lima Sá[1,Ψ], Diene de Borba Pacheco[1,Ψ], Leonardo Garcia Monte[1], Francine Alves Sinnott[1], Marina Amaral Xavier[1], Caroline Rizzi[1], Sibele Borsuk[2], Maria Elisabeth Aires Berne[3], Renato Andreotti[4], Cláudia Pinho Hartleben[1,*]

[1]Laboratório de Imunodiagnóstico and [2]Laboratório de Pesquisa em Doenças Infecciosas, Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brazil

[3]Departamento de Microbiologia e Parasitologia, Instituto de Biologia; Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brazil

[4]Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Gado de Corte, Campo Grande, Mato Grosso do Sul, Brazil

[Ψ] These authors contributed equally to the experimental work.

*** Corresponding author (mailing address): Laboratório de Imunodiagnóstico, Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, CEP 96010-900, Pelotas, RS, Brazil - P.O. Box 354. Phone: + 55 (53) 32757350**

E-mail: hartlebenclaudia@gmail.com

Abstract

Neosporosis is a disease caused by the apicomplexan parasite *Neospora caninum,* which is closely related to *Toxoplasma gondii*. *N. caninum* infection represents an important cause of reproductive failure in sheep, goats, horses, and cattle worldwide. The diagnosis of neosporosis is based on the detection of pathogen-specific antibodies in animal sera or the presence of tissue cysts. However, morphological similarities and serological cross-reactivity between *N. caninum* and *T. gondii* can result in the misdiagnosis. In this study, the *N. caninum* tachyzoite surface protein Ncp-43 was expressed in a recombinant form to elicit polyclonal antibodies (pAb) response. The pAb was purified and conjugated to horseradish peroxidase (HRP) or fluorescein isothiocyanate (FITC) to detect the recombinant and native Ncp-43 proteins, respectively. The pAb and pAb/HRP were able to recognize rNcp-43 by Dot blot and ELISA, and pAb/FITC immunolabeled the apical complex of tachyzoites. A blocking *enzyme-linked immunosorbent assay* (b-ELISA) was performed to evaluate pAb/HRP as a diagnostic tool. The mean percent inhibition for the positive and negative serum samples from cattle with neosporosis was significantly different ($P < 0.0001$). These results suggest that the pAb may bind to the same epitopes of Ncp-43 as anti-*N. caninum* antibodies in the positive samples tested. The b-ELISA using the pAb/HRP can facilitate diagnostic testing for neosporosis, since fewer steps are involved and cross-reactivity with secondary antibodies is avoided. In summary, this report describes the production of antibodies against *N. caninum*, and evaluates the potential of these tools for the development of new diagnostic tests for neosporosis.

Keywords: *Neospora caninum*, neosporosis, ELISA, Ncp-43, diagnosis.

Short Communication

Neosporosis is a disease caused by the apicomplexan parasite *Neospora caninum,* which is closely related to *Toxoplasma gondii* [10]. Infection by *N. caninum* causes neuromuscular disorders in dogs and reproductive failure in sheep, goats, horses, and cattle, resulting in economic losses worldwide [22,11]. The diagnosis of neosporosis is usually based on histopathological and immunohistochemical tests that identify the parasite in tissues or through serological analysis [3]. However, morphological similarities and serological cross-reactivity between *N. caninum* and *T. gondii* can result in the misdiagnosis of neosporosis [20]. The indirect fluorescent-antibody test (IFAT) was first applied to the diagnosis of neosporosis in [9], and has since been widely used as a standard test for serological analysis [9]. In recent years, alternative methods such as the *Neospora* agglutination test [7], the latex agglutination test [19], and *enzyme-linked immunosorbent assay* (ELISA) [11,7,9,21] have been proposed to improve the diagnosis of neosporosis. Despite the variety of strategies, most of these tests are based on the analysis of whole parasite lysates or fixed tachyzoites, and are therefore considered time-consuming and laborious [12]. Thus, single antigens such as parasite surface proteins provide an alternative for the detection of specific antibodies in infected hosts or for the generation of monospecific polyclonal antibodies that can be useful tools for the development of more specific tests [27]. Among these proteins, the antigenic domain of the *NcSRS2* gene (Ncp-43 protein) has been reported as an important antigen from *N. caninum* for use in diagnostic assays [6]. In addition, Ncp-43 is common to both the tachyzoite and bradyzoite stages, and was able to elicit parasite-specific antibodies in the serum of cattle [6,23], sheep [2], and dogs [4]. This report describes the production and evaluation of polyclonal antibodies (pAb) generated against the recombinant Ncp-43 protein (rNcp-43), and examines its potential for the diagnosis of neosporosis.

For recombinant protein production, the antigenic domain of *NcSRS2* gene (rNcp-43 protein), located in the distal C - terminal two thirds of the molecule, was amplified by PCR using primers F5' - CAC CAA AGA GTG GGT GAC TGG and R5' - GGT AAG CTT TGC ATC TCC TCT TAA CAC-3', cloned into pET100/D TOPO vector (Invitrogen Tech, Carlsbad, CA, USA) and used to transform *Escherichia coli* BL21 Star. The *E. coli* cells in the log phase ($OD_{600\ nm}$ = 0,6 – 0,8) were treated with 0.75 - mM isopropyl α-D-thiogalactoside (IPTG) for 3 h at 37 °C to induce expression of

fused fragments of *NcSRS2* gene. The protein was solubilized in a buffer containing 0.2% N-lauroyl sarcosine. rNcp-43 expression was confirmed by 10% SDS-PAGE and western blot using an anti-6 × histidine antibody (Sigma-Aldrich, USA). rNcp-43 was purified by immobilized metal ion affinity chromatography (IMAC) using Ni2+ Sepharose HisTrap columns, following the manufacturer's instructions (GE Healthcare, USA). Purified rNcp-43 was dialyzed against PBS (containing 0.1% glycine; pH 8.0) for approximately 16 h at 4 °C. Fractions of purified rNcp-43 were analyzed by 12% SDS-PAGE in reducing conditions, and final concentrations were determined using bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, USA) with bovine serum albumin as a standard.

Two 6-month-old male New Zealand rabbits were immunized with rNcp-43 following a 30-day adaptation period. For each rabbit, five subcutaneous injections were administered in the scapular area, alternating between the right and left sides. The first immunization dose contained 100 µg rNcp-43 and complete Freund's adjuvant (Sigma-Aldrich, USA). Subsequent immunizations were performed after 7, 14, 21, and 28 days using 100 µg rNcp-43 and incomplete Freund's adjuvant (Sigma-Aldrich, USA). Blood was collected prior to each immunization to determine antibody titers. After the last immunization, indirect ELISA was used to determine the rNcp-43 antibody titer. Hyperimmune serum was obtained from animals and purified by affinity chromatography using a protein A-Sepharose CL-4B column (GE Healthcare, USA) according to the manufacturer's instructions. Purification efficiency was evaluated by 10 % SDS-PAGE, and the final concentration was determined by spectrophotometry at 280 nm. The animals used in this study were treated in accordance with the guidelines recommended by Conselho Nacional de Controle de Experimentação Animal (CONCEA).

The detection of Ncp-43 in its recombinant and native forms was evaluated by ELISA, Dot blot, and direct and indirect immunofluorescence (IF). For direct assays, the pAb was conjugated to horseradish peroxidase (pAb/HRP) or fluorescein isothiocyanate (pAb/FITC) according to established procedures [15]. *ELISA*: Polystyrene ELISA microtiter plates (Nunc Polysorp; Nalge Nunc International, Rochester, USA) were coated with rNcp-43 (50 ng/well). The wells were washed and blocked with 5% nonfat milk in PBS, and serial dilutions (1:100 - 1:512,000) of pAb or pAb/HRP were added to the wells for 1 h at 37 °C. In parallel, control wells coated

with pAb only were treated with HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, USA) for 1 h at 37 °C. After repeated PBS washes, substrate solution (0.4 mg/mL o-phenylenediamine and 0.03% H_2O_2 in 0.1 M citrate buffer, pH 4.0) was added to the wells. The colorimetric reaction product was detected at an absorbance of 450 nm using a VICTOR X5 Multilabel Plate Reader (PerkinElmer, USA). *Dot blot*: Nitrocellulose membranes (GE Healthcare, UK) were coated with rNcp-43 (50 ng/spot) and incubated at 37 ºC until dry. The membranes were blocked with 5% nonfat milk in PBS, and incubated with pAb (1:6,000) or pAb/HRP (1:500) for 1 h at 37 °C. The membranes probed with pAb only were treated with HRP-conjugated anti-rabbit antibody for 1 h at 37 °C. After three 5-min washes with PBST (0.05% Tween 20), the membranes were incubated in substrate solution (6 mg diaminobenzidine, 0.03% H_2O_2, and 0.03% nickel sulfate in 50 mM Tris-HCl, pH 8.0). Preimmune and hyperimmune (after the last immunization) sera were used as controls. *IF*: The pAb and pAb/FITC were evaluated with Ncp-43 from *N. caninum* tachyzoites of strains Nc-1 [9], Nc-Goiás [13], and Nc-Liv [5], which were gifts from Débora Pereira Garcia and Andrea Caetano da Silva (Federal University of Goiás). The strains were propagated in Vero cells [9], and when 80% of cells infected with *N. caninum* tachyzoites showed cytopathic effects (based on cell integrity, typically 3-4 days p.i), the cell monolayer was removed by scraping, washed twice with PBS, and then centrifuged at 1,000 × *g* for 10 min. The cells were loaded onto chamber slides (ICN Biomedicals Inc., USA) for 1 h at 37 °C. The slides were blocked with 10% fetal bovine serum in PBS, washed twice with PBS, and coated with pAb or pAb/FITC for 1 h at 37 °C. The slides coated with pAb only were treated with the FITC-conjugated anti-rabbit antibody (Sigma-Aldrich, USA) for 1 h at 37 °C. The slides were washed twice with PBS and incubated for 1 h in a dark humid chamber at 37 °C. After PBS washes, a drop of mounting medium was added to the slide and immunolabeling was visualized by fluorescence microscopy (Olympus BX 51) with an excitation wavelength of 450 nm. Preimmune sera and *T. gondii* cells were used as negative controls.

To investigate the utility of the anti-Ncp-43 antibodies (pAb and pAb/HRP) for the serological diagnosis of neosporosis, a blocking *enzyme-linked immunosorbent assay* (b-ELISA) was performed. For this, polystyrene ELISA microtiter plates were sensitized and blocked as described above. Thirteen positive and eleven negative

undiluted serum samples (Laboratory of Parasitology/Federal University of Pelotas, RS, Brazil) from cattle previously tested by IF for neosporosis were added to the plates, which were incubated for 1 h at 37 ºC. The wells were washed three times with PBS and incubated with pAb (1:32,000) or pAb/HRP (1:500) for 1 h at 37 °C. The wells coated with pAb only were treated with the HRP-conjugated anti-rabbit antibody for 1 h at 37° C. The same substrate solution that was used for ELISA was added to the wells for the colorimetric reaction. The reactions were terminated by adding 2N H_2SO_4, and the optical density (OD) was measured at 492 nm using the VICTOR X5 Multilabel Plate Reader. The concentrations for the reagents used in the various steps of the ELISA procedures (*i.e.*, primary antibody, pAb, or pAb/HRP, and the coating antigen) were established by varying the concentration of the reagent added during a particular step while maintaining the conditions for all the other steps at a constant, except for the colorimetric reaction step. The percent inhibition for each dilution was determined by comparing the mean of each of the duplicate wells to the mean of duplicate control wells using the following formula: percent inhibition = [1 – (OD of sample – OD of buffer/OD of negative control – OD of buffer)] × 100 [26]. These experiments were performed in triplicate. All clinical samples were previously tested by IFAT according to established methods [24]. The Student's t-test was used to evaluate mean differences.

In this study, pAb against a single antigen (Ncp-43) from *N. caninum* was produced, purified and evaluated for its potential as a tool in neosporosis immunodiagnostic assays. The generation of antibodies using rabbits is a rapid and inexpensive process, while the use of polyclonal antibodies against an immunodominant epitope has advantages over the use of antibodies to whole antigens in terms of sensitivity and specificity [15]. In addition, pAbs are able to recognize different epitopes on the same antigen, which increases the chances of detection [15]. Accordingly, high titers of antibodies against rNcp-43 were detected by indirect ELISA testing of rabbit hyperimmune sera (1:52,000), b-ELISA (pAb, 1:32,000; pAb/HRP, 1:500), and Dot blot assay (pAb, 1:6,000; pAb/HRP, 1:500), with activity persisting after HRP conjugation (Figure 1).

Direct and indirect IF assays were performed to investigate the interaction of the pAb with native Ncp-43 on the parasite surface. Both the pAb (data not shown) and pAb/FITC (Figure 2) labeled the apical complex of tachyzoites, while no

immunoreactivity was observed when *T. gondii* cells (Figure 2A) or preimmune sera (data not shown) were used.

Since no therapy or effective vaccine is currently available for neosporosis, there is an urgent need to improve strategies to control this disease. Although serological methods have been developed for this purpose [1,11,14,16], most of these assays have limitations including low specificity and sensitivity [8], and some require the preparation of whole parasite lysate [3] or fixed tachyzoites [9], making antigen preparation laborious and the assays costly. b-ELISA is an effective method for pathogen-specific antibody detection, and has been widely used to monitor infectious diseases in animals [17,25,18]. This assay is superior to indirect ELISA as it does not require secondary antibodies specific to the immunoglobulins of the species being tested, and has the added advantage that serum containing IgM can be reliably tested using a single assay [25].

Serum samples from *Neospora*-infected animals were used in a b-ELISA assay in order to evaluate the ability of these antibodies to block the binding site of anti-Ncp-43 pAb. The mean percent inhibition differed significantly between the positive and negative serum samples tested ($P < 0.0001$; Figure 3). These results suggest that the pAb obtained in this study may bind to the same epitopes of Ncp-43 as anti-*N. caninum* antibodies from the species tested. The b-ELISA using an HRP-conjugated pAb can facilitate the performance of diagnostic tests, since fewer steps are involved and cross-reactivity with secondary antibodies is avoided. In conclusion, the anti-rNcp-43 pAb may be useful in the development of different kinds of tests for the diagnosis of neosporosis in livestock.

Acknowledgements

Funding for this study was provided by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Grant AUX-PE-PNPD-1513/2008) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Conflict of Interest

No competing financial interests exist.

References

1. Ahn HJ, Kim S, Kim DY et al (2003) ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. Korean J Parasitol 41:175-177

2. Andreotti R, Matos MF, Goncalves KN et al (2009) Comparison of indirect ELISA based on recombinant protein NcSRS2 and IFAT for detection of *Neospora caninum* antibodies in sheep. Rev Bras Parasitol Vet 18:19-22

3. Atkinson RA, Cook RW, Reddacliff LA et al (2000) Seroprevalence of *Neospora caninum* infection following an abortion outbreak in a dairy cattle herd. Aust Vet J 78:262-266

4. Bandini LA, Neto AF, Pena HF et al (2011) Experimental infection of dogs (*Canis familiaris*) with sporulated oocysts of *Neospora caninum*. Vet Parasitol 176:151-156

5. Barber JS, Holmdahl OJ, Owen MR et al (1995) Characterization of the first European isolate of *Neospora caninum* (Dubey, Carpenter, Speer, Topper and Uggla). Parasitol 111:563-568

6. Borsuk S, Andreotti R, Leite FP et al (2011) Development of an indirect ELISA-NcSRS2 for detection of *Neospora caninum* antibodies in cattle. Vet Parasitol 177:33-38

7. Canada N, Meireles CS, Carvalheira J et al (2004) Determination of an optimized cut-off value for the *Neospora* agglutination test for serodiagnosis in cattle. Vet Parasitol 121:225-231

8. Dong JH, Otsuki T, Kato T et al (2013) Development of two murine antibodies against *Neospora caninum* using phage display technology and application on the detection of *N. caninum*. PLoS One 8:e53264

9. Dubey JP, Hattel AL, Lindsay DS et al (1988) Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med Assoc 193:1259-1263

10. Dubey JP, Lindsay DS (1996) A review of *Neospora caninum* and neosporosis. Vet Parasitol 67:1-59

11. Dubey JP, Schares G (2006) Diagnosis of bovine neosporosis. Vet Parasitol 140:1-34

12. Dubey JP, Schares G (2011) Neosporosis in animals - the last five years. Vet Parasitol 180:90-108

13. García-Melo DP, Regidor-Cerrillo J, Ortega-Mora LM et al (2009) Isolation and biological characterisation of a new isolate of *Neospora caninum* from an asymptomatic calf in Brazil. Acta Parasitol 54:180-185

14. Gaturaga I, Chahan B, Xuan X et al (2005) Detection of antibodies to *Neospora caninum* in cattle by enzyme-linked immunosorbent assay with truncated NcSRS2 expressed in *Escherichia coli*. J Parasitol 91:191-192

15. Harlow E., Lane D (1988) Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press.

16. Hemphill A, Fuchs N, Sonda S et al (1999) The antigenic composition of *Neospora caninum*. Int J Parasitol 29:1175-1188

17. Kit S, Otsuka H, Kit M (1992) Blocking ELISA for distinguishing infectious bovine rhinotracheitis virus (IBRV)-infected animals from those vaccinated with a gene-deleted marker vaccine. J Virol Methods 40:45-56

18. Li X, Li G, Teng Q et al (2012) Development of a blocking ELISA for detection of serum neutralizing antibodies against newly emerged duck Tembusu virus. PLoS One 7:e53026

19. Moraveji M, Hosseini A, Moghaddar N et al (2012) Development of latex agglutination test with recombinant NcSAG1 for the rapid detection of antibodies to *Neospora caninum* in cattle. Vet Parasitol 189:211-217

20. Mugridge NB, Morrison DA, Heckeroth AR et al (1999) Phylogenetic analysis based on full-length large subunit ribosomal RNA gene sequence comparison reveals that *Neospora caninum* is more closely related to *Hammondia heydorni* than to *Toxoplasma gondii*. Int J Parasitol 29:1545-1556

21. Pare J, Hietala SK, Thurmond MC (1995) An Enzyme-linked-immunosorbent-assay (Elisa) for serological diagnosis of *Neospora* sp. Infection in Cattle. J Vet Diagn Invest 7:352-359

22. Piagentini M, Moya-Araujo CF, Prestes NC et al (2012) *Neospora caninum* infection dynamics in dairy cattle. Parasitol Res 111:717-721

23. Pinheiro AM, Costa MF, Paule B et al (2005) Serologic immunoreactivity to *Neospora caninum* antigens in dogs determined by indirect immunofluorescence, western blotting and dot-ELISA. Vet Parasitol 130:73-79

24. Pitel PH, Pronost S, Chatagnon G et al (2001) Neosporosis in bovine dairy herds from the west of France: detection of *Neospora caninum* DNA in aborted fetuses, seroepidemiology of *N. caninum* in cattle and dogs. Vet Parasitol 102:269-277

25. Shearer PL, Sharp M, Bonne N et al (2009) A blocking ELISA for the detection of antibodies to psittacine beak and feather disease virus (BFDV). J Virol Methods 158:136-140

26. Teixeira MFB, Esteves PA, Schmidt CS et al (2001) ELISA de bloqueio monoclonal para o diagnóstico sorológico de infecções pelo herpesvírus bovino tipo 1 (BHV-1)1. Pesq Vet Brasil 21:33-37

27. Zhang H, Lee EG, Yu L et al (2011) Identification of the cross-reactive and

species-specific antigens between *Neospora caninum* and *Toxoplasma gondii* tachyzoites by a proteomics approach. Parasitol Res 109:899-911

Fig. 1 Dot blot assay with the pAb or HRP-conjugated pAb, using rNcp-43 protein immobolized on nitrocellulose membrane. **a** pAb (1:6,000). **b** pAb/HRP (1:500). **c** Negative control (pooled preimmune serum). **d** Positive control (pooled hyperimmune serum).

Fig. 2 Detection of the apical complex of tachyzoites of *Neospora caninum* by direct immunofluorescence. Tachyzoites were fixed on microscope slides and probe with the FITC-conjugated pAb against rNcp-43 (arrows). **a** *T. gondii* cells were used as a negative control for antigen detection. **b-d** Strain Nc-1. **e-g** Strain Nc-goiás. **h-j** Strain Nc-Liv. Visualization was performed using a 100X objective on an Olympus BX51 fluorescence microscope. Scale bars 10 µm.

Fig. 3 Blocking ELISA using pAb/HRP as a diagnostic tool to detect antibodies in the serum of animals infected with *N. caninum*. Pools of positive (**a**) and negative (**b**) serum samples from cattle were used as sources of blocking antibodies. Mean differences were statistically significant (* P\ 0,0001).

Figure 1:

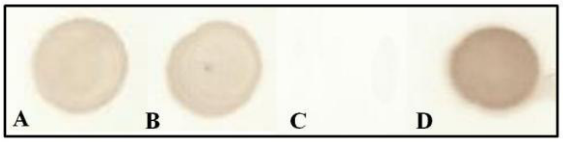


Figure 2:

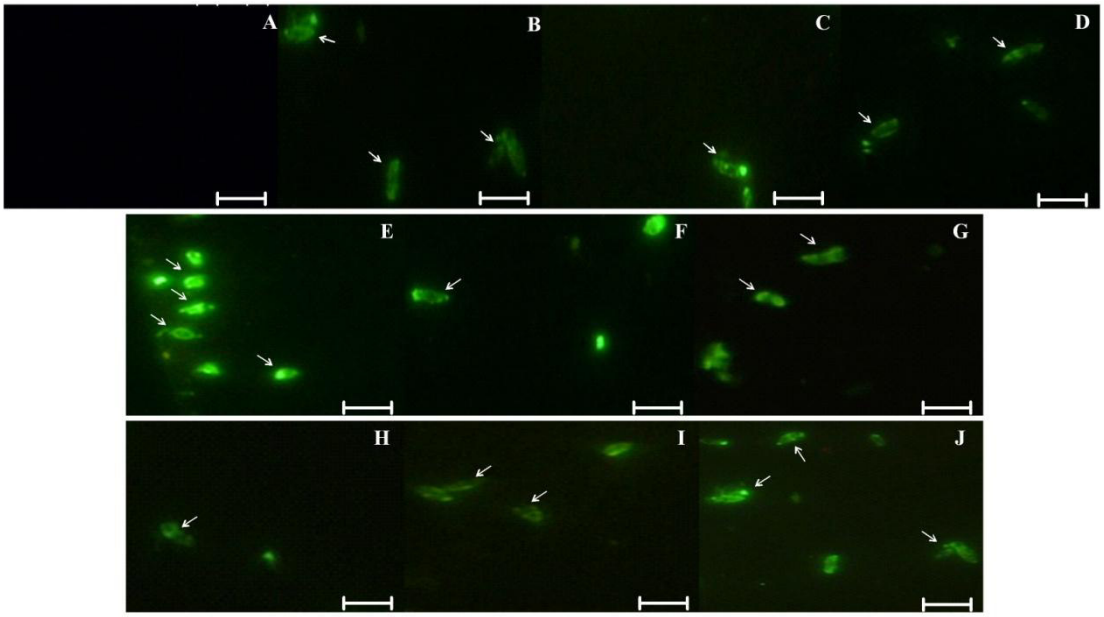
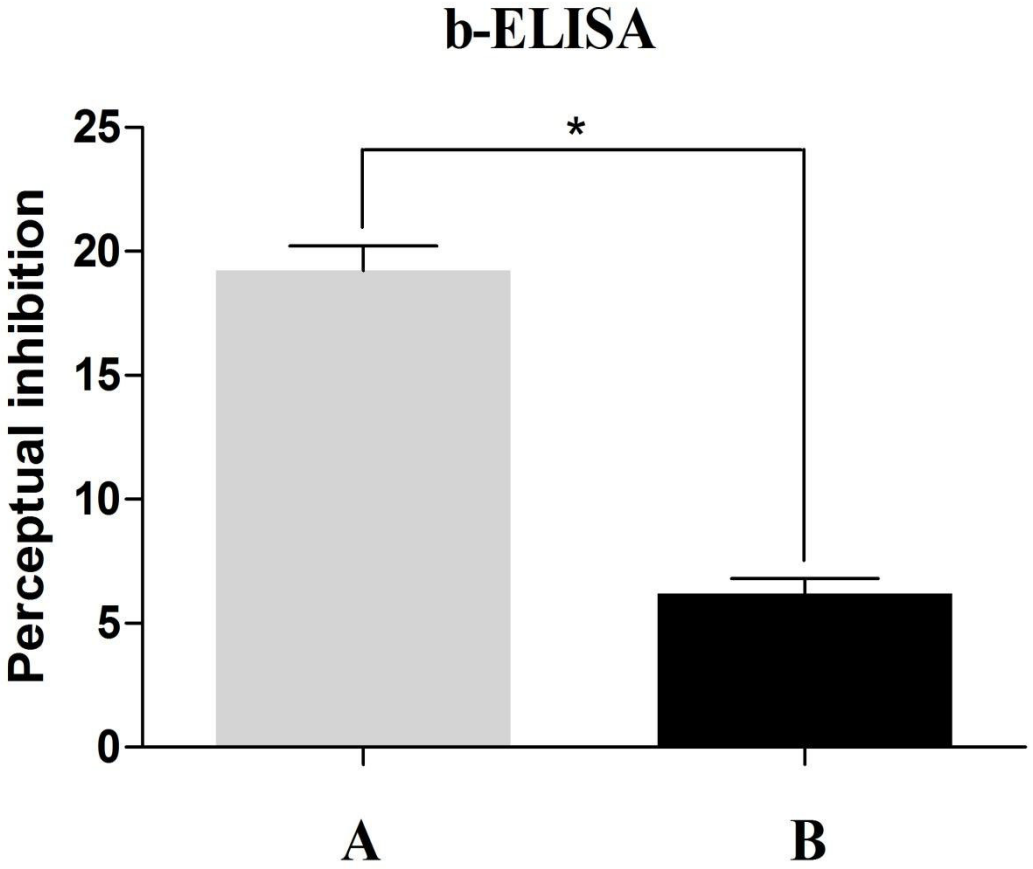


Figure 3:



4.1.3 Artigo 3 - Standardization of expression of hydrophobic surface protein SAG1 of *N. caninum* in *Escherichia coli*

Artigo a ser submetido na revista *Journal of Biological Methods*

Standardization of expression of hydrophobic surface protein SAG1 of n. caninum in Escherichia coli

Gizele L. Sá[1*], Bianca Sica Siedler[1], Camila B. Bender[1] and Sibele Borsuk[2]

[1]Laboratório de Imunodiagnóstico and [2]Laboratório de Pesquisa em Doenças Infecciosas, Núcleo de Biotecnologia-CDTec, Universidade Federal de Pelotas, Corredor da Embrapa s/n, CEP 96160-000, Capão do Leão, RS, Brasil.

[*]Corresponding author: gezelha@hotmail.com Phone: + 55 (53) 32757350

Abstract

Neospora caninum is a veterinary medically important pathogen of causing abortion in cattle and neuromuscular paralysis in dogs. The surface antigen 1 of *Neospora caninum* (Nc-SAG1) is an important candidate for the development of a diagnostic reagent for neosporosis. The use of this protein recombinant expressed in a prokaryotic system already presented satisfactory results in the diagnosis. However, currently the description theoretical the steps needed for obtaining a recombinant protein are greatly simplified, which often unfeasible the repeatability of the method. For this reason, in this work, we report a protocol of cloning, expression and purification of recombinant protein SAG1 of *N. caninum* (rNc-SAG1) in prokaryotic expression system using as a host cell *Escherichia coli* strains. In order to establish an effective protocol expression, the gene SAG1 *Neospora caninum* coded protein Nc-SAG1, was cloned and expressed in *E. coli* BL21 derivative strains: Star (DE3), BL21 pLysS (DE3), BL21 Rosetta (DE3) and Artic Express (DE3). Synthesis of rNc-SAG1 was induced with IPTG and tested at four different temperatures for 4 hr incubation stirring 180 rpm and overnight under the same conditions. The expression of the rNc-SAG1 protein was clearly observed only in the *E. coli* Artic Express (DE3) strain incubated 4 h at 4 °C, stirring 180 rpm. The insoluble fraction containing the inclusion bodies was further resuspended in buffer containing 8 M urea. The rNc-SAG1 protein solubilized was filtered and purified by nickel chromatography under denaturing conditions. The yield average of Nc-SAG1 recombinant obtained was 0,250 mg/L of *E. coli* culture. These results suggest that strain of *E. coli* Artic Express (DE3) can express *N. caninum* surface protein SAG1 (Nc-SAG1) when incubated 4 h at 4 °C, stirring 180 rpm.

Keywords: *Neospora caninum*, SAG1 and *Escherichia coli*

Short Communication

Neospora caninum is an intracellular Apicomplexan parasite that causes abortion in cattle [1]. This leads to significant financial losses world-wide [2]. Definitive diagnosis of neosporosis is achieved by demonstration of the parasite in tissues [3] by histological, immunohistochemical, and polymerase chain reaction (PCR) techniques on the brain or other tissues of aborted fetuses [4]. Serological tests are also a diagnostic tool currently available. Such tests include immunoblotting [5], indirect fluorescent-antibody test (IFAT) [1,6], agglutination test [7], the latex agglutination test [8] and enzyme-linked immunosorbent assays (ELISAs) [2,9–11]. Despite the variety of strategies, most of these tests are based on the analysis of whole parasite lysates or fixed tachyzoites, and are therefore considered time-consuming and laborious [1]. However, some of the serological methods allow the rapid processing of large numbers of samples which turns possible to evaluate the status of neosporosis in cattle herds [12]. Besides that, the combined use of different recombinant antigens in serological tests can represent an advantage, since they are amenable to large scale production and can be easily standardized [13]. In addition, the use of highly specific antigens may minimize the risk of cross-reactivity with other parasite species [14].

Surface proteins of intracellular pathogens are believed to play critical roles in infection. The *N. caninum* surface associate antigen 1 (Nc-SAG1) have been identified as major surface antigen proteins of *N. caninum* tachyzoites, and was demonstrated to be immune-dominant and involved in interactions between tachyzoite and host cell [15,16]. Furthermore, the identification of antibodies against Nc-SAG1 during pregnancy is associated with abortion risk [17]. When applied in a DNA vaccine format, Nc-SAG1 was able to reduce parasitic load in the brain of mice [18] . Furthermore its antigenicity was also demonstrated through recognition by sera from Neospora-infected animals [8,19,20]. In this sense, Nc-SAG1 was shown to be an effective candidate to diagnose *N. caninum* infection in cattle when it was used in serological test [21,22].

In the past decades, many reviews detailing the process of recombinant proteins expression have been published [23–25]. In the field of recombinant protein expression and purification, progress is continuously being made [26]. The choice of

the host cell for protein synthesis is determinant and without a doubt, *Escherichia coli* is the most widely used host cell for gene expression [27]. The advantages of using *E. coli* as the host organism include its unequalled fast growth kinetics [28], wit consequent facility in reaching high cell density cultures [29] fast and easy transformation with exogenous DNA [30] and availability of inexpensive components for rich complex media preparation [26]. These methodological data are widely applied in works that use recombinant proteins [31–33] as well as the diagnostic and the development of vaccines against neosporosis [8,19,34]. However currently the description theoretical the steps needed for obtaining a recombinant protein are greatly simplified, which often unfeasible the repeatability of the method. Since in practice, dozens of problems can occur from a poor growth of the host, inclusion bodies (IB) formation, protein inactivity and even not obtain any protein [26].

For this reason, in this work, we report a protocol of cloning, expression and purification of recombinant protein SAG1 of *N. caninum* (rNc-SAG1) in prokaryotic expression system using as a host cell *Escherichia coli* strains EDH5α, BL21 Star (DE3), BL21 pLysS (DE3) and Artic.

Materials and Methods

Escherichia coli thermo-competent cells

Strains of *Escherichia coli* EDH5α (Agilent Technologies), BL21 Star (DE3), BL21 pLysS (DE3), BL21 Rosetta (DE3) and Artic Express (DE3) (Agilent Technologies) were used in the preparation of termocompetentes cells used for cloning procedures and expression of Nc-SAG1 protein , according to Sambrook & Russell [35] with modifications. Cultures of each bacterial strain used was prepared from the ringing of fresh colonies, preciously seeded in medium Luria-Bertani containing on trypticase soy agar (aLB) and incubated at 37 ºC for 16-20 h in 5 mL Luria-Bertani broth (bLB) which was incubated overnight at 37 ºC, stirring 180 rpm. 100 µL of overnight culture in Luria-Bertani broth was transferred to 50 mL of bLB, incubated at 37 ºC stirring 180 rpm until they reached optical density ($OD_{600\ nm}$) of about 0.6. The resulting *E. coli* was harvested by centrifugation at 20.000 g x for 15 min. at 4 ºC and the

resulting pellets were resuspended with the aid de vortex in 40 mL of 50 mM CaCl2 solution, followed by 15 min. incubation on ice and sedimentation (20.000 g x for 15 min. at 4 ºC). The pellets resulting from each bacterial strain were resuspended in 3 mL 50 mM CaCl2 solution prepared in 20% glycerol and then stored at - 70 °C until use.

Escherichia coli electro-competent cells

Electro-competent cells were prepared according to established protocol (Sambrook & Russel, 2001) and used for the propagation of plasmid pBluescript. For such a *E. coli* DH5α (Agilent Technologies) culture was prepared from a colony replanting in fresh, pre-seeded aLB and incubated for 37 °C for 16-20 h in 10 ml of bLB was incubated overnight at 37 °C, stirring 180 rpm. From this culture, 5 ml were spiked into 500 ml of bLB and kept under the same conditions until reaching OD_{600nm} 0.6. Then the bacterial culture was cooled (4 °C) and centrifuged for 20 min. at 20.000 g × at 4 °C. The resulting pellet was washed twice with sterile milliQ water and once with 10% glycerol solution sterile at 4 ºC. The cells was harvested by centrifugation at 20.000 g x for 20 min. at 4 ºC between washes. The resulting pellet was gently resuspended in 1 ml of 10% sterile glycerol solution at 4 °C, aliquoted in cryotubes in a final volume of 70 µL, and stored at -70 until use.

Cloning of gene *SAG1* of *Neospora caninum*

The gene *SAG1* of *Neospora caninum* coded protein Nc-SAG1 (GenBank – acess AAD25091.1), was cloned with an in a commercial plasmid (Epoch Life Science, Inc.) to give the pBlueScript/Nc-SAG1. This plasmid was introduced by electroporation into *E. coli* DH5-α electrocompetent cells. The cells recombinants were selected in aLB medium containing 100 µg/mL of ampicillin (ampicillin 10 ng/mL - SERVA®). Colonies were analyzed by extracting plasmid DNA using a plasmid extraction Kit Quick Plasmid Miniprep (Invitrogen), followed by an enzymatic restriction of the plasmid DNA using restriction enzyme digestion with *EcoRI* and *BamHI* (Invitrogen) endonucleases. The restriction product were analyzed by agarose gel electrophoresis to check for the presence of a band of 2.958 pb corresponding to the

vector and a band of 941pb corresponding to the *SAG1* gene. *SAG1* was extracted and purified from agarose gel using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen), and inserted into the pAE expression vector using an enzyme T4 DNA ligase (invitrogen). The ligation reaction was transformed into the *E. coli* DH5-α electrocompetent cells and the fusion of the open reading frame of *SAG1*gene with the 6x histidine tag of vector was confirmed by screening colonies using phenol-chloroform. The resulting plasmid was designated as pAE/Nc-SAG1.

Expression of theNc-SAG1 protein in *E. coli*

TheNc-SAG1 protein was expressed with an upstream polyhistidine tag sequence in *E. coli.* For the expression were tested *E. coli* BL21 (DE3) derivative strains: Star (DE3), BL21 pLysS (DE3), BL21 Rosetta (DE3) and Artic Express (DE3) thermo-competent previously prepared, cultured in LB medium containing 100 µg/mL de ampicillin (ampicillin 10 ng/mL - SERVA®) and selection antibiotic of the strains recommended by manufacturer (Agilent Technologies). The plasmid pAE/Nc-SAG1 was introduced into *E. coli* thermo-competent cells by thermal shock. The cells recombinants were selected in aLB medium. Strains were individually cultured in 10 mL cLB at 37 ºC stirring 180 rpm until the OD_{600nm} level reached 0.6. Synthesis of Nc-SAG1 recombinant (rNc-SAG1) was induced with isopropyl-1-thio-ß-D-galactoside (IPTG) 1mM and tested at four different temperatures (Fig. 1) for 4 hr incubation stirring 180 rpm and overnight under the same conditions. The resulting *E. coli* were harvested by centrifugation at 1.500 g x for 10 min. at 4 ºC. The resulting pellets were eluted in TE buffer (10 mM Tris-HCl / 1 mM EDTA pH 8.0) and treated by sonication (3 x 45 s, 20 kHz, under cooling). After centrifugation under the same conditions, the supernatants and resulting pellets of each strain were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In order to identify the recombinant protein an anti-polyhistidine monoclonal antibody (Sigma-Aldrich) produced in mouse and anti-mouse conjugated peroxidase (Sigma-Aldrich) were used in a Western blotting (WB) as the primary and secondary antibody, respectively. For the negative control an aliquot of each *E. coli* strain, at different temperatures and incubation periods, were removed prior to induction with IPTG. The recombinant

protein Nc-SRS2 expressed with an upstream polyhistidine tag sequence was used as positive control in WB.

The *E. coli* strain that was able of expressing the rNc-SAG1 protein was transformed by thermal shock using plasmid pAE/Nc-SAG1 under the farming condition which shows best result. The resulting pellet was eluted in TE buffer, sonicated and centrifuged under the same conditions to separate the soluble (supernatant) and insoluble (pellet) fractions. Both as fractions were subjected SDS/PAGE and WB under the same conditions described above, to identify the location of rNc-SAG1. The protein present in insoluble fraction was solubilized in a buffer containing 8 M urea and incubated at 37 ºC for 1 h [36]. The rNc-SAG1 protein was filtered in nitrocellulose membrane filter 0.8 µM (Millipore) and purified by nickel resin affinity columns under denaturing conditions according to the manufacturer's instructions (GE Healtcare). The concentration of the protein suspension was determined using BCA Protein Assay Kit (Pierce^{TM}).

Results and Discussion

The expression of the recombinant Nc-SAG1 protein (≈ 30 kDa) was clearly observed only in the *E. coli* Artic Express (DE3) strain incubated 4 h at 4 °C, stirring 180 rpm (Fig. 2). In order to confirm that the ≈ 30 kDa protein present in the insoluble fraction was the rNc-SAG1 protein, a WB using monoclonal antibodies specific to the 6xHis tag (Fig. 3) was used. As expected, the ≈ 30-kDa protein was detected. The insoluble fraction containing the inclusion bodies was further resuspended in buffer containing 8 M urea. The rNc-SAG1 protein solubilized was filtered and purified by nickel chromatography under denaturing conditions. The yield average of Nc-SAG1 recombinant obtained was 0,250 mg/L of *E. coli* culture.

In some case the resulting protein in prokaryotic expression system does not have the correct refolding and so it does not have the activity it should [37]. rNc-SAG1 protein expressed in *E. coli* Artic Express (DE3) may be used in diagnostic assays since previous studies showed rNc-SAG1 produced in *E. coli* BL21 (DE3) keep your antigenicity [8,38]. Furthermore the denaturation of the tachyzoite's surface proteins of *N. caninum* did not prevent basal level of adhesion [39] this may indicate that the adhesion capacity of parasite/host cell independent of the parasite's protein

conformation. The *E. coli* expression system offers some advantages over other expression systems because the procedures of expression and purification are much easier [26]. Foreign gene expressed in *E. coli* often appears as insoluble form with inclusion bodies formation [40]. Indeed, the entire recombinant NcSAG1 was expressed in an insoluble form, which may have been due their hydrophobicity. The in silico analysis using the Vector NTI software of rNc-SAG1 sequence showed the presence of hydrophobic amino acids, thus the protein had the hydrophobic characteristic (Fig. 4).

The BL21 (DE3) and its derivatives are by far the most used strains for protein expression due to its characteristics that enable them high-level expression of heterologous proteins in *E. coli* [41]. Although all used strains in this work were derived from strain BL21 (DE3) only *E. coli* Artic Express (DE3) was able to express recombinant Nc-SAG1 protein when incubated at 4 h at 4 °C, stirring 180 rpm. Perhaps the expression of the recombinant protein induced the death most of the *E. coli* strains BL21 Star (DE3), BL21 pLysS (DE3) and BL21 Rosetta (DE3) strains due the possible toxicity of recombinant Nc-SAG1. This result is in agreement with the observations of cell death of *E. coli* BL21 DE3 when expressing membrane proteins [42]. NcSAG1 is identified as a surface antigen of *N. caninum* and confirmed to be an immunodominant antigen that is well conserved in geographically distant isolates [14]. Since the successful expression of rNc-SAG1 protein by *E. coli* Artic Express (DE3) strain probably due to this strain present, besides the common genetic features *E. coli* BL21 DE3, additional features enable direct cloning of cytotoxics proteins expression such as the co-expression of the cold-adapted chaperonins Cpn10 and Cpn60 from the psychrophilic bacterium *Oleispira* Antarctica. The Cpn10 and Cpn60 chaperonins from *O.* Antarctica have 74% and 54% amino acid identity to the *E. coli* GroEL and GroES chaperonins, respectively, and show high protein refolding activities at temperatures of 4 – 12 ºC. Furthermore, according to the manufacturer *E. coli* Artic Express (DE3), this strain displays Hte phenotype increases the transformation efficiency of the cells and the gene that encodes endonuclease I (*endA*) inactived. However even at low-temperature, which is represents one strategy for increasing the recovery of soluble protein [26,43], the rNc-SAG1 protein was completely expressed as an insoluble form and did not appear in the supernatant before solubilization and purification processes; as observed in

previous studies using strains cultured around 37 ºC [34,38,44]. The hydrophobic stretches in the polypeptide are present at high concentrations, high level expression, and therefore available for interaction with similar regions [40]. IB formation can be advantageous in many respects. Apart from the high product yields, resistance towards proteolytic cleavage, minimized product toxicity towards the host cell [45] and the relative ease of purification of the target protein from other contaminants are strong bio-processing advantages [46].

The rNc-SAG1 protein amount solubilized with urea 8 M and purified was 0,250 mg/L E. coli Artic Express (DE3) culture. Previous work who used the rNc-SAG1 protein expressed in *E. coli* BL21 (DE3) not present data concerning the production yield [8,19,34,38,47] which makes unfeasible compare the rNc-SAG1 production yield in different strains *E. coli*. However the average production other surface protein immunodominat named Nc-SRS2 was 1 mg/L of *E. coli* BL21 Star (DE3) culture [36,48]. This difference in performance may be due to different harmful effects that the heterologous proteins have on the host cell [26,42] and/or low temperature of cultivation resulting in slower growth which reduced synthesis rates can resulting in lower protein yields [43].

These results suggest that strain of *E. coli* Artic Express (DE3) can express *N. caninum* surface protein SAG1 (Nc-SAG1) when incubated 4 h at 4 °C, stirring 180 rpm. It is noteworthy that this protein probably present an incomplete folding which results in its inactivity. However, recombinant proteins produced in prokaryotic system may be used in diagnostic assays or the development of diagnostic tools as incomplete folding does not affect antigenicity. Thus, this protocol should be improved so that it can be applied by any laboratory for production of recombinant protein Nc-SAG1 intended for development of inputs and/or diagnosis.

Acknowledgements

Funding for this study was provided by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Grant AUX-PE-PNPD-1513/2008) and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Conflict of Interest

No competing financial interests exist.

References

1. Dubey JP, Schares G (2011) Neosporosis in animals--the last five years. Vet Parasitol 180: 90-108. S0304-4017(11)00384-0 [pii];10.1016/j.vetpar.2011.05.031 [doi].

2. Reichel MP, Alejandra Ayanegui-Alcerreca M, Gondim LF, Ellis JT (2013) What is the global economic impact of Neospora caninum in cattle - the billion dollar question. Int J Parasitol 43: 133-142. S0020-7519(12)00309-8 [pii];10.1016/j.ijpara.2012.10.022 [doi].

3. Bruhn FR, Daher DO, Lopes E, Barbieri JM, da Rocha CM, Guimaraes AM (2013) Factors associated with seroprevalence of Neospora caninum in dairy cattle in southeastern Brazil. Trop Anim Health Prod 45: 1093-1098. 10.1007/s11250-012-0330-y [doi].

4. Dubey JP, Jenkins MC, Kwok OC, Ferreira LR, Choudhary S, Verma SK, Villena I, Butler E, Carstensen M (2013) Congenital transmission of Neospora caninum in white-tailed deer (Odocoileus virginianus). Vet Parasitol 196: 519-522. S0304-4017(13)00145-3 [pii];10.1016/j.vetpar.2013.03.004 [doi].

5. Atkinson R, Harper PA, Reichel MP, Ellis JT (2000) Progress in the serodiagnosis of Neospora caninum infections of cattle. Parasitol Today 16: 110-114. S0169-4758(99)01604-X [pii].

6. Dubey JP, Hattel AL, Lindsay DS, Topper MJ (1988) Neonatal Neospora caninum infection in dogs: isolation of the causative agent and experimental transmission. J Am Vet Med Assoc 193: 1259-1263.

7. Canada N, Meireles CS, Carvalheira J, Rocha A, Sousa S, Correia Da Costa JM (2004) Determination of an optimized cut-off value for the Neospora agglutination test for serodiagnosis in cattle. Vet Parasitol 121: 225-231. 10.1016/j.vetpar.2004.02.025 [doi];S0304401704000767 [pii].

8. Moraveji M, Hosseini A, Moghaddar N, Namavari MM, Eskandari MH (2012) Development of latex agglutination test with recombinant NcSAG1 for the rapid detection of antibodies to Neospora caninum in cattle. Vet Parasitol 189: 211-217. S0304-4017(12)00208-7 [pii];10.1016/j.vetpar.2012.04.010 [doi].

9. Dubey JP, Schares G (2006) Diagnosis of bovine neosporosis. Vet Parasitol 140: 1-34. S0304-4017(06)00221-4 [pii];10.1016/j.vetpar.2006.03.035 [doi].

10. Byrem TM, Bartlett PC, Donohue H, Voisinet BD, Houseman JT (2012) Performance of a commercial serum ELISA for the detection of antibodies to Neospora caninum in whole and skim milk samples. Vet Parasitol 190: 249-253. S0304-4017(12)00302-0 [pii];10.1016/j.vetpar.2012.06.005 [doi].

11. Reichel MP, McAllister MM, Pomroy WE, Campero C, Ortega-Mora LM, Ellis JT (2014) Control options for Neospora caninum - is there anything new or are we going backwards? Parasitology 141: 1455-1470. S0031182014000158 [pii];10.1017/S0031182014000158 [doi].

12. Sa GL, Pacheco DB, Monte LG, Sinnott FA, Xavier MA, Rizzi C, Borsuk S, Berne ME, Andreotti R, Hartleben CP (2014) Diagnostic potential of anti-rNcp-43 polyclonal antibodies for the detection of Neospora caninum. Curr Microbiol 68: 472-476. 10.1007/s00284-013-0499-y [doi].

13. Khow O, Suntrarachun S (2012) Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pac J Trop Biomed 2: 159-162. 10.1016/S2221-1691(11)60213-X [doi];apjtb-02-02-159 [pii].

14. Howe DK, Sibley LD (1999) Comparison of the major antigens of Neospora caninum and Toxoplasma gondii. Int J Parasitol 29: 1489-1496. S0020-7519(99)00099-5 [pii].

15. Debache K, Guionaud C, Alaeddine F, Mevissen M, Hemphill A (2008) Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with Neospora caninum tachyzoites. Int J Parasitol 38: 1455-1463. S0020-7519(08)00144-6 [pii];10.1016/j.ijpara.2008.04.001 [doi].

16. Cannas A, Naguleswaran A, Muller N, Eperon S, Gottstein B, Hemphill A (2003) Vaccination of mice against experimental Neospora caninum infection using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology 126: 303-312.

17. Hiasa J, Kohara J, Nishimura M, Xuan X, Tokimitsu H, Nishikawa Y (2012) ELISAs based on rNcGRA7 and rNcSAG1 antigens as an indicator of Neospora caninum activation. Vet Parasitol 187: 379-385. S0304-4017(12)00072-6 [pii];10.1016/j.vetpar.2012.01.036 [doi].

18. Yoshimoto M, Otsuki T, Itagaki K, Kato T, Kohsaka T, Matsumoto Y, Ike K, Park EY (2015) Evaluation of recombinant Neospora caninum antigens purified from silkworm larvae for the protection of N. caninum infection in mice. J Biosci Bioeng 120: 715-719. S1389-1723(15)00152-8 [pii];10.1016/j.jbiosc.2015.04.002 [doi].

19. Wilkowsky SE, Bareiro GG, Mon ML, Moore DP, Caspe G, Campero C, Fort M, Romano MI (2011) An applied printing immunoassay with recombinant Nc-SAG1 for detection of antibodies to Neospora caninum in cattle. J Vet Diagn Invest 23: 971-976. 1040638711416845 [pii];10.1177/1040638711416845 [doi].

20. Otsuki T, Dong J, Kato T, Park EY (2013) Expression, purification and antigenicity of Neospora caninum-antigens using silkworm larvae targeting for subunit vaccines. Vet Parasitol 192: 284-287. S0304-4017(12)00546-8 [pii];10.1016/j.vetpar.2012.09.038 [doi].

21. Gaturaga I, Chahan B, Xuan X, Huang X, Liao M, Fukumoto S, Hirata H, Nishikawa Y, Takashima Y, Suzuki H, Fujisaki K, Sugimoto C (2005) Detection of antibodies to Neospora caninum in cattle by enzyme-linked immunosorbent assay with truncated NcSRS2 expressed in Escherichia coli. J Parasitol 91: 191-192. 10.1645/GE-267R1 [doi].

22. Liao M, Zhang S, Xuan X, Zhang G, Huang X, Igarashi I, Fujisaki K (2005) Development of rapid immunochromatographic test with recombinant NcSAG1 for detection of antibodies to Neospora caninum in cattle. Clin Diagn Lab Immunol 12: 885-887. 12/7/885 [pii];10.1128/CDLI.12.7.885-887.2005 [doi].

23. Makrides SC (1996) Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol Rev 60: 512-538.

24. Baneyx F (1999) Recombinant protein expression in Escherichia coli. Curr Opin Biotechnol 10: 411-421. S0958-1669(99)00003-8 [pii].

25. Stevens RC (2000) Design of high-throughput methods of protein production for structural biology. Structure 8: R177-R185. S0969212600001933 [pii].

26. Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in Escherichia coli: advances and challenges. Front Microbiol 5: 172. 10.3389/fmicb.2014.00172 [doi].

27. Rosano GL, Ceccarelli EA (2014) Recombinant protein expression in microbial systems. Front Microbiol 5: 341. 10.3389/fmicb.2014.00341 [doi].

28. Sezonov G, Joseleau-Petit D, D'Ari R (2007) Escherichia coli physiology in Luria-Bertani broth. J Bacteriol 189: 8746-8749. JB.01368-07 [pii];10.1128/JB.01368-07 [doi].

29. Shiloach J, Fass R (2005) Growing E. coli to high cell density--a historical perspective on method development. Biotechnol Adv 23: 345-357. S0734-9750(05)00046-7 [pii];10.1016/j.biotechadv.2005.04.004 [doi].

30. Pope B, Kent HM (1996) High efficiency 5 min transformation of Escherichia coli. Nucleic Acids Res 24: 536-537. l50353 [pii].

31. Mirahmadi H, Fallahi S, Seyyed Tabaei SJ (2016) Soluble recombinant merozoite surface antigen-142kDa of Plasmodium vivax: An improved diagnostic antigen for vivax malaria. J Microbiol Methods 123: 44-50. S0167-7012(16)30020-3 [pii];10.1016/j.mimet.2016.02.003 [doi].

32. Sonaimuthu P, Cheong FW, Chin LC, Mahmud R, Fong MY, Lau YL (2015) Detection of human malaria using recombinant Plasmodium knowlesi merozoire surface protein-1 (MSP-1(1)(9)) expressed in Escherichia coli. Exp Parasitol 153: 118-122. S0014-4894(15)00072-7 [pii];10.1016/j.exppara.2015.03.010 [doi].

33. Athmaram TN, Saraswat S, Misra P, Shrivastava S, Singh AK, Verma SK, Gopalan N, Behara PK, Rao PV (2013) Optimization of Dengue-3 recombinant NS1 protein expression in E. coli and in vitro refolding for diagnostic applications. Virus Genes 46: 219-230. 10.1007/s11262-012-0851-5 [doi].

34. Chahan B, Gaturaga I, Huang X, Liao M, Fukumoto S, Hirata H, Nishikawa Y, Suzuki H, Sugimoto C, Nagasawa H, Fujisaki K, Igarashi I, Mikami T, Xuan X (2003) Serodiagnosis of Neospora caninum infection in cattle by enzyme-linked immunosorbent assay with recombinant truncated NcSAG1. Vet Parasitol 118: 177-185. S0304401703004369 [pii].

35. Sambrook, J. and Russel, D. W. (2001) Molecular cloning: a laboratory manual. New York: Cold Spring Harbor.

36. Borsuk S, Andreotti R, Leite FP, Pinto LS, Simionatto S, Hartleben CP, Goetze M, Oshiro LM, Matos MF, Berne ME (2011) Development of an indirect ELISA-NcSRS2 for detection of Neospora caninum antibodies in cattle. Vet Parasitol 177: 33-38. S0304-4017(10)00676-X [pii];10.1016/j.vetpar.2010.11.026 [doi].

37. Martinez-Alonso M, Gonzalez-Montalban N, Garcia-Fruitos E, Villaverde A (2008) The Functional quality of soluble recombinant polypeptides produced in Escherichia coli is defined by a wide conformational spectrum. Appl Environ Microbiol 74: 7431-7433. AEM.01446-08 [pii];10.1128/AEM.01446-08 [doi].

38. Howe DK, Tang K, Conrad PA, Sverlow K, Dubey JP, Sibley LD (2002) Sensitive and specific identification of Neospora caninum infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clin Diagn Lab Immunol 9: 611-615.

39. Pereira LM, Yatsuda AP (2014) Comparison of an ELISA assay for the detection of adhesive/invasive Neospora caninum tachyzoites. Rev Bras Parasitol Vet 23: 36-43. S1984-29612014000100006 [pii].

40. Carrio MM, Villaverde A (2002) Construction and deconstruction of bacterial inclusion bodies. J Biotechnol 96: 3-12. S0168165602000329 [pii].

41. Daegelen P, Studier FW, Lenski RE, Cure S, Kim JF (2009) Tracing ancestors and relatives of Escherichia coli B, and the derivation of B strains REL606 and BL21(DE3). J Mol Biol 394: 634-643. S0022-2836(09)01139-5 [pii];10.1016/j.jmb.2009.09.022 [doi].

42. Miroux B, Walker JE (1996) Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260: 289-298. S0022-2836(96)90399-X [pii];10.1006/jmbi.1996.0399 [doi].

43. San-Miguel T, Perez-Bermudez P, Gavidia I (2013) Production of soluble eukaryotic recombinant proteins in E. coli is favoured in early log-phase

cultures induced at low temperature. Springerplus 2: 89. 10.1186/2193-1801-2-89 [doi];152 [pii].

44. Moraveji M, Hosseini A, Moghaddar N, Namavari MM, Eskandari MH (2012) Development of latex agglutination test with recombinant NcSAG1 for the rapid detection of antibodies to Neospora caninum in cattle. Vet Parasitol 189: 211-217. S0304-4017(12)00208-7 [pii];10.1016/j.vetpar.2012.04.010 [doi].

45. Greenshields AL, Knickle LC, Syvitski R, Douglas SE (2008) Strategies for recombinant expression of small, highly disulphide-bonded, cationic antimicrobial peptides. Protein Pept Lett 15: 985-994.

46. Fahnert B, Lilie H, Neubauer P (2004) Inclusion bodies: formation and utilisation. Adv Biochem Eng Biotechnol 89: 93-142.

47. Hecker YP, Coceres V, Wilkowsky SE, Jaramillo Ortiz JM, Morrell EL, Verna AE, Ganuza A, Cano DB, Lischinsky L, Angel SO, Zamorano P, Odeon AC, Leunda MR, Campero CM, Morein B, Moore DP (2014) A Neospora caninum vaccine using recombinant proteins fails to prevent foetal infection in pregnant cattle after experimental intravenous challenge. Vet Immunol Immunopathol 162: 142-153. S0165-2427(14)00258-X [pii];10.1016/j.vetimm.2014.11.001 [doi].

48. Andreotti R, Matos MF, Goncalves KN, Oshiro LM, Lima-Junior MS, Paiva F, Leite FL (2009) Comparison of indirect ELISA based on recombinant protein NcSRS2 and IFAT for detection of Neospora caninum antibodies in sheep. Rev Bras Parasitol Vet 18: 19-22. rbpv.01802004 [pii].

Figure 1: Schematic representation of the culture conditions tested after IPTG-induction of strains of *E. coli* BL21 (DE3).

Figure 2: Expression of rNc-SAG1 protein of *Neospora caninum* by *E.coli* BL21 (DE3) derivative strains. Western blotting analysis with soluble and insoluble fractions of IPTG-induced *E. coli* BL21 (DE3) cultures for 4 h in an incubator shaker at 180 rpm, using anti-6x His tag and anti-species-specific conjugated peroxidase as the primary and secondary antibody, respectively. **Panel A**: cultures incubated at 29 ºC. (1) Rosetta strain - soluble fraction (2) strain pLysS - soluble fraction (3) Star strain - soluble fraction (4) Rosetta strain - insoluble fraction (5) strain pLysS - insoluble fraction (6) Star strain - insoluble fraction (7) recombinant protein Nc-SRS2 (≈ 30 KDa). **Panel B**: cultures incubated at 37 ºC. (1) Rosetta strain - soluble fraction (2) strain pLysS - soluble fraction (3) Star strain - soluble fraction (4) Rosetta strain - insoluble fraction (5) strain pLysS - insoluble fraction (6) Star strain - insoluble fraction (7) recombinant protein Nc-SRS2 (≈ 30 KDa). **Panel C**: Artic Express starin (1) soluble fraction - maintained at 4 ºC (2) soluble fraction - maintained at 17 ºC (3) insoluble fraction - maintained at 4 ºC (4) insoluble fraction - maintained at 17 ºC (5) recombinant protein Nc-SRS2 (≈ 30 KDa).

Figure 3: Purification of rNC-SAG1 (≈ 30 kDa) using urea and imidazole. Western blotting of the product obtained by purification from *E. coli* Artic Express culture transformed with pAE/Nc -SAG1 plasmid and induced with IPTG using anti- 6xHis tag antibody: (1) Nc- SRS2 (≈ 30 kDa), (2) Artic transformed and not induced, (3) Artic pellet transformed and induced, (4) supernatant Artic transformed and induced, (5) pellet after solubilization, (6) supernatant after solubilization, (7) elution buffer containing 8 M urea, (8) elution containing 6 M urea buffer and 5 mM imidazole, (9) elution buffer containing 6 M urea and 10 mM imidazole, (10) elution buffer containing 6 M urea and 20 mM imidazole, (11) elution buffer containing 6 M urea and 250 mM imidazole, (12) elution buffer containing 6 M urea and 1 M imidazole, (13) sample passed through the purification column.

Figure 4: Hydrophobicity of Nc-SAG1 protein. The hydrophobic characteristic shown in the graphic was derived from the amino acid sequence of the open reading frame of the *Nc-SAG1* gene (GenBank – acess AAD25091.1) by using the Vector NTI software.

Figure 1

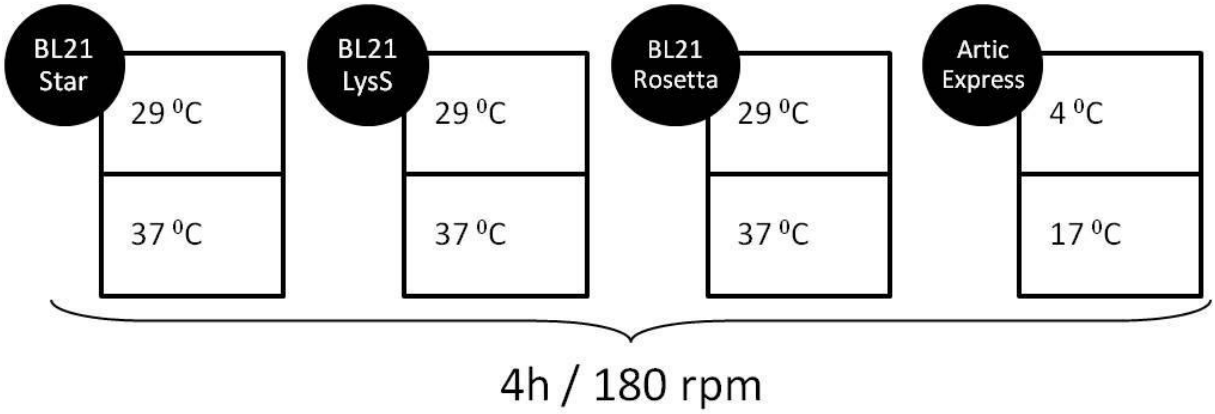

BL21 Star
29 °C
37 °C
BL21 LysS
29 °C
37 °C
BL21 Rosetta
29 °C
37 °C
Artic Express
4 °C
17 °C
4h / 180 rpm

Figure 2

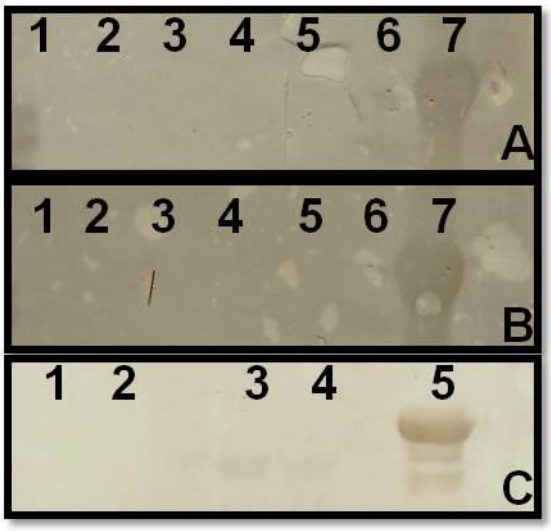
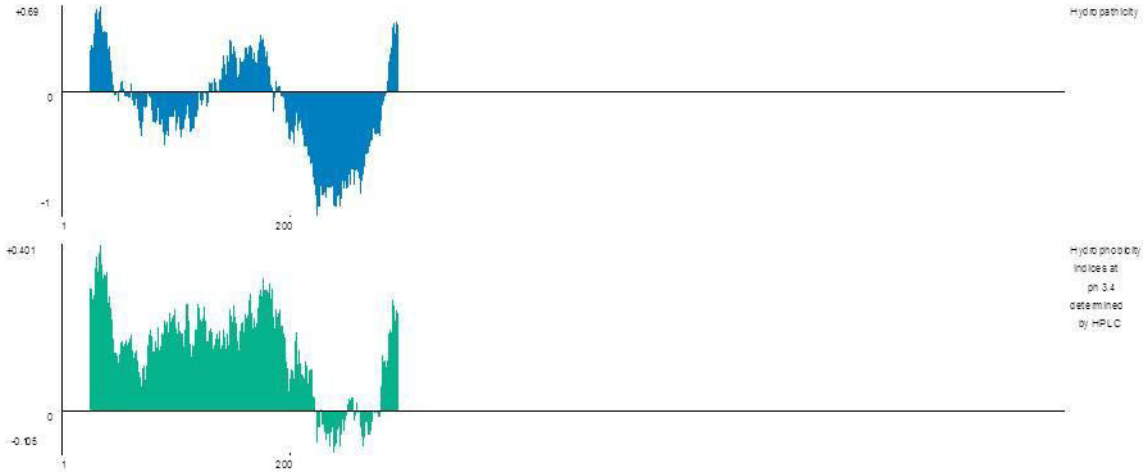


Figure 3

Figure 4


+0.69
0
-1
1
200
Hydropathicity
+0.401
0
-0.105
1
200
Hydrophobicity
Indices at
pH 3.4
determined
by HPLC

5 CONCLUSÃO GERAL

- Foi possível expressar as proteínas recombinantes Nc-p43 e Nc-p29 em *E. coli*.
- O anticorpo policlonal anti-rNcp-43 produzido reconhece os taquizoítos de *N. caninum*, e pode ser utilizado no diagnóstico direto e indireto da neosporose.
- É possível a utilização deste anticorpo, quando conjugado a FITC, em ensaios de detecção direta e específica do parasito.
- O b-ELISA utilizando o anticorpo policlonal conjugado a peroxidase (pAb/HRP) representa uma opção viável frente aos testes de diagnóstico disponíveis para a neosporose, uma vez que menos passos estão envolvidos na sua realização e seu formato evita a reatividade cruzada com anticorpos anti-espécie específicos.

6 REFERÊNCIAS

Adjogble, K.D., Mercier, C., Dubremetz, J.F., Hucke, C., Mackenzie, C.R., Cesbron-Delauw, M.F. & Däubener, W. (2004). GRA9, a new *Toxoplasma gondii* dense granule protein associated with the intravacuolar network of tubular membranes. International Journal of Parasitology. 34(11). p. 1255-1264.

Aguado-Martinez, A., Garcia, A.G., Schares, G., Castillo, V.R., García, A.F., Fernández, V.M. & Ortega-Mora, L.M. (2010). Characterisation of NcGRA7 and NcSAG4 proteins: immunolocalization and their role in the host cell invasion by *Neospora caninum* tachyzoites. Acta Parasitologica. 55(4). p. 304-312.

Ahn, H.J., Kim, S., Kim, D.Y., & Nam, H.W. (2003). ELISA detection of IgG antibody against a recombinant major surface antigen (Nc-p43) fragment of *Neospora caninum* in bovine sera. The Korean Journal of Parasitology. 41(3). p. 175-177.

Ahn, H.J., Song, K.J., Son, E.S., Shin, J.C. & Nam, H.W. (2001). Protease activity and host cell binding of the 42-kDa rhoptry protein from *Toxoplasma gondii* after secretion. Biochemical and Biophysical Research Communications. 287(3). p. 630-635.

Al-Qassab, S., Reichel, M. P. & Ellis, J. (2010). A second generation multiplex PCR for typing strains of *Neospora caninum* using six DNA targets. Molecular and Cellular Probes*,* 24(1). p. 20-26.

Alaeddine, F., Hemphill, A., Debache, K. & Guionaud, C. (2013). Molecular cloning and characterization of NcROP2Fam-1, a member of the ROP2 family of rhoptry proteins in *Neospora caninum* that is targeted by antibodies neutralizing host cell invasion in vitro. Parasitology. 140 (8). p. 1033-1050.

Alexander, D.L., Arastu-Kapur, S., Dubremetz, J.F. & Boothroyd, J.C. (2006). *Plasmodium falciparum* AMA1 binds a rhoptry neck protein homologous to TgRON4, a component of the moving junction in *Toxoplasma gondii*. Eukaryot Cell. 5(7). 1169-1173.

Alexander, D.L., Mital, J., Ward, G.E., Bradley, P. & Broothroyd, J.C. (2005). Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. PLoS Pathogens. 1(2). p. 137-149.

Almeria, S., Serrano, B., Yaniz, J.L., Darwich, L. & Lopez-Gatius, F. (2012). Cytokine gene expression profiles in peripheral blood mononuclear cells from *Neospora caninum* naturally infected dams throughout gestation. Vetereinary Parasitology. 183(3-4). p.237-243.

Almeria, S., López-Gatius, F., García-Ispierto, I., Nogareda, C., Bech-Sàbat, G., Serrano, B., Santolaria, P. & Yániz, J.L. (2009). Effects of crossbreed pregnancies on the abortion risk of Neospora caninum-infected dairy cows. Veterinary Parasitology. 163(4). p.323-329.

Alvarez-Garcia, G., Bueno, J.P., Bautista, M.O. & Mora, L.M.O. (2002). Pattern of recognition of *Neospora caninum* tachyzoite antigens by naturally infected pregnant cattle and aborted foetuses. Veterinary Parasitology. 107(1-2). p. 15-27.

Ancelin, M.L.,Calas, M., Vidal-Sailhan, V., Herbuté, S., Ringwald, P. & Via, H.J. (2003). Potent inhibitors of Plasmodium phospholipid metabolism with a broad spectrum of in vitro antimalarial activities. Antimicrobial Agents and Chemotherapy. 47(8). p. 2590-2597.

Andreotti, R., Barros, J.C., Pereira, A.R., Oshiro, L.M., Cunha, R.C., & Figueiredo Neto, L.F. (2010). Association between seropositivity for *Neospora caninum* and reproductive performance of beef heifers in the Pantanal of Mato Grosso do Sul, Brazil. Revista Brasileira de Parasitologia Veterinária. 19(2). p. 119-123.

Andreotti, R., Matos, M. F., Goncalves, K. N., Oshiro, L. M., Lima-Junior, M. S., Paiva, F. & Leite, F. L. (2009). Comparison of indirect ELISA based on recombinant protein NcSRS2 and IFAT for detection of *Neospora caninum* antibodies in sheep. Revista Brasileira de Parasitologia Veterinária. 18(2). p.19-22.

Andrianarivo, A. G., Anderson, M. L., Rowe, J. D., Gardner, I. A., Reynolds, J. P., Choromanski, L. & Conrad, P. A. (2005). Immune responses during pregnancy in heifers naturally infected with *Neospora caninum* with and without immunization. Parasitology Research. 96(1). p.24-3.

Angel, S.O., Matrajt, M & Echeverria, P.C. (2013). A review of recent patents on the protozoan parasite HSP90 as a drug target. Recent Patents on Biotechnology. 7(1). p. 2-8.

Barber, J. S. & Trees, A. J. (1998). Naturally occurring vertical transmission of *Neospora caninum* in dogs. International Journal for Parasitology. 28(1). p.57-64.

Barr, B. C., Conrad, P. A., Breitmeyer, R., Sverlow, K., Anderson, M. L., Reynolds, J., Chauvet, A. E., Dubey, J. P. & Ardans, A. A. (1993). Congenital Neospora infection in calves born from cows that had previously aborted Neospora-infected fetuses: four cases (1990-1992). Journal of the American Veterinary Medical Association. 202(1). p.113-117.

Baszler, T.V., Adams, S., Vander-Schalie, J., Mathison, B.A & Kostovic, M. (2001). Validation of a commercially available monoclonal antibody-based competitive-inhibition enzyme-linked immunosorbent assay for detection of serum antibodies to *Neospora caninum* in cattle. Journal of Clinical Microbiology. 39(11). p. 3851-3857.

Benavides, J., Katzer, F., Maley, S. W., Bartley, P. M., Canton, G., Palarea-Albaladejo, J., Purslow, C. A., Pang, Y., Rocchi, M. S., Chianini, F., Buxton, D. & Innes, E. A. (2012). High rate of transplacental infection and transmission of *Neospora caninum* following experimental challenge of cattle at day 210 of gestation. Veterinary Research. 10. p.43-83.

Benetti, A. H., Schein, F. B., Dos Santos, T. R., Toniollo, G. H., Da Costa, A. J., Mineo, J. R., Lobato, J., De Oliveira Silva, D. A. & Gennari, S. M. (2009). Inquiry of antibodies anti-*Neospora caninum* in dairy cattle, dogs and rural workers of the south-west region of Mato Grosso State. Revista Brasileira de Parasitologia Veterinária. 18(1). p.29-33.

Besteiro, S., Michelin, A., Poncet, J., Dubremetz, J.F. & Lebrun, M. (2009). Export of a *Toxoplasma gondii* rhoptry neck protein complex at the host cell membrane to form the moving junction during invasion. PLoS Pathogens. 5(2). p. e1000309.

Bjerkas, I., Mohn, S. F. & Presthus, J. (1984). Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Z.Parasitenkd. 70(2). p.271-274.

Bjorkman, C. & Uggla, A. (1999). Serological diagnosis of *Neospora caninum* infection. International Journal for Parasitology. 29(10). p.1497-1507.

Bohne, W., Heesemann, J. & Gross, U. (1994). Reduced replication of *Toxoplasma gondii* is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. Infection and Immunity. 62(5). p. 1761-1767.

Borsuk, S., Andreotti, R., Leite, F.P., Pinto, L.S., Simionatto, S., Hartleben, C.P., Goetze, M., Oshiro, L.M., Matos, M.F. & Berne, M.E. (2011). Development of an indirect ELISA-NcSRS2 for detection of *Neospora caninum* antibodies in cattle. Veterinary Parasitology. 177(1-2). p. 33-38.

Boysen, P., Klevar, S., Olsen, I. & Storset, A. K. (2006). The protozoan *Neospora caninum* directly triggers bovine NK cells to produce gamma interferon and to kill infected fibroblasts. Infection and Immunity. 74(2). p.953-960.

Breinich, M.S., Ferguson, D.J., Foth, B.J., Van Dooren, G.G., Lebrun, M., Quon, D.V., Striepen, B., Bradley, P.J., Frischknecht, F., Carruthers, V.B. & Meissner, M. (2009). A dynamin is required for the biogenesis of secretory organelles in *Toxoplasma gondii*. Current Biology. 19(4). p. 277-286.

Buxton, D., McAllister, M.M. & Dubey, J.P. (2002). The comparative pathogenesis of neosporosis. Trends Parasitology. 189(12). p. 546-552.

Byrem, T. M., Bartlett, P. C., Donohue, H., Voisinet, B. D. & Houseman, J. T. (2012). Performance of a commercial serum ELISA for the detection of antibodies to *Neospora caninum* in whole and skim milk samples. Veterinary Parasitology. 190(1-2). p.249-253.

Byren, I., Rege, S., Campanaro, E., Yankelev, S., Anastasiou, D., Kuropatkin, G. & Evans, R. (2012). Randomized controlled trial of the safety and efficacy of Daptomycin versus standard-of-care therapy for management of patients with osteomyelitis associated with prosthetic devices undergoing two-stage revision arthroplasty. Antimicrobial Agents and Chemotherapy**.** 56(11). p.5626-5632.

Cannas, A., Naguleswaran, A., Müller, N., Eperon, S., Gottstein, B. & Hemphill, A. (2003). Vaccination of mice against experimental *Neospora caninum* infection

using NcSAG1- and NcSRS2-based recombinant antigens and DNA vaccines. Parasitology. 126(4). p. 303-312.

Cardoso, J.M., Amakua, M., Santos, A.J., Araújob, U.S. & Gennari, S.M. (2012). A longitudinal study of *Neospora caninum* infection on three dairy farms in Brazil. Veterinary Parasitology. 187(3-4). p. 553-557.

Carruthers, V. & Boothroyd, J.C. (2007). Pulling together: an integrated model of *Toxoplasma* cell invasion. Current Opinion in Microbiology. 10(1). p. 83-89.

Carruthers, V.B. & Tomley, F.M. (2008). Microneme proteins in apicomplexans. Subcellular Biochemistry. 47. p. 33-45.

Cerede, O. Dubremetz, J.F., Soête, M., Deslée, D., Vial, H., Bout, D. & Lebrun, M. (2005). Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence. The Journal of Experimental Medicine. 201(3). p. 453-463.

Chahan, B., Gaturaga, I., Huang, X., Liao, M., Fukumoto, S., Hirata, H., Nishikawa, Y., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K., Igarashi, I., Mikami, T. & Xuan, X. (2003). Serodiagnosis of *Neospora caninum* infection in cattle by enzyme-linked immunosorbent assay with recombinant truncated NcSAG1. Veterinary Parasitology. 118(3-4). p.177-185.

Chi, J., VanLeeuwen, J.A., Weersink, A., & Keefe, G.P. (2002). Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, Mycobacterium avium subspecies paratuberculosis, and *Neospora caninum*. Preventive Veterinary Medicine. 55(2). p. 137-153

Cho, J.H., Chung, W.S., Song, K.J., Na, B.K., Kang, S.W., Song, C.Y. & Kim, T.S. (2005). Protective efficacy of vaccination with *Neospora caninum* multiple recombinant antigens against experimental *Neospora caninum* infection. The Korean Journal of Parasitology. 43(1). p. 19-25.

Corbellini, L. G., Smith, D. R., Pescador, C. A., Schmitz, M., Correa, A., Steffen, D. J. & Driemeier, D. (2006). Herd-level risk factors for *Neospora caninum* seroprevalence in dairy farms in southern Brazil. Preventive Veterinary Medicine. 74(2-3). p.130-141.

Costa, K.S., Santos, S.L., Uzêda, R.S., Pinheiro, A.M., Almeida, M.A., Araújo, F.R., McAllister, M.M. & Gondim, L.F. (2008). Chickens (*Gallus domesticus*) are natural intermediate hosts of *Neospora caninum*. International Journal for Parasitology. 38(2). p. 157-159.

Cowper, B., Matthews, S. & Tomley, F. (2012). The molecular basis for the distinct host and tissue tropisms of coccidian parasites. Molecular and Biochemical Parasitology. 186(1). p. 1-10.

Da Cunha Filho, N. A., Lucas, A. S., Pappen, F. G., Ragozo, A. M., Gennari, S. M., Junior, T. L. & Farias, N. A. (2008). Risk factors and prevalence of antibodies anti-*Neospora caninum* in urban and rural dogs from Rio Grande do Sul, Brazil. Revista Brasileira de Parasitologia Veterinária. 17(1). p.301-306.

Dautu, G., Ueno, A., Miranda, A., Mwanyumba, S., Munyaka, B., Carmen, G., Kariya, T., Omata, Y., Saito, A., Xuan, X. & Igarashi, M. (2008). *Toxoplasma gondii*: detection of MIC10 antigen in sera of experimentally infected mice. Experimental Parasitology. 118(3). p. 362-371.

Davison, H.C., Guy, C.S., McGarry, J.W., Guy, F., Williams, D.J., Kelly, D.F. & Trees, A.J. (2001). Experimental studies on the transmission of *Neospora caninum* between cattle. Research in Veterinary Science. 70(2). p. 163-168.

Debache, K., Alaeddine, F., Guionaud, C., Monney, T., Müller, J., Strohbusch, M., Leib, S.L., Grandgirard, D. & Hemphill, A. (2009). Vaccination with recombinant NcROP2 combined with recombinant NcMIC1 and NcMIC3 reduces cerebral infection and vertical transmission in mice experimentally infected with *Neospora caninum* tachyzoites. International jornal for Parasitology. 39(12). p. 1373-1384.

Debache, K., Guionaud, C., Alaeddine, F. & Hemphill, A. (2010). Intraperitoneal and intra-nasal vaccination of mice with three distinct recombinant *Neospora caninum* antigens results in differential effects with regard to protection against experimental challenge with *Neospora caninum* tachyzoites. Parasitology. 137(2). p. 229-240.

Debache, K., Guionaud, C., Alaeddine, F., Mevissen, M. & Hemphill, A. (2008). Vaccination of mice with recombinant NcROP2 antigen reduces mortality and cerebral infection in mice infected with *Neospora caninum* tachyzoites. International Journal for Parasitology. 38(12). p. 1455-1463.

Debache, K., Kropf, C., Schütz, C.A., Harwood, L.J., Käuper, P., Monney, T., Rossi, N., Laue, C., McCullough, K.C. & Hemphill, A. (2011). Vaccination of mice with chitosan nanogel-associated recombinant NcPDI against challenge infection with *Neospora caninum* tachyzoites. Parasite Immunology. 33(2). p. 81-94.

Dijkstra, T., Barkema, H. W., Eysker, M., Beiboer, M. L. & Wouda, W. (2003). Evaluation of a single serological screening of dairy herds for *Neospora caninum* antibodies. Veterinary Parasitology. 110(3-4). p.161-169.

Dijkstra, T., Barkema, H. W., Eysker, M., Hesselink, J. W. & Wouda, W. (2002). Natural transmission routes of *Neospora caninum* between farm dogs and cattle. Veterinary Parasitology. 105(2). p.99-104.

Dong, J., Otsuki, T., Kato, T. & Park, E.Y. (2012). Development of a diagnostic method for neosporosis in cattle using recombinant *Neospora caninum* proteins. BioMed Central Biotechnology.12. p. 19.

Doskaya, M., Caner, A., Can, H., İz, S.G., Gedik, Y., Döşkaya, A.D., Kalantari-Dehaghi, M. & Gürüz, Y. (2014). Diagnostic value of a Rec-ELISA using *Toxoplasma gondii* recombinant SporoSAG, BAG1, and GRA1 proteins in murine models infected orally with tissue cysts and oocysts. PLoS One. 9(9). p. e108329.

Dowse, T. & Soldati, D. (2004). Host cell invasion by the apicomplexans: the significance of microneme protein proteolysis. Current Opinion in Microbiology. 7(4). p. 388-396.

Dowse, T.J. & Soldati, D. (2005). Rhomboid-like proteins in Apicomplexa: phylogeny and nomenclature. Trends Parasitology. 21(6). p. 254-258.

Dubey, J. P. (2003). Review of *Neospora caninum* and neosporosis in animals. The Korean Journal of Parasitology. 41(1). p.1-16.

Dubey, J. P., Dorough, K. R., Jenkins, M. C., Liddell, S., Speer, C. A., Kwok, O. C. & Shen, S. K. (1998). Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture. International Journal for Parasitology. 28(8). p.1293-1304.

Dubey, J.P., Hattel, A., Lindsay, D. & Topper, M.J. (1988). Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. Journal of the American Veterinary Medical Association. 193(10). p. 1259-1263.

Dubey, J.P., Jenkins, M.C., Kwok, O.C., Ferreira, L.R., Choudhary, S., Verma, S.K., Villena, I., Butler, E. & Carstensen, M. (2013). Congenital transmission of *Neospora caninum* in white-tailed deer (*Odocoileus virginianus*). Veterinary Parasitology. 196(3-4). p. 519-522.

Dubey, J. P., Jenkins, M. C., Rajendran, C., Miska, K., Ferreira, L. R., Martins, J., Kwok, O. C. & Choudhary, S. (2011). Gray wolf (*Canis lupus*) is a natural definitive host for *Neospora caninum*. Veterinary Parasitology. 181(2-4). p.382-387.

Dubey, J. P. & Schares, G. (2006). Diagnosis of bovine neosporosis. Veterinary Parasitology . 140(1-2). p.1-34.

Dubey, J.P. & Schares, G. (2011). Neosporosis in animals--the last five years. Veterinary Parasitology. 180(1-2). p. 90-108.

Dubremetz, J.F. (2007). Rhoptries are major players in *Toxoplasma gondii* invasion and host cell interaction. Cellular Microbiology. 9(4). p. 841-848.

Dubremetz, J.F., Garcia-Réguet, N., Conseil, V. & Fourmaux, M.N. (1998). Apical organelles and host-cell invasion by Apicomplexa. International Journal for Parasitology. 28(7). p. 1007-1013.

Ellis, J., Goodswen, S., Kennedy, P. J. & Bush, S. (2012). The core mouse response to infection by *Neospora caninum* defined by gene set enrichment analyses. Bioinformatics and Biology Insights. 6. p.187-20.

Ellis, J., Miller, C., Quinn, H., Ryce, C. & Reichel, M.P. (2008). Evaluation of recombinant proteins of *Neospora caninum* as vaccine candidates (in a mouse model). Vaccine. 26(47). p. 5989-5996.

Fernandez-Garcia, A. Risco-Castillo, V., Zaballos, A., Alvarez-García, G. & Ortega-Mora, L.M. (2006). Identification and molecular cloning of the *Neospora caninum* SAG4 gene specifically expressed at bradyzoite stage. Molecular and Biochemical Parasitology. 146(1). p. 89-97.

Ferreirinha, P. Dias, J., Correia, A., Pérez-Cabezas, B., Santos, C., Teixeira, L., Ribeiro, A., Rocha, A. & Vilanova, M. (2014). Protective effect of intranasal immunization with *Neospora caninum* membrane antigens against murine neosporosis established through the gastrointestinal tract. Immunology. 141(2). p. 256-267.

Friedrich, N., Santos, J.M., Liu, Y., Palma, A.S., Leon, E., Saouros, S., Kiso, M., Blackman, M.J., Matthews, S., Feizi, T. & Soldati-Favre, D. (2010). Members of a novel protein family containing microneme adhesive repeat domains act as sialic acid-binding lectins during host cell invasion by apicomplexan parasites. The Journal of Biological Chemistry. 285(3). p. 2064-2076.

Ghalmi, F., China, B., Jenkins, M., Azzag, N. & Losson, B. (2014). Comparison of different serological methods to detect antibodies specific to *Neospora caninum* in bovine and canine sera. J Vet Diagn Invest. 26(1). p. 136-140.

Ghalmi, F., China, B., Ghalmi, A., Hammitouche, D. & Losson, B. (2012). Study of the risk factors associated with *Neospora caninum* seroprevalence in Algerian cattle populations. Research in Veterinary Science. 93. p. 655-661.

Gondim, L.F., Mcallister, M.M., Mateus-Pinilla, N.E., Pitt, W.C., Mech, L.D. & Nelson, M. E. (2004). Transmission of *Neospora caninum* between wild and domestic animals. Journal of Parasitology. 90(6). p.1361-1365.

Gondim, L.S., Abe-Sandes, K., Uzêda, R.S., Silva, M.S., Santos, S.L., Mota, R.A., Vilela, S.M. & Gondim, L.F. (2010). *Toxoplasma gondii* and *Neospora caninum* in sparrows (*Passer domesticus*) in the Northeast of Brazil. Veterinary Parasitology.168(1-2). p. 121-124.

Goodswen, S.J., Kennedy, P.J. & Ellis, J.T. (2013). A review of the infection, genetics, and evolution of *Neospora caninum*: from the past to the present. Infections Genetic and Evolution. 13 p.133-150.

Graewe, S., Rankin, K.E., Lehmann, C., Deschermeier, C., Hecht, L., Froehlke, U., Stanway, R.R. & Heussler, V. (2011). Hostile takeover by *Plasmodium*: reorganization of parasite and host cell membranes during liver stage egress. PLoS Pathogens. 7(9). p. e1002224.

Guionaud, C., Hemphill, A., Mevissen, M. & Alaeddine, F. (2010). Molecular characterization of *Neospora caninum* MAG1, a dense granule protein secreted into the parasitophorous vacuole, and associated with the cyst wall and the cyst matrix. Parasitology. 137(11). p. 1605-1619.

Haldorson, G.J., Stanton, J.B., Mathison, B.A., Suarez, C.E. & Baszler, T.V. (2006). *Neospora caninum*: antibodies directed against tachyzoite surface protein

NcSRS2 inhibit parasite attachment and invasion of placental trophoblasts in vitro. Experimental Parasitology. 112(3). p. 172-178.

Hasler, B. Stärk K, Gottstein, B., & Reist, M. (2008). Epidemiological and financial considerations for the control of *Neospora caninum* on Swiss dairy farms. Schweiz Arch Tierheilkd. 150(6). p. 273-280.

He, P., Li, J., Gong, P., Liu, C., Zhang, G., Yang, J., Tuo, W., Yang, B. & Zhang, X. (2013). *Neospora caninum* surface antigen (p40) is a potential diagnostic marker for cattle neosporosis. Parasitology Research. 112(5). p. 2117-2120.

Hehl, A.B., Lekutis, C., Grigg, M.E., Bradley, P.J., Dubremetz, J.F., Ortega-Barria, E. & Boothroyd, J.C. (2000). *Toxoplasma gondii* homologue of plasmodium apical membrane antigen 1 is involved in invasion of host cells. Infection and Immunity. 68(12). p. 7078-7086.

HEMPHILL, A. (1999). The host-parasite relationship in neosporosis. Advances in Parasitology. 43. p.47-104.

Hemphill, A., Debache, K., Monney, T., Schorer, M., Guionaud, C., Alaeddine, F., Mueller, N. & Mueller, J. (2013). Proteins mediating the *Neospora caninum*-host cell interaction as targets for vaccination. Frontiers in Bioscience. 5 (Elite Ed). p. 23-36.

Hemphill, A., Fuchs, N., Sonda, Gottstein, B. & Hentrich, B. (1997). Identification and partial characterization of a 36 KDa surface protein on *Neospora caninum* tachyzoites. 115. p. 371-380.

Hemphill, A., Fuchs, N., Sonda, S. & Hehl, A. (1999). The antigenic composition of *Neospora caninum*. International Journal for Parasitology. 29(8). p. 1175-1188.

Hemphill, A. & Gottstein, B. (1996). Identification of a major surface protein on *Neospora caninum* tachyzoites. Parasitology Research. 82(6). p. 497-504.

Hemphill, A., Vonlaufen, N. & Naguleswaran, A. (2006). Cellular and immunological basis of the host-parasite relationship during infection with *Neospora caninum*. Parasitology. 133(3). p. 261-278.

Hiasa, J., Kohara, J., Nishimura, M., Xuan, X., Tokimitsu, H. & Nishikawa, Y. (2012). ELISAs based on rNcGRA7 and rNcSAG1 antigens as an indicator of *Neospora caninum* activation. Veterinary Parasitology. 187(3-4). p. 379-385.

Hiasa, J., Nishimura, M., Itamoto, K., Xuan, X., Inokuma, H. & Nishikawa, Y. (2012). Enzyme-linked immunosorbent assays based on *Neospora caninum* dense granule protein 7 and profilin for estimating the stage of neosporosis. Clin Vaccine Immunol. 19(3). p. 411-417.

Holec-Gasior, L., Ferra, B., Hiszczyńska-Sawicka, E. & Kur, J. (2014). The optimal mixture of *Toxoplasma gondii* recombinant antigens (GRA1, P22, ROP1) for diagnosis of ovine toxoplasmosis. Veterinary Parasitology. 206(3-4). p. 146-152.

Howe, D.K., Crawford,M.C., Lindsay, D. & Sibley, L.D. (1998). The p29 and p35 immunodominant antigens of *Neospora caninum* tachyzoites are homologous to the family of surface antigens of *Toxoplasma gondii*. Infection and Immunity. 66(11). p. 5322-5328.

Howe, D.K. & Sibley, L.D. (1999). Comparison of the major antigens of *Neospora caninum* and *Toxoplasma gondii*. International Journal for Parasitology. 29(10). p. 1489-1496.

Howe, D.K., Tang, K., Conrad, P.A., Sverlow, K., Dubey, J. P. & Sibley, L. (2002). D. Sensitive and specific identification of *Neospora caninum* infection of cattle based on detection of serum antibodies to recombinant Ncp29. Clinical and Diagnostic Laboratory Immunology. 9(3). p.611-615.

Ibrahim, H. M. (2013). Seroprevalence of *Neospora caninum* antibodies in chicken samples from Delta Egypt using a recombinant NcSAG1 protein-based ELISA. The Egyptian Journal of Immunology. 20(10). p.29-37.

Innes, E.A. (2010). A brief history and overview of *Toxoplasma gondii*. Zoonoses Public Health. 57(1). p. 1-7.

Innes, E.A., Andrianarivo, A.G., Bjorkman, C., Williams, D.J. & Conrad, P.A. (2002). Immune responses to *Neospora caninum* and prospects for vaccination. Trends Parasitology. 18(11). p.497-504.

Innes, E.A. & Vermeulen, A.N. (2006). Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*. Parasitology. 133(S). p. 145-168.

Ismael, A.B., Poisson, I.D., Lebrun, M., Dubremetz, J.F., Bout, D. & Mévélec, M.N. (2006). Mic1-3 knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice. J Infect Dis. 194(8). p. 1176-1183.

Jia, L.J., Zhang, S.F., Qian, N.C., Xuan, X.N., Yu, L.Z., Zhang, X.M. & Liu, M.M. (2013). Generation and immunity testing of a recombinant adenovirus expressing NcSRS2-NcGRA7 fusion protein of bovine *Neospora caninum*. The Korean Journal of Parasitology. 51(2). p. 247-253.

Jimenez-Ruiz, E., Alvarez-García, G., Aguado-Martínez, A., Salman, H., Irache, J.M., Marugán-Hernández, V. & Ortega-Mora, L.M. (2012). Low efficacy of NcGRA7, NcSAG4, NcBSR4 and NcSRS9 formulated in poly-epsilon-caprolactone against *Neospora caninum* infection in mice. Vaccine. 30(33). p. 4983-4992.

Jimenez-Ruiz, E., Bech-Sàbat, G., Alvarez-García, G., Regidor-Cerrillo, J., Hinojal-Campaña, L. & Ortega-Mora, L.M. (2013). Specific antibody responses against *Neospora caninum* recombinant rNcGRA7, rNcSAG4, rNcBSR4 and rNcSRS9 proteins are correlated with virulence in mice. Parasitology. 140(5). p. 569-579.

Jung, C., Lee, C.Y. & Grigg, M.E. (2004). The SRS superfamily of Toxoplasma surface proteins. International Journal for Parasitology. 34(3). p.285-296.

Kaasch, A.J. & Joiner, K.A. (2000). Protein-targeting determinants in the secretory pathway of apicomplexan parasites. Current Opinion in Microbiology. 3(4). p. 422-428.

Kamga-Waladjo, A.R. et al. (2010). Seroprevalence of *Neospora caninum* antibodies and its consequences for reproductive parameters in dairy cows from Dakar-Senegal, West Africa. Tropical Animal Health and Production. 42(5). p. 953-959.

Keller, N., Naguleswaran, A., Cannas, A., Vonlaufen, N., Bienz, M., Björkman, C., Bohne, W. & Hemphill, A. (2002). Identification of a *Neospora caninum* microneme protein (NcMIC1) which interacts with sulfated host cell surface glycosaminoglycans. Infection and Immunity. 70(6). p. 3187-3198.

Keller, N., Riesen, M., Naguleswaran, A., Vonlaufen, N., Stettler, R., Leepin, A., Wastling, J.M. & Hemphill, A. (2004). Identification and characterization of a *Neospora caninum* microneme-associated protein (NcMIC4) that exhibits unique lactose-binding properties. Infection Immunity. 72(8). p. 4791-4800.

Khan, I.A., Schwartzman, J.D., Fonseka, S. & Kasper, L.H. (1997). *Neospora caninum*: role for immune cytokines in host immunity. Experimental Parasitology**.** 85(1). p.24-34.

Khow, O. & Suntrarachun, S. (2012). Strategies for production of active eukaryotic proteins in bacterial expression system. Asian Pacific Journal of Tropical Biomedicine. v.2(2). p. 159-162.

King, J.S., Slapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T. & Windsor, P.A. (2010). Australian dingoes are definitive hosts of *Neospora caninum*. International Journal for Parasitology. 40(8). p.945-950.

Kobayashi, T., Narabu, S., Yanai, Y., Hatano, Y., Ito, A., Imai, S. & Ike, K. (2013). Gene cloning and characterization of the protein encoded by the *Neospora caninum* bradyzoite-specific antigen gene BAG1. Journal of Parasitology. 99(3). p. 453-458.

Köhler, S., Delwiche, C.F., Denny, P.W., Tilney, L.G., Webster, P., Wilson, R.J., Palmer, J.D. & Roos, D.S. (1997). A plastid of probable green algal origin in Apicomplexan parasites. Science. 275(5305). p. 1485-1489.

Latif, B.M. & Jakubek, E.B. (2008). Determination of the specificities of monoclonal and polyclonal antibodies to *Neospora*, *Toxoplasma* and *Cryptosporidium* by fluorescent antibody test (FAT). Tropical Biomedicine. 25(3). p.225-231.

Lee, E.G. Kim, J.H., Shin, Y.S., Shin, G.W., Kim, Y.R., Palaksha, K.J., Kim, D.Y., Yamane, I., Kim, Y.H., Kim, G.S., Suh, M.D. & Jung, T.S. (2005). Application of proteomics for comparison of proteome of *Neospora caninum* and *Toxoplasma gondii* tachyzoites. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences. 815(1-2). p. 305-314.

Lee, E.G. Kim, J.H., Shin, Y.S., Shin, G.W., Suh, M.D., Kim, D.Y., Kim, Y.H., Kim, G.S. & Jung, T.S. (2003). Establishment of a two-dimensional electrophoresis

map for *Neospora caninum* tachyzoites by proteomics. Proteomics. 3(12). p. 2339-2350.

Lekutis C., Ferguson D.J., Grigg M.E., Camps M. & Boothroyd J.C. (2001). Surface antigens of *Toxoplasma gondii*: variations on a theme. International Journal of Parasitology. 31(12). p.1285-1292.

Liao M., Xuan X., Huang X., Shirafuji H., Fukumoto S., Hirata H., Suzuki H. & Fujisaki K. 2005. Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. Parasitology. 130(5). p.481-488.

Liao, M., Zhang, S., Xuan, X., Zhang, G., Huang, X., Igarashi, I. & Fujisaki, K. (2005). Development of rapid immunochromatographic test with recombinant NcSAG1 for detection of antibodies to *Neospora caninum* in cattle. Clinical and Diagnostic Laboratory Immunology. 12(7). p.885-887.

Lindsay, D.S. & Dubey, J.P. (2000). Determination of the activity of diclazuril against *Sarcocystis neurona* and *Sarcocystis falcatula* in cell cultures. Journal of Parasitology. 86(1). p. 164-166.

Liu, G., Cui X., Hao P., Yang D., Liu J. & Liu Q. (2013). GRA 14, a novel dense granule protein from *Neospora caninum*. Acta Biochimica et Biophysica Sinica. 45(7). p. 607-609.

Locatelli-Dittrich, R., Soccol, V.T., Richartz, R.R., Gasino-Joineau, M.E., Vinne, R. & Pinckney, R. D. (2001). Serological diagnosis of neosporosis in a herd of dairy cattle in southern Brazil. Journal of Parasitology. 87(6). p.1493-1494.

Lovett, J.L., Howe, D.K. & Sibley, L.D. (2000). Molecular characterization of a thrombospondin-related anonymous protein homologue in *Neospora caninum*. Mol Biochem Parasitol. 107(1). p. 33-43.

Lyons, R. E.; Mcleod, R.; Roberts, C. W. (2002). *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. Trends Parasitology. 18(5). p.198-201.

Mac Kenzie, W.R., Hoxie, N.J., Proctor, M.E., Gradus, M.S., Blair, K.A., Peterson, D.E., Kazmierczak, J.J., Addiss, D.G., Fox, K.R. & Rose, J.B. (1994). A massive outbreak in Milwaukee of cryptosporidium infection transmitted through the public water supply. The New England Journal of Medicine. 331(3). p.161-167.

Malik, M. & Nitiss, J.L. (2004). DNA repair functions that control sensitivity to topoisomerase-targeting drugs. Eukaryotic Cell. v.3, n.1, p.82-90, 2004.

Marugan-Hernandez, V., Alvarez-Garcia, G., Risco-Castillo, V., Regidor-Cerrillo, J. & Ortega-Mora, L.M. (2010). Identification of *Neospora caninum* proteins regulated during the differentiation process from tachyzoite to bradyzoite stage by DIGE. Proteomics.10(9). p.1740-1750.

Marugan-Hernandez, V., Alvarez-Garcia, G., Tomley, F., Hemphill, A., Regidor-Cerrillo, J. & Ortega-Mora, L.M. (2011). Identification of novel rhoptry proteins in *Neospora caninum* by LC/MS-MS analysis of subcellular fractions. Journal of Proteomics. 74(5). p.629-642.

Mcallister, D. & Latham, S. (2002). Neospora 2001. Trends Parasitology. 18(1). p.4-5.

Mcallister, M.M., Bjorkman, C., Anderson-Sprecher, R. & Rogers, D. G. (2000). Evidence of point-source exposure to *Neospora caninum* and protective immunity in a herd of beef cows. Journal of the American Veterinary Medical Association 217(6). p.881-887.

Mcallister, M. M.; Dubey, J. P.; Lindsay, D. S.; Jolley, W. R.; Wills, R. A.; Mcguire, A. M. (1998). Dogs are definitive hosts of *Neospora caninum*. International Journal for Parasitology. 28(9). p.1473-1478.

Mccann, C.M., Mcallister, M.M., Gondim, L.F., Smith, R.F., Cripps, P.J., Kipar, A., Williams, D. J. & Trees, A. J. (2007). *Neospora caninum* in cattle: experimental infection with oocysts can result in exogenous transplacental infection, but not endogenous transplacental infection in the subsequent pregnancy. International Journal for Parasitology. 37(14). p.1631-1639.

Mcguire, A.M., Mcallister, M.M., Jolley, W.R. & Anderson-Sprecher, R.C. (1997). A protocol for the production of *Neospora caninum* tissue cysts in mice. Journal of Parasitology. 83(4). p.647-651.

Melo, D.P., Da Silva, A.C., Ortega-Mora, L.M., Bastos, S.A. & Boaventura, C.M. (2006). Prevalence of antibodies anti-*Neospora caninum* in bovines from Anapolis and Goiania microregions, Goias, Brazil. Revista Brasileira de Parasitologia Vetrinária. 15(3). p.105-109.

Mercier, C., Adjogble, K.D., Daubener, W. & Delauw, M.F. (2005). Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? International Journal for Parasitology. 35(8). p.829-849.

Miller, C.M., Quinn, H.E., Windsor, P.A. & Ellis, J.T. (2002). Characterisation of the first Australian isolate of *Neospora caninum* from cattle. Australian Veterinary Journal. 80(10). p.620-625.

Moraveji, M., Hosseini, A., Moghaddar, N., Namavari, M.M. & Eskandari, M.H.(2012). Development of latex agglutination test with recombinant NcSAG1 for the rapid detection of antibodies to *Neospora caninum* in cattle. Veterinary Parasitology. 189, (2-4). p.211-217.

Moura, A.B., Souza, A.P., Sartor, A.A., Bellato, V., Pisetta, G.M. Teixeira, E.B. & Eusser Junior, A. (2011). *Neospora caninum* antibodies and risk factors in dogs from Lages and Balneário Camboriú. Santa Catarina. 63. p.262-265.

Munhoz, A.D., Flausino, W., Silva, R.T., Almeida, C.R. & Lopes, C.W. (2006). Distribution of anti-*Neospora caninum* antibodies in dairy cows at municipalities of Resende and Rio Claro in the State of Rio de Janeiro, Brazil. Revista Brasileira de Parasitologia Veterinária. 15(3). p.101-104.

Naguleswaran, A., Alaeddine, F., Guionaud, C., Vonlaufen, N., Sonda, S., Jenoe, P., Mevissen, M., Hemphill, A. (2005). *Neospora caninum* protein disulfide

isomerase is involved in tachyzoite-host cell interaction. International Journal for Parasitology. 35(13). p.1459-1472.

Naguleswaran, A., Cannas, A., Keller, N., Vonlaufen, N., Bjorkman, C., Hemphill, A. (2002). Vero cell surface proteoglycan interaction with the microneme protein NcMIC(3) mediates adhesion of *Neospora caninum* tachyzoites to host cells unlike that in *Toxoplasma gondii*. International Journal for Parasitology. 32(6). p.695-704.

Nam, H. W. (2009). GRA proteins of *Toxoplasma gondii*: maintenance of host-parasite interactions across the parasitophorous vacuolar membrane. The Korean Journal of Parasitology. 47(Supply). p.S29-S37.

Nasir, A., Lanyon, S.R., Schares, G. & Reichel, M.P. (2011). Sero-prevalenceof *Neospora caninum* and *Besnoitia besnoiti* in South Australian beef and dairy cattle. Veterinary Parasitology.

Nishikawa, Y., Tragoolpua, K., Makala, L., Xuan, X. & Nagasawa, H. (2002). *Neospora caninum* NcSRS2 is a transmembrane protein that contains a glycosylphosphatidylinositol anchor in insect cells. Veterinary Parasitology. 109(3-4). p.191-201.

Orozco, M. A., Morales, E. & Salmeron, F. (2013). Characterization of the inflammatory response in the uteri of cows infected naturally by *Neospora caninum*. Journal of Comparative Pathology. 148(2-3). p.148-156.

Pena, H.F., Soares, R.M., Ragozo, A.M., Monteiro, R.M., Yai, L.E., Nishi, S.M. & Gennari, S.M. (2007). Isolation and molecular detection of *Neospora caninum* from naturally infected sheep from Brazil. Veterinary Parasitology. 147(1-2) p.61-66.

Penarete-Vargas, D.M., Mevelec, M.N., Dion, S., Seche, E., Dimier-Poisson, I. & Fandeur, T. (2010). Protection against lethal *Neospora caninum* infection in mice induced by heterologous vaccination with a mic1 mic3 knockout *Toxoplasma gondii* strain. Infection and Immunity. 78(2). p.651-660.

Pereira-Bueno, J., Quintanilla-Gozalo, A., Perez-Perez, V., Espi-Felgueroso, A., Alvarez-Garcia, G., Collantes-Fernandez, E. & Ortega-Mora, L. M. Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. Veterinary Parasitology.111(2-3). p.143-152.

Pescador, C.A., Corbellini, L. G., Oliveira, E. C., Raymundo, D.L. & Driemeier, D. (2007). Histopathological and immunohistochemical aspects of *Neospora caninum* diagnosis in bovine aborted fetuses. Veterinary Parasitology. 150(1-2). p.159-163.

Peters, M., Lutkefels, E., Heckeroth, A.R. & Schares, G. (2001). Immunohistochemical and ultrastructural evidence for *Neospora caninum* tissue cysts in skeletal muscles of naturally infected dogs and cattle. International Journal for Parasitology. 31(10). p.1144-1148.

Piagentini, M., Moya-Araujo, C.F., Prestes, N.C. & Sartor, I.F. (2012). *Neospora caninum* infection dynamics in dairy cattle. Pararitology Rearch. 111(2). p.717-721.

Pinitkiatisakul, S., Friedman, M., Wikman, M., Mattsson, J.G., Lovgren-Bengtsson, K., Stahl, S. & Lunden, A. (2007). Immunogenicity and protective effect against murine cerebral neosporosis of recombinant NcSRS2 in different iscom formulations. Vaccine. 25(18). p.3658-3668.

Pinitkiatisakul, S., Mattsson, J.G. & Lunden, A. (2008). Quantitative analysis of parasite DNA in the blood of immunized and naive mice after infection with *Neospora caninum*. Parasitology. 135(2). p.175-182.

Pollo-Oliveira, L., Post, H., Acencio, M.L., Lemke, N., Van Den Toorn, H., Tragante, V., Heck, A.J., Altelaar, A.F. & Yatsuda, A.P. (2013). Unravelling the *Neospora caninum* secretome through the secreted fraction (ESA) and quantification of the discharged tachyzoite using high-resolution mass spectrometry-based proteomics. Parasites & Vectors. 6(1). p.335.

Proctor, M.E., Blair, K.A. & Davis, J.P. (1998). Surveillance data for waterborne illness detection: an assessment following a massive waterborne outbreak of Cryptosporidium infection. Epidemiology and Infection. 120(1). p.43-54.

Ragozo, A.M.A., Vânia.S.O., Paula, V.S.O., Souza, S.L.P., Bergamaschi, D.P. & Gennari, S.M. (2003). Ocorrência De Anticorpos Anti-*Neospora Caninum* em Soros Bovinos Procedentes de Seis Estados Brasileiros. Revista Brasileira de Parasitologia Veterinéria. 12(1). p.33-37.

Ramamoorthy, S., Sanakkayala, N., Vemulapalli, R., Duncan, R.B., Lindsay, D.S., Schurig, G.S., Boyle, S.M., Kasimanickam, R. & Sriranganathan, N. (2007). Prevention of lethal experimental infection of C57BL/6 mice by vaccination with Brucella abortus strain RB51 expressing *Neospora caninum* antigens. International Journal for Parasitology. 37(13). p.1521-1529.

Ravindran, S. & Boothroyd, J. C. (2008). Secretion of proteins into host cells by Apicomplexan parasites. Traffic.9(5). p.647-656.

Regidor-Cerrillo, J., Alvarez-Garcia, G., Pastor-Fernandez, I., Marugan-Hernandez, V., Gomez-Bautista, M. & Ortega-Mora, L.M. (2012). Proteome expression changes among virulent and attenuated *Neospora caninum* isolates. Journal of Proteomics. 75(8). p.2306-2318.

Reichel, M.P., Alejandra Ayanegui-Alcerreca, M., Gondim, L.F. & Ellis, J.T. (2013). What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. International Journal for Parasitology. 43(2). p.133-142.

Reichel, M.P. & Ellis, J.T. (2009). *Neospora caninum*--how close are we to development of an efficacious vaccine that prevents abortion in cattle? International Journal for Parasitology. 39(11). p.1173-1187.

Reichel, M.P., Ellis, J.T. & Dubey, J.P. (2007). Neosporosis and hammondiosis in dogs. Journal of Small Animal Practice. 48(6). p.308-312.

Reichel, M.P., Mcallister, M.M., Pomroy, W.E., Campero, C., Ortega-Mora, L.M. & Ellis, J.T. (2014). Control options for *Neospora caninum* - is there anything new or are we going backwards? Parasitology.141(11). p.1455-1470.

Reichmann, G., Dlugonska, H. & Fischer, H.G. (2002). Characterization of TgROP9 (p36), a novel rhoptry protein of *Toxoplasma gondii* tachyzoites identified by T cell clone. Molecular and Biochemical Parasitology. 119(1). p.43-54.

Reiss, M., Viebig, N., Brecht, S., Fourmaux, M.N., Soete, M., Di, C. M., Dubremetz, J. F. & Soldati, D. (2001). Identification and characterization of an escorter for two secretory adhesins in *Toxoplasma gondii*. The Journal of Cell Biology. 152(3). p.563-578.

Risco-Castillo, V., Fernandez-Garcia, A., Zaballos, A., Aguado-Martinez, A., Hemphill, A., Rodriguez-Bertos, A., Alvarez-Garcia, G. & Ortega-Mora, L.M. Molecular characterisation of BSR4, a novel bradyzoite-specific gene from *Neospora caninum*. International Journal for Parasitology. 37(8-9). p.887-896.

Rocchi, M.S., Bartley, P.M., Inglis, N.F., Collantes-Fernandez, E., Entrican, G., Katzer, F. & Innes, E.A. (2011). Selection of *Neospora caninum* antigens stimulating bovine CD4+ve T cell responses through immuno-potency screening and proteomic approaches. Veterinary Research. 42. p.91.

Rodrigues, A.A., Gennari, S.M., Aguiar, D.M., Sreekumar, C., Hill, D.E., Miska, K.B., Vianna, M.C. & Dubey, J.P. (2004). Shedding of *Neospora caninum* oocysts by dogs fed tissues from naturally infected water buffaloes (*Bubalus bubalis*) from Brazil. Veterinary Parasitology. 124(3-4). p.139-150.

Rojo-Montejo, S., Collantes-Fernandez, E., Perez-Zaballos, F., Rodriguez-Marcos, S., Blanco-Murcia, J., Rodriguez-Bertos, A., Prenafeta, A. & Ortega-Mora, L.M. (2013). Effect of vaccination of cattle with the low virulence Nc-Spain 1H isolate of *Neospora caninum* against a heterologous challenge in early and mid-gestation. Veterinary Research. 44. p.106.

Romand, S., Thulliez, P. & Dubey, J.P. (1998). Direct agglutination test for serologic diagnosis of *Neospora caninum* infection. Parasitology Research. 84(1). p.50-53.

Romero, J.J., Perez, E. & Frankena, K. (2004). Effect of a killed whole *Neospora caninum* tachyzoite vaccine on the crude abortion rate of Costa Rican dairy cows under field conditions. Veterinary Parasitology. 123(3-4). p.149-159.

Sa, G.L., Pacheco, D.B., Monte, L.G., Sinnott, F.A., Xavier, M.A., Rizzi, C., Borsuk, S., Berne, M.E., Andreotti, R. & Hartleben, C. P. (2014). Diagnostic potential of anti-rNcp-43 polyclonal antibodies for the detection of *Neospora caninum*. Current Microbiology. 68(4). p.472-476.

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audige, L. & Gottstein, B. A (2001). Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. Veterinary Parasitology.102(1-2). p.1-15.

Sambrook, J. & Russell, D.W. (2001). Molecular cloning: a laboratory manual. 3rd ed., New York: Cold Spring Harbor.

Santos, J.M. & Soldati-Favre, D. (2011). Invasion factors are coupled to key signalling events leading to the establishment of infection in apicomplexan parasites. Cellular Microbiology. 13(6). p.787-796.

Schares, G., Barwald, A. & Conraths, F. J. (2005). Adaptation of a surface antigen-based ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 52(1). p.45-48.

Schares, G., Zinecker, C.F., Schmidt, J., Azzouz, N., Conraths, F.J., Gerold, P. & Schwarz, R.T. (2000). Structural analysis of free and protein-bound glycosyl-phosphatidylinositols of *Neospora caninum*. Molecular and Biochemical Parasitology. 105(1). p.155-161.

Selahi, F., Namavari, M., Hosseini, M.H., Mansourian, M. & Tahamtan, Y. Development of a disperse dye immunoassay technique for detection of antibodies against *Neospora caninum* in cattle. The Korean Journal of Parasitology. 51(1). p.129-132.

Sengupta, P.P., Balumahendiran, M., Raghavendra, A.G., Honnappa, T.G., Gajendragad, M.R. & Prabhudas, K. (2012). Prevalence of *Neospora caninum* antibodies in dairy cattle and water buffaloes and associated abortions in the plateau of Southern Peninsular India. Tropical Animal Health and Production. 45(1). p.205-210.

Shibahara, T., Kokuho, T., Eto, M., Haritani, M., Hamaoka, T., Shimura, K., Nakamura, K., Yokomizo, Y. & Yamane, I. (1999). Pathological and immunological findings of athymic nude and congenic wild type BALB/c mice experimentally infected with *Neospora caninum*. Veterinary Pathology. 36(4). p.321-327.

Shin, Y.S., Shin, G.W., Kim, Y.R., Lee, E.Y., Yang, H.H., Palaksha, K.J., Youn, H.J., Kim, J.H., Kim, D.Y., Marsh, A.E., Lakritz, J. & Jung, T.S. (2005). Comparison of proteome and antigenic proteome between two *Neospora caninum* isolates. Veterinary Parasitology,134(1-2). p.41-52.

Sinnott, F.A., Monte, L.G., Collares, T.F., De Matos, B.M., Pacheco, D.B., Borsuk, S., Andreotti, R. & Hartleben, C.P. (2015). Blocking ELISA using recombinant NcSRS2 protein for diagnosing bovine neosporosis. Current Microbiology. 70(3) p.429-432.

Sohn, C.S., Cheng, T.T., Drummond, M.L., Peng, E.D., Vermont, S.J., Xia, D., Cheng, S.J., Wastling, J.M. & Bradley, P. J. (2011). Identification of novel proteins in *Neospora caninum* using an organelle purification and monoclonal antibody approach. PLoS.One. 6(4). p.e18383.

Song, K.J., Yang, Z., Chong, C.K., Kim, J.S., Lee, K.C., Kim, T.S. & Nam, H.W. (2013). A rapid diagnostic test for toxoplasmosis using recombinant antigenic N-

terminal Half of SAG1 linked with intrinsically unstructured domain of gra2 protein. The Korean Journal of Parasitology. 51(5). p.503-510.

Staska, L.M., Davies, C.J., Brown, W.C., Mcguire, T.C., Suarez, C.E., Park, J.Y., Mathison, B.A., Abbott, J.R. & Baszler, T.V. (2005). Identification of vaccine candidate peptides in the NcSRS2 surface protein of *Neospora caninum* by using CD4+ cytotoxic T lymphocytes and gamma interferon-secreting T lymphocytes of infected holstein cattle. Infection and Immunity. 73(3). p.1321-1329.

Staska, L.M., Mcguire, T.C., Davies, C.J., Lewin, H.A. & Baszler, T.V. *Neospora caninum*-infected cattle develop parasite-specific CD4+ cytotoxic T lymphocytes. Infection and Immunity. 71(6). p.3272-3279.

Takashima, Y., Takasu, M., Yanagimoto, I., Hattori, N., Batanova, T., Nishikawa, Y., & Kitoh, K. (2013). Prevalence and dynamics of antibodies against NcSAG1 and NcGRA7 antigens of *Neospora caninum* in cattle during the gestation period. The Journal of Veterinary Medical Science. 75(11). p.1413-1418.

Tonkin, M.L., Crawford, J., Lebrun, M.L. & Boulanger, M.J. *Babesia divergens* and *Neospora caninum* apical membrane antigen 1 structures reveal selectivity and plasticity in apicomplexan parasite host cell invasion. Protein Science. 22(1). p.114-127.

Trees, A.J. & Williams, D.J. (2005). Endogenous and exogenous transplacental infection in *Neospora caninum* and *Toxoplasma gondii*. Trends Parasitology. 21(12). p.558-561.

Triglia, T., Healer, J., Caruana, S.R., Hodder, A.N., Anders, R.F., Crabb, B.S. & Cowman, A. F. (2000). Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. Molecular Microbiology. 38(4). p.706-718.

Tyler, J.S., Treeck, M. & Boothroyd, J.C. (2011). Focus on the ringleader: the role of AMA1 in apicomplexan invasion and replication. Trends Parasitology. 27(9). p.410-420.

Uchida, M., Nagashima, K., Akatsuka, Y., Murakami, T., Ito, A., Imai, S. & Ike, K. (2013). Comparative study of protective activities of *Neospora caninum* bradyzoite antigens, NcBAG1, NcBSR4, NcMAG1, and NcSAG4, in a mouse model of acute parasitic infection. Parasitology Research. 112(2). p.655-663.

Uchida, Y., Ike, K., Kurotaki, T., Ito, A. & Imai, S. (2004). Monoclonal antibodies preventing invasion of *Neospora caninum* tachyzoites into host cells. The Journal of Veterinary Medical Science. 66(11). p.1355-1358.

Uzeda, R.S., Schares, G., Ortega-Mora, L.M., Madruga, C.R., Aguado-Martinez, A., Corbellini, L.G., Driemeier, D. & Gondim, L.F. (2013). Combination of monoclonal antibodies improves immunohistochemical diagnosis of *Neospora caninum*. Veterinary Parasitology. 197(3-4). p.477-486.

Vanleeuwen, J.A. et al. (2010). Risck factors associated with *Neospora caninum* seropositivity in randomly sampled Canadian dairy cows and herds. Preventive Veterinary Medicine. 93(2-3). p. 129-138.

Vianna, M.C., Sreekumar, C., Miska, K.B., Hill, D.E. & Dubey, J.P. (2005). Isolation of *Neospora caninum* from naturally infected white-tailed deer (*Odocoileus virginianus*). Veterinary Parasitology. 129(3-4). p.253-257.

Vonlaufen, N., Guetg, N., Naguleswaran, A., Muller, N., Bjorkman, C., Schares, G., Von, B. D., Ellis, J. & Hemphill, A. (2004). In vitro induction of *Neospora caninum* bradyzoites in vero cells reveals differential antigen expression, localization, and host-cell recognition of tachyzoites and bradyzoites. Infection and Immunity. 72(1). p.576-583.

Weiss, L.M., Ma, Y.F., Takvorian, P.M., Tanowitz, H.B. & Wittner, M. (1998). Bradyzoite development in *Toxoplasma gondii* and the hsp70 stress response. Infection and Immunity. 6697). p.3295-3302.

Weston, J.F., Heuer, C. (2012). Williamson, N. B. Efficacy of a *Neospora caninum* killed tachyzoite vaccine in preventing abortion and vertical transmission in dairy cattle. Preventive Veterinary Medicine. 103(2-3). p.136-144.

WHO. (2014). WHO procedures for certification of malaria elimination. WHO/The Weekly Epidemiological Record (WER). 89(29). p.321-325.

Widerstrom, M., Schonning, C., Lilja, M., Lebbad, M., Ljung, T., Allestam, G., Ferm, M., Bjorkholm, B., Hansen, A., Hiltula, J., Langmark, J., Lofdahl, M., Omberg, M., Reuterwall, C., Samuelsson, E., Widgren, K., Wallensten, A. & Lindh, J. (2014). Large outbreak of *Cryptosporidium hominis* infection transmitted through the public water supply, Sweden. Emerging Infectious Diseases journal. 20(4). p.581-589.

Wilkowsky, S.E., Bareiro, G.G., Mon, M.L., Moore, D.P., Caspe, G., Campero, C., Fort, M. & Romano, M.I. (2011). An applied printing immunoassay with recombinant Nc-SAG1 for detection of antibodies to *Neospora caninum* in cattle. Journal of Veterinary Diagnostic Investigation. 23(5). p.971-976.

Williams, D.J., Guy, C. S., Smith, R. F., Ellis, J., Bjorkman, C., Reichel, M. P. & Trees, A. J. (2007). Immunization of cattle with live tachyzoites of *Neospora caninum* confers protection against fetal death. Infection and Immunity. 75(3). p.1343-1348.

Williams, D.J., Hartley, C.S., Bjorkman, C. & Trees, A.J. (2009). Endogenous and exogenous transplacental transmission of *Neospora caninum* - how the route of transmission impacts on epidemiology and control of disease. Parasitology. 136(14). p.1895-1900.

Williams, D.J. & Trees, A.J. (2006). Protecting babies: vaccine strategies to prevent foetopathy in *Neospora caninum*-infected cattle. Parasite Immunology. 28(3). p.61-67.

Woodcroft, B.J., Mcmillan, P.J., Dekiwadia, C., Tilley, L. & Ralph, S. A. (2012). Determination of protein subcellular localization in apicomplexan parasites. Trends Parasitology. 28(12). p.546-554.

Wu, X. N., Lin, J., Lin, X., Chen, J., Chen, Z.L. & Lin, J. Y. (2012). Multicomponent DNA vaccine-encoding *Toxoplasma gondii* GRA1 and SAG1 primes: anti-Toxoplasma immune response in mice. Parasitology Research. 111(5). p.2001-2009.

Yin, J., Qu, G., Cao, L., Li, Q., Fetterer, R., Feng, X., Liu, Q., Wang, G., Qi, D., Zhang, X., Miramontes, E., Jenkins, M., Zhang, N. & Tuo, W. (2012). Characterization of *Neospora caninum* microneme protein 10 (NcMIC10) and its potential use as a diagnostic marker for neosporosis. Veterinary Parasitology. 187(1-2). p.28-35.

Zhang, H., Compaore, M.K., Lee, E.G., Liao, M., Zhang, G., Sugimoto, C., Fujisaki, K., Nishikawa, Y. & Xuan, X. (2007). Apical membrane antigen 1 is a cross-reactive antigen between *Neospora caninum* and *Toxoplasma gondii*, and the anti-NcAMA1 antibody inhibits host cell invasion by both parasites. Molecular and Biochemical Parasitology. 151(2). p.205-212.

Zhang, H., Lee, E.G., Yu, L., Kawano, S., Huang, P., Liao, M., Kawase, O., Zhang, G., Zhou, J., Fujisaki, K., Nishikawa, Y. & Xuan, X. (2011). Identification of the cross-reactive and species-specific antigens between *Neospora caninum* and *Toxoplasma gondii* tachyzoites by a proteomics approach. Parasitology Research. 109(3). p.899-911.