UNIVERSIDADE FEDERAL DE PELOTAS
Programa de Pós-Graduação em Parasitologia

Dissertação

Produção e caracterização de um anticorpo policlonal monoespecífico contra rNcp43 para o diagnóstico da neosporose

Gizele Lima de Sá

Pelotas, 2012

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Produção e caracterização de um anticorpo policlonal monoespecífico contra rNcp43 para o diagnóstico da neosporose

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RESUMO

SÁ, Gizele Lima de. **Produção e caracterização de um anticorpo policlonal monoespecífico contra rNcp43 para o diagnóstico da neosporose**. 2012. 36f. Dissertação (Mestrado) – Programa de Pós-Graduação em Parasitologia. Universidade Federal de Pelotas, Pelotas.

A neosporose é considerada uma doença de distribuição mundial, causada pelo protozoário apicomplexa *Neospora caninum*, causador de desordens neuromusculares em cães e abortos em bovinos, o que o torna um patógeno de relevância na bovinocultura. O diagnóstico desta enfermidade pode ser realizado através da identificação do parasito em cortes histológicos ou pela detecção de anticorpos específicos. No entanto, os métodos sorológicos aplicados podem ser dificultados por reações cruzadas com outros parasitos apicomplexas, como *Toxoplasma gondii*. Antígenos específicos do parasito utilizados para detecção de anticorpos ou na produção de insumos biológicos para a detecção de taquizoítos podem melhorar a especificidade e a sensibilidade dos testes de diagnóstico e de estudos da biologia do parasito. Entre os antígenos específicos de *Neospora*, destaca-se a proteína de superfície imunodominante NcSRS2 (Nc-p43), presente tanto em taquizoítos quanto em bradizoítos. Neste estudo, a proteína Nc-p43 foi produzida em sua forma recombinante (rNc-p43), através da inserção do gene *NcSRS2* no vetor de clonagem *pET100/DTOPO*, o qual foi utilizado para transformar a bactéria *Escherichia coli* BL21 Star. A proteína rNc-p43 foi avaliada quanto a reatividade com soros imunes de animais naturalmente infectados das espécies bovina, ovina e canina; e utilizada para imunizar camundongos da linhagem BALB/c para a produção de um anticorpo policlonal (pAb). O anticorpo anti rNc-p43 (pAb/rNc-p43) foi conjugado com isotiocianato de fluoresceína (FITC) e avaliado quanto a reação com a proteína nativa na superfície do parasito por imunofluorescência. A proteína rNc-p43 foi reconhecida por anticorpos anti – *N. caninum* presente nos soros imunes, através de ELISA e Dot blot e foi capaz de gerar anticorpos contra o antígeno rNc-p43. O pAb/rNc-p43 reagiu com a proteína rNc-p43 em ELISA indireto e Western blotting, detectou taquizoítos de *N. caninum* em imunofluorescência indireta e direta, apresentando um padrão de fluorescência somente no complexo apical do parasito, mantendo sua afinidade mesmo após sua conjugação com FITC. O pAb/rNc-p43 não apresentou reação cruzada com *T. gondii*. Os resultados deste estudo sugerem que a rNc-p43 obtida e o pAb gerado podem ser úteis no desenvolvimento de testes de diagnóstico baseados na detecção de anticorpos específicos e do antígeno presente na superfície do parasito.

Palavras chave: *Neospora caninum*. rNc-p43. Anticorpo policlonal.

ABSTRACT

SÁ, Gizele Lima de. **Produção e caracterização de um anticorpo policlonal monoespecífico contra rNcp43 para o diagnóstico da neosporose**. 2012. 36f. Dissertação (Mestrado) – Programa de Pós-Graduação em Parasitologia. Universidade Federal de Pelotas, Pelotas.

Neosporosis is considered a disease of worldwide distribution. It is caused by the apicomplexa protozoan *Neospora caninum*, responsible for neuromuscular disorders in dogs and abortions in bovines, which makes it an important pathogen in cattle breeding. The diagnosis of this disease can be accomplished by identifying the parasites by histological sections or by detection of specific antibodies. However, serological methods can be hampered by cross-reactivity with other apicomplexa parasites, such as *Toxoplasma gondii*. Parasite-specific antigens used for detection of antibodies or in the production of antiserum for the detection of tachyzoites can improve the specificity and sensitivity of diagnostic tests and studies of the biology of the parasite. Among specific antigens of *Neospora* genus, *NcSRS2* (Nc-p43) which is an immunodominant surface protein stands out. It is present in tachyzoites as well as bradyzoites. In this study, Nc-p43 protein was produced in its recombinant form (rNc-p43), by inserting the gene *NcSRS2* in the cloning vector *pET100/DTOPO,* which was used to transform *Escherichia coli* BL21 Star. rNc-p43 protein was evaluated for reactivity with immune sera from naturally infected bovine, ovine and canine species, and used to immunize BALB/c mice for the production of a polyclonal antibody (pAb). rNc-p43 (pAb/rNc-p43) antibody was conjugated to fluorescein isothiocyanate (FITC) and the reactivity with the native protein on the surface of the parasite was evaluated by immunofluorescence. rNc-p43 protein was recognized by anti – *N. caninum* present in immune sera by ELISA and dot blot and it was able to generate antibodies against the p43 antigen. The pAb/rNc-p43 reacted with the rNc-p43 protein in indirect ELISA and Western blotting, detecting *N. caninum* tachyzoites in direct and indirect immunofluorescence, with a fluorescence pattern only in the apical complex of the parasite, maintaining affinity even after conjugation with FITC. pAb/rNc-p43 showed no cross-reactivity with *T. gondii*. The results of this study suggest that the rNc-p43 obtained and the pAb produced can be useful in developing diagnostic tests based on the detection of specific antibodies and the antigen present on the surface of the parasite.

Key Words: *Neospora caninum*. rNc-p43. Polyclonal antibody

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LISTA DE ABREVIATURAS

CPE – Efeito citopático.

DAB - 3,3'-tetrahydrochloride.

DMEM – Meio Dulbecco's modified essentia.

ELISA - Enzyme-Linked Immunoabsorbent Assay.

ELISA/rNcp43 – ELISA utilizando a proteína recombinante como antígeno.

FCS – Fetal calf serum (Soro fetal bovino).

Fig – Figura.

FITC – Isotiocianato de fluoresceína.

g – Força da gravidade.

h – Hora.

H_2O_2 – Peróxido de hidrogênio.

IFI – Imunofluorescência indireta.

IFTA – (Indirect Fluorescent Antibody Test) Imunofluorescência indireta.

IgG – Imunoglobulina G.

IHC – Imunohistoquímica.

IPTG - Isopropil α-D-thiogalactoside.

M – Molar.

mAbs – Anticorpos monoclonais.

mg/L – Miligramas por mililitros.

min – Minutos.

NcSRS2 (Ncp43) – Proteína de superfície imunodominante presente em taquizoítos e bradizoítos.

nm – Nanômetro.

OD – Densidade óptica.

OPD - o-Phenylenediamine.

pAb – Anticorpo policlonal.

pAb/rNcp43 – Anticorpo policlonal contra a proteína recombinante Nc-p43.

pAb/rNcp43 – FITC - Anticorpo policlonal contra a proteína recombinante Nc-p43 conjugado a isotiocianato de fluoresceína.

PBS – Tampão fosfato.

PBS-NM – Tampão fosfato com 5% de leite desnatado.

PBS-T – Tampão fosfato com 0.05% de Tween 20.

POase – Horse radish peroxidase.

rNcp43 – Proteína Nc-p43 na sua forma recombinante.

SDS – Dodecil sulfato de sódio.

SNC – Sistema nervoso central.

UFPel – Universidade Federal de Pelotas.

µg – Micrograma.

µL – Microlitro.

µm- Micrômetro.

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1. INTRODUÇÃO GERAL

A neosporose é uma enfermidade causada pelo protozoário do filo apicomplexa *Neospora caninum*, classe Sporozoea da família Sarcocystidae, subfamília Toxoplasmatinae. Este protozoário até o ano de 1988 foi confundido com *Toxoplasma gondii*, devido à similaridade morfológica e biológica entre os mesmos. Contudo, o agente foi isolado na Noruega a partir de amostras biológicas de cães por Bjerkas et al. (1984) e sua característica antigênica foi descrita por Dubey et al. (1988a) diferenciando-o definitivamente de *T. gondii.* A doença ocorre de forma clinica importante em bovinos e cães, hospedeiros intermediário e definitivo do parasito, respectivamente, e tornou-se uma patologia de relevância devido aos prejuízos econômicos causados a bovinocultura, sendo relacionada a falhas reprodutivas e diminuição da produtividade (DUBEY, 1999; TREES, 1999). No entanto, a infecção natural foi demonstrada através do isolamento do parasito em outros animais como ovelhas, cabras, veados, rinocerontes e cavalos; e anticorpos contra *N. caninum* já foram detectados em soros de búfalos, raposas, coiotes, camelos e felinos (DUBEY, 2003). O potencial zoonótico deste parasito foi avaliado por ensaios sorológicos em diferentes populações humanas aparentemente sadias (NAM et al., 1998; TRANAS, 1999) e em indivíduos imunocomprometidos (LOBATO et al., 2006), tendo sido detectados anticorpos específicos contra *N. caninum*. Embora não haja relatos sobre a infecção humana sua ocorrência não esta descartada (GRAHAM et al., 1999).

O *N. caninum* é um parasito intracelular obrigatório com predileção pelo sistema nervoso central (SNC) dos hospedeiros e apresenta três estágios evolutivos: taquizoítos, bradizoítos e oocistos (DUBEY, 2003). A infecção do hospedeiro intermediário ocorre pela ingestão de oocistos levando a uma infecção sistêmica, e o hospedeiro definitivo desenvolve uma infecção intestinal ao alimentar-se da carcaça de hospedeiros intermediários. Os oocistos não esporulados quando eliminados nas fezes dos hospedeiros definitivos não são infectantes, medem de 10 a 11µm de diâmetro, são esféricos ou subesféricos contendo um esporonte central. Decorridos três dias no ambiente os oocistos esporulam, tornando-se infectantes (MACLLISTER et al., 1998). Na forma esporulada apresentam dois esporocistos que medem 8,4 X 6,1µm, e cada esporocisto contém quatro esporozoítos, medindo 7-8 X 2-3µm (LINDSAY et al., 1999). Os esporozoítos liberados invadem as células da parede

intestinal e passam a ser chamados de taquizoítos, multiplicando-se rapidamente por endodiogenia podendo apresentar forma ovóide, lunar ou globular e medir cerca de 6 x 2µm, formando pseudocistos os quais se rompem liberando taquizoítos. Neste estágio, após 5min de contato com outras células hospedeiras (células neurais, macrófagos, fibroblastos, células endoteliais, monócitos, hepatócitos e células epiteliais do tubo neural), o parasito é capaz de penetrar a célula ativamente (HEMPHILL; GOTTSSTEIN, 1996). Após a multiplicação no interior da célula e posterior rompimento celular, novos taquizoítos são liberados, mantendo a infecção tecidual. Contudo, alguns taquizoítos ao penetrarem na célula hospedeira se diferenciam em bradizoítos, exibindo uma multiplicação lenta por endodiogenia, medindo de 6 a 8 X 1 a 1,8µm e apresentam as mesmas organelas do estágio de taquizoíto, exceto pela menor quantidade de roptrias e estrutura mais delgada (BJERKAS; DUBEY, 1991). Estas formas estão contidas no interior de cistos teciduais, os quais possuem formas ovais com diâmetro de até 107µm, tendo sido observados no tecido cerebral, medula espinhal, nervos e retina (DUBEY et al., 1988b). A espessura da parede cística varia de acordo com o tempo de infecção podendo chegar a 4µm. Cistos com bradizoítos são oralmente infecciosos e estão intimamente ligados à transmissão vertical e horizontal da infecção. A reativação dos cistos no hospedeiro intermediário, caracterizada pela reconversão ao estágio de taquizoíto, pode ocorrer durante a gravidez, devido a imunossupressão, levando a infecção da placenta e do próprio feto (INNES et al., 2002; QUINN et al., 2002).

Na neosporose bovina, a principal via de transmissão é a via transplacentária exógena e endógena (TREES; WILLIANS, 2005). A maioria dos terneiros que nascem de mães previamente infectadas são portadores da infecção, a qual pode perdurar durante toda vida do animal (ANDERSON et al., 1997). Portanto, uma vaca cronicamente infectada pode transmitir a infecção em sucessivas gestações (DUBEY, 2003). No entanto, sabe-se que a infecção em bovinos pode também se estabelecer pela ingestão de oocistos esporulados por via horizontal contidos em água ou alimento contaminado (DE MAREZ et al., 1999). A transmissão lactogênica de *N. caninum* já foi demonstrada experimentalmente em terneiros recém-nascidos, alimentados com taquizoítos adicionados ao colostro, mas não há evidências de que isso ocorra naturalmente (DAVISON et al., 2001), apesar da demonstração de DNA do parasito no leite de fêmeas lactantes naturalmente infectadas (MOSKWA et al.,2003). Hospedeiros definitivos alimentados com

taquizoítos adicionados ao leite não apresentaram evidências de infecção, mas quando alimentados com tecidos de terneiros contendo cistos eliminaram oocistos não esporulados nas fezes, não sendo detectados anticorpos anti *N. caninum* antes de 38 dias pós infecção experimental (DIJKSTRA et al., 2001). Esta via de transmissão foi associada a fazendas com evidencia de infecção por *N. caninum*, onde os cães consumiam placenta e defecavam no pasto e nas silagens armazenadas, enquanto nas fazendas sem evidências de infecção esta prática não ocorria com freqüência (DIJKSTRA et al.,2001). Estes experimentos levam a conclusão de que os cães são provavelmente infectados por fluidos fetais ou restos de placenta expelidos por bovinos infectados, podendo infectar bovinos sadios horizontalmente pela liberação de oocistos, sendo um dos fatores que permite a persistência do parasito por muitos anos em um rebanho. Apesar de o parasito infectar uma ampla gama de hospedeiros, podendo causar danos a várias espécies, cães e bovinos são as espécies mais significativamente afetadas, sendo o aborto o principal sinal clínico observado em bovinos adultos (BARR et al., 1998) e desordens neuromusculares em cães (DUBEY, 2003) e terneiros até 4 meses de idade (MEERSCHMAN et al., 2005). Vacas infectadas podem abortar com qualquer idade a partir do terceiro mês até o final da gestação, e aquelas soropositivas para *N. caninum* são mais suscetíveis ao aborto do que as soronegativas (THURMOND; HIETALA, 1996).

As conseqüências de uma infecção por *N. caninum*, em uma fêmea durante a gestação, seja por uma transmissão transplacentária exógena (por ingestão de oocisto esporulado pela fêmea) ou endógena (reativação de cistos contendo bradizoítos), podem ser: abortos, natimortos, nascimento de terneiros debilitados e infectados ou mesmo clinicamente sadios. Assim, cerca de 95% dos terneiros provenientes de vacas infectadas podem nascer clinicamente sadios, ainda que soropositivos, e somente uma pequena parcela dos fetos sucumbe à infecção a ponto de ser abortado. A ocorrência ou não de aborto é influenciada pela idade do feto, a taxa de parasitemia materna e de características particulares da cepa de *N. caninum* (INNES et al., 2002). Assim, pelo menos em bovinos, a idade e conseqüentemente a maturidade imunológica do feto foi associada ao resultado da infecção (BUXTON et al, 2002).

O diagnóstico *post mortem* da neosporose pode ser realizado pela detecção parasitológica através da histopatologia (SAGER et al., 2001), imuno-histoquímica

(PETERS et al., 2001), isolamento *in vitro* ou *in vivo* (DUBEY et al.,1998) ou pela detecção do DNA do parasito por métodos moleculares (ELLIS, 1998); e o *ante mortem*, pela observação de oocistos a fresco, em exame de fezes de hospedeiros definitivos (MACLLISTER et al., 1998), pela detecção de anticorpos específicos contra *N. caninum* presente no soro de animais infectados (HEMPHILL et al., 2000) ou métodos moleculares aplicados a fluidos biológicos (SCHATZBERG et al., 2003, GONDIM et al., 2004, FERRE et al., 2005). Dentre os diagnósticos desenvolvidos, aqueles aplicados a detecção do parasito em tecidos tornam-se inviáveis em nível de rebanho, por apresentarem dificuldade no seu desenvolvimento, baixa especificidade e sensibilidade (DEMEERSCHMAN et al., 2005, DUBEY et al., 2006). Resultados anteriores corroboram com esta afirmativa, uma vez que em experimentos utilizando bovinos, não foram constatadas lesões histológicas ou parasitos pela imunohistoquímica, utilizando anticorpos anti-antígenos totais, no entanto o *N. caninum* foi isolado a partir de amostras teciduais em cultivo celular (STENLUND et al., 1997; DAVINSON et al., 1997; SAWADA, 2000).

Por este motivo, os testes sorológicos são amplamente aplicados em levantamentos epidemiológicos. Entre os testes que detectam anticorpos, a imunofluorescência indireta (IFI) é considerada como teste de referência (DUBEY et al., 1988a). Outros formatos de testes foram descritos para identificar anticorpos específicos em soros animais, tais como *Enzyme-Linked Immunoabsorbent Assay* (ELISA) (AHN 2003; BJORKMAN; UGGLA, 1999) e teste de aglutinação (*Neospora* Agglutination Test) (ROMAND et al., 1998). Porém, os ensaios ELISA desenvolvidos apresentaram menor especificidade quando comparados a IFI, pois se baseiam na utilização de antígenos solúveis totais do parasito, incluindo antígenos intracelulares, enquanto que a IFI utiliza taquizoítos fixados intactos, prevalecendo os antígenos de superfície do parasito (BJORKMAN; UGGLA, 1999).

O estudo da antigenicidade do parasito sugeriu que os antígenos imunodominantes, específicos das espécies pertencentes ao filo Apicomplexa a qual compreende o gênero *Neospora,* encontram-se na superfície celular e testes sorológicos que possuem como alvo estes antígenos apresentam menor percentual de reações cruzadas com parasitos do mesmo filo (BJORKMAN; UGGLA, 1999). Os antígenos imunodominantes NcSAG1 (Nc-p29) e NcSRS2 (Nc-p43) tem sido descritos como os principais alvos para o desenvolvimento de vacinas e de ensaios diagnósticos mais sensíveis e específicos (HOWE et al., 1998). A Nc-p43, expressa

pelo gene *NcSRS2*: é uma proteína de superfície imunodominante presente nos estágios de taquizoítos e bradizoítos (HEMPHILL et al., 1997), é altamente imunogênica (HOWE et al., 1998), conferiu proteção eficaz frente à infecção e a transmissão vertical em camundongos fêmeas gestantes (NISHIKAWA et al., 2001) e estimulou a produção de anticorpos específicos em experimentos de imunização conduzidos em cães (NISHIKAWA et al., 2000). Ainda, quando utilizada para o diagnóstico da neosporose bovina apresentou alta sensibilidade e especificidade (BORSUK et al., 2010), e anticorpos monoclonais e policlonais gerados contra esta proteína, foram capazes de inibir a adesão e invasão de células hospedeiras *in vitro* (HALDORSON et al., 2006).

Neste contexto, a utilização da proteína Nc-p43 para o desenvolvimento de ferramentas de diagnóstico ou em estudos de desenvolvimento de vacinas, torna-se promissor, uma vez que anticorpos gerados contra este antígeno podem ser utilizados como insumos biológicos em ensaios moleculares ou imunológicos, ou em estudos de inibição da adesão e invasão de células hospedeiras *in vitro* e *in vivo* pelo parasito.

A hipótese deste estudo foi de que o antígeno Nc-p43, na sua forma recombinante, pode ser utilizado em testes de diagnóstico e na produção de anticorpos capazes de reconhecer a proteína nativa exposta na superfície da membrana do *N. caninum*. Nesta perspectiva, o objetivo deste estudo foi produzir a proteína Nc-p43 em sua forma recombinante e utilizar a rNc-p43 obtida para gerar um anticorpo policlonal monoespecífico (pAb); caracterizar o pAb por Imunofluorescência Indireta, *Imunoblot* e ELISA; avaliar o pAb produzido quando conjugado a fluoróforos o que possibilitará a identificação específica do parasito em amostras clínicas e cortes de tecido.

A dissertação está apresentada na forma de artigo científico, a qual foi submetida para publicação no periódico **Journal of Veterinary Science**, portanto esta formatado conforme as exigências deste periódico.

2. ARTIGO

Neospora caninum Ncp43: PROTEIN EXPRESSION AND MONOSPECIFIC POLYCLONAL ANTIBODY GENERATION FOR DIAGNOSTIC APPLICATION

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Abstract

Neospora caninum is an important veterinary pathogen that causes abortion in cattle and neuromuscular disease in dogs however causes natural infections in several other ruminant species, including goats, sheep and deer. The diagnosis of neosporosis is based on antigen detection by histopathology and immunohistochemistry, and on specific antibodies detection against *Neospora,* among that the standard Indirect Fluorescent Antibody Test (IFTA), and diagnostic test formats using native proteins from the parasite, such as ELISA and agglutination test. The recently of a major surface protein Ncp43 was obtained on it recombinant form (rNcp43) to detect specific antibodies in infected hosts and to immunize BALB/c mice to generate murine polyclonal antibody (pAb) for antigen detection in ELISA and fluorescence approaches. The rNcp43 obtained was able to detect anti *N. caninum* antibodies in bovine, sheep and canine sera with both ELISA and Dot blot, and induced antibodies generation against Ncp43. pAb anti rNcp43 detected *N. caninum* tachyzoites by label only apical complex of the parasite, and it activity remained after enzyme and fluorophore conjugation. Results of this study suggest that rNcp43 obtained and pAb generated can be useful for the development of diagnostic tests based on both antibodies and antigen detection.

Key Words: *Neospora caninum;* rNcp43; polyclonal antibody

Introduction

Neospora caninum do not only causes neuromuscular infections in dogs its definitive host (*Canis familiaris*), but also abortion, stillbirth and infection in cattle [12]. Natural infections of the parasite have been identified in several other species of ruminants, including goats, sheep and deer [10], which results in losses livestock [3].

The diagnosis of neosporosis in infected host is usually based on histopathology and immunohistochemistry (IHC) of the aborted fetus; however, in some cases the fetal tissue is not available. The alternative is by specific antibodies detection against *N. caninum*, whereas the presence of antibodies in animal serum indicates the presence or recent infection with the parasite [6].

Serological methods are often used in seroepidemiological studies of *N.caninum* in animals [13]. Among tests for antibodies detection, indirect fluorescent Antibody Test (IFTA) is considered as standard [11]. Other diagnostic test formats were described to identify antibodies in animal sera such as ELISA [1,6] and agglutination test [27]. However, the ELISA developed are less specific than IFTA, due the use of total soluble parasite antigens, including intracellular antigens, since IFTA uses fixed intact tachyzoites and is possible to detect only surface antigens of the parasite [6].

The use of recombinant antigens can improve ELISA sensitivity and specificity by using a reduced number of antigens. In addition, recombinant antigens are produced more easily in large quantities and better standardized for serological assays development [13].

Studies are currently focused on surface antigens that characterize species of phylum Apicomplexa, which includes the genus *Neospora*, and also provide lower percentage of cross-reactivity with parasites of the phylum [6]. Among *Neospora* immunodominant membrane proteins, NcSRS2 (Ncp43) being both tachyzoites and bradyzoites stages [24] and reported as a successful alternative to detect antibodies present in bovine sera, supporting the use of this protein in enzyme immunoassays [7].

Polyclonal and monoclonal antibodies against *N. caninum* were developed with the aim of improving the vaccine and diagnostic [5,32,20]. In a recently work, a panel of specific monoclonal antibodies (mAbs) to *N. caninum*, and mAbs generated recognized a variety of protein compartments from Neospora including the micronemes, rhoptries (body and neck), dense granules, mitochondrion, apicoplast, inner membrane complex, and parasite surface, useful in vaccine and diagnostic development were reported [29].

In this study we report the usefulness of rNcp43 from *N. caninum* to identify infect host and generate a monospecific polyclonal antibody. The rNcp43 was recognized by antibodies from infected animals. The polyclonal antibody (pAb) recognized rNcp43 on different approaches, it recognized the native protein on the surface of *N. caninum* and did not react with *Toxoplasma gondii* tachyzoites suggesting it could be a useful diagnostic tool for neosporosis.

Materials and Methods

Parasites

N. caninum (NC-1) strain [11] was a gift from Dr.ª Débora Pereira Garcia (Federal University Goias), and was used to prepare an antigen formulation for Immunofluorescent assays. The parasites were propagated in Vero cells maintained

in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), at 37ºC with 5% CO_2. When 80% of the Vero cells that had been infected with *N. caninum* tachyzoites showned citopaticeffect (CPE), the cell monolayers were removed by scraping, were washed twice with phosphate-buffered saline (PBS) solution, and then centrifuged at 1000×g for 10min.

Sera

The sera used in this experiment were kindly provided by Laboratory of Parasitology from Federal University of Pelotas. Thirty cattle, 30 sheep, and 20 dog sample sera were used, half of each one negative and half were positive to *N. caninum*, and further stored at -20ºC until analysis. All samples were tested in the Indirect Fluorescent Antibody test (IFAT) to determine the positive and negative sera according to what has been established by Pitel *et al.* [26]. To obtain the positive control (C^+) of each species used in serological reactions of this study an indirect ELISA was performed in triplicate with the positive serum samples, where samples had averaged absorbance values twice larger than the negative control reaction were combined in pool, and the negative control (C^-) used was normal mouse serum.

Recombinant *NcSRS2*

The antigenic domain of NcSRS2 (Ncp-43), 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' and cloned into pET100/D TOPO vector (Invitrogen Tech, Carlsbad, CA, USA). The recombinant plasmid (pET100/D-TOPO/NcSRS2) was used for transformation into *Escherichia coli* BL21 Star (Invitrogen Tech, Carlsbad, CA, USA). The *E. coli* cells in the log phase were treated with 0.75-mM isopropyl α-D-thiogalactoside (IPTG) for 3 hat 37ºC to induce expression of fused fragments of *NcSRS2*. According Borsuk *et al.* [7], the recombinant *NcSRS2* expression was confirmed by SDS-PAGE and Western blotting using anti-6×His alkaline phosphatase conjugate (1:10,000) (Sigma Chemicals, USA). Antibody-reacting protein bands were revealed using 3,3'-tetrahydrochloride (DAB) and H_2O_2. The protein was purified using affinity chromatography on a HiTrap chelating column (GE Healthcare, UK) charged with Ni^{2+} ions. The protein was solubilized in a buffer containing 0.2% N-lauroyl sarcosine. Subsequently, the concentration and purity of recombinant *NcSRS2* were determined using a BCA kit (Pierce, Rockford, IL, USA) and SDS-PAGE, respectively.

Polyclonal Antibodies

Polyclonal Antibody (pAb) to recombinant protein Ncp43 (rNcp43) were prepared as previously described [9]. Briefly, 100µg of rNcp43 was emulsified with an excess of complete Freund's adjuvant (Sigma Aldrich Co., St. Louis, MO) and injected intraperitoneally on days 0, 7, 14 and 21, into two 6-week-old BALB/c mice treated with pristane on day 6. The pAb was purified by affinity chromatography on a protein A-Sepharose CL-4B column (GE Healthcare Company, USA) according to manufacturer's instructions. Purification efficacy was evaluated by SDS-PAGE and final concentration was measured by spectrophotometry at 280 nm. Purified pAb was stored at -20ºC. The reaction pAb against rNcp43 was evaluated by immunoenzymatic assays, and native protein recognition by direct and indirect immunofluorescence, using *Toxoplasma gondii* tachyzoites to detected cross-reactivity. To investigate how would protein conjugation affect pAb performance, a

conjugate of pAb/rNcp43 and POase (pAb/rNcp43- POase) and a conjugate of pAb/rNcp43 and FITC were produced following established procedures [31,33]. The study protocol was approved and maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation of the UFPel (registration number in the CEEA: 9577).

Direct and Indirect Enzime-Linked Immunosorbent Assays (ELISA)

Polystyrene 96-well microtiter plates (Polysorp Nunc, USA) were coated overnight at 4ºC with 50ng/well of recombinant protein NcSRS2 (rNcp43) in 0.05-M carbonate-bicarbonate buffer (pH9.6). The plates were then washed three times using 0.01-M PBS with 0.05% Tween 20 (PBS-T) and blocked using 0.01-M PBS with 5% nonfat milk at 37ºC for 1h. After three washes with PBS-T, both positive and negative control sera and serum samples of each species were added in duplicate, diluted at 1:100 in 0.01-M PBS with 5% nonfat milk and incubated at 37ºC for 1h. After three washes, 100µL/well of anti-bovine IgG (diluted at 1:4000), anti-sheep IgG (diluted at 1:2000) and anti-dog (diluted at 1:5000) conjugated to peroxidase (Sigma Chemicals, USA), diluted in 0.01-M PBS with 5% nonfat milk were added, followed by incubation at 37ºC for 1h. After five washes, 100µL of the substrate (*o*-phenylenediamine dihydrochloride; OPD tablets, Sigma Chemicals, USA) in phosphate-citrate buffer (0.4mg/mL) containing 0.04% of 30% (v/v) hydrogen peroxide, pH 5.0, were added to each well and the plates were incubated in the dark at room temperature for 15min. Optical density (OD) was read at 450 nm in an ELISA reader (VICTOR^{TM}X5/2030Multilabel Reader - PerkinElmer). For experiments testing pAb/rNcp43 and pAb/rNcp43 – POase reactivity to rNcp43 the same ELISA conditions were used excepting addition of anti-mouse peroxidase conjugate (Sigma Chemicals, USA) and pAb/rNcp43 peroxidase conjugate respectively.

Dot blot

Nitrocellulose membrane (GE Healthcare, UK) was coated with 76ng of rNcp43, incubated at 37ºC to dry, blocked with PBS 5% nonfat milk (PBS-NM) for 1h at 37ºC and than incubated with pAb/rNcp43 diluted 1:20 at 37ºC for 1h. The membrane was washed three times with 0.05% PBS-T for 5 min each, incubated with anti-mouse peroxidase conjugate (Sigma) diluted 1:5000 in PBS-NM at 37ºC for 1h, and washed five times with 0.05% PBS-T. Blot was visualized after the addition of the substrate/chromogen solution (H_2O_2/4-chloro-1-naphtol). Normal mouse serum and positive control bovine were used as negative and positive control, respectively.

Direct and Indirect Fluorescent Antibody Test

The antigen for Indirect Fluorescent Antibody Test (IFAT) was prepared as following: cells infected with *N. caninum* tachyzoites were diluted in PBS buffer in order to obtain ~1×10^6 tachyzoites/mL, then 20–30µL (~30,000 tachyzoites) were added to each slide well. The slides were dried at 37ºC and stored at −20ºC until use. Different dilution of pAb in PBS buffer, ranging from 1:5 to 1:100 were added to slide and incubated for 45 min at 37ºC, washed twice with PBS and coated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma Chemicals, USA) at a dilution of 1:100 in PBS buffer and incubated for 1 h in a dark humid chamber for 45 min at 37ºC. Each glass slide included negative and positive control sera added of anti-bovine IgG fluorescein isothiocyanate-conjugated (Sigma Chemicals, USA). Evans blue dye was used in sample buffer. For experiments testing pAb/rNcp43 and pAb/rNcp43 – FITC reactivity to native protein the same IFAT conditions were used

excepting by addition of anti-mouse fluorescein isothiocyanate-conjugated (Sigma) and pAb/rNcp43 fluorescein isothiocyanate conjugated respectively; slides containing *Toxoplasma gondii* tachyzoites were used to investigated pAb/rNcp43 cross-reactivity following manufacturer's instructions (kit Imuno-con Toxo, WAMA Diagnóstica, Brasil). After washing with PBS a drop of mounting medium was added and a cover slip was sealed with acrylic. Labeling was visualized by fluorescent microscopy (Olympus BX 51) with excitation wavelength of 450 nm. These experiments were repeated three times.

Results

Cloning of expression of recombinant NcSRS2 (rNcp43)

E. coli BL21 Star transformed with the expression plasmid pET100/D-TOPO/nSRS2 expressed a recombinant protein of the expected size (~29 kDa). Purification of rNcp43 from *E. coli* by affinity chromatography was highly efficient, resulting in approximately 1mg/L of rNc-p43. A single band was observed when the protein was submitted to SDS-PAGE (Fig. 1A) and Western blot (Fig. 1B).

Antigenic properties of rNcp43

The rNcp43 was recognized by antibodies present in naturally infected animal sera. Out of 15 cattle, 15 sheep and 10 canine positive samples, all of them reacted in ELISA/rNcp43. The averaged absorbance values at 450nm were 1,236; 0,624 and 0,322 for cattle, sheep and canine sera, respectively. The antigenicity of rNcp43 was evaluated in ELISA by using serum sample from animals naturally infected. The averaged absorbance of negative sera of all species studied was at least twice lower compared with the average shown by the positive sera (Fig. 2).

pAb

The pAb/rNcp43 obtained was evaluated in immunoenzymatic assays and immunofluorescence. To determine pAb recognition of rNcp43, indirect ELISA and Dot blot were carried out. pAb/rNcp43 showed titers of 1,280 in indirect ELISA (Fig. 3A), detected immobilized protein on Dot blot (Fig. 3B) and it activity remained after peroxidase conjugation (data not show). pAb/rNcp43 labeled *N. caninum* tachyzoites both direct and indirect immunofluorescence (Fig. 4) and did not label *T. gondii* tachyzoites. Fluorescent reactivity patterns exhibited by the pAb/rNcp43 exposed to the *N. caninum* consisted of only apical complex fluorescent.

Discussion

In this study we evaluated the potential immunogen and antigen rNcp43 protein produced in *E. coli.* For that purpose the antigenic domain of NcSRS2 (Ncp-43), located in the distal C-terminal two thirds of the molecule, was obtained according Borsuk *et al.* [7]. Despite of successful cloning and expression of rNcp43 in baculovirus expression system [23], the use of *E. coli* was described by Howe *et al.* [19] to expression of rNcp43. Thereafter, studies of rNcp43 expressed in *E. coli* have been reported for diagnostic purposes using indirect ELISA in cattle [30,21,7] and sheep sera [4]. Recently, a commercial Sandwich Enzyme-Linked Immunosorbent [14] was developed for cattle and dog neosporosis diagnosis.

The rNcp43 antigen obtained is recognized by antisera from animals naturally infected by *N. caninum* and was able to generate a monospecific pAb/rNcp43 that

recognized native protein from tachyzoites. Monoclonal and polyclonal antibodies against SRS2 native protein of *N. caninum* were described by Haldorson e*t al.* [15] and were able to block the invasion of tachyzoites into both trophoblasts and Vero cells. Monospecific polyclonal antibodies generated against Ncp43 in its recombinant form were not described yet.

Indirect and direct immunofluorescence was carried out to explore pAb/rNcp43 potential in differentiation of protozoan tachyzoites. Anti-rNcp43 pAb labeled the apical complex of *N. caninum* tachyzoites both primary antibody and conjugated to FITC, and did not attach to *Toxoplasma gondii*. Polyclonal antibodies have been successfully used in immunofluorescence based diagnostic tests for cryptosporidiosis [25,8], giardiasis [2,34] and toxoplasmosis [28,22] also caused by protozoan. The antibody reported here provided evidence of its usefulness to differentiate tachyzoites of *N. caninum* from *T. gondii* in tissue and biological fluids.

Immunoenzymatic assays were used to investigate pAb reaction against rNcp43. pAb/rNcp43 reacted with the protein antigen on Dot blot and indirect ELISA. Furthermore, pAb/rNcp43 maintained antibody activity after conjugation with peroxidase suggesting it is suitable for two-site immunoassays such as sandwich ELISA and useful in immunohistochemical methods. Since a commercial Sandwich Enzyme-Linked Immunosorbent utilize specific antibody for neosporosis diagnosis [14], the polyclonal antibody developed is a reagent suitable for enzymatic assay.

There are two antigens majority on surface of tachyzoites and bradyzoites of approximately 29kDa and 35kDa designated Ncp36 and Ncp43 respectively [18,17]. Among these, Ncp43 is considered most likely involved in the adhesion and invasion process [17,24], it is present in dense granules and rhoptries [16] and has been the mostly cited antigen in diagnostic studies in cattle [30,21,7], sheep [4] and dog [14].

These findings are promising for diagnostic assays development whereas rNcp43 obtained could be used in neosporosis serological surveys for cattle, dog and sheep species. pAb/rNcp43 – FITC is a potential tool for tachyzoites detection on biological fluids and tissues. Moreover, the pAb/rNcp43 described here is suitable for further diagnostic studies using immunoseparation techniques or chromatographic assays, and in immune protective studies.

In conclusion, the results reported in this work demonstrated that rNcp43 expressed in *E. coli* is recognized by antibodies from naturally infected animals and were able to generate a monospecific polyclonal antibody that recognized native protein from *N. caninum* tachyzoites. pAb against this target will be useful in different test formats for diagnosis of neosporosis.

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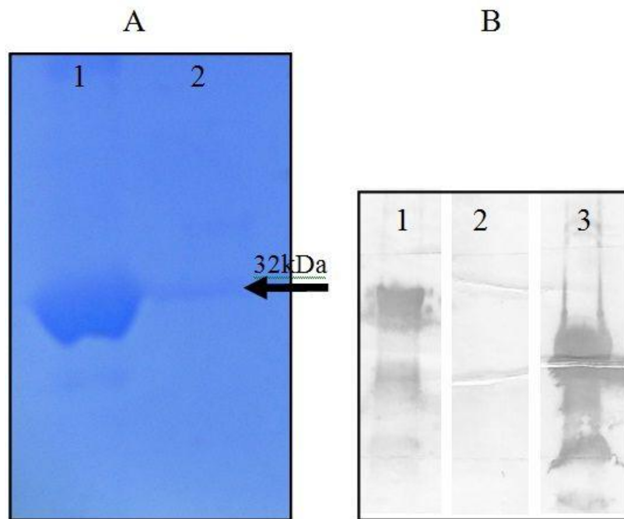

Fig. 1. Evaluation of rNcp43expression in *E. coli* BL21 Star. (A) 12% SDS-PAGE under reducing conditions, stained with Coomassie brilliant blue. [1] rNcp43 (~29kDa); [2] positive control LipL32 (32kDa). (B) Detection of fused fragment of *NcSRS2* by Western blot analysis upon separation in 12% SDS-PAGE. [1] rNcp43 (~29kDa) of *N. caninum* [2] negative control - extract of *E. coli* BL21Star [3] positive control - Ag85B (32.4 kDa) of *Mycobacterium bovis*.

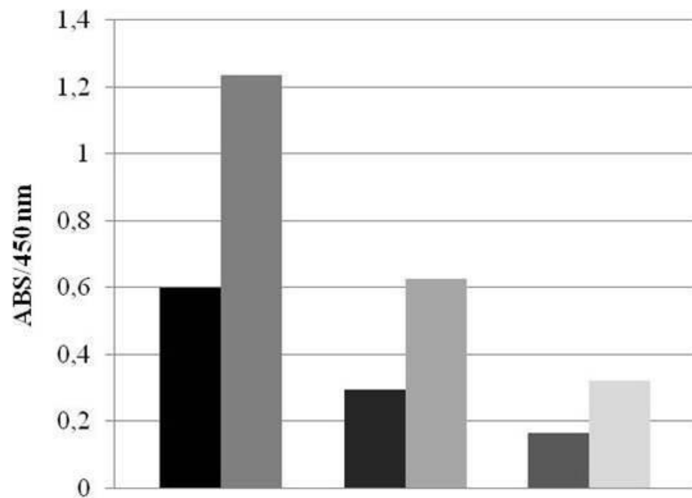

Fig. 2. Antibodies to *N. caninum* rNcp43 in naturally infected animals. Average absorbance values of animals species studied in ELISA/rNcp43. (■) negative sera of cattle, (■) positive sera of cattle, (■) negative sera of sheep, (■) positive sera of sheep, (■) negative sera of dog (■) positive sera of dog.

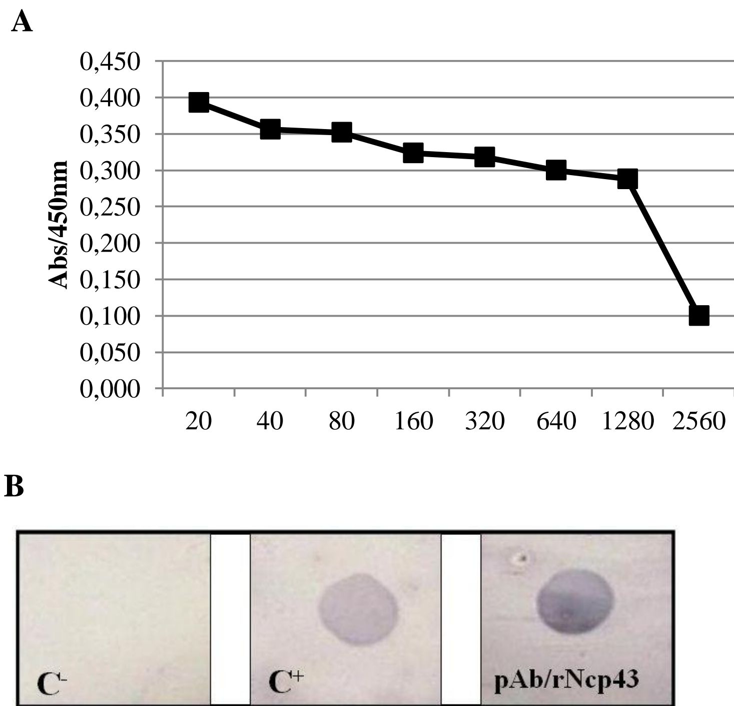

Fig. 3. Polyclonal antibody reaction against rNcp43 (pAb/rNcp43). (A) pAb/rNcp43 dilute 1:20 to 1:2,560 in Enzyme-linked Immunosorbent assay using rNcp43. (B) Dot blot assay using rNcp43. [C^-] mouse normal serum diluted 1:100, [C^+] hyperimmune bovine serum diluted 1:100, [pAb/rNcp43] pAb diluted 1:20.

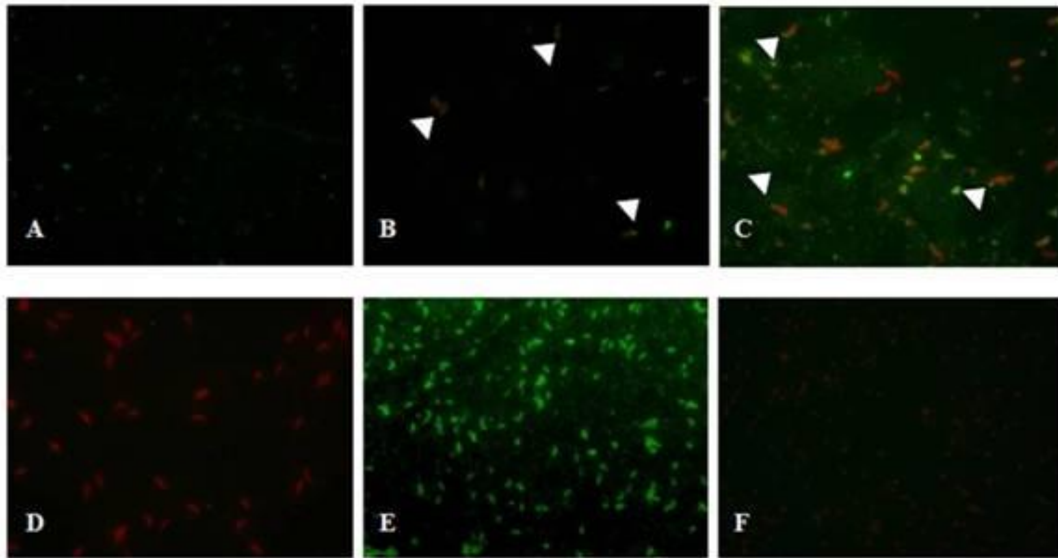

Fig. 4. Immunofluorescence assay probed with polyclonal antibody (pAb/rNcp43). Panel A and B: Indirect immunofluorescence (IFAT) using pAb/rNcp43 visualized 40X and 100X objective on an Olympus BX 51 fluorescent microscope. Panel C: Direct immunofluorescence using pAb/rNcp43-FITC. Panel D: Indirect immunofluorescence probing pAb/rNcp43 *T. gondii* tachyzoites. Panel E: Positive control. Panel F: negative control.

3. CONCLUSÃO

Estes resultados são promissores para o desenvolvimento ensaios de diagnóstico já que a proteína rNc-p43 obtida reagiu com anticorpos presentes em soros das espécies bovina, ovina e canina, infectados naturalmente. O pAb/rNc-p43 - FITC é uma ferramenta em potencial para a detecção de taquizoítos em fluidos biológicos e tecidos. Além disso, o pAb/rNc-p43 aqui descrito é adequado para estudos de diagnóstico através de técnicas de cromatografia ou immuno-separação, e em estudos de resposta imunidade.

Em conclusão, os resultados reportados neste trabalho demonstrou que rNcp43 expressa em E. coli é reconhecido por anticorpos de animais infectados naturalmente e foram capazes de gerar um anticorpo policlonal monoespecífico que reconheceu a proteína nativa de taquizoítos de N. caninum, portanto o pAb contra esta alvo pode ser útil em diferentes formatos de testes para o diagnóstico da neosporose.

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