CLONING AND EXPRESSION OF AN ANTIGENIC DOMAIN OF A MAJOR SURFACE PROTEIN (NC-P43) OF *Neospora caninum**

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ABSTRACT:- LIMA JUNIOR, M.S.DA C.; ANDREOTTI, R.; CAETANO, A.R.; PAIVA, F.; MATOS, M. DE F.C. [**Cloning and expression of an antigenic domain of a major surface protein (Nc-p43) of *Neospora caninum.***] Clonagem e expressão de um domínio antigênico da proteína majoritária de superfície (Nc-p43) de *Neospora caninum. Revista Brasileira de Parasitologia Veterinária*, v. 16, n. 2, p. 61-66, 2007. Embrapa Gado de Corte, BR 262, Km 4, CP 154; Campo Grande, MS 79002-970, Brazil. E-mail: andreott@cnpgc.embrapa.br

Neospora caninum is an obligate intracellular protozoan that can infect domestic and wild canids, as well as ruminants and equines. It was described in 1988 as causing neuromuscular alterations and death in dogs. Recently, *N. caninum* has been the focus of considerable attention for its large impact on the dairy industry, given the economic losses related to breeding failures and to a decrease in productivity. ELISA diagnosis of neosporosis has not been widely used in Brazil, mostly because of the assay's cost, and thus the distribution of the disease in the country is not well known. In order to evaluate its ability to react with sera from infected animals from the state of Mato Grosso do Sul, an antigenic determinant domain of a major surface protein (Nc-p43) was produced. The antigenic domain, located in the distal 2/3 region of the C-terminus, was amplified by polymerase chain reaction. The DNA fragments were cloned into pet100/D-TOPO vectors. The recombinant plasmids were transformed into *Escherichia coli* of the BL21 Star (DE3) strain and induced to express the fused fragment of Nc-p43 as a 29-kDa protein that, when assayed with bovine *Neospora*-positive serum from a regional sample, was sensitive for identification by immunoblotting. This Nc-p43 fragment may be of use in additional studies targeted at diagnosing *N. caninum* infection and at evaluating the immunoprotection conferred by the protein fragment to animal hosts.

KEYWORDS: *Neospora caninum*, Nc-p43, antigen, recombinant protein.

RESUMO

Neospora caninum é um protozoário intracelular obrigatório que pode infectar canídeos domésticos e selvagens, ruminantes e eqüinos, tendo sido descrito em 1988 por Dubey et al. como causador de alterações neuromusculares e morte em cães. Recentemente, *N. caninum* vem recebendo atenção especial por seu grande impacto na indústria de leite, acarretando perdas econômicas relacionadas com falhas reprodutivas e com o decréscimo da produtividade. O diagnóstico de neosporose com teste ELISA não é amplamente utilizado no Brasil, principalmente devido ao custo do ensaio, e por isso o *status* da distribuição da neosporose nas diferentes regiões do país não é suficientemente conhecido. Com o objetivo de avaliar a habilidade de reagir com soros de animais infectados provenientes de Mato Grosso do Sul, foi produzida uma região de domínio com um determinante antigênico de uma importante proteína de superfície (Nc-p43). O domínio antigênico dessa proteína está localizado na região 2/3 distal do C-terminal e foi amplificado por reação de polimerase em cadeia. O fragmento de DNA foi clonado em um vetor pet100/D-TOPO. O plasmídio recombinante foi transformado em *Escherichia coli* da variedade BL21 Star (DE3) e induzido a expressar o fragmento protéico de Nc-p43 como uma proteína de 29 kDa que, quando em contato com soro positivo para *Neospora*, mostrou-se sensível na identificação por *immunoblotting*. Tal fragmento de Nc-p43 pode ter uso em futuros estudos voltados

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ao diagnóstico de infecção por *N. caninum* e à avaliação de sua imunoproteção a possíveis hospedeiros.

PALAVRAS-CHAVE: *Neospora caninum*, Nc-p43, antígenos, proteína recombinante.

INTRODUCTION

Neospora caninum is a protozoan of the phylum Apicomplexa, class Sporozoasida, family Sarcocystidae, subfamily Toxoplasmatinae, that can infect domestic and wild canids, as well as ruminants and equines. It was first described as causing neuromuscular alterations and death in dogs (DUBEY et al., 1988). Domestic dogs, which have been considered the definitive hosts of this parasite, shed oocysts in their feces after ingesting tissue cysts of *N. caninum* (MCALLISTER et al., 1998; LINDSAY et al., 1999). However, more recent studies have detected the presence of oocysts of *N. caninum* in the feces of coyotes (*Canis latrans*), which also came to be regarded as definitive hosts of the protozoan (GONDIM et al., 2004).

Studies on the prevalence of anti-*Neospora caninum* antibodies have revealed that neosporosis has a wide geographical distribution, having been reported in many parts of the world, including Australia, New Zealand, Europe, Korea, Japan, Thailand, and the Americas. Dogs and cattle (both beef and dairy) are the chief species exposed to this parasite and can be equally affected (ANDERSON et al., 2000).

In Brazil, surveys based on indirect fluorescence antibody test (IFAT) titers of 1:50 or higher showed prevalences of 26.55% in dogs in Campo Grande (OLIVEIRA et al., 2004), of 8.3% in a sample of 136 dogs older than six months in the state of Rondônia (CAÑON-FRANCO et al., 2003), and of 21.6% in a sample of 134 dogs living on dairy-cattle farms in the State of Paraná (SOUZA et al., 2002). Gennari et al. (2002), found frequencies of 10% for domiciled dogs and 25% for stray ones in the State of São Paulo.

Meira Santos et al. (1999) obtained a prevalence of 18% for dogs in the State of Bahia, whereas Belo et al. (1999) found 35% for stray dogs in the State of São Paulo.

Concerning bovine neosporosis in Brazil, the occurrence of seropositive cattle in six states was as high as 23.6%, with values increasing in animals over 24 months of age (RAGOZO et al., 2003). In the state of Mato Grosso do Sul, seropositivity ranged from 7.7% in bovines without a history of miscarriage to 43% in those with it (ANDREOTTI et al., 2004).

Recently, *N. caninum* has been the focus of considerable attention for its large impact on the dairy industry, given the economic losses related to breeding failures and to a decrease in productivity (DUBEY, 2003). ELISA diagnosis of neosporosis has not been widely used in Brazil, mostly because of the assay's cost - one reason why the status of neosporosis distribution in the country is not well known - but also because cross-reactivity can prevent ELISA from distinguishing between apicomplexan parasites, particularly when unpurified antigens are used.

The surface proteins of apicomplexan parasites are often immunodominant and seem to be of particular interest as a diagnostic tool and/or as vaccine antigens (BULOW; BOOTHROYD, 1991; LUNDEN et al., 1997). Many surface proteins of *N. caninum* have been identified, including Nc-p43 (HEMPHILL; GOTTSTEIN, 1996), p29 and p35 (HOWE et al., 1998), and p38 (SCHARES et al., 2000). Although their biological functions have not been described, indirect evidence is available that at least one of these antigens (Nc-p43) is involved in the parasite's attachment to host cells (HEMPHILL, 1996). The complete coding region of Nc-p43 has been determined (NISHIKAWA et al., 2000, 2001).

One antigenic domain of Nc-p43 from *N. caninum* is located in the distal 2/3 region of the C-terminus (SON et al., 2001) and has been detected by ELISA (AHN et al., 2003). However, the antigenicity of these epitopes across different *N. caninum* isolates and their ability to react with sera from infected animals from the State of Mato Grosso do Sul have not yet been determined.

The present paper reports the results of the cloning and expression of the distal 2/3 region of the C-terminus Nc-p43 protein from an *N. caninum* Nc-1 isolate. Also, the ability of this antigen to recognize IgG on positive bovine sera sampled at regional scale were tested.

MATERIAL AND METHODS

Parasite: *Neospora caninum* tachyzoites of the Nc-1 strain were maintained in Vero cells (CRL 6318, ATCC-CCL8L, Rockville, MD) in DMEM supplemented with 10% FBS (Sigma).

Collection of samples: Blood samples were supplied by a bovine serum bank and stored at 2-8 °C until analysis. Six samples each of *Neospora*-positive and *Neospora*-negative bovine sera, respectively, determinated by IFAT were used.

Indirect fluorescence antibody test (IFAT): *Neospora caninum* (Nc-1) (DUBEY et al., 1988) and *T. gondii* (RH) were grown in Vero cells with a protozoal culture medium consisting of Eagle's medium (Sigma) supplemented with 100 IU/mL penicillin and 100 mg/mL streptomycin at 37 °C at 5% CO_2 in a humidified incubator. The parasites were maintained in serum-free medium. After approximately 80% of the host cells had been infected with parasite clusters, the monolayers were trypsinized and the medium containing parasites was collected. IFAT slides were prepared with whole tachyzoites of *N. caninum* and *T. gondii*, as described by Gupta et al. (2002). IFAT was used to detect anti-*N. caninum* and *T. gondii* antibodies in the samples. The rabbit anti-bovine IgG conjugate was purchased from VMRD (USA). The samples were tested at a dilution of 1:100 and the positive ones were pooled for Western Blot analysis (LOCATELLI-DITTRICH, 2002).

Polymerase chain reaction (PCR) and amplification of the Nc-p43 gene fragment: Total DNA was extracted from the tachyzoites using DNAzol (Invitrogen Tech, Carlsbad, CA) and used as template. PCR was performed with CAC CAA AGA GTG GGT GAC TGG A as the forward primer and GGT AAG

CTT TGC ATC TCC TCT TAA CAC as the reverse one. The forward primer was made using a CACC sequence in the 5′ region to attach to the pET100/D-TOPO vector plus the primer sequence designed for the DNA segment (AHN et al., 2003).

Amplified DNAs were cloned into pet100/D-TOPO vectors (Invitrogen Tech, Carlsbad, CA) and sequenced with T7 and reverse T7S. The specific oligonucleotide primers were synthesized according to the coding sequence of GenBank accession number U93870.

To determine orientation (sense/anti-sense) of the insert in the plasmid, primer T7 from the vector was used as the forward primer and GGT AAG CTT TGC ATC TCC TCT TAA CAC from the insert as the reverse one. They were respectively used to evaluate the clones by PCR in terms of correct orientation for subcloning and expression.

Construction of recombinant plasmids and expression of fusion proteins: The amplified DNAs were inserted into pet100/D-TOPO vectors (Invitrogen Tech, Carlsbad, CA),. which were then used to transform *E. coli* of the TOP10 strain (Invitrogen Tech, Carlsbad, CA). Once the correct orientation of the insert was confirmed, plasmids were inserted by transformation into *E. coli* (BL21 strain) (Invitrogen, Carlsbad, CA).

E. coli cells in the log phase (O.D. 0,6) were treated with 0.5-mM isopropyl α-D-thiogalactoside (IPTG) for 3 h at 30°C to induce expression of fusion proteins.

Western Blot: It was performed according to the method described by Towbin et al. (1979). Protein extracts were separated on 12% SDS-PAGE gels stained with Coomassie blue and transferred onto nitrocellulose (NC) sheets (Schleicher and Schuell, Keene, NH). The recombinant protein was confirmed based on molecular weight on SDS-PAGE and Western blot.

HisG antibody was used to detect expression of the recombinant fusion protein. For the determination of the antigenic domain, the NC sheet was blocked by 10% rabbit serum in PBS/0.05% Tween-20 overnight. NC was then incubated with 1:25-diluted bovine serum, that were tested in advanced as positive or negative for *Neospora* diagnostic by IFAT (BAE et al., 2000), followed by incubation with 1:250-diluted peroxidase-conjugated rabbit anti-bovine IgG antibody (Sigma, Saint Louis, MO). After that, NC was soaked in substrate - DAB solution (Sigma, Saint Louis, MO) - for 15 min and washed with water. Six positive and six negative bovine serum samples were used to react with the recombinant protein as antigens in the Western blotting.

RESULTS AND DISCUSSION

With the set of primers used to amplify DNA from tachyzoites, a fragment of 732 base pairs (Fig. 1) was obtained by PCR from the Nc-1 strain of *N. caninum*. The fragment was confirmed based on its nucleotide sequence, GenBank accession number EF469765 (Fig. 3).

DNA sequencing data of the partial Nc-p43 gene of the *N. caninum* Nc-1 strain (Fig. 3) revealed a 99% identity with NcSRS - GenBank accession AY940488 - (HALDORSON et al., 2005) and a 97% identity with Nc-p43 - GenBank accession U93870 (HEMPHILL; GOTTSTEIN, 1996).

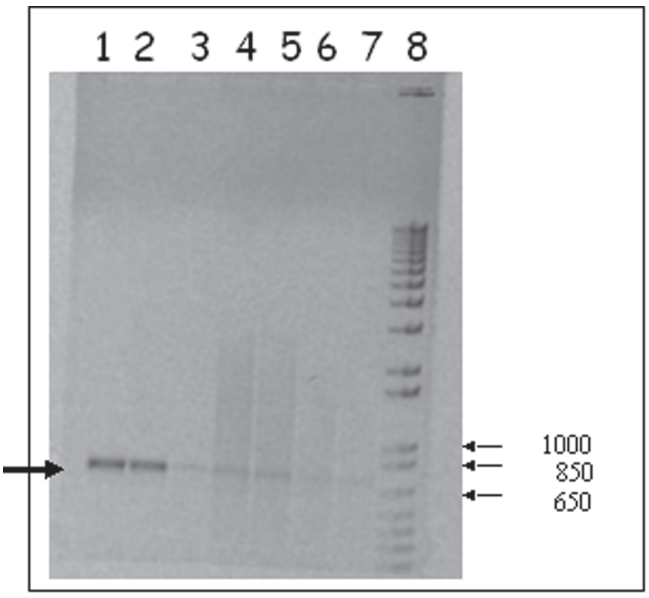

Figure 1. PCR-amplified partial Nc-p43–coding gene of *N. caninum*, Nc-1 strain. Numerals on the right indicate base pairs. Separation in 1.2% agarose gel using Taq polymerase (1-2), platinum Pfx DNA polymerase (3-7), and molecular ladder (8). Arrow on the left indicates DNA amplification.

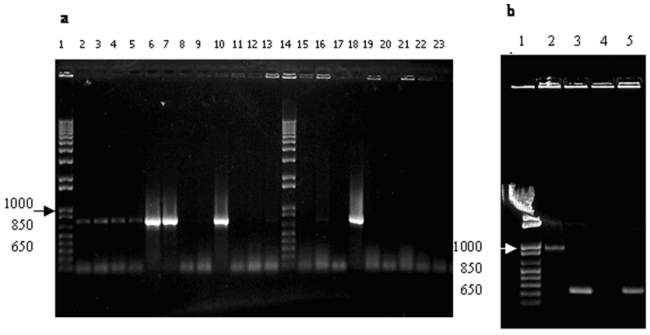

Figure 2. **(a)** PCR-amplified Nc-p43–coding gene fragments of plasmid in different *E. coli* clones isolated. Forward primer: CAC CAA AGA GTG GGT GAC TGG A; reverse primer: GGT AAG CTT TGC ATC TCC TCT TAA CAC (Ahn et al., 2003). DNA separation in 1.2% agarose gel. Numerals on the top designate: 1- molecular ladder and others (2-23) the colony tested. **(b)** PCR-amplified Nc-p43–coding gene fragments of plasmid in different *E. coli* clones isolated for identification of insert orientation. Primers: T7 from vector; GGT AAG CTT TGC ATC TCC TCT TAA CAC as reverse primer. DNA separation in 1.2% agarose gel. Numerals on the top designate: 1- molecular ladder and the others (2-5) the colony tested. Arrow indicates the correct orientation.

The positive clones were selected by PCR using the same set of primers, as shown in Fig 2a. Primer T7 from the vector (as the forward primer) and GGT AAG CTT TGC ATC TCC TCT TAA CAC (as the reverse one) were used to evaluate the clones by PCR in terms of correct orientation for subcloning and expression (Fig 2b).

The total amount of purified protein was 1.7 mg/L from transformed *E. coli* culture and, the recombinant protein,

CACCAAAGAGTGGGTGACTGGAACTCTTCAACAGGGAATAAGGATTACAATTCCCGACGAGCA
CTACCCAGCTACGTCGAAAGCTTTCCGTGTTGGTTGTAAGGCGGGTAAAAATGTTTGTTTGCTC
AATGTGTATGTTCAGAGTAGGGAATCAGAGGTGATCGGACAGGTGGCGCATTGTGCTTATAGTT
CCAACGTACGTCTGCGTCCAATCACTGTGAATCCAGAAAACAACGGAGTGACATTAATTTGCGG
ACCAGACGGGAAGGCGTTCCCGGATGATTACATGAACCACCATTGTACGGAACTAGACGAATG
TAAAGAACGCCCTTATTCCGCCGTGTTTCCTGGGTTCTCCAGTAGTTTTTGGACTGGAGAAGCTT
CCGGCGTAGCCGGGGCGACACTGACCATTCCCAAGGACCAGTTTCCTTCGACAGCACAGACCA
TCTATTTGGGCTGCACTGGTCACCCGGATGATAAACAGGTCACCTGTGTGGTTCCAGTAAATAT
AGAAGAAGTTGCAAAACCAGCAGGGGCCGGATCGAACCCAGGTGGTGGTTCGCAACCGGACC
AATCCTCGGAGAAACGCGACGGGGAACAGGTAAACAAAGGCAAACCTCCCACTGGCGGATCC
GGCGGAGCAACAACTGGAAAACAAAATGCATCCCAAAACGCGAAGGACAAGGGGGAAACAGG
AGGAGAGAACGGCGATTCCCCCGTGTTAAGAGGAGATGCAAAGCTTACC

Figure 3. Nucleotide sequence of the Nc-p43 fragment inserted in the plasmid vector. The sequence of the forward primer is underlined. CACC, the specific site of the primer, was matched with the vector and provided the direction of the insert. The other nucleotides were part of the original gene sequence (GenBank accession number EF469765).

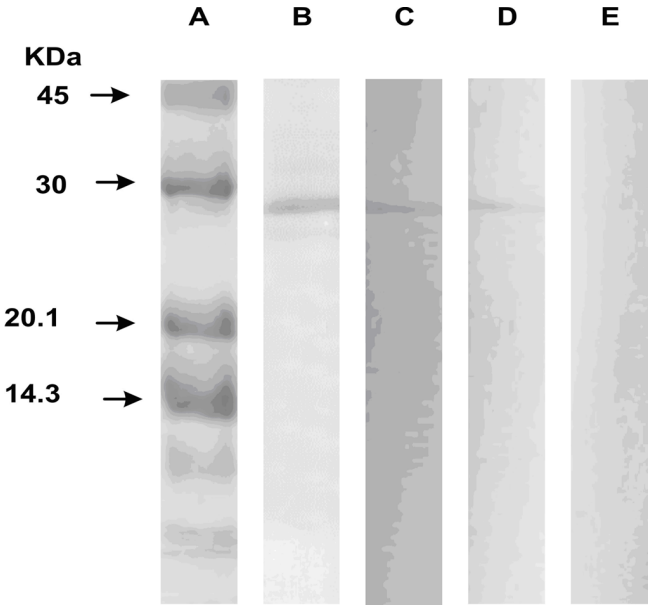

Figure 4. **(A)** Molecular weights of markers. **(B)** Coomassie blue–stained SDS-PAGE (12% gel) of the expressed Nc-p43 fragment, purified by affinity chromatography in nickel column, showing a major band of 29 kDa. **(C)** Western blot detecting HisG tag. **(D)** Western blot detection of the Nc-p43 recombinant protein with bovine *Neospora caninum*–positive serum. **(E)** Western blot detection of Nc-p43 recombinant protein with bovine *Neospora caninum*–negative serum. Positive and negative bovine sera were determinated by IFAT.

showed a estimated molecular weight of 29 kDa by SDS-PAGE (Fig. 4B).

The insert received an N-terminal 6xHis tag through the vector pet100/D-TOPO and incorporated an additional 3 kDa to the original 26-kDa molecular weight of the main sequence reported by Ahn et al. (2003). This tag region was used to demonstrate by Western blotting that the protein is recombinant (Fig. 4C). All the positive bovine sera reacted against the protein, but no reaction occurred with the negative serum used as control. (Figures 4D and 4E show a positive and a negative serum sample, respectively.)

Although the partial nucleotide sequence of the Nc-p43–coding gene revealed a sequence homology to the SRS-2 gene of *T. gondii* (HEMPHILL et al., 1997; HOWE et al., 1998), cross-reactivity of this antigen with *T. gondii* seems to be negligible, as determined by immunoblotting (BJERKAS et al., 1994; PARE et al., 1998; BAE et al., 2000).

Changes in amino acids occur in the antigenic domain of the distal 2/3 region of the C-terminus Nc-p43 protein. The extracts of the KBA-2 strain were more suitable as a diagnostic antigen than were those of Nc-1 for *N. caninum* infection in Korea, though no differences were found in the morphology of the parasites (BAE et al., 2000). Also, no pathologic features caused by the infection were observed in the hosts (KIM et al., 2000). Therefore, interaction of parasite antigen and antibody can be better understood when regional conditions are taken into account.

The truncated NcSRS2 protein expressed in *E. coli* has been evaluated by ELISA, allowing for clear distinction between known *N. caninum*-positive and negative sera from cattle (GATURAGA et al., 2005).

Although the recombinant protein produced by Nc-p43 recognizes *Neospora*-positive bovine sera, it does not recognize negative sera colleted in cattle from the State of Mato Grosso do Sul, suggesting the possibility of using this protein as antigen in diagnosis. Further immunological studies are needed to reveal this potential feature.

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