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Diagnostic Potential of Anti-rNcp-43 Polyclonal Antibodies for the Detection of *Neospora caninum*

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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.

Gizele Lima de Sá and Diene de Borba Pacheco contributed equally to the experimental work.

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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 [11, 22]. 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) [7, 9, 11, 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 F: 5′-CAC CAA AGA GTG GGT GAC TGG and R: 5′-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 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*-Lauroylsarcosine. 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 using Ni^{2+} Sepharose HiTrap 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 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.

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 °C 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

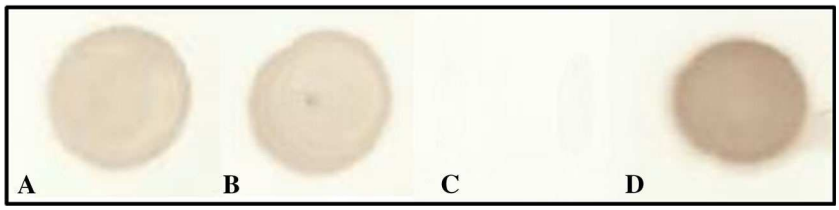

Fig. 1 *Dot blot* assay with the pAb or HRP-conjugated pAb, using rNcp-43 protein immobilized on nitrocellulose membrane. **a** pAb (1:6,000). **b** pAb/HRP (1:500). **c** Negative control (pooled preimmune serum). **d** Positive control (pooled hyperimmune serum)

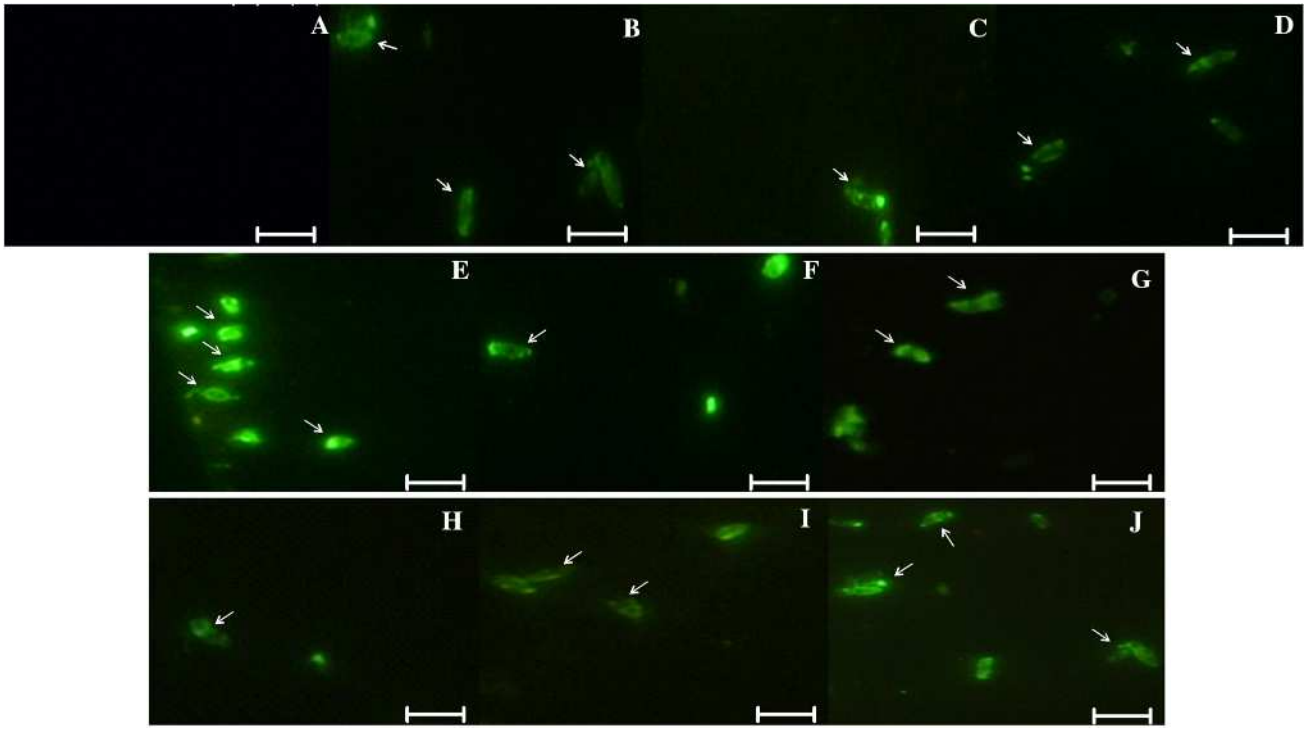

Fig. 2 Detection of the apical complex of tachyzoites of *N. caninum* by direct immunofluorescence. Tachyzoites were fixed on microscope slides and probed with the FITC-conjugated pAb against rNcp-43 (arrows). **a** *T. gondii* cells were used as a negative control for antigen detection. **b–d** Strain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

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 BX51) 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 °C. The wells were washed three times with PBS and incubated with pAb (1:32,000) or pAb/

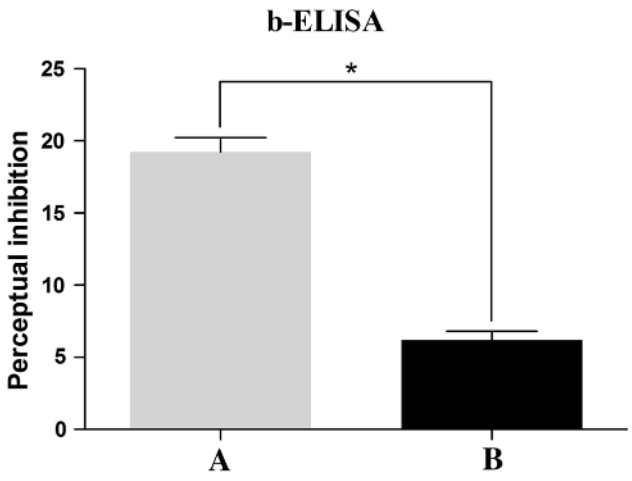

Fig. 3 Blocking ELISA using pAb/HRP as a diagnostic tool to detect antibodies in the serum of animals infected with *N. caninum*. Pools of positive (**a**) and negative (**b**) serum samples from cattle were used as sources of blocking antibodies. Mean differences were statistically significant (*$P < 0.0001$)

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 2 N 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 (Fig. 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 (Fig. 2) labeled the apical complex of tachyzoites, while no immunoreactivity was observed when *T. gondii* cells (Fig. 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, 18, 25]. 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 ($P < 0.0001$; Fig. 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.

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