Full Article

Comparison of indirect ELISA based on recombinant protein NcSRS2 and IFAT for detection of *Neospora caninum* antibodies in sheep

Comparação entre ELISA baseado no antígeno recombinante NcSRS2 e RIFI para detecção de anticorpos de *Neospora caninum* em ovinos

Renato Andreotti[1]*; Maria de Fátima Cepa Matos[2]; Kelly Noda Gonçalves[2]; Leandra Marla Oshiro[2];
Manoel Sebastião da Costa Lima-Junior[2]; Fernando Paiva[2]; Fábio Leivas Leite[3]

[1]Embrapa Gado de Corte, Campo Grande - MS, Brazil

[2]Universidade Federal de Mato Grosso do Sul – UFMS

[3]Universidade Federal de Pelotas – UFPel

Abstract

Neospora caninum, an Apicomplexan parasite that can causes abortion, is responsible for considerable economic and reproductive losses in livestock. The purpose of the present study was to determine whether recombinant NcSRS2 is a suitable indirect ELISA antigen for determining specific immune response to *N. caninum* in sheep. A total of 441 serum samples were subjected to IFAT and rNcSRS2 based-ELISA, with both tests performing similarly. The sensitivity and specificity of indirect ELISA were 98.6 and 98.3%, respectively. The kappa index shows 0.98 concordance between the two tests, which is considered excellent. Seroprevalences of 30.8 and 32.0% were detected by IFAT and indirect ELISA, respectively, showing these tests did not differ significantly on this measure (p > 0.05). Serological analysis showed that HisG tag was detected by Western Blotting recognizing rNcSRS2 protein. The potential value of rNcSRS2-based ELISA as a highly specific and sensitive tool for serological diagnosis is also supported by the strong agreement found between IFAT and ELISA. The results support the potential use of recombinant protein NcSRS2 as an antigen in indirect ELISA in sheep.

Keywords: NcSRS2, ELISA, IgG, diagnosis, ovine.

Resumo

Neospora caninum é um parasito Apicomplexa que pode causar abortos e é reconhecido como agente importante responsável por perdas econômicas e reprodutivas. Este estudo avaliou a proteína recombinante NcSRS2 como antígeno para ELISA indireto na determinação de resposta imune para *N. caninum* em ovinos. 441 amostras de soro foram analisadas por IFAT e ELISA indireto com rNcSRS2 e ambos os testes revelaram comportamento similar. A sensibilidade e especificidade de ELISA indireto foram 98,6 e 98,3%, respectivamente. O índice kappa mostrou uma concordância entre os dois testes com valor de 0,98, que é considerado excelente. Prevalências de 30,8 e 32,0% detectadas por IFAT e ELISA indireto, respectivamente, mostraram que os testes não diferiram significativamente nesse aspecto (P > 0,05). A análise sorológica revelou que os anticorpos específicos da cauda de histidina reconheceu por Western Blotting a proteína recombinante NcSRS2. O valor potencial do ELISA indireto baseado no antígeno rNcSRS2 como ferramenta altamente específica e sensível para diagnóstico sorológico é também reforçado pela alta concordância dos valores obtidos com IFAT e com ELISA indireto. Esses resultados respaldam o uso potencial da proteína rNcSRS2 como antígeno em ELISA indireto em ovinos.

Palavras-chave: NcSRS2, ELISA, IgG, diagnóstico, ovinos.

Introduction

Neospora caninum, an apicomplexan protozoan, was first identified by Dubey et al. (1988) and has been described worldwide. It can infect a wide range of intermediate animals and has been recognized as an important causative agent of economic and reproductive losses in the livestock industry, especially in dairy cattle (DUBEY, 2003).

Detection of *N. caninum* specific antibodies in bovine serum indicates that an animal is infected with the parasite. Selective culling can be a strategy to eliminate or reduce infection in a herd (BJORKMAN; UGGLA, 1999).

The parasite's surface protein NcSRS2 has been regarded as a candidate for a vaccine capable of providing immunity against *N. caninum* (INNES et al., 2002). The protein has proved functionally involved in the adhesion process and in host cell invasion (HEMPHILL, 1996). Anti-NcSRS2 monoclonal antibodies (mAbs) may inhibit invasion of *N. caninum* in host cells in vitro (NISHIKAWA et al., 2000).

In mice, NcSRS2 expressed in recombinant vaccinia virus has reduced parasite load in tissues following challenge (NISHIKAWA et al., 2001a), while also decreasing congenital transmission in females vaccinated before pregnancy and challenged during pregnancy (NISHIKAWA et al., 2001b). Haldorson et al. (2005) demonstrated that, in its native form, NcSRS2 induced protection against *N. caninum* congenital transmission in mice. In addition, NcSRS2, as a surface antigen of *N. caninum*, has potential use in the serological diagnosis of *N. caninum* infection (AHN et al., 2003; GATURAGA et al., 2005).

In sheep, despite the availability of serological tests for neosporosis to identify infected dams, it is also important to determine those in a herd at risk of abortion. In Brazil, serological diagnosis of neosporosis in sheep relies solely on the indirect fluorescent antibody test (IFAT), which is not suitable for large-scale use. On the other hand, the enzyme-linked immunosorbent assay (ELISA) can be helpful in studies on reproductive failure related to neosporosis. The purpose of the present study was to determine whether recombinant NcSRS2 can prove a useful ELISA antigen to elicit a specific immune response to *N. caninum* in sheep.

Material and Methods

1. Blood samples

Blood samples were collected from a total female herd in a breeding production farm in the city of Campo Grande, Central-Western Brazil.

Blood samples were drawn by venous puncture from 441 adult sheep from a single farm. Sera from the samples were stored at –20 °C until use. To evaluate humoral immune response, individual sera were subjected three times to NcSRS2-specific IgG ELISA and IFAT.

2. Parasites

Neospora caninum isolate NC-1 (DUBEY et al., 1988) was used to challenge mice and prepare an antigen formulation for immunization. The parasites were propagated in Vero cells maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), at 37 °C with 5% CO_2. When 80% of Vero cells had been infected with *N. caninum* tachyzoites, the cell monolayers were removed by scraping, twice washed with phosphate-buffered saline (PBS) solution and then centrifuged at 1000 rpm for 10 minutes.

3. Indirect fluorescence antibody test (IFAT)

Samples were subjected to IFAT to detect the presence of antibodies. The antigen was produced by culturing tachyzoites of *N. caninum* of strain NC-1 (DUBEY et al., 1988) in Vero cells (OLIVEIRA et al., 2004). Fluorescent isothyocianate (FITC) sheep conjugate (Sigma) was used and the samples were tested at a dilution of 1:50 (Paré et al., 1995). Each slide included negative and positive control sera. Sera were analyzed at a dilution of 1:50 defined as the cut-off, using the method described by Trees et al. (1993).

4. Recombinant NcSRS2

The antigenic domain of NcSRS2, located in the distal C-terminal two thirds of the molecule, was amplified by polymerase chain reaction according to Ahn et al. (2003).

The amplified DNAs were cloned 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 for transformation into *E. coli* BL21 Star (DE3) (Invitrogen Tech, Carlsbad, CA). *E. coli* cells in the log phase were treated with 0.75-mM isopropyl α-D-thiogalactoside (IPTG) for 4.5 hours at 30 °C to induce expression of fused fragments of NcSRS2 (LIMA-JUNIOR et al., 2007).

The protein was purified using a nickel-nitrilotriacetic acid (Ni-NTA) purification system (Invitrogen Tech, Carlsbad, CA) and the recombinant protein was confirmed by molecular weight measurements using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting.

5. Indirect enzyme-linked immunosorbent assays (ELISA)

ELISA 96-well plates (ref. 3590, Costar, USA) were adsorbed for 12 hours at 4 °C with 40 ng.well^{-1} of rNcSRS2 proteins diluted in a suitable buffer (1-M $Na_2HPO_4.2H_2O$, 0.8-M $C_6H_6O_7.H_2O$, pH 4.0). The plates were then stored at –20 °C. After thawing, they were washed five times with PBS with 0.1% phosphate buffered saline/Tween (PBST) and blocked with DMEM, pH 7.2, with 2% γ-globulin-free equine serum and 5% slim milk for 4 hours at 4 °C. After five washes with PBST, positive and negative sera (in quadruplicate) and test sera (in duplicate) diluted at 1:1000 in PBST were incubated for 30 minutes at 37 °C. The plates were then washed five times with PBST, and 100 μL.well^{-1} of rabbit anti-ovine IgG horseradish peroxidase conjugate (ref. A-7414, Sigma, USA) diluted at 1:5000 in PBST were added. The plates were incubated as described and, after 5 washes with PBST, *o*-phenylene diamine dihydrochloride (OPD, Sigma)/H_2O_2 was added as chromogen substrate. The reaction was stopped by adding 2.5-N H_2SO_4 and the results were read at 490 nm on an ELISA reader (Bio-Tek, USA). Cut-offs were defined as the mean optical density (OD) of 25 negative control sera plus three standard deviations.

6. Statistical analysis

Data were analyzed by applying the chi-square test with a 95% confidence interval. ELISA sensitivity and specificity were compared with those of IFAT using 441 ovine sera positive for *N. caninum* antibodies, where IFAT was considered as gold standard. Kappa concordance, positive and negative predictive values and accuracy were estimated as well. Test performance was demonstrated by the ROC (receiver operating characteristic) curve.

Results and Discussion

The recombinant protein NcSRS2 obtained from *E. coli* culture was affinity-purified in nickel-charged agarose resin with yield of 1,7 mg.L^{-1} of medium. SDS-PAGE revealed a 29-kDa protein, whereas Western Blotting using HisG tag antibody confirmed the recombinant nature of the protein (Figure 1).

With the set of primers used to amplify DNA from tachyzoites, a fragment of 732 base pairs (375-1107) was obtained based on sequence of EF469765 accession number from GenBank. The sequence and expression was developed as described by Lima-Junior et al. (2007).

Indirect ELISA was standardized with optimal antigen, sera, and conjugate dilutions. The cut-off was determined using rNcSRS2 as antigen in testing sera for ELISA test and comparing by ROC curve with those previously validated by IFAT. The standard deviation of the mean value of the negative samples was added to the mean value of these samples. A resulting mean value of 0.119 was thus obtained, corresponding to three times the standard deviation (Figure 2a).

A total of 441 serum samples were analyzed using IFAT as a gold standard and rNcSRS2-based ELISA and both tests had similar performance. The sensitivity and specificity of indirect ELISA were 98.6 and 98.3%, respectively (Figure 2b).

The predictive value considered as a post-test probability revealed a positive predictive value (PPV) of 98.5%. It shows the proportion of true-positives among all individuals with a positive test. On the other hand, the proportion of true-negatives among all individuals with a negative test considered as the negative predictive value (NPV) was 98.3%.

The accuracy estimate, that shows the proportion of correct result of a diagnosis test, i.e., the proportion between true-positives and negatives in relation to all possible results, measures how much the obtained estimate on the ELISA test is related to the "real value" of the parameter that here is the IFAT test and was 0.98. The kappa index, that shows the concordance between two tests, was 0.98, which is considered excellent.

Table 1. Prevalence of anti-*Neospora caninum* antibodies in sheep, according to indirect ELISA and IFAT.

	Sera tested		
	Positive	Total	% positive
IFAT	136	441	30.8[a]
ELISA	141	441	32.0[a]

[a]Nonsignificant difference (chi-square test, 5% level of significance).
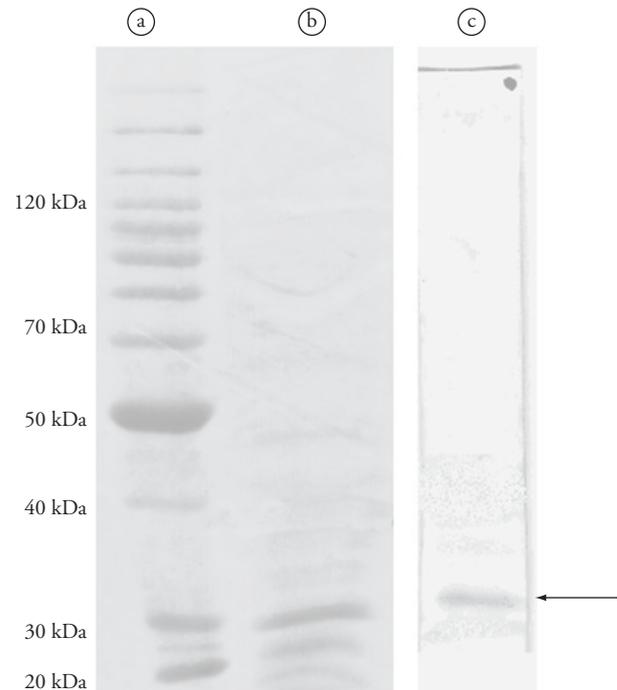
Figure 1. a) Molecular weights of markers. b) Coomassie blue-stained SDS-PAGE (12% gel) of the expressed NcSRS2 fragment purified by affinity chromatography in nickel column, showing a 29-kDa band. c) HisG tag detected by Western Blotting.
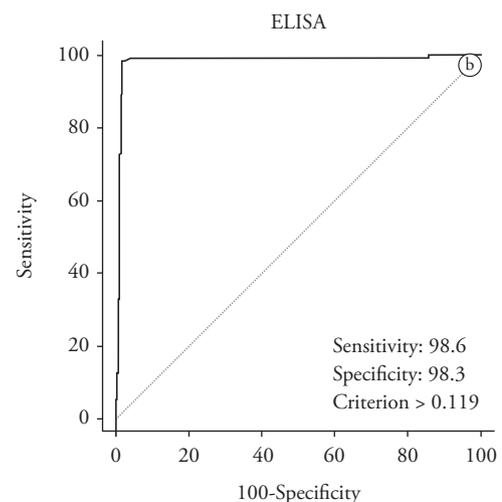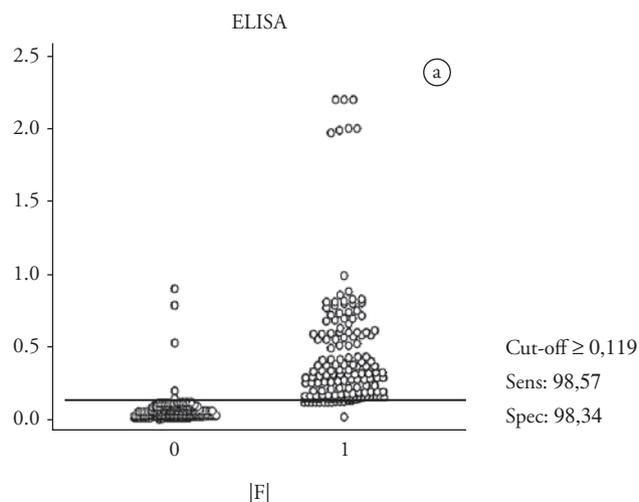
Figure 2. a) Plot of ROC analysis for *Neospora caninum* ELISA- IFAT. Optimal cut-off that provides the highest sensitivity and specificity. b) ROC plot with the area under the curve and 95% confidence interval between 97.6 and 99.9.

Seroprevalences of 30.8 and 32.0%, as measured by IFAT and indirect ELISA, respectively (Table 1), showed that the tests did not differ significantly in performance ($p > 0.05$). The herd, even though not previously evaluated for its breeding profile, was thus shown to harbor cases of *N. caninum* infection, the extent of which should be determined.

For cattle sampled in the state of Mato Grosso do Sul (OSHIRO et al., 2007), prevalences of 14.9% (449/2488) and 69.8% (143/205) were found for animals and herds, respectively.

Seven-week-old female BALB/c mice developed specific antibodies 14 days after initial immunization with rNcSRS2, as detected by indirect ELISA (GONÇALVES et al., 2008).

IFAT has been regarded as the reference test for the development of other assays for the detection of *N. caninum* antibodies (BJORKMAN; UGGLA, 1999). While in the present study the antigen was a 29-kDa rNcSRS2 protein from the distal C-terminal two thirds of the native molecule, Liu et al. (2007), in order to increase the solubility of the recombinant protein, removed the N-terminal hydrophobic signal peptide from the NcSRS2 gene to create a tNcSRS2 gene. The recombinant antigen used here, and the one described by Liu et al. (2007), clearly discriminated between positive and negative sera.

When ELISA tests based on crude extracts or recombinant proteins were developed to detect *Neospora*-specific antibodies in cattle, no cross-reaction was seen between those produced by *Neospora* infection and those by *Toxoplasma*, *Cryptosporidium, Sarcocystis, Eimeria*, or *Babesia* (HOWE et al., 1998; SCHARES et al., 2002).

Western blotting revealed that the 29-kDa protein NcSRS2 does not react with sera positive for *Toxoplasma gondii* (data not shown), a finding in accordance with that reported by Liu et al. (2007), which did not find cross-reaction with this infectious agent in tNcSRS2-based ELISA.

The high specificity and sensitivity of rNcSRS2-based ELISA make it potentially suitable for serological diagnosis. Further support for this potential is provided by the high agreement between IFAT and ELISA detection rates and by the fact that Western blotting with rNcSRS antibody recognizes both antigens (rNcSRS2 and native protein).

It is important to remember that the recombinant protein purification pattern that is going to be used as antigen should be observed and the breakdown of this production should be evaluated in relation to the possibility to show a background, considering that *E. coli* proteins contaminants and unspecific reactions may occur. The antigen lot standardization will guarantee that these undesirable reactions will not happen on the diagnosis tests routinely offered.

Unlike IFAT, ELISA allows for simultaneously testing a large number of samples, making it a good candidate for serological diagnosis in sheep using NcSRS2 as the antigen.

Acknowledgments

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