PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 41:e06814, 2021
DOI: 10.1590/1678-5150-PVB-6814

Original Article
Livestock Diseases

Cloning and expression of *Neospora caninum* rhoptry 2 and its potential as an immunobiological for neosporosis control[1]

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ABSTRACT.- dos Santos A.G.Jr., Conrad N.L., Piraine R.E.A., Cunha R.C., Andreotti R. & Leite F.P.L. 2021. **Cloning and expression of *Neospora caninum* rhoptry 2 and its potential as an immunobiological for neosporosis control**. *Pesquisa Veterinária Brasileira 41:e06814, 2021*. Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96010-900, Brazil. E-mail: fleivasleite@gmail.com

The protozoan *Neospora caninum* is known worldwide as one of the main causes of abortion in cattle. During infection, rhoptry proteins present in the apical complex of the parasite play important roles in adhesion and parasitophorous vacuole formation. The use of *N. caninum* ROP2 in experimental vaccines has shown promising protective results. In our study we performed cloning and expression in *Escherichia coli* of an antigenic portion of *N. caninum* ROP2. The recombinant protein (rROP2) was obtained in insoluble form, and the purified protein showed a size of approximately 18kDa. Even being a small truncate NcROP2 region, it was possible to conserve the antigenic epitopes which were recognized by bovine serum naturally infected with *N. caninum*. Vaccination with rROP2 on aluminum hydroxide adjuvant induced high levels of rROP2-specific IgG antibodies capable of recognizing native protein in tachyzoite lysates. In conclusion, our approaches were effective in obtaining the rROP2 protein, which induced specific mouse immune response and was also recognized by sera from *N. caninum* naturally infected cattle. These results suggest that it is a promising antigen for the development of neosporosis subunit vaccines as well as a suitable antigen for use in immunodiagnosis.

INDEX TERMS: Cloning, *Neospora caninum*, rhoptry 2, immunobiology, neosporosis, protozoa, abortion, vaccine, diagnosis.

RESUMO.- [**Clonagem e expressão de rhoptry 2 *Neospora caninum* e seu potencial como um imunobiológico para o controle de neosporose.**] O protozoário *Neospora caninum* é conhecido mundialmente como uma das principais causas de aborto em bovinos. Durante a infecção, as proteínas rhoptry presentes no complexo apical do parasita desempenham papel importante na adesão e formação de vacúolos parasitóforos. O uso de ROP2 de *N. caninum* em vacinas experimentais tem mostrado resultados de proteção promissores. Em nosso estudo, realizamos a clonagem e expressão em *Escherichia coli* de uma porção antigênica de *N. caninum* ROP2. A proteína recombinante (rROP2) foi obtida na forma insolúvel, e a proteína purificada apresentou tamanho aproximado de 18kDa. Mesmo sendo uma pequena região truncada de NcROP2, foi possível conservar os epítopos antigênicos que foram reconhecidos pelo soro de bovinos naturalmente infectados com *N. caninum*. A vacinação com rROP2 adsorvida no adjuvante de hidróxido de alumínio induziu altos níveis de anticorpos IgG anti-rROP2, capazes de reconhecer a proteína nativa em lisados de taquizoítos. Em conclusão, nossas abordagens foram eficazes na obtenção da proteína rROP2, que induziu resposta imune específica em camundongos e também foi reconhecida por soros de bovinos naturalmente infectados com *N. caninum*. Estes resultados sugerem que rROP2 é um antígeno promissor para o desenvolvimento de vacinas de subunidades de neosporose, bem como um antígeno adequado para uso em imunodiagnóstico.

TERMOS DE INDEXAÇÃO: Clonagem, rhoptry 2, *Neospora caninum*, imunobiologia, neosporose, protozoa, aborto, vacina, diagnóstico.

[1] Received on January 7, 2021.
Accepted for publication on February 16, 2021.

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INTRODUCTION

Neosporosis is described worldwide as the principal parasitic disease in cattle, and it is estimated to be responsible for economic losses in cattle industries worldwide (Reichel et al. 2013). *Neospora caninum*, the responsible agent, is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa (Dubey & Schares 2011). The main clinical manifestation of bovine neosporosis is abortion, which is caused by the transplacental transmission of *N. caninum* to the fetus (Qian et al. 2017). Effective control methods that could prevent parasite spread are necessary. Vaccination is the most promising prophylactic measure for the prevention and control of infectious diseases. Analyses of different control strategies carried out so far indicate that vaccination as the most cost-effective approach to manage *N. caninum* infection (Reichel & Ellis 2006).

Vaccination with attenuated *N. caninum* tachyzoites were able to protect mice and cattle against neosporosis (Williams et al. 2007, Bartley et al. 2008). Nevertheless, despite the effectiveness, vaccination with live attenuated strains has considerable drawbacks due to possible regression to a virulent status (Reichel & Ellis 2006) as well as shelf-life and logistical issues (Weber et al. 2013). A vaccine, based on lysated whole parasite, was evaluated and some studies observed mice protection from *N. caninum* infection or vertical transmission (Liddell et al. 1999, Ribeiro et al. 2009). However, other studies showed that this immunization technique conferred insufficient protection or even exacerbated the infection in murine model (Lundén et al. 2002, Teixeira et al. 2005) and failed to prevent vertical transmission in cattle (Andrianarivo et al. 2000).

The use of organelle proteins, such as micronemes, rhoptries, and dense granules, whose contents are involved in tachyzoite-host cell interactions (Weber et al. 2013), has been the great focus of recent *N. caninum* research (Debache et al. 2008, Tahmoorespoor et al. 2013). The potential vaccinal antigens are selected based on their immunogenic capacity, implications in the process of adhesion/invasion and their ability to protect against experimental challenge (Debache et al. 2009, Monney et al. 2011). Some recombinant *N. caninum* proteins have been tested as potential vaccinal candidates with promising although variable efficacy (Monney et al. 2012, Pastor-Fernández et al. 2015, Ferreirinha et al. 2014).

The protein ROP2 of *N. caninum* is a rhoptry protein located in the apical complex and is present in all parasitic developmental stages, it actively participates in the invasion of the host cell and in formation of parasitophorous vacuoles (Dubremetz 2007, Talevich & Kannan 2013). Vaccines based on NcROP2 have shown promising results in challenge studies (Debache et al. 2008, Pastor-Fernández et al. 2015) when used in association with other proteins (Debache et al. 2009, 2010). Chimeras produced were able to protect mice against challenge, however, inducing an insufficient immune response against vertical transmission (Monney et al. 2011). In this study we aimed to clone and express a *N. caninum* rhoptry 2 to be used as an immunobiological tool for neosporosis control.

MATERIALS AND METHODS

Production of recombinant proteins and structure perdition. The nucleotide sequence of the NcROP2 gene used in the construction of the protein was based on the sequence deposited on GenBank under the number HM587954 (Monney et al. 2011). Specific primers were constructed for amplification of a part of the truncate NcROP2 nucleotide (tROP2) sequence referring to amino acids 172 to 340 and cloning into the pAE vector (Ramos et al. 2004). The PCR products were inserted into the restriction sites of the *Xho*I and *Eco*RI enzymes so that the frame allowed expression of the products with a 6-histidine tag (Fig.1). The cloning products were also sequenced (ACTGene Analysis). The *Escherichia coli* strain BL21 (DE3) (Invitrogen) was used for expression of the recombinant proteins. Induction was performed with 1mM IPTG for 4 h at 37°C. Bacteria were recovered by centrifugation, sonicated, and eluted in 8M urea. Solubilized recombinant proteins were purified using the ÄKTA Primer (GE Healthcare) system. Desalting of the samples was carried out in a Desalting 5mL HiTrap column (GE Healthcare), and concentration using dialysis membranes (cut-off 14 kDa, INLAB). Subsequently the proteins were analyzed using SDS-PAGE. The concentration of the purified protein was determined using the commercial BCA Protein Assay kit (GE Healthcare). Purified protein aliquots were stored at -70°C until use. I-TASSER software was used to generate the 3D predicted model from amino acid sequences (Roy et al. 2010).

Western blot. The purified proteins were subjected to SDS-PAGE and electro-transferred to a nitrocellulose membrane (GE Healthcare). The membrane was incubated with blocking solution (PBS-T with 5% milk powder) for 1 h at 37°C. Afterwards, the membrane was cropped and each strip was incubated separately for 1 h at 37°C with one of the following primary antibodies: anti-histidine (1:5.000) or bovine serum positive for neosporosis (1:400) previously confirmed by indirect immunofluorescence reaction (IFAT). They were then used at the same dilution (1:5.000), as secondary: anti-mouse and anti-bovine antibodies respectively. The reactions were revealed with a solution containing DAB (0.6mg diaminobenzidine, 0.03% nickel sulfate, 50mM Tris-HCl pH 8.0 and 15µl hydrogen peroxide). The *E. coli* BL21 (DE3), a non-transformed strain, was used as the negative control.

Vaccination. A total of 18 Balb/c isogenic female mice, 28 days old and weighing 16-21g, were randomly divided into two groups of 9 animals. Mice in Group 1 were inoculated with a vaccine formulated

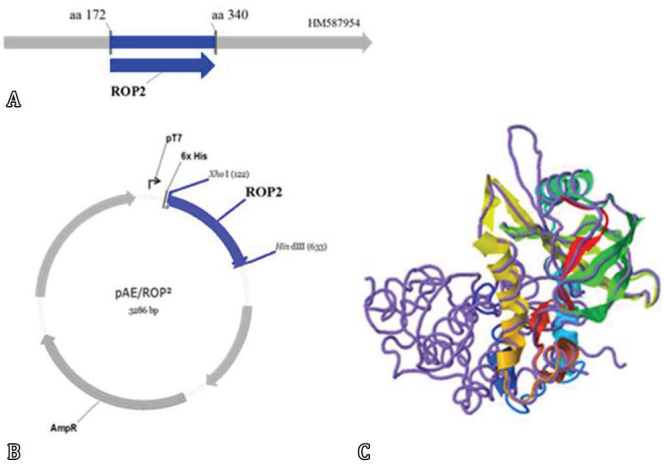

Fig.1. Silico analysis. (**A**) The amino acid sequences for Rop2 (168 amino acids) are indicated (blue line), (**B**) schematic representation of the pAE/ROP2 vector expression (from Vector NTI Advance 10), (**C**) 3D predicted model from amino acid sequences using I-TASSER software.

with 25µg of protein/dose adsorbed in 10% aluminum hydroxide [$Al(OH)_3$; Sigma Aldrich)] as adjuvant and Group 2 were inoculated with 0.9% saline solution plus 10% aluminum hydroxide. The mice were inoculated by intramuscular injection (0.2ml) on day 0 and received a booster on day 14 of the experiment. Blood samples were collected by the submandibular puncture on days 0, 7, 14, 28, 35, 42 and 60. After collection, serum was separated, labeled and stored at -20°C until analysis.

All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 9651) of the "Universidade Federal de Pelotas" (UFPel). The UFPel-CEEA agreement is approved by the Brazilian National Council for Animal Experimentation Control (CONCEA).

Indirect enzyme-linked immunosorbent assays. Total serum IgG antibodies against rROP2 were quantified by an indirect enzyme-linked immunosorbent assay (ELISA) following Dummer et al. (2014), with modifications. Briefly, the plates (Polysorp Surface, Nunc, Sigma-Aldrich) were coated with 100µl of a suspension containing 0.1mg/ml^{-1} of rROP2, or a *Neospora caninum* tachyzoites (1×10^6) suspension lysed, both diluted in carbonate-bicarbonate at pH 9.6 at 4°C for 18 h. The plates were washed three times with phosphate buffer solution at pH 7.6 (PBS) containing 0.5% Tween 20 (PBS-T). Sera diluted in PBS-T (1:100) were added to the wells in duplicate (100µl/well) and incubated at 37°C for 60 min. The plates were then washed three times with PBS-T. Then, 50µl of peroxidase-conjugated anti-mouse IgG (Dakopatts A/S), or anti-mouse IgG1 or IgG2 isotype (Sigma-Aldrich), diluted 1:4,000 in PBS-T was added to each well, and the plates were incubated at 37°C for 60 min. Immediately afterward, the plates were washed five times with PBS-T, and then 50µl of o-phenylenediamine (OPD, Sigma-Aldrich) chromogenic substrate was added, and the mixture was allowed to react in the dark for 15 min at room temperature, then a 100µl stop solution (sulfuric acid 3%) was added. Absorbance readings were conducted using a microplate reader MR 700 (Dynatech Microplate Reader) at 492nm, and the results were expressed as the total IgG increase.

To check if rROP2 is recognized by neosporosis positive serum, an ELISA was performed. Bovine sera were tested, 5 positive and 5 negatives for *N. caninum*, previously characterized by IFAT and provided by Embrapa beef cattle (Campo Grande, Brazil) diluted 1:200 in PBS-T. The secondary antibody peroxidase-conjugated anti-bovine IgG (Sigma-Aldrich) was diluted 1:5.000 in PBS-T. This assay was performed following protocol described above. To accurately assess the sensitivity and specificity of the diagnostic test, the results of 10 samples previously confirmed as positive and negative by IFAT, defined as the cut-off point, using the method previously described (Pare et al. 1995) were analyzed for Receiver Operating Characteristic (ROC) using the MedCalc software (version 16.4.3)[4].

RESULTS

PCR amplification of the NcROP2 gene partial sequence, yielded a fragment of the expected size (522 bp), encoding the expression cassette of 179 amino acids, including fusion with the 6xHis tag (Fig.1A and B). The sequence was cloned successfully and the transformation of the pAE/ROP2 vector in the *Escherichia coli* strain TOP10F resulted in several clones. Next, cloned vectors were sent to sequencing in order to confirm the insertion of the NcROP2 gene. The sequencing result was subjected to the BLAST tool (Zhang et al. 2000), revealing sequences identical to those reported (GenBank accession number: HM587954). The rROP2 protein structural model prediction, using I-TASSER software, has demonstrated that the recombinant structure has kept a similar conformation to the native protein (Fig.1C).

The ROP2 recombinant protein, expressed by *E. coli* BL21 (DE3) strain, was detected in insoluble form (Fig.2A). This expression process yielding ~125µg/L of culture. The rROP2 expression was confirmed by Western blot using a monoclonal anti-His antibody which recognized the recombinant protein, showing a band of 18kDa size and rROP2 protein was also detected by positive serum from *Neospora caninum* infected bovine (Fig.2B).

Mice immunized with rROP2 adsorbed in 10% aluminum hydroxide presented anti-rROP2 antibodies ($P<0.05$) at 14 days post inoculation (PI). The boost induced a significant ($P<0.05$) increase of total IgG, being stable until the day 60 PI (Fig.3A). The rROP2 humoral response presented a significant higher ($P<0.05$) IgG1 production compared with IgG2a (Fig.3B). Sera from rROP2 immunized mice also recognized the native protein, ROP2, in the tachyzoite lysates, since 21 days post inoculation (Fig.3C).

The ELISA showed that rROP2 protein was recognized by *N. caninum* positive sera and was not recognized by negative sera (Fig.4A), resulting in a specificity of 100% and a sensitivity of 100% (Fig.4B).

DISCUSSION

In this study we select a *Neospora caninum* rhoptry 2 protein (ROP2) to be used as antigen to develop an experimental neosporosis vaccine. The amplification of the NcROP2 gene result in a fragment of 522pb, the same size was estimated in silico by Software Vector NTI® Advance 10, indicating that the expression occurred for the whole cassette. A small ROP2 protein *Escherichia coli* expressed portion (18kD), as described in this study methodology, did not compromise the main antigenic epitopes present in the native protein, since it was recognized by positive serum from *N. caninum* infected bovine (Fig.2B), thus confirming epitope prediction analysis (Monney et al. 2011).

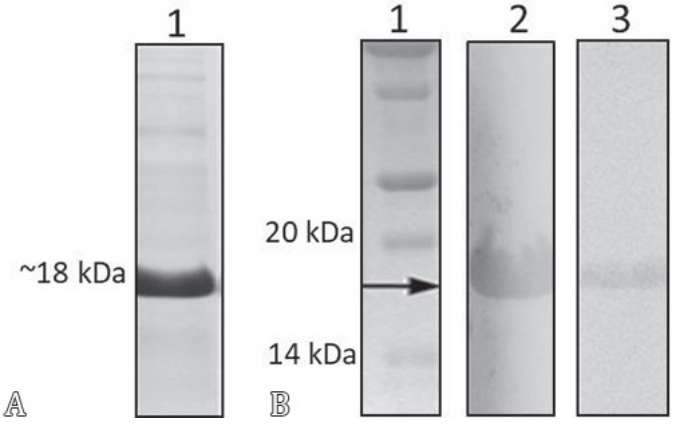

Fig.2. Characterization of rROP2. (**A**) SDS-PAGE, purified rROP2 (1). (**B**) Western blot, molecular weight marker (1), probed with anti-His (2), probed with anti-*Neospora caninum* antibodies from *N. caninum* infected bovine (3).

[4] Available at <http://www.medcalc .org>

To induce specific and effective antibody production against a pathogen, recombinant vaccine antigens need to preserve epitopes with identity similar to the native protein. The loss of original conformation among *N. caninum* epitopes has been reported as a possible cause of low protection in murine challenge models (Srinivasan et al. 2007). The ROP2 recombinant protein was able to induce IgG specific to rROP2 which also recognized the native protein, ROP2, in the tachyzoite lysates (Fig.3C), confirming that the construct-maintained epitopes similar to those of the native proteins in vaccinated mice. The rROP2 immunogenic potential agrees with the observed by Debache et al. (2008) using a rROP portion of 43kDa. Besides the reduction in the amino acid number of the protein expressed in this study, it has kept the high immunogenicity, in vaccinated mice, yielding high titers of antibodies until day 60 PI.

The predominant of IgG1 response in the mouse is indicative of a Th2-like response. Aluminum hydroxide as adjuvant in the vaccine polarize to a Th2 response by inhibiting IL-12 from DCs (Coffman 2010, Mori et al. 2012). Previous studies with chimeras composed of ROP2 and different *N. caninum* recombinant antigen, such as microneme protein NcMIC3, showed that protection against *N. caninum* infection in a murine model, was associated with IgG1 antibody response (Cannas et al. 2003, Monney et al. 2011). A Th2 immune response obtained in our study agree with others research, suggesting that a Th2 response is important for protection against fetal infection and consequently abortion. In this sense a Th2-type immune response is ideal, since it is physiologically modulated, and has been shown that can control proliferation and spread of *N. caninum* during gestation (Innes 2007, Debache et al. 2009).

Studies using the *N. caninum* protein NcSRS2 expressed in *E. coli* (Borsuk et al. 2011) and in *Pichia pastoris* (Pinheiro et al. 2013) presented 96 and 97.8% specificity, respectively. Our study, using rROP 2 protein as antigen, showed a specificity and sensitivity of 100% (Fig.4). However, more sera need to be evaluated to confirm rROP potential to use as a tool to

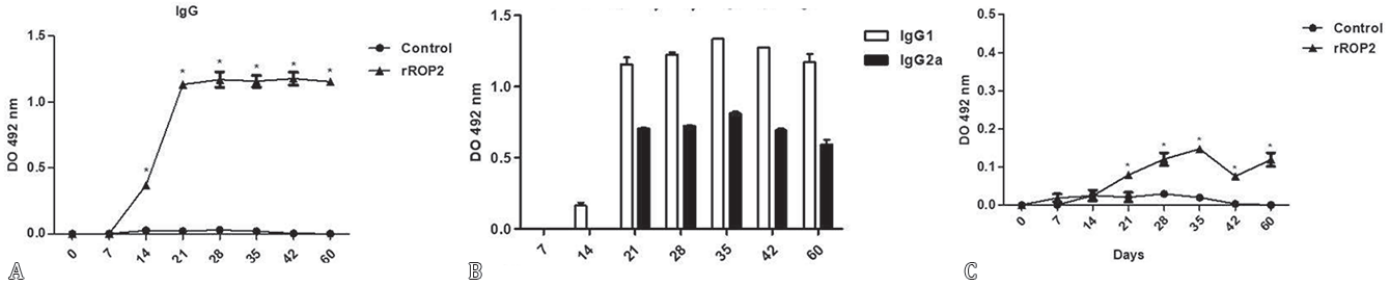

Fig.3. IgG dynamics. The data represent the mean ELISA values (± standard error) of total serum IgG from mice inoculated with rROP2. (**A**) Total IgG dynamic against rROP2. (**B**) IgG1 e IgG2a dynamics. (**C**) Total IgG dynamic against lysed *Neospora caninum* antigen. The statistical analysis was performed by two-way ANOVA followed by the Bonferroni post-test. Asterisks (*) indicate significant difference (P<0.05) between the mice inoculation with rROP2 (inoculated days 0 and14) and the control groups (P<0.001) on days 14, 28, 35, 42 and 60.

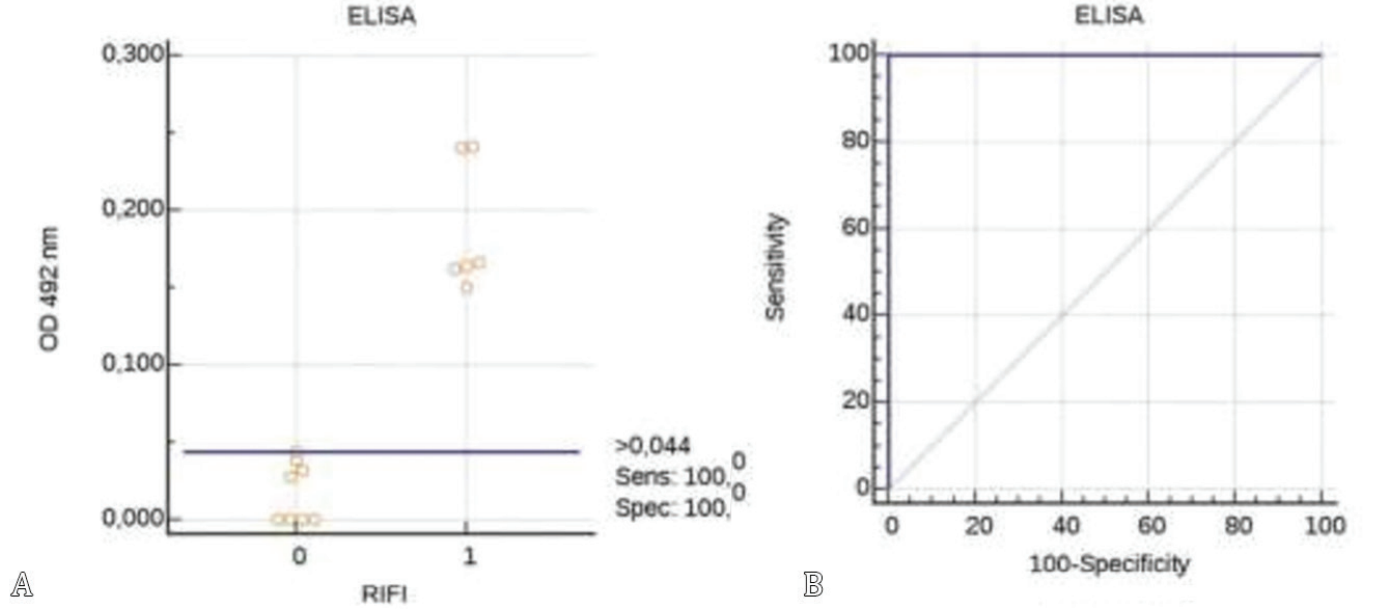

Fig.4. Receiver operating characteristic (ROC) of ELISA-rROP2 using 5 positive and 5 negative sera. (**A**) Distribution frequency of the absorbance results for positive (1) and negative (0) sera. The absorbance value (0.044) obtained in ELISA was established as the cutoff point. (**B**) ROC curve. Area under the curve = 1.000; 95% confidence interval between 0.768 and 1.000.

neosporosis diagnosis. The use of recombinant antigen can increase the specificity of the diagnostic test, when compared to commercial ELISA and IFAT, where crude antigen is present, and leads to decreases in specificity due to false positive results through cross-reactions with other Apicomplexa (Higa et al. 2000). The use of IFAT as a diagnostic standard test for *N. caninum* is both expensive and subjective, which limits its use in large-scale investigations (Dubey & Schares 2006, Borsuk et al. 2011). The availability of a serological test, using the rROP2 as antigen described here, could be of easy implementation, contributing to neosporosis epidemiological studies.

CONCLUSIONS

The results obtained in this study demonstrate that the rROP2 fraction and the methodology used allows to obtain an optimum protein yield and a product with a similar conformation to native protein, being capable of high immunogenicity and antigenic.

Our approaches were effective to express a truncated rROP2, suggesting that it is a promising antigen for use as an immunobiological, as a vaccine antigen, or for immunodiagnosis in the control of neosporosis.

Acknowledgements.- We would also thank to "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior" (CAPES) - Brazil - Finance Code 001 and "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq) for scholarships. Alceu G. Santos Junior and Neida Lucia Conrad contributed equally to this work.

Conflict of Interest.- The authors declare that they have no conflict of interest.

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