Expression of *Neospora caninum* NcSRS2 surface protein in *Pichia pastoris* and its application for serodiagnosis of *Neospora* infection

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Neospora caninum is considered a major cause of abortion in cattle worldwide. The antigenic domain of NcSRS2 in *N. caninum* is an important surface antigen present in the membrane of this parasite. In the present study, the *Pichia pastoris* expression system proved to be a useful tool for the production of recombinant protein. The truncated NcSRS2 gene (by removal of the N-terminal hydrophobic sequence), was cloned in the vector pPICZalphaB, and integrated on the genome of the methylotrophic yeast *P. pastoris*. Subsequently, the NcSRS2 protein was expressed, purified, and characterized using naturally infected cattle sera and Mab 6xhistag. The recombinant protein NcSRS2 was present in the supernatant of the culture, where later it was concentrated and purified using ammonium sulfate (~100 mg/ml). An indirect immunoenzymatic assay (ELISA) was performed using cattle sera from endemic *N. caninum* area.

Keywords: NcSRS2, *Pichia pastoris*, Expression

Introduction

Neospora caninum is the causative agent of neosporosis. The economic importance of *N. caninum* infections in cattle is attributed to abortion costs of increased animal disposals,¹ and decreasing milk production.² Worldwide, Brazil has the second largest cattle herd and is the largest beef exporter.³ Yet, the country still has major economic losses related to livestock diseases, which can affect weight gain, reproductive rate, or even cause loss of the animal. Losses of this type are not insignificant reaching an index of up to 27.34% in the state of Mato Grosso do Sul, which concentrates the majority of the total national cattle herd.⁴ While Neosporosis is important for these losses economically, there are few studies about the actual impact of the disease in Brazilian herds.

NcSRS2 is an immunodominant surface protein present in bradyzoites and tachyzoites of *N. caninum*.^{5,6} It has potential for use in specific serological diagnoses of neosporosis. The antigenic domain of the protein NcSRS2 has been determined and is localized in the C-terminal 2/3 parts.⁷ The protein has also been expressed using prokaryotic systems in several studies;^{8–10} however, *Escherichia coli*, which is used extensively as a host for

heterologous recombinant protein expression, has limitations that include yield, folding, and post-translational modifications.^{11,12}

The methylotrophic yeast, *Pichia pastoris* has been used effectively for expression of heterologous proteins.¹³ The main characteristics of protein production with *P. pastoris* are its capacity to promote post-translation modifications, and its secretion of heterologous proteins in soluble form.¹⁴ The use of this yeast gives advantages over prokaryotic expression systems, which include easy genetic manipulation and fast growth in relatively simple culture media, allowing for expansion into large-scale protein production.^{15,16} In addition, it is well known that this eukaryotic system preserves characteristics of the recombinant antigen, e.g. glycosylation, that might be important in its recognition by the immune system. As such, this platform was chosen for production of a recombinant *N. caninum* antigen.

Materials and Methods

Cattle sera

The cattle sera used came from beef cattle breeding region of Brazil, and the main breed is *Bos Taurus*. The peripheral blood was collected from the jugular vein of adult bovine, using a 19 g needle attached to

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Vacutainer tubes (Becton-Dickinson, Rutherford, NJ, USA) and stored at -20°C until use. The sera was confirmed as positive or negative by IFAT using the method previously described.^{17,18}

Bacterial strains and growth conditions

Neospora caninum isolate NC-1¹⁵ was used to prepare the antigen for this study. The parasite was propagated in Vero cells using Dulbecco's modified essential medium supplemented with 10% fetal calf serum (FCS), at 37°C in a humidified atmosphere of 5% CO_2 in air. When 80% of the Vero cells had been destroyed by the tachyzoites, the monolayer cells were removed and washed twice with phosphate-buffered saline (PBS) solution at $1000 \times g$ centrifugation for 10 minutes.

E. coli strain TOP10 (Invitrogen) was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37°C with the addition of zeocin at $25 \mu\text{g/ml}$. *P. pastoris* strain X33 (Invitrogen, Carlsbad, CA, USA) was grown in yeast extract peptone dextrose medium (1% yeast extract, 2% peptone, and 2% D-glucose) supplemented with $100 \mu\text{g/ml}$ of zeocin at 28°C .

Cloning NcSRS2

The C-terminal DNA sequences coding for the NcSRS2 gene (Genbank accession no. JQ410454.1) were amplified by PCR using primers F5'- CGG AAT TCC CAA AGA GTG GGT GAC TGG AAC and R5'- GCT CTA GAC ATG CAT CTC CTC TTA ACA CGG G. The PCR product was cleaved with *EcoRI* and *XbaI* before in-frame ligation of the fragment to the pPICZalphaB vector (Invitrogen), which had been previously digested with the same enzymes. The ligation reaction was transformed into *E. coli* TOP10 competent cells. The pPICZalphaB/NcSRS2 was propagated in *E. coli* TOP10, and the plasmids isolated using GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Salt Lake City, Utah, USA). The identity of the inserts was determined by DNA sequencing using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA analysis systems — MegaBACE 500 (GE Healthcare).

The recombinant plasmid (pPICZalphaB/NcSRS2) was linearized with *PmeI* enzyme (New England BioLabs, Ipswich, MA, USA). The linear plasmid DNA was then purified by phenol-chloroform extraction, and *P. pastoris* competent cells were transformed by electroporation ($25 \mu\text{FD}$, 200 Ohms, and 2.0 kV) with $10 \mu\text{g}$ of the linear plasmid DNA.

Screening for expression of recombinant NcSRS2

Approximately 100 colonies were plated onto buffered methanol complex medium (BMMY: 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.00004% biotin, 0.5% methanol, 100 mM potassium phosphate, and 2%

agar; pH 6.0). After 48 hours of incubation at 28°C , expression of NcSRS2 was induced with 1% methanol and evaluated at 48 hours. Expression of the recombinant protein was confirmed by colony blotting. Briefly, a nitrocellulose membrane (Hybond ECL; GE Healthcare) was placed on the surface of each Petri dish, and in direct contact with the colonies for 1 hour at 28°C . Any adherent matter was removed from the membrane by washing with PBS-T [PBS, pH 7.4, 0.05% (v/v) Tween 20]. After blocking (PBS-T, 5% non-fat dried milk), the membrane was incubated for 1 hour at room temperature with anti-His-6X tag (Sigma Aldrich, St. Louis, MO, USA), and diluted at 1 : 10 000 with PBS containing 5% skimmed milk (PBS-SM) at room temperature for 1 hour. The membrane was washed three times with PBS-T for 5 minutes each, and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Sigma Aldrich), diluted at 1 : 5000 with PBS-SM at room temperature for 1 hour. The reacting bands were revealed using 3,3'-diaminobenzidine and H_2O_2 .

The presence of the *ncsrs2* gene in the *P. pastoris* genome was confirmed by colony PCR. Crude genomic DNA extracts were prepared by boiling selected recombinant yeast clones in water. PCR was performed (similarly to the primers described above) using the crude genomic DNA extracts as the template. The PCR products were analyzed by horizontal gel electrophoresis and visualized with GelRed (Biotium, Hayward, CA, USA).

Expression of NcSRS2 protein in *P. pastoris* X33

A recombinant clone (positive by dot blotting and by colony PCR) was selected and inoculated into a 3 l baffled fermenter containing 1 l of BMMY broth. The culture was incubated at 28°C , for approximately 48 hours until an OD_{600} of 2–6 was reached. Expression was induced by the addition of methanol at a 1% final concentration. Samples (supernatant and cells) were collected at the following times: 24, 48, 72, 96, 120, and 144 hours. Finally, the media were harvested at $10\,000 \times g$ for 10 minutes, and the supernatant was stored at -70°C . The expression of the recombinant protein was analyzed by dot blotting using the anti-hisx6tagMab (Sigma).

Purification and concentration of rNcSRS2

The secreted NcSRS2 recombinant protein was concentrated and purified by precipitation using a 20% ammonium sulfate saturation that was added to the culture supernatant at 4°C , and increased up to final concentrations of 30, 40, 50, 60, and 70%. The precipitated proteins were collected by centrifugation at $10\,000 \times g$ for 15 minutes at 4°C , suspended in PBS buffer, and then dialyzed in deionized water for 72 hours. The protein concentrations in the culture supernatants, concentrates, and purified protein

samples were determined using a BCA kit (Pierce, Rockford, IL, USA).

Western blotting (WB)

The ability of the recombinant protein to interact specifically with neosporosis-positive sera was determined by WB using sera collected from neosporosis-positive cattle previously defined by IFAT. Purified recombinant NcSRS2 and an unrelated recombinant protein (negative control) were used for WB analysis of positive and negative bovine sera. The samples were mixed with sodium dodecyl sulfate gel-loading buffer (100 mM Tris-HCl at pH 6.8, 100 mM 2-mercaptoethanol, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, and 20% glycerol) under reducing conditions. The samples were heated at 100°C for 10 minutes, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Afterwards, the proteins in the gel were electrically transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked with PBS-SM for 1 hour at room temperature and incubated with positive or negative cattle sera diluted at 1:100 with PBS-SM at room temperature for 1 hour. The membrane was washed three times with PBS-T for 5 minutes each and incubated with horseradish peroxidase-conjugated anti-bovine IgG (Sigma) diluted at 1:4000 with PBS-SM at room temperature for 1 hour. The reacting bands were revealed using 3,3'-diaminobenzidine and H₂O₂.

Indirect enzyme-linked immunosorbent assays (ELISAs)

The 96-well polystyrene microtiter plates (Polysorp, Nunc, Rochester, NY, USA) were coated overnight at 4°C with 50 ng/well of recombinant protein NcSRS2 in 0.05-M carbonate-bicarbonate buffer (pH 9.6). The plates were then washed three times using 0.01M PBS with 0.05% Tween 20 (PBS-T), and blocked using 0.01M PBS with 5% non-fat milk at 37°C for 1 hour. After three washes with PBS-T, the positive and negative control sera and serum samples, all in duplicate, were diluted to 1:100 in 0.01M PBS-T and incubated at 37°C for 1 hour. After three washes, anti-bovine IgG conjugated to peroxidase (Sigma), and diluted at 1:4000 in 0.01M PBS-T, 100 µl/well was added, which was followed by incubation at 37°C for 1 hour. After five washes, 100 µl of the substrate (*o*-phenylenediamine dihydrochloride; OPD tablets, Sigma) in phosphate-citrate buffer (0.4 mg/ml) containing 0.04% of 30% (v/v) hydrogen peroxide, pH 5.0, was added to each well, and the plates were incubated in the dark, and at room temperature for 15 minutes, and then 50 µl of stop buffer (1 N H₂SO₄) was added. Mean optical density (OD) at 492 nm was determined for all test wells using a microtiter plate reader (Multiskan

Figure 1 Dot blotting of *P. pastoris* supernatant transformed with pPICZalphaB/NcSRS2 and cultured in fermenter using the anti-hisx6tagMab. (C+): positive control (NcSRS2 expressed in *E. coli*); (C-): negative control (*P. pastoris* X33 untransformed). Days 1, 2, 3, 4, 5, and 6 are the induction periods for NcSRS2 expression with 1% of methanol.

MCC/340 MKII), and intra-plate ELISA was performed.

Statistical analysis

To accurately assess the ELISA for diagnostic specificity, sensitivity, cutoff, and predictive values, the results from the 139 bovine sera (confirmed positive and negative samples) were subjected to receiver operating characteristic (ROC) analysis using MedCalc statistical software (version 10.3.0.0) (www.medcalc.be).

Results

Plasmid construction

The DNA sequences that encode for the protein NcSRS2 (732 bp) were amplified by PCR and cloned into the *P. pastoris* expression vector pPICZaB. Of the 100 *P. pastoris* colonies screened for expression, about 30% were recognized by a monoclonal antibody (Mab) specific to the 6XHis tag at the C-terminus of the recombinant protein. Colony PCR was used to confirm the presence of the insert in the expression vector of the *P. pastoris* genome. The clones exhibiting the highest expression levels were selected for further expression studies.

Recombinant protein purification and concentration

The NcSRS2 was secreted to supernatant of the culture of *P. pastoris* transformed with pPICZalphaB/NcSRS2, which contains the alpha-factor signal sequence, allowing secretion of the recombinant protein. The concentration of NcSRS2 in the culture supernatant was found to increase with time (Fig. 1) and recombinant proteins of the expected size were observed, NcSRS2 (30 kDa) (Fig. 2).

The supernatant containing the secreted NcSRS2 was collected and purified or concentrated by ammonium sulfate precipitation. The optimal salt concentration for NcSRS2 was 40–50%. The recombinant proteins were dialyzed to remove the ammonium sulfate and then analyzed by dot blotting

Figure 2 WB of *P. pastoris* expressing NcSRS2. MW: full range rainbow molecular weight marker (GE), 1 – recombinant NcSRS2. The bands were revealed with bovine sera positive for neosporosis diluted at 1 : 100 and anti-bovine IgG conjugated to peroxidase diluted at 1 : 4000.

(Fig. 3A), which were reactive using sera from positive cattle and from mice inoculated with recombinant protein NcSRS2 purified from *E. coli*. The yield obtained was 100 mg/l after concentration with 50% ammonium sulfate.

Antigenicity of the recombinant NcSRS2 protein

The antigenicity of the purified protein was evaluated with positive cattle sera for neosporosis by WB. The protein NcSRS2 expressed in *P. pastoris* was recognized by the sera, showing that the antigenic properties were conserved when the eukaryotic system was used for expression (Fig. 2).

ELISA-NcSRS2, sensitivity, and specificity

The NcSRS2 produced in *P. pastoris* was used for standardization of ELISA-NcSRS2 which was achieved using 139 bovine sera (94 positive and 45 negative) previously determined by IFAT, and were evaluated using ELISA-NcSRS2. Correlation between the two diagnostic tools was assessed using ROC analysis. Figure 4A shows the frequency distribution of both the positive and negative IFAT samples. Based on ROC analysis (Fig. 4B), a mean bovine sera ELISA OD value of 0.42 was chosen as the threshold to distinguish between positive and negative samples, yielding a specificity of 97.8% and a sensitivity of 100%.

Discussion

The NcSRS2 surface protein is common to both the tachyzoite and bradyzoite stages, and shows potential for use in *N. caninum* serological infection diagnosis.^{6,19–22} The protein has been expressed in systems that include prokaryotic and eukaryotic systems based on the baculovirus,^{8–10,22} however, *P. pastoris* has never been used to express proteins from *N. caninum*.

P. pastoris is important for the production of recombinant proteins.²³ The use of this yeast provides advantages over prokaryotic expression systems, since easy genetic manipulation, and fast growth in relatively simple culture media allow for its expansion to industrial scale protein production, all this besides having a strong induction promoter in methanol.¹⁶

In this work, a C-terminal fragment of NcSRS2 was successfully cloned and expressed in *P. pastoris* methylotrophic yeast. In previous studies, there are no reports concerning the amount of protein obtained after purification; our group has already produced NcSRS2 in *E. coli*,⁸ with yields of between 3.2 and 8 mg/l (unpublished data). In this study, we report the expression in *P. pastoris* resulting in yields of over 100 mg/l for rNcSRS2 after concentration with 50% ammonium sulfate, without the need for subsequent solubilization, and/or re-folding steps. This procedure allowed us to evaluate the applicability of a low cost and simpler purification.²⁴

Several proteins from parasites have been expressed in *P. pastoris* systems.^{25–31} Some recombinant antigens were used as vaccine, where VAR2CSA-DBL1 recombinant can be targeted by vaccination and may have application for pregnancy malaria vaccine development.³² Mice challenged with live cells of *T. gondii* have also been protected after immunization with recombinant SAG2.²⁶ Protective immunity against lethal malaria infection in mice has been induced by cholera toxin B subunit glycoprotein expressed in yeast.³³ Partial protection has been obtained using *P. falciparum* HGXPRT expressed in *P. pastoris*, after immunization of mice that were challenged with *P. yoeli*,³⁴ and msp1.³⁵ Antigens from

Figure 3 Dot blotting of NcSRS2 purification/precipitation. (A) Precipitation of NcSRS2 of supernatant culture with 30, 40, 50, 60, and 70% of ammonium sulfate. (B) Positive control (NcSRS2 purified from *E. coli*) and negative control (*P. pastoris* X33 untransformed). Line 1 — bovine serum positive to neosporosis; Line 2 — mice serum positive to recombinant NcSRS2; Line 3 — Mab anti-Hisx6Tag.

Schistosoma japonicum have been evaluated as well as vaccine.³⁶

Use in diagnosis has also been assessed; SAG2 expressed in *P. pastoris* was used for toxoplasmosis diagnosis.^{27,37} Recombinant cathepsin B-1 protease has been used as a serodiagnostic antigen for human opisthorchiasis showing sensitivity and specificity (ELISA test) of 67 and 81%, respectively.³⁸ Cysteine protease of *Clonorchis sinensis* has also been used for serological diagnosis of clonorchiasis with a sensitivity of 91.7% and a specificity of 97.6%.³⁹

Several reports have suggested that recombinant proteins produced in *E. coli* exhibit failures during protein folding, which interferes when these proteins are used in diagnosis, or as a recombinant vaccine.⁴⁰

NcSRS2 expressed in *P. pastoris* has been recognized by neosporosis positive bovine sera, showing that it conserves its antigenic properties when the eukaryotic expression system is used. The NcSRS2 recombinant was then evaluated using indirect ELISA with cattle sera from positive and negative animals. The results for specificity and sensitivity were 97.8 of 100%, respectively.

We believe that this is the first report of the use of the methylotrophic yeast *P. pastoris* to express the NcSRS2 protein of *N. caninum*. The method provided satisfactory expression (great amounts of protein), and an easy purification strategy. The protein proved useful for the diagnosis of neosporosis in cattle using indirect ELISA.

Figure 4 Receiver operating characteristic (ROC) analysis of the ELISA-NcSRS2 using 94 confirmed positive and 45 assuredly negative sera. (A) Distribution frequency of confirmed positive (1) and confirmed negative (0) sera. Samples were considered positive when cutoff values were greater than or equal to 0.42 ELISA absorbance (mean values). (B) ROC plot. Area under curve=0.999 (0.003); 95% confidence interval between 0.971 and 1.000.

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