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Research Article

The Development of an Indirect ELISA Test Using the Recombinant Protein NcSRS2 to Detect anti-*Neospora Caninum* Antibodies in Humans

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Abstract

Objective

The objective of this study was the development and standardization of an indirect serological test (ELISA) using the NcSRS2 recombinant protein for anti-*Neospora caninum* antibodies detection in human serum.

Material and Methods

Blood samples were collected from HIV patients in reference centers in Campo Grande, MS, Mato Grosso do Sul (Brazil), during the period from November 2009 to June 2011. After collection the samples were centrifuged for serum obtainment and tested with ELISA (Enzyme Linked Immuno Sorbent Assay) and IFAT (Indirect fluorescence antibody test) for anti-*N. caninum* IgG detection. The purified NcSRS2 recombinant protein was analyzed with Western blotting, using human serum samples that tested positive and negative for *N. caninum*.

Results

209 human blood samples were analyzed with the NcSRS2 for ELISA standardization, being 49 positive and 160 negative, according to IFAT. The correlation between the two diagnostic methods was evaluated with the ROC analysis. Of the 160 negative IFAT samples, 8 were positive when ELISA was used. To confirm this result, Western blotting was used. The positive predictive value was of 91,3%, and the negative predictive value was of 100%. The sensibility value of the ELISA test was of 100%, and specificity value was of 90,5%.

Conclusions

Using the NcSRS2 recombinant protein, the sensibility and specificity were of 100% and 90%, respectively, when analyzed

in conjunction with the IFAT test, which is currently considered the standard test. The Western blotting confirmed the positive reaction between the protein and serum samples. Therefore, the data suggests that ELISA is a promising method that may be used in the serological diagnosis and seroepidemiology of this parasite in humans considering that it can be done on a larger scale and with less subjectivity.

Keywords: *Neospora caninum*; ELISA; HIV; AIDS; Recombinant Protein

Introduction

Neosporosis is a disease caused by the *Neospora caninum* protozoan, an intracellular parasite belonging to the same family of *Toxoplasma gondii*, being confused with it until 1988, when it was described and classified [1]. This parasite is responsible for bovine abortions and canine neuromuscular diseases [2]. It is present in many countries and there is a variety of intermediary hosts in its life cycle, including domestic and wild animals, being transmitted vertically or horizontally [3].

Analyzing the epidemiological data, mainly based on serological diagnosis, it is possible to affirm that this parasite is present worldwide [4]. The presence of specific antibodies in human serum means that the individual may have had exposition to the parasite [5]. According to MacCann et al. [6], individuals may be contaminated with *N. caninum* by the accidental ingestion of oocysts present in food, or also by ingesting poorly cooked or raw meat with oocysts.

After the experience of Barr et al. [7], where tachyzoites were inoculated in *Rhesus* female monkeys, non-human primates, and wounds similar to those caused by *T. gondii* transplacental infections appeared, research in human beings was initiated, such as the one performed by Tranas et al. [8], which confirmed anti-*N. caninum* antibody presence in humans in California. Afterwards, many studies have been made, such as the one by Lobato et al. [5] in immunocompromised patients, and Oshiro et al. [9] in HIV (Human Immunodeficiency Virus) patients, both indicating the possibility of an opportunistic infection. Ibrahim et al. [10] verified seropositivity in pregnant women in Egypt, who also suggested the similarity with *T. gondii* regarding the infection of healthy individuals, where mostly are asymptomatic.

The increase of *N. caninum* biology and epidemiology would not have been possible without the use of serological tests. There are various diagnosis methods, and within them the IFAT is considered the gold standard [11]. In addition, the ELISA presents an advantage in comparison to others: it allows the performance of a large scale test, its non-subjectivity and the use of specific Ag recombinant [12].

With the identification of *N. caninum* antigens, the possibility of improving the specificity of serological tests using them increased [13]. One of these examples is the NcSRS2 recombinant protein, a surface antigen that is directly linked to the process of adhesion and invasion of the host cell. This protein was identified and isolated in infected animal serum [14].

Serological tests with the immunodominant protein such as the NcSRS2 increases the chances of repeatability, reproducibility and specificity if compared to a diagnostic system with various antigens, such as the surface antigens in IFAT. Based on the NcSRS2 surface protein, this study has the objective of standardizing an indirect serological essay to identify anti-*N. caninum* antibodies in human serum.

Material and Methods

Blood samples

Blood samples were collected from HIV patients in reference centers in Campo Grande, MS, during the period of November 2009 to July 2011. The study was previously approved by the Human Research Ethics Committee (CEP) of the Universidade Federal de Mato Grosso do Sul (Opinion n. 1941/11).

Each sample consisted of 10 mL of blood, collected from the cephalic vein and stored in tubes without anticoagulant, being identified by a healthcare professional. After collection, the samples were centrifuged to obtain the serum and stored at -20 °C until the performance of the serological test.

Parasites

The *N. caninum* isolate Nc-1[1] was used to prepare the antigen for this study. The parasite was propagated in vero cells using Dulbecco's Modified Essential Medium (DMEM), supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2. When 80% of the vero cells had been destroyed by *N. caninum* tachyzoites, then one layer was removed and washed twice with phosphate-buffered saline (PBS) solution, with centrifugation at 1,000 × *g* for 10 min.

Indirect fluorescence antibody test (IFAT)

Serum samples were tested by IFAT for IgG anti-*N. caninum* detection. The cutoff point was considered to be 1:50 (PBS, pH 7. 2) for *N caninum* [5]. The IFAT technique and the blades for IFAT were prepared according to Oshiro et al. [15] Bovine serum samples, knowingly positive or negative, were used as control in all blades. The anti-human IgG fluorescent conjugates were used with a dilution of 1:5,000 (conjugate with fluorescein isothiocyanate, Sigma-Aldrich). The blades were observed using a microscope equipped for fluorescence (epi-illumination system) with an objective of 40×. Only the fluorescent reactions around the entire periphery of the parasite were considered positive. Reactions were considered negative when the parasites on the blade did not present fluorescence, or the fluorescence was located only on one end, what is characterized as "polar coloration" or "apical reaction". The samples with total tachyzoite peripheral fluorescence were considered positive [16].

Recombinant NcSRS2

The protein NcSRS2 was expressed as described by Andreot-

ti et al [17]. The amplified DNAs were cloned into pet100/D-TOPO vectors (Invitrogen Tech, Carlsbad, CA), which were used to transform *Escherichia. coli* TOP10 strains (Invitrogen Tech, Carlsbad, CA). Once the correct orientation of the insert was confirmed, the recombinant plasmid was transformed into *E. coli* BL21 Star (DE3) (Invitrogen Tech, Carlsbad, CA). *Escherichia coli* cells in the log phase were treated with 0.75 mM isopropylα-D-thiogalactoside (IPTG) for 4.5 hours at 30 °C to induce the expression of the used NcSRS2 recombinant protein [18].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.

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Indirect enzyme-linked immunosorbent assays (ELISA)

ELISA 96-well plates (SPL, Life Sciences) were adsorbed for 12 hours at 4 °C with 33,5 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). After thawing, they were washed three times with PBS with 0.1% phosphate buffered saline/Tween (PBST) and blocked with 5% nonfat powder milk. Samples of serum, positive and negative (in duplicate), were diluted at 1:100 in PBST and incubated for 1 hour at 37 °C. The plates were then washed three times with PBST, and 100 µL well of rabbit anti-human IgG antibody conjugated with peroxidase (Sigma Chemicals, EUA) diluted at 1:2,500 in PBST were added. The plates were incubated, as described previously, and, after 5 washes with PBST, *o*-phenylenediamine dihydrochloride (OPD, Sigma Chemicals, EUA) /H_2O_2 was added as a chromogen substrate. The reaction was stopped by adding 2.5 N H_2SO_4 and the results were read at 490 nm on a Bioclin mindray MR – 96A ELISA reader.

Western blotting

The purified NcSRS2 recombinant protein was homogenized with a SDS-PAGE gel buffer under reducing conditions. The samples were left at 95°C during 10 minutes and separated in SDS-PAGE %15. The separated proteins in gel were electro transferred to nitrocellulose membranes (GE Healthcare, UK). The membranes were blocked with PBS containing %5 of skim powdered milk (LD) during 30 minutes at room temperature and incubated with positive and negative human serum (1:1000 with PBS+LD), and left overnight at room temperature. Then, the nitrocellulose membrane was washed 3 times, during 5 minutes each, with PBST. After, the membrane was incubated with anti-IgG antibodies conjugated with peroxidase (Sigma Chemicals, EUA), diluted at 1:2,000 with PBS+LD at room temperature for 90 minutes. The reaction was revealed using 3,3›-tetrahydrochloride and H_2O_2.

Statistical analysis

To accurately assess the assay for diagnostic specificity, sensitivity, cutoff and predictive value, the results from the 209 human sera confirming positive and negative samples were subjected to receiver operating characteristic (ROC) analysis using the MedCalc statistical software version 10.3.0.0.

Results

IFAT, ELISA-NcSRS2 and Western blotting

For the IFAT test blades with a parasite concentration of 1.0 × 10^6 tachyzoites/mL were prepared. The produced NcSRS2 protein presented a 1.340 µg/mL yield, enough to standardize the ELISA and Western blotting tests.

209 human blood samples were analyzed for the ELISA test with the NcSRS2 recombinant protein standardization, being 49 positive and 160 negative, according to IFAT. To determine the best antigen, serum and conjugate dilution, plates with concentration curves were used to choose the best reading of the reaction with the substrate. The correlation between the two diagnosis methods was evaluated using ROC.

Of the 160 negative IFAT samples, 8 were above the cutoff point of 0,11, being positive for the ELISA test. Of the 39 positive IFAT samples, only 2 were on the cutoff point for negative ELISA. To confirm these results, the Western blotting (Figure 1) with the protein and sera of positive and negative samples were used.

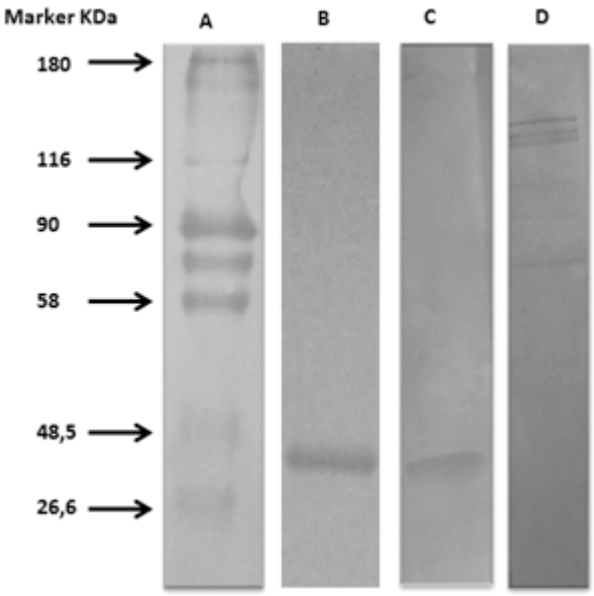

Figure 1. (A) Molecular weight Standard Pre Blue stained Marker (SDS7B2 / Sigma®). (B) SDS-PAGE gel (15%) stained with Coomassie blue NcSRS2 containing the recombinant protein fragment with a 29KDa.(C) (pool of positive samples) Western blotting detecting anti-human IgG peroxidase-conjugated antibodies from human serum positive for *Neospora caninum.* (D) (negative sample) Western blotting using anti-human IgG peroxidase-conjugated antibodies from human serum negative for *Neospora caninum*. Human sera positive and negative to *Neospora caninum* used for Western blotting were previously tested by IFAT and ELISA tests.

The seroprevalence found was of 24.4% and the positive predictive value for this prevalence was of 91.3%, while the negative predictive value was of 100%. The sensibility value of the ELISA test was of 100%, while the specificity value was of 90.5%, according to Figure 2.

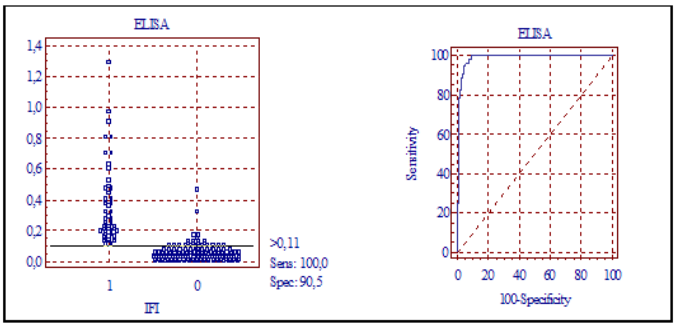

Figure 2. ROC analysis on the ELISA-NcSRS2 test using 51 confirmed positive and 158 confirmed negative human serum samples (as shown using the indirect fluorescence antibody test). (a) Frequency distribution of the confirmed positive (1) and confirmed negative (0) serum samples. The samples were considered positive when the cutoff values were greater than or equal to a mean ELISA absorbance value of 0.11. (b) ROC plot. Area under curve = 0.986; 95% confidence interval from 0.959 to 0.997.

Discussion

The presence of anti-*N. caninum* antibodies in humans was firstly reported by the end of the XX century, in California, by Tranas et al. [8], presenting a 6.7% seropositivity in blood donors. In 2006, Lobato et al. [5], in addition to immunocompromised patients, also reported anti-*N. caninum* antibodies in newborns and healthy individuals, with a rate of 5% and 6%, respectively, suggesting an environmental contamination, and not an infection.

In 2009, Benetti et al. [19] reported a 10.5% seropositivity in rural workers located in rural properties with a high level of bovines positive for *N. caninum*. In Egypt, pregnant women sera were analyzed, with a positivity rate of 7.92%, being also reported a simultaneous infection of *T. gondii* and *N. caninum* in 5.94% of the samples [10]. Oshiro et al. [9] also reported the existence of this same co-infection in HIV positive patients, with a rate of 25.2%.

The *N. caninum* infection rate in patients with immunological problems, specifically the HIV positive ones, has been decreasing with the creation of new drugs, and their survival rate has been increasing [20]. Nonetheless, when the immunity decreases due to various factors, opportunistic infections may appear, having diverse causes, such as fungi, bacteria, viruses and protozoans, amongst others [21].

For some years, there have been reports of opportunistic infection cases in HIV patients, and some of these may have been caused by the *T. gondii* protozoan. There are also reports of infections by *N. caninum* [22] such as the one made by Lobato et al. [5], informing the presence of anti-*N caninum* antibodies in immunocompromised patients. Oshiro et al. [9] also mentioned the presence of *N. caninum* positive sera in these patients, with a rate of 26.1%. This demonstrates the importance for these patients to, in addition to *T. gondii,* consider *N. caninum* as an opportunistic parasite.

The use of a diagnosis method that allow for large scale testing would increase the chance of possible quick parasite identification. Despite not being the gold standard for *N. caninum* identification, the ELISA would enable this agility [12]. With advanced technology and studies about the *N. caninum*, there have been more opportunities to develop more advanced diagnosis methods, be it for the discovery of a wide variety of antigens [13], or by the production of recombinant proteins.

As an example, it has been proved that the immunodominant surface protein NcSRS2 is present in the already identified isolates, at the moment of parasite-host interaction and in the sera of animals infected during the parasitaemia. In addition, the use of only one recombinant protein in the ELISA test would increase the test's specificity.

There have already been reports of indirect serological methods, particularly the ELISA, using this recombinant protein produced in *baculovirus* [14]. Andreotti et al. [17] and Borsuk et al. [12] have already produced the protein in *E. coli* for bovine and ovine serological diagnosis, respectively. In 2013, Pinheiro et al. [23] obtained the NcSRS2 protein from *Pichia pastoris* for the development of a serological diagnosis method for bovines in an endemic area in Rio Grande do Sul. Recently, in 2014, Sinnot et al. [24] developed a blocked ELISA test based on the NcSRS2, also for bovines, and in 2015, Pinheiro et al. [25] developed a test for ovine and dogs using the expression platform in *P. pastoris*.

In this study we used the recombinant protein NcSRS2 expressed in *E. coli* for the development of an indirect serological test (ELISA) for the detection of anti-*N. caninum* antibodies in humans. The sensibility and specificity were of 100% and 90%, respectively, when the data was compared with IFAT, which is considered the gold standard for parasites. Western blotting confirmed the positive reaction between proteins and the positive serum samples. With these results, we can suggest that the use of ELISA to diagnose neosporosis in humans is a promising method, since it can be automatized, it is fast and it eliminates the IFAT subjectivity.

Conflict of interest

The authors declare that they do not have any conflicts of interest.

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