ORIGINAL ARTICLE

Anti-neosporal IgG and IgE antibodies in Canine Neosporosis

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Impacts

- The present study shows the immune response of IgG and IgE antibodies in dogs infected with *Neospora caninum* in Brazil.
- The usefulness of a Urea-ELISA using a soluble *N. caninum* antigen to measure the avidity of IgG anti-neosporal antibodies is presented.
- The substitution of IFAT by both ELISA and Urea-ELISA using this antigen in the serology of canine neosporosis is proposed, which should improve the control of this infection.

Keywords:
Neospora caninum; *Toxoplasma gondii*; dog; immune response; IgG; IgE; antibody avidity

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Summary

Neospora caninum infection provokes neurological disorders, recurrent abortion and death in dogs and cattle. Dogs are both intermediate and definitive host of *N. caninum*. Thus, the development of sensitive and specific immunoassays to diagnose canine neosporosis is essential to control this disease. This work investigated serum anti-neosporal IgG and IgE antibodies in 140 dogs represented by 30 healthy animals (group I), 11 dogs showing acute *N. caninum* infection (group II), 50 urban dogs with serological evidence of canine neosporosis in indirect fluorescent antibody test (IFAT) (group III) and 49 urban dogs without clinical and laboratory evidences of neosporosis (group IV). Enzyme-linked immunosorbent assay (ELISA) and western immunoblotting, both using a soluble *N. caninum* tachyzoite antigen (SNA), investigated these two isotypes of antibodies, while a Urea-ELISA measured the avidity of the IgG antibodies. Anti-*Toxoplasma gondii* IgG antibodies were also investigated in the animals. Anti-neosporal IgG was found in all animals from groups II and III, whereas 32.7% (16/49) of dogs from group IV were reactive. IgG antibodies of low avidity were demonstrated in dogs from group II (median 35.3%), while animals from groups III and IV had IgG antibodies of high avidity (medians of 61.5% and 61.7% respectively). IgE antibodies were found in four (13.3%) and five (16.6%) dogs from groups III and IV respectively. Dogs presenting acute infection (group II) or chronic infection (group III) had IgG antibodies to several neosporal antigens, mainly of 29–30 and 35 kDa, while 13 of 16 dogs from group IV recognized antigens from 14 to 170 kDa. Antibodies to *T. gondii* were detected in 36 of 50 (72%) sera from group III and 25 of 49 (51%) sera from group IV. We concluded that IgG-ELISA and Urea-ELISA with SNA may substitute for IFAT in both laboratory routine and epidemiological studies of canine neosporosis.

Introduction

Neosporosis is a parasitic disease caused by the coccidian protozoan *Neospora caninum* (Bjerkås et al., 1984; Dubey et al., 1988). It provokes neurological disorders, abortion and death in dogs, cattle and other animal species (Dubey et al., 2002). Dogs are both the intermediate and definitive host of *N. caninum*, shedding neosporal oocysts after infection (Dubey et al., 2002).

Neosporosis is diagnosed by serological methods that are mainly represented by indirect fluorescent antibody test (IFAT), and by direct agglutination test (DAT) and by enzyme-linked immunosorbent assay (ELISA) (Björkman and Uggla, 1999). Polymerase chain reaction (PCR) may also be employed for laboratory diagnosis of neosporosis (Dubey, 2003), and *N. caninum* cysts in tissues from infected animals may be demonstrated by haematoxylin–eosin staining, and more specifically identified by immunohistochemical techniques using anti-neosporal monoclonal antibodies.

Anti-neosporal IgG antibodies are commonly investigated by IFAT in epidemiological studies on canine neosporosis (Björkman and Uggla, 1999; Hemphill and Gottstein, 2000). This test has a high specificity, but its low sensitivity may produce false-negative results in serum samples containing low antibody titres. Thus, more sensitive immunoassays must be employed to diagnose canine neosporosis, allowing the efficient identification of acute and chronic infection.

There is no detailed study on the use of crude neosporal antigen in IgG-ELISA, nor any report on the usefulness of IgG avidity measurement to diagnose dogs acutely or chronically infected with *N. caninum*. As well, the involvement of IgE antibodies in the immune response in this parasitic disease, as happens in human toxoplasmosis (Wong et al., 1993), and canine and human leishmaniasis (Atta et al., 1998; Almeida et al., 2005) is still unknown.

This work investigated the usefulness of a soluble antigen of *N. caninum* to detect dog anti-neosporal IgG and IgE antibodies in indirect ELISA and western immunoblotting and also the efficiency of IgG avidity measurement to identify chronic *N. caninum* infection.

Material and Methods

The serological screening of neosporosis was carried out using serum samples from 140 dogs, which were classified into four groups. Group I was constituted by 30 healthy dogs that were bred in controlled conditions, while 11 dogs, which were orally inoculated with tissue cysts of *N. caninum* (30–35 days infection) and shed neosporal oocysts, made up group II. Fifty dogs (22 pet and 28 stray dogs) from Salvador and Lauro de Freitas (Bahia, Brazil) presenting a positive IFAT were group III, while 49 IFAT negative and asymptomatic dogs (32 pet and 17 stray dogs) from these same localities were group IV.

Anti-neosporal IgG antibodies were investigated by IFAT with tachyzoites of the Nc-Bahia strain of *N. caninum*, which was previously isolated from an infected dog from Salvador (Bahia, Brazil) (Gondim et al., 2001). They were maintained in Vero cells cultured in Dulbecco Minimal Essential Medium supplemented with 10% equine serum. To obtain the parasites, the Vero cells were first washed with phosphate buffered saline (PBS) (150 mM NaCl containing 10 mM phosphate, pH 7.2) and then mechanically disrupted. After, the tachyzoites were purified by gel filtration using a small column of Sephadex^{TM} G-25 (Pharmacia, Uppsala, Sweden).

Anti-toxoplasmal IgG antibodies were investigated in IFAT using tachyzoites obtained from the peritoneal fluid from young Swiss albino mice infected with the AS-28 strain of *T. gondii*. They were washed with PBS and purified by gel filtration as above.

Both *N. caninum* and *T. gondii* tachyzoites were used in IFAT after fixation on slides, for 30 min at 37°C. To use in neosporal IFAT, dog sera were diluted at 1/50 in PBS. They were used diluted at 1/16 to investigate *T. gondii* antibodies. The primary immune reactions in both tests were carried out for 30 min at 37°C. Afterwards, the slides were thrice washed with PBS and incubated at 37°C for 30 min with a goat IgG anti-dog IgG FITC conjugate (Sigma Chemical Co., St Louis, MO, USA) diluted at 1/32 in PBS containing 0.01% Evans blue. The preparations were again washed with PBS, mounted with 90% glycerin in carbonate buffer, pH 9.2, and evaluated for fluorescence using a microscopic enhancement of 400X. Serum samples that attempted to react were serially diluted and tested again to find their titres. These titres were expressed as the reciprocal of the highest dilution giving a positive reaction.

IgG-ELISA was performed with a soluble *N. caninum* antigen (SNA) obtained from Vero cell cultured tachyzoites. To obtain soluble extract, previously washed tachyzoites were first disrupted for 5 min in the cold with 6 mM Nonidet P-40 in 50 mM Tris(hydroxymethylaminomethane)–HCl buffer, pH 7.2, containing 150 mM NaCl. After centrifugation at 3000 ***g*** for 30 min at 4°C, a clear supernatant containing SNA was used in ELISA polystyrene plates (Nunc, Maxsorp, Denmark) in the concentration of 500 ng protein/microwell, in 100 μl of pH 9.2 carbonate buffer, after overnight incubation at 4°C. Following antigen coating, the wells were thrice washed with PBS and incubated for 1 h at 37°C with PBS containing 1% gelatin (PBS-G) to block their residual free reactive

sites. Anti-neosporal IgG antibodies were added to the wells with 100 μl of serum diluted at 1/200 in PBS-G, for 1 h at 37°C. Afterwards, the wells were washed three times with PBS-T and incubated in the same conditions with a goat IgG anti-dog IgG peroxidase conjugate (Sigma Chemical Co.), which was previously diluted at 1/1000 in PBS-T/G. The wells were again washed with PBS-T and the immune reactions revealed with 100 μl of citrate–phosphate buffer containing hydrogen peroxide plus TMB (3,3′, 5,5′-tetramethilbenzidine). After 30 min at 22–25°C, the reactions were stopped with 50 μl of 2N sulphuric acid and the absorbances measured at 450–600 nm using a Diamedix BP-12 ELISA reader (Diamedix Corporation, Miami, FL, USA). ELISA titre was expressed as absorbance.

Anti-neosporal IgE antibodies were investigated in 11 sera from group II and in 30 sera from groups III and IV, respectively, by an indirect ELISA using polystyrene wells also coated with 500 ng protein of SNA. This assay was carried out with 100 μl of 1/2 diluted serum in PBS-T/G, for 18 h at 4°C. The reactions with goat IgG anti-dog IgE peroxidase conjugate (Bethyl Laboratories Inc., Montgomery, TX, USA) were performed with 100 μl of this reagent diluted at 1/10 000, during 1 h incubation at 37°C. The immune reactions were revealed as for IgG-ELISA.

The avidity of anti-neosporal IgG antibodies was investigated in IgG-ELISA with SNA using urea. Thus, immediately after the primary reaction with anti-neosporal antibodies, the wells were first treated with 6 M urea in PBS-T for 5 min at room temperature and then washed two times with PBS-T. The avidity was calculated comparing the percentage of residual absorbance of the well treated with urea against a control without such a treatment, both using the same serum.

Western immunoblotting was performed with SDS-PAGE fractionated SNA polypeptides (10% SDS-polyacrylamide gel electrophoresis without reducing agent 250 V, 30 mA) which were electro transferred to a 0.45 μm nitrocellulose membrane using a current of 2 mA/cm^2 of gel. Membrane strips containing these *N. caninum* antigens (0.5 cm wide) were previously treated with PBS-T containing 1% skimmed milk (PBS-T/M) and incubated with sera diluted at 1/50 in this medium, for 2 h at 37°C. Separately, IgE antibodies were investigated incubating the strips for 18 h at 4°C in 1/2 diluted sera. To detect IgG or IgE antibodies, the immune reactions with either goat IgG anti-dog IgG or goat IgG anti-dog IgE peroxidase conjugates diluted at 1/500 and 1/1000, respectively, were developed for 90 min at 37°C. The immunoblots were revealed with hydrogen peroxide plus 4-chloro-1-α-naphtol as usual.

The cut-off of the immunoassays (IgG-ELISA, 0.097 and IgE-ELISA, 0.193) was statistically determined using 30 and 20 sera from group I respectively (Frey et al., 1998). ELISA absorbance and avidity measurement were expressed as median. ELISA sensitivity and specificity were calculated as previously advised (Greiner and Gardner, 2000). A Chi-squared test evaluated the frequency of positive results in toxoplasmal and neosporal IgG-IFAT. The results of ELISA in the four groups were compared by the Kruskal–Wallis test, whereas the *U*-test of Mann–Whitney was used to compare anti-neosporal IgG antibody titre and % avidity in groups II, III and IV. Correlation analysis used the Spearman rank order test. The Kappa index of IgG-ELISA was calculated using IFAT as reference. Statistical significance was attributed at $P < 0.05$.

Results

IgG antibodies and avidity

Anti-neosporal IgG antibodies were not detected by IFAT in two groups (I and IV). The sera from group II and 49 sera from group III reacted in this test (titre ranging from 50 to 800 and from 50 to 1600 respectively).

Antibodies to *T. gondii* were not detected in groups I and II, while 36 of 50 (72%) sera from group III and 25 of 49 (51%) sera from group IV had these immunoglobulins. There was no difference in the frequency of seropositivity for anti-toxoplasmal antibodies in the groups III and IV (χ^2 test, $P > 0.05$).

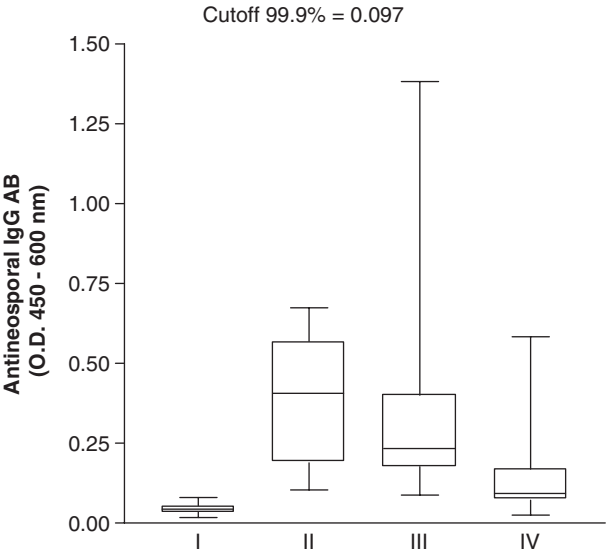

Fig. 1. Anti-neosporal IgG antibodies detected by ELISA in sera from uninfected dogs from group I (cut-off), from experimentally infected dogs from group II, and also from naturally infected dogs from groups III and IV respectively (Kruskal–Wallis test, $P < 0.0001$).

All sera (100%) from group II reacted in ELISA (median 0.406), while 49 of 50 (98%) sera from group III and 16 of 49 (32.7%) sera from group IV were also reactive in this immunoassay (median titres of 0.233 and 0.092 respectively). Groups II and III presented similar titres in ELISA, both differing from group IV (Fig. 1).

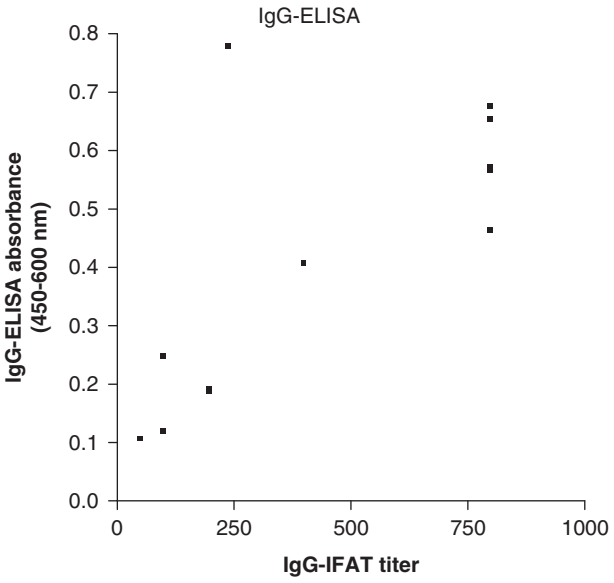

Fig. 2. Correlation between the titres of IgG-ELISA and IgG-IFAT obtained with sera from experimentally infected dogs from group II (Spearman test, $r = 0.910$, $P < 0.0001$).

The sensitivity and specificity of IgG-ELISA were both 100% in the groups I and II, while 98% sensitivity and 80% specificity were observed on IFAT with the four groups.

The Kappa index of IgG-ELISA was 1.0 in the groups I and II, 0.66 in the groups III and IV, and 0.88 with the four groups. There was a strong correlation between the titres of IFAT and IgG-ELISA in the group II (Fig. 2).

IgG antibodies of low avidity were detected in sera from group II (median titre, 35.3%), while high avidity IgG antibodies were detected in sera from both group III (median titre, 61.5%) and IV (median titre, 61.7%) (Fig. 3).

IgE antibodies

Anti-neosporal IgE antibodies were rarely found in the groups evaluated. There were no IgE antibodies in sera from group II, while four (13.3%) sera from group III and five (16.6%) sera from group IV had low titre of these antibodies (Fig. 4).

Western immunoblotting

Sera from group II recognized 11 antigens of *N. caninum* in the following frequency: 29, 30 and 35 kDa (100%), 33 kDa (81.8%), 84 and 97 kDa (72.4%), 40 kDa (63.6%), 55 and 170 kDa (45.5%), 60 kDa (27.3%) and 77 kDa (9.1%). Sera from group III also reacted against

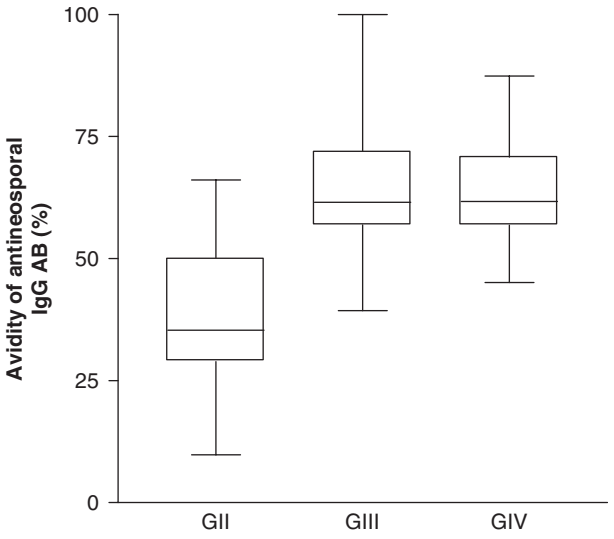

Fig. 3. Avidity measurement of anti-neosporal IgG antibodies in sera from experimentally infected dogs from group I and from naturally infected dogs from groups III and IV (Mann–Whiney test, $P < 0.0001$).

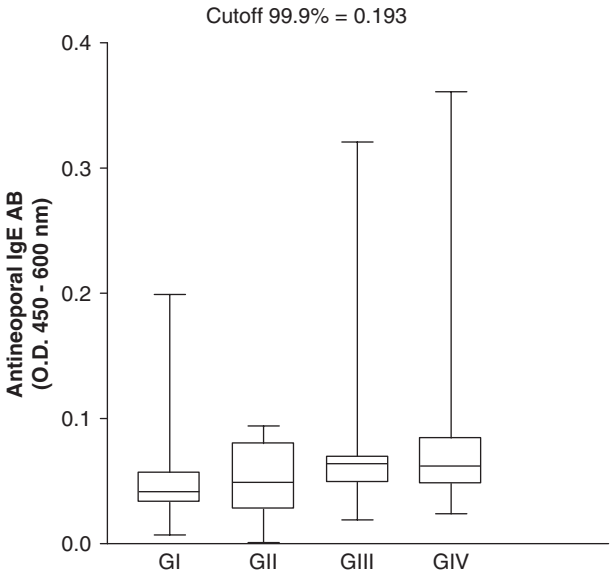

Fig. 4. Titres of anti-neosporal IgE antibodies in sera from uninfected dogs from group I (cut-off), from experimentally infected group II and from naturally infected groups III and IV.

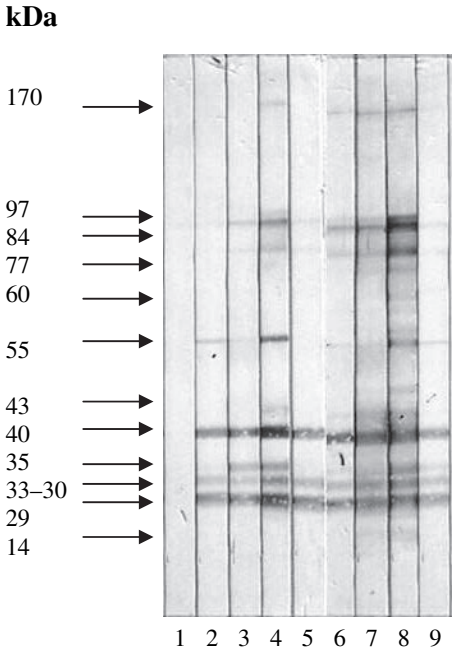

Fig. 5. Western immunoblotting analysis of SNA polypeptides recognized by antineosporal IgG antibodies. Strip 1 is a negative control, strips 2–6 represent sera from dogs experimentally infected (group II), while strips 7–9 are immunoblots obtained with sera from naturally infected dogs (groups III and IV).

these neosporal antigens. Thirteen of 16 sera from group IV mainly recognized antigens of 14, 30, 40, 43, 55, 60, 77, 84 and 170 kDa, and only one reacted with the polypeptide of 35 kDa (Fig. 5). There was no immune reaction with sera that tested negative in both IFAT and ELISA, nor any immune reaction between SNA reactive sera and Vero cell extract.

IgE antibodies from sera from groups III and IV weakly recognized neosporal polypeptides of 14, 29, 60, 77 or 177 kDa (data not shown).

Discussion

In this work, we evaluated the usefulness of ELISA with a soluble antigen of *N. caninum* to detect serum anti-neosporal IgG and IgE antibodies in dogs experimentally or naturally infected by this parasite. Additionally, we analysed the avidity of the anti-neosporal IgG antibodies produced in these two types of infection and detected in this assay.

IgG-ELISA with SNA when compared with IFAT presented both excellent sensitivity and specificity in the diagnosis of acute experimental infection. There was a strong correlation between IgG-ELISA and IFAT titres in acutely infected dogs, demonstrating the presence of surface immunodominant antigens in SNA. However, IgG-ELISA was more sensitive than IFAT to diagnose asymptomatic neosporosis in naturally infected dogs.

There was no cross immune reaction between anti-toxoplasmal IgG antibodies and neosporal antigens in SNA-ELISA. Such a finding was demonstrated by negative results in SNA-ELISA with sera that only contained antibodies for *T. gondii*.

Urea-ELISA with SNA was very efficient in detecting acute *N. caninum* infection and in demonstrating dog anti-neosporal IgG antibodies of low avidity. In addition, this test confirmed the presence of chronic infection in IFAT negative dogs having low titres of anti-neosporal IgG antibodies of high avidity.

The immune response to SNA in both acute and chronic infection was mainly represented by IgG antibodies against the immunodominant Ncp29 and Ncp35 proteins (Hemphill and Gottstein, 1996; Howe et al., 1998; Howe and Sibley, 1999; Marsh et al., 1999; Mineo et al., 2001). However, polypeptides of 14, 30, 40, 43, 55, 60, 77, 84 and 170 kDa were also recognized by IgG antibodies from naturally infected dogs that were IFAT negative, demonstrating their usefulness to diagnose canine neosporosis. Antibodies for these *Neospora* antigens were demonstrated in other animal species naturally infected with *N. caninum* (Bjerkås et al., 1994; Osawa et al., 1998).

Anti-neosporal IgE antibodies were observed in a few sera, always in low titre. Their antigenic reactivity against SNA was mainly represented by weakly stained immunoblots of 14, 29, 60, 77 or 177 kDa. However, these reactions may be caused by crossed immune reactions between IgE antibodies against carbohydrate epitopes and neosporal glycoproteins, as already shown for glycosylated leishmanial antigens (Atta et al., 2004), demonstrating that anti-neosporal IgE antibodies cannot be used as serum markers of dog *N. caninum* infection. On the other hand, they showed that neosporal antigens preferentially elicit IgG immune response, in both acute and chronic *N. caninum* infection.

We concluded that SNA-ELISA is a more sensitive test than IFAT for diagnosing canine neosporosis and that avidity measurement is an important tool for identifying this disease in chronically infected dogs presenting low titres of anti-neosporal IgG antibody.

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