Experimental infection of dogs (*Canis familiaris*) with sporulated oocysts of *Neospora caninum*

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ARTICLE INFO

ABSTRACT

Neospora caninum is widely distributed in the world and this parasite is one of the major causes of abortion in cattle. Dogs and coyotes are definitive hosts of *N. caninum* and several species of domestic and wild animals are intermediate hosts. Dogs can become infected by the ingestion of tissues containing cysts and then excrete oocysts. It is not yet known whether sporulated oocysts are able to induce a patent infection in dogs, i.e. a shedding of *N. caninum* oocysts in feces. The objective of this study was to experimentally examine the infection of dogs by sporulated oocysts. The oocysts used in the experiment were obtained by feeding dogs with brain of buffaloes (*Bubalus bubalis*) positive for anti-*N. caninum* antibodies by indirect fluorescent antibody test (IFAT $\geq$200). Oocysts shed by these dogs were confirmed to be *N. caninum* by molecular methods and by bioassay in gerbils, and sporulated *N. caninum* oocysts were used for the oral infection of four dogs. The dogs were 8 weeks old and negative for antibodies to *N. caninum* and *Toxoplasma gondii*. Dogs 1 and 4 received an inoculum of 10,000 sporulated oocysts each; dog 2 an inoculum of 5000 sporulated oocysts and dog 3 received 1000 sporulated oocysts of *N. caninum*. The total feces excreted by these dogs were collected and examined daily for a period of 30 days. No oocysts were found in their feces. The dogs were monitored monthly for a 6-month period to observe a possible seroconversion and when this occurred the animals were eliminated from the experiment. Dogs 1 and 4 seroconverted 1 month after the infection with titer, in the IFAT, of 1600 and 800, respectively; the other two dogs presented no seroconvertion during the 6-month period. Dogs 1 and 2 were euthanized 180 days after infection and were examined for the detection of *N. caninum* in tissues (brain, muscle, lymph node, liver, lung, heart and bone marrow) by immunohistochemistry and PCR with negative results in both techniques. Bioassay in gerbils with brain of these dogs was also performed and again the results were negative. In conclusion, dogs infected with sporulated oocysts of *N. caninum* were not able to shed oocysts in feces. However, a higher dose of infection stimulated the production of antibodies against *N. caninum* in the dogs.

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1. Introduction

Neospora caninum is a protozoan parasite originally reported in dogs (Bjerkås et al., 1984) and is the major infectious cause of abortion in cattle worldwide (Anderson et al., 1991; Dubey, 2003).

Dogs may start to shed oocysts 5 days after ingestion of tissues of experimentally or naturally infected animals and sporulation occurs within 24–72 h (Lindsay et al., 1999). The number of oocysts shed varies with the type of tissue ingested, age and immune status of dogs (Gondim et al., 2001, 2005; Lindsay et al., 1999). Dogs may shed oocysts in feces but without detectable seroconversion; thus, negative serology does not confirm the dog as free of a patent infection with *N. caninum* (Lindsay et al., 1999; Schares et al., 2005). The infection in dogs can occur by ingestion of tissue cysts, which highlights the importance of carnivorism (Dijkstra et al., 2001; Gondim et al., 2002, 2005; Lindsay et al., 1999), and by tachyzoites, which transplacentally reach the fetus during pregnancy in infected females (Dubey and Lindsay, 1989; Cole et al., 1995).

Ruminants may become infected through ingestion of sporulated oocysts (McCann et al., 2007), that contaminate water and food. However, it is not known whether dogs can be infected by this route (Dubey et al., 2007). The aim of this study was to investigate the possibility of infection of dogs by the ingestion of sporulated oocysts of *N. caninum*.

2. Materials and methods

2.1. Collection of oocysts of N. caninum

Three 8-weeks-old dogs (A, B and C) were experimentally infected with brain of seropositive buffaloes (*Bubalus bubalis*) examined for the presence of antibodies anti-*N. caninum* by indirect fluorescent antibody test (IFAT). All buffaloes were male from the same farm, breed and age (around 36 months old). Dogs A, B and C consumed brain of a different buffalo, with antibody titer of 400, 200 and 200, respectively. Two dogs (A and B) were immunosuppressed with 80 mg methylprednisolone acetate (Depo-Medrol®, Pfizer, Brazil), intramuscularly, 3–4 days post infection (PI). Fecal samples from each dog were daily and individually examined for the detection of *Neospora*-like oocysts from day 5 to 30 PI using a standard sucrose flotation technique (Ogassawara and Benassi, 1980).

The total fecal volume of each dog was daily weighed, homogenized and 1 g was mixed with 12 ml concentrated sucrose ($d = 1.205$ g/cm^3), sieved and transferred to 15 ml tubes. After centrifugation ($450 \times g$, 10 min), two drops of the supernatant were recovered and transferred to a slide and light microscopically examined at 1000-fold magnification for *N. caninum* oocysts detection.

For sporulation, fecal samples containing oocysts were mixed with a solution of 2% potassium dichromate (w/v) in Petri dishes at 27 °C for 14 days, and then stored at 4 °C for further use. Sporulated oocysts stored for 3 months or less were used to inoculate the dogs.

2.2. Molecular and biological characterization of inoculated oocysts

The oocysts were submitted to PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) using Hsp400F and Hsp400R primers based on the Hsp70 gene (Monteiro et al., 2007). The restriction enzymes used were *Stu*I and *Kpn*I according to Monteiro et al. (2008); *Stu*I enzyme cleaves *N. caninum*, forming two bands (150 + 250 bp) and *Kpn*I cleaves the PCR products from *Hammondia heydorni* oocysts (136 + 264 bp). The analysis of the amplified products was performed using electrophoresis in agarose gel 2.5% (w/v) with running buffer 0.5× TBE (0.045 M Tris-borate and 0.001 M EDTA, pH 8.0). Positive controls were extracted from known samples of *N. caninum* (Nc-1 strain) and oocysts of *H. heydorni* (stock HheBR) that were previously characterized by molecular methods (Monteiro et al., 2008) and compared to sampled material.

A group of three gerbils (*Meriones unguiculatus*) was used as control of the viability of the oocysts of *N. caninum*. Each gerbil was orally inoculated with 1000 oocysts and followed for a period of 60 days. After this period the gerbils were anesthetized intraperitoneally with 0.015 ml of 2% xylazine (Kensol®, Konig, Brazil) associated with 0.03 ml of ketamine 10% (Vetaset®, Fort Dodge, Brazil) and intracardiac blood was collected. After the collection, the gerbils were euthanized with 0.2 ml of T61® (Intervet, Brazil) and a section of the brain and heart was collected for the detection of the parasite by real-time PCR and immunohistochemistry (IHC). In addition, samples of spleen, liver and skeletal muscles were analyzed by IHC. Direct examination of brain sections, using smear slides and coverslips, were performed to detect tissue cysts of *N. caninum*.

The blood of the gerbils was tested for the presence of anti-*N. caninum* antibodies by IFAT (Dubey et al., 1988) at 1:50 and 1:100 using secondary fluorescein isothiocyanate labeled anti-gerbil antibodies (Immunology Consultants Laboratory, USA) and intact tachyzoites of *N. caninum* (strain NC-1) as antigen, and by Western blotting (WB) at 1:100 according to Schares et al. (2005).

2.3. Experimental infection of dogs

Four 2-month-old dogs (1, 2, 3 and 4), mixed-breed, from 1.0 to 2.0 kg body weight, seronegative for *N. caninum* (IFAT ≤50) and *Toxoplasma gondii* (IFAT ≤16) were selected. These dogs were housed individually, fed commercial food and received water *ad libitum* and were dewormed and vaccinated against parvovirus, parainfluenza, corona virus, leptospirosis and canine distemper (Duramune® Max5CvK – Fort Dodge, Brazil) prior the infection with *N. caninum* oocysts.

The oocysts obtained from the bioassay in dogs were counted in a Neubauer chamber and four aliquots were separated: two aliquots of 1×10^4 oocysts, one aliquot of 5×10^3 and another of 1×10^3 oocysts. Each of these aliquots was inoculated orally into a dog, as follows: dog 1 and dog 4 were infected with 10^4 oocysts, dog 2 with 5×10^3 oocysts and dog 3 with 1×10^3 oocysts of *N. can-*

inum. The bioassay of gerbils started on the same day the dogs were infected, as described in Section 2.2.

The feces of the dogs were individually collected from day 5 to 30 PI and kept under refrigeration at 4 °C until the coproparasitological examination for the detection of *Neospora*-like oocysts. After this period, a serological monitoring of dogs was performed monthly up to 6 months PI for detection of antibodies anti-*N. caninum*. The dogs that became positive were removed from the study. The sera of the dogs were analyzed by IFAT using a cutoff point of 1:50 (Cheadle et al., 1999; Mineo et al., 2001), by WB at 1:100 with immunodominant antigens present in total extract of tachyzoites (Schares et al., 2005), and with a native p38-antigen (NcSRS2) purified by immune-affinity chromatography from tachyzoite lysates (Schares et al., 2000).

Animal management and veterinarian procedures with gerbils and dogs were approved and are in accordance to the Animal Use Ethics Committee of the Faculty of Veterinary Medicine of the University of São Paulo (protocol number 1207/2007).

2.4. Necropsy of dogs

Six months after inoculation with oocysts, dogs 1 and 2 were euthanized for detection of *N. caninum*. Tissues (brain, muscle, lymph nodes, liver, lung, heart and bone marrow) were collected for IHC and PCR targeting the Nc-5 gene (Sreekumar et al., 2004).

A section of the brain (approximately 10 g) was separated to perform bioassay in gerbils (Dubey and Lindsay, 2000). The brains were individually macerated using a mortar and pestle with 15 ml of trypsin (0.25%), transferred to centrifuge tubes and incubated for 50 min at 37 °C with mixing every 10 min. Then the material was washed with PBS to remove the trypsin, by centrifugations (2000 × *g* for 10 min at 4 °C). Brains samples of dogs 1 and 2 were intraperitoneally inoculated to groups of two gerbils per brain. These animals were followed for 90 days and euthanized as described in Section 2.2. The brain, heart, liver, lung, lymph nodes, muscle and bone marrow of the gerbils were collected and examined by PCR and, the serum was tested for the presence of anti-*N. caninum* antibodies. Direct examination of the brain for the detection of cysts of *N. caninum* was also conducted.

The tissues of the dogs were also examined for the presence of the parasite by histopathological techniques, staining with hematoxylin–eosin and IHC (Corbellini et al., 2000).

2.5. Real-time PCR

DNA for real-time PCR was extracted from brain and heart samples of each gerbil. Tissues were frozen liquid nitrogen and homogenized using a mortar and pestle and incubated with DNAzol® (DNAZOL® Reagent, GIBCO, USA) until cellular lyses was complete. Subsequently the material was centrifuged at 10.000 × *g* for 10 min to remove cellular debris. Absolute ethanol was used to precipitate nuclear material and subsequently extracted from the supernatant. After several washes with 70% ethanol

the DNA was solubilized into 8 mM of NaOH solution. DNA concentration and quality were estimated by a spectrophotometer reading at 260 and 280 nm (BioPhotometer, Eppendorf, USA).

Fifty nanograms of DNA were used in each amplification reaction using universal real-time PCR buffer (TaqMan Universal PCR Master Mix, Applied Biosystems, USA) and a specific set of primers and probe (Sense: 5′-GCGGACGTGTCGTTGGTTG-3′, Anti-sense: 5′-GTTCACACACTATAGCCACAAACAA-3′, Probe 5′-VIC CCTGCGGCAGCAAGGCTCCTT-TAMRA-3′) for the Nc5 gene of *N. caninum* (accession number AY665719) and a endogenous control 18S (Eukariotic 18S rRNA Endogenous Control, Applied Biosystems, USA). DNA extract from tachyzoites of *N. caninum* (NC-1 strain) and Milli-Q water served as positive and negative controls, respectively.

Amplifications were performed using 7500 Real Time PCR System (Applied Biosystems, USA) equipment and subjected to 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and, 60 °C for 1 min. Amplification curves were analyzed by Sequence detection software (SDS, v1.3, Applied Biosystems, USA).

Reactions were considered positive if the fluorescent signal increased during amplification and negative if the fluorescent signal remained at the same basal level observed during the beginning of the amplification cycles.

2.6. Immunohistochemical analysis (IHC)

Tissue sections stained with hematoxylin and eosin (HE) were employed for the histopathogical examination. *N. caninum* detection was performed by an IHC test using anti-*N. caninum* primary antibody (VMRD, Pullman, USA).

The tissues were initially incubated in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidase. Antigen retrieval was performed using 0.1% trypsin for 10 min at 37 °C followed by heat (microwave on full power 2 min) with the slides immersed in citrate buffer pH 6.0 (1 l of distilled water, 2.1 g C$_6$H$_8$O$_7$, adjusted to pH 6.0 with 0.5% NaOH). Nonspecific labeling was reduced by applying 5% reduced-fat milk for 15 min. The primary antibody was applied for 1 h at room temperature (37 °C) followed by 20 min with linking solution (LSAB Kit, Universal, K0690, Dako Corporation, Carpinteria, CA) and for 25 min with streptavidin-peroxidase, using phosphate-buffered saline washings between steps. Labeling was performed using the chromogen 3,5-diaminobenzidine tetra-hydrochloride as a substrate (DAB, Dako K3468, USA) for 5–10 min. Hematoxylin applied for 1 min was used as a counter stain (Corbellini et al., 2000).

2.7. Detection of antibodies (IFAT, WB)

2.7.1. IFAT of gerbil and dog serum

Gerbil and dog sera were tested for anti-*N. caninum* antibodies by Indirect fluorescence antibody tests (Dubey et al., 1988) using secondary fluorescein isothiocyanate labeled anti-gerbil and anti-dog antibodies (Immunology Consul-
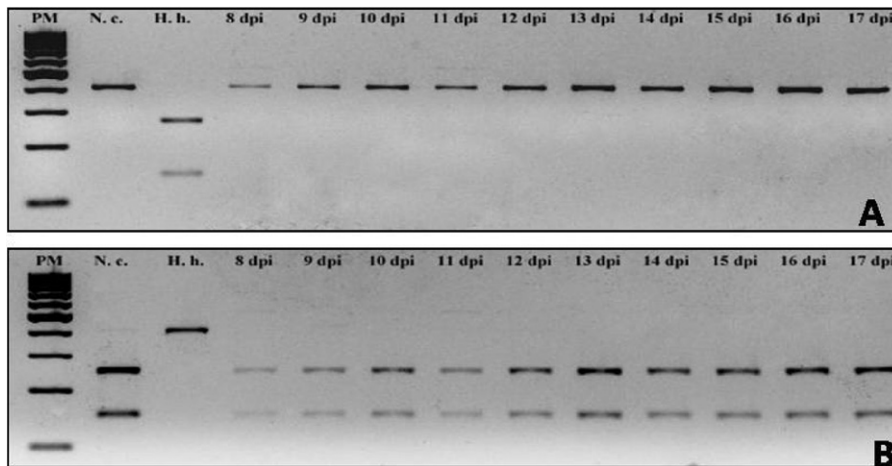
Fig. 1. Agarose gel electrophoresis of the PCR-RFLP products amplified from *Hammondia*-like oocysts using primers directed to the HSP 70 coding sequences and the restriction enzymes *Kpn*I and *Stu*I from 8th to 17th dpi. (A) *Kpn*I enzyme – N.c.: positive control for *N. caninum* (400 bp); H.h.: positive control for *H. heydorni* (136 + 264 bp). (B) *Stu*I enzyme – N.c.: positive control for *N. caninum* (150 + 250 bp); H.h.: positive control for *H. heydorni* (400 bp). PM: molecular weight; weight marker (100 bp).

tants Laboratory and Sigma, USA) and intact tachyzoites of *N. caninum* (strain NC-1) as antigen. The cutoff was set at 1:50.

2.7.2. Western blotting (WB)

The Nc-1 strain of *N. caninum* (Dubey et al., 1988) was maintained in Vero cell cultures and purified following protocol of Schares et al. (1998, 1999). Tachyzoites obtained from the culture were frozen at −80 °C until needed for WB.

Affinity chromatography employing monoclonal antibodies mAb 4.15.15, as described by Schares et al. (2000), was used to purify *N. caninum* surface antigens p38 (NcSRS2).

Whole tachyzoite antigens of *N. caninum* and purified surfaced antigens (Schares et al., 1998) were used for the WB test. Pellets containing 4×107 *N. caninum* tachyzoites and purified p38 (0.05 μg) were incubated in buffer (2% [w/v] SDS, 10% [v/v] glycerol, 62 mM Tris–HCl, pH 6.8) for 1 min at 94 °C and separated in polyacrylamide gel (SDS) at 12% [w/v] (60 mm × 70 mm × 1 mm in size). The antigens were subsequently transferred to PVDF membranes (Immobilon-P, Millipore, USA) which, after the transfer, were blocked with PBS-TG (PBS with 0.05% [v/v]), Tween 20 (Sigma, USA) and 2% liquid fish gelatin (v/v) (Serva, Germany). The membranes were cut into strips for later examination.

When using total antigen of *N. caninum*, the reactivity of sera with immunodominant antigens of *N. caninum* tachyzoites (Nc-IDA) of 29, 30, 33 and 37 kDa was recorded (Schares et al., 2000). For the WB employing the purified p38 protein (NcSRS2) reactivity of the sera with a single band at 38 kDa was recorded.

To detect anti-*N. caninum* antibodies, incubations of the WB strips followed methodology as described by Schares et al. (1998) with modifications. Sera from the gerbil and dogs were diluted at 1:100 in PBS-TG and serum from an experimentally infected gerbil and from dog with congen-

ital infection was used as positive control (Schares et al., 2005).

3. Results

3.1. Shedding of oocysts

One of the immunosuppressed dogs (A) died 5 days PI and the other (B) shed *Neospora*-like oocysts 8–17 days PI. Dog (C) which had received no immunosuppressive treatment did not shed oocysts.

3.2. Molecular and biological characterization of the inoculum

The oocysts shed by dog B were tested separately, according to the day of shedding, and were identified as *N. caninum* (Fig. 1) by PCR-RFLP. The three gerbils of the bioassay, which were inoculated with oocysts, presented anti-*N. caninum* antibodies by IFAT ($\geq$100) and by WB on day 60 PI. By real-time PCR, brains were positive, whereas hearts were negative. Cysts in the brain of gerbils were not observed by direct examination.

3.3. Experimental infection of dogs with oocysts

None of the dogs inoculated with sporulated oocysts of *N. caninum* shed oocysts in the feces during the observation period of 30 days. Dogs 1 and 4, which received the largest number of oocysts (10^4), presented anti-*N. caninum* antibodies 30 days PI with IFAT titer of 1600 and 800, respectively, and the dog 2 and dog 3, which had received 5×10^3 and 1×10^3 oocysts, respectively, did not seroconvert during the 6 months of observation (Table 1). The WB showed bands which represented specific reactions with immunodominant antigens present in the total extract of tachyzoites, as well as with purified p38 (NcSRS2) antigen, confirming all IFAT findings (Fig. 2).

Table 1
Antibody detection and shedding of oocysts in dogs orally inoculated with *N. caninum* sporulated oocysts.

Dog no.	No. of inoculated oocysts	Seroconversion (dpi)	IFAT titer	Western blotting	Oocysts in feces
1	10,000	30	1600	P	N
2	5000	N	<50	N	N
3	1000	N	<50	N	N
4	10,000	30	800	P	N

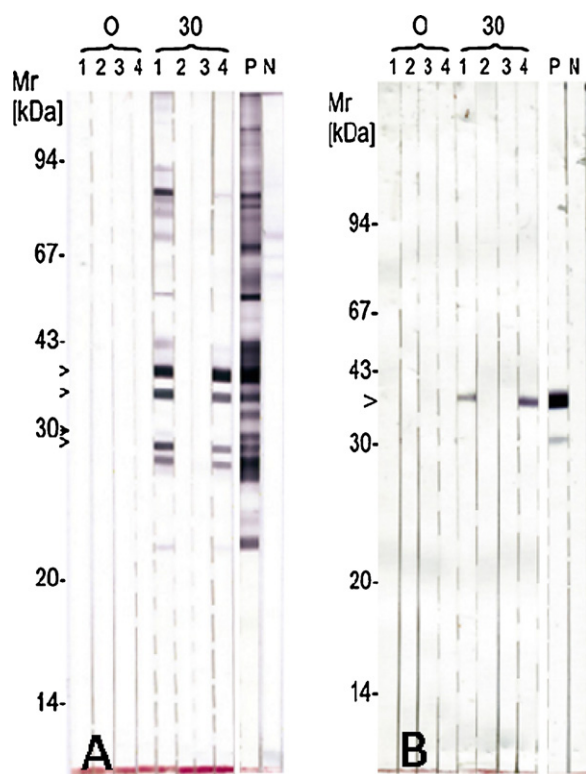dpi: days post infection; N: negative; P: positive.

Fig. 2. *Neospora caninum*-specific antibody reactions of dogs (1, 2, 3 and 4) inoculated with *N. caninum* oocysts at day of inoculation (0) and 30 days after inoculation (30). (A) The sera of two dogs (1, 4) reacted with immunodominant antigens of 30, 33 and 37 kDa, 30 days after inoculation; (B) the sera of two dogs (1, 4) reacted with immunodominant antigens of purified p38 (NcSRS2), 30 days after inoculation. P: positive control; N: negative control; Mr: molecular weight.

3.4. Necropsy of dogs

All the tissues from dogs 1 and 2, were tested negative for *N. caninum* by real-time PCR and by IHC. The bioassay of gerbils that were infected with the brain of those dogs was negative by direct examination, PCR and serology.

4. Discussion

The elimination of oocysts by a dog infected with brain of *B. bubalis* confirming previous findings that buffaloes are *N. caninum* intermediate hosts (Rodrigues et al., 2004). The observation that not all infected dogs eliminate oocysts could be due the use of different buffaloes as source of infection. Some of the buffaloes could be more heavily infected

than others and no information regarding the *N. caninum* isolates was available.

The viability of *N. caninum* oocysts obtained from donor dogs was confirmed by molecular and biological methods. During the bioassay in gerbils, the presence of serum antibodies (IFAT and WB) and the DNA (real time PCR) in gerbil tissues confirmed the viability of the *N. caninum* oocysts used as inocula for dogs.

The oral uptake of *N. caninum* oocysts by dogs caused no patent infection with shedding of oocysts; however, the finding of *N. caninum*-specific antibodies seemed to be dose dependent, since only dogs that were inoculated with the higher dose of oocysts (10^4) seroconverted.

The results of the present study suggest that an oral infection of dogs with *N. caninum* oocysts is not able to induce a patent infection. This is in contrast to the observations in *T. gondii*-infected cats where the ingestion of oocysts of *T. gondii* is capable of inducing infection and shedding of oocysts in feces. The pre-patent period is longer (18 days or more) and few cats shed small amounts oocysts in (Dubey, 2006) when the route of infection is by ingestion of oocysts. Thus, for *N. caninum* as for other coccidians that form cysts (such as *Sarcocystis* spp. and *T. gondii*), the most efficient route of transmitting the agent is by carnivorism, when the definitive host ingests tissue cysts containing bradyzoites (Dubey, 2001, 2006).

In this study, the dogs received doses ranging from 10^3 to 10^4 oocysts and only the dogs that received 10^4 oocysts seroconverted. These doses can be considered high, as naturally or experimentally infected dogs shed few oocysts in feces (Basso et al., 2001, 2009; McGarry et al., 2003; McInnes et al., 2006; Pena et al., 2007; Schares et al., 2005; Šlapeta et al., 2002), so under natural conditions the chances for a dog to ingest a high number of oocysts are low.

It is known that a dose of 10^3 oocysts of *N. caninum* was able to cause infection and death in gerbils 6–13 days PI (Dubey and Lindsay, 2000). De Marez et al. (1999) infected calves with oocysts of *N. caninum* (10^4 and 10^5) and all animals seroconverted 4 weeks PI and DNA were detected by PCR. Trees et al. (2002) infected pregnant cows with 600 oocysts of *N. caninum* and this dose was sufficient to infect these animals, as confirmed by PCR and seroconversion 3 weeks PI, but this dose was unable to cause abortion and there was no evidence of transplacental transmission, indicating the interference of infectious dose in the clinical signs observed.

It is known that the absence of antibodies does not mean that the dog is free from infection (Lindsay et al., 1999). Dogs experimentally infected can shed oocysts without detection of antibodies for at least 6 months (Dijkstra et al., 2001; Lindsay et al., 1999; Rodrigues et al., 2004) and it

has already been reported the seroconversion in infected dogs at least seven months PI (Gondim et al., 2002). In the present study dogs infected with lower doses remained seronegative until the end of the experimental period.

Due to the predilection of the parasite for the central nervous system, bioassay in gerbil was performed with the brain of a seropositive and a seronegative dog. PCR and IHC were also performed with the brain and other tissues (muscle, lymph node, liver, lung, heart and bone marrow) of these dogs; however, results from both dogs were negative.

In conclusion the results provide evidence that in dogs infected with high doses of sporulated *N. caninum* oocysts no patent infection but a specific antibody response against the tachyzoites of the parasite is induced. This suggests that these dogs may have become intermediate host of *N. caninum*.

Acknowledgements

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