

Assessment of experimental infection for dogs using *Gallus gallus* chorioallantoic membranes inoculated with *Neospora caninum*

Avaliação da infecção experimental de cães com membranas córion-alantóides de *Gallus gallus* inoculadas com *Neospora caninum*

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Abstract

The aim of this study was to evaluate parasitism kinetics and tissue lesions in the first week of infection by *Neospora caninum* in dogs fed *Gallus gallus* chorioallantoic membranes (CMs) previously infected *in ovo*. Five two-month-old pups were used. Each dog was given five CMs that were previously infected with *N. caninum* via the oral route. Four animals were euthanized in the first week of infection. All four dogs had their stools examined one week prior to and up to the day they were euthanized. The stools of the uneuthanized dog were collected for 30 days. After euthanasia, organ sections were utilized for histopathology, immunohistochemistry, indirect immunofluorescent tissue reactions, PCR and real-time PCR to detect parasites. Necropsy revealed that the small and large intestines, spleen, and lungs were affected. No oocysts or *N. caninum* DNA were identified in the stool samples. Real-time PCR was the most sensitive technique used to detect the protozoa in tissues, which were identified in 41% of the analyzed samples. Our results indicate that an experimental model using previously infected CMs appears to be a useful model for the study of the host-parasite relationship during the infection's acute phase.

Keywords: Neosporosis, acute phase, infection, real-time PCR, immunohistochemistry.

Resumo

O objetivo deste estudo foi avaliar a cinética de parasitismo e lesões teciduais, na primeira semana de infecção por *Neospora caninum*, em cães alimentados com membranas corioalantóicas (MCs) de *Gallus gallus*, previamente infectadas *in ovo*. Foram utilizados cinco filhotes de dois meses de idade. Cada cão recebeu cinco MCs previamente infectadas com *N. caninum*, por via oral. Quatro animais foram eutanasiados na primeira semana de infecção. Todos os quatro cães tiveram suas fezes examinadas uma semana antes e até o dia em que foram eutanasiados. O cão que não foi eutanasiado teve suas fezes colhidas durante 30 dias. Depois da eutanásia fragmentos de órgãos foram processados para histopatologia, imuno-histoquímica, reação de imunofluorescência indireta em tecidos, PCR e PCR em tempo real para detecção do parasito. A necropsia revelou que os intestinos delgado e grosso, baço e pulmões foram os órgãos afetados. Oocistos de *N. caninum* não foram identificados nas amostras de fezes. A PCR em tempo real foi a técnica mais sensível para detectar o protozoário nos tecidos, sendo identificados em 41% das amostras analisadas. Os nossos resultados indicam que o modelo experimental utilizando MCs evidenciou ser um bom modelo para estudar a relação parasito-hospedeiro durante a fase aguda da infecção.

Palavras-chaves: Neosporose, fase aguda, infecção, PCR em tempo real, imonu-histoquímica.

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Introduction

Since its discovery by Bjerkas et al. (1984), many studies have been conducted to better understand the infection of dogs by the parasite *Neospora caninum*. Most dogs are symptom-free, irrespective of the antibody titers observed (SICUPIRA et al., 2012), whereas, unlike cattle, horizontal infection has been characterized as the primary route of parasite dissemination (DUBEY; SCHARES, 2011). In this context, it has been observed that bradyzoites can infect and induce oocyst production (DIJKSTRA et al., 2001; GONDIM et al., 2002) and that the ingestion of oocysts induces antibody production only (BANDINI et al., 2011). Furthermore, whether tissues containing only tachyzoites can be orally infectious to animals is not known (DUBEY; SCHARES, 2011).

Experimentally, oocyst elimination has been reported in dogs fed infected bovine and murine tissue (GONDIM et al., 2002), embryonated egg membranes (FURUTA et al., 2007), bovine placenta (DIJKSTRA et al., 2001) and buffalo tissue (RODRIGUES et al., 2004). The quantity of eliminated oocysts, however, is directly associated with the intermediate host (GONDIM et al., 2002), canine age and reinfection status (GONDIM et al., 2005), the parasite's tropism for certain tissues (CAVALCANTE et al., 2011), and the concentration of parasites in the ingested tissue.

Because the elimination of oocysts in coccidia precedes the merogonous and gametogonous phase, attempts have been made to identify these *N. caninum* phases in dogs, but without success (CEDILLO et al., 2008). In light of this, the objective of this study was to evaluate the parasitism kinetics and tissue lesions in the first week after *N. caninum* infection in dogs fed *Gallus gallus* chorioallantoic membranes previously infected *in ovo*.

Materials and Methods

We used *N. caninum* tachyzoites of isolate NC-1 (DUBEY et al., 1988) cultivated in CV-1 cells (African green monkey kidney fibroblasts) kept according to Furuta et al. (2007). Specific pathogen-free (SPF) embryonated chicken eggs (Hy-Line do Brasil Ltda, Nova Granada, SP, Brazil) were used in these experiments. They were accommodated in a rotary incubator and kept at $37.7 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity. Every day from the 7th, the eggs were taken to the egg candler to diagnose embryo viability. On the 10th day, 30 eggs were inoculated with 1×10^6 *N. caninum* tachyzoites. On the 8th day after infection (DAI), the eggs were assessed and those that had viable embryos were opened. After confirmation of the infective forms in the chorioallantoic membranes by direct microscopy, they were immersed in an antibiotic/antibiotic solution containing penicillin (10000 U), streptomycin (100 µg) and B amphotericin (25 µg) per mL (Invitrogen, USA) and kept under refrigeration at 4°C , until they were offered to the dogs.

Five two-month-old pups were used. All pups were from the same litter, and the bitch was negative for anti-*N. caninum* antibodies. The pups were accommodated in individual stalls and vaccinated against canine distemper, parvovirus, leptospirosis, influenza, infectious hepatitis and coronavirus. The pups were dewormed (Praziquantel 5 mg/kg and Pyrantel Pamoate 14.4 mg/kg

single dose and repeated after 15 days) and treated with giardicid (Metronidazole, 10 mg/kg, twice a day for 5 days). Each dog was submitted to anti-*Babesia canis*, anti-*Ehrlichia canis*, anti-*Toxoplasma gondii*, anti-*N. caninum* and anti-*Leishmania chagasi* antibody detection, all of which were found to be seronegative. This treatment was furthered with a diet based on commercial feed, without the possibility of raw meat ingestion or of any other feed to avoid possible coccidian infection. The stalls were cleaned daily and disinfected with a solution of formaldehyde at 1% and sodium hypochlorite at 2%. All animals were submitted to a physical and clinical assessment.

Each dog was given five chorioallantoic membranes previously infected with *N. caninum* via the oral route. Four animals were euthanized in the first week of infection (3rd, 4th, 5th and 6th DAI). All four dogs had their stools examined for oocysts one week prior to and up to the day they were euthanized. The dog that was not euthanized (the control) had its stools collected and assessed for 30 DAI. This dog was kept as control to verify the possibility of oocysts elimination as well as seroconversion after the 1st week of infection. All dogs received 5 mL of 2% sodium bicarbonate via the oral route 15 minutes prior to ingestion of the membranes. The animals were euthanized following sedation with 0.2% acepromazine (0.04 mg/kg) and then anesthesia with thiopental sodium (12.5 mg/kg), via intravenous infusion of 10% potassium chloride. The study was approved by the Ethics Committee for Animal Use with protocol number CEUA/Unesp-Jaboticabal 025713/2009.

The total volume of feces collected in 24 h was homogenized daily. The centrifugal fluctuation method with sugar solution (density of 1.26 g/cm^3) was used for the stool test. Five grams of feces was used, and the protocol used was similar to that of Gondim et al. (2002), with the exception of the final precipitate, which was re-suspended in a 2% potassium dichromate solution regardless of the microscopy result that was kept under aeration for three days and stored at -20°C until the molecular assay.

Anti-*N. caninum* antibodies were detected in serum with an indirect fluorescent antibody test, according to Yamane et al. (1997), using tachyzoites of the NC-1 strain with a cutoff point of 1:25. The dogs had their blood sampled at infection day zero and again on the day of euthanasia. The control dog had its blood taken weekly for 30 days.

Fragments of submandibular, mesenteric and popliteal lymph nodes, tonsils, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, biliar vesicle, tongue, heart, lungs, liver, spleen, kidney, pancreas, brain and cerebellum were packed in 10% buffered formaldehyde for 24 hours and subsequently transferred to 70% ethanol for histopathology and immunohistochemistry (IHC) according to Furuta et al. (2007) with some modifications. For IHC, the antigen retrieval was performed in buffered citrate solution (pH 6.0) in a water bath at 95°C for 30 minutes, and endogenous peroxidase was blocked in methyl alcohol solution with hydrogen peroxide at 8% for 20 minutes, after we used polyclonal serum from a naturally infected bovine (MUNHOZ et al., 2011) as the primary antibody at a 1:1000 dilution. The Indirect immunofluorescent reaction in tissues was performed according to Furuta (2008).

Fragments (20-50 mg) of organs were kept at -70°C , and DNA was extracted with a DNeasy kit (Qiagen™) according to the manufacturer's recommendations. For DNA extraction from dog stool samples, we used DNASTool kit (Qiagen™). Before adding Proteinase K, we performed 8 freezing and unfreezing cycles (-70°C , 96°C) lasting one minute each, followed by shaking with 0.5 mm glass beads for 5 minutes. PCR was conducted using the primers Np6 (5'-CAG TCA ACC TAC GTC TTC T-3') and Np21 (5'-GTG CGT CCA ATC CTG TAA C-3'), whose amplicon is approximately 328 base pairs (YAMAGE et al., 1996). The amplification reactions were performed according to Munhoz et al. (2011).

Real-time PCR was performed using System SYBR Green, and the oligonucleotide sequences used were NC5-F1 (5'-AAA CAG GAG GAG AGA ACG GCG ATT-3') and NC5-R1 (5'-AGT ACG CAA AGA TTG CCG TTG CAG-3') (FURUTA, 2008). The amplification reactions were performed with a final volume of 25 μL . A plate with 96 wells was employed, to which we added 12.5 μL of platinum Green™ qPCR SuperMix UDG with ROX (Applied Biosystems™), 0.4 μM of each oligonucleotide initiator and 200 ng of DNA of each the sample, and the final volume was adjusted with sterile double distilled water. All samples were analyzed in triplicate. After pipetting all of the reagents, the plate was sealed and placed in the thermocycler. The cycles were 1x (50°C for 2 min.), 1x (95°C for 10 min.) and 40x (95°C for 15 s, 60°C for 1 min.). We used NC-1 strain tachyzoite DNA as a positive control in the Real-time PCR and conventional PCR and sterile double distilled water as a negative control.

Results

During the entire experiment, none of the dogs showed any specific or non-specific clinical signs associated with the disease,

with the exception of blackened stools. Necropsy revealed that the small intestine (duodenum, jejunum and ileum) and the large intestine (colon and cecum), spleen and lungs were the organs affected (Figure 1). In the lungs, we observed small areas with hemorrhagic points and multifocal emphysema of all lobes. Intense splenomegaly with congestion and white pulp hyperplasia were found in the spleen, whereas in the small intestine, there was intense hemorrhage throughout the segment, especially in the duodenum, with moderate thickening of the mucous membrane associated with hyperplasia of the aggregated lymphoid tissues.

The main microscopy findings were in the small intestine (duodenum): diffuse, moderate infiltrate of mononuclear cells and eosinophils between the mucous membrane and muscularis mucosae, severe degeneration/scaling of the villi and very reactive lymphoid aggregates. The findings in the large intestine revealed a discrete infiltrate of mononuclear cells between the mucous membrane and the muscularis mucosae. In the lungs, we observed the presence of hemoglobin pigments, emphysema, thickening of the septa with congestion and multifocal infiltrate of mononuclear cells (Figure 2) and hepatization and alveolar serous effusion. In the spleen, intense reactivity of the white pulp and congestion were present, whereas in the liver, there was vacuolation characterized by steatosis and hydropic degeneration associated with inflammatory infiltrate that was characterized by neutrophils in the portal and focal centrilobular region.

In the IHC assay, immunoreactivity was observed in the lungs, duodenum and jejunum on the 3rd DAI; in the lungs and spleens on the 4th and 5th DAI; in the cerebellum, lungs, spleen, jejunum, tonsils, and mesenteric, submandibular and popliteal lymph nodes on the 6th DAI (Table 1, Figure 3). Parasite DNA was not amplified by PCR in the samples used in this analysis. No oocysts or *N. caninum* DNA were identified in the stool samples analyzed, and no dogs seroconverted to positive during the experiment.

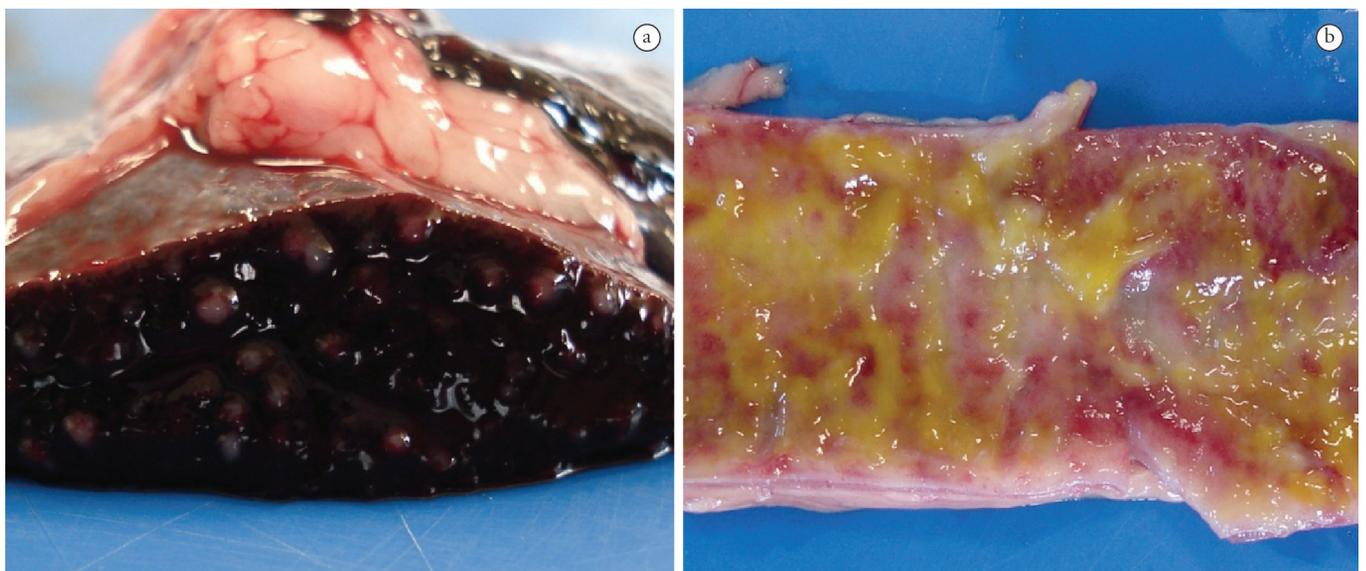


Figure 1. Necropsic findings in dogs experimentally infected with *Neospora caninum*. a) Spleen showing white pulp hyperplasia in an animal on the 4th DAI; b) Section of jejunum with thickening of mucous membrane and agglomerated petechia distributed throughout the mucous membrane (5th DAI).

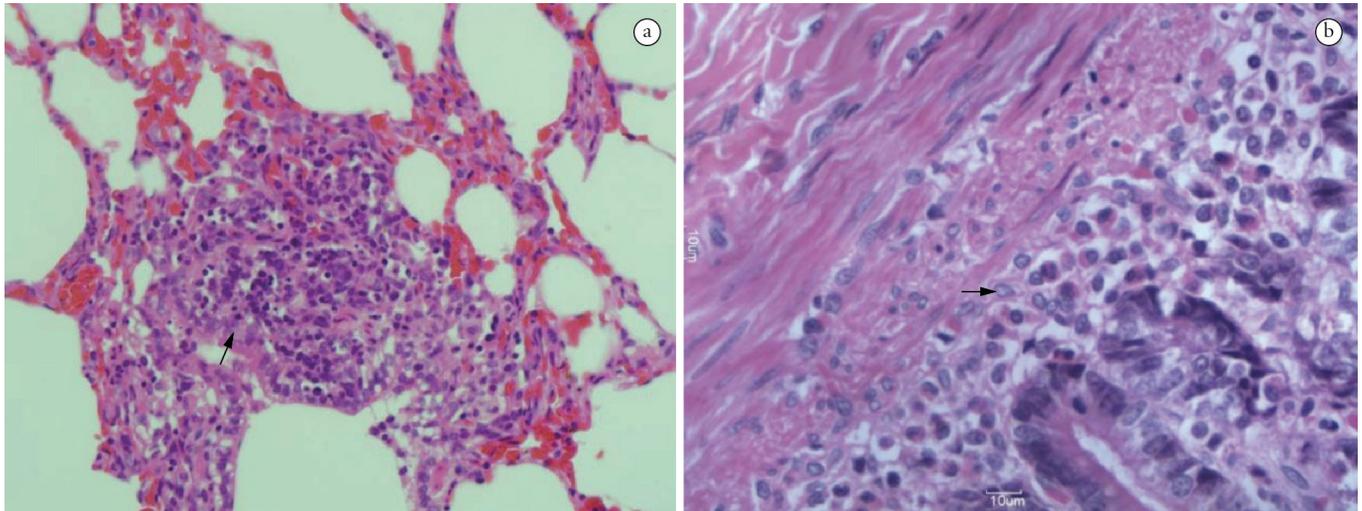


Figure 2. Histopathological alterations in the organs of dogs experimentally infected with *Neospora caninum*. a) Parenchymal consolidation associated with congestion and infiltrate inflammatory in the pulmonary interstitium on the 3rd DAI (→, 400X); b) Small intestine with moderate infiltrate characterized by mononuclear cells and eosinophils located in the submucosa and the muscularis mucosae on the 6th DAI (→, 400x), H&E.

Table 1. Detection of *Neospora caninum* in the tissues of experimentally infected dogs using immunohistochemistry and real time PCR techniques.

Organs	Animal I (3 rd DAI)		Animal II (4 th DAI)		Animal III (5 th DAI)		Animal IV (6 th DAI)	
	IHQ ^a	Real Time ^b	IHQ	Real Time	IHQ	Real Time	IHQ	Real Time
Brain	Negative	+++ ^c	Negative	+++	Negative	Negative	Negative	+++
Cerebellum	Negative	++	Negative	+++	Negative	Negative	Positive	+
Liver	Negative	++	Negative	+++	Negative	+	Negative	Negative
Heart	Negative	+++	Negative	+++	Negative	+++	Negative	+++
Lungs	Positive	+++	Positive	Negative	Positive	Negative	Positive	Negative
Stomach	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Esophagus	Negative	Negative	Negative	+++	Negative	Negative	Negative	Negative
Spleen	Negative	++	Positive	++	Positive	Negative	Positive	Negative
Kidney	Negative	+++	Negative	+	Negative	++	Negative	Negative
Mesenteric lymph nodes	Negative	Negative	Negative	++	Negative	Negative	Positive	+
Popliteal lymph nodes	Negative	Negative	Negative	Negative	Negative	++	Positive	Negative
Submandibular lymph nodes	Negative	+++	Negative	+++	Negative	Negative	Positive	+
Pancreas	Negative	+++	Negative	+++	Negative	+++	Negative	+++
Tonsils	Negative	+++	Negative	Negative	Negative	Negative	Positive	Negative
Biliar Vesicle	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Duodenum	Positive	+++	Negative	++	Negative	Negative	Negative	Negative
Proximal jejunum	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Medial jejunum	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
Distal jejunum	Positive	Negative	Negative	+++	Negative	Negative	Positive	Negative
Ileum	Negative	+++	Negative	Negative	Negative	Negative	Negative	Negative
Cecum	Negative	++	Negative	Negative	Negative	Negative	Negative	Negative
Colon	Negative	+++	Negative	Negative	Negative	Negative	Negative	+++
Tongue	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative

^aImmunohistochemistry; ^bReal-time PCR and ^cThree positive samples (+++); two positive samples (++) and one positive sample (+) via real-time PCR.

Real-time PCR was the most sensitive technique used to detect the protozoa in tissues, and identified them in 41% (38/92) of the samples analyzed. The technique detected *N. caninum* in all of the euthanized animals. The heart and pancreas were 100% positive in every animal (Table 1). Finally, we observed immunoreactivity

of the parasite in indirect immunofluorescence of the liver, lung, spleen, stomach, duodenum and pancreas tissue on the 3rd DAI; lung, spleen, mesenteric and popliteal lymph node tissue on the 4th DAI; heart, kidney and spleen tissue on the 5th DAI; and lung, spleen, pancreas, jejunum and colon tissue on the 6th DAI. With

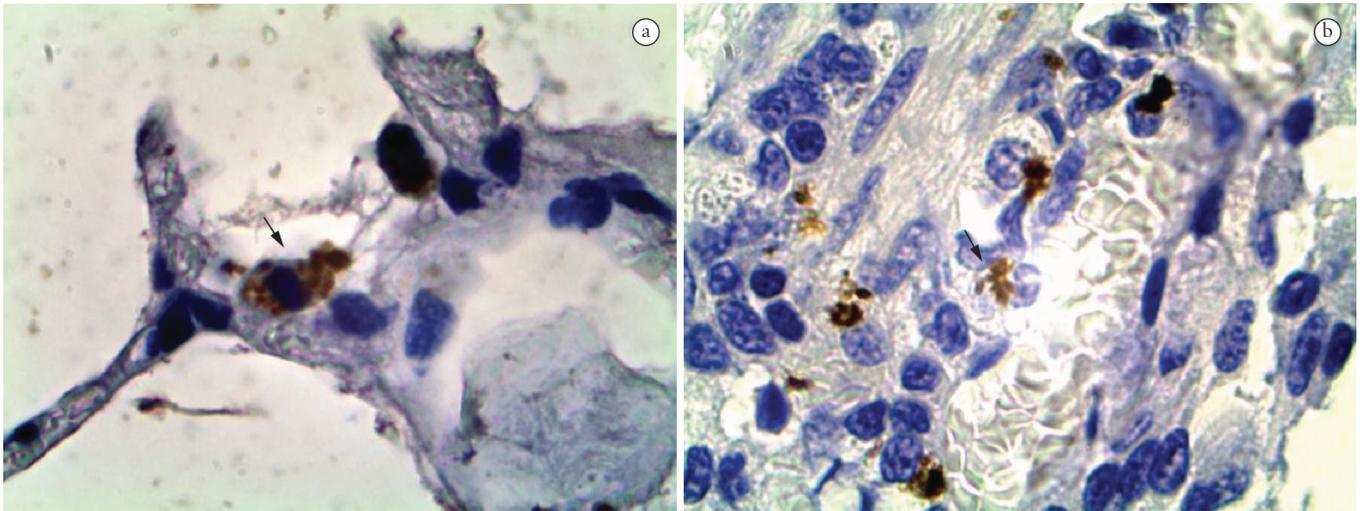


Figure 3. Immunohistochemical markings in organs of dogs experimentally infected with *Neospora caninum*. a) Pulmonary interstitium on the 3rd DAI (→, 1000X); b) Small intestine in the muscularis mucosae on the 6th DAI (→, 1000X).

the exception of proliferative forms, no merogonic or gametogonic forms of *N. caninum* were observed in the epithelial cells of the intestinal mucous membrane.

Discussion

The lack of clinical signs in infected dogs demonstrates that the infection tends to take a subclinical course even when there are macroscopic and microscopic alterations induced by the parasite. Dijkstra et al. (2001) and Cedillo et al. (2008) did not observe any clinical signs or lesions during necropsy or histopathology associated with the infection in dogs fed milk containing tachyzoites or in naturally infected placenta and fetuses, respectively. It is probable that the post-infection period used in these studies to euthanize the dogs may have influenced the findings because in the present study, the animals were euthanized in the first week of infection, which characterizes its acute phase. Furthermore, Dijkstra et al. (2001) believe that the lack of evidence of infection in dogs fed milk and tachyzoites may have occurred due to their elimination by stomach pepsin and hydrochloric acid (DUBEY et al., 1998), a possibility that was controlled for in the present study by prior ingestion of bicarbonate by the dogs, as recommended by Furuta et al. (2007).

The PCR results demonstrated that there was insufficient sensitivity to identify the parasite in dog tissue during the infection's acute phase, even in tissues with macroscopic and histopathological alterations. Such results may be, in part, due to the irregular distribution of the parasite (DE MAREZ et al., 1999), low parasitism (DIJKSTRA et al., 2001) or the location of the parasite in sporadic tissue areas (RODRIGUES et al., 2004) that are associated with the quantity of tissue used for DNA extraction.

Dijkstra et al. (2001) suggest that a strict location of the sexual forms of *N. caninum* in the intestine would justify the lack of parasites observed in the tissues; however, as previously mentioned, it is more likely that low parasitism and irregular distribution are the best hypotheses because the use of other associated techniques,

such as IHC, indirect immunofluorescent staining of tissues and especially real-time PCR, made it possible to identify the parasite in a number of tissue samples from animals in this study. This migration was systemic – we identified the parasite in the brains, cerebella, lungs, hearts, spleens and pancreases of virtually all animals – and rapid, as the parasite's DNA was identified in the brain after 72 hours with the use of real-time PCR.

The elimination of oocysts in dogs fed chicken chorioallantoic membranes was observed by Furuta et al. (2007). The main advantage of this protocol is that only the inoculation of *N. caninum* is guaranteed thus avoiding the risk of mixed infections with related coccidia (CAVALCANTE et al., 2011) and providing a good quantity of infected tissue to the definitive host, enabling more elimination of oocysts (DIJKSTRA et al., 2001; GONDIM et al., 2005).

However, the lack of visualization of oocysts in stools (of both euthanized animals and the control) and the lack of observation of merogonous and gametogonous forms are similar to the results found by Cedillo et al. (2008). These authors associated the possibility of autolysis or low parasitism with the lack of signs of infection, which differs from the present study because once the membranes were collected, they were immediately placed in PBS (pH 7.2), assessed for the viability of the infecting forms by microscopy, and were then offered to the dogs. Stool PCR was performed with the purpose of identifying DNA in dog feces in the event that there was little elimination of the oocysts (less than one oocyst per gram of feces), which was therefore not detectable by microscopy.

One hypothesis for the lack of seroconversion is the ingestion of a low parasitic load (CAVALCANTE et al., 2011). However, it is likely that the parasite stimulates cell immunity, especially in the intestine because the reinfection of dogs with tissues containing cysts results in a lack or reduced number of eliminated oocysts (DIJKSTRA et al., 2001, GONDIM et al., 2005). This is supported by our study because the uneuthanized dog that did

not seroconvert, as has been observed by Furuta et al. (2007) and Dijkstra et al. (2001).

Our results, therefore, indicate that an experimental model using previously infected chorioallantoic membranes may not be ideal for the production of oocysts or to visualize merogonous and gametogonous forms; however, this model did result in the rapid dissemination of the parasite resulting in important tissue alterations, showing it to be a useful model for the study of the host-parasite relationship during the infection's acute phase. Furthermore, this work demonstrated that in situations where there is no visualization of cysts and a likely low parasitic load, there is need for other diagnostic techniques used in conjunction with PCR or, if possible, other more sensitive methods such as real-time PCR, to minimize the possibility of false negative results.

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