**NOTE** *Parasitology*

# Detection of *Nesopora caninum*-Specific DNA from Cerebrospinal Fluid by Polymerase Chain Reaction in a Dog with Confirmed Neosporosis

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ABSTRACT. A one-month male Greyhound dog presented with a swinging gait of the hindlimbs, and later developed muscular atrophy of the femoral region and hyperextension of hindlimbs. The dog had positive serum IFAT titers to *Neospora caninum*, but a negative titer in the cerebrospinal fluid (CSF). *N. caninum*-specific DNA was amplified from the CSF using a semi-nested polymerase chain reaction assay. Clusters of protozoa in biopsied muscle fibers were subsequently confirmed as *N. caninum* tachyzoites by immunohistochemical examination. Early recognition and treatment are necessary for effective recovery of clinical canine neosporosis, but antemortem diagnosis is difficult. We suggest that the detection of parasite deoxyribonucleic acid in the CSF is a useful antemortem diagnostic method in facilitating treatment of this disease.

KEY WORDS: antemortem diagnosis, canine, *Neospora caninum,* neosporosis, PCR.

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A canine neuromuscular disease caused by a protozoan parasite that was morphologically similar, yet serologically different, to *Toxoplasma gondii* was first described in Norway [3]. This parasite was also detected in dogs from the U.S.A. and was given the name *Neospora caninum* four years later [7]. Subsequently, clinical neosporosis in dogs has been reported in several other countries [1, 2, 6, 18]. Although the presence of dogs infected with *N. caninum* has previously been recognized by serological investigations in Japan [17, 23], there has previously been only one report of canine neosporosis with clinical signs [27].

Early recognition and treatment are necessary to ensure that dogs recover from clinical neosporosis [1, 16, 25]. However, antemortem diagnosis of neosporosis is difficult [10, 11]. Definitive diagnosis is generally based on postmortem demonstration of the organism in tissue, blood, or cerebrospinal fluid (CSF) [19]. Thus, the detection of organisms or their specific genes will be useful for making a definitive antemortem diagnosis.

We describe a case report of confirmed neosporosis in a Greyhound dog in Japan and the use of polymerase chain reaction (PCR) as an effective method for facilitating the treatment of this disease.

A swinging gait of the hindlimbs was observed in a one-month-old male Greyhound dog by examination of the postural reaction containing proprioception, placing reaction, hopping reaction, wheel barrow reaction, and extensor postural thrust reaction (Fig. 1A). Meanwhile, it was reported that some juvenile Greyhound show dogs exhibited clinical signs of polyneuropathy, similar to that observed in neosporosis. Affected dogs also exhibited exercise intolerance and walking difficulties such as high stepping gait and 'bunny-hopping', but postural reactions seemed unaffected in the early stages of the disease [5]. These clinical signs were different from those observed in the dog in our case report. No abnormality was found based on brain neurological examination. The dog could maintain a standing posture, but showed paraparesis of the hindlimbs. The spinal reflexes, containing extended carpi radialis, biceps, patella, cranial tibialis and gastrocnemius reflexes of the dog exhibited normal reactions. The dog was born by Caesarian section, and had no littermates. A good appetite and stable mental status was observed, along with an absence of anamnesis.

Twelve days after the first medical examination (FME), the dog remained healthy and had a normal appetite. However, muscular atrophy of the femoral region and hyperextension of the hindlimbs were observed (Fig. 1B). A brain neurologic examination was conducted again, but no abnormality was found. X-ray findings of the hindlimb bones were normal. Postural reactions of the hindlimbs also disappeared, and the spinal, patella, cranial tibialis, and gastrocnemius reflexes for hindlimbs, had diminished or were absent. Hematology of the dog indicated a mild lymphocytosis (Table 1). Among serum biochemical findings (Table 2), elevated values, except creatine kinase (CK), were potentially associated with the age of this dog,

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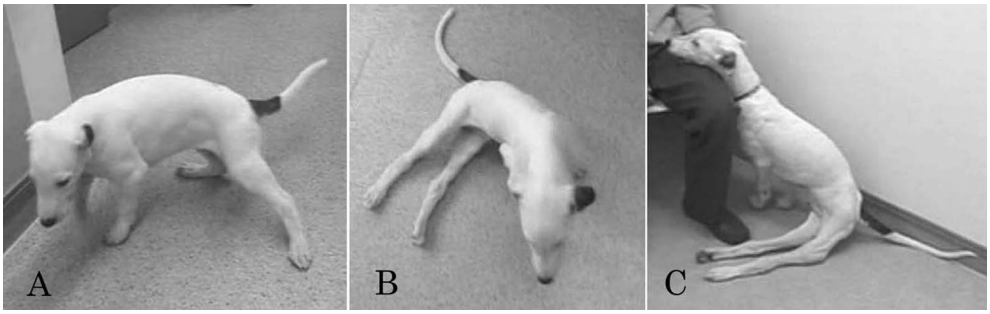


Fig. 1. A Greyhound dog with bilateral hindlimb extensor rigidity. (A) At first medical examination (FME). (B) At the 12th day after FME. (C) At the 94th day after FME.

Table 1. Hematological findings in a Greyhoud dog with clinical neosporosis

| Analyte | Results |
|---|---|
| Red blood cell ($\times 10^6/\mu l$) | 4.73 |
| White blood cell ($/\mu l$) | 14,200 |
| Neutrophil (%) | 54 |
| Lymphocyte (%) | 39 |
| Monocyte (%) | 4 |
| Eosinophil (%) | 3 |
| Basophil (%) | 0 |

Table 2. Serum biochemical findings in a Greyhoud dog with clinical neosporosis

| Analyte | Results |
|---|---|
| Total protein (g/d*l*) | 4.4 |
| Albumin (g/d*l*) | 2.6 |
| Alanin aminotranferase (U/I) | 65 |
| Alkaline phosphatase (U/I) | 651 |
| Aspartate transaminase (U/I) | 43 |
| Total bilirubin (mg/d*l*) | 0.4 |
| Total cholesterol (mg/d*l*) | 180 |
| Globulin (mg/d*l*) | 137 |
| Blood urea nitrogen (mg/d*l*) | 6 |
| Creatine kinase (U/I) | 1193 |
| Creatinine (mg/d*l*) | 0.4 |
| Phosphorus (mg/d*l*) | 9.3 |
| Calcium (mg/d*l*) | 11.8 |
| Sodium (mEq/*l*) | 143 |
| Potassium (mEq/*l*) | 4.2 |
| Chloride (mEq/*l*) | 113 |

although calcium, potassium, alkaline phosphatase, and CK values were especially high. Consecutive treatment with ampicillin (20 mg/kg bid) for 10 days, ketoprofen (1 mg/kg sid) for five days and a vitamin formulation for 10 days, did not improve the health of the dog.

Twenty-one days after the FME, a presumed diagnosis of neosporosis was made based on the symptoms, and we conducted histopathological, serological, and genetic examinations. Treatment using clindamycin (11 mg/kg tid) for 72 days, sulfa trimethoprim (14 mg/kg bid) for 72 days, and aprofen (4.4 mg/kg sid) for 56 days was subsequently initiated. After two months of consecutive treatments, paralysis of the dog's hindlimbs had not improved. Although the treatment was discontinued, the clinical symptoms of the dog did not become worse. Therefore, the treatment was completed at the 94th day after the FME (Fig. 1C). The dog died for unknown reasons 2.5 years later.

Serum and CSF from the affected dog and serum from its mother were examined for antibodies against *N. caninum* using the indirect fluorescent antibody technique (IFAT) as described by Dubey *et al.* [8, 9]. The dog had positive serum IFAT titers to *N. caninum* (1:3,200), but a negative titer (<1:50) in the CSF. The mother of this dog also had positive serum antibodies (1:800). In contrast, both dogs had a negative latex agglutination titer to *T. gondii* (Toxotest-MT 'Eiken', Eiken Chemical Co., Ltd., Tokyo, Japan) in the sera and CSF. The dog in this study appeared to be congenitally infected with *N. caninum*, on account of high IFAT antibody levels to the parasite observed in both the dog (1:3,200) and its mother (1:800).

For molecular analysis, total deoxyribonucleic acid (DNA) was isolated from blood and CSF using a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was used to detect *N. caninum* DNA using specific primers $Np21^+$ (5'-CCC AGT GCG TCC AAT CCT GTA AC-3') and $Np6^+$ (5'-CTC GCC AGT CAA CCT ACG TCT TCT-3'), which amplified a 337 bp product of the Nc-5 gene [21, 28]. In order to improve the sensitivity of this PCR, semi-nested internal primers Np7 (5'-GGG TGA ACC GAG GGA GTT G-3') and $Np6^+$, which amplified a 232 bp product, were used to form a semi-nested assay. Both assays were performed using the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, with a final extension of 72°C for 10 min. The 232 bp fragment was sequenced with an automated DNA sequencer (SQ-5500E; Hitachi, Ibaraki, Japan). BLAST searching of the DNA Data Bank of Japan database for homologous sequences was used to identify the sequence. PCR using the semi-nested primer pair $Np6^+$/Np7 detected *N. caninum*-specific DNA only in the CSF of the dog 21 days after FME, but not from blood samples on the 21st, 50th, and 66th days after FME or

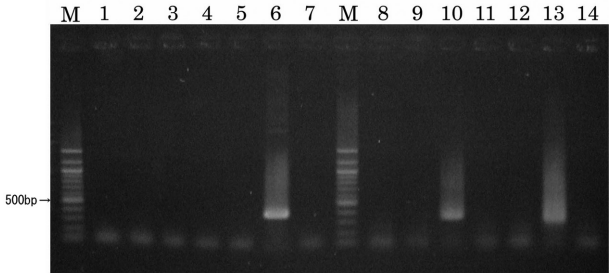


Fig. 2. Semi-nested PCR amplification of the *N. caninum* specific Nc-5 gene from DNA extracted from blood and CSF from the *N. caninum* infected dog and its mother. All PCR reactions were performed in a 50 $\mu l$ volume containing 0.5 $\mu$g DNA, 0.2 mM dNTP mixture, 1× PCR buffer containing 2.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Greiner bio-one, Co., Ltd., Germany). Lane M: DNA marker (100 bp ladder); lanes 1 and 8: blood of the dog collected 21 days after FME; lanes 2 and 9: blood of the dogs' mother collected 21 days after FME; lanes 3 and 10: CSF from the dog collected 21 days after FME; lanes 4 and 11: blood collected from the dog 50 days after FME; lanes 5 and 12: blood collected 66 days after FME; lanes 6 and 13: positive control (*N. caninum* Nc-1 tachyzoite); lanes 7 and 14: negative control (distilled water). $Np21^+/6^+$ primers were used for lanes 1–7 and $Np7/6^+$ for lanes 8–14. A *N. caninum*-specific DNA fragment was observed in lane 10.

from the mother on the 21st day (Fig. 2). The PCR product sequence was 99% homologous to AY459289, EU686398, EU073599 and X84238 based on BLAST searching.

For histopathological and immunohistochemical examinations, muscle tissue samples of the indurated portion in the femoral region of the quadriceps were taken by biopsy. Tissues were fixed in 10% neutral buffered formalin, and processed for routine histology. Paraffin-embedded sections (5 $\mu$m thickness) were cut, and stained with hematoxylin and eosin (HE), before being examined microscopically. The sections were also used for immunohistochemical examination using an automated slide stainer (Histostainer; Nichirei, Tokyo, Japan). Immunohistochemistry was performed based on the streptavitin-biotin-peroxidase complex technique (Nichirei) with primary mouse monoclonal antibodies against NcSAG1 for tachyzoite and NcBSR4 and NcSAG4 for bradyzoite. These antibodies were made by our laboratory in the previous manner [26]. Microscopically, the muscle lesion was characterized by severe diffused cellular infiltration. These inflammatory cells primarily consisted of lymphocytes, plasma cells, macrophages, together with some neutrophils and eosinophils. Muscle bundles appeared atrophied and denatured (Fig. 3A). Occasionally, clusters of protozoa, assumed to be *N. caninum*, were observed in the muscle fibers (Fig. 3B). From the results of the immunohistochemical examination using monoclonal antibodies to NcSAG1, NcBSR4, and NcSAG4, they were confirmed to be *N. caninum* tachyzoites (Fig. 3C).

Clinical signs for the dog in our report were pathognomonic based on the appearance of disease five weeks after birth, hyperextension of the hindlimbs, atrophy of the quadriceps, and a remarkable CK increase. Presumptive diagnosis was made on the basis of the signs mentioned above, and consequently examinations including IFAT of serum and CSF were conducted to confirm our tentative diagnosis. Demonstration of *N. caninum* antibodies in the serum can help to confirm diagnosis in dogs that exhibit clinical signs of neosporosis [9, 10]. Detection of high levels of antibodies in the CSF is reported as a more useful aid for neosporosis diagnosis, since the antibody titers of the CSF are generally lower than blood antibody titers [1]. However, the present dog had no IFAT antibodies against *N. caninum* in the CSF (<1:50), although a high antibody titer was observed in the serum (1:3,200).

Diagnosis based on parasite DNA from the CSF proved to be more reliable method. There are several reports that confirmed the presence of *N. caninum* in canine CSF by microscopy or the detection of parasite DNA by PCR [7, 12–14, 20, 22, 24]. While visual identifications of organisms in CSF were accomplished following immunosuppressive corticosteroid and cyclosporine therapies [12, 13], specific *N. caninum* DNA was detected in CSF by PCR, even if the

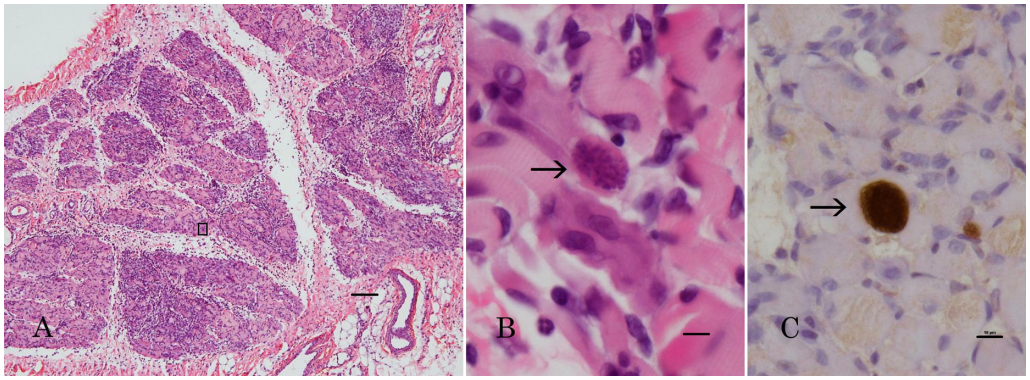


Fig. 3. Sections of muscle lesion with myositis and clusters of *N. caninum* in the muscle fibers of the indurated portion in the femoral region of the quadriceps by biopsy. (A) HE staining for section of the femoral muscle region with myositis. Bar=100 $\mu$m. (B) HE staining for *N. caninum* organisms in the square of (A). Bar=10 $\mu$m. (C) Immunohistochemical staining for *N. caninum* tachyzoites using a monoclonal antibody to NcSAG1. Bar=10 $\mu$m.

organism was not found directly by light microscopy in the CSF [14, 22, 24]. Neither protozoa in the CSF by light microscopy, nor IFAT antibodies in the CSF, were detected in our study, however, *N. caninum*-specific DNA was detected using semi-nested PCR. The specific DNA was isolated from CSF with no IFAT titer and not from the blood with a high titer, which was assumed to be because *N. caninum* could escape from the blood to central nervous system including CSF before the appearance of *N. caninum*-specific antibodies in a similar manner to that observed for *T. gondii*. The parasite is able to determine a delicate balance between parasitism and the immune response of the host [4]. Additionally, the immunohistochemical examination proved that the dog had progressive neosporosis, as clusters of protozoa in the muscle fibers by biopsy were confirmed to be *N. caninum* tachyzoite, rapid proliferation stage. These examinations, the detection of *N. caninum*-specific DNA fragment from CSF and the immunohistochemical examination, led to a definitive diagnosis of neosporosis for this dog. Definitive diagnosis is based on demonstration of the parasite in the tissues, blood, or CSF [19]. Therefore, the detection of protozoan DNA from CSF by PCR should be considered to be a useful in rapid diagnostic method for progressive clinical canine neosporosis.

Although antemortem diagnosis is important to initiate treatment during the early stages of neosporosis, it is reported to be difficult [10, 11]. Thate and Laanen [25] reported that the success rate of neosporosis therapy is usually low and depends on the interval between the onset of signs and the initiation of therapy. Treatment is most effective in early stages before muscular contracture has occurred [15], and all the recovery cases were due to early antemortem diagnosis and treatment [1, 16, 20, 25]. The initiation of early treatment with early diagnosis is crucial to the treatment of this disease. From the present study, the dog with clinical signs should be started the treatment for neosporosis by the tentative diagnosis using the serological examination of the serum or the reliable diagnosis using the detection of *N. caninum*-specific DNA in CSF, and confirmed by the subsequent immunohistochemical examination with specific antibodies as definitive diagnosis. The above mentioned manner is thought to be the most realistic and effective at present. It is ensured that detection of parasitic DNA in the CSF could become one of the antemortem diagnosis for onset of treatment of the disease.

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REFERENCES

1. Barber, J. S. and Trees, A. J. 1996. Clinical aspects of 27 cases of neosporosis in dogs. *Vet. Rec.* **139**: 439–443. [Medline] [CrossRef]
2. Basso, W., Venturini, M. C., Bacigalupe, D., Kienast, M., Unzaga, J. M., Larsen, A., Machuca, M. and Venturini, L. 2005. Confirmed clinical *Neospora caninum* infection in a boxer puppy from Argentina. *Vet. Parasitol.* **131**: 299–303. [Medline] [CrossRef]
3. Bjerkås, I., Mohn, S. F. and Presthus, I. 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. *Z. Parasitenkd.* **70**: 271–274. [Medline] [CrossRef]
4. da Silva, R. C. and Langoni, H. 2009. *Toxoplasma gondii*: host-parasite interaction and behavior manipulation. *Parasitol. Res.* **105**: 893–898. [Medline] [CrossRef]
5. Drögemüller, C., Becker, D., Kessler, B., Kemter, E., Tetens, J., Jurina, K., Jäderlund, K. H., Flagstad, A., Perloski, M., Lindblad-Toh, K. and Matiasek, K. 2010. A deletion in the N-myc downstream regulated gene 1 (*NDRG1*) gene in Greyhounds with polyneuropathy. *PLoS ONE* **5**: e11258. [Medline] [CrossRef]
6. Dubey, J. P. 2003. Review of *Neospora caninum* and neosporosis in animals. *Korean J. Parasitol.* **41**: 1–16. [Medline] [CrossRef]
7. Dubey, J. P., Carpenter, J. L., Speer, C. A., Topper, M. I. and Uggla, A. 1988. Newly recognized fatal protozoan disease of dogs. *J. Am. Vet. Med. Assoc.* **192**: 1269–1285. [Medline]
8. Dubey, J. P., Hattel, A. L., Lindsay, D. S. and Topper, M. I. 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *J. Am. Vet. Med. Assoc.* **193**: 1259–1263. [Medline]
9. Dubey, J. P. and Lindsay, D. S. 1996. A review of *Neospora caninum* and neosporosis. *Vet. Parasitol.* **67**: 1–59. [Medline] [CrossRef]
10. Dubey, J. P., Dorough, K. R., Jenkins, M. C., Liddell, S., Speer, C. A., Kwok, O. C. and Shen, S. K. 1998. Canine neosporosis: clinical signs, diagnosis, treatment and isolation of *Neospora caninum* in mice and cell culture. *Int. J. Parasitol.* **28**: 1293–1304. [Medline] [CrossRef]
11. Dubey, J. P., Vianna, M. C., Kwok, O. C., Hill, D. E., Miska, K. B., Tuo, W., Velmurugan, G. V., Conors, M. and Jenkins, M. C. 2007. Neosporosis in Beagle dogs: clinical signs, diagnosis, treatment, isolation and genetic characterization of *Neospora caninum*. *Vet. Parasitol.* **149**: 158–166. [Medline] [CrossRef]
12. Gaitero, L., Anor, S., Montoliu, P., Zamora, A. and Pumarola, M. 2006. Detection of *Neospora caninum* tachyzoites in canine cerebrospinal fluid. *J. Vet. Intern. Med.* **20**: 410–414. [Medline]
13. Galgut, B. I., Janardhan, K. S., Grondin, T. M., Harkin, K. R. and Wight-Carter, M. T. 2010. CASE REPORT: Detection of *Neospora caninum* tachyzoites in cerebrospinal fluid of a dog following prednisone and cyclosporine therapy. *Vet. Clin. Pathol.* **39**: 386–390. [Medline] [CrossRef]
14. Garosi, L., Dawson, A., Couturier, J., Matiasek, L., de Stefani, A., Davies, E., Jeffery, N. and Smith, P. 2010. Necrotizing cerebellitis and cerebellar atrophy caused by *Neospora caninum* infection: magnetic resonance imaging and clinicopathologic findings in seven dogs. *J. Vet. Intern. Med.* **24**: 571–578. [Medline] [CrossRef]
15. Hay, W. H., Shell, L. G., Lindsay, D. S. and Dubey, J. P. 1990. Diagnosis and treatment of *Neospora caninum* infection in a dog. *J. Am. Vet. Med. Assoc.* **197**: 87–89. [Medline]
16. Heckeroth, A. R. and Tenter, A. M. 2007. Immunoanalysis of three litters born to a Doberman bitch infected with *Neospora caninum*. *Parasitol. Res.* **100**: 837–846. [Medline] [CrossRef]
17. Kubota, N., Sakata, Y., Miyazaki, N., Itamoto, K., Bannai, H., Nishikawa, Y., Xuan, X. and Inokuma, H. 2008. Serological survey of *Neospora caninum* infection among dogs in Japan through species-specific ELISA. *J. Vet. Med. Sci.* **70**: 869–872. [Medline] [CrossRef]
18. Lindsay, D. S. and Dubey, J. P. 2000. Canine neosporosis. *J. Vet.*

*Parasitol.* **14**: 1–11.
19. Lyon, C. 2010. Update on the diagnosis and management of *Neospora caninum* infections in dogs. *Top. Companion Anim. Med.* **25**: 170–175. [Medline] [CrossRef]
20. McGlennon, N. J., Jefferies, A. R. and Casas, C. 1990. Polyradiculoneuritis and polymyositis due to a toxoplasma-like protozoan: diagnosis and treatment. *J. Small Anim. Pract.* **31**: 102–104. [CrossRef]
21. Müller, N., Zimmermann, V., Hentrich, B. and Gottstein, B. 1996. Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. *J. Clin. Microbiol.* **34**: 2850–2852. [Medline]
22. Peters, M., Wagner, F. and Schares, G. 2000. Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. *Parasitol. Res.* **86**: 1–7. [Medline] [CrossRef]
23. Sawada, M., Park, C. H., Kondo, H., Morita, T., Shimada, A., Yamane, I. and Umemura, T. 1998. Serological survey of antibody to *Neospora caninum* in Japanese dogs. *J. Vet. Med. Sci.* **60**: 853–854. [Medline] [CrossRef]
24. Schatzberg, S. J., Haley, N. J., Barr, S. C., deLahunta, A., Olby, N., Munana, K. and Sharp, N. 2003. Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. *Am. J. Vet. Res.* **64**: 1507–1513. [Medline] [CrossRef]
25. Thate, F. M. and Laanen, S. C. 1998. Successful treatment of neosporosis in an adult dog. *Vet. Q.* **20**: S113–S114. [Medline]
26. Uchida, Y., Ike, K., Kurotaki, T., Ito, A. and Imai, S. 2004. Monoclonal antibodies preventing invasion of *Neospora cainum* tachyzoites into host cells. *J. Vet. Med. Sci.* **66**: 1355–1358. [Medline] [CrossRef]
27. Umemura, T., Shiraki, K., Morita, T., Shimada, A., Haritani, M., Kobayashi, M. and Yamagata, S. 1992. Neosporosis in a dog: the first case report in Japan. *J. Vet. Med. Sci.* **54**: 157–159. [Medline] [CrossRef]
28. Yamage, M., Flechtner, O. and Gottstein, B. 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain "cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). *J. Parasitol.* **82**: 272–279. [Medline] [CrossRef]