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INFECTIOUS DISEASE

Neospora caninum: the First Demonstration of the Enteroepithelial Stages in the Intestines of a Naturally Infected Dog

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Summary

A 1.5-month-old Kangal breed puppy from a dairy cattle farm died after showing severe diarrhoea and incoordination. Necropsy examination revealed multifocal pulmonary consolidation and necrosis and fibrinohaemorrhagic enteritis. Microscopically, there was necrotic and purulent bronchopneumonia, myocarditis and non-purulent encephalitis. In the jejunum and ileum there was villous atrophy and crypt hyperplasia with oocyst-like and schizont-like structures in the epithelia. Immunohistochemically, *Neospora caninum* antigen was detected in association with the intestinal protozoal structures, degenerative neurons and areas of necrosis in the lungs and heart. Polymerase chain reaction confirmed that the organism was *N. caninum* and not *Toxoplasma gondii*. The seroprevalence for *N. caninum* was 74.2% (49/66 animals) for the cattle and 57.1% (4/7 animals) for dogs on this farm. This report documents fatal systemic neosporosis and enteroepithelial stages of *N. caninum* in a naturally infected puppy. To the authors' knowledge, this is the first definition of intestinal neosporosis in a naturally infected dog as well as the first evidence of fatal canine neosporosis in Turkey.

Keywords: dog; enteroepithelial stages; *Neospora caninum*; neosporosis

Neospora caninum infection was first reported in dogs with skin lesions and systemic pathology in 1988 (McAllister *et al.*, 1998). Canine neosporosis must be distinguished from disease caused by morphologically similar protozoal agents such as *Toxoplasma gondii* and *Hammondia hammondi* (Lindsay and Dubey, 2000; Dubey, 2003; Dubey *et al.*, 2006). Dogs and coyotes are the only definitive hosts of *N. caninum*, shedding the oocysts following enteroepithelial schizogony and gametogony (McAllister *et al.*, 1998; Lindsay *et al.*, 1999). Several reports describe the shedding of *N. caninum* oocysts in the faeces of naturally infected dogs (Lindsay and Dubey, 2000; Dubey, 2003; Gondim *et al.*, 2005; Dubey and Schares, 2011). Oocyst shedding is also reported in experimental studies; for example, puppies given calf brains and avian chorio-allantoic membranes containing *N. caninum* infective stages shed millions of oocysts for 2–3 days in their faeces (Cedillo *et al.*, 2008; Munhoz *et al.*, 2013). Although it is likely that the enteroepithelial stages of *N. caninum* develop in the dog intestine, neither schizogony nor gametogony have yet been demonstrated in this tissue.

The intestinal form of canine neosporosis would seem to be largely subclinical, but systemic fatal cases have been reported in young puppies (Barber and Trees, 1996; Lindsay and Dubey, 2000; Dubey, 2003; Basso *et al.*, 2005; Gondim *et al.*, 2005). According to one previous report, there is no gender or breed predisposition in dogs (Lindsay and Dubey, 1989). To date, the range of histopathological findings recorded in canine neosporosis include meningoencephalitis, pneumonia, polymyositis and radiculoneuritis (Barber and Trees, 1996;

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Dubey, 2003; Dubey and Schares, 2011). The present case report records, for the first time, the enteroepithelial developmental stages of *N. caninum*, together with other systemic lesions, in a naturally infected puppy.

A 1.5-month-old Kangal breed puppy, raised on a dairy cattle farm, died after showing severe diarrhoea and incoordination. The abortion rate among cattle on this farm was 5% and *N. caninum* seroprevalance was 74.2% (49/66 animals) in cattle and 57.1% (4/7 animals) in dogs. The puppy reported here was also seropositive. Clinical neosporosis was diagnosed in a calf on this farm and one seropositive bitch had a history of abortion (Ocal *et al.*, 2014).

The puppy was subjected to necropsy examination and tissue samples were collected and fixed in 10% neutral buffered formalin for 48 h. The tissues were processed routinely, embedded in paraffin wax, sectioned (4–5 μm) and stained with haematoxylin and eosin (HE), Masson's trichrome and periodic acid–Schiff (PAS). Slides were examined by light microscopy (Olympus BX51, Tokyo, Japan) and digital photomicrographs were taken. Fresh samples of spleen, lymph node and brain were taken during the necropsy examination and stored at −20°C until analyzed.

Immunohistochemistry (IHC) was performed using a commercial immunoperoxidase kit (Invitrogen, Carlsbad, California, USA). Briefly, tissue sections mounted on electrostatic adhesive slides were dewaxed in xylene and hydrated through graded alcohols. The sections were boiled in citrate buffer (pH 6.0) for 30 min for the retrieval of *N. caninum* antigens. Subsequently, endogenous peroxidase activity was inhibited by the use of H_2O_2 3% in methanol and non-specific labelling was blocked by pre-incubation with normal goat serum. Primary *N. caninum* monoclonal antibody (210/70 NC, VMRD Inc., Pullman, Washington, USA) was added at a dilution of 1 in 10,000 for 60 min. Sections were then incubated with biotinylated secondary antibody, labelled with horseradish peroxidase and, finally, AEC chromogen–substrate solution. Serial sections were examined for *T. gondii* antigen using polyclonal rabbit anti-*T. gondii* antibody (Haziroglu *et al.*, 2003) and for canine parvovirus (CPV) antigen using commercial monoclonal antibody (SC-57961; Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Positive control slides included sections of the brain of an *N. caninum*-infected calf, the liver of a *T. gondii*-infected mouse and the ileum of a dog with CPV infection. For negative controls, phosphate buffered saline was used instead of each primary antibody and normal mouse serum was used as a control for *N. caninum* and CPV antibodies.

For PCR analyses, DNA was isolated from samples of the lung, liver and spleen using a commercial DNA isolation kit (Qiagen, Valencia, California, USA). The presence of DNA was confirmed by electrophoresis in a 2% agarose gel and spectrophotometric quantification. Semi-nested PCR was performed using the *NC-5* gene and the Np21–Np6 and Np9–Np10 primers (Müller *et al.*, 1996). For the first stage of the semi-nested PCR, a reaction mix in a volume of 50 μl, comprising 150 ng of target DNA, 2 mM $MgCl_2$, 10× reaction solution (50 mM KCl, 10 mM Tris–HCl [pH 8.3], 0.1% Triton ×100), 10 pmol of each primer, 200 μM of each dNTP and 2 units of Taq DNA polymerase, was prepared. After the first denaturation at 95°C for 5 min, 35 cycles of denaturation were performed, each at 95°C for 30 sec, followed by annealing at 57°C for 30 sec and two extension stages, the first at 72°C for 60 sec and the second at 72°C for 7 min. For the second stage of the semi-nested PCR, a PCR reaction mix was prepared using 2 μl of the first PCR product and the aforementioned constituents at the proportions indicated above. The second stage of the procedure involved an initial denaturation at 95°C for 5 min, 35 denaturation cycles at 95°C for 30 sec, annealing at 56°C for 30 sec, an initial extension phase at 72°C for 60 sec and a second extension phase at 72°C for 7 min. The PCR products were analyzed by separating them in a 1.8% agarose gel.

Necropsy examination revealed multifocal pulmonary consolidation and necrosis and fibrinohaemorrhagic enteritis. Microscopically, there was necrotic and purulent bronchopneumonia, acute catarrhal, haemorrhagic and necrotic enteritis, non-purulent interstitial myocarditis and non-purulent meningoencephalitis. In the ileum, there were erosions of the epithelial layer with villous atrophy and fusion. The crypts were atrophic, cystic and necrotic in appearance. In the lamina propria there was marked infiltration of eosinophils and macrophages in addition to congestion and multifocal haemorrhages. These lesions were suggestive of CPV; however, numerous schizont-like and oocyst-like structures were also observed in the intestinal mucosa and crypt epithelia (Figs. 1 and 2). In some areas, crypt epithelium contained granular structures (10–15 μm) on the apical side of the cells (Fig. 3). These schizont-like structures showed pale blue granular staining on Masson's trichrome stain and were PAS negative. Immunohistochemically, they were strongly labelled for *Neospora* antigen. Additionally, IHC revealed numerous developmental stages characteristic of schizonts within necrotic debris and cystic crypts (Fig. 3). Weakly labelled microgamont-like structures were observed and these appeared as very small spots

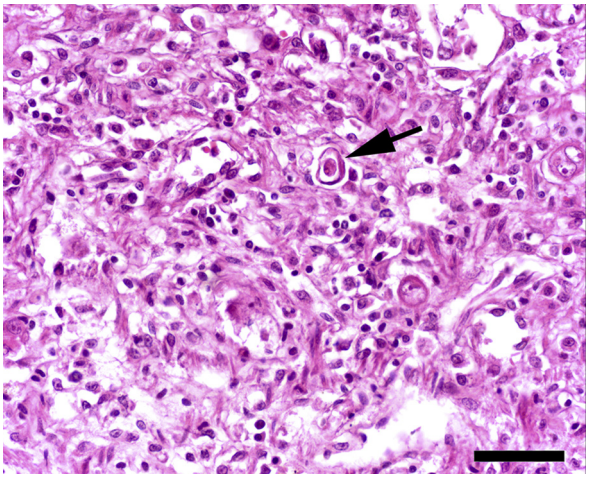

Fig. 1. Zygote and oocyst-like forms of *N. caninum* in the ileal epithelium (arrow). HE. Bar, 100 µm.

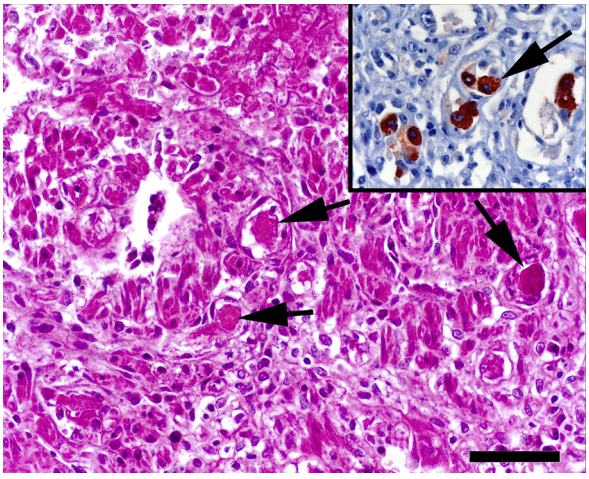

Fig. 3. Schizont-like structure of *N. caninum* in crypt epithelium (arrows). HE. Bar, 100 µm. Inset: strong labelling of schizonts in crypt epithelium. IHC.

with flagella-like structures (Fig. 4). Intracellular oocysts also showed weak immunopositivity for *N. caninum*, but *N. caninum* antigen was not found in interstitial tissue. *N. caninum* antigen was also detected in degenerative neurons and in foci of necrosis in the lung and heart. There was no labelling for *T. gondii* or CPV antigens. The 224 base pair amplification products from PCR were consistent with the *N. caninum Nc5* gene and all samples from the lungs, liver and spleen contained *N. caninum* DNA.

Since the first description of *N. caninum* oocysts in naturally infected dog faeces, several reports have described oocyst shedding as an important horizontal transmission route for intermediate hosts of neosporosis (McAllister *et al.*, 1998; Lindsay *et al.*, 1999; Dubey, 2003; Gondim *et al.*, 2005; Dubey and Schares, 2011; Ocal *et al.*, 2014). It has also been shown that dogs given *N. caninum* tachyzoites and tissue cysts may shed millions of oocysts after 2–8 days. However, though it is highly likely that asexual and sexual developmental stages of neosporosis probably take place in the small intestines of dogs, to date there is no report confirming the presence of those stages (Hemphill *et al.*, 2009). An attempt to demonstrate schizogony and gametogony of *N. caninum* in in-vitro cultured canine duodenal cells also failed (Hemphill *et al.*, 2009). In this duodenal cell culture model, Hemphill *et al.* (2009) inoculated *N. caninum* bradyzoites and tachyzoites onto the intestinal epithelium, but although the infective stages successfully entered the epithelial cells, particularly through the microvillus containing apical side, no stage conversion

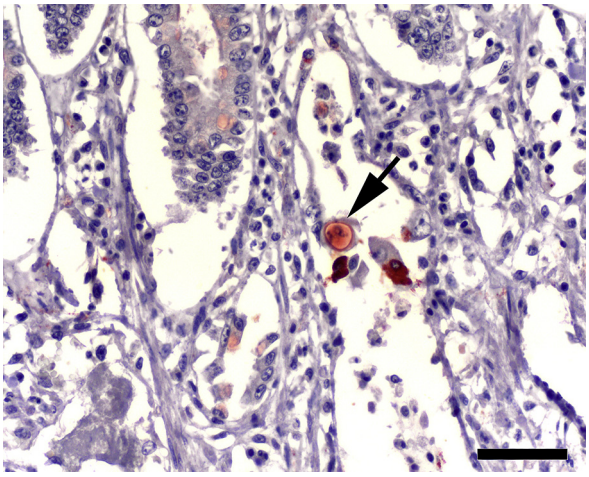

Fig. 2. Strong labelling of an oocyst of *N. caninum* (arrow). IHC. Bar, 60 µm.

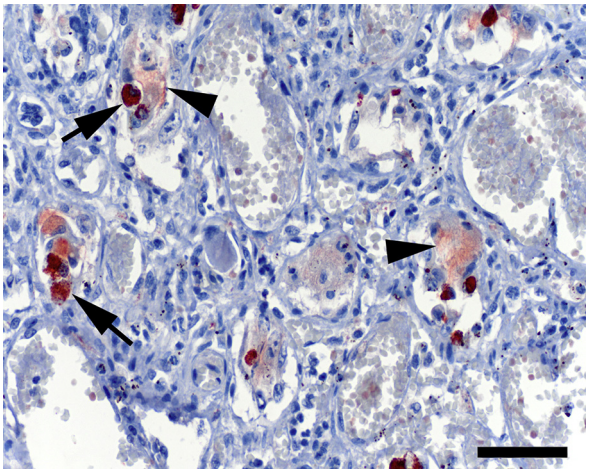

Fig. 4. Labelled schizonts and flagella-like microgamonts showing weak *N. caninum* antigen positivity (arrowhead) and strong labelling of schizonts (arrows). IHC. Bar, 100 µm.

occurred during the 10 day incubation time. The authors concluded that this study should be repeated using an immortal duodenal cell line for prolonged times in order to observe stage conversion from tachyzoite to schizont.

T. gondii, which shows genetic and morphological resemblance to *N. caninum*, has been shown to have five different schizogony stages (A–E) in the cat intestine. Stages A, B and C are the initial proliferative stages (30–40 μm in size) and they have been considered as temporary stages, while D and E schizonts are smaller (8–12 μm) and are seen just before stage conversion to gametocytes (Dubey, 2010). In the present case, schizogony stages observed in the ileal mucosa resembled type D and E schizonts of *T. gondii*. While schizonts were easily observed in the HE-stained slides, microgamonts were recognized only after IHC. However, macrogamonts of the parasite were not identified, even with PAS staining (Dubey, 2010). Unfortunately, it was not possible to perform an ultrastructural examination in this case. In the intestinal mucosa of this puppy, intraepithelial schizonts showed strong immunoreactivity for *N. caninum* while the immunoreaction was relatively weak in microgametocytes and oocysts. This difference can be explained by the different structural proteins expressed by the different developmental stages of the parasite. The commercial *N. caninum* antibody may have different affinity for common antigens shared by tachyzoites, microgamonts and schizonts. A previous report demonstrated that the intestinal developmental stages (e.g. schizont and gametocyte) of *T. gondii* may cross-react with tachyzoite-specific primary antibody in immunoperoxidase tests (Taka *et al.*, 1999). Otherwise, the lack of labelling of interstitial macrophages and the epithelial localization of the protozoon is consistent with these stages not being tachyzoites (Dubey, 2010).

Neosporosis is generally asymptomatic in dogs that are the intermediate and definitive hosts of the parasite (Barber and Trees, 1996; Lindsay and Dubey, 2000; Dubey *et al.*, 2006). There are no reports of pathological findings in the small intestine related to neosporosis. In the present case there were numerous schizonts of *N. caninum* within the intestinal epithelium and these were associated with epithelial necrosis, desquamation, villous atrophy and crypt necrosis. Severe mucosal lesions were also prominent clinically and on gross necropsy examination. The intestinal lesions were characterized as fibrinohaemorrhagic enteritis and the severity of the observed epithelial necrosis appeared to be related to the number of schizonts present. This mucosal damage caused by *N. caninum* resembles the intestinal lesions caused by other coccidian protozoons such as *Eimeria* spp. and *Isospora* spp. and *T. gondii* (Dubey *et al.*, 2006; Dubey, 2010). Canine coronavirus and rotavirus infections can also cause mucosal lesions, but these viruses generally localize to the apical site of the epithelia and the mucosal lesions are frequently limited to epithelial desquamation and mild crypt necrosis.

Whether gender, breed or age influences canine neosporosis is unknown (Cedillo *et al.*, 2008), but some reports have shown puppies to be at higher risk than adults (Gondim *et al.*, 2005). How dogs become infected naturally by *N. caninum* is not known, but some reports describe ingestion of cattle tissue (Dubey and Schares, 2011). In the present case, as the diagnosis of neosporosis was made after necropsy examination, neither the presence of *N. caninum* oocysts in the faeces nor seropositivity was investigated.

In conclusion, neosporosis should be considered as a differential diagnosis for canine enteritis and IHC may be helpful in recognizing the causative agent. To the authors' knowledge, this report provides is the first description of intestinal neosporosis in naturally infected dogs and the first evidence of fatal neosporosis in Turkey.

Acknowledgments

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