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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