Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
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;
Dubey, 2003; Dubey and Scharfs, 2011). The present case report records, for the first time, the enteroeplithelial 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 seroprevalence 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 H2O2 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 horse-radish 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 MgCl2, 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 they appeared as very small spots
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 Scharcs, 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...
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 the first description of intestinal neosporosis in naturally infected dogs and the first evidence of fatal neosporosis in Turkey.

**Acknowledgments**

This study was presented at the Cutting Edge Pathology Congress in Berlin in August 2014 as a poster presentation. The project was partly supported by Kırıkkale University Scientific Research Council (project number: 2008/44). We thank Dr. M. Fatih Bozkurt, Afyon Kocatepe University, for supplying CPV antibody.

**References**

Barber JS, Trees AJ (1996) Clinical aspects of 27 cases of neosporosis in dogs. *Veterinary Record*, 139, 439–443.

Basso W, Venturini MC, Bacigalupe D, Kienast M, Unzaga JM et al. (2005) Confirmed clinical *Neospora caninum* infection in a boxer puppy from Argentina. *Veterinary Parasitology*, 131, 299–303.

Cedillo C JR, Martínez M JJ, Santacruz AM, Banda RVM, Morales SE (2008) Models for experimental infection of dogs fed with tissue from fetuses and neonatal cattle naturally infected with *Neospora caninum*. *Veterinary Parasitology*, 154, 151–155.

Dubey JP (2003) Review of *Neospora caninum* and neosporosis in animals. *Korean Journal of Parasitology*, 41, 1–16.

Dubey JP (2010) *Toxoplasmosis of Animals and Humans*, 2nd Edt. CRC Press, Boca Raton.

Dubey JP, Chapman JL, Rosenthal BM, Mense M, Schueler RL (2006) Clinical *Sarcocystis neurona*, *Sarcocystis canis*, *Toxoplasma gondii*, and *Neospora caninum* infections in dogs. *Veterinary Parasitology*, 137, 36–49.
Dubey JP, Schares G (2011) Neosporosis in animals — the last five years. Veterinary Parasitology, 180, 90–108.
Gondim LFP, McAllister MM, Gao L (2005) Effects of host maturity and prior exposure history on the production of Neospora caninum oocysts by dogs. Veterinary Parasitology, 134, 33–39.
Haziroglu R, Altintas K, Atasever A, Gulbahar MY, Kul O et al. (2003) Pathological and immunohistochemical studies in rabbits experimentally infected with Toxoplasma gondii. Turkish Journal of Veterinary and Animal Science, 27, 285–293.
Hemphill A, Vonlaufen N, Golaz JL, Burgener IA (2009) Infection of primary canine duodenal epithelial cell cultures with Neospora caninum. Veterinary Parasitology, 95, 372–380.
Lindsay DS, Dubey JP (1989) Immunohistochemical diagnosis of Neospora caninum in tissue sections. American Journal of Veterinary Research, 50, 1981–1983.
Lindsay DS, Dubey JP (2000) Canine neosporosis. Journal of Veterinary Parasitology, 14, 1–11.
Müller N, Zimmermann V, Hentrich B, Gottstein B (1996) Diagnosis of Neospora caninum and Toxoplasma gondii infection by PCR and DNA hybridization immunoassay. Journal of Clinical Microbiology, 34, 2850–2852.
Munhoz AD, Mineo TWP, Alessi AC, Lopes CWG, Machado RZ (2013) Assessment of experimental infection for dogs using Gallus gallus chorioallantoic membranes inoculated with Neospora caninum. Revista Brasileira de Parasitologia Veterinaria, 22, 565–570.
Ocal N, Atmaca HT, Albay MK, Deniz A, Kalender H et al. (2014) A new approach to Neospora caninum infection epidemiology: neosporosis in integrated and rural dairy farms in Turkey. Turkish Journal of Veterinary and Animal Science, 38, 161–168.
Taka A, Omata Y, Ohsawa T, Koyama T, Kanda M et al. (1999) Antibody reactivity in mice and cats to feline enteric stages of Toxoplasma gondii. Veterinary Parasitology, 83, 73–78.