NOTE *Parasitology*

Detection of *Neospora caninum* from Farm-Bred Young Blue Foxes (*Alopex lagopus*) in China

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ABSTRACT. *Neospora caninum* has been detected in several wild mammalian species, i.e., deers, coyotes, dingoes, and foxes. Farm-bred foxes were rarely reported to be affected by the parasite. In this study, we detected for the first time the infection of *N.caninum* in the farm-bred young blue foxes (*Alopex lagopus*) in China. Neospora-like tissue cysts were observed in brains and kidneys of the foxes by histopathological and immunohistochemical examinations. One hundred and three sera from the clinically normal vixens were tested for the presence of *N.caninum* and *Toxoplasma gondii* antibodies by two commercial ELISA test kits. Twenty-eight of 103 (27.2%) sera were positive for *N. caninum* and 1 serum (0.97%) was positive for *T. gondii*. A portion of the Nc5 gene of *N. caninum* was amplified from the DNA extracted from the fox brains by semi-nested PCR, further confirmed the existence of *N. caninum* among the farm-bred fox herd in China.

KEY WORDS: *Alopex lagopus*, *Neospora caninum*, seroprevalence.

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Neospora caninum is an obligate intracellular protozoan parasite with a worldwide distribution [9]. Since its first recognition in dogs in Norway and the description of a new genus *Neospora* and species *Neospora caninum* [3, 6], neosporosis has emerged as a serious disease causing fatal neuromuscular disease in dogs and abortion or stillbirth in cattles and other mammals of veterinary importance [2]. The full life-cycle of this parasite has not yet been elucidated. Tachyzoites, tissue cysts, and oocysts are considered to be the three infectious stages in its life-cycle [5]. Blue foxes (*Alopex lagopus*) are members of the *Canidae* family which are widely bred in China. Previous epidemiological studies have indicated that foxes have been exposed to *N. caninum*, and they can serve as intermediate hosts as well as definitive hosts [1, 11], though attempts to induce oocyst production have been unsuccessful [10]. Seroepidemiological studies have indicated that *N. caninum* were prevailing among red foxes in several countries [7]. However, meager information is available on the prevalence of *N. caninum* among foxes in China. In this study, serological, histopathologic, immunohistochemical and molecular evidence are presented, which implicate *N. caninum* for the first time in China as the main aetiological agent causing paralysis and death in farm-bred young blue foxes.

The foxes were bred in a farm located in Hebei province, China. The farm had more than 200 newborn foxes in 2007, the morbility of the group was more than 60%, and the mortality of the affected foxes exceeded 50%. After excluding the infection of a variety of pathogens such as pathogenic bacteria (e.g. *E. coli, Staphylococcus* and *Salmonella*) or viruses (e.g. fox distemper virus, parvovirus and encephalitis virus), a total of 103 fox sera were collected to test for antibody of *N. caninum* (c-ELISA VMRD Laboratories, Pullman, Washington, U.S.A.) and *T. gondii* (Anti-*Toxoplasma* IgG Antibody ELISA Kit, ZhuHai S.E.Z. HaiTai Biological Pharmaceuticals, Ltd, China) using commercial ELISA kits according to the manufacturer's recommendations.

Necropsy of blue foxes were performed according to the guidelines of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Samples of brain, heart, lung, liver, spleen, kidney and lymph node were fixed in 10% neutral buffered formalin at necropsy and processed routinely for histological examination. Fixed tissues were embedded in paraffin, sectioned, stained with haematoxylin and eosin (HE), Periodic Acid Schiff (PAS) and Giemsa, respectively. Immunohistochemistry was employed to study the above of formalin-fixed paraffin-embedded specimens. The primary antibodies were canine antisera against *N. caninum* and mouse antisera against *T. gondii* (kindly provided by Dr. Xuenan Xuan from Japan). *N. caninum*-negative canine sera were used to test non-specific background staining.

DNA was extracted from brain tissues of the young blue foxes. The genomic Nc5 region was selected as the target sequence for DNA amplification by semi-nested PCR using the *N. caninu*m-specific primer pair Np6/21, followed by amplification with secondary primers Np6/7 [12].

Of the 103 sera tested, twenty-eight reacted with *N. caninum*. Antibody to *T. gondii* was only found in one of serum

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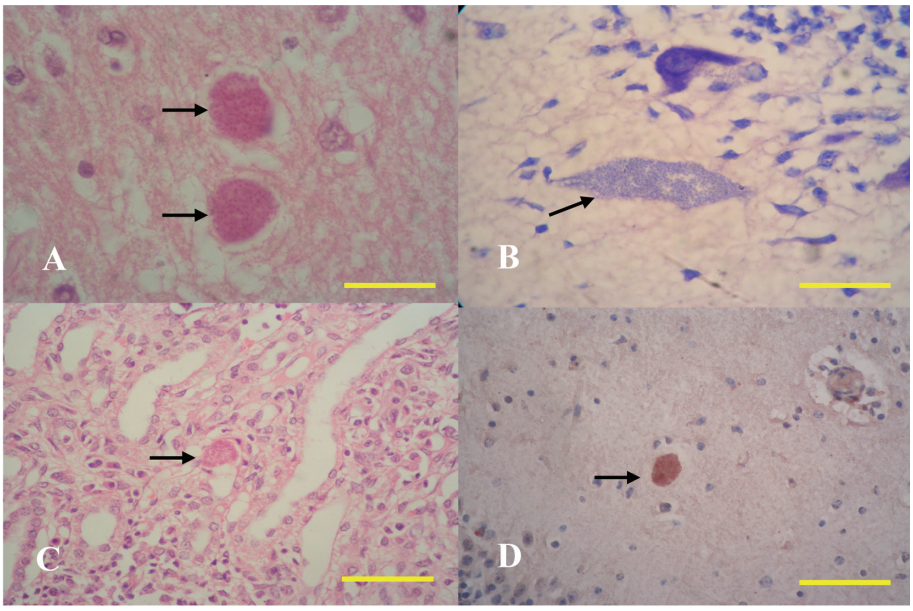

Fig. 1. Photomicrographs of formalin-fixed paraffin-embeded tissue sections. (A) *N. caninum* cysts in the cerebrum. HE stain. (B) A clump of tachyzoites of *N. caninum* around granular layer of the cerebellum. Giemsa stain. (C) *N. caninum* cyst in the renal tubules. PAS stain. (D) Tissue cyst in the cerebrum stained positively with the canine anti-*N. caninum* antisera. Scale bar in A and B=20 μm; scale bar in C and D=50 μm.

samples, which was negative reaction for *N. caninum*. Seropositive five foxes with *N. caninum* were small in size with clinical signs of coarse fur, inappetence, emaciation, fever, and particularly neurologic signs including depression, ataxia and paralysis. At necropsy, small foci of infarcts on the edge of the spleen were observed. There were visible foci of necrosis and rugosity in the surface of the kidney. The boundary between medulla and cortex was unclear. Cerebral pia mater was coarse, and hydrocephalus was visible in all five foxes.

Markedly congestion, small foci of necrosis were visible in the sections of the cerebrum, midbrain, cerebellum and spinal cord. Multifocal nonsuppurative encephalitis was observed, which consisted of perivascular cuffings of colloid cells. Many Neospora-like tissue cysts, round in shape and 25–100 μm in diameter with walls up to 3 μm thick, were found around the inflammatory foci in the cerebrum (Fig. 1A), and medulla oblongata. A cluster of tachyzoites were also found in the brain tissue (Fig. 1B). Immunohistochemically, tissue cysts in the cerebrum stained positively with the canine anti-*N. caninum* antisera (Fig. 1D), but showed no significant reaction to the mouse anti-*T. gondii* antisera and *N. caninum*-negative canine sera which exclude the possibility of non-specific background staining.

Following amplification of DNA from five foxes using *N. caninum* seminested PCR, 227bp products were observed (Fig. 2) from the positive control and four of five foxes (No. 2 sample was negative). Two PCRs using primers specific for *N. caninum* failed to yield an amplification products from negative control (*T. gondii* DNA and water). DNA sequencing of gel-cleaned PCR products and alignment of Nc5 sequences revealed 97–99% similarity to the Nc5 sequence of *N. caninum* (data not shown), thus providing a definitive genomic identification of the infection.

PCR plays an important role in the diagnosis of *N. caninum* infection. Specific partial Nc5 sequence of *N. caninum* was amplified by PCR from positive control and DNA of four foxes, PCR for *T. gondii* was negative. Since we observed Neospora-like tissue cysts or tachyzoites in all the five fox brains, the omit of detection by PCR may due to non-uniform distribution or very low number of parasites in tissues [4].

According to the previous documents, tissue cysts are more likely to form in the central nervous system rather than other tissues [6]. To our surprise, *N. caninum* tissue cysts have also been found in the renal tubules (2 of 5 foxes) with PAS staining (Fig. 1C). The thickness of the cyst wall in the tissues was the same as previously reported [8]. No cysts were observed in other tissues.

Recently, the first identification of *N. caninum* infection in aborted bovine foetus in China was reported [13], yet there was no report on *N. caninum* infection in foxes. The results of our study verified for the first time the prevalence of *N. caninum* in china. More fox sera need to be collected from other regions to delineate the significance of *N. caninum* as the main aetiological agent causing paralysis and death in farm-bred young blue foxes in China. This work is now carrying on in our laboratory.

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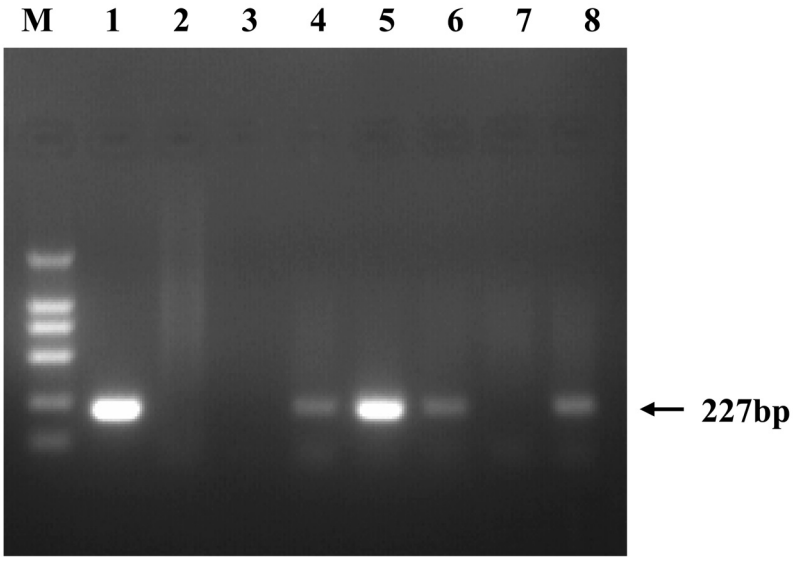

Fig. 2. Semi-nested PCR for *N. caninum* using Np6/Np7 primers. M=2 kb DNA ladder; Lane 1, Positive control (DNA of the Nc-1 isolate of *N. caninum*); Lane 2, Negative control (DNA of *T. gondii*); Lane 3, Negative control (Water); Lanes 4–8, DNA extracted from the fox brains, we note the specific amplification products of 227 bp in positive control and lanes 4, 5, 6, 8.

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