First molecular detection of *Neospora caninum* in red fox (*Vulpes vulpes*) brain sample in Türkiye

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ABSTRACT

The red fox is the wild carnivore with the widest distribution in the world. Thus, this animal acts as intermediate and final host for many parasite species. *Neospora caninum* is one of the most important protozoan agents causing abortion in cattle, sheep and goats in the world. The final hosts of *N. caninum* are domestic dogs and wild canids such as wolves and coyotes, while its intermediate hosts are domestic ruminants and many warm-blooded animals, including red foxes. The aim of this study was to research *N. caninum* in brain samples of three red foxes obtained from wildlife in Türkiye by using PCR. At the end of the study *N. caninum* DNA was detected in one of three brain samples. To the best of our knowledge, with this study, *N. caninum* was detected for the first time in a red fox brain sample in Türkiye.

The red fox is the wild carnivore with the broadest range both in the world and in Türkiye (9). They are final and intermediate hosts of many parasites and travel long distances due to their feeding and nest-seeking behaviors. They spread parasites to animal and human settlements. Therefore, it is very important to determine which parasites red foxes act as both intermediate and final hosts (11, 12, 15).

Neospora caninum is a coccidian protozoan in the Toxoplasmatidae family. The final host of *N. caninum* is canids (dog, wolf, coyote) and its intermediate hosts are warm-blooded animals such as cattle, small rodents, red foxes, and poultry (6). It is transmitted to active final hosts with consumption of infected intermediate host tissues. Infection of intermediate hosts occurs horizontally by oral ingestion of sporulated oocysts, or vertically in pregnant animals by transplacental transmission of tachyzoites to the offspring (6, 7).

Serological (ELISA, IFAT, agglutination test and immunoblotting) and molecular (PCR, real-time PCR) methods are used for the diagnosis of *N. caninum* in intermediate hosts (6). Although serological diagnostic methods are used in large-scale epidemiological studies, they may cause detection of false positives due to cross-reaction with some agents such as *Hammondia heydorni* (7). Molecular-based methods, on the other hand, have higher sensitivity and specificity than serological diagnostic methods. Thus, molecular-based methods are preferred in many studies examining *N. caninum* in intermediate hosts (1, 2, 6, 17).

Neospora caninum was detected in red foxes in Ireland (16), Spain (1), Belgium (3), Czechia (10), and Romania (17). However, there is no data on the presence and prevalence of *N. caninum* in red foxes in Türkiye. Purpose of this study was to investigate *N. caninum* in red fox brains obtained from wildlife by PCR and to

contribute to the literature by determining the state of the parasite in wildlife in Türkiye, where there is very little data.

The study material was composed with three red fox brains, two from Yozgat and one from Ankara. Total brain samples from red foxes were extracted as described by Munson et al. (13) and stored in a deep freezer at -20°C until use. Brains were examined for the presence of *N. caninum* with the digestion method described by Dubey et al. (6) with little modifications.

Brain samples were placed in the blender and mixed by adding 200 mL of Acid-Pepsin solution (6). The Blender was run at the highest speed for 30 seconds and the brain samples were homogenized. Homogenized samples were transferred to a beaker containing acid-pepsin solution at 37°C and digested in a heated magnetic stirrer for 5-6 hours until it completely dissolved. Undigested tissue pieces were removed by filtration through 250 micron sieves. The resulting mixture was left to sedimentation. At the end of the process, the upper liquid part was removed. The underlying sediment was mixed with PBS (pH: 7.4, Sigma Aldrich®, Germany) and transferred into 50 mL falcon tubes. The sedimentation process was repeated at least 5 times in falcon tubes to deport the acid-pepsin solution. Each time, the liquid above the sediment was removed and the remaining sediment was diluted with PBS and vortexed. The obtained sediment was stored in a deep freezer at -20°C until used for DNA isolation.

Genomic DNA was extracted from 200 µl sediment by using QIAamp® DNA Mini Kit (Qiagen®, Germany) according to the manufacturer's instructions and stored at -20°C until used in PCR. The primer pairs Np21 (5'-GTGCGTCCAATCCTGTAAC-3') and Np6 (5'-CAGTCAACCTACGTCTTCT-3'), which amplify the Nc-5 region of *N. caninum* at a size of 328 bp, were used in this study (18). PCR master mix with total volume of 25 µL was prepared with 14.375 µL DNase, RNase-free sterile distilled water, 2.5 µL 10× Taq Buffer with KCl, 2.5 µL 25 mM MgCl2, 1 µL dNTP mix (10 mM), 1 µL of each primers (10pmol/µL) 0.125 µL Taq DNA Polymerase (5 U/µL, ThermoScientific) and 2.5 µL template DNA. PCR was carried out as described by Gondim et al. (8).

The 9 µL of PCR product was mixed with 1 µL of 10× Blue Juice TM Gel Loading Buffer (Invitrogen®, Lithuania) and loaded onto a gel (1.5%) stained with SYBR™ Safe DNA Gel Stain (Invitrogen, USA). Electrophoresis was performed in a gel tank with 0.5×TBE buffer solution at 90 volts for 40 minutes, then the agarose gel was checked for the presence of specific bands in a UV transilluminator (3UV Benchtop Transilluminator, UVP®, Canada) and photographed.

A 328 bp product which is compatible with *N. caninum* was detected in one of the samples obtained from Ankara (Figure-1).

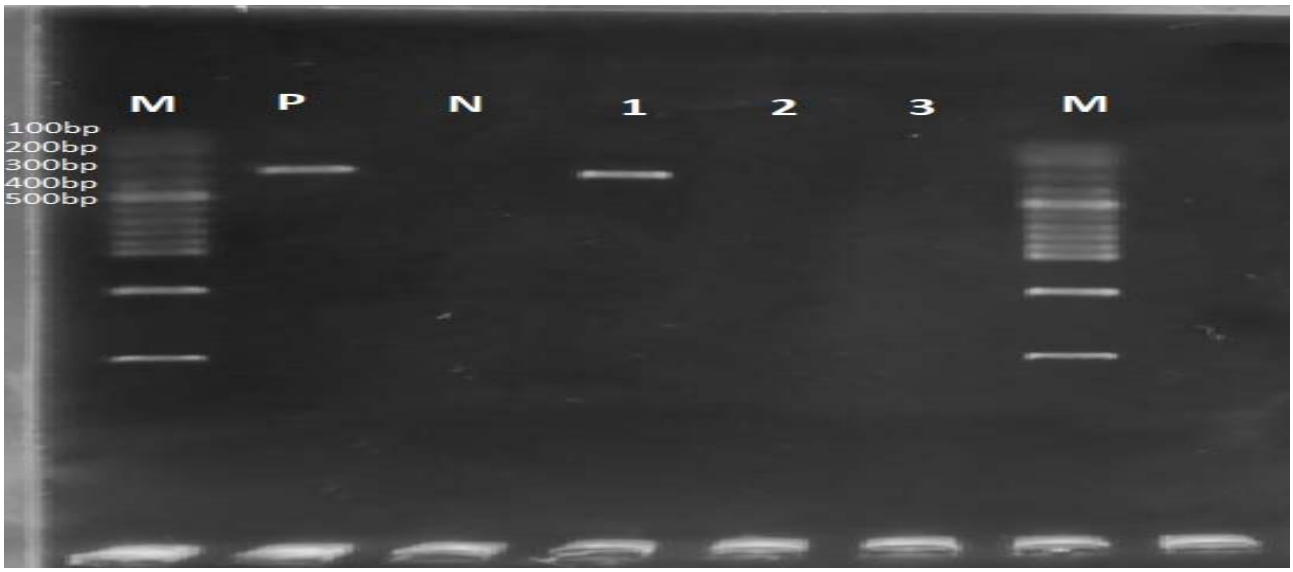


Figure 1. PCR results of *Neospora caninum* NC-5 region.
M: Marker
P: Positive Control
N: Negative Control
1: Positive Sample
2-3: Negative Samples.

Molecular-based methods (PCR and modifications) are important diagnostic tools for the identification of *N. caninum* in wild animals (6, 7). Molecular prevalence of *N. caninum* in the brain of red foxes was detected as 10.7% (13/122) in Spain (1), 5.96% (9/151) in Ireland (16), 4.8% (4/83) in Great Britain (2), 6.6% (20/304) in Belgium (3), 4.61% (7/152) in Czech Republic (10), and 0.54% (1/182) in Romania (17). In this study, we detected the *N. caninum* DNA in one of the three red fox brain samples which were coming from Yozgat and Ankara provinces, in Central Anatolia, Türkiye.

Neospora caninum is one of the most important protozoan agents that can cause abortion in farm animals such as cattle, sheep and goats and fatal neurological infections in canids (6, 7). The annual economic losses caused by the agent in cattle farms are 1.298 billion dollars globally (14) and it is estimated to be 40.5 million dollars in Türkiye (4). There are two separate cycles in the life cycle of *N. caninum*. One of them is the domestic cycle between domestic dogs and farm animals while the other is the sylvatic cycle that develops between wild canids (wolves, coyotes) and warm-blooded animals (deer, antelope, red fox and small rodents) (6). The presence of *N. caninum* in red foxes obtained from wild life in Türkiye was investigated for the first time by PCR in this study.

Red foxes are important intermediate hosts of *N. caninum* in the wildlife. Nowadays the population size of these animals is increasing especially in European countries due to the oral rabies vaccination program (9). With this increase, the parasites carried by the red foxes reach the wider geographical areas which are in contact with farm animals. Therefore, the determination of parasites in foxes is becoming a more important issue to prevent economic losses in farm animals and to protect public health (5, 9).

In conclusion, with this study, to best of our knowledge we detected the *N. caninum* in a red fox brain for the first time in Türkiye. We think that it is so important to detect the wild intermediate and final hosts of *N. caninum* for clear understanding the epidemiology and the control of the disease.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions (ÖRNEKTİR)

AB, BC, CD and DE conceived and planned the experiments. AB and DE carried out the experiments. AB, CD and DE planned and carried out the simulations. AB, BC, CD and DE contributed to sample preparation. AB, BC, CD and DE contributed to the interpretation of the results. AB took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethics statement

Permission to examine parasitic diseases in fox samples was obtained from Veterinary Control Central Research Institute Local Ethics Committee with the decision numbered 2017/02.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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