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Short communication

Prevalence of *Hepatozoon canis* infection in dogs from the area of Lublin Voivodship

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Abstract

Canine hepatozoonosis is a tick-borne protozoal disease. Two species of *Hepatozoon* may infect dogs: *Hepatozoon americanum* and *H. canis*. The aim of the paper was to attempt to detect the genetic material of *H. canis* in blood samples collected from dogs suspected to suffer from tick-borne diseases. 107 samples were tested with the use of the real-time PCR technique (Vcheck M Bionote analyser), of which 99 were collected from dogs which never left Polish territory (group 1) and 8 from dogs which spent the holidays with their owners in Turkey (group 2). DNA of *H. canis* was detected in 1 dog in group 1 (with *Ixodes ricinus* infestation), and in 2 dogs in group 2 (with *Ripicephalus sanguineus* infestation). The results obtained indicate that infections with *H. canis* should be taken into account and included in the differential diagnosis of vector-borne diseases in dogs in Poland, and the accurate identification of the infection agent is crucial for developing the correct treatment regimen and prognosis.

Keywords: *Hepatozoon canis*, dogs, PCR, Poland, vector-borne diseases

Introduction

Canine hepatozoonosis is a disease caused by *Hepatozoon*-apicomplexan parasites (family: *Hepatozoidae*) phylogenetically closely related to the piroplasms. All the *Hepatozoon* spp. share a basic life cycle that includes sexual development and sporogony in a haematophageous invertebrate definitive host, and merogony followed by gametogony in a vertebrate intermediate host (Baneth 2011). Two species of these parasites may infect dogs: *H. americanum* and *H. canis*. Canine hepatozoonosis caused by *H. canis* is a common infection

of dogs reported originally from the Old World and more recently also from South and North America, while *H. americanum* infections are reported exclusively in North America (Baneth 2011). *Hepatozoon* transmission takes place by ingestion of the definitive host (*R. sanguineus)* containing *Hepatozoon* oocysts by the intermediate host (dogs). It has been shown that some species of *Hepatozoon* are also transmitted by the predation of one vertebrate upon another infected vertebrate host (Smith 1996). Another mode of transmission was presented by Schäfer et al. (2022) who have confirmed that vertical transmission is a possible route of *H. canis* infection in dogs. The authors demonstrated the DNA of the pathogen in the stillborn puppy, as well as in the seven surviving puppies born by bitch infected by discussed parasites.

Clinical cases associated with infection by *H. canis* in dogs in Poland have been described for the first time previous year (Tołkacz et al. 2023). Unfortunately, the authors have not identified the source of infection. This raises a question of whether the invasions were accidental or *H. canis* infections in dogs in Poland occur but are not diagnosed. To answer this question, it is necessary to perform molecular monitoring towards *H. canis* infections. Confirmation of infections due to these parasite species is also important from a clinical point of view, because different species of *Hepatozoon* need different therapy protocols.

The aim of the paper was to attempt to detect the genetic material of *Hepatozoon* in blood samples collected from dogs suspected to suffer from tick-borne diseases.

Materials and Methods

The sample material contained whole blood taken from 107 dogs with anaemia confirmed by haematological examination, submitted to the Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin (Poland) between May 2023 and September 2023, to conduct the analysis for selected tick-borne diseases (babesiosis, anaplasmosis, ehrlichiosis, haemotropic mycoplasmosis, leishmaniasis, hepatozoonosis, bartonellosis). The study was conducted in accordance with the Directive of the European Parliament on the protection of animals used for scientific purposes (Directive 2010/63/EU). All owners of the dogs agreed to include them in the study. Blood sampling was a part of the clinical procedure – no Ethic Commission Agreement was required.

Each sample was labelled with a unique number without providing details about the owner of the dog. The examined samples have been collected from 99 dogs that have never left the territory of Poland (group 1), as well as from 8 dogs that returned from Turkey, where they spent holidays with their owners (group 2).

All blood samples were analysed in a Vcheck M Bionote analyser which isolated whole blood DNA, and amplified the DNA of *Leishmania* spp., *Babesia* spp., *Mycoplasma haemocanis*, *Hepatozoon* spp., *Ehrlichia canis, Anaplasma* spp., *Rickettsia rickettsi,* and *Bartonella* spp. in real-time PCR (Canine Vector 8 Panel). All DNA samples positive for *Hepatozoon* were additionally amplified and sequenced according to the procedure described by Tołkacz et al. (2023). PCR was performed using the forward primer Hep1 (5′-CGCGAAATTACCCAATT-3′) and reverse primer Hep2 (5′-CAGACCGGTTACTTTYAGCAG-3′) that amplified the fragment of 18S ribosomal RNA gene of *Hepatozoon* spp., with a size of 666-bp. Each reaction mixture (50 µL) contained 100 µM of each dNTP, 1.6 mM of MgCl2, 0.25 µM of each primer, 2.5 U of Taq DNA polymerase, and 5 µL of DNA template. PCR amplification was performed using a programmable thermal cycler (Biometra, Goettingen, Germany) with the following program: initial denaturation at 92°C for 2 min, 50 cycles of denaturation at 92°C for 60 s, annealing at 57°C for 60 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 5 min. The DNA sequence was determined on both strands using the same primers employed for PCR at a DNA sequencing core facility (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). DNA sequences were assembled and edited using SeqMan (DNAstar, Lasergene, Madison, USA), and Clustal V alignments to the published *H. canis* 18S rRNA gene: OP587280-OP587285 from Poland, MK757802 from Germany, KJ572976 from Hungary, GU371447 from Italy, and MG077085 from Turkey .

Results and Discussion

Out of 107 samples tested with the PCR method, the DNA of *Babes*ia was detected in twenty six, *Anaplasma phagocytophilum* in six, and *H. canis* in three blood samples (Fig. 1). No other pathogens were detected in the study. The animals infected with *B. canis* showed symptoms typical for babesiosis i.e. pale mucus membranes, apathy, anorexia, and bloody urine. The six dogs with *Anaplasma* infection did not show any signs of the disease. The three *H. canis* positive dogs (all males, mixed breed) came from the territory of Lubelskie Voivodeship (one dog from group 1 and two dogs from group 2), and showed symptoms

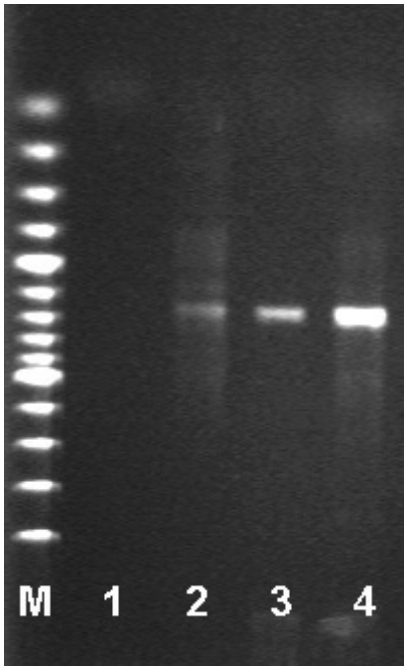

Fig. 1. PCR amplification of a partial sequence of *Hepatozoon canis* 18S RNA gene (product size: 660 bp) in blood samples from a Polish dog. (M) molecular weight marker = 100 bp; (1) negative control; (2-4) positive samples obtained in own study.

of severe apathy, anorexia, and muscles weakness. The dog that never left territory of Poland (aged 6 years) came from the village located near Lublin, while the two other dogs (aged 1 and 7 years) came from area of the city.

The Ct values of *H. canis* amplicons read from the amplification curve in real-time PCR fluctuated around 25-28 cycles.

The sequences of these three products showed a similarity of 98.2-100% with other analysed *H. canis* sequences from GenBank and were deposited under accession numbers: PP464233 (one sequence from dog of group 1) and PP464234 (two sequences from dogs of group 2).

In two dogs that returned from holidays, *R. sangiuneus* ticks were found on their skin. In the dog that never left the territory of Poland, infestation with *I. ricinus* was confirmed.

H. canis is transmitted by *R. sanguineus* ticks, which are found only occasionally in Poland (Szymanski 1979). The fact that in one infected dog, the only tick species which was found was *I. ricinus* indicates that more studies are needed to confirm the role of this tick species in the transmission of hepatozoonosis (Baneth 2011). Of course it should be remembered that the host needs to eat the vector to be infected with *Hepatozoon* species. It means that the presence of *Ixodes* tick on animals already infected is not a strong indicator of a possibility of a vector role.

The transfers of tick species out of areas of their natural distribution are divided into natural transfers (e.g., migration of ticks on hosts) and accidental transfers (e.g., resulting from the transport of livestock animals, trade in exotic animals, and transfers on animals during travel). It is important to monitor the occurrence of unknown tick species on hosts in Poland. Lack of the data how (and with which vector) the patient contracted the disease means that the reported cases of hepatozoonosis in terms of the epidemiology of the disease still remains a question.

Our results indicate that infections with *H. canis* should be taken into account and included in the differential diagnosis of vector-borne diseases (especially in dogs with anaemia). So far, the disease has been diagnosed in Polish dogs only sporadically; however, it has been reported in countries neighbouring Poland: Ukraine (Hamel et al. 2013), the Czech Republic (Mitkova et al. 2017), Slovakia (Majláthová et al. 2007) and Germany (Helm et al. 2020).

The diagnosis of hepatozoonosis cannot be achieved in each case in a straightforward manner by examining stained blood smears. Microscopic examination of the blood smears reveals the presence of *Hepatozoon* gamonts in the neutrophils of dogs with parasitaemia ranging from a few percent up to 50%, and subclinical infection to mild disease is usually associated with low parasitaemia (1–5%), so in the case of a suspicion of the disease it is advisable to perform a PCR assay with complementary primers for *Hepatozoon* genes (Baneth 2011).

The accurate identification of the species of parasite that causes the infection is crucial for developing the correct treatment regimen and prognosis. *H. canis* infection is treated with imidocarb dipropionate at 5-6 mg/kg every 14 days until parasites are no longer present in the blood (Baneth and Weigler 1997). In the described cases, the dogs were treated successfully with imidocarb.

The observations presented above indicate that hepatozoonosis should be considered in the differential diagnosis of vector-borne diseases. The cases of this infection, due to increased travelling of owners with pets in endemic areas for hepatozoonosis, and climate changes in many parts of the world including Poland, will probably be noted more and more often in the near future in areas free of this disease so far.

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