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Case Report

First case of clinical canine hepatozoonosis in Ukraine

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ABSTRACT

Canine hepatozoonosis is caused by the tick-borne protozoans *Hepatozoon canis* and *Hepatozoon americanum* (Apicomplexa, Hepatozoidae). While *H. americanum* is widespread in the south-central and south-eastern United States, *H. canis* is documented in Europe, Africa, Asia, and South America, and is transmitted to dogs by ingesting infected ticks, primarily *Rhipicephalus sanguineus*. In Europe, this vector is mainly distributed in the Mediterranean region but endemic areas within Central Europe have also been reported, possibly facilitated by climate change.

A mixed-breed male puppy, ~3–7 months-old, evacuated from Kherson City, Ukraine, was admitted to a private veterinary clinic in Kyiv in August 2023. There was no information about the owner and history of the animal. Physical examination evidenced fever, anorexia, lethargy, mild dehydration, and pale mucous membranes. Alopecia, purulent skin ulcers, and pruritus were also observed. On day 1 of admission, a 34 % hematocrit and elevated leukocyte counts, particularly granulocytes and lymphocytes, were observed. *H. canis* gamonts were detected in stained blood smears and confirmed by specific PCR. Treatment was initiated with imidocarb at 6.6 mg/kg i.m. at 15-day intervals for six weeks, and doxycycline 10 mg/kg orally once daily for two weeks. Blood counts improved between towards day 16, with initial increase and subsequent normalization of hematological parameters. *Hepatozoon*-PCR was still positive on day 16. The dog made a full recovery, and no further tests were done thereafter. The systemic clinical signs were likely parasite-induced. *H. canis* and its vector *R. sanguineus* are being increasingly reported in Europe outside of their original geographical distribution, the Mediterranean basin. This case confirms the geographical expansion of this protozoan parasite.

1. Introduction

The agents of hepatozoonosis (Apicomplexa, Adeleida, Hepatozoidae) are infecting different animal species [17] and especially carnivores [3,15,26]. Canine hepatozoonosis is caused by two species, *Hepatozoon americanum*, restricted to North America [39], and *H. canis*, which is common in the Mediterranean region in Europe. *H. canis* was also detected in cats [5]. The brown dog tick, *Rhipicephalus sanguineus* sensu lato, is considered the main vector for *H. canis* [6,20,21].

However, oocysts of *H. canis* were also observed in the hemocoel of *Haemaphysalis longicornis* and *Ha. flava* [32]. Over the past decade, there have been increasing reports of the presence of *H. canis* in other European countries, far from the Mediterranean basin [38]. The severity of hepatozoonosis depends on the level of infection and varies from sub-clinical to life-threatening disease. The meront stage can cause lesions in different organ systems (hepatitis, pneumonia, myositis) and the hematological system (non-regenerative anemia) [4,7,39]. Pruritic dermatitis localized mainly in the dorsal portions of the neck and thorax

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has been described in a *H. canis*-infected puppy [16]. Typical clinical signs of hepatozoonosis may include lethargy, weakness, deterioration of body condition, pallor of the mucous membranes, and changes in hematological parameters [4,35]. The acute course of hepatozoonosis in puppies is often associated with an underdeveloped immune system [35].

In the Ukraine, information on the presence of *H. canis* is limited [2,22], as well as on its transmission vectors. Here, to the authors' knowledge, we describe the first documented and confirmed case of hepatozoonosis with clinical signs in a puppy in Ukraine.

2. Case presentation

A mixed-breed male puppy evacuated from Kherson City, Ukraine (Fig. 1), was admitted to a private veterinary clinic in Kyiv in August 2023. No information about the puppy's owner or history was available. The estimated age was between three and seven months. The following was noted upon physical examination: hyperthermia, anorexia, lethargy, mild dehydration, and pale mucous membranes. The puppy also had the following skin-related conditions: alopecia, purulent skin ulcers, and pruritus. No ticks were found during the examination.

On day 1, a thin blood smear was prepared [19], then fixed and stained with a commercial kit (LEUCODIF 200, BLT00083, Erba Lachema s.r.o., Czech Republic). Microscopic studies were performed using a Nikon, Eclipse Ci-L plus microscope. *Hepatozoon* sp. gamonts were detected in the cytoplasm of neutrophils and monocytes (Fig. 2). We also detected single white blood cells containing two gamonts (Fig. 3). The average size of the *Hepatozoon* sp. gamonts was estimated. The maximum length and width of 100 gamonts and their nuclei were measured with the help of the scale bar provided by the microscopy imaging software (NIS-Elements D5.02.03) during microscopy (Nikon, Eclipse Ci-L plus). The mean length of the gamonts \pm standard deviation (SD) was $9.28 \pm 0.57 \mu\text{m}$ and the mean \pm SD width $4.07 \pm 0.50 \mu\text{m}$ (shape index 2.3). The mean length of gamont nuclei \pm SD was $4.16 \pm 0.63 \mu\text{m}$ and the mean \pm SD width $2.76 \pm 0.40 \mu\text{m}$ (shape index 1.5). In this blood smear observed under a digital slide scanning system NanoZoomer S360 (Hamamatsu, Shizuoka, Japan), 84.1 % of leukocytes contained *Hepatozoon* sp. gamonts. No other hemoparasites were observed in blood smears.

Complete blood cell count parameters are shown in Table 1. White blood cells (WBC) on day 1 were higher than reference intervals. Meanwhile, red blood cells (RBC), hemoglobin (HGB) and hematocrit (HCT) were below the reference intervals.

Additionally, serum samples were tested for the presence of antibodies to *Anaplasma phagocytophilum* and *Ehrlichia canis* (MegaELISA® ANAPLASMA phagocytophilum and MegaELISA® EHRlichia canis, MEGACOR, Austria). Both tests resulted negative, as well as a test for canine parvovirus antigen (CPV Ag, BioNote, Inc., Republic of Korea).

EDTA-blood samples were subjected to DNA extraction using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Conventional

polymerase chain reaction was performed using primers H14Hep-a18SFw (5'-GAAATAACAATACAAGGCTTAAAATGCT-3') and H14Hepa18SRv (5'-GTGCTGAAGGAGTCTGTTTATAAGA-3') targeting a fragment of the 18S ribosomal RNA (rRNA) of *Hepatozoon* spp. [23]. DNA amplification was done under the following conditions: 95 °C for 15 min followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min. Final extension was performed at 72 °C for 5 min. The DNA was additionally tested for cyst-forming coccidia parasites using the primers Sarco F and Sarco R according to the previous published protocols [9] and the results were negative. The amplicons were separated by 1.5 % agarose gel electrophoresis (stained with ethidium bromide) and observed under UV light. Amplicons with high DNA quality and quantity were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research Corp., Irvine, USA) and sequenced by a commercial laboratory (Microsynth AG, Balgach, Switzerland) using the same primers employed for PCR amplification.

A BLAST analysis (<https://blast.ncbi.nlm.nih.gov>) of the obtained 561 base-pair sequence with trimmed primer regions (GenBank Accession Number PQ315997) showed that the sequence was 100 % identical (coverage and identity) to reported *H. canis* entries from Europe, the Americas, and Asia (e.g., Accession Nr. PP574309, AY150067, KX712123, KP216462, KM115969, KM116003, KX816958, KM057841, KU569168, AY461375, OR814217, OR814220, OR814224, LC556379, MN393911, MK645959, OL774788 and LC169075).

Microscopical examinations and molecular tests confirmed a *H. canis* infection; therefore, the puppy (weighing 3.8 kg) was treated with imidocarb (Imidopiran®) at 6.6 mg/kg i.m. at 15-day intervals for six weeks and doxycycline 10 mg/kg orally once daily for three weeks from day 1.

On day 16 post admission and treatment initiation, the blood counts improved compared to the day 1, with initial elevation and subsequent normalizing of various leukocyte counts, along with stable (decreased) erythrocyte, and (normal) thrombocyte levels.

Analysis of three blood smears revealed rare *H. canis* gamonts in neutrophils on day 16. The PCR test for *Hepatozoon* sp. on day 16 was also still positive.

After 6 weeks and the last injection of Imidopiran®, the puppy was clinically recovered, and therefore no more blood smear PCRs to detect the presence of *H. canis* DNA were performed after day 16. We contacted the new owners on day 185 after the first admission and the start of treatment to ask about the animal's condition. It turned out that the animal's condition was satisfactory, there were no complaints about its health, and the owners sent an up-to-date photo of the animal (Fig. 4). The systemic clinical signs were therefore probably caused by the *H. canis* infection, while the etiology of the skin alterations remained unresolved.

3. Discussion

This is the first registered clinical case of hepatozoonosis in a dog with *H. canis* confirmed by microscopic and molecular methods in Ukraine. The sensitivity of the molecular methods according to previous studies is significantly higher than that of microscopy [25]. However, most PCR studies focused on the 18S rRNA locus need further nucleotide sequencing for definitive identification at the species level [10,14,15,27]. Although other markers, in particular 16S rRNA and ITS-1, are used to identify species of the genus *Hepatozoon* in different animal species, they have not been widely used for genotyping and molecular systematics. So, genomic data and new molecular markers are urgently needed for the implementation of effective strategies to detect and control *Hepatozoon* infections [29,37].

Hepatozoon canis is predominantly found in the Mediterranean region of Europe, in Africa, Asia and South America. However, in recent years, there have been increasing reports of *H. canis* infections in Europe occurring in regions outside the Mediterranean basin. The number of infected *H. canis* dogs increased in Poland [38] and as well as in the



Fig. 1. The direction of the puppy's relocation on the map.

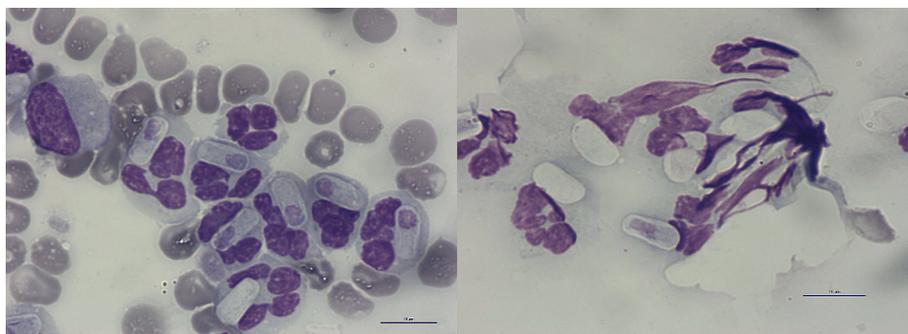


Fig. 2. *Hepatozoon canis* gamonts in segmented neutrophils from the blood smear of a puppy on day 1, stained with LEUCODIF 200.

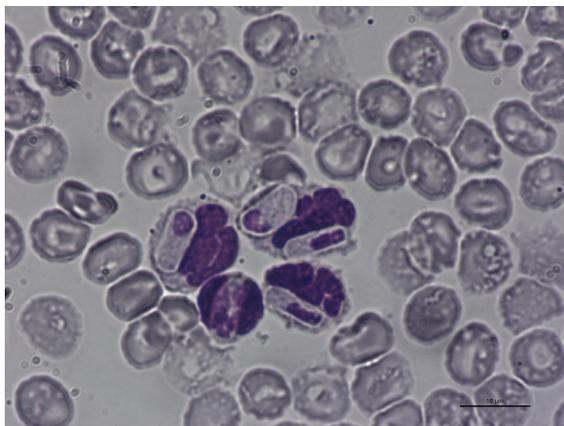


Fig. 3. Two *Hepatozoon canis* gamonts within a neutrophil in the blood smear of a puppy on day 1, stained with LEUCODIF 200.

Table 1

Complete blood cell count parameters on Day 1 and Day 16 according to hematological analyzer MicroCC-20 Plus (HTI, USA).

Parameter	Day 1 06.08.2023	Day 16 21.08.2023	Reference interval	Units
WBC (white blood cells)	50.2 ↑	10.5	6.0–17.0	$\times 10^9/l$
Lymphocytes	9.7 ↑	6.1 ↑	0.8–5.1	$\times 10^9/l$
MID (white blood cells except lymphocytes or granulocytes)	3.0 ↑	1.0	0.0–1.8	$\times 10^9/l$
GRA (granulocytes)	37.5 ↑	3.4 ↓	4.0–12.6	$\times 10^9/l$
RBC (red blood cells)	4.8 ↓	5.3 ↓	5.5–8.5	$\times 10^{12}/l$
HGB (hemoglobin)	103.0 ↓	114.0	110.0–190.0	g/l
MCH (mean cell volume)	21.6	21.5	20.0–25.0	p8
HCT (hematocrit)	34.0 ↓	37.1 ↓	39.0–56.0	%
PLT (platelet count)	162.0	197.0	117.0–460.0	$\times 10^9/l$
MPV (mean platelet volume)	9.0	8.6	7.0–12.9	fL

other countries bordering Ukraine: Hungary [24], Romania [11,12], Moldova [13] and Slovakia [31], while in other countries *Hepatozoon*-like infection rate has remained similar for decades [5]. According to Baneth and Weigler [4], *H. canis* infection level can vary not only depending on the territory but also on the season. The highest level of infection in dogs occurs from May to November, which coincides with the period when our case was detected (August).

The prevalence of *H. canis* is not well investigated in Ukraine, but infections have been previously reported. In Spring 2011 in Kyiv, *H. canis* was isolated from an EDTA-blood sample from one of 23 dogs

(1/23) [22]. Another study reported a high infection rate of *H. canis* infection in dogs that were moved from Ukraine to shelters in Poland in 2022 [2]. In this study among 53 dogs and 1 cat, *H. canis* DNA was identified in 27 dogs (51 %, 95 % CI 36.8–64.9) and one cat. However, since the authors were unaware from which region(s) these animals were moved, they could not establish the territorial prevalence of the parasitic agent in Ukraine [2].

During the examination of the puppy on the first day of admission to the clinic, no ticks or other ectoparasites were found. This means that the animal could have been infected either by (1) ingestion of a definitive host (most probably a *R. sanguineus* s.l. tick) or (2) in utero. According to previous studies, *R. sanguineus* s.l. ticks have been found on the coasts of the Black and Azov Seas [34], which coincides with the geographical location of Kherson, from where the puppy was moved in August. Transplacental transmission of *H. canis* is also possible, as shown in cats [5] and dogs [36].

The appearance of signs of the disease at an early age (in absence of ticks) also might indicate transplacental infection. In this case, one of the clinical signs may be skin lesions, in which, even in the absence of microscopically visible gamonts of *H. canis*, the presence of its DNA in skin biopsies can be detected [16]. According to another study [38], crusted skin lesions, particularly on the nose and in the perianal region, along with clinical signs such as weakness, lethargy, inappetence, generalized lymphadenopathy, tissue oedema and oozing, were observed in one of three *H. canis*-positive puppies aged 12–15 weeks, with transplacental transmission not excluded and *Babesia vulpes* co-infection. The latter case shows parallels with our findings; however, no co-infections were identified in our investigation. In the case we described, on day 1 of admission, the puppy also had alopecia of unknown etiology, purulent skin ulcers and itching, which disappeared by day 16. On the other hand, stress may have been a possible factor contributing to the high parasite burden observed, as the puppy was relocated to Kyiv from Kherson, and the latter had been in the epicenter of hostilities and a full-scale war for more than a year and a half, at the time of puppy's move. Finally, relocation could have been an additional stress factor and resulted in clinical signs before the animal was referred to a veterinary clinic.

Prompt diagnosis is difficult because parasite stages may become detectable only about one week after the onset of clinical signs. Therefore, it is possible to detect the parasite's gamonts by examining stained blood smears of the animals. Various methods and diagnostic kits are used for staining, among them are May-Grunwald Giemsa stain, Hemacolor stain and others. Different dyes can cause a noticeable difference in the staining of both *Hepatozoon* gamonts and blood cells [30]. Buffy coat is the most recommended material for such research [25,36], but is not always available, especially in case of suspected babesiosis, where a blood smear and whole blood for detection of pathogen DNA are mainly used for diagnosis. *H. canis* gamonts infect white blood cells, and are mainly found in monocytes [30] and neutrophils. The quantity of *H. canis*-infected white blood cells ranges from less than 1 % till 100 % and this level correlates with the severity of the disease [4]. In our case,

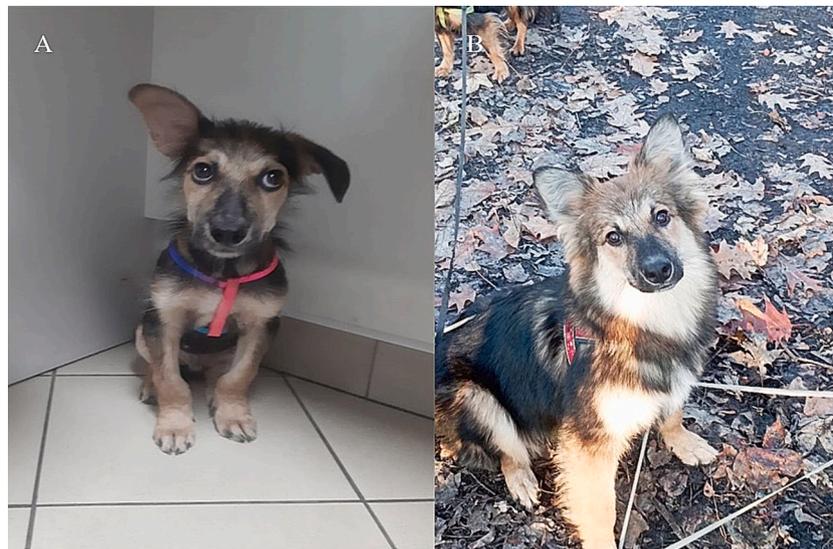


Fig. 4. Puppy on the day 1 (A) and day 185 (B).

the puppy with 84.1 % of leukocytes infected with *Hepatozoon canis* showed all the signs mentioned by the authors earlier, namely hyperthermia, anorexia, lethargy, mild dehydration, and pale mucous membranes [4,7].

According to the results of Aktas et al. [1] the measurements of *H. canis* gamonts were in the range of $9.28\text{--}11.56\ \mu\text{m} \times 4.22\text{--}6.70\ \mu\text{m}$. Compared with our results, the length of the parasite is completely consistent with the data of other researchers, but the width of the agent is slightly smaller. Despite this, the shape index of gamonts length and width is still the same [5]. It is important to note that in this case not only single, but occasionally even two gamonts in one neutrophil were found, which was not reported in the other publications. At the same time, deformation of the blood cells was visible, which is typical for parasitization by large-sized gamonts [39].

Hematological changes in the presence of *H. canis* in dogs are quite variable and correlate with the level and time of infection. According to Paiz et al. [33] leukocytosis was found in 39.1 % of the dogs with positive blood smears in Brazil. Leukocytosis as well as low values of hemoglobin and hematocrit are suggestive of acute inflammatory response to *H. canis* [35,36]. The latter is consistent with the changes in complete blood cell count parameters we observed in day 1.

Timely treatment may resolve clinical signs, but is often unable to completely eliminate the parasite, leading to a chronic carrier status. There are no officially approved medicines for dogs with clinical signs of hepatozoonosis [36]. The use of imidocarb dipropionate, toltrazuril/emodepside and clindamycin did not lead to elimination of *H. canis* gamonts when these compounds were used in different groups of naturally infected dogs [7,18]. At the same time, *H. canis* DNA remained detectable during the months of the study. This may be due to the localization of pathogens in tissues and especially in the bone marrow, where the bioavailability of the above compounds is minimal [18].

The most used compounds for the treatment of dogs with clinical signs of hepatozoonosis are imidocarb dipropionate [36] and doxycycline [28,35]. However, according to the literature, it is possible to use them in combination with tetracycline [4], toltrazuril, or trimethoprim-sulfonamide [7]. Imidocarb in combination with doxycycline was also effective to resolve clinical hepatozoonosis due to *Hepatozoon felis* in a cat from Austria [8]. The combination of imidocarb dipropionate and doxycycline was also used in our case and was effective to reach a clinical improvement. However, evaluation of new protocols for *H. canis* infection treatment is essential.

The present case of hepatozoonosis in a puppy is important for both scientists and veterinarians, as it highlights the need for awareness of

vector-borne diseases and its expanding geographical distribution, as well as appropriate diagnosis and management of this disease and its vectors, ixodid ticks.

4. Conclusions

In this study, we describe for the first time a case of *H. canis* infection in a puppy with clinical signs in the Ukraine. The level of infection of blood leukocytes was 84.1 %. Since such cases of clinical manifestations of the disease have not been previously reported in Ukraine, our study will help veterinary practitioners diagnose and treat canine hepatozoonosis. Additionally, we aimed to create an impetus for the wider study of the prevalence of *H. canis* among dogs, cats, and wild carnivores as well as among vectors of the disease, ticks.

CRediT authorship contribution statement

Maryna Galat: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Diana Gliga:** Writing – review & editing, Investigation. **Vladyslava Storozhuk:** Writing – original draft, Investigation. **Oleksandr Paraska:** Writing – review & editing, Data curation. **Olena Semenko:** Writing – review & editing, Investigation. **Sergii Honcharov:** Writing – review & editing, Investigation. **Hans-Peter Fuehrer:** Writing – review & editing. **Gaston Moré:** Writing – review & editing, Investigation. **Walter Basso:** Writing – review & editing, Supervision, Methodology, Investigation. **Britta Lundström-Stadelmann:** Writing – review & editing, Supervision. **Caroline F. Frey:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Ethical statement

The authors declare that ethical report is not necessary because the study did not include any experimentation on animals.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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