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Babesia spp. and *Hepatozoon* spp. infections in wolves (*Canis lupus*) in Lithuania

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Abstract

European grey wolves (*Canis lupus*) may act as reservoirs or incidental hosts for several protozoan parasites, including *Babesia* spp. and *Hepatozoon* spp., which are relevant for both wildlife and domestic animals. This study aimed to investigate the occurrence of *Babesia* spp. and *Hepatozoon* spp. in free-ranging wolves from Lithuania and to characterise the detected parasites using molecular and phylogenetic methods. Screening for the presence of protozoan pathogens was performed by testing spleen samples from 70 wolves with PCR. Overall, 59 of 70 wolves (84.3%) were infected with at least one pathogen. *Hepatozoon canis* was highly prevalent, detected in 58 wolves (82.9%), while *Babesia canis* was found in 21 individuals (30.0%). The co-infection of *B. canis* and *H. canis* was found in 20 wolves (28.6%). This study provides the first molecular evidence of *B. canis* and *H. canis* infections in wolves from Lithuania, representing, to the best of our knowledge, the northernmost record of these pathogens in European wolf populations.

Keywords: grey wolves; *Canis lupus*; *Hepatozoon canis*; *Babesia canis*; Lithuania

Introduction

The European grey wolf (*Canis lupus*) plays an important ecological role as a top predator, contributing to the maintenance of natural ecosystems balance (Walker et al. 2018). In addition to their ecological role, wolves can act as potential reservoirs and carriers of various pathogens circulating in wildlife populations (Battisti et al. 2020, Wymazał et al. 2024). In recent years, studies on vector-borne pathogens in wolves have increased across Europe (Battisti et al. 2020, Hodžić et al. 2020, Kuručki et al. 2022, Tołkacz et al. 2023, Wymazał et al. 2024, Fayos et al. 2025, Remesar et al. 2025). As urban areas expand and wild animals increasingly interact with humans and domestic animals, the risk of pathogen transmission commonly associated with wildlife is growing (Mackenstedt et al. 2015). *Babesia* spp. and *Hepatozoon* spp. are tick-borne protozoan parasites that infect both domestic and wild animals (Baneth 2011, Baker et al. 2024).

Babesia spp. are intraerythrocytic parasites of the phylum Apicomplexa that infect the red blood cells of various mammals. These parasites are primarily transmitted through the bite of infected ixodid ticks, with vector species varying depending on the *Babesia* species involved (Gray et al. 2010, Martínez-García et al. 2021). In Europe, *Babesia canis*, the most commonly reported species in canids, is transmitted primarily by *Dermacentor reticulatus* ticks (Mierzejewska et al. 2018). Other species, such as *Babesia capreoli* and the badger-associated *Babesia* sp., have also been detected in wild carnivores, including wolves, foxes, and badgers (Battisti et al. 2020, Ortuño et al. 2022). Although domestic dogs are considered the primary hosts for *B. canis*, studies in free-ranging wolves have revealed that they can also be infected, suggesting a potential role for wolves as reservoirs (Solano-Gallego et al. 2016, Wymazał et al. 2024, Fayos et al. 2025). The reported prevalence of infection in wolves varies considerably by region (Battisti et al. 2020, Fayos et al. 2025).

Hepatozoon spp. are apicomplexan protozoan parasites that primarily infect leukocytes and muscle fibres in canids. Transmission occurs through the ingestion of infected ticks, most commonly *Rhipicephalus sanguineus* (Baneth 2011). In Europe, *Hepatozoon canis* is the predominant species reported in both wild and domestic canids. While domestic dogs are considered the primary host, increasing evidence shows that wild carnivores, including foxes, jackals, and wolves, are frequently infected (Battisti et al. 2020, Helm et al. 2020, Kuručki et al. 2022, Tołkacz et al. 2023, Kuručki et al. 2024). Prevalence in wolves varies widely across studies, with particularly high infection rates reported in southern Europe (Battisti et al. 2020, Hodžić et al. 2020, Tołkacz et al. 2023).

In the Baltic region, including Lithuania, studies on vector-borne protozoan pathogens in wolves are still lacking. To date, no comprehensive investigations of the occurrence of *Babesia* spp. and *Hepatozoon* spp. in free-ranging wolves have been conducted in the Baltic States.

Considering the ecological role of wolves as potential reservoirs for pathogens circulating at the wildlife–domestic–human interface, such studies are essential for both wildlife health monitoring and broader epidemiological surveillance. Therefore, this study aimed to investigate the occurrence of *Babesia* spp. and *Hepatozoon* spp. in wolves in Lithuania, to perform molecular identification and phylogenetic analysis of the detected pathogens, and to evaluate the prevalence of co-infections.

Material and methods

Sample collection and DNA extraction

Spleen samples were obtained from 70 legally hunted wolves between 2018 and 2024. The grey wolves originated from ten districts of Lithuania (Figure 1). Genomic DNA was isolated from spleen tissue using a Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) according to the manufacturer's protocol. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Lithuania). At that point the samples were adjusted to a working concentration of 100 ng/µL. All extracted DNA was stored at –20°C until subsequent molecular analyses.

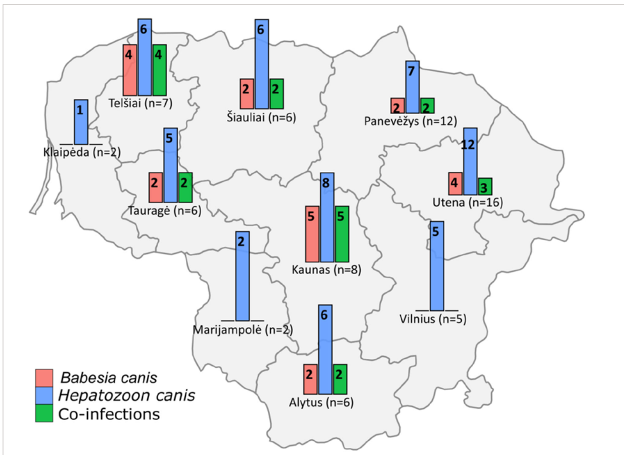

Figure 1. District-level infection rates in Lithuania for *Babesia canis*, *Hepatozoon canis*, and co-infections

Three mini bars per county show % positive; numbers inside bars indicate positive counts (shown only if > 0), and county labels include sample size ($n = x$).

Amplification of pathogen DNA

All samples were analysed for the presence of *Babesia* spp. and *Hepatozoon* spp., using conventional and nested PCR assays (Table 1). For *Babesia* detection, a nested PCR targeting the *18S rRNA* gene was performed using two sets of primers, BS1/BS2 and PiroA/PiroC. Each 20 µL reaction mixture for both PCR rounds contained 1x MyTaq Mix (Bioline, USA), 0.5 µM of each primer, 2U of MyTaq Polymerase (Bioline, USA), nuclease-free water, and 2 µL of DNA template for the first round, or 2 µL of the first-round PCR product for the second round. For *Hepatozoon* detection, the *18S rRNA* gene was targeted using primers HepF and HepR. A 20 µL reaction mixture contained 1x DreamTaq Green Master Mix (Thermo Fisher Scientific, Lithuania), 0.5 µM of each primer, nuclease-free water, and 2 µL of DNA template.

Each PCR assay included both positive and negative controls. For the negative control, nuclease-free water was used, while pathogen DNA samples previously verified by sequencing served as positive controls. Amplified products were visualised on 1.5% agarose gels stained with ethidium bromide. Bands corresponding to positive samples were excised and purified using the GenJet PCR purification kit (Thermo Fisher Scientific, Vilnius, Lithuania). After that point, they were sent to Macrogen Europe B.V. (Amsterdam, The Netherlands) for sequencing.

Data analysis

The map of infection rates was generated using R, version 4.3.1 (R Core Team 2023), while the obtained sequence data were processed and edited with the MEGA X software package (Kumar et al. 2018). Sequence similarity searches were carried out using BLASTn in GenBank (Altschul et al. 1990, 1997, Camacho et al. 2009, Sayers et al. 2019, National Centre for Biotechnology Information n.d.), and phylogenetic relationships were inferred using the Maximum Likelihood method with 1,000 bootstrap replicates. Representative sequences from this study were deposited in GenBank under accession numbers PX227340 and PX232811 for the *18S rRNA* gene of *Babesia canis*, and PX227314–PX227317 for the *18S rRNA* gene of *Hepatozoon canis*.

Table 1. Primers and PCR protocols used for amplification of tick-borne pathogens in the present study

Pathogens	Target gene, size	Primer sequence 5′→3′	PCR conditions	Reference
Babesia spp.	*18S rRNA*, ~ 380 bp	PCR1 BS1: GACGGTAGGGTATTGGCCT BS2: ATTCACCGGATCACTCGATC	94°C 3 min, 35x (94°C 60 s, 58°C 60 s, 72°C 90 s), 72°C 3 min	Armstrong et al. 1998
		PCR2 PiroA: AATACCCAATCCTGACACAGGG PiroC: CCAACAAAATAGAACCAAAGTCCTAC	94°C 3 min, 35x (94°C 60 s, 64°C 60 s, 72°C 90 s), 72°C 3 min	
Hepatozoon spp.	*18S rRNA*, ~ 625 bp	PCR HepF: ATACATGAGCAAAATCTCAAC HepR: CTTATTATTCCATGCTGCAG	95°C 5 min, 35x (94°C 60 s, 53°C 30 s, 72°C 60 s), 72°C 10 min	Inokuma et al. 2002

Results

Of the 70 wolves examined, 59 (84.3%) were infected with at least one pathogen. *Hepatozoon* spp. was the most prevalent, detected in 58 individuals (82.9%), while *Babesia* spp. was found in 21 wolves (30.0%). A total of 20 (28.6%) of the *Babesia*-positive wolves were co-infected with *Hepatozoon* spp.

Phylogenetic analysis revealed the presence of two parasite species, *Babesia canis* and *Hepatozoon canis*, in Lithuanian wolves. Two genotypes of *B. canis* were identified (Figure 2). The first genotype sequence (PX227340), representing 17 individuals, showed 100% identity with *B. canis* sequences previously reported from canids, equids, and ticks in various European countries. The second genotype sequence (PX232811), representing four individuals, showed identity with *B. canis* sequences previously reported in GenBank from animals of the canid family (golden jackal, red fox, dog) and from ticks. Meanwhile, four genotypes of *H. canis* were identified (PX227314–PX227317) (Figure 3). The *H. canis* genotypes detected in wolves showed 100% identity to sequences obtained from carnivores and ticks in Europe.

Infected wolves were detected in all sampled districts, but prevalence varied geographically. The highest proportion of *B. canis*-positive wolves was recorded in the districts of Kaunas (5/8; 62.5%) and Telšiai (4/7; 57.1%), while only negative samples were found in Klaipėda (0/2; 0.0%), Marijampolė (0/2; 0.0%), and Vilnius (0/5; 0.0%) (Figure 1). In contrast, *H. canis* prevalence reached 100% in five districts: Alytus, Kaunas, Marijampolė, Šiauliai, and Vilnius. The lowest proportion of *H. canis* was observed in Klaipėda (1/2; 50.0%). It should be emphasised that differences in sample size among districts may influence the observed variation in infection rates.

Discussion and conclusions

This study provides the first molecular evidence of *B. canis* and *H. canis* in European wolves from Lithuania, representing, to the best of our knowledge, the northernmost record of these pathogens in wolf populations reported to date. Previously, such infections in wolves had only been documented further south, including recent records from Poland (Tołkacz et al. 2023, Wymazał et al. 2024).

In the present study, *B. canis* was detected in 30.0% of examined wolves. The prevalence of *B. canis* in wolves is highly variable across Europe, ranging from 0.9% in Italy to 3.1–6.3% in Spain (Fayos et al. 2025, Remesar et al. 2025), 10% in the Czech Republic (Daněk et al. 2023), 18% in Poland (Wymazał et al. 2024), and 31.8% in Croatia (Beck et al. 2017). Several other *Babesia* species have occasionally been detected in wolves, but generally at lower prevalence than *B. canis*. *Babesia capreoli*, a parasite commonly associated with cervids, has been identified

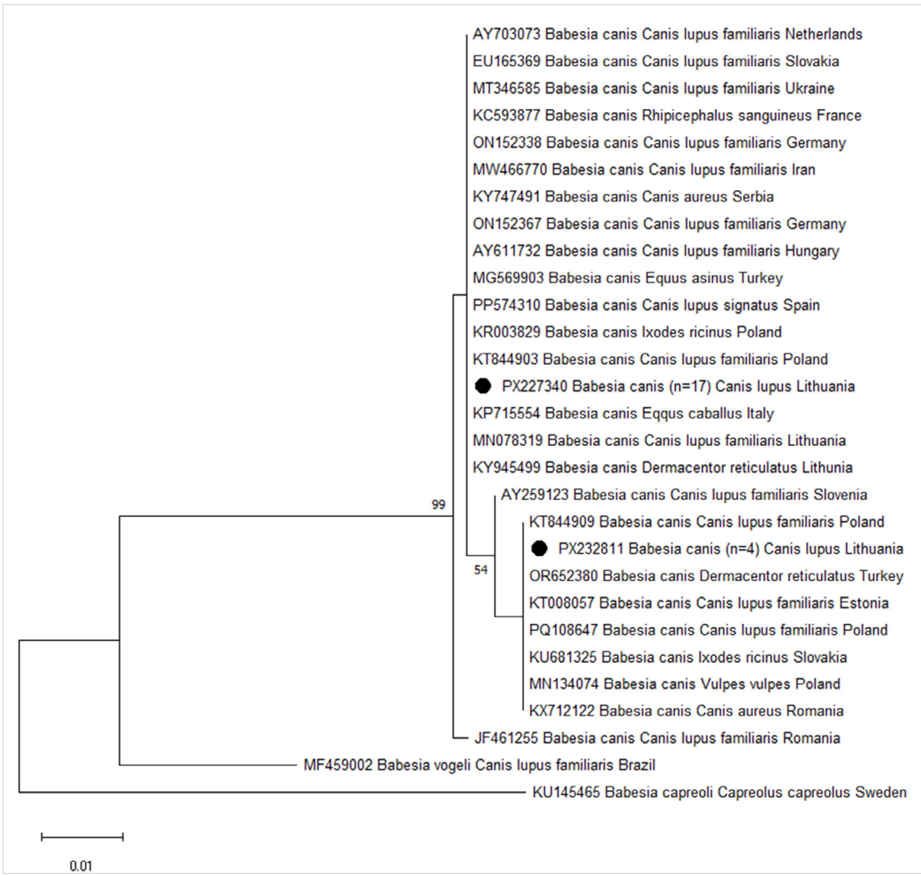

Figure 2. Phylogenetic tree of partial sequences of the *18S rRNA* gene of *Babesia canis*

Samples isolated from wolves in this study are marked with •. The number of samples represented by the sequence is given in parentheses ($n = x$).

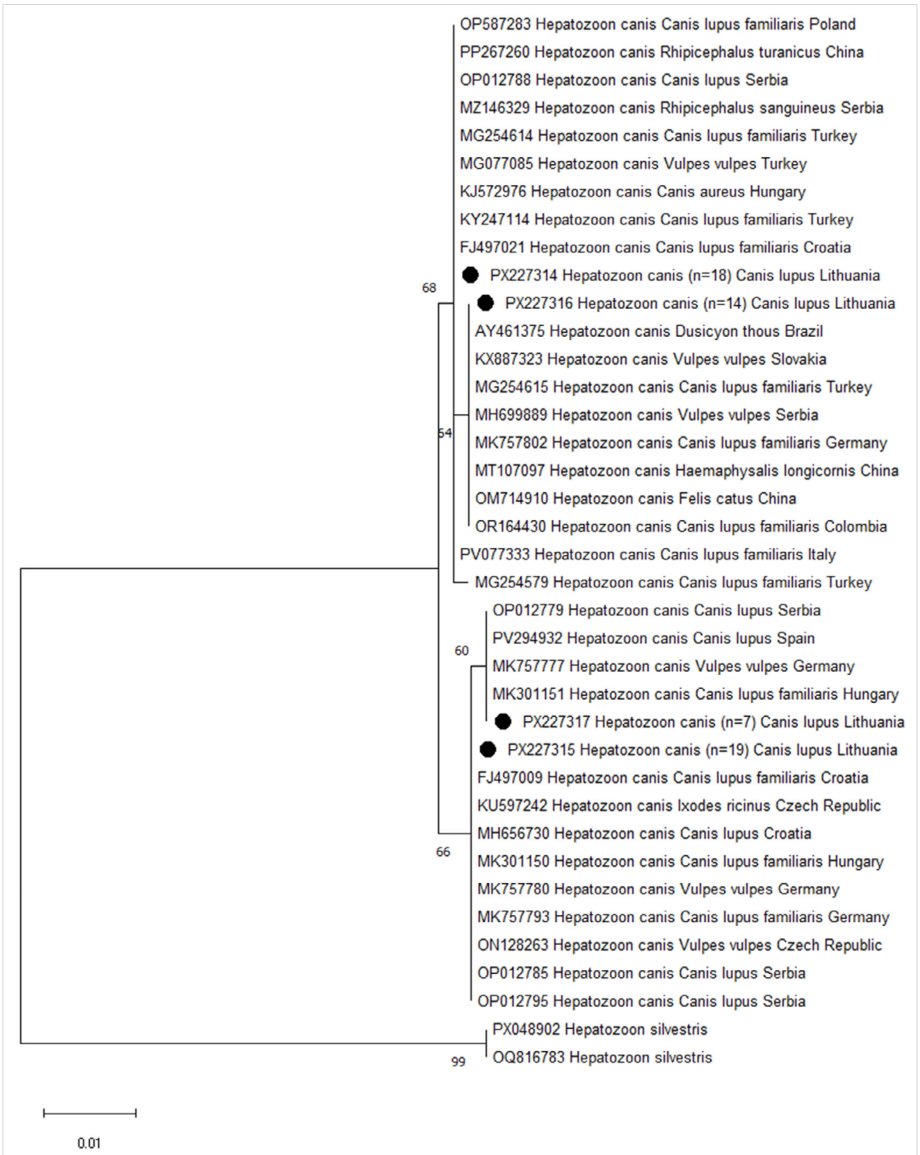

Figure 3. Phylogenetic tree of the *18S rRNA* gene of *Hepatozoon canis*

Samples isolated from wolves in this study are marked with •. The number of samples represented by the sequence is given in parentheses ($n = x$).

in wolves in Italy (9.1%) and Spain (1.6%) (Battisti et al. 2020, Remesar et al. 2025). A single case of badger-associated *Babesia* sp. type A has been reported in a wolf from Italy, indicating potential cross-species transmission among sympatric carnivores (Santoro et al. 2019).

Babesia canis is transmitted mainly by the ornate dog tick, *Dermacentor reticulatus*, which is widespread across large parts of Central and Eastern Europe, including Lithuania (Mierzejewska et al. 2018, Radzijevskaja et al. 2018). The presence of both the parasite in wolves and its competent vector in the region strongly suggests that local transmission cycles are maintained in the sylvatic environment. Given that *D. reticulatus* also feeds on the blood of domestic dogs, the overlap of vector habitats with wolf territories may facilitate circulation of the parasite between wild and domestic hosts, highlighting the epidemiological importance of wolves in areas where the vector is abundant.

In this study, *H. canis* was detected in 82.9% of wolves, representing a remarkably high prevalence compared with reports from other European countries. Similarly, high infection rates have been documented in wolves from southern regions, including Italy (75.8–92%) (Battisti et al. 2020, Remesar et al. 2025), Spain (93–96.3%) (Fayos et al. 2025, Remesar et al. 2025), Portugal (86%) (Remesar et al. 2025), and Serbia (57.9%) (Kuručki et al. 2022). In contrast, studies from Central Europe, such as those from Poland and Germany, reported lower prevalence rates (35% and 46%, respectively) (Hodžić et al. 2020, Tołkacz et al. 2023). The detection of *H. canis* at such a high frequency in Lithuania suggests that this parasite is well established in the local wolf population.

The brown dog tick, *Rhipicephalus sanguineus*, is recognised as the principal vector of *H. canis* in Europe (Baneth 2011). However, its presence has not been documented in Lithuania or the neighbouring Baltic countries. Instead, other tick species occurring in the region may contribute to parasite transmission. Previous studies have shown that *H. canis* DNA has been detected in extra tick species, including *Haemaphysalis concinna*, *Dermacentor marginatus*, *D. reticulatus*, and *Ixodes ricinus*, suggesting their possible role in the transmission of this pathogen (Hornok et al. 2013, 2022). Notably, three species of the mentioned ticks: *I. ricinus*, *D. reticulatus* and *H. concinna*, are observed in Lithuania (Paulauskas et al. 2015, Paulauskas et al. 2016, Paulauskas et al. 2020). While these findings suggest a broader range of potential vectors, experimental confirmation of their competence is still lacking.

Co-infections with *B. canis* and *H. canis* were observed in this study (28.6%). Similar cases have been reported in southern Europe, including a recent description of a fatal case of babesiosis in an Iberian wolf (*Canis lupus signatus*) co-infected with *H. canis* (Fayos et al. 2025, Remesar et al. 2025). Co-infections frequently occur in wildlife, and the simultaneous presence of multiple pathogens may weaken the host's immune system, thereby facilitating disease development (Fayos et al. 2025). However, in our study, the health status of the examined wolves was not available, and therefore, the potential clinical impact of these infections could not be evaluated.

These findings emphasise the epidemiological relevance of wolves in the circulation of vector-borne pathogens in the Baltic region. Further research combining pathogen detection, vector ecology, and health assessments of wolves and other wildlife is essential for a better understanding of the dynamics of these infections.

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