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RESEARCH ARTICLE

First Molecular Identification of *Babesia ovis* in Dogs: An Unexpected Host

Mehmet Can Ulucesme[1], Aleyna Karoglu[1,2], Begum Barutcuoglu[1], Munir Aktas[1] and Sezayi Ozubek[1*]

[1]Department of Parasitology, Faculty of Veterinary Medicine, University of Fırat, Elazığ 23200, Türkiye
[2]Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, United Kingdom
*Corresponding author: sozubek@firat.edu.tr

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A B S T R A C T

Babesiosis, a tick-borne disease caused by various *Babesia* species, is primarily associated with livestock but has increasingly been reported in dogs worldwide. This research investigates the prevalence of *Babesia* species in shelter dogs located in Diyarbakır province, Türkiye. Blood samples from 80 asymptomatic dogs were processed for DNA extraction, followed by nested PCR (nPCR) to amplify the 18S *rRNA* gene of *Babesia* (*B.*) and *Theileria* (*T.*) species. Sequencing and subsequent phylogenetic analysis of the positive cases identified *B. ovis* in 9 dogs (11.2%) and *B. vogeli* in 2 dogs (2.5%), marking the first molecular confirmation of *B. ovis* in dogs. Besides the two species of *Babesia*, *Toxoplasma gondii* was also detected incidentally from one sample. These findings provide novel evidence of *B. ovis* infection in dogs, highlighting potential cross-species transmission. The study underscores the critical need for continuous surveillance and molecular diagnostics to enhance our knowledge of tick-borne pathogen in dogs and their potential zoonotic risks. Further research is essential to clarify the role of dogs in the *B. ovis* transmission dynamics and to develop more targeted control strategies.

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INTRODUCTION

Babesia (*B.*) was first identified by Romanian scientist Victor Babes in the late nineteenth century while investigating febrile hemoglobinuria in cattle. The parasite was subsequently described in dogs in Italy just seven years after this initial discovery (Piana and Galli-Valerio, 1895). Different *Babesia* species can cause canine babesiosis, which appears clinically as haemolysis, anemia, thrombocytopenia, haemoglobinuria and fever (Solano-Gallego *et al.*, 2016; Zygner *et al.*, 2023). In dogs, various genetically characterized *Babesia* species such as *B. vogeli*, *B. canis*, *B. rossi*, and *B. gibsoni* have been identified, all of which belong to the *Babesia* sensu stricto group. In addition to these, other *Babesia* species like *B. vulpes*, *B. conradae*, and *B. negevi* have also been detected in dogs (Solano-Gallego *et al.*, 2016; Jalovecka *et al.*, 2019; Baneth *et al.*, 2020). *Babesia* species in canids are morphologically classified into two primary groups: large forms, measuring between 2.5-5.0µm, and small forms, ranging from 1.0-2.5µm. This classification is based on the morphology of the parasite during its intra-erythrocytic stages. Among these species, *B. canis*, *B. vogeli*, and *B. rossi* fall into the 'large' *Babesia* category, while *B. gibsoni*, *B. vulpes*, *B. conradae*, and *B. negevi* are classified as 'small' *Babesia* species (Birkenheuer, 2021). A recently identified species, *B. negevi*, has been documented in dogs from Israel, Palestine, and Jordan (Baneth *et al.*, 2020; Far *et al.*, 2021).

Depending on the *Babesia* species and the host's immune status, infection can result in mild, moderate, or severe disease. *Haemaphysalis elliptica* is the vector for *B. rossi*, which causes the most severe form of the disease in domestic dogs. This tick species is found exclusively in sub-Saharan Africa. It's prevalent particularly in regions like South Africa and Nigeria, and is likely widespread across the sub-Saharan area (Zygner *et al.*, 2023). *B. vogeli* infections are often mild or asymptomatic in adult dogs, but may lead to severe anemia in young animals. This widely distributed parasite of *Babesia* species is transmitted by *Rhipicephalus* (*R.*) *sanguineus* (Solano-Gallego *et al.*, 2016). Infections from *B. canis* are typically less severe than those from *B. rossi*, but both species have the potential to cause acute babesiosis. *B. canis* (sensu stricto), transmitted by *Dermacentor reticulatus*, was once limited to Europe but has recently been reported in China (Matijatko *et al.*, 2012). Infections caused by *B. negevi* in dogs are characterized by anemia, thrombocytopenia, pale mucous membranes, as reported in all known cases. Although the vector for *B. negevi* is still unidentified, *Ornithodoros tholozani* is suspected (Baneth *et al.*, 2020).

Molecular investigations in Türkiye have detected the presence of *B. canis*, *B. gibsoni*, and *B. vogeli* in dogs (Aktas and Ozubek, 2017; Bilgin *et al.*, 2019; Ceylan *et al.*, 2021), with clinical cases associated with *B. canis* (Gokce *et al.*, 2013) and *B. gibsoni* (Gülanber *et al.*, 2006). Additionally, *B. rossi* has been detected in *Haemaphysalis parva* ticks collected from humans (Orkun *et al.*, 2014) and wild boars (Orkun and Karaer, 2017). In foxes, *B. vulpes*, a species known to cause clinical infections in dogs, has also been reported (Orkun and Karaer, 2017). Furthermore, a novel, unnamed *Babesia* species was recently detected in dogs in Türkiye (Aktas and Ozubek, 2017).

The aim of this study was to identify and genetically characterize *Babesia* species infecting shelter dogs in Türkiye. By using molecular techniques and sequencing, we aimed to confirm the presence and identify the species, thereby enhancing the understanding of their epidemiology and supporting the development of targeted control measures for canine babesiosis in the region.

MATERIALS AND METHODS

Study area: The study was conducted between May and July 2023 in Diyarbakir province, located in the eastern part of Türkiye (Fig. 1). The harsh continental climate of this region is characterized by hot summers, with July temperatures often reaching 35°C. The considerable difference in temperature between winter and summer in Diyarbakir highlights the region's strong continental climate, characterized by hot summers and cold winters. The average annual temperature is normally between 17 and 18°C, with an annual precipitation 350 to 400mm (Toprak *et al*., 2009).

Sampling: Eighty (80) blood samples were collected from dogs at Ovabag Veterinary Clinic in the Diyarbakır region, Türkiye. These dogs, which had recently been adopted from shelters and were having routine medical examinations, showed no clinical signs and were classified as healthy or asymptomatic. Blood samples were collected in K3-EDTA and stored at -20°C until DNA extraction. Each dog was also carefully inspected for tick infestation, but no ticks were detected on any of the animals.

Molecular analysis: A commercial kit (PureLink™ Genomic DNA Mini Kit [Invitrogen Corporation, Carlsbad, CA, USA]) was used to extract the genomic DNA from these blood samples and the manufacturer's protocol was followed. The extracted DNA was then quantified using a NanoDrop® ND-2000 UV/Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Dog DNA was confirmed in all samples using the primers CANF (5'-CTTGTCACGGTAAGGTTC-3') and CANR (5'-CTGATGTATTTCCTGCACCAAG-3') (Criado-Fornelio *et al.*, 2003). To screen for *Theileria* and *Babesia* species, the extracted DNA underwent to nPCR using the primers for the primary amplification of an *18S rRNA* gene fragment, approximately 1700 bp in size (Oosthuizen *et al.*, 2008). This step was designed to improve the quality of the amplification. The piroplasm *18S rRNA* gene's hypervariable V4 region was then amplified by nPCR using RLB-F2 (5′-GACACAGGGAGGTAGTGACAAG-3′) and RLBR2 (5′-CTAAGAATTTCACCTCTGACAGT-3′) primers, yielding an amplified fragment of approximately 400 bp (Georges *et al.*, 2001). PCR reactions were carried out using the PCR Sprint system (Sensoquest, Göttingen, Germany). The PCR protocol began with an initial denaturation step at 94°C for 2 minutes. This was followed by two cycles of 20 seconds at 94°C, 30 seconds at 67°C, and 30 seconds at 72°C. The annealing temperature was then decreased by 2°C after every two cycles until reaching a final temperature of 57°C. Next, 40 additional cycles were conducted, with each cycle comprising 20 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C. The process concluded with a final extension step at 72°C for 7 minutes. Due to the high sensitivity of nPCR, stringent negative control tests were employed. For the positive control in the nPCR, *T. annulata* (AY508463) was used, while for the negative control, DNA samples from sheep, dogs, and cattle that had been molecularly tested negative for *Babesia* spp., *Theileria* spp., and *Anaplasma* spp. were used. To verify the amplified samples identified through the nPCR analysis, all positive samples underwent sequence analysis. The resulting nucleotide sequences (~400 bp) were compared to sequences in the NCBI database through BLAST analysis.

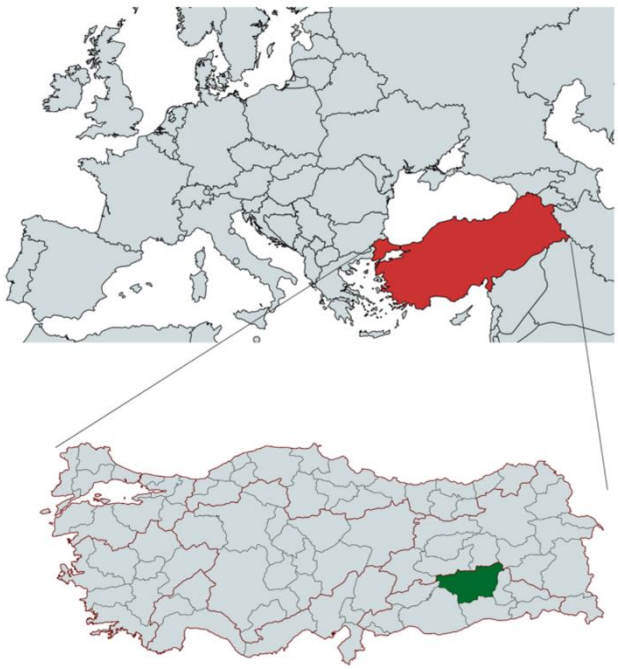

Fig. 1: Geographic location of the study area. The map highlights Türkiye in red, located in the southeastern region of Europe. Diyarbakır province, where the study was conducted, is shown in green in the inset map of Türkiye. Figure was created with mapchart.net.

Phylogenetic analysis: In this study, the phylogenetic analyses of the *B. ovis* and *B. vogeli 18S rRNA* gene sequences obtained were performed using the MEGA11 software (Tamura *et al.*, 2021). A phylogenetic tree was constructed based on a maximum likelihood phylogeny using the Tamura 3-parameter model (Tamura, 1992) with 1000 bootstrap replicates.

RESULTS

Among the 80 blood samples examined, *B. ovis* was detected in 9 (11.2%) dogs and *B. vogeli* in 2 (2.5%) dogs. Incidentally, *T. gondii* was also identified in one dog sample (1.2%).

DNA sequence analyses in the present study showed that 9 samples (11.2%) had *B. ovis* DNA. The *18S rRNA* gene partial sequences from the *B. ovis* isolates found in this study showed 100% nucleotide identity with one another. They also showed 100% identity with sequences of *B. ovis* isolated from sheep (MN493112.1), horse (MG569902.1), and cat (OR984759.1) in Türkiye, goat (KF681515.1) in Albania, and sheep (OL744565.1) in Iran. *B. vogeli* isolates found in this study had 99–100% nucleotide similarity with the isolates of *B. vogeli* found in dogs from Egypt (OP604259.1; LC651124.1), Brazil (MN823213.1), and France (MK495837.1). Furthermore, BLAST analysis revealed that one sample was identified as *T. gondii* with 99-100% similarity to isolates obtained from cats in Iran (LC416238.1) and sheep in China (KX008031.1) (Table 1). Since *B. ovis* is routinely studied in our laboratory, the detection of *B. ovis* in dog samples initially raised concerns about potential contamination. In order to resolve this, the blood samples' genomic DNA was extracted again, and PCR analysis was conducted once more using Apex 2x Red Taq Master Mix (Genesee Scientific, San Diego, USA) (Fig. 2). After PCR, 12 samples were sent for sequence analysis, and the results showed that all samples were 100% identical to the previous sequences. Furthermore, all positive samples were examined using sheep-specific primers [OaITGAM-F (5′-TGGATGGACTGGTAGACTTG-3′) and OaITGAM-R (5′-GGGTCACGACATTCATACAC-3′)] that target the integrin alpha M gene in order to rule out the presence of sheep DNA (Erster *et al.*, 2016). It was confirmed that no sheep DNA was present.

Phylogenetic analysis was conducted to determine the evolutionary relationships among various *Babesia* species, including *B. ovis* and *B. vogeli* which were identified in this study and highlighted in the constructed phylogenetic tree. The analysis utilized the MEGA 11 software, employing the Tamura 3-parameter model with *Plasmodium falciparum* (GenBank accession no. M19172.1) as an outgroup to root the tree (Tamura, 1992). This approach helped delineate the genetic relationships and evolutionary distinctions within the *Babesia* group. The phylogenetic analysis revealed that *B. ovis* and *B. vogeli*, while clustering distinctly within the *Babesia* group, occupy unique positions in different clades. Notably, *B. ovis*, which is typically associated with sheep, has also been detected in unexpected hosts such as cats and horses, in addition to its presence in canine blood samples. This indicates a broader host range than previously recognized. The high bootstrap values (above 50%) confirm the reliability of these findings. Moreover, *B. vogeli* forms a consistent cluster with sequences from various global regions, demonstrating a stable genetic identity that spans its widespread geographic distribution (Fig. 3).

DISCUSSION

The present study provides the first molecular proof of *B. ovis* in canine blood samples, representing a significant progress in understanding the epidemiology of tick-borne diseases. Traditionally, *B. ovis* has been associated with clinical infections in sheep, leading to severe economic losses in livestock due to its impact on health and productivity. The detection of *B. ovis* in dogs, an unexpected host, raises critical questions regarding the dynamics of *Babesia* transmission and its potential implications for animal health. This finding is consistent with previous reports of *B. ovis* in other unexpected hosts, such as donkeys, horses and cats (Ozubek and Aktas, 2018a; Ceylan *et al.*, 2021, 2024). Molecular studies have also demonstrated that certain *Theileria* and *Babesia* species, traditionally found in livestock, can infect dogs. For instance, *T. annulata*, a protozoan parasite primarily infecting cattle and causing tropical theileriosis, has been detected in dogs in Iran (Bigdeli *et al.*, 2012), Türkiye (Aktas *et al.*, 2015), and Tunisia (Rjeibi *et al.*, 2016). Similarly, *T. orientalis*, typically benign in cattle but pathogenic in certain genotypes, has been reported in dogs in Iran and Myanmar (Gholami *et al.*, 2016; Bawm *et al.*, 2021). Other examples include *T. luwenshuni* and *T. uilenbergi*, both known to cause severe clinical symptoms in sheep but detected in dogs in Iran (Gholami *et al.*, 2016) and China (Wu *et al.*, 2023), respectively. *T. ovis*, widespread in sheep and goats and generally considered apathogenic (Stuen, 2020), has been reported in dogs in Iran (Gholami *et al.*, 2016) and Kyrgyzstan (Altay *et al.*, 2023). Furthermore, one of the most pathogenic species of *Babesia* in cattle (Ozubek *et al.*, 2020), *B. bigemina*, has been detected in dogs in Mexico

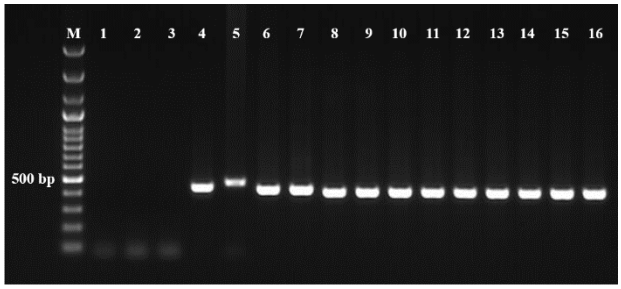

Fig. 2: Agarose gel electrophoresis results from nPCR. Lane M: DNA ladder; Lane 1: Negative control (DNA from dog); Lane 2: Negative control (DNA from sheep); Lane 3: Negative control (DNA from cow); Lane 4: Positive control-*T. annulata* (AY508463); Lane 5: Isolate k44 *T. gondii* (PQ202301); Lane 6: Isolate k72 *B. vogeli* (PQ202300); Lane 7: Isolate k23 *B. vogeli* (PQ202299); Lane 8: Isolate k36 *B. ovis* (PQ202298); Lane 9: Isolate k35 *B. ovis* (PQ202297); Lane 10: Isolate k27 *B. ovis* (PQ202296); Lane 11: Isolate k26 *B. ovis* (PQ202295); Lane 12: Isolate k24 *B. ovis* (PQ202294); Lane 13: Isolate k22 *B. ovis* (PQ202293); Lane 14: Isolate k21 *B. ovis* (PQ202292); Lane 15: Isolate k19 *B. ovis* (PQ202291); Lane 16: Isolate k18 *B. ovis* (PQ202290). The marker indicates a 500 bp size.

Table 1: Isolate accession numbers and blast analysis results for *Babesia* and *Toxoplasma gondii* species identified in the study.

Isolates	Accesion number	BLAST Analysis
K18	PQ202290	100% *Babesia ovis* (MN493112.1)
K19	PQ202291	100% *Babesia ovis* (MN493112.1)
K21	PQ202292	100% *Babesia ovis* (MN493112.1)
K22	PQ202293	100% *Babesia ovis* (MN493112.1)
K24	PQ202294	100% *Babesia ovis* (MN493112.1)
K26	PQ202295	100% *Babesia ovis* (MN493112.1)
K27	PQ202296	100% *Babesia ovis* (MN493112.1)
K35	PQ202297	100% *Babesia ovis* (MN493112.1)
K36	PQ202298	100% *Babesia ovis* (MN493112.1)
K23	PQ202299	100% *Babesia vogeli* (OP604259.1)
K72	PQ202300	100% *Babesia vogeli* (OP604259.1)
K44	PQ202301	100% *Toxoplasma gondii* (LC416238.1)

(Bravo-Ramos *et al.*, 2022). These findings highlight the surprisingly broad host range that certain parasites can exhibit and suggest that transient presence of *B. ovis* DNA in unexpected hosts could be more common than previously assumed. However, the parasitological and epidemiological significance of these presences of *B. ovis*, particularly their impact on primary hosts, requires further investigation.

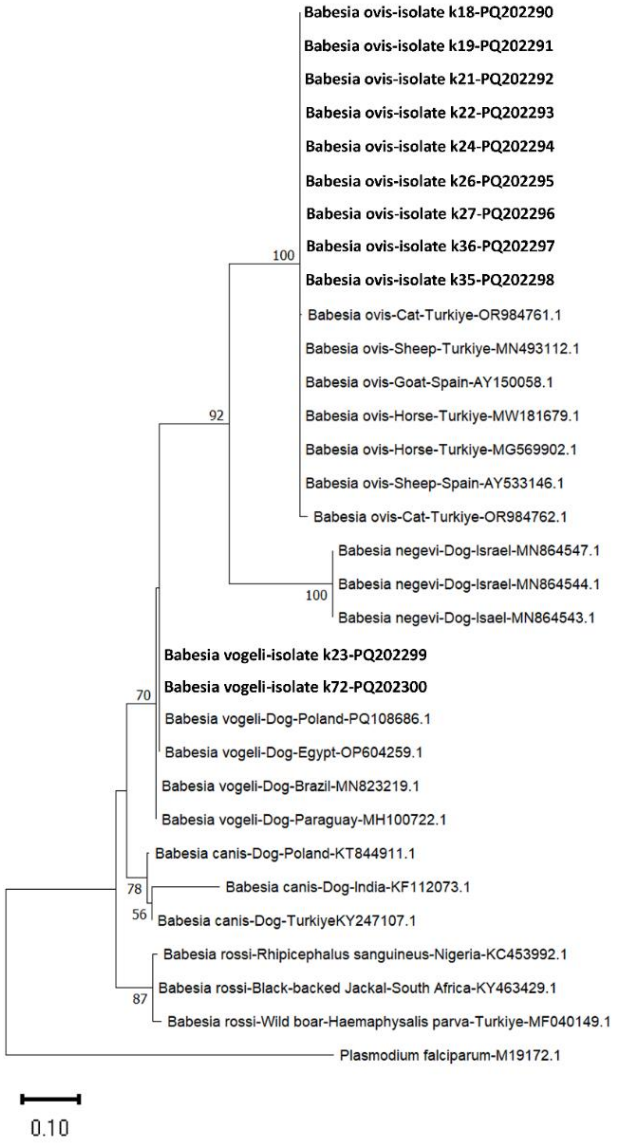

Fig. 3: Phylogenetic relationships of *Babesia* species, including *B. ovis* and *B. vogeli* (highlighted in bold), as identified in this study. The phylogenetic tree was constructed using Mega 11 software utilizing the Tamura 3-parameter model. *Plasmodium falciparum* (GenBank accession no. M19172.1) was used as an outgroup to root the tree. Bootstrap values, calculated from 1000 replicates and displayed at branch nodes, indicate the percentage of times the taxa clustered together in the bootstrap test; values are shown only for percentages above 50%. GenBank accession numbers are provided next to the species names. The scale bar represents nucleotide substitutions per site, illustrating the evolutionary distances among the species.

The presence of *B. ovis* in 9 out of 80 dogs (11.2%) indicates a notable prevalence of this parasite in the canine population of Diyarbakır province, Türkiye. This finding is particularly intriguing given that *B. ovis* is typically associated with sheep. The phylogenetic analysis confirmed that the *B. ovis* sequences from dogs were identical to those from sheep in Türkiye, Albania, and Iran. In the Diyarbakır province, where this study was conducted, *B. ovis* is known to be prevalent in sheep (Bozan *et al.*, 2024). However, there is no data in this study regarding whether the dogs had any direct or indirect contact with infected sheep. Investigating the role of dogs in the epidemiology of *B. ovis* transmission, including their potential contribution as reservoirs or incidental hosts, is essential for a comprehensive understanding of disease dynamics. *R. sanguineus*, is a three-host tick primarily associated with dogs but is also known to feed on other hosts, including humans. It is recognized as a vector for various pathogens, such as *Coxiella burnetii*, *Babesia canis*, *Ehrlichia canis*, *Rickettsia conorii*, and *Rickettsia rickettsii*, which are of significant veterinary and medical concern (Dantas-Torres, 2010). While it has been shown that *R. sanguineus* cannot transmit *B. ovis* transovarially (Razmi and Nouroozi, 2010), the possibility of transstadial transmission remains unclear. Notably, *B. ovis* DNA has been detected in *R. sanguineus* collected from infested small ruminants in Türkiye (Ma *et al.*, 2023) and in unfed adults and nymphs *R. sanguineus* (Ozubek and Aktas, 2018b). These findings suggest that *R. sanguineus* may possess vector capacity for *B. ovis*, and further studies should be conducted. Additionally, it can be speculated that dogs might acquire the parasite through the consumption of meat from dead animals that succumbed to babesiosis in the wild. Although there is no direct evidence for such transmission, this possibility warrants further investigation and could provide new insights into the transmission dynamics of *B. ovis* in non-traditional hosts. Investigating this aspect is crucial, as it could have significant epidemiological implications in areas where the tick and susceptible hosts coexist, potentially affecting control measures for *B. ovis*.

Moreover, in this study, *B. vogeli* was detected, confirming the presence of these pathogens in the region. The detection of both species in asymptomatic samples suggests the possibility of co-circulation and mixed infections within the local canine population. These results are in compliance with prior reports from Türkiye, where *B. vogeli* have been identified in symptomatic and asymptomatic dogs (Gülanber *et al.*, 2006; Gokce *et al.*, 2013; Aktas *et al.*, 2015; Aktas and Ozubek, 2017). The presence of these pathogens emphasizes the need for continuous surveillance and precise diagnostic methods to inform effective management and control strategies for canine babesiosis in the region.

An unexpected outcome of this study was the detection of *T. gondii*. Although the study's primers were designed to amplify *Theileria* and *Babesia* species, it has been reported that they occasionally detected other protozoans including *Hepatozoon* and *Sarcocystis* (Moustafa *et al.*, 2017). The protozoan parasite *T. gondii* is capable of infecting a variety of warm-blooded animals, including dogs. Infections in dogs are both epidemiologically and clinically significant because through their fur, mouth, or paws they can mechanically transfer *T. gondii* oocysts to people, leading to potential zoonotic risks for humans (Dubey *et al.*, 2020). Although the presence of *T. gondii* in this study may be incidental, it underscores the need for careful interpretation of molecular findings, particularly when unexpected results arise. *Toxoplasma gondii* is also known to cause clinical

toxoplasmosis in dogs (Calero-Bernal and Gennari, 2019), and its detection in this context suggests that molecular techniques targeting one pathogen can inadvertently reveal the presence of others. Determining the frequency of these pathogens in canine populations is critical for assessing the risk of zoonotic transmission and developing effective control methods.

Conclusions: In summary, this study presents the first molecular proof of *B. ovis* in dogs, raising the possibility of transmission between species. The presence of *B. vogeli* in asymptomatic dogs highlights the risk of subclinical infections in the region. Additionally, the unexpected detection of *T. gondii* underscores the need for cautious interpretation of molecular results and awareness of zoonotic risks. These findings emphasize the importance of continuous surveillance and targeted control strategies for managing tick-borne diseases in both canine and livestock populations. More investigation is necessary to better understand all the factors affecting the dynamics of *B. ovis* transmission and to create effective control and prevention measures.

Authors contribution: Conceptualization was carried out by MCU, BB, AK, SO, MA; the methodology was designed by MCU, BB, AK, SO, MA; validation was performed by MCU, BB, AK, SO, MA; formal analysis was conducted by MCU, BB, AK, SO, MA; the investigation was led by MCU, BB, AK, SO, MA; SO prepared the original draft, while the review and editing were also done by SO; project administration was handled by BB, AK, SO. All authors reviewed and approved the final version of the manuscript, contributed to data interpretation, and critically revised the manuscript for significant intellectual content.

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Conflicts of interest: The authors declare no conflicts of interest.

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