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Research Article

Detection of *Babesia bigemina* Cases in Dogs in Rural Areas/Türkiye by Molecular Methods

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Abstract

Babesiosis, a very important vector disease, has also been detected in humans and animals. This study was carried out on 120 whole blood samples taken from Cankiri Municipality Animal Care and Rehabilitation Center (Türkiye) between December 2021 and 2022 in Cankiri. In this study, venous blood samples taken by a veterinarian were used for diagnosis. The 18S rRNA gene region was examined with conventional PCR analysis and the positive results were subjected to sequence analysis. As a result of sequence analysis, these positive cases were determined to be *Babesia bigemina***.** Additionally, as a result of statistical analysis, it was found that dogs detected positive for *B. bigemina* did not give significant results according to age, gender or symptoms. *B. bigemina* detected in this study were registered in the NCBI database with 7 (Acc no: OQ186727), 12 (Acc no: OQ186720), 19 (Acc no: OQ186721), 21 (Acc no: OQ186722), 23 (Acc no: OQ18723), 28 (Acc no: OQ186724), 30 (Acc no: OQ186725), and 70 (Acc no: OQ186726) numbers. The *B. bigemina* species detected in dogs in this study is actually seen in farm animals. This study is the first detection report of *B. bigemina* in dogs in Turkey. As a result, *Babesia* species seen in dogs need to be reconsidered and investigated with the development of molecular techniques.

Keywords: *Babesia, Babesia bigemina*, Babesiosis, Dog

Introduction

Babesiosis is a tick-borne zoonotic disease caused by the *Babesia* protozoan parasite that infects red blood cells. Babesiosis, which is a very important vector disease in terms of public health, has also been detected in many different animal species, including humans. Transmission by ticks often occurs in tropical and subtropical regions during the warmer months [1].

Babesiosis is a disease described first in cattle [2,3]. In Turkiye, babesiosis cases were detected in cattle in 1969 [4]. Babesiosis in dogs were also reported in Europe [5]. *Babesia* spp. infection can be observed in dogs with lethargy, anorexia, fever, jaundice, hemolytic anemia, hemoglobinuria or bilirubinuria, weight loss, and death [6,7].

According to their size, *Babesia* species are divided freely into two groups: small *Babesias* containing *B. microti, B. gibsoni*, etc., and large *Babesias* containing *B. bovis, B. canis*, etc. Only the trophozoites of *B. divergens* are small and are in the same group as the larger *Babesias* [8]. The causative agents of canine babesiosis are the larger *Babesias* (3-5 µm in size), *B. canis* and the smaller (1-3 µm in size) *B. gibsoni* [8]. With the increase in molecular studies, *B. canis* was thought to have three subspecies: *B. canis rossi, B. canis canis,* and *B. canis vogeli*. However, it is thought that they may be in a different group due to important differences in clinical, geographical distribution, and vector specificities.

In addition, three small *Babesia* species, such as *B. gibsoni, B. conradae,* and *B. microti,* are known to infect dogs. Unlike *Babesias* encountered in dogs, a large-formed *Babesia* species related to *B. bigemina* has been reported from North Carolina in the United States [10-12].

This study aims to detect the 18S rRNA gene for *Babesias* in blood samples of stray dogs in rural areas of Türkiye and to perform sequence analysis of those found positive.

Material and Methods

Ethical Statement

This study was approved by the Local Ethics Committee of the Veterinary Control Center Research Institute (Etlik/Ankara) (Approval no: 2021/23).

Sample Collection

This study was carried out under veterinary supervision on 120 whole blood samples taken from Cankiri Municipality Animal Care and Rehabilitation Center between December 2021 and 2022. The blood collected was taken into 5 mL tubes from the venous blood of the dogs coming to this center under the supervision of a veterinarian and used for the diagnosis of the animals. The demographic information of the dogs was also recorded by the veterinarian.

Molecular Analysis

DNA extraction: A commercial kit (Gene MATRIX series, Quick Blood Lot No. F/280921) advised extracting 120 EDTA blood samples from dogs using a spin column. The obtained DNA was stored at -20ºC until the conventional PCR study.

Primer design for detection of DNA and conventional PCR: Using forward and reverse primers, the 18S rRNA-specific gene region was aimed in standard PCR. The 18S rRNA primers were made in Turkiye at BM laboratories. The 18S rRNA primers we used in conventional PCR analysis are capable of detecting *Hepatozoon* spp., *Babesia* spp., *Theleria* spp., and *Hemovilia mauritacana*. PCR mix, for a total of 45 µL for each sample: Distilled water is 33.75 µL, 10 x PCR buffer is 5 µL, Mg Cl2 (25 mM) is 3 µL, d NTP mix (10 mM) is 1 µL, Primer I (10 pmol) is 1 µL, Primer II (10 pmol) is 1 µL, and Taq DNA polymerase (5 U/µL) is 0,25 µL. For this purpose, BJ1 (5'-GTCTTGTAATTGGAATGATGG-3') and BN2 (5'-AGTTTATGGTTAGGACTACG-3') primers and protocols were used for the amplification of the 18S rRNA gene region [13].

A 45 µL of this master mix was dispensed into the tubes, and 5 µL of sample DNA was added to each tube to bring the total reaction volume to 50 µL. Samples prepared according to this mixture were placed in a thermal cycler for 5 min at 95ºC, 30 sec at 95ºC, 45 sec at 52ºC, 45 sec at 72ºC, and 5 min at 72ºC for reproduction.

After that, PCR products were detected by 2% agarose gel electrophoresis. In this study, 100 bp ladders were used for identification, and fragments falling in the range of 400-500 bp were examined. Suspicious positives falling between 400 and 500 bp in the target region were subjected to sequence analysis. As a result of sequence analysis, *B. bigemina* was detected. The appearance of 400–500 bp regions was considered positive, and DNA sequence analysis was performed on positive samples.

Sequencing and Phylogenetic Analyses

All conventional PCR-positive samples were sequenced in one direction at a commercial sequencing service provider (BM Laboratories, Türkiye). Nucleotide sequences were analyzed using the online nucleotide Blast system (National Center for Biotechnology Information, www.blast.ncbi.nlm.nih.gov/Blast). Phylogenetic analysis was determined using the Maximum Likelihood method and the Tamura-Nei model [14]. When drawing the *B. bigemina* phylogenetic tree, approaches like the reference strains, where the reference strains came from, which countries they were studied in, and how far apart they were from each other were also tested. This analysis involved eight (7, 12, 19, 21, 23, 28, 30, 70) nucleotide sequences. Evolutionary analyses were conducted in MEGA X [15].

Statistical Analysis

Statistical analyses of data were performed using SPSS version 26 software. Descriptive features are given using frequencies and percentages. The difference between the groups in terms of these frequencies was compared using the Chi-square test. A value of $P<0.05$ was accepted as the significance limit.

Results

Demographic data obtained from dogs in this study and positivity rates according to conventional PCR results are given in *Table 1*. The dogs observed in this study were mostly male (51.67%) and one-year-old (56.67%). The most common symptom among the dogs in this study was redness (18.33%), itching (17.5%), and cachexia (9.17%). As a result of statistical analysis, *B. bigemina* positivity rates did not yield significant results according to age, gender, and symptoms ($P>0.001$).

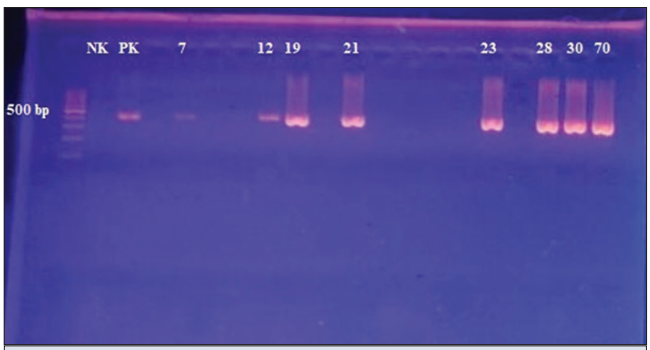

Fig 1. Conventional PCR gel image of *Babesia bigemina* in whole positive samples

Table 1. *Distribution of demographic data according to Babesia bigemina positivity (n=120)*

Demographic Data of Dogs		*Babesia* spp.		Total (%)
		Positive	Negative	
Age	1	5	63	68 (56.67)
	2	2	35	37 (30.83)
	3	0	12	12 (10)
	4	1	2	3 (2.55)
Gender	Male	3	59	62 (51.67)
	Female	5	53	58 (48.33)
Lesion	Alopecia	2	5	7 (5.83)
	Diarrhea	0	1	1 (0.83)
	Cachexia	0	11	11 (9.17)
	Itching	0	21	21 (17.5)
	Itching Alopecia	0	2	2 (1.67)
	Redness	2	20	22 (18.33)
	Redness Alopecia	0	2	2 (1.67)
	Vomiting	0	2	2 (1.67)
	Vomiting diarrhea	0	2	2 (1.67)
	Breaking nails	0	1	1 (0.83)
	None	4	45	49 (40.83)
Total		8 (6.67)	112 (93.33)	120 (100)

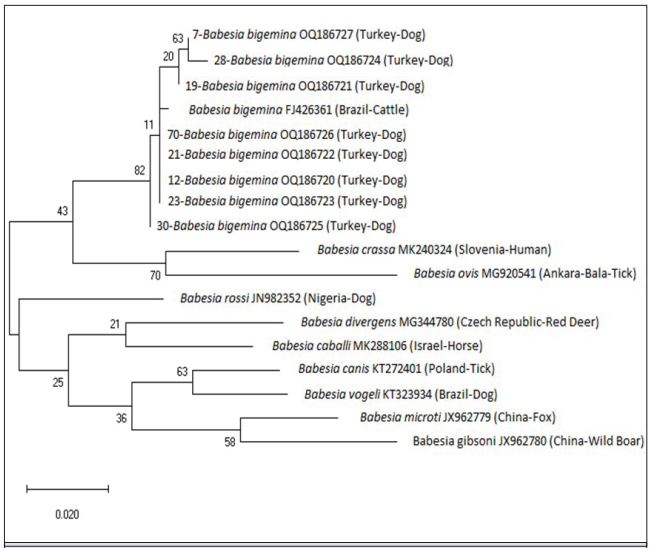

Fig 2. Phylogenetic tree of *Babesia bigemina* specimens found in this study

Of the 120 dogs tested, 8 were positive by conventional PCR *(Fig. 1)*. *B. bigemina* was found in eight of the positive samples (7, 12, 19, 21, 23, 28, 30, 70) in the sequence analysis performed (BM laboratories, Türkiye). These positive data were registered in the NCBI database as 7 (Acc no: OQ186727), 12 (Acc no: OQ186720), 19 (Acc no: OQ186721), 21 (Acc no: OQ186722), 23 (Acc no: OQ18723), 28 (Acc no: OQ186724), 30 (Acc no: OQ186725), and 70 (Acc no: OQ186726) *(https://www.ncbi.nlm.nih.gov/)*. The phylogenetic tree was drawn with reference strains and given in *Fig. 2*. When blasted with the NCBI database, all samples detected positive in dogs were investigated for their relationship with the *Babesia* species detected so far. It was found to be 100% compatible only with *B. bigemina*. The *B. bigemina* species detected in this study are closely related to the *B. bigemina* species, which was also detected in cattle in Brazil. *Babesia* species in this study are in the same clade and in the same branch *(Fig. 2)*.

Discussion

Babesiosis is a protozoan disease. Babesiia species cause hemoglobinuria, and can be infectious in dogs as well as cattle and sheep [16]. Although *Babesia* is an important human problem in the world, it continues to pose a threat to veterinary medicine. This threat is also important in terms of economic losses. Accordingly, this parasite has been poorly studied compared to its malaria-causing relative, *Plasmodium* [17].

Studies on dogs in Türkiye were conducted in Istanbul *(B.canis, B. vogeli, B. rossi)* [18], in Kayseri *(B. canis canis, B. gibsoni, B. canis vogeli)* [19], in Konya *(B. canis vogeli)* [20], in Erzurum *(B. canis)* [21], and in Diyarbakır *(Babesia spp.,*

B. canis, B. vogeli) [22]. *B. canis, B. vogeli, B. rossi,* and *B. gibsoni* are the most common *Babesia* species in dogs in our country and in the world. However, other *Babesia* species can be detected, apart from those frequently seen in dogs. The severity of the disease depends on the type of *Babesia* [6]. The 18S rRNA primers we used in conventional PCR analysis are capable of detecting *Hepatozoon* spp., *Babesia* spp., *Theleria* spp., and *Hemovilia mauritacana*. Suspicious positives detected after sequence analysis were found to be *B. bigemina*. This is the report of *B. bigemina* in dogs from Turkiye. This infective agent was determined to be *B. bigemina* when positive reactions detected were blasted with the NCBI database. A review of previous studies revealed that an unnamed species of *Babesia* closely related to *B. bigemina* has been reported from North Carolina in the United States [10, 23].

As a result, it is possible for dogs to become infected with unexpected *Babesia* species. The emergence of extensive molecular techniques and the rise of rigorous research support this. Thus, *Babesia* infections in dogs gradually began to be clarified. In addition, another study that mentions the presence of *B. bigemina* in dogs shows this. In this study, this agent was detected in canines living on cattle farms [24]. It should be noted that *B. bigemina* bearing ectoparasites can be shared between livestock and wildlife. This may explain the distribution, but the distribution of tick-borne *Babesia* species in dogs is still not fully understood [25].

B. bigemina, one of the larger *Babesia* seen in cattle, is carried *by Boophilus* spp., *R. sanguineus, R. bursa, R. evertsi, R. microplus, R. annulatus, Haemaphysalis* spp., *Amblyomma parvum* [25-29]. Although there is a wide variety of these ticks in Europe, it cannot be assessed that how many of these ticks dogs carry and how many of them have infections. Interestingly, *B. bigemina* infections were detected in dogs in this study. The fact that *B. bigemina* can be carried by many different ticks may indicate that the infection can also be seen in dogs. One of the limitations of this study is that tick samples, if any, were not collected from the dogs that were drawn blood.

It has been reported that merozoites circulating in the blood can be transmitted to a healthy host by direct blood transfusion. In America, Australia, and Europe, *B. gibsoni* has been found to be transmitted vertically and through wounds, (fighting dogs), saliva, or blood draws [2]. This issue also makes us think of other unreported *Babesia* species. In this study, it was determined that dogs with *B. bigemina* were in close contact with each other and became close by fighting.

Babesia species that affect dogs are thought to be of zoonotic importance [30]. However, human babesiosis is a rare disease, and data on the causative protozoan species is quite lacking. Zoonotic babesiosis is caused by *B. divergens* transmitted from cattle and *B. microti* transmitted from rodents. Therefore, it is thought that *B. bigemina* detected in this study is not zoonotic. However, this needs further investigation. Detection of a species originating from cattle is rare in dogs, and its zoonotic character should be investigated [2].

Although molecular methods are expensive and complex, they are preferred because of their high specificity and sensitivity. The use of PCR is becoming increasingly common nowadays. Species identification is also more reliable in PCR [31].

Since the animals investigated in this study were stray dogs, the findings, although clinically apparent, were not distinctive for babesiosis. Typical signs of *Babesia* were not observed in these dogs. Although jaundice is a common symptom of babesiosis infection, it is a rare symptom in dogs [32] and also not detected in this study.

As a result, the *B. bigemina* species detected in dogs in this study is normally seen in farm animals. With this study *B. bigemina* was detected in dogs in Türkiye. *Babesia* species that can infect dogs should be reconsidered with the development of molecular techniques and researches should be increased. By emphasizing the atypical cases encountered, the causes and consequences should be investigated, and the issue should be carefully considered.

Availability of Data and Materials

The authors declare that the data and materials are available on request from the corresponding author (B. Yucesan).

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Ethical Statement

This study approved by the Local Ethics Committee of the Veterinary Control Center Research Institute (Etlik/Ankara) (Approval no: 2021/23).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

OO and BY planned the study and designed the experiments, OO, BY and YY helped write the article and laboratory process, ZO helped collect samples during fieldwork, and BY, YY helped with data analyses. All authors read and approved the final version of the manuscript.

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