Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
30 (2): 161-170, 2024
DOI: 10.9775/kvfd.2023.30303

Research Article

Prevalence and Molecular Characterization of Tick-Borne Pathogens in Dogs in Northeast Anatolia Region, Türkiye [1]

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[1] *Presented (oral presentation) at 23. National Parasitology Congress, 30 October-3 November 2023), Antalya, Türkiye*
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How to cite this article?

Taşçı GT, Aydın N, Ölmez N, Yiğit M, Işık ME, Vatansever Z: Prevalence and molecular characterization of tick-borne pathogens in dogs in Northeast Anatolia Region, Türkiye. *Kafkas Univ Vet Fak Derg,* 30 (2): 161-170, 2024.
DOI: 10.9775/kvfd.2023.30303

Article ID: KVFD-2023-30303
Received: 24.07.2023
Accepted: 06.01.2024
Published Online: 19.01.2024

Abstract

This study was carried out to determine the prevalence and molecular characterization of tick-borne pathogens (TBP) *(Babesia* spp., *Theileria* spp., and *Hepatozoon* spp.) in asymptomatic dogs in the northeast Anatolia region of Türkiye. Blood samples of 400 clinically healthy dogs were analyzed using PCR and RLB techniques. TBP prevalence and the relationship between habitat, gender, age, and breed of dogs were determined by using the R package prevalence (version 0.2.0.) and the Pearson chi-square statistics. *Babesia* spp., *Theileria* spp., *Hepatozoon* spp. DNAs were detected in 13% (52/400) of the dogs by PCR, while the prevalence of TBPs was 24.25% (CI: 20.24-28.62) by subsecuantial RLB assay. Sequence analysis of two *Theileria ovis*, two *Babesia canis* and two *Hepatozoon canis* isolates showed 100% identity with the corresponding reference isolates. In this study, *Theileria ovis* positivity was detected for the first time in dogs in the northeast Anatolia region of Türkiye.

Keywords: Dog, Molecular characterization, Prevalence, Tick-borne pathogens

Introduction

Ticks transmit a variety of pathogens to dogs, including *Hepatozoon* spp., *Babesia* spp., and *Theileria* spp., threatening animal health and causing severe economic losses [1-4].

Canine babesiosis is a disease transmitted by *Dermacentor, Rhipicephalus*, and *Haemaphysalis* ticks and caused by large (5 x 2.5 µm) *(Babesia canis, B. vogeli, B. rossi*, and *Babesia* sp.) and small (2 x 1.5 µm) *(B. gibsoni, B. conradae*, and *B. microti-like*) *Babesia* species. In studies conducted in different countries around the world, *Babesia* spp. infections in dogs have been reported with varying prevalence rates [1-3,5-12].

Theileriosis in dogs is manifested with clinical signs including fever, anemia, thrombocytopenia, immune-mediated syndrome, bleeding tendencies, lethargy, pale mucous membranes and corneal opacity [5,9,13]. Several *Theileria* species such as *Theileria annae*, *T. sable, T. luwenshuni, T. buffeli*, *T. orientalis, T. ovis, T. annulata,* and *T. equi* have been reported in dogs [4,5,8,9,13-19].

Hepatozoonosis is a protozoal infection in dogs caused by *Hepatozoon canis* and *H. americanum*. While *H. canis* is transmitted by *Rhipicephalus sanguineus s.l.* ticks, *H. americanum* is transmitted by *Amblyomma maculatum*. In studies conducted in Türkiye and different countries around the world, the prevalence of hepatozoonosis has been found to be at least 1% and at most 57.8%. Only *H. canis* species was found in dogs in Türkiye [1,3,20-29].

Epidemiologic data on hepatozoonosis, babesiosis, and theileriosis in dogs are limited for the northeast Anatolia region. In this study, it was aimed to determine the prevalence and molecular characterization of tick-borne pathogens (TBPs) (*Babesia* spp., *Theileria* spp., and *Hepatozoon* spp.) in dogs in this region of Türkiye.

Material and Methods

Ethical Statement

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Approval no: KAÜ-HADYEK: 2020-121).

Study Area

Mountains, plateaus, plains, and rivers occupy a large place in Kars, Ardahan, and Iğdır provinces, which cover an area of approximately 20.000 km^2 in the northeast Anatolia region of Türkiye. The region is located in a strategically important geographical position in terms of bordering Armenia, Iran, Nakhchivan and Georgia. Its economy is mainly based on animal husbandry, especially pasture-based livestock production. Many dogs in the region are also bred to guard the herds. In Kars and Ardahan provinces, the autumn and winter months are cold, while the summer months are cool and rainy. The dry season is not very common because it can rain in all seasons. In Iğdır, there is a temperate climate due to the microclimate created by the earth formations. While the average annual temperature is 4.7°C in Kars and 3.7°C in Ardahan, it is around 12.2°C in Iğdır. In addition to parasitic diseases transmitted by ticks, the distribution of tick species that transmit pathogens also varies regionally and can be found at high rates in different regions of Türkiye [30].

Ardahan, Kars, and Iğdır provinces have large pastures and plateaus because of their geographical location in the northeastern Anatolia region of Türkiye. The economy of the region is primarily based on agriculture and livestock grazing. In the rural parts of the region, dogs are bred in almost every home mainly to guard the livestock. The urban/peri-urban population of dogs in the region is mostly consisted of stray animals. According to the Animal Protection Law (No: 5199) in Turkey, stray animals should be collected in shelters, sterilized, vaccinated, and left to the place where they were found, which are mostly in the streets or periurban areas of the cities. Unfortunately, such regulations do not fulfill the expectations to reduce the number of stray animals. On the contrary, they lead to an increase in the population of stray animals that lack health control.

This study was carried out in the rural and urban/peri-urban areas where dogs were previously diagnosed with parasitic tick-borne infection (records of Kafkas University Faculty of Veterinary Medicine Clinics) [31,32] in the provinces of Kars, Ardahan, and Iğdır in the northeast Anatolian region of Türkiye.

Collection of Blood Samples

Blood samples were taken from *Vena cephalica antebrachii* of 400 asymptomatic dogs from various habitats, ages, genders, and breeds, randomly selected from 22 locations (*Fig. 1*). The location, gender, breed, and age of each animal were recorded.

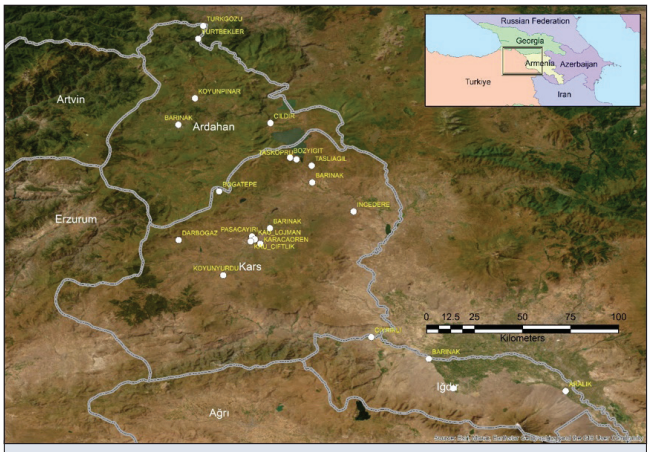

Fig 1. The map of study area

DNA Extraction and PCR Analysis of Blood Samples

DNA was extracted from blood samples using a commercial kit (EcoSpin Blood Genomic DNA Kit, Cat. No: EcoBGD-50x, Türkiye) according to the manufacturer's instructions. DNA samples were stored at -20°C until use. Touchdown PCR was performed on genomic DNA samples using specific primers for TBPs in a thermal cycler (Biometra, Analytik Jena, Germany). The RLBF2 (5'-GACACAGGGAGGTAGTGACAAG-3') and RLBR2 (biotin-5'-CTAAGAATTTCACCTCTGACAGT-3') primers, which amplify the 460-540 bp fragment of the V4 region of the *18SSSU rRNA* gene for *Theileria* spp. and *Babesia* spp., and Hep-F (5′-ATACATGAGCAAAATCTCAAC-3′) and Hep-R (biotin-5′-CTTATTATTCCATGCTGCAG-3′) primers, which amplify the 666 bp fragment of the *18S rRNA* gene for *Hepatozoon* spp., were used.

A total volume of 25 µL reaction mixture containing 8.5 µL nuclease-free water, 12.5 µL master mix (MyTaq, Bioline), 1 µL reverse primer, 1 µL forward primer, and 2 µL of template DNA was used. Positive and negative controls (nuclease-free water) were used to check for contamination [8,23,28,29,33-35]. The PCR products were analyzed on a 1.5% agarose gel using 0.5X TBE buffer and visualized by staining Ethidium bromide under ultraviolet light.

Reverse Line Blotting (RLB)

Species-specific probes were synthesized by a commercial company (Macrogen, Korea), and the RLB hybridization technique was performed for TBPs with some modifications [30,33]. Lyophilized probes were diluted with DNase- and RNase-free bi-distilled water at a concentration of 100 pmol/µL. The PCR products were perpendicularly bouned to the membrane in order to detect TBPs. The nucleotide sequences of the probes used in RLB were shown in *Table 1.*

Table 1. *Nucleotide sequences of probes used in RLB*

Probs	Sequences (5'-3')	References
Babesia catch all 1	ATT AGA GTG TTT CAA GCA GAC	[15]
Babesia catch all 2	ACT AGA GTG TTT CAA ACA GGC	[15]
Theileria annulata	CCT CTG GGG TCT GTG CA	[33]
Theileria/Babesia catchall	TAA TGG TTA ATA GGA (AG)C(AG) GTT G	[33]
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA	[34]
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT	[34]
Babesia vogeli	AGC GTG TTC GAG TTT GCC	[36]
Babesia rossi	CGG TTT GTT GCC TTT GTG	[36]
Babesia canis	TGC GTT GAC CGT TTG AC	[36]
Babesia canis 2	TGG TTG GTT ATT TCG TTT TCG	[36]
Babesia canis canis	TGC GTT GAC GGT TTG AC	[36]
Theileria equi	TTC GTT GAC TGC GYT TGG	[36]
Hepatozoon catch all	GCT TTG TAA TTG GAA TGA TAG A	[36]
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG	[36]
Anaplasma- Ehrlichia catch all	GGG GGA AAG ATT TAT CGC TA	[36]
Anaplasma marginale	GAC CGT ATA CGC AGC TTG	[36]
Anaplasma ovis	ACC GTA CGC GCA GCT TG	[36]
Anaplasma centrale	TCG AAC GGA CCA TAC GC	[36]
Anaplasma phagocytophilum V1	TTG CTA TAA AGA ATA ATT AGT GG	[36]
Anaplasma phagocytophilum V2	GAA CGG ATT ATT CTT TGT AGC	[36]
Theileria genus-specific	GTT GAA TTT CTG CT(A/G) CAT (C/T)GC	[37]
Theileria ovis	TTT TGC TCC TTT ACG AGT CTT TGC	[37]
Babesia ovis	GCG CGC GGC CTT TGC GTT TAC T	[37]
Babesia gibsoni	CTG CGT TGC CCG ACT CG	[38]
Babesia conradae	CGT TCC CTT CGG GGC	[38]
Theileria annae	CTT ATC ATT AAT TTC GCT TCC GAA CG	[38]
Hepatozoon canis	GCA TAT TCA GGA CTT TTA CTT TGA	[38]

Sequence Analysis

In order to confirm and characterize the species, DNA of the RLB positive samples were subjected to PCR (BTS18SF2/BTS18SR2 and HepF/HepR primers), and resulting products were purified (QIAquick® PCR Purification Kit, Qiagen) and then bidirectionally sequenced (Sanger Dideoxy Sequencing Method). Sequences were oriented, edited, and aligned using Geneious Prime software. The sequences were compared for similarity to sequences deposited in the GenBank database *(www.ncbi.nlm.nih.gov/BLAST).*

Statistical Analysis

Statistical evaluation was performed using Pearson chi-square, Fisher's exact test, and SPSS 20.0 software to determine the relationship between infections and locations. Prevalence values were estimated with R package prevalence (version 0.2.0.) developed by Devleesschauwer et al.[39]. R package apparent prevalence (AP) was calculated using Jeffreys confidence intervals as in the package. True prevalence (TP) was calculated with perfect test assumption and a uniform prior beta distribution in a Bayesian framework with the following model developed using the package: Model {x ~ dbin (AP, n) AP <- SE * TP + (1 - SP) * (1 - TP), SE <- 1, SP <- 1, TP ~ dbeta (1, 1) }.

Results

This study was carried out between April 2021-October 2021, and a total of 22 locations (4 from Iğdır, 8 from Ardahan, and 10 from Kars) were visited. Fifty-two (13%) of 400 PCR products were found positive for *Babesia, Theileria*, and *Hepatozoon* spp. by agarose gel electrophoresis, while this rate was 24.25% (97/400) after RLB hybridization assay. The apparent prevalence of the

tested pathogens was calculated as 3.5% for *T. ovis*, 13% for *B. canis*, and 7.75% for *H. canis*. Mixed infections were not detected in any of the samples tested in the northeastern Anatolia region of Türkiye *(Table 2)*.

In the study, the highest prevalence of *B. canis* was detected as 5.55% (CI: 1.90-12.66) in KafkasX, 6.66% (CI: 1.41-19.70) in the 2-year group, 6.66% (CI: 2.29-15.07) in females and 4.00% (CI: 1.13-10.28) in shelter dogs in Iğdır.

The highest prevalence of *B. canis* in Kars was detected as 39.24% (CI: 29.01-50.23) in KafkasX, 31.68% (CI: 23.22-41.17) in the 3-year and older group, 38.46% (CI: 27.34-50.58) in females, and 71.42% (CI: 57.82-82.58) in shelter dogs.

The prevalence of *B. canis* was highest as 10.34% (CI: 3-25.09) in KafkasX, 10% (CI: 2.13-28.38) in the 2-year group, 3.44% (CI: 0.72-10.60) in males, and 50.00% (CI: 16.68-83.31) in shelter dogs in Ardahan.

The highest prevalence of *H. canis* was detected as 20.83% (CI: 11.24-33.81) in KangalX, 20% (CI: 8.80-36.66) in the 2-year group, 20.77% (CI: 12.89-30.81) in males, and 17.33% (CI: 10.07-27.05) in shelter dogs in Iğdır.

The highest prevalence of *H. canis* in Kars was detected as 3.63% (CI: 0.76-11.15) in KangalX, 2.12% (CI: 0.23-9.51) in the 1-year group, 3.07% (CI: 0.64-9.50) in females, and 2.5% (CI: 0.70-6.52) in rural dogs.

The prevalence of *H. canis* was highest as 10% (CI: 4.28-19.45) in KangalX, 10% (CI: 2.89-24.34) in the 1-year group, 8.33% (CI: 2.40-20.59) in females, and 7.95% (CI: 3.62-14.98) in rural dogs in Ardahan.

The highest prevalence of *T. ovis* was detected as 9.72% (CI: 4.45-18.14) in KafkasX, 10.52% (CI: 5.10-18.88) in the 3-year and older group, 7.79% (CI: 3.31-15.35) in males, and 9.33% (CI: 4.27-17.45) in shelter dogs in Iğdır.

The highest prevalence of *T. ovis* in Kars was detected as 5.71% (CI: 1.20-17.09) in mixed-breed, 4.76% (CI: 0.51-20.17) in the 2-year group, 3.07% (CI: 0.64-9.50) in females, and 3.33% (CI: 1.13-7.72) in rural dogs.

The prevalence of *T. ovis* was highest as 3.44% (CI: 0.37-15.00) in KafkasX, 4.54% (CI: 0.95-13.79) in the 3-year

Table 2. *Distribution of infectious agents in blood samples according to RLB results*

Study Area		***Theileria ovis***	***Babesia canis***	***Hepatozoon canis***
Iğdır	City Center/Shelter	5	2	12
	Aralik Town Center			
	City Center	2	1	1
	Tuzluca/Ciyrikli	1	2	8
Ardahan	City Center/Shelter			
	Posof/Yurtbekler		1	
	Posof/Turkgozu		2	
	Hanak/Koyunpinar			
	Cildir/Town Center			1
	Cildir/Tasliagil			2
	Cildir/Taskopru			4
	Cildir/Bozyigit	2		
Kars	City Center/Shelter		35	
	City Center/Karacaoren			
	Akyaka/Incedere			2
	Arpacay/Shelter			
	City Center/Pasacayiri	2		
	Selim/Koyunyurdu			
	Selim/Darbogaz		1	
	City Center/Bogatepe	2		1
	City Center/University lodging		6	
	City Center/University farm		2	
Total		**14**	**52**	**31**

Table 3. *The distrubition of TBPs according to the habitat, breed, age and gender of dogs.*

Study Area		Breed						Age						Gender				Habitat			
		KangalX		KafkasX		Mixed		≤1		2		3≥		Male		Female		Rural		Shelter	
		PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB	PCR	RLB
Iğdır	City Center/Shelter																			15	23
	Aralik Town Center																				
	City Center	4	5	11	18				1	6	7	9	15	9	15	6	8				
	Tuzluca/Ciyrikli	5	6	2	3		2	4	4		1	3	6	5	8	2	3	7	11		
Ardahan	City Center/Shelter																				
	Posof/Yurtbekler				1								1				1		1		
	Posof/Turkgozu				2						2				2				2		
	Hanak/Koyunpinar																				
	Cildir/Town Center		1						1								1		1		
	Cildir/Tasliagil	1	2								1	1	1		1	1	1	1	2		
	Cildir/Taskopru	1	3		1				2			1	2	1	3		1	1	4		
	Cildir/Bozyigit	1	1		1							1	2	1	2			1	2		
Kars	City Center/Shelter	1	5	11	25	3	5	4	8		3	11	24	6	11	9	24			15	35
	Akyaka/ Incedere	1	1				1		1			1	1			1	2	1	2		
	Arpacay/Shelter																				
	City Center/ Pasacayiri					1	2					1	2			1	2	1	2		
	Selim/ Koyunyurdu	1	1					1	1					1	1			1	1		
	Selim/Darbogaz	1	1									1	1			1	1	1	1		
	City Center/Bogatepe	1	2	1	1					1	1	1	2	2	3			2	3		
	City Center/ University Lodging			5	6							5	6	5	6			5	6		
	City Center/ University Farm					1	1					1	1	1	1			1	1		
Total		17	28	30	58	5	11	9	18	7	15	36	64	31	53	21	44	22	39	30	58

***Table 4**. The infection rates of TBPs by PCR and RLB according to the habitat, breed, age and gender of the dogs*

Risk Factors		*Babesia canis*				*Theileria ovis*				*Hepatozoon canis*				PCR		
		N	Pos	MLE%/CI	P	N	Pos	MLE%/CI	P	N	Pos	MLE%/CI	P	N	Pos	P
Age	≤1	108	11	10.18/5.78-17.32	P>0.05	108	0	0/0-3.43	P<0.05	108	7	6.48/3.17-12.77	P>0.05	108	9	P>0.05
	2	71	7	9.85/4.85-18.98		71	1	1.40/0.24-7.55		71	7	9.85/4.85-18.98		71	7	
	3≥	221	34	15.38/11.22-20.72		221	13	5.88/3.46-9.80		221	17	7.69/4.85-11.97		221	36	
Breed	KangalX	163	8	4.90/2.50-9.38	P<0.05	163	2	1.22/0.33-4.3	P>0.05	163	18	11.04/7.10-16.77	P>0.05	163	17	P>0.05
	KafkasX	180	38	21.11/15.78-27.64		180	9	5.00/2.65-9.22		180	11	6.11/3.44-10.61		180	30	
	Mix	57	6	10.52/4.91-21.12		57	3	5.26/1.80-14.36		57	2	3.50/0.96-11.92		57	5	
Gender	Male	239	22	9.20/6.15-13.54	P<0.05	239	10	4.18/2.28-7.52	P>0.05	239	21	8.78/5.81-13.05	P>0.05	239	31	P>0.05
	Female	161	30	18.63/13.37-25.35		161	4	2.48/0.97-6.21		161	10	6.21/3.40-11.05		161	21	
Habitat	Rural	270	14	5.18/3.11-8.51	P<0.05	270	7	2.59/1.26-5.25	P>0.05	270	18	6.66/4.25-10.29	P>0.05	270	22	P<0.05
	Shelter	130	38	29.23/22.09-37.55		130	7	5.38/2.63-10.69		130	13	10.00/5.93/16.35		130	30	

***N:** Number of samples, **Pos:** Positive samples, **MLE:** Maximum likelyhood estimate, **CI:** Confidence interval*

and older group, 3.44% (CI: 0.72-10.60) in males, and 2.27% (CI: 0.47-7.09) in rural dogs in Ardahan.

The distribution and infection rates of TBPs according to the habitat, breed, age, and gender of the dogs were summarized in *Table 3* and *Table 4*.

Analysis of the overall infection rates, it is seen that *B. canis* infections are concentrated in the east and north, while *H. canis* and *T. ovis* infections are concentrated in the south and east of the study area *(Fig. 2)*.

Relevant gene regions or genotypes of *B. canis*, *T. ovis*, and *H. canis* species detected by RLB were bidirectionally sequenced using BTS18SF2/BTS18SR2 and HepF/HepR primers by a commercial company (BM labosis, Türkiye), and compared with the sequences available in GenBank. In sequence analysis, the *18S rRNA* gene, which is widely used in the identification of *Babesia* and *Theileria* species, was targeted. The isolates belonging to *Babesia* and *Theileria* species and genotypes were amplified using Nbab1F/Nbab1R and BTS18SF2/BTS18SR2 primers, and approximately 1600 bp and 1400 bp length amplification products were obtained, respectively.

In this study, three different pathogens *(T. ovis* in two samples, *B. canis* in two samples, and *H. canis* in two samples) were detected. The raw versions of the relevant sequences were analyzed using the Geneious Prime program and consensus sequences were formed. When compared with the GenBank database; Kars City Center-Shelter 1 (Accession number: OR652378) and Kars City Center-Shelter 20 (Accession number: OR652379) sequences of the present study were found to be identical, and as a result of BLAST analysis, the sequences were found to be 100% identical to the *B. canis* sequences previously reported from Kars (KF499115), Erzurum (KY247107, KY247106, KY247105, MT703876, KP745630, MN704759), Romania (KX711222, MW939359, HQ662634), China (MK571831,

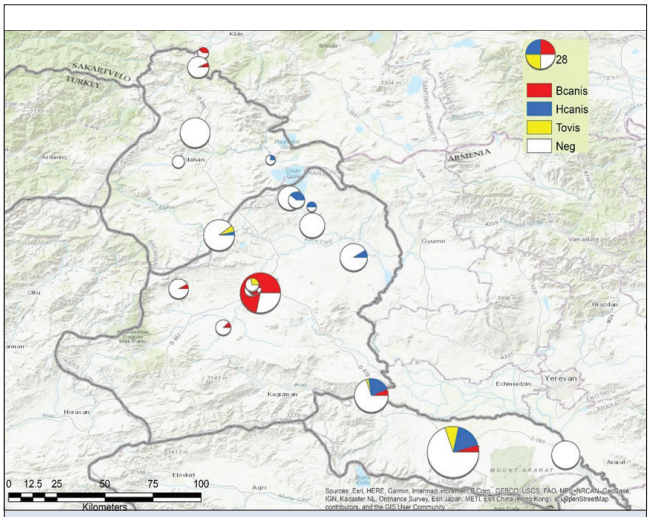

Fig 2. The overall infection rates of TBPs in study area

MK256974, MH143391), Kazakhstan (MK070118), Russia (AY649326, AY962186) and Estonia (KT008057). The *B. canis* sequences reported from Kayseri were found to be 99.86% (MG569903, 2 nucleotide differences) and 98.96% (KJ513199, KJ513200, KJ513201, 4 nucleotide differences) similar.

The two sequences named Bogatepe 18 (Accession number: OR652381) and Iğdır City Center-Shelter 28 (Accession number: OR652382) were found to be identical each other and showed 100% compatible with *T. ovis* sequences reported from China (FJ603460) and Türkiye (AY50845, KT851434, KT851435, KT851433, KT851429). Additionally, these findings were determined to be 99.92% similar to some sequences reported from Türkiye, with only 1 nucleotide difference (MN493111, KT851436).

The two sequences named Iğdır City Center-Shelter 26 (Accession number: OR652383) and Iğdır Tuzluca 17 (Accession number: OR652384) were found to be identical and 100% compatible with the *H. canis* sequences reported from Konya (KX641899), Samsun (KX588232), Ankara (MG077087, MG254611, MG254622, MG254594), Croatia (KT736298), Iran (KX880506, KX880503, KX880502, KT736298), Pakistan (MG209580), and Zambia (LC331054).

Discussion

Several factors including the existing vector population, identification of new vectors, host/reservoir population, animal transfer from endemic areas, climatological changes and the implementation of vector control programs, play a role in the epidemiology of vector-borne diseases [3]. Studies have shown that the northeast Anatolia region of Türkiye has a suitable ecosystem for tick population and a high potential to transmit pathogens [30-32].

Babesiosis in dogs is caused by both large and small *Babesia* species worldwide [2,6,31,40]. Canine babesiosis was first reported in 1935, and *B. vogeli* and *B. gibsoni* were also detected in Türkiye [41]. *Babesia canis* was first reported in the Kars region by Gökçe et al.[31]. In another study conducted in the Kars region [32], *B. canis* was detected in 28 out of 53 (52.8%) dogs by PCR. In studies conducted in Türkiye and different countries around the world, canine babesiosis has been found at rates ranging from 0.1% to 96% [1-3,5-11,41-43]. In addition, *B. canis* has previously been reported in equids in this study area [44]. In this study conducted in the northeast Anatolia region, the prevalence of *B. canis, T. ovis*, and *H. canis* was estimated as 13% (CI: 9.97-16.55), 3.5% (CI: 2.01-5.64) and 7.75% (CI: 5.43-10.67), respectively by RLB. When the study results are evaluated on a provincial scale, the TBP infection rate in Iğdır was 16.05% (22/137) by PCR and 24.81% by RLB (CI: 18.16-32.52). In Kars, the infection rate was 15.97% (27/169) by PCR and 30.17% (CI: 23.63-37.39) by RLB, while it was determined at a rate of 3.19% (3/94) by PCR and 12.76% (CI: 7.17-20.60) by RLB in Ardahan region. While Kars and Ardahan are geographically similar, Iğdır has a different structure compared to these two provinces. In Kars and Iğdır, the severity of the TBP infection has become apparent. The local study on dog tick infestation [45] revealed the presence of potential TBP vectors. Considering the density of tick population, the higher infection rate in the Kars and Iğdır regions compared to the Ardahan region can be attributed to the increased exposure to tick attacks.

TBPs can be seen in all breeds of dogs. Compared to other breeds, German Shepherds and Komondor dogs are more predisposed to TBP infections [2]. In studies [5,7,46] investigating whether dog breeds affect the prevalence of babesiosis, American Pit Bull Terriers were found to be susceptible to *B. gibsoni* infection. Birkkenheuer et al.[47] also stated that the treatment of babesiosis is very challenging, and Tosa Inu and American Staffordshire Terrier breeds are more susceptible than other breeds. As a result of this investigation, the overall infection rates of TBPs were 10.42% (17/163) in KangalX, 16.66% (30/180) in KafkasX and 8.77% (5/57) in mixed-breed dogs by PCR. The prevalence of babesiosis was 4.90% (CI: 2.34-9.04) in KangalX, 21.11% (CI: 15.63-27.50) in KafkasX, and 10.52% (CI: 4.51-20.41) in mixed-breed dogs by RLB. In this region, KafkasX and KangalX dogs are generally bred to guard ruminant herds followed by mixed breeds, and *B. canis* was predominantly detected in KafkasX dogs in all provinces.

According to Solano-Gallego et al.[2], adults are more predisposed to TBP infections than puppies. In a study conducted by Aktaş et al.[8], it was found that age was not a statistically significant factor in canine babesiosis. In our study, the infection rate of TBPs was 8.33% (9/108) in the 1-year group, 9.85% (7/71) in the 2-year group, and 16.28% (36/221) in the 3-year and older group by PCR. Meanwhile the prevalence of *B. canis* was 10.18% (CI: 5.53-16.93) in the 1-year group, 9.85% (CI: 4.51-18.38) in the 2-year group, and 15.38% (CI: 11.09-20.57) in the 3-year and older group by RLB. Although there is unequal distribution among age groups, the prevalence of babesiosis was found to increase with age in RLB results (P>0.05). When infection rates were compared by provinces, *B. canis* infection was most frequently detected in the 3-year and older group in Kars, and in the 2-year group in Ardahan and Iğdır. This situation was once again linked to the density of tick population in the provinces [45].

When the distribution of TBPs was evaluated by gender, there was no significant difference in the prevalence between female and male dogs. However the infection

was more commonly found in male dogs [2,3,28]. In our study, the infection rate of TBPs was 12.97% (31/239) in males and 13.04% (21/161) in females by PCR and the prevalence of babesiosis was 22.17% (CI: 17.26-27.75) in the male group and 27.32% (CI: 20.89-34.57) in the female group by RLB. Although it was statistically insignificant (P>0.05), the distribution of *B. canis* according to gender was found to be concentrated in females in Kars and Iğdır, and males in Ardahan.

Considering the habitat of dogs, in a study, the difference in infection rate of stray and shelter dogs was found insignificant (P>0.05) [8]. However, our study revealed that the infection rate of TBPs was 8.14% in rural and 23.07% in shelter dogs as determined by PCR (P<0.05). Additionally, the prevalence of *B. canis* was found to be 7.50% (CI: 3.77-13.23) in rural and 71.42% (CI: 57.82-82.58) in shelter dogs in Kars, 3.40% (CI: 0.96-8.81) in rural and 50% (CI: 16.68-83.31) in shelter dogs in Ardahan, 3.22% (CI: 0.67-9.94) in rural and 4% (CI: 1.13-10.28) in shelter dogs in Iğdır by RLB (P<0.05). The dogs were not properly cared for and their living conditions were inadequate. They were not received proper protection and control program in terms of TBPs. These findings are overlapped with both the study conducted by Taşçı et al.[45] and the data of this study. In the study conducted by Taşçı et al.[45], it was determined that shelter dogs were more heavily infested with ticks than owned dogs. It has been observed that shelter dogs are infected with *B. canis* at a much higher rate than rural dogs.

Previous studies in Türkiye and various countries around the world have reported the prevalence of hepatozoonosis in dogs to be 1-57.8% [1,3,20,22-29,32,42,48]. In the northeastern Anatolia region of Türkiye, the prevalence of hepatozoonosis in dogs was determined to be 7.75% (CI: 5.43-10.67) by RLB within the reference range. When the results of the study were evaluated at the provincial level, the infection rate of hepatozoonosis was 15.32% (CI: 10.04-22.05) in Iğdır, 1.77% (CI: 0.50-4.66) in Kars, and 7.44% (CI: 3.39-14.06) in Ardahan by RLB. The reason why this determined hepatozoonosis prevalence rate was lower than other regions of Türkiye is believed to be due to both the exposure to tick attacks and the biology of the pathogen. In a study conducted in this region [45], it was determined that *Rhipicephalus sanguineus s.l.*, the vector of *H. canis*, was abundant in Iğdır. Compared to other provinces, the higher hepetozoonosis infection rate in Iğdır could be attributed to the vector tick population density.

Although theileriosis is not a specific infection of dogs, it has a global distribution in dogs. The prevalence of *Theileria* species was found at different rates in previous studies. *T. luwenshuni*, *T. ovis,* and *T. buffeli* were determined in sheepdogs (13%) in Iran [14]. In a study conducted in Myanmar [17], 3 dogs were found to be infected with *T. orientalis*, 3 with *T. buffeli*, 2 with *T.cf. velifera*, 1 with *T. luwenshuni* and 1 with *Theileria sp*. The prevalence of *T. annulata* was found to be 10.78% in Pakistan [4], 1% in Türkiye [8], and 32.5% in Iran [41]. *T. annae* was found to be 3% in Croatia [5], 62.5% in Spain [9], *T. sable* was found to be 13% in Nigeria [15], and *T. equi* was found to be 1.3% in Croatia [5], 19% in France [13], 4% in Nigeria [15], 25.81% in Egypt [18], *T. orientalis* was found to be 0.1% in China [49], while *T. ovis* was found to be 4.7% in South Africa [36], 0.29% in Kyrgyzstan [50]. The mentioned species are typically found in cattle, horses, and sheep as their hosts. However, *T. ovis* was detected at a rate of 3.5% (CI: 2.01-5.64) in dogs in this study. The difference between the infection rates could be attributed to factors such as the treatment for parasitic diseases, geographical locations, number of blood samples and breeding purposes of the dogs.

In conclusion, the health of dogs is not sufficiently taken care of in the northeast Anatolia region. The number of shelter dogs is quite high due to reasons such as lack of infrastructure, unconsciousness, and inadequacy of municipal services. Many rural dogs are bred carelessly and unconsciously. Nowadays, with increasing technological developments, non-specific *Theileria* species have been detected in different hosts. *T. ovis,* an apathogenic agent of sheep, was encountered molecularly for the first time in dogs in this region. Although this situation does not give us information about the actual host of the parasite, it only allowed us to detect the parasite in dogs. The detection of *T. ovis* in dogs indicates a different picture of theileriosis than traditionally thought. Although the findings of our and other studies [5,8,9,15,36,41,50] do not prove that dogs which are used to manage the sheep and cattle herds are hosts of *Theileria* species, they can be interpreted to contribute to the epidemiology of theileriosis. According to our findings, because of close living dogs and ruminant hosts, the lack of host specificity of some species suggests that *T. ovis* may be determined in different hosts and dogs may be natural carriers or reservoirs of *T. ovis.* The results of the study revealed that TBP infection rates were found to be higher in shelter dogs compared to rural dogs. This indicates that the care and rehabilitation conditions of shelter dogs are not healthy enough.

More comprehensive studies should be conducted on the pathogenicity of species or genotypes, distribution of vector ticks, and molecular epidemiology of the detected parasites, and to protect dog health, an effective prevention and control program against parasites in dogs should be implemented as soon as possible.

Declarations

Availability of Data and Materials: The data and materials of this study are available from the corresponding author (G.T. Taşçı).

Acknowledgements: Positive controls were provided by Prof. Dr. Esin GÜVEN (Atatürk University), Prof. Dr. Alparslan YILDIRIM and Assoc. Prof. Dr. Arif ÇİL (Erciyes University), Prof. Dr. Nuran AYSUL (Aydın Adnan Menderes University), Assoc. Prof. Dr. Mehmet Fatih AYDIN (Karamanoğlu Mehmetbey University) and Assoc. Prof. Dr. Ömer ORKUN (Ankara University). We thank to all researchers.

Financial Support: This study was supported by the Scientific and Technological Research Institute of Türkiye (TUBITAK) (Project No: 120 O 868).

Conflict of Interest: The authors declared that there is no conflicts of interest.

Author Contributions: GTT: Design of study, Collection, DNA extraction and PCR analysis of blood samples; NA: Design of study, Reverse Line Blotting; NÖ: Design of study, DNA extraction and PCR analysis of blood samples; MY: Collection, DNA extraction and PCR analysis of blood samples; MEI: Collection, DNA extraction and PCR analysis of blood samples; ZV: Reverse Line Blotting

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