Parasites & Vectors

RESEARCH

Open Access

Molecular investigation of bacterial and protozoal pathogens in ticks collected from different hosts in Turkey

Ahmet Efe Köseoğlu[1], Hüseyin Can[1*], Mervenur Güvendi[1], Sedef Erkunt Alak[1], Çağrı Kandemir[2], Turğay Taşkın[2], Samiye Demir[3], Gülşah Akgül[4], Aysu Değirmenci Döşkaya[5], Muhammet Karakavuk[6], Mert Döşkaya[5], Adnan Yüksel Gürüz[5] and Cemal Ün[1]

Abstract

Background: The emergence of tick-borne disease is increasing because of the effects of the temperature rise driven by global warming. In Turkey, 19 pathogens transmitted by ticks to humans and animals have been reported. Based on this, this study aimed to investigate tick-borne pathogens including *Hepatozoon* spp., *Theileria* spp., *Babesia* spp., *Anaplasma* spp., *Borrelia* spp., and *Bartonella* spp. in tick samples ($n = 110$) collected from different hosts (dogs, cats, cattle, goats, sheep, and turtles) by molecular methods.

Methods: To meet this objective, ticks were identified morphologically at the genus level by microscopy; after DNA isolation, each tick sample was identified at the species level using the molecular method. Involved pathogens were then investigated by PCR method.

Results: Seven different tick species were identified including *Rhipicephalus sanguineus*, *R. turanicus*, *R. bursa*, *Hyalomma marginatum*, *H. anatolicum*, *H. aegyptium*, and *Haemaphysalis erinacei*. Among the analyzed ticks, *Hepatozoon* spp., *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. were detected at rates of 6.36%, 16.3%, 1.81%, and 6.36%, respectively while *Borrelia* spp. and *Bartonella* spp. were not detected. *Hepatozoon* spp. was detected in *R. sanguineus* ticks while *Theileria* spp., *Babesia* spp., and *Anaplasma* spp. were detected in *R. turanicus* and *H. marginatum*. According to the results of sequence analyses applied for pathogen positive samples, *Hepatozoon canis*, *Theileria ovis*, *Babesia caballi*, and *Anaplasma ovis* were identified.

Conclusion: *Theileria ovis* and *Anaplasma ovis* were detected for the first time to our knowledge in *H. marginatum* and *R. turanicus* collected from Turkey, respectively. Also, *B. caballi* was detected for the first time to our knowledge in ticks in Turkey.

Keywords: *Hepatozoon canis*, *Theileria ovis*, *Babesia caballi*, *Anaplasma ovis*

*Correspondence: huseyin.can@ege.edu.tr
[1] Molecular Biology Section, Department of Biology, Faculty of Science, Ege University, Izmir, Turkey
Full list of author information is available at the end of the article

Background

Ticks are obligate blood-sucking arachnid ectoparasites belonging to the Ixodida suborder that feeds on a wide variety of wild and domestic vertebrates excluding fish [1–3]. To date, 896 tick species identified are classified into three families: Ixodidae (hard ticks, 702 species), Argasidae (soft ticks, 193 species), and Nuttalliellidae (*Nuttalliella namaqua*, 1 species) [3]. After mosquitoes,

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 2 of 11

ticks are the second most common pathogen vectors worldwide [1, 4, 5], and tick-borne diseases (TBDs) are increasingly threatening animal and human health along with causing economic losses [3, 6].

To date, 19 tick-borne pathogens have been reported in Turkey [7]. Among them, causative agents of hepatozoonosis, theileriosis, babesiosis, anaplasmosis, bartonellosis, and Lyme disease are frequently investigated in both ticks and their vertebrate hosts in Turkey and other countries. Hepatozoon, an apicomplexan intraerythrocytic genus of parasites represented by > 300 species, and belonging to the Hepatozoidae family, is commonly detected in tetrapod vertebrates and numerous hematophagous invertebrates [8, 9]. Among these species, *H. canis* and *H. felis* cause canine and feline hepatozoonosis, respectively, which are important in the veterinary field [10]. In Turkey, *H. canis* was detected in *R. sanguineus* collected from dogs and *Haemaphysalis parva* collected from red foxes [11, 12]. *Theileria* is another apicomplexan parasite that belongs to the order Piroplasmida, and it infects many domestic and wild ruminant animals. *Theileria parva*, *T. annulata*, and *T. orientalis* infect cattle [10, 13], while *T. lestoquardi*, *T. luwenshuni*, *T. separata*, and *T. ovis* infect small ruminants such as sheep and goats [14, 15]. In Turkey, *T. ovis* has been detected in *R. bursa*, *R. turanicus*, and *R. sanguineus* collected from sheep and goats [16, 17]. *Babesia* is another important apicomplexan parasite that belongs to the order Piroplasmida, which is transmitted by ticks and infects the red blood cells of various mammals such as cattle, sheep, horses, dogs, and rodents [10]. The vectors of *Babesia* parasites transmitted to humans and animals are the Ixodid tick species [18]. *Babesia caballi* together with *T. equi* causes equine piroplasmosis disease in horses and is transmitted by *Dermacentor*, *Rhipicephalus*, and *Hyalomma* [19–21]. Among bacterial pathogens, *Anaplasma* is a gram-negative bacterium that is a member of the family Anaplasmataceae in the order Rickettsiales, and it affects human and animal health by causing tick-borne diseases with species such as *A. phagocytophilum*, *A. centrale*, *A. marginale*, *A. bovis*, *A. platys*, and *A. ovis* [22–24]. Although *A. ovis* infects sheep, goats, and wild ruminants in Africa, Asia, Europe, and the US, and shows less pathogenicity than other *Anaplasma* species, it is the main species causing anaplasmosis in small ruminants with subclinical infections [25, 26]. *Borrelia* (*Borreliella*) is another bacterial pathogen in the Spirochaetaceae family that causes tick-borne Lyme borreliosis disease and is transmitted by *Ixodes* tick vectors in Europe, the Far East, and North America [27–29]. *Borrelia burgdorferi* (*s.l.*) includes 20 species, and 9 of them are known as human and animal pathogens [29]. In addition to ticks, *Bartonella*, which is another bacterial pathogen, can be transmitted by fleas and lice and can infect domestic and wild mammals, including humans. Twenty-three species have been identified, and 13 of them have been found to be associated with human diseases [30, 31]. Among *Bartonella* species, *B. henselae* and *B. clarridgeiae* cause cat-scratch disease, while *B. quintana* causes trench fever disease [31, 32]. In Turkey, *B. henselae and B. clarridgeiae* have been reported in domestic cats [33].

In this study, we aimed to investigate tick-borne pathogens including *Hepatozoon* spp., *Theileria* spp., *Babesia* spp., *Anaplasma* spp., *Borrelia* spp., and *Bartonella* spp. in tick samples collected from different hosts from the İzmir, Aydın, Şanlıurfa, and Siirt provinces of Turkey by PCR method.
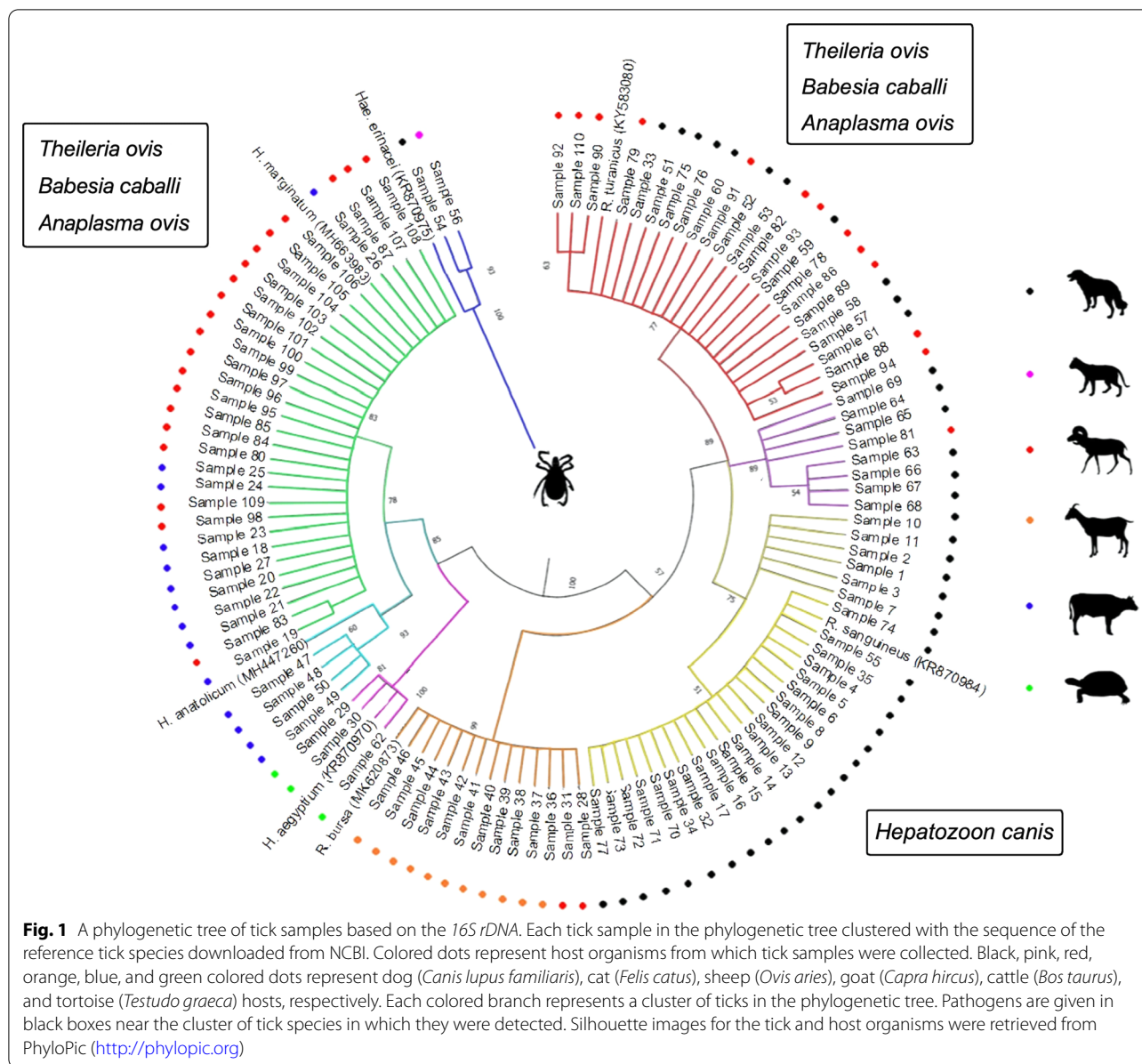
Methods

Morphological identification of tick samples

Tick samples ($n = 110$) were collected from a variety of host organisms [dog ($n = 46$), cat ($n = 1$), cattle ($n = 14$), goat ($n = 11$), sheep ($n = 35$), turtles ($n = 3$)] in four provinces [İzmir ($n = 57$), Aydın ($n = 7$), Şanlıurfa ($n = 16$), Siirt $= 30$] of Turkey between May 2016 and July 2020 (Figs. 1 and 2). Each tick sample was collected from a different host, and all of them were selected randomly. The collections were conducted with the owners' consent. Tick samples attached to the animals were collected, and most of them were fully engorged but some were semi-engorged. Also, all of ticks were adults except for a single tick sample that was a nymph (sample no. 62). Ticks were removed by a sterile pair of forceps and kept in separate vials filled with 70% ethanol and morphologically identified at the genus level under a stereo-microscope using identification keys as described [34–36].

Molecular identification of tick samples

DNA was isolated from each tick sample by a commercial DNA extraction kit (Qiagen) according to the protocol of the manufacturer. Before DNA isolation, each tick sample that was kept in ethanol was rinsed twice with sterile distilled water, dried on a sterile filter, and transferred to a 1.5-ml tube containing tissue lysis buffer and proteinase K. Later, the ticks were cut in to small pieces using a sterile scalpel and homogenized by a sterile micro-pestle. The obtained homogenate was incubated at 55 °C until all small tissue pieces had been lysed, and then the DNA isolation protocol was continued.

In the molecular identification of tick samples, the mitochondrial *16S rDNA* was amplified using 5′-CCG GTCTGAACTCAGATCAAGT-3′ and 5′-CTGCTCAAT GATTTTTTAAATTGCTGTGG-3 primers as described by Mendell et al. [37]. The 25 μl PCR reaction included 2 μl template DNA, 1 μl primers (0.4 mM each), 12.5 μl DreamTaq PCR Master Mix (2×, Thermo Scientific), and

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 3 of 11

Fig. 1 A phylogenetic tree of tick samples based on the *16S rDNA*. Each tick sample in the phylogenetic tree clustered with the sequence of the reference tick species downloaded from NCBI. Colored dots represent host organisms from which tick samples were collected. Black, pink, red, orange, blue, and green colored dots represent dog (*Canis lupus familiaris*), cat (*Felis catus*), sheep (*Ovis aries*), goat (*Capra hircus*), cattle (*Bos taurus*), and tortoise (*Testudo graeca*) hosts, respectively. Each colored branch represents a cluster of ticks in the phylogenetic tree. Pathogens are given in black boxes near the cluster of tick species in which they were detected. Silhouette images for the tick and host organisms were retrieved from PhyloPic (http://phylopic.org)

8.5 µl nuclease-free water. The PCR was performed using the following protocol: 5 min initial denaturation step at 95 °C, followed by 10 cycles of 1 min at 92 °C, 1 min at 48 °C, and 90 s at 72 °C; 32 cycles of 1 min at 92 °C, 35 s at 54 °C, and 90 s at 72 °C; and a final extension of 7 min at 72 °C. All PCR products were separated in 1% agarose gel, stained with nucleic acid dye (SafeView) and visualized.
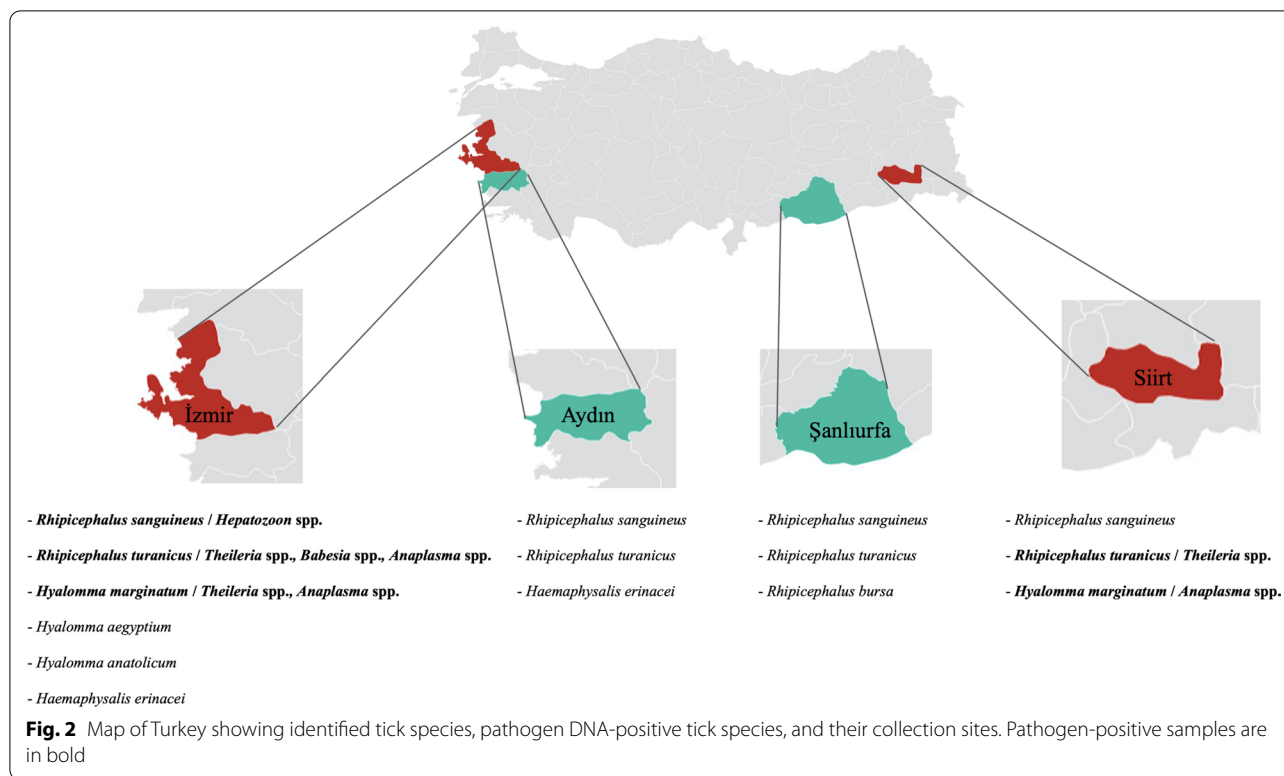
Molecular investigation of pathogens by PCR

For detection of *Hepatozoon* spp., *Babesia*/*Theileria* spp., *Anaplasma* spp., *Borrelia* spp., and *Bartonella* spp., the used PCR methods, target genes, primers, expected PCR product sizes, and reaction and amplification conditions are shown in Table 1. For each PCR, DreamTaq PCR Master Mix (2×, Thermo Scientific) was used.

Sequencing

PCR products belonging to ticks and positive pathogen samples were sequenced, and the generated sequences were aligned and edited by MEGA7.0 software. Later, a BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed by comparing with the reference samples in National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) to identify ticks and tick-borne pathogen species.

Köseoğlu *et al. Parasites Vectors* (2021) 14:270

Page 4 of 11

Fig. 2 Map of Turkey showing identified tick species, pathogen DNA-positive tick species, and their collection sites. Pathogen-positive samples are in bold

Phylogenetic analysis

For species identification of tick samples and pathogens, phylogenetic analysis was conducted after a model test selection. Accordingly, the mitochondrial *16S rDNA* gene region (375 bp) of tick samples was aligned in MEGA7.0, and the phylogenetic tree was reconstructed by the maximum Likelihood method using the Kimura 2 (K2) model with 1000 bootstrap replications. As reference *16S rDNA* sequences, *R. sanguineus* (KR870984), *R. turanicus* (KY583080), *R. bursa* (MK620873), *H. marginatum* (MH663983), *H. anatolicum* (MH447260), *H. aegyptium* (KR870970), and *Hae. erinacei* (KR870975) deposited in NCBI database (https://www.ncbi.nlm.nih.gov) were used. The *18S rDNA* gene region (613 bp) of *H. canis* samples was aligned in MEGA7.0, and the phylogenetic tree was reconstructed by the minimum evolution method using the Tamura 3-parameter (T92) model with 1000 bootstrap replications. As reference *18S rDNA* sequences, *H. canis* (MH922768.1), *H. felis* (AB771571.1), and *Toxoplasma gondii* (L24381.1; as an outgroup) deposited in NCBI database (https://www.ncbi.nlm.nih.gov) were used. *18S rDNA* gene region (1034 bp) belonging to *T. ovis* and *B. caballi* samples was aligned in MEGA7.0, and the phylogenetic tree was reconstructed by the maximum likelihood method using the Tamura three-parameter gamma distribution (T92+G) model with 1000 bootstrap replications. As

reference *18S rDNA* sequences, *T. ovis* (FJ603460.1), *T. annulata* (KF429800.1), *T. parva* (L02366.1), *B. caballi* (EU888901.1), *B. bigemina* (KM046917.1), *B. microti* (LC127372.1), and *Plasmodium falciparum* (JQ627152.1; as an outgroup) species deposited in the NCBI database (https://www.ncbi.nlm.nih.gov) were used. *Msp4* gene sequence (321 bp) of *A. ovis* sample was aligned with reference sequences, and a phylogenetic tree was reconstructed by the maximum likelihood method using the Kimura-2 (K2) model with 1000 bootstrap replications. As reference *Msp4* sequences, *A. ovis* (KU497712.1), *A. phagocytophilum* (KC847317.1), and *A. marginale* (KU497715.1) species deposited in NCBI database (https://www.ncbi.nlm.nih.gov) were used.

Statistical analysis

Molecular detection proportions between the Aegean region and Southeastern Anatolia region as well as between tick species were computed, and comparison of the proportions was performed by the chi-square test using the PASW Statistics 18 software. Statistically significant differences were determined at $P < 0.05$.

Results

Morphological and molecular identification of ticks

Among 110 tick samples, morphological analysis showed that there were three genera including

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 5 of 11

Table 1 PCR methods, target genes, primers, expected PCR product sizes, and reaction and amplification conditions as well as references used for detection of tick-borne pathogens

Organism	Molecular diagnostic method	Target gene	Primer	PCR product size (bp)	Reaction content	Reaction conditions	References
Hepatozoon spp.	Conventional PCR	*18S rDNA*	5′-ATACATGAG CAAAATCTC AAC-3′ 5′-CTTATTATTCCA TGCTGCAG-3′	650	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 2 µl DNA)	95 ℃ 5 min, 34× (95 ℃ 30 s, 53 ℃ 30 s, 72 ℃ 90 s), 72 ℃ 5 min	[11, 55]
Theileria spp. *Babesia* spp.	Nested PCR 1	*18S rDNA*	5′-GTGAAACTG CGAATGGCTCAT TAC-3′ 5-AAGTGATAAGGT TCACAAAACTTC CC-3	1609–1523	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 1 µl DNA)	94 ℃ 1 min, 30× (98 ℃ 10 s, 55 ℃ 15 s, 68 ℃ 45 s), 68 ℃ 5 min	[10]
	Nested PCR 2		5′-GGCTCATTA CAACAGTTATAG TTTATTTG-3′ 5′-CGGTCCGAA TAATTCACC GGAT-3′	1544–1454			
Anaplasma spp.	Nested PCR 1	*Msp4*	5′-ATGAATTAC AGAGAATTGCTT GTAGG-3′ 5-TTAATTGAAAGC AAATCTTGCTCC TATG-3′	849	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 5 µl DNA)	94 ℃ 4 min, 40× (94 ℃ 30 s, 55 ℃ 30 s, 72 ℃ 30 s), 72 ℃ 10 min	[56]
	Nested PCR 2		5′-CTATTGGYG- GNGCYAGAGT-3′ 5-GTTCATCGAAAA TTCCGTGGTA-3′	381		94 ℃ 4 min, 40× (94 ℃ 30 s, 56 ℃ 30 s, 72 ℃ 30 s), 72 ℃ 10 min	
Borrelia spp.	Nested PCR 1	*OspA*	5′-CTTGAAGTT TTCAAAGAA GAT-3′ 5′-CAACTGCTG ACCCCTCTA AT-3′	487	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 2 µl DNA)	95 ℃ 5 min, 40× (95 ℃ 15 s, 55 ℃ 30 s, 72 ℃ 45 s), 72 ℃ 5 min	[57]
	Nested PCR 2		5′-ACAAGAGCA GACGGAACC AG-3′ 5′-TTGGTGCCATTT GAGTCGTA-3′	350		95 ℃ 5 min, 40× (95 ℃ 15 s, 58 ℃ 30 s, 72 ℃ 45 s), 72 ℃ 5 min	
Bartonella spp.	Nested PCR 1	*16S-23S rDNA ITS*	5′-AAGTCGTAA CAAGGT-3′ 5′-TACTGGTKCGC TATCGGTCA-3′	1800	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 5 µl DNA)	95 ℃ 5 min, 40× (94 ℃ 30 s, 40 ℃ 45 s, 72 ℃ 4 min), 72 ℃ 10 min	[58]
	Nested PCR 2		5′-TTTCTCTTTCTT CAGATGATG-3 ′5′-AAAGCAGGT GCTCTCCCAGAA AGCAGGTGCTCT CCCAG-3	587	25 µl reaction volume (12.5 µl PCR master mix, 1 µl primers, 2 µl DNA)	95 ℃ 5 min, 30× (94 ℃ 30 s, 60 ℃ 30 s, 72 ℃ 40 s), 72 ℃ 5 min	

Rhipicephalus ($n = 71$; 64.5%), *Hyalomma* ($n = 37$; $n =$ 33.6%), and *Haemaphysalis* ($n = 2$; 1.8%). According to NCBI-BLAST and phylogenetic analysis results, *R. sanguineus* ($n = 27$; 24.5%), *R. turanicus* ($n = 31$; 28.1%), *R. bursa* ($n = 13$; 11.8%), *H. marginatum* ($n = 30$; 27.2%), *H. anatolicum* ($n = 4$; 3.6%), *H. aegyptium* ($n = 3$;

2.72%), and *Hae. erinacei* ($n = 2$; 1.8%) were identified (Fig. 1).

Molecular detection rates of involved pathogens

At least one pathogen DNA was amplified from 31 of 110 (28.1%) tick samples studied. Three tick samples (3/110;

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 6 of 11

2.72%) including *R. turanicus* and *H. marginatum* species were detected to carry DNA of at least two different pathogens. Tick species that were detected to harbor the pathogen DNA were *R. turanicus, H. marginatum,* and *R. sanguineus*. The detection rates of pathogen DNA were 25.9% (7/27), 32.2% (10/31), and 46.6% (14/30) for *R. sanguineus, R. turanicus,* and *H. marginatum,* respectively. *Hepatozoon* spp. was detected in *R. sanguineus* collected from dogs in İzmir, and the detection rate of *Hepatozoon* spp. DNA was 6.36% (7/110). Six of them

were successfully sequenced and identified as *H. canis* (Table 2). *Theileria* spp. was detected in 18 tick samples whereas *Babesia* spp. in two tick samples. DNA detection rates were 16.3% (18/110) and 1.81% (2/110) for *Theileria* spp. and *Babesia* spp., respectively. Both were detected in *R. turanicus* and *H. marginatum* collected from sheep (Table 2). *Babesia* spp. was found in tick samples collected from İzmir whereas *Theileria* spp. in İzmir as well as Siirt. Among these positive samples, 15 samples were successfully sequenced, and 13 of them were identified as

Table 2 The detected tick-borne pathogen species along with tick species, host organism, locality, detection method, percentage of nucleotide identity, and molecular detection rate

Pathogen species	Sample no.	Tick species	Locality	Host organism	Molecular diagnostic method	Accession number, percentage of nucleotide identity	Molecular detection rate
Hepatozoon canis	3	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	LC428208.1; 100%	6.36% (7/110)
Hepatozoon canis	4	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	MH615006.1; 99%	
Hepatozoon canis	6	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	LC018209.1; 99%	
Hepatozoon canis	7	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	LC018209.1; 100%	
Hepatozoon canis	11	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	LC018209.1; 100%	
Hepatozoon canis	14	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	LC428208.1; 100%	
Hepatozoon spp.	15	*Rh. sanguineus*	İzmir	Dog	Conventional PCR	[a]	
Theileria ovis	78	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	16.3% (18/110)
Theileria ovis	79	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	85	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	88	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	89	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	90	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	91	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	92	*Rh. turanicus*	Siirt	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	94	*Rh. turanicus*	İzmir	Sheep	Nested PCR	MN493111.1; 100%	
Theileria spp.	95	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Theileria ovis	97	*Hy. marginatum*	İzmir	Sheep	Nested PCR	MN493111.1; 100%	
Theileria ovis	99	*Hy. marginatum*	İzmir	Sheep	Nested PCR	MN493111.1; 100%	
Theileria spp.	100	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Theileria ovis	101	*Hy. marginatum*	İzmir	Sheep	Nested PCR	MN493111.1; 99.9%	
Theileria spp.	103	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Theileria spp.	104	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Theileria ovis	105	*Hy. marginatum*	İzmir	Sheep	Nested PCR	MN493111.1; 100%	
Theileria spp.	107	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Babesia caballi	109	*Hy. marginatum*	İzmir	Sheep	Nested PCR	MN629354.1; 100%	1.81% (2/110)
Babesia caballi	110	*Rh. turanicus*	İzmir	Sheep	Nested PCR	MN629354.1; 100%	
Anaplasma spp.	83	*Hy. marginatum*	Siirt	Sheep	Nested PCR	[a]	6.36% (7/110)
Anaplasma spp.	84	*Hy. marginatum*	Siirt	Sheep	Nested PCR	[a]	
Anaplasma spp.	86	*Hy. marginatum*	Siirt	Sheep	Nested PCR	[a]	
Anaplasma ovis	94	*Rh. turanicus*	İzmir	Sheep	Nested PCR	MN307492.1; 99.67%	
Anaplasma spp.	97	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Anaplasma spp.	98	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	
Anaplasma spp.	99	*Hy. marginatum*	İzmir	Sheep	Nested PCR	[a]	

[a] Indicates that sequence data were not obtained

T. ovis, while 2 were identified as *B. caballi*. *Anaplasma* spp. were detected in seven tick samples including *H. marginatum* and *R. turanicus* collected from sheep in İzmir and Siirt. The detection rate of *Anaplasma* spp. DNA was 6.36% (7/110) in tick samples analyzed. Among positive samples, a single positive sample detected in *R. turanicus* was successfully sequenced and identified as *A. ovis* (Table 2). *Borrelia* spp. and *Bartonella* spp. were not detected among tick samples analyzed.

The percent identity rate among *H. canis* isolates varied from 99.05 to 100%, whereas for *T. ovis* isolates, it varied from 99.9 to 100%. The percent identity rate was 100% for *B. caballi* isolates.

The pathogen DNA positivity proportion detected in the Aegean region was statistically significantly higher than in the Southeastern Anatolia region ($P < 0.05$). Also, no statistically significant relationship was found in terms of the pathogen detection proportion among pathogen-positive tick species ($P > 0.05$).
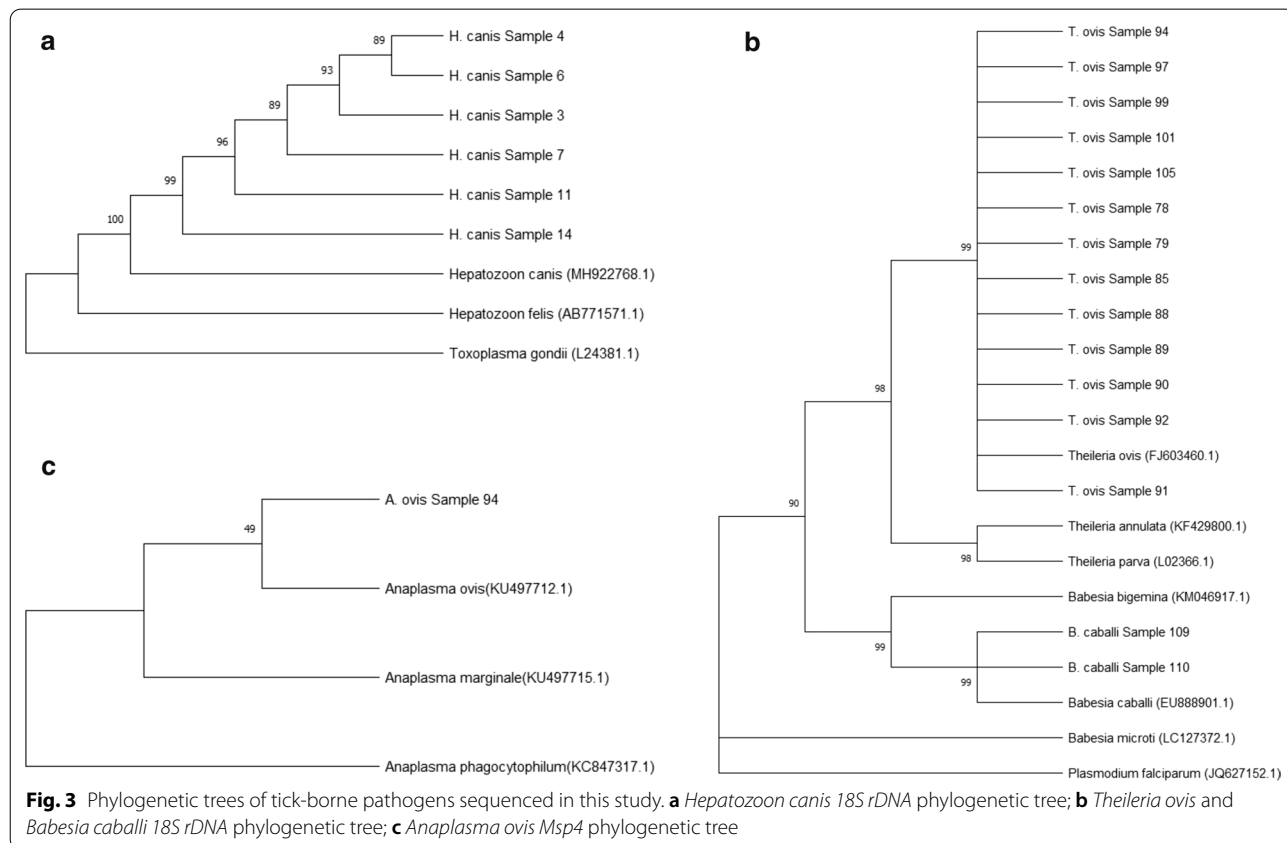
Phylogenetic trees

All analyzed species clustered with their own reference sample, and unexpected branches containing different tick species were not observed in the phylogenetic tree (Fig. 1). In addition, although eight tick sequences

(samples 63, 64, 65, 66, 67, 68, 69, and 81) clustered in the same proximity to both *R. turanicus* and *R. sanguineus* reference species in the phylogenetic tree (Fig. 1), these sequences matched with *R. turanicus* in the BLAST analysis. Similarly, unexpected or mixed branches containing different species were not observed in phylogenetic trees constructed for each pathogen (Fig. 3).

Discussion

The number of ticks has increased in suitable ecological habitats because of the rising temperature driven by global warming. To date, 19 tick-borne pathogens were reported in Turkey [7]. Although prevalence studies conducted for tick-borne pathogens are present in Turkey (Table 3), the number of these studies is insufficient in the Aegean region of Turkey, and thus the prevalence of tick-borne pathogens is incompletely known in this region. The present study aimed to investigate the important tick-borne pathogens in tick samples collected from four different provinces of Turkey especially involving the Aegean region. For this aim, identification of tick samples was conducted and *R. sanguineus* (24.5%), *R. turanicus* (28.1%), and *H. marginatum* (27.2%) were confirmed as the most prevalent tick species. Previous studies conducted in Turkey demonstrated that *R. turanicus* and *H.*

Fig. 3 Phylogenetic trees of tick-borne pathogens sequenced in this study. **a** *Hepatozoon canis 18S rDNA* phylogenetic tree; **b** *Theileria ovis* and *Babesia caballi 18S rDNA* phylogenetic tree; **c** *Anaplasma ovis Msp4* phylogenetic tree

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 8 of 11

Table 3 Molecular prevalence of *Hepatozoon canis*, *Theileria ovis*, and *Anaplasma ovis*, detected in previous studies, in ticks from Turkey

Pathogen species	Locality	Host organism	Tick species	Diagnostic method	Prevalence	References
Hepatozoon canis	Diyarbakır	Dog	*Rh. sanguineus*	Conventional PCR	20.58% (14/68 pool)	[11]
	Ankara	Red fox	*Hae. parva*	Conventional PCR	33.3% (1/3 pool)	[12]
Theileria ovis	Elazığ	Sheep, Goat	*Rh. bursa*	Conventional PCR	19.27% (37/192 individual)	[16]
	Bolu, Kastamonu, Çorum, Samsun, Tokat, Giresun, Bayburt	Sheep, Goat	*Rh. bursa*	Conventional PCR	2.37% (10/49 pool)	[17]
		Sheep, Goat	*Rh. turanicus*	Conventional PCR	2.27% (13/70 pool)	[17]
		Sheep, Goat	*Rh. sanguineus*	Conventional PCR	1.47% (1/6 pool)	[17]
Anaplasma ovis	Ankara	Boar, Rabbit, Fox	*Rh. turanicus*, *De. marginatus*, *Hae. parva*, *Hy.* spp., *Hy. marginatum*, *Hy. excavatum*, *Hy. aegyptium*	Conventional PCR	0% (0/445 individual and 0/102 pool)	[49]
	Ankara, Bolu, Kırşehir	Cattle, Sheep, Goat, Dog	*Rh. sanguineus*, *Rh. turanicus*, *Rh. bursa*, *De. marginatus*, *Hy. marginatum*, *Hy. excavatum*, *Hy. anatolicum*, *Hae. parva*, *Hae. inermis*, *Ix. ricinus*	Conventional PCR	0% (0/75 adult female individual and 0/151 larval pool)	[50]
	Ankara	Sheep	*Rh. sanguineus*	Conventional PCR	0.82% (2/242 individual)	[51]

marginatum were prevalent, in line with our study except for *R. sanguineus* [38, 39].

In addition, such important protozoan and bacterial pathogens also have been investigated in this study; 35.9% of tick samples collected from the Aegean region were pathogen DNA-positive whereas 23.9% of ticks collected from the Southeastern Anatolia region were pathogen DNA-positive. *Hepatozoon* spp. was detected by conventional PCR with a rate of 6.36% in *R. sanguineus* collected from dogs in İzmir province of Turkey and identified as *H. canis* (Table 2). Although *R. sanguineus* is known to be the main vector for *H. canis*, the vectoring possibilities of other tick species including *R. turanicus*, *Hae. longicornis*, *Hae. flava*, and *Amblyomma ovale* have been reported [40–42]. In Turkey, *H. canis* was detected by conventional PCR with a prevalence of 33.3% in *Hae. parva* collected from red foxes in Ankara while it was detected by conventional PCR with a prevalence of 20.58% in *R. sanguineus* collected from dogs in Diyarbakır [11, 12] (Table 3). In addition to Turkey, *H. canis* was detected by conventional PCR in adult *R. sanguineus* species and nymph *Ixodes ricinus* collected from dogs in Italy with prevalences of 33% and 5%, respectively [43]. These findings show that *Hae. parva* also can harbor the *H. canis* in Turkey in addition to the main vector, *R. sanguineus*.

In this study, *Theileria* spp. was detected by nested PCR with a rate of 16.3% in *R. turanicus* and *H. marginatum* collected from sheep in İzmir and Siirt provinces

and identified as *T. ovis* (Table 2). Also, the present study is the first reporting the detection of *T. ovis* in *H. marginatum* in addition to *R. bursa*, *R. turanicus*, and *R. sanguineus* in Turkey. For example, *T. ovis* was detected by conventional PCR with a prevalence of 19.27% in *R. bursa* collected from sheep and goats in Elazig province of Turkey [16] (Table 3), and it was known that *T. ovis* was the most common *Theileria* species in small ruminants and its vector was *R. bursa* in the Mediterranean basin and Turkey [14, 16, 44, 45]. However, another work conducted in the Black Sea region of Turkey (Bolu, Kastamonu, Çorum, Samsun, Tokat, Giresun, and Bayburt) detected *T. ovis* by conventional PCR in *R. turanicus* and *R. sanguineus* in addition to *R. bursa* collected from sheep and goats with prevalences of 2.27%, 1.47%, and 2.37%, respectively [17] (Table 3).

In this study, *Babesia* spp. was detected by nested PCR with a rate of 1.81% in *H. marginatum* and *R. turanicus* collected from sheep in İzmir and identified as *B. caballi*. This was the second important first report in this study, demonstrating the presence of *B. caballi* in two different tick species in Turkey (Table 2). A previous study conducted in Erzurum province of Turkey screened *B. caballi* by multiplex PCR in horses but was not detected [20] while another study detected it with a prevalence of 3% by PCR in horses in Ankara [46]. Also, no report has detected *B. caballi* in ticks in Turkey. Apart from Turkey, *B. caballi* was detected by conventional PCR with a prevalence of 7.6% in *R. evertsi evertsi* from horses and

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 9 of 11

donkeys in Nigeria while it was detected by nested PCR with a prevalence of 12.9% in *Dermacentor nuttalli* from horses in Mongolia [47, 48]. Also, *B. caballi* was detected with a prevalence of 14.55% in 2017 while it was detected with a prevalence of 27.59% in 2018 by real-time PCR in *R. bursa* collected from vegetation in Italy [21].

In this study, *Anaplasma* spp. was detected by nested PCR with a rate of 6.36% in *H. marginatum* and *R. turanicus* collected from sheep in the Siirt and İzmir provinces of Turkey, and only one sample detected in *R. turanicus* could be sequenced and identified as *A. ovis* (Table 2). This was the third important first report in this study demonstrating the presence of *A. ovis* in *R. turanicus* in Turkey (Table 2). In Turkey, *A. ovis* was not detected by conventional PCR in ticks collected from several animals in Ankara [49] (Table 3). Similarly, a previous work comprising Ankara, Bolu, and Kırşehir provinces did not detect *A. ovis* by conventional PCR in several ticks collected from animals including cattle, sheep, goats, and dogs [50] (Table 3). However, another study detected *A. ovis* by PCR with a prevalence of 0.82 (2/242) in *R. sanguineus* collected from sheep in Ankara [51] (Table 3). Apart from Turkey, *A. ovis* was detected by conventional PCR with a prevalence of 2.3% in *Hae. longicornis* collected from sheep in China while it was detected by conventional PCR with a prevalence of 2.4% in *D. nuttalli* collected from sheep and goats in Mongolia [23, 52]. Also, *A. ovis* was detected by real-time PCR with a prevalence of 20.3% in *R. bursa* collected from goats in France [26].

Interestingly, *Borrelia* spp. and *Bartonella* spp. were not detected among tick samples analyzed in the present study. Indeed, *Borrelia* spp. has been detected at high prevalence rates in tick samples analyzed in previous studies conducted in Turkey. For example, *Borrelia* spp. was detected by nested PCR with a prevalence of 20% in *R. turanicus* collected from boars in Ankara [49]. A different study conducted in İstanbul detected *Borrelia* spp. by conventional PCR with prevalence rates of 44% and 39% in adult and nymph forms of *H. aegyptium*, respectively, which were collected from tortoises (*Testudo graeca*) [53]. Also, *B. burgdorferi* was detected by nested PCR with prevalence rates of 38.7% and 11.4% in İstanbul and Kırklareli provinces, respectively [54]. In addition, to date, the molecular prevalence of *B. henselae* has only been reported in domestic cats in Turkey [7, 33], and no molecular prevalence study is available on the existence of *Bartonella* spp. in ticks.

This study has some limitations. First is the small sample size. A more comprehensive study with more samples can enable reaching a more precise pathogen detection rate as well as detect the *Borrelia* spp. and *Bartonella* spp. in these study areas. The other is the use of a single molecular diagnostic method. In addition to this method, the use of microscopy and culture methods or additional molecular methods such as RNA analyses indicating pathogen-related gene expression can indicate that ticks were infected with the detected pathogens and these pathogens could be transmitted by ticks to vertebrate hosts.

Conclusions

Hepatozoon canis, T. ovis, B. caballi, and *A. ovis* were detected among tick samples studied contrary to *Borrelia* spp. and *Bartonella* spp. Overall, at least one pathogen DNA was detected in 28.1% of tick samples studied. Among these findings, three of them are of great importance for studies conducted in this field in Turkey. The first one is the first detection of *T. ovis* in *H. marginatum* whereas the second one is the first detection of *A. ovis* in *Rh. turanicus,* and the third one is the first detection of *B. caballi* in both *H. marginatum* and *R. turanicus* species, which have veterinary and medical importance*.*

Availability of data and materials
All sequences obtained from pathogens were deposited into GenBank (National Center for Biotechnology Information Search database). Provided GenBank Accession numbers are as follows: MW810676, MW810677, MW810626, MW810627, MW810628, MW810629, MW810630, MW810631, MW810474, MW810475, MW810476, MW810477, MW810478, MW810479, MW810480, MW810481, MW810482, MW810483, MW810484, MW810485, MW810486, and MW821793.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Molecular Biology Section, Department of Biology, Faculty of Science, Ege University, Izmir, Turkey. [2]Department of Animal Science, Faculty of Agriculture, Ege University, Izmir, Turkey. [3]Zoology Section, Department of Biology, Faculty of Science, Ege University, Izmir, Turkey. [4]Department of Internal Medicine, Faculty of Veterinary Medicine, Siirt University, Siirt, Turkey. [5]Department of Parasitology, Faculty of Medicine, Ege University, Izmir, Turkey. [6]Ödemiş Technical Training College, Ege University, Izmir, Turkey.

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 10 of 11

References

1. Nava S, Guglielmone AA, Mangold AJ. An overview of systematics and evolution of ticks. Front Biosci. 2009;14:2857–77. https://doi.org/10.2741/3418.
2. Kaufman WR. Ticks: physiological aspects with implications for pathogen transmission. Ticks Tick Borne Dis. 2010;1:11–22. https://doi.org/10.1016/j.ttbdis.2009.12.001.
3. Wang T, Zhang S, Pei T, Yu Z, Liu J. Tick mitochondrial genomes: structural characteristics and phylogenetic implications. Parasit Vectors. 2019;12:451. https://doi.org/10.1186/s13071-019-3705-3.
4. Fivaz B, Petney T, Horak I. Tick vector biology: medical and veterinary aspects. Berlin: Springer Science & Business Media; 1992.
5. de la Fuente J, Estrada-Pena A, Venzal JM, Kocan KM, Sonenshine DE. Overview: ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci. 2008;13:6938–46. https://doi.org/10.2741/3200.
6. Jongejan F, Uilenberg G. The global importance of ticks. Parasitology. 2004;129:3–14. https://doi.org/10.1017/s0031182004005967.
7. Inci A, Yildirim A, Duzlu O, Doganay M, Aksoy S. Tick-borne diseases in Turkey: a review based on one health perspective. PLoS Negl Trop Dis. 2016;10:e0005021. https://doi.org/10.1371/journal.pntd.0005021.
8. Smith TG. The genus Hepatozoon (Apicomplexa: Adeleina). J Parasitol. 1996;82:565–85.
9. Baneth G, Samish M, Shkap V. Life cycle of *Hepatozoon canis* (Apicomplexa: Adeleorina: Hepatozoidae) in the tick *Rhipicephalus sanguineus* and domestic dog (*Canis familiaris*). J Parasitol. 2007;93:283–99. https://doi.org/10.1645/GE-494R.1.
10. Masatani T, Hayashi K, Andoh M, Tateno M, Endo Y, Asada M, et al. Detection and molecular characterization of *Babesia*, *Theileria*, and *Hepatozoon* species in hard ticks collected from Kagoshima, the southern region in Japan. Ticks Tick Borne Dis. 2017;8:581–7. https://doi.org/10.1016/j.ttbdis.2017.03.007.
11. Aktas M, Özübek S, Ipek DNS. Molecular investigations of *Hepatozoon* species in dogs and developmental stages of *Rhipicephalus sanguineus*. Parasitol Res. 2013;112:2381–5. https://doi.org/10.1007/s00436-013-3403-6.
12. Orkun Ö, Nalbantoğlu S. *Hepatozoon canis* in Turkish red foxes and their ticks. Vet Parasitol Reg Stud Rep. 2018;13:35–7. https://doi.org/10.1016/j.vprsr.2018.03.007.
13. Morrison WI, McKeever DJ. Current status of vaccine development against *Theileria* parasites. Parasitology. 2006;133:169–87. https://doi.org/10.1017/S0031182006001867.
14. Altay K, Aktaş M, Dumanli N. *Theileria* infections in small ruminants in the east and southeast Anatolia. Turkiye Parasitol Derg. 2007;31:268–71.
15. Azmi K, Al-Jawabreh A, Abdeen Z. Molecular detection of *Theileria ovis* and *Theileria equi* in livestock from Palestine. Sci Rep. 2019;9:11557. https://doi.org/10.1038/s41598-019-47965-0.
16. Aktas M, Altay K, Dumanli N. PCR-based detection of *Theileria ovis* in Rhipicephalus bursa adult ticks. Vet Parasitol. 2006;140:259–63. https://doi.org/10.1016/j.vetpar.2006.04.005.
17. Aydin MF, Aktas M, Dumanli N. Molecular identification of *Theileria* and *Babesia* in ticks collected from sheep and goats in the Black Sea region of Turkey. Parasitol Res. 2015;114:65–9. https://doi.org/10.1007/s00436-014-4160-x.
18. Teal AE, Habura A, Ennis J, Keithly JS, Madison-Antenucci S. A new real-time PCR assay for improved detection of the parasite *Babesia microti*. J Clin Microbiol. 2012;50:903–8. https://doi.org/10.1128/JCM.05848-11.
19. Englund L, Pringle J. New diseases and increased risk of diseases in companion animals and horses due to transport. Acta Vet Scand Suppl. 2003;100:19–25.
20. Guven E, Avcioglu H, Deniz A, Balkaya İ, Abay U, Yavuz Ş, et al. Prevalence and molecular characterization of *Theileria equi* and *Babesia caballi* in jereed horses in Erzurum, Turkey. Acta Parasitol. 2017;62:207–13. https://doi.org/10.1515/ap-2017-0025.
21. Romiti F, Magliano A, Antognetti V, Manna G, Cersini A, Scicluna MT, et al. Investigation of Ixodid ticks as vectors of *Babesia caballi* and *Theileria equi*
(Protozoa: Apicomplexa) in central Italy. J Vector Ecol. 2020;45:25–31. https://doi.org/10.1111/jvec.12370.
22. Aktas M, Vatansever Z, Altay K, Aydin MF, Dumanli N. Molecular evidence for *Anaplasma phagocytophilum* in *Ixodes ricinus* from Turkey. Trans R Soc Trop Med Hyg. 2010;104:10–5. https://doi.org/10.1016/j.trstmh.2009.07.025.
23. Peng Y, Qi M, Jian F, Wang J, Lv Y, Wei J, et al. Molecular identification of tick-borne pathogens in tick *Haemaphysalis longicornis* from sheep in Henan, China. Turk J Vet Anim Sci. 2017;41:51–5. https://doi.org/10.3906/vet-1507-103.
24. Wang J, Zhang Y, Wang X, Cui Y, Yan Y, Wang R, et al. A loop-mediated isothermal amplification assay targeting 16S rRNA gene for rapid detection of *Anaplasma phagocytophilum* infection in sheep and goats. J Parasitol. 2017;103:187–92. https://doi.org/10.1645/16-158.
25. Liu Z, Peasley AM, Yang J, Li Y, Guan G, Luo J, et al. The *Anaplasma ovis* genome reveals a high proportion of pseudogenes. BMC Genomics. 2019;20:69. https://doi.org/10.1186/s12864-018-5374-6.
26. Cabezas-Cruz A, Gallois M, Fontugne M, Allain E, Denoual M, Moutailler S, et al. Epidemiology and genetic diversity of *Anaplasma ovis* in goats in Corsica, France. Parasit Vectors. 2019;12:3. https://doi.org/10.1186/s13071-018-3269-7.
27. Kar S, Yilmazer N, Midilli K, Ergin S, Alp H, Gargili A. Presence of the zoonotic *Borrelia burgdorferi* sl. and *Rickettsia* spp. in the ticks from wild tortoises and hedgehogs. Clin Exp Health Sci. 2011;1:166–70.
28. Yang J, Guan G, Niu Q, Liu Z, Li Y, Liu J, et al. Development and application of a loop-mediated isothermal amplification assay for rapid detection of *Borrelia burgdorferi* s. l. in ticks. Transbound Emerg Dis. 2013;60:238–44. https://doi.org/10.1111/j.1865-1682.2012.01335.x.
29. Mendoza-Roldan JA, Colella V, Lia RP, Nguyen VL, Barros-Battesti DM, Iatta R, et al. *Borrelia burgdorferi* (sensu lato) in ectoparasites and reptiles in southern Italy. Parasit Vectors. 2019;12:35. https://doi.org/10.1186/s13071-019-3286-1.
30. Diaz MH, Bai Y, Malania L, Winchell JM, Kosoy MY. Development of a novel genus-specific real-time PCR assay for detection and differentiation of *Bartonella* species and genotypes. J Clin Microbiol. 2012;50:1645–9. https://doi.org/10.1128/JCM.06621-11.
31. Staggemeier R, Pilger DA, Spilki FR, Cantarelli VV. Multiplex SYBR® green-real time PCR (qPCR) assay for the detection and differentiation of *Bartonella henselae* and *Bartonella clarridgeiae* in cats. Rev Inst Med Trop Sao Paulo. 2014;56:93–5. https://doi.org/10.1590/S0036-46652014000200001.
32. Morozova OV, Cabello FC, Dobrotvorsky AK. Semi-nested PCR detection of *Bartonella henselae* in *Ixodes persulcatus* ticks from Western Siberia, Russia. Vector Borne Zoonotic Dis. 2004;4:306–9. https://doi.org/10.1089/vbz.2004.4.306.
33. Celebi B, Kilic S, Aydin N, Tarhan G, Carhan A, Babur C. Investigation of *Bartonella henselae* in cats in Ankara, Turkey. Zoonoses Public Health. 2009;56:169–75. https://doi.org/10.1111/j.1863-2378.2008.01170.x.
34. Walker JB, Keirans JE, Horak IG. The Genus *Rhipicephalus* (Acari, Ixodidae): a guide to the brown ticks of the world. revised. Cambridge: Cambridge University Press; 2000.
35. Estrada-Peña A, Bouattour A, Camicas JL, Walker AR. Ticks of domestic animals in the Mediterranean region. Zaragoza: University of Zaragoza; 2004.
36. Apanaskevich DA, Horak IG. The genus *Hyalomma* Koch, 1844: V. Re-evaluation of the taxonomic rank of taxa comprising the *H.(Euhyalomma) marginatum* Koch complex of species (Acari: Ixodidae) with redescription of all parasitic stages and notes on biology. Int J Acarol. 2008;34:13–42. https://doi.org/10.1080/01647950808683704.
37. Mendell NL, Reynolds ES, Blanton LS, Hermance ME, Londoño AF, Hart CE, et al. Detection of *Rickettsiae*, *Borreliae*, and *Ehrlichiae* in ticks collected from Walker County, Texas, 2017–2018. Insects. 2019;10:315. https://doi.org/10.3390/insects10100315.
38. Karasartova D, Gureser AS, Gokce T, Celebi B, Yapar D, Keskin A, et al. Bacterial and protozoal pathogens found in ticks collected from humans in Corum province of Turkey. PLoS Negl Trop Dis. 2018;12:e0006395. https://doi.org/10.1371/journal.pntd.0006395.
39. Keskin A, Keskin A, Bursali A, Tekin S. Ticks (Acari: Ixodida) parasitizing humans in Corum and Yozgat provinces, Turkey. Exp Appl Acarol. 2015;67:607–16. https://doi.org/10.1007/s10493-015-9966-4.

Köseoğlu *et al. Parasites Vectors*   (2021) 14:270

Page 11 of 11

40. Otranto D, Dantas-Torres F, Weigl S, Latrofa MS, Stanneck D, Decaprariis D, et al. Diagnosis of *Hepatozoon canis* in young dogs by cytology and PCR. Parasit Vectors. 2011;4:55. https://doi.org/10.1186/1756-3305-4-55.

41. Giannelli A, Lia RP, Annoscia G, Buonavoglia C, Lorusso E, Dantas-Torres F, et al. *Rhipicephalus turanicus*, a new vector of *Hepatozoon canis*. Parasitology. 2017;144:730–7. https://doi.org/10.1017/S003118201600250X.

42. de Castro DL, Rubini AS, dos Santos PK, Metzger B, de Paula Antunes JM, Martins TF, et al. Investigation of tick vectors of *Hepatozoon canis* in Brazil. Ticks Tick Borne Dis. 2013;4:542–6. https://doi.org/10.1016/j.ttbdis.2013.07.006.

43. Giannelli A, Ramos RAN, Dantas-Torres F, Mencke N, Baneth G, Otranto D. Experimental evidence against transmission of Hepatozoon canis by *Ixodes ricinus*. Ticks Tick Borne Dis. 2013;4:391–4. https://doi.org/10.1016/j.ttbdis.2013.03.001.

44. Sevinc F, Xuan X. Major tick-borne parasitic diseases of animals: a frame of references in Turkey. Eurasian J Vet Sci. 2015;31:132–42.

45. Uilenberg G. International collaborative research: significance of tick-borne hemoparasitic diseases to world animal health. Vet Parasitol. 1995;57:19–41. https://doi.org/10.1016/0304-4017(94)03107-8.

46. Güçlü HZ, Karaer KZ. Detection of *Babesia caballi* (Nuttall, 1910) and *Theileria equi* (Syn. Babesia equi, Laveran, 1901) by the polymerase chain reaction (PCR) in show and sport horses in the region of Ankara. Türkiye Parazitol Derg. 2007;31:89–93.

47. Onyiche TE, Taioe MO, Ogo NI, Sivakumar T, Biu AA, Mbaya AW, et al. Molecular evidence of *Babesia caballi* and *Theileria equi* in equines and ticks in Nigeria: prevalence and risk factors analysis. Parasitology. 2020;147:1238–48. https://doi.org/10.1017/S0031182020000992.

48. Battsetseg B, Xuan X, Ikadai H, Bautista JLR, Byambaa B, Boldbaatar D, et al. Detection of *Babesia caballi* and *Babesia equi* in *Dermacentor nuttalli* adult ticks. Int J Parasitol. 2001;31:384–6. https://doi.org/10.1016/s0020-7519(01)00120-5.

49. Orkun Ö, Çakmak A. Molecular identification of tick-borne bacteria in wild animals and their ticks in Central Anatolia, Turkey. Comp Immunol Microbiol Infect Dis. 2019;63:58–65. https://doi.org/10.1016/j.cimid.2018.12.007.

50. Orkun Ö. Molecular investigation of the natural transovarial transmission of tick-borne pathogens in Turkey. Vet Parasitol. 2019;273:97–104. https://doi.org/10.1016/j.vetpar.2019.08.013.

51. Aktas M, Altay K, Dumanli N, Kalkan A. Molecular detection and identification of *Ehrlichia* and *Anaplasma* species in ixodid ticks. Parasitol Res. 2009;104:1243–8. https://doi.org/10.1007/s00436-009-1377-1.

52. Enkhtaivan B, Narantsatsral S, Davaasuren B, Otgonsuren D, Amgalanbaatar T, Uuganbayar E, et al. Molecular detection of *Anaplasma ovis* in small ruminants and ixodid ticks from Mongolia. Parasitol Int. 2019;69:47–53. https://doi.org/10.1016/j.parint.2018.11.004.

53. Güner ES, Hashimoto N, Kadosaka T, Imai Y, Masuzawa T. A novel, fast-growing *Borrelia* sp. isolated from the hard tick *Hyalomma aegyptium* in Turkey. Microbiology (Reading). 2003;149:2539–44. https://doi.org/10.1099/mic.0.26464-0.

54. Sen E, Uchishima Y, Okamoto Y, Fukui T, Kadosaka T, Ohashi N, et al. Molecular detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes ricinus* ticks from Istanbul metropolitan area and rural Trakya (Thrace) region of north-western Turkey. Ticks Tick Borne Dis. 2011;2:94–8. https://doi.org/10.1016/j.ttbdis.2011.03.004.

55. Cardoso L, Cortes HC, Eyal O, Reis A, Lopes AP, Vila-Viçosa MJ, et al. Molecular and histopathological detection of *Hepatozoon canis* in red foxes (*Vulpes vulpes*) from Portugal. Parasit Vectors. 2014;7:113. https://doi.org/10.1186/1756-3305-7-113.

56. Yang J, Liu Z, Niu Q, Liu J, Han R, Liu G, et al. Molecular survey and characterization of a novel *Anaplasma* species closely related to *Anaplasma capra* in ticks, northwestern China. Parasit Vectors. 2016;9:603. https://doi.org/10.1186/s13071-016-1886-6.

57. Wills MKB, Kirby AM, Lloyd VK. Detecting the lyme disease spirochete, *Borrelia burgdorferi*, in ticks using nested PCR. J Vis Exp. 2018;132:56471. https://doi.org/10.3791/56471.

58. Hercik K, Hasova V, Janecek J, Branny P. Molecular evidence of *Bartonella* DNA in ixodid ticks in Czechia. Folia Microbiol (Praha). 2007;52:503–9. https://doi.org/10.1007/BF02932111.

Publisher's Note