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Research paper

Molecular detection of a novel *Babesia* sp. and pathogenic spotted fever group rickettsiae in ticks collected from hedgehogs in Turkey: *Haemaphysalis erinacei*, a novel candidate vector for the genus *Babesia*

Ömer Orkun*, Ayşe Çakmak, Serpil Nalbantoğlu, Zafer Karaer

Department of Parasitology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

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ABSTRACT

In this study, a total of 319 ticks were obtained from hedgehogs (*Erinaceus concolor*). All ticks were pooled into groups and screened by PCR for tick-borne pathogens (TBPs). PCR and sequence analyses identified the presence of a novel *Babesia* sp. in adult *Haemaphysalis erinacei*. In addition, the presence of natural transovarial transmission of this novel *Babesia* sp. was detected in *Ha. erinacei*. According to the *18S rRNA* (nearly complete) and partial *rRNA* locus (*ITS-1/5.8S/ITS-2*) phylogeny, it was determined that this new species is located within the *Babesia* sensu stricto clade and is closely related to *Babesia* spp. found in carnivores. Furthermore, the presence of three pathogenic spotted fever group (SFG) rickettsiae was determined in 65.8% of the tick pools: *Rickettsia sibirica* subsp. *mongolitimonae* in *Hyalomma aegyptium* (adult), *Hyalomma* spp. (larvae), *Rhipicephalus turanicus* (adult), and *Ha. erinacei* (adult); *Rickettsia aeschlimannii* in *H. aegyptium* (adult); *Rickettsia slovaca* in *Hyalomma* spp. (larvae and nymphs) and *H. aegyptium* (adult). To our knowledge, this is the first report of *R. sibirica mongolitimonae* in *H. aegyptium, Ha. erinacei*, and *Rh. turanicus*, and the first report of *R. slovaca* in *H. aegyptium*. In addition, the presence of a single *Hemolivia mauritanica* haplotype was detected in *H. aegyptium* adults. Consequently, the presence of a novel *Babesia* sp. has been identified in a new candidate vector tick species in this study. Additionally, three SFG rickettsiae that cause infections in humans were identified in ticks collected from hedgehogs. Therefore, environmental wildlife monitoring for hedgehogs should be carried out for ticks and tick-borne pathogens in the region. Additionally, studies regarding the reservoir status of hedgehogs for the aforementioned pathogens must be carried out.

1. Introduction

Wildlife plays an important role in the lifecycle of tick-borne diseases (TBDs). Certain wild animals can actively participate in the lifecycle by serving both as suitable hosts for vector ticks and reservoirs for the pathogens (Dantas-Torres et al., 2012; Pfäffle et al., 2013; Tomassone et al., 2018). An important wild animal for ticks is the hedgehog. The habitats of these nocturnal insectivores are closely related to the habitats of both humans and domestic animals in both rural and urban areas. Furthermore, some people raise them as pets in their homes. Additionally, intercountry and even intercontinental hedgehog trading occurs (Pfäffle et al., 2013; Riley and Chomel, 2005). However, it is well known that the hedgehogs are suitable hosts for both immature and/or mature stages of some tick species in nature and they may contribute to the continued presence of certain tick species in some habitats (Dziemian et al., 2014; Földvári et al., 2011; Pfäffle et al., 2011).

It is always a matter of interest whether these insectivores are a potential source of TBDs for both humans and domestic animals due to the close relationship between their habitats (Földvári et al., 2011; Gern et al., 1997; Jahfari et al., 2017; Silaghi et al., 2012; Skuballa et al., 2007; Speck et al., 2013). While the number of studies on the potential role of hedgehogs is limited, most have been performed in recent years. In particular, TBDs are at the head of the list because hedgehogs serve as suitable hosts for some tick species that may also infest humans and domestic animals (for example, *Ixodes ricinus*) (Dziemian et al., 2014; Földvári et al., 2011). To date, the presence of some significant pathogens has been identified in hedgehogs and/or their ticks (Bitam et al., 2006; Földvári et al., 2014; Gern et al., 1997; Hoogstraal, 1979; Jahfari et al., 2017; Kozuch et al., 1967; Silaghi et al., 2012; Skuballa et al., 2007; Speck et al., 2013). Moreover, hedgehogs have been implicated as a reservoir of *Anaplasma phagocytophilum, R. helvetica,* and

* Corresponding author at: Department of Parasitology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey.
E-mail address: omerorkun@yahoo.com.tr (Ö. Orkun).

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different genospecies located in the *B. burgdorferi* sensu lato complex (Földvári et al., 2014; Gern et al., 1997; Silaghi et al., 2012; Skuballa et al., 2007; Speck et al., 2013). Thus, it is understood that hedgehogs can serve as a host for certain TBPs that can cause important diseases in humans and animals, and as amplifying hosts for vector tick species (Dziemian et al., 2014; Földvári et al., 2011; Pfäffle et al., 2013, 2011; Riley and Chomel, 2005). In Turkey, data about ticks that infest hedgehogs and the pathogens in these ticks are limited. To date, an unidentified *Rickettsia* spp. in one *I. ricinus* collected from a hedgehog in Istanbul province (Kar et al., 2011) and Crimean-Congo hemorrhagic fever (CCHF) virus in one *Hyalomma aegyptium* collected from a hedgehog in Tokat province (Ekici et al., 2013) have been reported. Thus, new studies of TBPs in hedgehogs living in Turkey and their ticks are needed. Therefore, we planned a preliminary study to investigate the presence of certain TBPs in ticks collected from hedgehogs living in their natural habitats in rural and urban regions of Ankara province, Turkey. For this purpose, we investigated the presence of *Anaplasma* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Hepatozoon/Hemolivia* spp., and SFG rickettsiae in the collected ticks by PCR. Additionally, we characterized a novel *Babesia* sp. in ticks detected in this study.

2. Materials and methods

2.1. Tick collection, morphological identification of ticks and further biological processes

Study materials were obtained between 2014 and 2015 and 2017–2018 in Ankara province, Turkey. A total of 12 Southern white-breasted hedgehogs (*E. concolor*) living in their natural habitats were examined for tick infestations. From these, eight hedgehogs were caught by the local beekeepers with the aim of keeping them away from their honeybee hives in a rural region (Çubuk district, at different dates). These animals were included in this study. We carefully examined each hedgehog for the presence of ticks, which were removed from the animals in their natural habitat using fine forceps (Fig. 1). Collected ticks were maintained alive in separate vials with perforated caps and individual labels. The examined hedgehogs were immediately released by the beekeepers to the most suitable habitats approximately five kilometers away from their honeybee colonies. No medical intervention was carried out on the animals. The remaining hedgehogs that were found wounded or sick by people in urban areas (central districts) of Ankara and brought to our faculty for treatment were checked for tick infestation. The same collection processes were carried out on these hedgehogs. All collected ticks were transported under the appropriate conditions (inside the air-permeable vials at room temperature and on the same day) to the Protozoology and Entomology Laboratory of Ankara University for identification and further processing. Live ticks were immediately processed upon arrival at the laboratory.

Fig. 1. A female *Hyalomma aegyptium* feeding on a hedgehog.

All collected ticks were identified morphologically under a stereomicroscope according to taxonomic keys (Apanaskevich, 2003; Apanaskevich and Horak, 2008; Filippova, 1997). While adult ticks were identified at the species level, larvae and nymphs collected from hedgehogs were identified at the genus level to avoid misidentification due to lack of distinguishing features. Five fully engorged female ticks obtained from hedgehogs were maintained alive in an incubator in the laboratory under suitable conditions (26–28 °C and 80–85% relative humidity) until egg production and larval hatching. Additionally, a fully engorged *Hyalomma* nymph obtained from a hedgehog was incubated under suitable conditions (28 °C and 80–85% relative humidity) and identified later as an unfed, adult tick. New generations were identified at the species level and included in the study as unfed ticks to demonstrate the presence of the natural transovarial and transstadial pathogen transmission. Following identification, all ticks were stored at −80 °C until DNA extraction.

2.2. Molecular and phylogenetic analyses

Obtained ticks were separated and grouped according to the degree of engorgement, body size, and developmental stage. Each group included the same species gathered from the same individual host or female tick. Ticks were pooled into groups of 1–40 ticks. Each tick was first washed in 70% ethanol, then rinsed in sterile distilled water, and dried on sterile filter paper to avoid environmental contamination. Ticks were homogenized using a SpeedMill PLUS homogenizer (Analytikjena, Jena, Germany) according to the manufacturer's instructions. Genomic DNA was extracted from the homogenized tick pools using a BlackPREP tick DNA/RNA kit (Analytikjena) following the manufacturer's instructions. DNA extracts were stored at −20 °C until PCR analysis. Prior to running PCR analyses to detect microbial agents, an initial tick mitochondrial *16S rDNA* control PCR was performed on every pool to determine whether PCR inhibition was present (Black and Piesman, 1994). The primers for this control (16S + 1 and 16S-1) amplify an approximately 460 base pairs (bp) fragment. Only positive samples were further analyzed for the presence of tick-borne microorganisms.

Ticks/pools were screened for the presence of DNA from *Anaplasma* spp., *Babesia* spp., *Borrelia burgdorferi* sensu lato, *Hepatozoon/Hemolivia* spp., and *Rickettsia* spp. Initially, a *Babesia* genus-specific conventional PCR was implemented using primers BJ1 and BN2, which amplify a 411–452 bp fragment of the 18S ribosomal RNA (*18S rRNA*) gene (Casati et al., 2006). Subsequently, the near-full-length *18S rRNA* gene (~1700 bp) of three new specimens, which tested positive for *Babesia* spp. by the first PCR, were amplified using primers Nbab_1F and TB Rev. (Matjila et al., 2008; Oosthuizen et al., 2008). A section (~940 bp) of the *rRNA* locus (part of the *18S rRNA*, complete internal transcribed spacer 1 (*ITS1*), complete *5.8S rRNA*, complete *ITS2*, partial *28S rRNA* gene) of these three specimens was also amplified using primers ITS _F and ITS_R (Schmid et al., 2008). In addition, two PCRs were carried out to amplify an approximately 550 bp fragment of the mitochondrial cytochrome *b* gene (*COB*) from the *Babesia* spp. positive samples using COB-F and COB-R primers (Tian et al., 2013) and HSP70for and HSP70rev primers, which amplify an approximately 353 bp fragment of the heat shock protein 70 gene (*hsp70*) of *Babesia* spp. (Blaschitz et al., 2008). Besides, Rickettsial DNA was screened by PCR with the primers

Rp CS.409d and *Rp* CS. 1258n, which amplify a 750 bp fragment of the citrate synthase gene (*gltA*) (common to the whole *Rickettsia* genus) (Roux et al., 1997). Each tick positive for *gltA* was also tested for the outer membrane protein A gene (*ompA*) of *Rickettsia* spp. using the primers Rr. 190.70 and Rr. 190.701, which amplify a 629–632 bp fragment, allowing the differentiation of closely-related strains (Fournier et al., 1998). For the detection of *B. burgdorferi* sensu lato species, a nested PCR, which amplifies an approximately 200 bp fragment of the *5S–23S rDNA* intergenic spacer (*IGS*), was performed using two sets of primers (RIS1-RIS2 and RIS3-RIS4) (Postic et al., 1994; Sen et al., 2011). For the detection of *Anaplasma* spp., two conventional PCRs, which amplify fragments (851 bp for *A. marginale/A. ovis* and 849 bp for *A. phagocytophilum*) of the major surface protein 4 gene (*msp4*), were carried out using two sets of primers (*A. marginale/A. ovis*: MSP45-MSP43 primers and *A. phagocytophilum*: MAP4AP5-MSP4AP3 primers) (de la Fuente et al., 2007). For the detection of *Hepatozoon/Hemolivia* spp., a conventional PCR, which amplifies an approximately 666 bp fragment of the *18S rRNA* gene, was carried out using primers HepF and HepR (Inokuma et al., 2002). All PCR parameters used in this study are given in Supplementary file 1. Negative (sterile DNase-RNase-free water) and positive (DNA from *B. bigemina, B. divergens, R. montanensis, B. burgdorferi* sensu lato, *A. ovis, A. phagocytophilum, Hepatozoon canis*) controls were included in all PCRs. We also performed pre-PCRs with various dilutions (1–1/100) of the positive controls to avoid false negative results due to low copy numbers of protozoan and bacterial genes.

The successfully amplified PCR products were purified using a QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Purified DNA was bi-directionally sequenced using a BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol. Automated fluorescence sequencing was performed with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The obtained nucleotide sequences were compared to the registered GenBank sequences using BLAST (http://www.ncbi.nlmn.nih.gov/BLAST). The sequences were edited and aligned using CLUSTAL W (Thompson et al., 1994) in BioEdit software (Hall, 1999). The most appropriate nucleotide substitution model based on the Akaike Information Criterion (AIC) for each gene was determined with jModeltest v.0.1.1 (Posada, 2008). Phylogenetic relationships between the sequences were inferred using the maximum likelihood method (ML), as implemented in raxmlGUI v1.5 beta (Silvestro and Michalak, 2012; Stamatakis, 2014) with the general-time-reversible (GTR) model of nucleotide substitution and 1000 bootstrap iterations (ML + thorough bootstrap). Phylogenetic trees were visualized in FigTree v1.4.3 (Rambaut, A. University of Edinburg, Edinburg UK, http://tree.bio.ed.ac.uk/software/figtree/). The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers MH500058–84 for *Rickettsia* spp. and HM497190–99 for *Hemolivia* spp. Furthermore, the new sequences of the species designated *Babesia* sp. Ankara were submitted with accession numbers MH504112 to MH504117.

3. Results

3.1. Identification of the collected ticks

A total of 12 hedgehogs were obtained during the study, of which nine were from the rural area and three from the urban area. No tick infestation was detected in any of hedgehogs obtained from the urban area and only flea infestation was detected in two of these. Tick infestations were present in six of the nine hedgehogs living in rural areas. In addition, fleas had infested three hedgehogs, and mites had infested one hedgehog. In this study, ectoparasites other than ticks found on the hedgehogs were not identified and examined for pathogenic microorganisms. A total of 164 ticks were collected from these six hedgehogs. The collected ticks were identified morphologically as *H. aegyptium* (32♂, 18♀), *Hyalomma* spp. (nymph) ($n = 30$), *Hyalomma* spp. (larvae) ($n = 45$), *Haemaphysalis erinacei* (4♂, 2♀), and *Rhipicephalus turanicus* (21♂, 12♀) (Supplementary file 2). Of these, five fully engorged female ticks (two *H. aegyptium*, two *Rh. turanicus,* and one *Ha. erinacei*) were incubated for ovipositing. Although all females produced eggs, one *H. aegyptium* female died after laying only a few eggs, from which no larvae hatched. Successful larvae hatching occurred in the eggs produced by the remaining four females. Forty unfed larvae obtained from each female tick were randomly selected and included in the study as one tick pool. Furthermore, one engorged *Hyalomma* sp. (nymph) that was incubated to molt into the adult stage hatched and matured successfully. This tick was then identified as *H. marginatum* (male) and included as individual tick in the study (Supplementary file 2).

3.2. Results of molecular analyses

3.2.1. PCR detections

A total of 319 ticks divided into 41 pools were included for molecular analysis in this study. All tick pools were screened by PCR for TBPs. PCR analyses revealed the presence of DNA belonging to *Babesia* spp. in three (7.3%) tick pools (*Ha. erinacei*, two partially fed adults and one unfed larvae), *Rickettsia* spp. in 27 (65.8%) tick pools (18 *H. aegyptium* adult, five *Hyalomma* spp. (nymph), one *Hyalomma* spp. (larvae), two *Rh. turanicus* adults, and one *Ha. erinacei* adult pool), and *Hemolivia* spp. in 10 (23.3%) tick pools (*H. aegyptium* adults). Of these, nine *H. aegyptium* pools and one *Ha. erinacei* pool were found to have a mixture of *Rickettsia/Hemolivia* spp. and *Babesia/Rickettsia* spp., respectively (Supplementary file 2). In contrast, no *Anaplasma* spp., *Borrelia* spp. or *Hepatozoon* spp. DNA was detected in the obtained tick samples.

3.2.2. Sequencing and phylogenetic analyses of Babesia spp.

Analysis of the partial *Babesia 18S rRNA* PCR (with primers BJ1 and BN2) revealed that three sequences obtained from the *Ha. erinacei* pools belong to same *Babesia* species and have no identical sequence in the GenBank database. The most closely related sequence by BLAST analysis (with 96.9% identity, 475/490 bp) was *B. rossi* clone RLB1501/cl3 (accession no. KY463434) obtained from a black-backed jackal (*Canis mesomelas*) in South Africa. This considerable nucleotide difference (15 different nucleotides) and the presence of natural transovarial transmission in a novel putative vector (*Ha. erinacei*) suggested the presence of a novel *Babesia* sp. Thus, the near-full-length *18S rRNA* gene of the three specimens was sequenced using primers Nbab_1F and TB Rev., resulting in an approximately 1.500 bp product. Using BLAST, we determined that the three sequences had no identical sequence in GenBank and the most closely related sequence (98.6–98.7% identity, 1470/1490 bp) was *B. rossi* (*B. rossi* clone RLB1501/cl3) (Table 1). Subsequently, a PCR, amplifying approximately 960 bp of the *rRNA* locus of *Babesia* spp. (part of *18S rRNA*; complete *ITS1*, *5.8S rRNA*, and *ITS2*; part of *28S rRNA*) was performed for these three isolates. The three isolates gave positive bands between 750 and 800 bp, whereas positive controls (*B. bigemina* and *B. divergens*) gave 950 bp bands. By sequencing, we obtained an approximately 760 bp length nucleotides (include partial *18S rRNA*; complete *ITS1*, 5.8S *rRNA*, and *ITS2*; and part of *28S rRNA*) belonging to these new isolates. BLAST analyses revealed no identical sequences and the isolates have 81.8–82.8% nucleotide identity (651/786 bp with 43 gaps) to *B. rossi* (*B. rossi* strain NGR clone 99, accession no. JN982348; *B. rossi*, accession no. AF394535) (Table 1). The differences were primarily located in the *ITS1* and *ITS2* genes. Additionally, BLAST homology searches determined the most closely related sequence, with 96.7% identity (621/642 bp) to be *Babesia presentii* (accession no. AY272048) obtained from a domestic cat in Israel (Table 1). However, this comparison could be performed using approximately 640 bp including part of *18S rRNA*;

Table 1
The pathogens detected in this study and their levels of nucleotide similarity with other sequences.

Detected pathogens		Sequenced genes	Tick species (no. positive pools)	Nucleotide identity percentage	GenBank accession nos.
Babesia spp.	*Babesia* sp. Ankara	*18S rRNA*[+]	*Haemaphysalis erinacei* (3)	98.6–98.7[a]	MH504115-17
		Part of *rRNA* locus[*]	*Haemaphysalis erinacei* (3)	96.7[b], 81.8–82.8[c,d]	MH504112-14
Rickettsia spp.	*Rickettsia sibirica mongolitimonae*	*ompA*	*Hyalomma aegyptium* (8)	99.1–99.8[e]	MH500058, 62, 64, 70–72, 80, 82
		ompA	*Hyalomma* spp. (larvae) (2)	99.6–99.8[e]	MH500059, 61
		ompA	*Rhipicephalus turanicus* (2)	99.8[e]	MH500063, 79
		ompA	*Haemaphysalis erinacei* (1)	99.8[e]	MH500060
	Rickettsia aeschlimannii	*ompA*	*Hyalomma aegyptium* (9)	100[f,g]	MH500067, 73–78, 81, 83
	Rickettsia slovaca	*ompA*	*Hyalomma* spp. (nymph) (3)	100[h,i]	MH500065, 63, 65
		ompA	*Hyalomma* spp. (larvae) (1)	100[j,k]	MH500066
		ompA	*Hyalomma aegyptium* (1)	100[h,i]	MH500084
Hemolivia spp.	*Hemolivia mauritanica*	*18S rRNA*	*Hyalomma aegyptium* (10)	99.8-100[l,m]	MH497190–99

[+] Near full length *18S rRNA* gene.
[*] Partial *18S rRNA*, complete *ITS1*, complete *5.8S rRNA*, complete *ITS2*, partial *28S rRNA* gene.
[a] *Babesia rossi* clone RLB1501/cl3, accession no. KY463434.
[b] *Babesia presentii*, accession no. AY272048.
[c] *Babesia rossi* strain NGR clone 99, accession no. JN982348.
[d] *Babesia rossi*, accession no. AF394535.
[e] *Rickettsia sibirica* subsp. *mongolitimonae* isolate Hma-Adana, accession no. KY513920.
[f] *Rickettsia aeschlimannii* strain TR/Orkun-H.nymph88/Ankara, accession no. JQ691729.
[g] *Rickettsia aeschlimannii* strain TR/Orkun-H.aegyp85/Ankara, accession no. JQ691727.
[h] *Rickettsia slovaca* isolate Kuqa 01, accession no. KX506733.
[i] *Rickettsia slovaca* isolate BB-50/Elma-D.marg, accession no. KF791234.
[j] *Rickettsia slovaca* strain WB3/Dm Pavullo, accession no. HM161776.
[k] *Rickettsia slovaca* strain TR/Orkun-D.marg79/Ankara, accession no. JQ691724.
[l] *Hemolivia mauritanica* isolate SY-45-10, accession no. KF992707.
[m] *Hemolivia mauritanica* isolate IQ-4-10, accession no. KF992700.

complete *ITS1* and *5.8S rRNA*; and partial *ITS2*, because the *B. presentii* sequence registered in GenBank is shorter than our sequences. Interestingly, no successful amplifications were obtained from this new *Babesia* sp. with the primers for hsp70 and COB genes, although the positive controls (*B. bigemina* and *B. divergens*) gave positive bands at the expected lengths. Phylogenetic analyses using the maximum likelihood method of the *18S rRNA* gene and the partial *rRNA* locus were performed to place the new *Babesia* sp. in the taxon and determine its genetic relationship with other piroplasmids (Figs. 2 and 3). Consequently, we determined that this new *Babesia* sp. locates within the *Babesia* sensu stricto clade (true Babesias) and is closely related to *Babesia* spp. found in carnivores. At this stage, the novel *Babesia* sp. obtained from *Ha. erinacei* ticks was tentatively named "*Babesia* sp. Ankara."

3.2.3. Sequencing of Rickettsia spp. and Hemolivia spp.

The presence of *Rickettsia* spp. was detected in 27 tick pools using the *gltA* primers. The *ompA* gene region was successfully amplified from all *gltA*-positive samples, and the *ompA* positive samples were sequenced. BLAST homology search results revealed the presence of *Rickettsia sibirica* subsp. *mongolitimonae* in eight *H. aegyptium*, two *Hyalomma* spp. (larvae), two *Rh. turanicus* and one *Ha. erinacei* pool. The sequences had 99.1–99.8% identity to a sequence previously obtained from a *H. marginatum* collected from a patient in Adana province, Turkey (accession no. KY513920). The presence of *Rickettsia aeschlimannii* was detected in nine *H. aegyptium* pools. According to BLAST analyses, the obtained sequences are identical to sequences obtained from a *H. aegyptium* in Ankara (accession no. JQ691727) and from a *Hyalomma* spp. (nymph) collected from a human in Ankara (accession no. JQ691729). Finally, *Rickettsia slovaca* was detected in three *Hyalomma* spp. (nymph), one *Hyalomma* spp. (larvae), and one *H. aegyptium* pool. BLAST analyses revealed that the obtained sequences are identical to sequences obtained from two *D. marginatus* collected from humans in Ankara (accession nos. KF791234 and JQ691724), two *D. marginatus* in Italy (accession nos. HM161776 and HM161770), and from a *Melophagus ovinus* in China (accession no. KX506733) (Table 1).

Finally, the presence of *Hemolivia* spp. was detected in 10 *H. aegyptium* pools. A single *Hemolivia mauritanica* haplotype was detected in all positive pools. BLAST analyses revealed that the obtained isolates have 99.8–100 identity to sequences obtained from tortoises in Syria (accession no. KF992707) and Iraq (accession no. KF992700) (Table 1).

4. Discussion

The prevalence and geographical distribution of TBDs continues to increase. New tick-borne epidemics have emerged in some regions, resulting in many people to become sick and even die (e.g. CCHF in Turkey). In addition, TBDs cause significant economic losses especially to the livestock industry (Dantas-Torres et al., 2012; Estrada-Peña and De La Fuente, 2014; Jongejan and Uilenberg, 2004). Therefore, the determination of the following major drivers in the ecotone, in which the ticks are involved, is necessary. (1) To identify vector ticks and the relevant natural reservoir host, (2) to identify amplificatory natural hosts for vector ticks, (3) to identify of risk factors, and (4) to create early warning systems (Estrada-Peña and De La Fuente, 2014; Gortazar et al., 2015; Pfäffle et al., 2013; Tijsse-Klasen et al., 2014). Hedgehogs may be appropriate ecotone candidates (Jahfari et al., 2017; Skuballa et al., 2007) because it is known that they have a wide geographical distribution (Riley and Chomel, 2005), and serve as suitable natural hosts for both immature and/or adult forms of certain tick species (Földvári et al., 2011; Hoogstraal, 1959). In addition, some tick species that feed on hedgehogs may also infest humans and domestic animals (Guglielmone et al., 2014). Therefore, hedgehogs potentially participate in the lifecycle of certain TBPs. Furthermore, the habitats of the hedgehogs in both rural and urban areas are generally intertwined with the habitats of both humans and livestock, which is an important reason for the necessity of investigating these animals (Pfäffle et al., 2013; Riley and Chomel, 2005). Although studies have shown that hedgehogs (mainly *Erinaceus europaeus* and *Erinaceous roumanicus*) can be a reservoir for certain pathogens (e.g. *A. phagocytophilum*) in European countries (Földvári et al., 2014; Gern et al., 1997; Silaghi et al., 2012; Speck et al., 2013), the knowledge about the role of hedgehogs

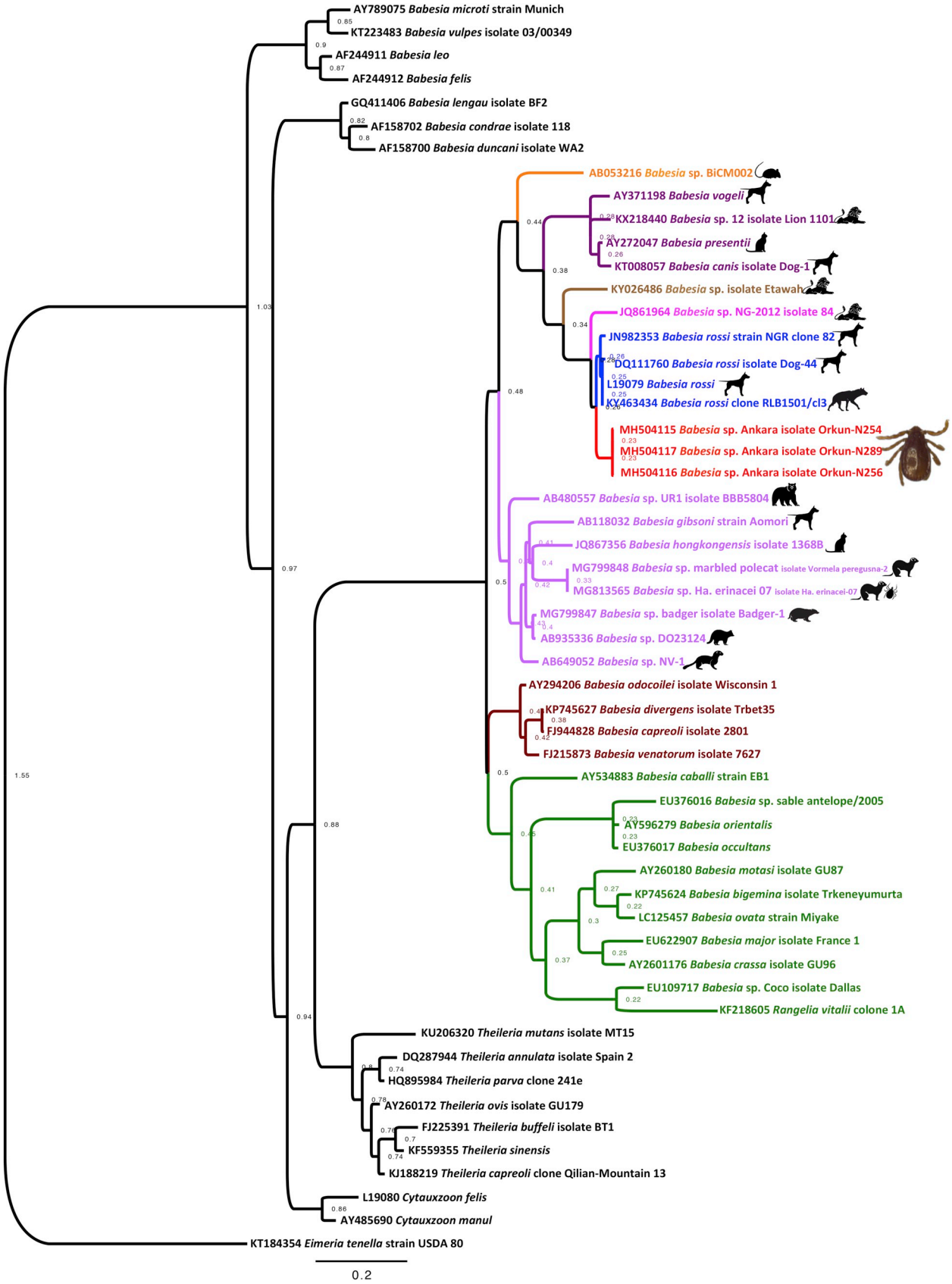

Fig. 2. Phylogenetic tree based on aligned sequences of *18S rRNA* gene (near full length) of the piroplasmida with *Eimeria tenella* as outgroup and constructed by using ML method calculated under the GTR + I + G substitution model. The novel babesial sequences obtained in this study are shown in red color. Symbols added to the end of the sequences indicate the obtained host or tick species. GenBank accession numbers of sequences are given before species names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

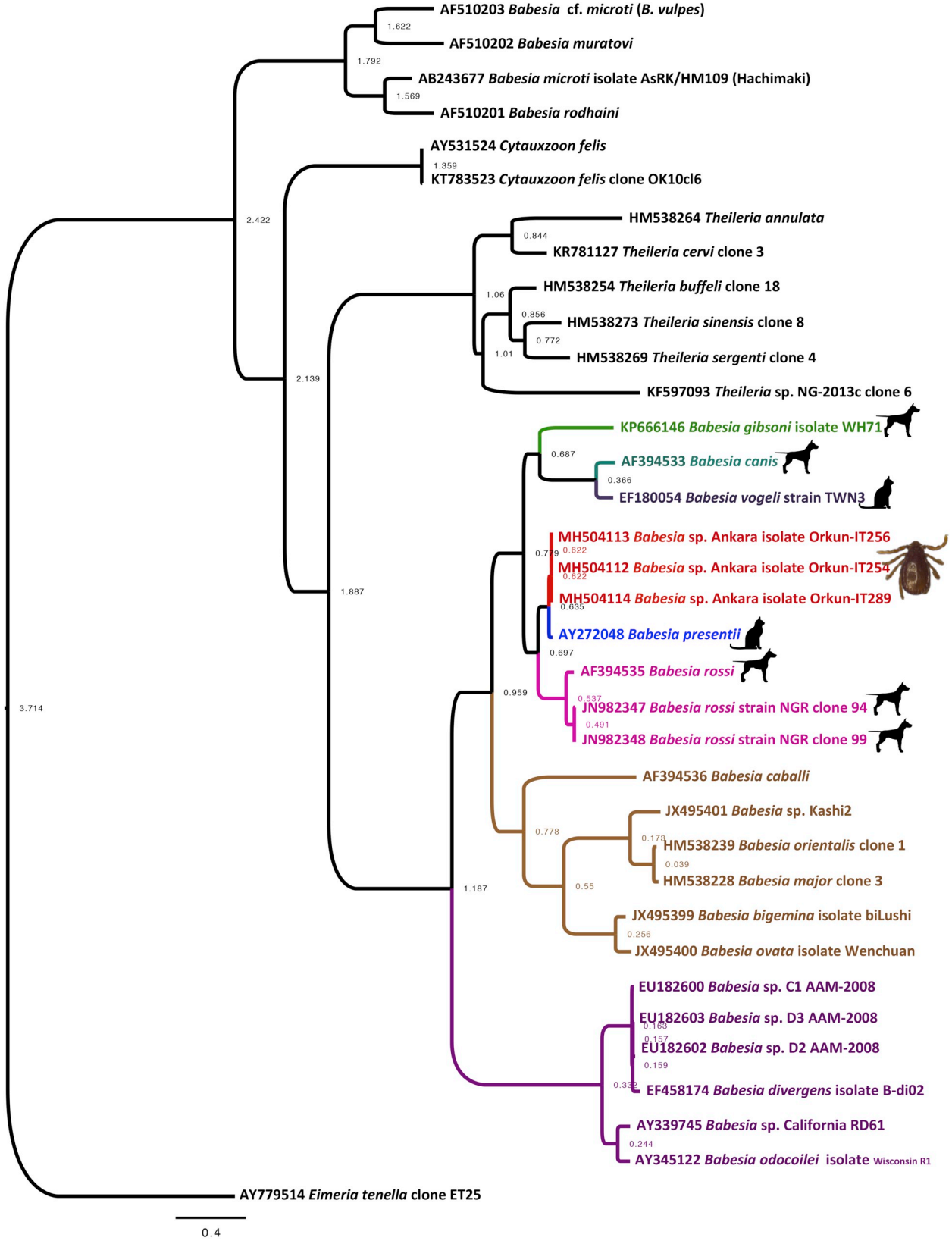

Fig. 3. Phylogenetic tree based on aligned sequences of the partial *rRNA locus* (Partial *18S rRNA*, complete *ITS1*, complete *5.8S rRNA*, partial *ITS2*) of the piroplasmida with *Eimeria tenella* as outgroup and constructed by using ML method calculated under the GTR + I + G substitution model. The novel babesial sequences obtained in this study are shown in red color. Symbols added to the end of the sequences indicate the obtained host or tick species. GenBank accession numbers of sequences are given before species names. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(especially *E. concolor*) in the ecology of TBPs is still limited or unknown in many countries. One such country is Turkey. Turkey has suitable habitats and climatic conditions for many tick species, some of which (e.g. *H. marginatum, H. anatolicum, I. ricinus, Rh. bursa,* etc.) are vectors of many human and animal TBDs (Inci et al., 2016).

Focusing on the studies that looked for TBPs in ticks on *E. concolor*, in a study conducted in Tokat province of Turkey, a total of 54 ticks were collected from road-killed *E. concolor* hedgehogs. These ticks were identified as *H. aegyptium, H. marginatum, H. scupense,* and *Rh. turanicus*. The presence of CCHF virus was reported in one partially fed *H. aegyptium* in this study (Ekici et al., 2013). Another study reported the presence of *Rickettsia massiliae* in two *Ha. erinacei* collected from an *E. concolor* in central Israel (Waner et al., 2014). In our study, a total of 164 ticks were collected from six *E. concolor*. We identified five different tick-borne microorganisms, one of which is a novel species.

Our molecular and phylogenetic analyses revealed the presence of a novel *Babesia* sp. (*Babesia* sp. Ankara) in *Ha. erinacei* ticks and demonstrated natural transovarial transmission of this *Babesia* sp. in *Ha. erinacei* larvae. Certain genetic regions, some of which contain important information on differentiation for all members of genus *Babesia*, were sequenced to place the species within the *Babesia* taxon (Figs. 2 and 3). We placed the *Babesia* sp. Ankara in the *Babesia* sensu stricto clade, which includes only "true babesias." Indeed, the presence of transovarial transmission in ticks confirms this finding. Currently, the susceptible vertebrate host of *Babesia* sp. Ankara is not known, but we found this novel *Babesia* sp. has a close genetic relationship to *Babesia* spp. of carnivores. *B. rossi*, the most pathogenic *Babesia* sp. for dogs (Schnittger et al., 2012), and *B. presentii*, which is described in cats (Baneth et al., 2004), are the most genetically close babesias. *B. presentii* has been described in a cat in Israel, there are no reports on *B. presentii* apart from the first description, and the vector tick species is unknown (Baneth et al., 2004). *B. rossi,* is particularly prevalent in Africa and it is mainly transmitted by *Ha. elliptica* (formerly *Ha. leachi*) (Schnittger et al., 2012). In addition, outside of Africa, *B. rossi* has been reported in *Ha. parva* ticks collected from wild boars and a human in Turkey (Orkun et al., 2014a; Orkun and Karaer, 2017). In the comparisons of *Babesia* sp. Ankara and *B. rossi* sequences from Turkey, we detected that *Babesia* sp. Ankara has 3.5–3.7% different (17–18 nucleotide differences within 489 bp) nucleotides compared to the *B. rossi* isolates (accession nos. MF040146, MF040148, MF040149) based on a partial *18S rRNA* gene sequence. Furthermore, the detection of *Babesia* sp. Ankara in both *H. erinacei* adult and unfed larvae suggest that this tick species is a strong competent vector candidate. This is the first detection of a *Babesia* sp. in unfed *Ha. erinacei* ticks, and there is important evidence that this tick could be a vector. There is only one very recent report related to the existence of a *Babesia* sp. in partially fed *Ha. erinacei* ticks. The presence of unclassified *Babesia* sp. DNA has been reported in *Ha. erinacei* ticks collected from a marbled polecat (*Vormela peregusna*), which was infected by the same *Babesia* sp., obtained from the China-Kazakhstan border (Liu et al., 2018). Hedgehogs, foxes, martens, and weasels are known to be suitable natural hosts for both immature and mature forms of *Ha. erinacei*, which is an endophilic tick and has a three-host lifecycle (Petney et al., 2017). *Ha. erinacei* adults have been detected in owned and street cats and foxes in Turkey (Orkun, 2015; Ö. Orkun, unpublished data). Therefore, these carnivores can be suitable vertebrate hosts for *Babesia* sp. Ankara, as they all exist in the study area (all except weasels have been confirmed by one of us, Ö. Orkun, during the field studies). However, it is necessary to determine the natural vertebrate host of *Babesia* sp. Ankara with field and experimental studies. Our future investigations will focus on this.

Along with the novel *Babesia* sp., this study revealed the presence of *R. aeschlimannii, R. sibirica mongolitimonae,* and *R. slovaca*, all of which are human pathogens, in ticks collected from hedgehogs. Of these, *R. sibirica mongolitimonae* was found to be most abundant. *R. sibirica mongolitimonae,* which causes lymphangitis-associated rickettsiosis (LAR) in humans, is mainly transmitted by *Hyalomma* spp. and *Rhipicephalus* spp. (Parola et al., 2013). In Turkey, this bacterium has been found in *H. marginatum* collected from a patient with rickettsial infection (Kuscu et al., 2017), in *Hyalomma* spp. (nymph) collected from wild boar (Orkun and Çakmak, 2019), and in *H. marginatum*, *H. excavatum*, *Rh. bursa,* and *Ha. parva* ticks collected from humans (Karasartova et al., 2018; Keskin and Bursali, 2016). In the current study, *R. sibirica mongolitimonae* was detected in *H. aegyptium*, *Hyalomma* spp. (larvae), *Rh. turanicus*, and *Ha. erinacei* ticks. To our knowledge, this is the first report of *R. sibirica mongolitimonae* in ticks collected from hedgehogs and in *H. aegyptium, Ha. erinacei*, and *Rh. turanicus*. This report does not give precise information about whether these tick species are competent vectors, and the reservoir status of hedgehogs is not yet known for this bacterium. We can, however, say that the hedgehogs and/or certain ticks deserve additional studies to determine whether they play a role in the lifecycle of *R. sibirica mongolitimonae*. In addition, both nymphs and adults of *H. aegyptium* often infest humans in Turkey (Orkun et al., 2014a; Vatansever et al., 2008). Therefore, *R. sibirica mongolitimonae* infection could be implicated in febrile patients infested by *H. aegyptium*. Another rickettsial bacterium detected in this study was *R. aeschlimannii*. This bacterium, which is transmitted mainly by ticks belonging to genus *Hyalomma*, causes a febrile disease in humans (Parola et al., 2013). The presence of *R. aeschlimannii* has been reported in ticks collected from both humans and domestic and wild animals in around the study area (Orkun et al., 2014a,b; Orkun and Çakmak, 2019). However a human case has yet to be reported in Turkey. Potential reasons for the absence of human patients could be patient neglect in the region, or the disease may have been treated with antibiotics without laboratory diagnosis and official notification. Serological screening studies for this bacterium should be performed for people living in the region for confirmation because tick-borne rickettsial diseases are not among the obligatory diseases to be reported in Turkey. Natural transovarial and transstadial transmission of *R. aeschlimannii* in *H. marginatum* ticks has also been demonstrated in previous studies conducted in Turkey. Therefore, *H. marginatum* plays a major role in the transmission of this bacterium in Turkey (Orkun, 2018; Orkun and Çakmak, 2019). In the current study, *R. aeschlimannii* was only detected in *H. aegyptium* adults. Previous studies have shown that *H. aegyptium* may be a vector of *R. aeschlimannii* (Bitam et al., 2009; Orkun et al., 2014a,b); nevertheless, our study confirmed the presence of this bacterium in *H. aegyptium* collected from hedgehogs. Additionally, *H. aegyptium* often infest humans (Orkun et al., 2014a; Vatansever et al., 2008), so this tick species should also be considered in *R. aeschlimannii* infections in infested humans living in the area. Finally, *R. slovaca* DNA was detected in ticks in this study. *R. slovaca*, which is characterized by "scalp eschars and neck lymphadenopathy following tick bites" (SENLAT), is mainly transmitted by *D. marginatus* and *D. reticulatus* ticks (Parola et al., 2013). Although *R. slovaca* has been reported in *D. marginatus* ticks collected from humans, domestic, and wild animals in the region (Orkun et al., 2014a,b; Orkun and Çakmak, 2019), no human case has been reported in Turkey. *R. slovaca* DNA has also been found in blood of a wild boar in Turkey (Orkun and Çakmak, 2019). In the current study, we detected *R. slovaca* DNA in *Hyalomma* spp. (larvae and nymph) and *H. aegyptium* adult collected from hedgehogs. Thus, we demonstrated the presence of *R. slovaca* in ticks collected from hedgehogs, and in *H. aegyptium* for the first time. The majority of studies conducted worldwide have shown that *D. marginatus* and *D. reticulatus* ticks play a significant role in the transmission of *R. slovaca* (Parola et al., 2013). However, we found no ticks belonging to the genus *Dermacentor* on hedgehogs. Immature of *D. marginatus* ticks are known to feed on hedgehogs in nature (Guglielmone et al., 2014), so a previous hedgehog-mediated transition of the rickettsial DNA is possible. Therefore, research to determine the roles of hedgehogs and *Hyalomma* ticks in the lifecycle of *R. slovaca* lifecycle is needed.

We detected the presence of *H. mauritanica*, which is a parasitic protozoon species of tortoises, in 50% of all *H. aegyptium* pools (10 of 20 pools) collected from hedgehogs. *H. mauritanica* is mainly transmitted

by *H. aegyptium* ticks (Siroký et al., 2004). In Turkey, this protozoon has been found in tortoise (*Testudo graeca*) blood and *H. aegyptium* ticks collected from tortoises (Široký et al., 2009). Additionally, *H. mauritanica* DNA has been reported in *Hyalomma* spp. (nymph) collected from humans in Çorum province of Turkey (Karasartova et al., 2018). Our study confirms the presence of *H. mauritanica* in *H. aegyptium* in Turkey. Furthermore, the detection of this protozoon in *H. aegyptium* adults collected from hedgehogs indicates that immature forms of these ticks have previously fed on tortoises in the region. Therefore, hedgehogs and tortoises, which are both considered amplifying hosts for *H. aegyptium*, exist in the same ecotone. Additionally, hedgehogs can play a role in the biology of this protozoon as a suitable host for *H. aegyptium* and should, therefore, be considered in the ecology of the disease.

Consequently, our study shows that hedgehogs participate in the lifecycle of some TBPs by serving as suitable hosts for certain vector ticks in Turkey. The most important findings of this study are the identification of a novel *Babesia* sp. and putative vector tick species for genus *Babesia*. Furthermore, the current study demonstrates that hedgehogs can carry ticks infected by human pathogenic SFG rickettsiae, such as *R. aeschlimannii*, *R. sibirica mongolitimonae*, and *R. slovaca*. Thus, these insectivores, which can share their habitats with humans in rural and urban areas, must be taken into consideration in the ecology of these diseases. Uncontrolled hedgehog populations in habitats shared by humans and animals may pose a risk to health. Wildlife disease monitoring is an effective method of controlling TBDs (Gortazar et al., 2015; Tomassone et al., 2018). Our study reveals that hedgehogs should be included in wildlife monitoring programs. Therefore, more comprehensive research to determine the role of hedgehogs and their ticks in the ecology of TBPs in Turkey is needed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.028.

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