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Short communication

Molecular investigation of the natural transovarial transmission of tick-borne pathogens in Turkey

Ömer Orkun

Department of Parasitology, Faculty of Veterinary Medicine, Ankara University, 06110 Ankara, Turkey

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ABSTRACT

This study aimed to investigate the presence of the natural transovarial transmission of tick-borne pathogens in unfed larvae obtained from engorged female ticks from domestic animals in Turkey. Larvae (n = 4530, 151 pools) obtained from 75 engorged female ticks and female carcasses were screened for the presence of certain tick-borne pathogens by PCR. The presence of transovarial transmission of *Babesia occultans* was detected in *Hyalomma marginatum* and *Hy. excavatum*, while *Ba. ovis* in *Rhipicephalus bursa*. *Theileria annulata* was detected only in *Hy. excavatum* and *Rh. turanicus* female carcasses, but not in their examined progenies. Additionally, *Rickettsia aeschlimannii* and *Rickettsia raoultii* were detected in *Hy. marginatum* and *Dermacentor marginatus* females, respectively, and all their examined larvae. Besides, *Ri. slovaca* was detected in a *De. marginatus* female carcass and its one of two examined larvae pools. The presence of mixed *Ba. occultans* and *Ri. aeschlimannii* infection was also determined in an *Hy. marginatum* female and its larvae. This is the first demonstration of transovarial transmission of *Ba. occultans* in naturally infected *Hy. excavatum*. These data suggested that *Hy. excavatum* may act as vector in the natural cycle of *Ba. occultans*.

1. Introduction

Tick-borne diseases are a significant threat to human and animal health. Tick-borne outbreaks can reach serious levels and can cause deaths in humans and heavy economic losses in livestock (Jongejan and Uilenberg, 2004). Therefore, it is necessary to carry out detailed studies on ticks and tick-borne pathogens (TBPs) (Dantas-Torres et al., 2012). Microorganisms can be transmitted via various routes in ticks. The main transmission routes are horizontal (host to tick and vice versa), transstadial (from tick stage to tick stage), transovarial (from female ticks to their progenies), venereal (by tick mating), and co-feeding (Parola and Raoult, 2001; Turell, 2007; Chauvin et al., 2009). Some pathogens have only one transmission route except for the horizontal transmission, such as *Theileria annulata* (*Th. annulata*), which is only transmitted transstadially in vector ticks (Mehlhorn and Schein, 1984), whereas Crimean-Congo hemorrhagic fever (CCHF) virus can be transmitted via all aforementioned routes (Turell, 2007). However, at least one of these routes along with the horizontal transmission must be present in a tick for it to be considered a competent vector (Kahl et al., 2002; Pfaffle et al., 2013). In addition, if a tick species can transmit a pathogen both transovarially (with high transmission rate) and transstadially, this species is not only a vector but also a reservoir for that pathogen. Therefore, the distribution of TBPs is determined by the vector ticks (Parola and Raoult, 2001).

Vertical or transovarial transmission (ToT) is an important route of transmission for some TBPs. A mated ixodid female tick, feeding on an infected host, engorges and leaves the hosts to lay eggs. Pathogens found in the midgut of the female tick can infect and multiply in its reproductive organs (mainly ovaries). In this way, the pathogen is transmitted to the next generation. via this transmission route, an infected female can produce hundreds or even thousands of infected larvae, each of which can serve as a source of infection (Burgdorfer and Varma, 1967; Chauvin et al., 2009; Socolovschi et al., 2009). Transovarial transmission is one of the most distinctive characteristics of the genus *Babesia* (*Babesia* sensu stricto), which is a protozoan that causes disease in humans and animals (Uilenberg, 2006). This mode of transmission is the main difference between *Babesia* species and other piroplasmidae such as *Theileria* (Mehlhorn and Schein, 1984; Chauvin et al., 2009). In addition, ToT occurs in some spotted fever group (SFG) rickettsiae (Parola and Raoult, 2001; Socolovschi et al., 2009) and rarely in some *Borrelia* spp. (Rollend et al., 2013). However, there are limited data on the transmission routes for many combinations of TBPs and tick species. To determine the biology of TBPs, the transmission routes of these pathogens in their vector ticks must be identified (Salman and Tarres-Call, 2013). Therefore, this study focusses on the presence of TBPs in unfed larvae obtained from engorged female ticks

E-mail address: omerorkun@yahoo.com.tr.

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collected from domestic animals in Turkey with the aim to demonstrate whether natural ToT exists or not.

2. Materials and methods

2.1. Sample collection, morphological identification of ticks, and incubation of female ticks

Ticks were collected from domestic animals (cattle, sheep, goats, and dogs) in Ankara, Bolu and Kırşehir provinces of Turkey from 2015 to 2017. The bodies of the animals were carefully checked, and ticks were removed from the animal by forceps. The collected ticks were placed in separate, labeled tubes with perforated caps. All live ticks were transported on the same day and under ambient conditions to the Protozoology and Entomology Laboratory of Ankara University for further processing. Only engorged female ticks were selected for this study. The ticks were identified morphologically to species level using a stereomicroscope (Stemi 2000-C, Zeiss, Germany) equipped with an AxioCam digital camera (Zeiss) and ZEN software and taxonomic keys described by Filippova (1997); Walker et al. (2000), and Apanaskevich and Horak (2005, 2008). At the same time, male ticks near the females were used to identify engorged female ticks at the species level, but they were not included in the study. Following morphological identification, engorged females were transferred to individual glass flasks with cotton caps and incubated at 22–28 °C and 80–90% relative humidity (depending on the species) until they oviposited and the larvae hatched. Unfed larvae were harvested from each female tick. Only the laid female carcasses and their larvae were used for PCR. All samples were stored at −80 °C until nucleic acid extraction.

2.2. Molecular analyses

2.2.1. Nucleic acid extraction, homogenization, and cDNA synthesis

Only female carcasses and their progenies (the unfed larvae) were processed to test for ToT. Sixty larvae were randomly selected from each female tick and divided into two pools of thirty. An additional pool of 30 larvae from a single *Rhipicephalus bursa* (*Rh. bursa*) was also included. When females had finished laying, they were included in the study and analyzed individually. Female carcasses and their larvae were first washed in 70% ethanol and then rinsed in sterile distilled water before being dried on sterile filter paper to avoid environmental contamination (such as eggshell, feces, etc.). Each larval pool and female carcass were homogenized in a bead-containing tube using a SpeedMill PLUS cooling homogenizer (Analytikjena, Jena, Germany) following the manufacturer's instructions. Total DNA/RNA were simultaneously extracted from the homogenized samples using a BlackPREP tick DNA/RNA kit (Analytikjena) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the RNA using a Transcriptor high-fidelity cDNA synthesis kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The extracted and synthesized nucleic acids were stored at −80 °C until PCR analysis.

2.2.2. PCR, sequencing, and phylogenetic analyses

Prior to running PCR analyses to detected pathogens, an initial tick specific control PCR for the amplification of 16S rDNA gene (Black and Piesman, 1994) was performed on every samples to determine whether an inhibition was present. Only the positive samples were further analyzed for TBPs. All larval pools and female carcasses were screened by PCR for *Anaplasma* spp., *Babesia* spp., *Borrelia* spp., *Rickettsia* spp., and CCHF virus. All PCR parameters used in this study are given in Table 1 in detail. Negative controls (Sterile DNase/RNase-free water) and positive controls (DNA from *Anaplasma ovis* (*An. ovis*), *An. phagocytophilum*, *Babesia bigemina* (*Ba. bigemina*), *Borrelia burgdorferi* sensu lato (*Bo. burgdorferi* s. l.), and *Rickettsia montanensis* (*Ri. montanensis*), and cDNA from CCHF virus RNA) were included in all PCR runs. Pre-PCRs were also performed with the positive controls at different dilutions (1:1 – 1:100) to avoid false negative results occurring due to low copy numbers of target genes.

The amplified products were purified from 1.5% agarose gels 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) following the manufacturer's protocol. Automated fluorescence sequencing was performed with an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). Obtained nucleotide sequences were compared with registered GenBank sequences using BLAST analysis (www.ncbi.nlmn.nih.gov/BLAST). The sequences were edited and aligned using BioEdit software (Hall, 1999). The jModeltest version 0.1.1 was used to determine the most appropriate nucleotide substitution model on the Akaike Information Criterion for the sequence data (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) models 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).

3. Results

3.1. Identification of the collected female ticks and harvesting of larvae

A total of 124 engorged female ticks were collected from domestic animals during the study. These were 108 females from 71 cattle, seven females from six sheep, six females from three goats, and three females from a shepherd dog. The ticks were identified as *Hyalomma excavatum* (*Hy. excavatum*) ($n = 33$, 31 from cattle and two from goats), *Hy. marginatum* ($n = 30$, from cattle), *Hy. anatolicum* ($n = 3$, from cattle), *Rh. turanicus* ($n = 30$, 26 from cattle and four from goats), *Rh. sanguineus* ($n = 3$, from one dog), *Rh. bursa* ($n = 2$, from sheep), *Haemaphysalis parva* (*Ha. parva*) ($n = 8$, five from cattle and three from sheep), *Ha. inermis* ($n = 1$, from cattle), *Dermacentor marginatus* (*De. marginatus*) ($n = 8$, six from cattle and two from sheep), and *Ixodes ricinus* (*Ix. ricinus*) ($n = 6$, from cattle) (Table 2).

Of the 124 engorged females, 97 females produced eggs, whereas 27 females did not. Larvae emerged from the eggs of 75 egg-laying females, and no larvae emerged from the eggs of 22 females (Table 2).

3.2. Results of molecular analyses

3.2.1. PCR detections

Hatched larvae from the 75 females were harvested, and 151 pools of larvae ($n = 4530$) and 75 female carcasses were used for PCR analyses. All extracted DNA samples were positive for the tick 16S rDNA and subjected to PCRs to detect the presence of *Anaplasma* spp., *Babesia* spp., *Borrelia* spp., *Rickettsia* spp., and CCHF virus, and then all positive amplicons were sequenced. As a result of the PCR analyses, *Babesia* spp. were detected in three female carcasses (one *Hy. marginatum*, one *Hy. excavatum,* and one *Rh. bursa*) and their seven larval pools, *Theileria* spp. in six female carcasses (five *Hy. marginatum*, and one *Rh. turanicus*) using primers designed for *Babesia* 18S rRNA gene. *Rickettsia* spp. were detected in six female carcasses (four *Hy. marginatum* and two *De. marginatus*) and their eleven larval pools. In addition, *Babesia* spp. and *Rickettsia* spp. were detected as a mixed infection in a *Hy. marginatum* female carcass and its larval pools (Table 2 and 3). *Anaplasma* spp., *Borrelia* spp., and CCHF virus were not detected in any larvae pools. Additionally, no potential cross-contamination was detected between the used positive controls and the detected pathogens.

Table 1
PCR parameters for DNA amplification of target organisms.

Target organism	Target gene region	Type of PCR	Primers (F, R)	Product size (bp)	Cycling parameters	Reference
Tick	16S rDNA	Conventional	16S + 1 16S-1	~460	94 °C 2 m, 35 cycles [94 °C 30 s, 56 °C 30 s, 72 °C 1 min], 72 °C 10 min	Black and Piesman, 1994
Babesia spp.	18S rRNA	Conventional	BJ1 BN2	411-452	94 °C 2 m, 40 cycles [94 °C 30 s, 55 °C 30 s, 72 °C 1 min], 72 °C 10 min	Casati et al., 2006
Anaplasma marginale /A. ovis	*msp4*	Conventional	MSP45 MSP43	851	94 °C 2 m, 40 cycles [94 °C 30 s, 60 °C 30 s, 72 °C 1 min], 72 °C 10 min	de la Fuente et al., 2007
A. phagocytophilum	*msp4*	Conventional	MAP4AP5 MSP4AP3	849	94 °C 2 m, 40 cycles [94 °C 30 s, 54 °C 30 s, 72 °C 1 min], 72 °C 10 min	de la Fuente et al., 2007
Rickettsia spp.[a]	*gltA*	Conventional	*Rp* CS.409d *Rp* CS. 1258n	750	94 °C 2 m, 40 cycles [94 °C 30 s, 54 °C 30 s, 72 °C 1 min], 72 °C 10 min	Roux et al., 1997
	ompA	Conventional	Rr. 190.70 Rr. 190.701	629-632	94 °C 2 m, 40 cycles [94 °C 30 s, 53 °C 30 s, 72 °C 1 min], 72 °C 10 min	Fournier et al., 1998
Borrelia spp.	5S-23S rDNA	Nested	RIS1-RIS2 RIS3-RIS4	226-266 ~200	94 °C 2 m, 30 cycles [94 °C 30 s, 52 °C 30 s, 72 °C 45 min], 72 °C 10 min (the first round) 94 °C 2 m, 35 cycles [94 °C 30 s, 50 °C 30 s, 72 °C 45 min], 72 °C 10 min (the second round)	Postic et al., 1994 Sen et al., 2011
CCHF virus (Europe 1 clade)	S-segment	Nested	Eecf-F1 - Eecf-R1 Eecf-F2 - Eecf-R2	307 211	94 °C 2 m, 35 cycles [94 °C 30 s, 60 °C 30 s, 72 °C 45 min], 72 °C 10 min (the first round) 94 °C 2 m, 35 cycles [94 °C 30 s, 57 °C 30 s, 72 °C 45 min], 72 °C 10 min (the second round)	Midilli et al., 2009
CCHF virus (Europe 2 clade)	S-segment	Nested	Gre-F1 - Gre-R1 Gre-F2 - Gre-R2	593 469	94 °C 2 m, 35 cycles [94 °C 30 s, 57 °C 30 s, 72 °C 45 min], 72 °C 10 min (the first round) 94 °C 2 m, 35 cycles [94 °C 30 s, 57 °C 30 s, 72 °C 45 min], 72 °C 10 min (the second round)	Midilli et al., 2009

[a] Rickettsial DNA was first detected by a conventional PCR, which amplify a fragment of the citrate synthase gene (*gltA*) of *Rickettsia* spp. Subsequently, *gltA*-positive samples were also screened for the outer membrane protein A gene (*ompA*) of Rickettsia spp. to differentiate between closely related species.

3.2.2. Sequencing and phylogenetic analyses of the detected pathogens

Sequence and phylogenetic analyses revealed *Ba. occultans* in two *Hy. marginatum* female carcasses (female ticks were collected from cattle in Kızılcahamam and Çubuk districts of Ankara province) and all their examined larval pools (four pools), and in one *Hy. excavatum* female carcass (this female was collected from cattle in Kalecik district of Ankara) and all its examined larvae pools (two pools). On BLAST analysis, all *Ba. occultans* sequences obtained in this study were identical to *Ba. occultans* isolate Trender 1 (accession no. KP745626) obtained from cattle in Turkey, *Ba. occultans* isolate Ba-212 (accession no. MF040147) obtained from *Hyalomma* spp. (nymph) collected from a hare in Turkey, and *Ba. occultans* isolate 58 (accession no. HQ331478)

Table 2
Female ticks collected from hosts, the obtained larvae, and PCR positivity.

Tick species	Hosts in which female ticks collected	Locations (no of collected ticks)	No. of total female ticks incubated	No. of successfully laid females	No. of larvae pools[a] (No. of total larvae used)	PCR Positivity		
						No. of *Babesia*-positive female carcasses – larvae pools (%)	No. of *Theileria*-positive female carcasses – larvae pools (%)	No. of *Rickettsia*-positive female carcasses – larvae pools (%)
Hy. marginatum	Cattle	Ankara (25) Bolu (4) Kırşehir (1)	30	20	40 (1200)	2 (10%)[b] - 4 (10%)[b]	0	5 (25%)[b] - 10 (25%)[b]
Hy. excavatum	Cattle	Ankara (31)	31	25	50 (1500)	1 (4%) - 2 (4%)	5 (20%) - 0	0
	Goat	Ankara (2)	2	1	2 (60)	0	0	0
Hy. anatolicum	Cattle	Ankara (3)	3	2	4 (120)	0	0	0
De. marginatus	Cattle	Ankara (5) Kırşehir (1)	6	4	8 (240)	0	0	2 (50%) - 3 (37.5%)
	Sheep	Ankara (2)	2	1	2 (60)	0	0	0
Rh. bursa	Sheep	Ankara (2)	2	1	3 (90)	1 (100%) - 3 (100%)	0	0
Rh. turanicus	Cattle	Ankara (25) Kırşehir (1)	26	17	34 (1020)	0	1 (5.8%) - 0	0
	Goat	Ankara (4)	4	3	6 (180)	0	0	0
Rh. sanguineus	Dog	Ankara (3)	3	1	2 (60)	0	0	0
Ha. parva	Cattle	Ankara (3) Kırşehir (2)	5	0	–	–	–	–
	Sheep	Ankara (2) Kırşehir (1)	3	0	–	–	–	–
Ha. inermis	Cattle	Bolu (1)	1	0	–	–	–	–
Ix. ricinus	Cattle	Bolu (6)	6	0	–	–	–	–
Total			124	75	151 (4530)	4 (5.3%) – 9 (5.9%)[c]	6 (8%) – 0	7 (9.3%) – 13 (8.6%)

[a] Each larvae pool includes 30 unfed larvae.
[b] *Babesia* spp. and *Rickettsia* spp. were detected as mixed in a *Hy. marginatum* larvae pool.
[c] The 0.6% difference is due to the number of the examined larvae pool in *Rh. bursa*, so it can be ignored.

Table 3
Babesia spp., *Rickettsia* spp., and *Theileria* spp. detected in this study and their level of nucleotide similarity with other isolates.

Detected pathogens		Tick species (No. positive female carcasses - larvae pools)	Locations (district/province)	Nucleotide identity percentage	GenBank accession nos.
Babesia spp.	*Ba. occultans*	*Hy. marginatum* (1 - 2)	Kızılcahamam/Ankara	100[a,b,c]	MG920538; MK713819, 20
		Hy. marginatum (1 - 2)	Çubuk/Ankara	100[a,b,c]	MG920539; MK713826, 27
		Hy. excavatum (1 - 2)	Kalecik/Ankara	100[a,b,c]	MG920540; MK713821, 22
	Ba. ovis	*Rh. bursa* (1 - 3)	Polatlı/Ankara	100[d]	MG920541; MK713823-25
Theileria spp.	*Th. annulata*	*Hy. excavatum* (5 - 0)	Kalecik/Ankara	100[e,f]	MK713828, 30-34
		Rh. turanicus (1 - 0)	Kalecik/Ankara	100[e,f]	MK713829
Rickettsia spp.	*Ri. aeschlimannii*	*Hy. marginatum* (2 - 4)	Kızılcahamam/Ankara	100[g,h]	MG920559, 60; MK726324, 25, 28, 29
		Hy. marginatum (2 - 4)	Mengen/Bolu	100[g,h]	MG920562, 64; MK726322, 23, 32, 33
		Hy. marginatum (1 - 2)	Çubuk/Ankara	100[g,h]	MG920561; MK726330, 31
	Ri. raoultii	*De. marginatus* (1 - 2)	Çamlıdere/Ankara	100[i,j]	MG920563; MK726326, 27
	Ri. slovaca	*De. marginatus* (1 - 1)	Kızılcahamam/Ankara	100[k,l]	MK726320, 21

[a] *Babesia occultans* isolate Trender 1, accession no. KP745626.
[b] *Babesia occultans* isolate Ba-212, accession no. MF040147.
[c] *Babesia occultans* isolate 58, accession no. HQ331478.
[d] *Babesia ovis* isolate tick20.3D, accession no. KT587794.
[e] *Theileria annulata* isolate Azerbijan, accession no. MK183002.
[f] *Theileria annulata* isolate CEK20, accession no. MG569892.
[g] *Rickettsia aeschlimannii* isolate Ro-775, accession no. MF379308.
[h] *Rickettsia aeschlimannii* isolate Ro-718, accession no. MF379306.
[i] *Rickettsia raoultii* strain IM16, accession no. CP019435.
[j] *Rickettsia raoultii* strain Khabarovsk, accession no. CP010969.
[k] *Rickettsia slovaca* isolate Ro-714, accession no. MF379304.
[l] *Rickettsia slovaca* isolate BB-131/Polat-D.marg, accession no. KF791246.

obtained from *Hy. marginatum* in Tunisia. *Babesia ovis* was detected in a *Rh. bursa* female carcass (this female was collected from a sheep in Polatlı district of Ankara) and all its examined larval pools (three pools). Based on BLAST analysis, the *Ba. ovis* sequence obtained in this study was identical to *Ba. ovis* isolate tick20.3D (accession no. KT587794) obtained from *Rh. bursa* collected from a sheep in Palestine (Table 3 and Fig. 1). Besides, *Th. annulata* was also detected in five *Hy. excavatum* and one *Rh. turanicus* carcasses (these females were collected from cattle in Kalecik district of Ankara), but all the larvae were negative. Based on BLAST analysis, the *Th. annulata* sequences obtained in this study were identical to *Th. annulata* isolate Azerbijan (accession no. MK183002) obtained from a cow in Iran and *Th. annulata* isolate CEK20 (accession no. MG569892) obtained from a donkey in Turkey (Table 3 and Fig. 1). In addition, *Ri. aeschlimannii* was detected in five *Hy. marginatum* female carcasses (these females were collected from cattle in Kızılcahamam and Çubuk districts of Ankara province and in Mengen district of Bolu province) and all the examined larvae pools (ten). Of these, one *Hy. marginatum* female carcass and its larvae pools, which were positive for *Ri. aeschlimannii*, which was obtained from Kızılcahamam, were found co-infected with *Ba. occultans*. BLAST analyses revealed that all *Ri. aeschlimannii* sequences obtained in this study were identical to *Ri. aeschlimannii* isolates Ro-775 (accession no. MF379308) obtained from *Hy. marginatum* collected from a hare in Turkey and *Ri. aeschlimannii* isolates Ro-718 (accession no. MF379306) obtained from *Hyalomma* spp. (nymph) collected from a hare in Turkey. Also, *Ri. raoultii* was detected in an *De. marginatus* female carcass (this female was collected from a cow in Çamlıdere district of Ankara) and its larvae pools (two pools). *Rickettsia raoultii* sequences obtained in this study were identical to *Ri. raoultii* strain IM16 (accession no. CP019435) obtained from a human in Inner Mongolia, and *Ri. raoultii* strain Khabarovsk (accession no. CP010969) obtained from *De. silvarum* in Russia. Besides, *Ri. slovaca* was detected in a *De. marginatus* female carcass (this female was collected from a cow in Kızılcahamam district of Ankara) and its one of two larvae pools. The other larvae pool obtained from this female tick was negative for *Ri. slovaca*. All *Ri. slovaca* sequences obtained in this study were identical to *Ri. slovaca* isolate Ro-714 (accession no. MF379304) obtained from *De. marginatus* collected from a wild boar in Turkey and *Ri. slovaca* isolate BB-131/Polat-D.marg (accession no. KF791246) obtained from *De. marginatus* collected from a human in Turkey (Table 3 and Fig. 2).

Detailed information about the collected ticks and nucleotide similarities of the obtained *Babesia* spp., *Rickettsia* spp., and *Theileria* spp. sequences are given in Tables 2 and 3. The phylogenetic trees were constructed using 18S rRNA sequences of *Babesia/Theileria* spp. and *ompA* sequences of *Rickettsia* spp. and the newly obtained sequences are illustrated in Figs. 1 and 2, respectively. The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers MG920538-41, MG920559-64, MK713819-34, and MK726320-33.

4. Discussion

Transovarial transmission-competent tick species are accepted as both competent vectors and reservoirs if their life stages can suck blood from suitable vertebrate hosts and can successfully transmit the pathogens to their next stages (Kahl et al., 2002; Parola and Raoult, 2001). Hypothetically, tick species that can transmit pathogens transovarially are important vectors for relevant pathogens. However, it is important to investigate the presence of natural ToT in addition to experimental studies. Although a tick species can transmit a certain pathogen transovarially and/or transstadially in experimental studies, the same tick may not play an important role in the transmission of that pathogen due to the life stages of the tick not naturally feeding on appropriate reservoirs or susceptible hosts. Therefore, such a tick would not be considered a natural vector of the pathogen (Kahl et al., 2002). One of the best examples is that *Hy. rufipes* which is not considered a natural vector of *Th. annulata* because their immature forms do not naturally prefer to feed on cattle. However, this species is able to transmit *Th. annulata* experimentally (Jongejan et al., 1983). Therefore, studies investigating the presence of the natural transmission routes have significant potential to contribute to the ecology of TBPs.

This study detected that two species of *Babesia* can be transmitted transovarially in certain tick species. The first is *Ba. occultans*, which was identified in both *Hy. marginatum* and *Hy. excavatum* larvae.

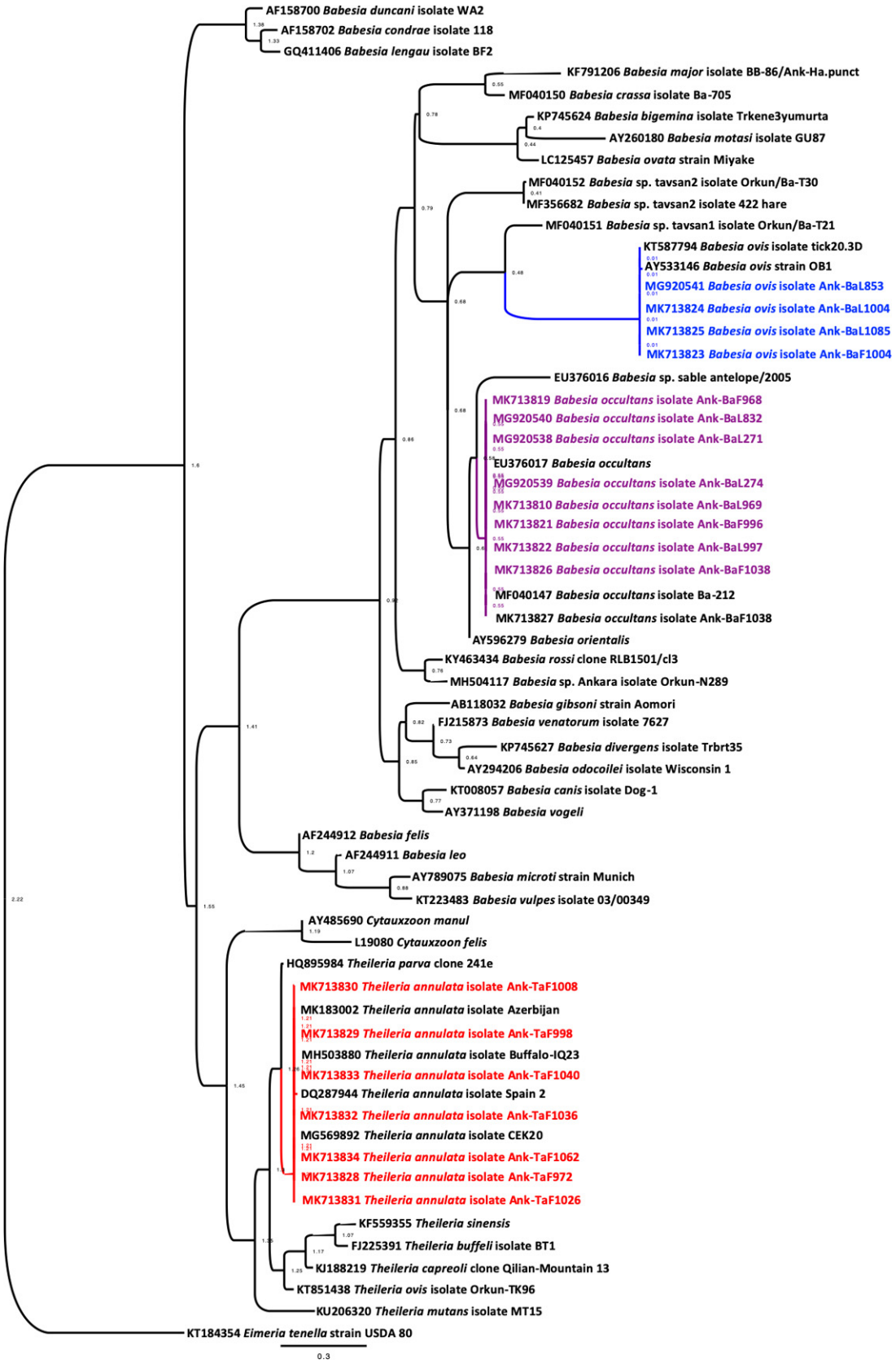

Fig. 1. Phylogenetic tree based on aligned partial sequences of 18S rRNA gene of the piroplasmida with *Eimeria tenella* as outgroup and constructed by using ML method calculated under the GTR + I + G substitution model. The sequences obtained in this study are shown as colored. GenBank accession numbers of sequences are given before species names.

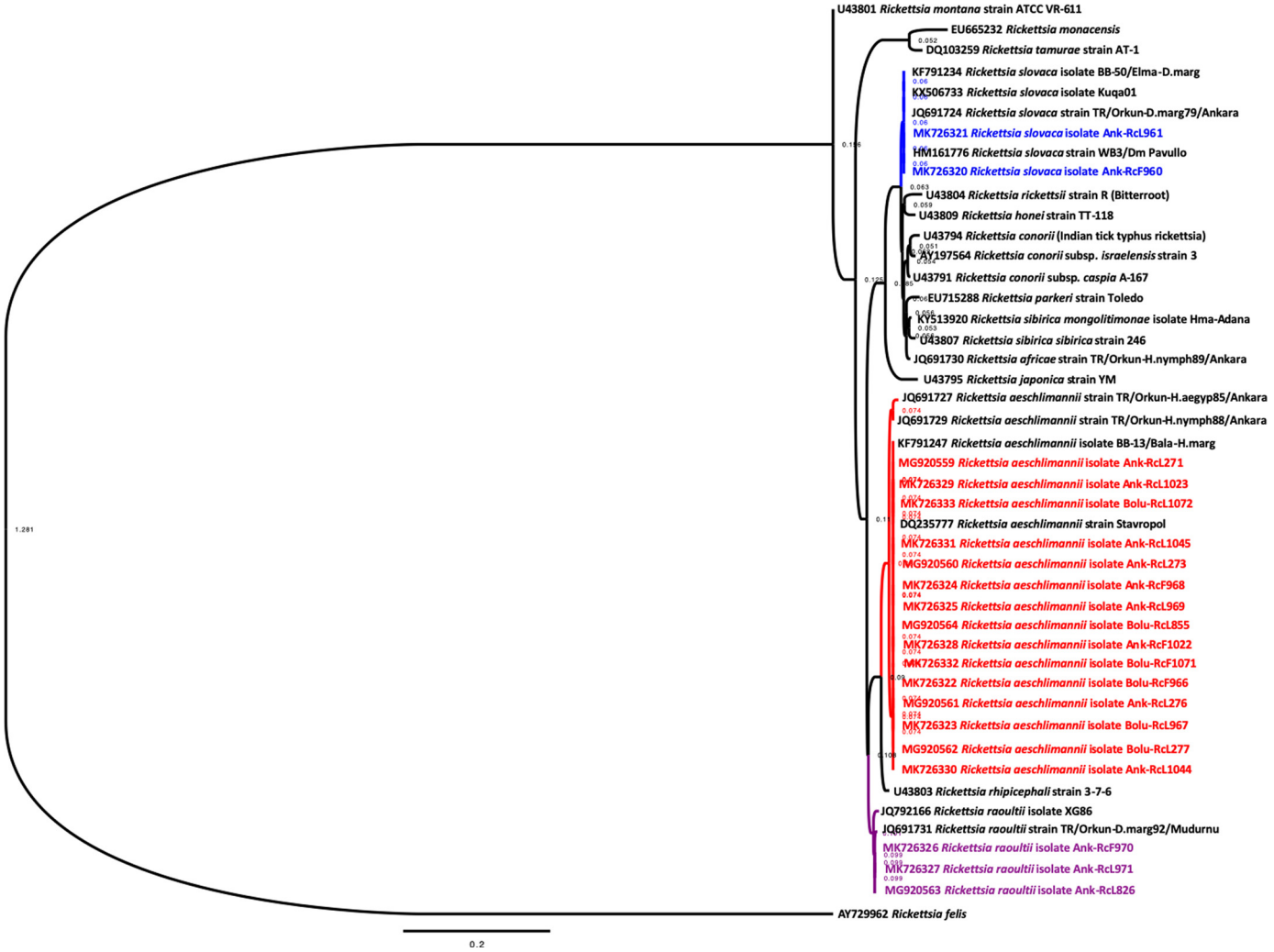

Fig. 2. Phylogenetic tree based on aligned sequences of the rickettsial *ompA* gene and constructed by using ML method calculated under the GTR + G substitution model. The rickettsial sequences obtained in this study are shown as colored. GenBank accession numbers of sequences are given before species names.

Babesia occultans has been shown to be transmitted transovarially and transstadially in *Hy. rufipes* (Gray and De Vos, 1981). Investigations showing the transmission routes of *Ba. occultans* in tick species are limited. In addition to the transmission routes in *Hy. rufipes* demonstrated by Gray and De Vos (1981), a Turkish study reported the presence of *Ba. occultans* in egg samples obtained from four *Hy. marginatum* and one *Rh. turanicus* females collected from cattle and unfed *Hy. marginatum* ticks collected from the field (Aktas et al., 2014). Thus, *Ba. occultans* can be transmitted transovarially and transstadially in *Hy. marginatum* ticks (Aktas et al., 2014). The current study showed that in addition to *Hy. marginatum*, *Ba. occultans* can be transmitted transovarially to the larvae of *Hy. excavatum*. Thus, this study demonstrated for the first time the ToT of *Ba. occultans* in naturally infected *Hy. excavatum*. Moreover, both *Hy. marginatum* and *Hy. rufipes*, which can transmit *Ba. occultans*, are two host ticks in their natural life cycle (Apanaskevich and Oliver, 2014). The adult forms of these species infest various ungulates (especially cattle), while the immature forms (larvae and nymphs) infest small to medium-sized mammals (e.g., hares, hedgehogs, etc.) and birds (e.g., ground-feeding birds, migratory birds etc.) (Apanaskevich and Oliver, 2014; Orkun, 2015). Although capable of transmitting the pathogens, the host preferences of these tick species can dramatically reduce the likelihood of transmitting *Ba. occultans* to cattle. Besides, *Hy. excavatum* can develop via two- or three-host life cycles (Apanaskevich and Oliver, 2014). Both adult and immature *Hy. excavatum* can infest cattle, especially when they develop in the two-host life cycle. This two-host life cycling of *Hy. excavatum* was detected in cattle in some villages of the research area (Orkun, 2015). Therefore, *Hy. excavatum* can be more efficient vector than *Hy. marginatum* and *Hy. rufipes* in the natural transmission of the disease. The results of this study supported the hypothesis that *Hy. excavatum* may act as a competent vector in the natural cycle of *Ba. occultans*.

The other *Babesia* species displaying ToT in this study is *Ba. ovis*. This protozoon, which is highly pathogenic in small ruminants, is mainly transmitted by *Rh. bursa* and is widespread in Africa, Asia, and Europe (Schnittger et al., 2012). *Babesia ovis* has been reported in small ruminants (Sevinc et al., 2013) and *Rh. bursa* collected from them in Turkey (Altay et al., 2008). In addition, *Ba. ovis* has been reported in *Rh. bursa* ticks collected from Anatolian wild sheep (*Ovis gmelinii anatolica*) in Turkey (Orkun et al., 2016). *Rhipicephalus bursa* is known to play a major role in the transmission of *Ba. ovis,* which is transmitted transovarially (Buscher et al., 1988). Here, this study confirmed the ToT of *Ba. ovis* in *Rh. bursa* tick of Turkey and emphasized that this tick species should be considered as the primary source of the disease in the region.

Besides, the presence of *Th. annulata* was identified in *Hy. excavatum* and *Rh. turanicus* females collected from cattle using primers designed for *Babesia* 18S rRNA gene. Contrary to this, all examined larvae pools obtained from the *Th. annulata*-positive female ticks were negative. Actually, this is an expected result because there is no ToT mode of *Th. annulata* in ticks (Mehlhorn and Schein, 1984).

In addition to *Babesia* species, ToT of *Ri. aeschlimannii* and *Ri. raoultii*, which are defined as pathogenic SFG rickettsiae of humans, was observed in *Hy. marginatum* and *De. marginatus*, respectively. Both rickettsial species were detected in both female carcasses of the relevant ticks and their all examined larvae pools. *Rickettsia aeschlimannii* was originally isolated from *Hy. marginatum* ticks collected in Morocco in 1992 (Beati et al., 1997). The presence of this bacterium, which is transmitted mainly by *Hyalomma* spp., has been reported in Africa, Asia, and Europe (Parola et al., 2013). *Rickettsia aeschlimannii* has also been reported in *Hy. aegyptium, Hy. marginatum, Hy. excavatum, Hyalomma* spp. (nymph), and *Rh. bursa* ticks collected from humans (Gargili et al., 2012; Orkun et al., 2014a, b) and *Hy. aegyptium*, *Hy. marginatum*, *Hyalomma* spp. (nymph), and *Rh. turanicus* ticks collected from domestic and wild animals in Turkey (Orkun et al., 2014b; Orkun and Cakmak, 2019; Orkun et al., 2019). Additionally, the presence of *Ri. aeschlimannii* has been reported in unfed *Hy. aegyptium* and *Hy. marginatum* ticks in Turkey (Orkun et al., 2014a, b; Orkun and Cakmak, 2019). Limited data exist regarding the transmission routes of *Ri. aeschlimannii* in ticks. Matsumoto et al. (2004) found this pathogen in larvae obtained from *Hy. marginatum* females and in unfed *Hy. rufipes* nymph and adults. The current study supported the results of Matsumoto et al. (2004) and demonstrated that *Ri. aeschlimannii* can be transmitted transovarially in *Hy. marginatum* ticks. Furthermore, this study detected an infection rate of 25% (in five larvae pools obtained from 20 females) for *Ri. aeschlimannii* in *Hy. marginatum* females. These data suggested that *Hy. marginatum* is an important vector for *Ri. aeschlimannii*. The ToT of *Ri. aeschlimannii* in *Hy. marginatum* demonstrated in this study and the previous demonstration of the transstadial transmission of the pathogen in the same tick species (Orkun and Cakmak, 2019) showed that *Hy. marginatum* is not only vector, but also reservoir for *Ri. aeschlimannii* in Turkey. Although no human cases related to *Ri. aeschlimannii* have been reported in Turkey, this bacterium should be considered in patients who have been bitten by *Hy. marginatum*. Additionally, the distribution of this pathogen may be wider than previously reported in Turkey because both transovarial and transstadial transmission exist in *Hy. marginatum* ticks of Turkey and also it is a common tick species in the country.

Rickettsia raoultii, an another SFG rickettsiae detected in this study, can cause scalp eschar and neck lymphadenopathy after tick bite (SENLAT) syndrome in humans. This pathogen, which is mainly transmitted by *Dermacentor* spp., has been found in Asia, Europe, and North Africa. *Dermacentor marginatus* and *De. reticulatus* play major roles in the transmission of *Ri. raoultii* in Europe (Parola et al., 2013). The presence of this bacterium has also been reported in *De. marginatus* ticks collected from humans and cattle in Turkey (Gargili et al., 2012; Orkun et al., 2014b). *Rickettsia raoultii* can be transmitted via transovarial and transstadial routes in *De. marginatus, De. reticulatus, De. silvarum,* and *De. nuttalli* ticks (Samoilenko et al., 2003; Samoylenko et al., 2009). The results of the current study are consistent with previous studies demonstrating natural ToT in *De. marginatus*. Furthermore, *De. marginatus* appears to be one of the most important *Ri. raoultii* vectors in Turkey. Therefore, *Ri. raoultii* should be taken into consideration in patients who have been bitten by *De. marginatus* and have SENLAT syndrome, although no human cases have been reported in Turkey to date.

Additionally, *Ri. slovaca*, the other *Dermacentor*-related SFG rickettsiae detected in the current study, can also cause SENLAT syndrome in humans, like *Ri. raoultii*. *Dermacentor marginatus* and *De. reticulatus* ticks are accepted to be main vector of this pathogen in Europe (Parola et al., 2013). The presence of this rickettsial pathogen has also been reported in *De. marginatus* ticks collected from humans and domestic and wild animals in Turkey (Orkun et al., 2014a, b; Orkun and Cakmak, 2019). Besides, *Ri. slovaca* DNA has been detected in blood of a free-living wild boar in Turkey (Orkun and Cakmak, 2019). It has been reported that *Ri. slovaca* can be transmitted transovarially with 100% ToT rate in *De. marginatus* ticks (Rehacek, 1984; Socolovschi et al., 2009). However, the current study determined the presence of *Ri. slovaca* in one of two larvae pools obtained from an infected *De. marginatus*. In fact, the presence of similar findings with wide range of ToT rate has also been reported in some other SFG rickettsiae (Burgdorfer and Brinton, 1975; Samoylenko et al., 2009; Socolovschi et al., 2009). At this stage, additional investigations are required to determine the range of ToT rate of *Ri. slovaca* in *De. marginatus*.

The current study shows that five pathogenic tick-borne microorganisms are naturally transmitted transovarially at different ToT rates in ticks of Turkey. Of these five, ToT of *Ba. occultans* in *Hy. excavatum* was demonstrated for the first time. Additionally, the ToT of pathogenic *Ri. aeschlimannii*, *Ri. raoultii* and *Ri. slovaca* has been confirmed in *Hy. marginatum* and *De. marginatus* species, respectively. Finally, these rickettsial species are circulating in ticks with ToT in Turkey; therefore, these bacteria should not be neglected in patients who have been bitten by the relevant ticks.

Declaration of Competing Interest

The author declares no conflict of interest.

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