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MOLECULAR CHARACTERIZATION OF *ANAPLASMA PHAGOCYTOPHILUM* IN IXODID TICKS IN KAYSERİ REGION IN TURKEY*

KAYSERİ YÖRESİNDEKİ IXODID KENE POPÜLASYONLARINDA *ANAPLASMA PHAGOCYTOPHILUM*'UN MOLEKÜLER KARAKTERİZASYONU

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ABSTRACT

The study was conducted to investigate the presence of *Anaplasma phagocytophilum* in ixodid ticks collected from cattle in Kayseri region of Turkey using Real Time PCR and to characterize positive isolates based on 16S rRNA gene region. DNA was extracted from 265 adult ticks. Nested PCR analyses were performed using *Anaplasma phagocytophilum*-specific primers amplifying a 641 bp fragment of 16S rRNA gene region. Real Time PCR analysis revealed positive results in one *H. marginatum* and *R. turanicus* sample. DNA sequences were submitted to GenBank and analyzed by pairwise and multiple sequence alignments with other *A. phagocytophilum* strains in GenBank to investigate the phylogeny. The phylogenetic analysis revealed that *A. phagocytophilum* isolates collected from *H. marginatum* and *R. turanicus* samples in Kayseri region clustered into three main groups (A, B, and C) with previously reported isolates from the world. A and B groups showed high homology, whereas C group had an average genetic variation of 0.2%. The average genetic differences between A and B groups were 10.8±2.0 and 13.0±2.7% between A and C groups, while the average genetic difference between B and C groups was 13.8±2.8%. In conclusion, this study provides scientific data on molecular prevalence and genetic characteristics of *A. phagocytophilum* in tick samples in Turkey.

Keywords: *Anaplasma phagocytophilum,* Kayseri, molecular characterization, tick, 16S rRNA

ÖZ

Bu çalışma, Kayseri yöresindeki sığırlardan toplanmış ixodid kenelerde *Anaplasma phagocytophilum*'un Real Time PCR'la araştırılması ve pozitif izolatların 16S rRNA gen bölgesi yönünden karakterize edilmesi amacıyla yapılmıştır. Bu amaçla sığırlardan toplanmış 265 ergin ixodid keneden genomik DNA ekstrakte edilmiştir. DNA ekstraksiyonu sonrası 16S rRNA gen bölgesinin 641 bp'lik kısmını amplifiye eden *Anaplasma phagocytophilum* spesifik primerlerle nested PCR analizleri yapılmıştır. Real Time PCR'la bireysel olarak incelemesi yapılan örneklerden bir *H. marginatum* ve bir *R. turanicus* örneğinde pozitiflik saptanmıştır. DNA dizilerinin GenBank kayıtları gerçekleştirilmiş ve mevcut diğer bazı *A. phagocytophilum* suşları ile hizalamaları yapılarak filogenisi araştırılmıştır. Filogenetik analiz sonucu Kayseri yöresindeki sığırlardan toplanmış *H. marginatum* ve *R. turanicus* örneklerinde belirlenen *A. phagocytophilum* izolatları ile Dünya'dan ve Türkiye'den daha önce GenBank'a girilmiş izolatların 3 ana dalda (A, B ve C kümeleri) kümelendikleri belirlenmiştir. A ve B gruplarındaki *A. phagocytophilum* izolatlarının homolog oldukları, C grubunda yer alan izolatların ise kendi aralarında ortalama %0,2'lik genetik farklılığın bulunduğu belirlenmiştir. A grubundaki izolatların B grubundakilerle %10,8±2,0, C grubundakilerle ise %13,0±2,7; B grubundaki izolatların C grubundaki izolatlarla arasındaki ortalama genetik farklılık %13,8±2,8 saptanmıştır. Sonuç olarak bu çalışma ile Kayseri yöresindeki sığırlardan toplanmış kene örneklerinde *A. phagocytophilum*'un moleküler yaygınlığı ve genetik karakterleri hakkında bilimsel veriler elde edilmiştir.

Anahtar kelimeler: *Anaplasma phagocytophilum,* Kayseri, moleküler karakterizasyon, kene, 16S rRNA

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INTRODUCTION

Anaplasma phagocytophilum is a rickettsial pathogen that is emerging as an important medical and veterinary concern, transmitted by ticks. It is a gram-negative bacterium that is found intracellularly in the blood cells or endothelial cells of blood vessels in humans and animals throughout the world. The primary vectors of *A. phagocytophilum* belong to the *Ixodes persulcatus* complex, which includes *I. ricinus* in Europe, *I. persulcatus* in Asia, and *I. scapularis* and *I. pacificus* in the US. Other tick species, such as *I. trianguliceps, I. ventalloi, I. hexagonus*, and *R. turanicus*, have also been described as vectors worldwide (1). In Turkey, recent studies have found *A. phagocytophilum* in ticks at a prevalence between 2.7% and 17.5%, with regional differences. The pathogen has been found in *I. ricinus* ticks as well as in *Haemaphysalis sulcata, Hyalomma marginatum*, and *H. excavatum* ticks, suggesting that these tick species may be involved in *A. phagocytophilum* transmission in Turkey. Despite this evidence, anaplasmosis is not considered an endemic disease in Turkey, and little information is available regarding the ecologic and epidemiologic features of the disease in this region (2).

In 2001, taxonomic revision combined *Ehrlichia phagocytophila* and *Ehrlichia equi*, previously known as the agent of human granulocytic ehrlichiosis (HGE), into a single species named *A. phagocytophilum*. Despite this reorganization, different strains of *A. phagocytophilum* have unique genetic and phenotypic characteristics that differentiate them from pathogenic strains (3). *Anaplasma phagocytophilum* has been found to display genetic diversity in studies analyzing its 16S rRNA genes. HGA agents possessing unique 16S rRNA sequences exhibit differing biological and ecological traits, such as their ability to cause disease and specificity for certain vectors. Further genetic variations have been identified in gene regions such as *groEL, msp2, msp4,* and *Anka*, as well as 16S rRNA, in *A. phagocytophilum* samples taken from ticks and mammals (4).

Microscopic examination is a challenging technique for identifying *A. phagocytophilum* in clinically healthy animals. Therefore, diagnostic approaches primarily focus on serological and PCR-based assays. However, PCR techniques are preferred due to their higher specificity compared to serological methods, which may produce cross-reactivity. PCR assays offer several benefits, such as rapid results, high sensitivity and specificity, and the ability to quantify the parasite load. Multiple PCR methods have been developed for detecting *A. phagocytophilum*. Real-time PCR assays are particularly advantageous because they provide fast and quantitative analysis and are performed in a closed system to prevent carry-over contamination (5).

The genetic characterization of the tick-borne pathogens is quickly progressing and providing new avenues for the development of novel control strategies for both tick infestations and their associated pathogens. To elucidate host preference and epidemiological diversity, molecular characterization of *A. phagocytophilum* isolates has been undertaken. The most often used gene was the 16S rRNA gene region in the epidemiological and molecular characterization studies since different pathogenic potential of distinct 16S rRNA gene variants of *A. phagocytophilum* has been proposed (4). Although the 16S rRNA exhibits a minor degree of variation in their nucleotide sequences, several genetic variants have been determined in sequences obtained from several hosts and ticks in different geographical regions. All these reasons make 16S rRNA gene fascinating targets for genetic studies, making prevalent high quality sequence data available (4).

The aim of this research was to examine and identify the *A. phagocytophilum* isolates in various tick species collected from cattle in Turkey through sequencing of the 16S rRNA gene and TaqMan real-time PCR assay targeting the msp2 gene region. We hypothesized that different tick species, apart from the primary vector tick *I. ricinus*, may be potential vectors for genetically diverse *A. phagocytophilum* strains in Turkey.

MATERIALS AND METHODS

Tick collection

In this study, 265 adult ixodid ticks were collected from cattle in different locations within the Kayseri region in Central Anatolia, Turkey, between April and September 2015. The collected ticks included 60 *R. annulatus*, 25 *R. turanicus*, 8 *R. bursa*, 21 *Hae. parva,* 53 *H. marginatum*, 32 *H. anatolicum*, 36 *H. excavatum*, and 30 *H. detritum*. The ticks were morphologically identified and preserved in 70% ethanol until DNA extraction (6).

DNA extraction from ticks

All ticks were examined separately. The DNA was extracted from each individual tick using the AxyPrep Multisource Genomic DNA Miniprep Kit (AP-MN-MS-GDNA-250, Axygen Biosciences, USA) following the manufacturer's instructions and stored at -20°C until PCR. The DNA concentration and purity of each tick specimen were determined by spectrophotometry (ASP -3700, ACT Gene). DNA samples were diluted to a final concentration of 50ng/µL.

Real Time PCR assay

A TaqMan real-time PCR assay was used to detect the presence of *A. phagocytophilum* in ixodid tick samples. The *msp2* gene was targeted using the primers ApMSP2 F (5'-ATGGAAGGTAGTGTTGGTTATGGTATT-3') and ApMSP2r (5'-TTGGTCTTGA AGCGCTCGTA-3') along with the ApMSP2 P HEX (5'-TGG TGCCAGGGTTGA GCTTGAGATTG-3') probe (7). The reaction mixture consisted of 25 µL, containing 2X TaqMan PCR Master Mix, 20 µM of each primer, 5 µM of TaqMan probe, nuclease-free water and 5 µL of DNA. The reaction protocol was as follows: 10 minutes at 95◦ C, followed by 40 cycles of 15 seconds at 95◦ C and 60 seconds at 60◦ C. The test was performed using the Stratagene Mx 3005P real-time PCR system.

Amplification of partial sequences from the 16S rRNA gene

Nested PCR was performed to amplify a 641-bp fragment of the 16S rRNA gene of *A. phagocytophilum*. At the first PCR, EC9 (5'-TACCTTGTTACGACTT-3') and EC12A (5'-TGATCCTGGCTCAGAACGAACG-3') primers, which were amplifying a 1462-bp fragment were used (8). At nested PCR step, SSAP2f (5'-GCTGAATGTGGGGATAAT TTAT-3') and SSAP2r (5'-ATGGCTGCTTCCTTTCGGTTA-3') primers were performed (8). The reaction mixture for both steps consisted of 25 µL containing 5 µl 10XPCR buffer, 2 mM $MgCl_2$, 1 µM of each primer, 0,5 mM dNTP,

1,25U Taq DNA polymerase, and 50ng/µl template DNA. The thermocycler program involved an initial denaturation step at 94 ◦ C for 5 minutes, followed by 40 cycles of denaturation at 94 ◦ C for 30 seconds, annealing at 52 ◦ C for 30 seconds, and extension at 72 ◦ C for 60 seconds during the first PCR stage. For the nested PCR step, the program began with an initial denaturation at 94 ◦ C for 5 minutes, followed by 40 cycles of denaturation at 94 ◦ C for 60 seconds, annealing at 55 ◦ C for 60 seconds, and extension at 72 ◦ C for 60 seconds. The PCR products were then separated via electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV light.

Cloning, sequencing, and phylogenetic analysis

After amplification, the PCR products were purified using the High Pure PCR Product Purification Kit from Roche (Switzerland). The purified PCR products were then cloned and sequenced. The identity of the resulting sequences was determined using multiple alignments by the Basic Local Alignment Search Tool (BLAST) and Geneious 6.1.6 (9). Phylogenetic analyses were performed using the maximum likelihood algorithm. The most suitable substitution model for sequence evolution has been determined in jModelTest v.0.1.1. The GTR+I model, which shows the lowest AIC (Akaike Information Criterion, correction) score, was used for constructing the phylogenetic tree. The Maximum Likelihood (ML) analysis was performed using the PhyML plugin in the Geneious software. Bootstrap tests with 1000 replicates were used to construct phylogenetic clusters, and evolutionary distances were computed using the Kimura 2 parameter method. The analyses were conducted in MEGA 6.0 (10). The resulting sequences of the *A. phagocytophilum* 16S rRNA gene were added to the GenBank database and assigned the accession numbers KU925346 and KU925347.

Ethical Statement

Since this study was carried out on ticks, Ethics Committee Approval is not required.

RESULTS

Tick identification and real time PCR results

A total of 265 adult ticks were collected from cattle in Kayseri region and they were examined for *A. phagocytophilum* by TaqMan real time PCR. The overall prevalence of *A. phagocytophilum* in ticks collected from cattle in the Central Anatolia region of Turkey was 0.8% (2/265) (Table I). Of the ticks, one *H. marginatum* (1.9%) and one *R. turanicus* (4.0%) were determined as *A. phagocytophilum* positive (Figure I). Both positive samples were determined in adult female ticks. Of the 265 adult ticks, 41 (15.5%) were male, while 224 (84.5%) were female.

Phylogenetic analyses

The comparison of partial 16S rRNA gene sequences revealed that the strains obtained from *H. marginatum*

Table I. The prevalence of *A. phagocytophilum* in ticks by real time PCR

Tick species	n	Prevalence in ticks	
		n	%
Rhipicephalus annulatus	60	0	0
Rhipicephalus turanicus	25	1	4.0
Rhipicephalus bursa	8	0	0
Haemaphysalis parva	21	0	0
Hyalomma marginatum	53	1	1.9
Hyalomma anatolicum	32	0	0
Hyalomma excavatum	36	0	0
Hyalomma detritum	30	0	0
Total	265	2	0.8

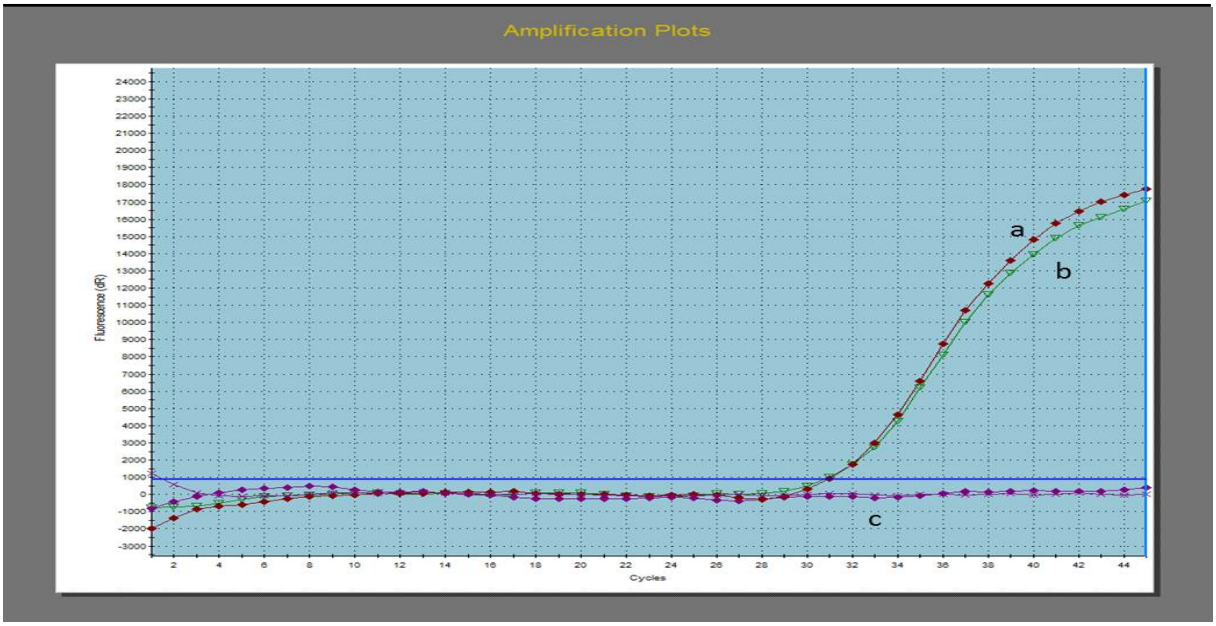

Figure I. TaqMan probe based real time PCR results in ixodid ticks. a, b: Positive samples, c: Negative samples

(TRAphagHmar isolate) and *R. turanicus* (TRAphagRtur isolate) ticks belong to *A. phagocytophilum*. Phylogenetic analysis of these TRAphagHmar and TRAphagRtur isolates, along with other selected sequences available in GenBank, resulted in the division of the phylogenetic tree into three main clusters (A, B, and C clusters) (Figure II).

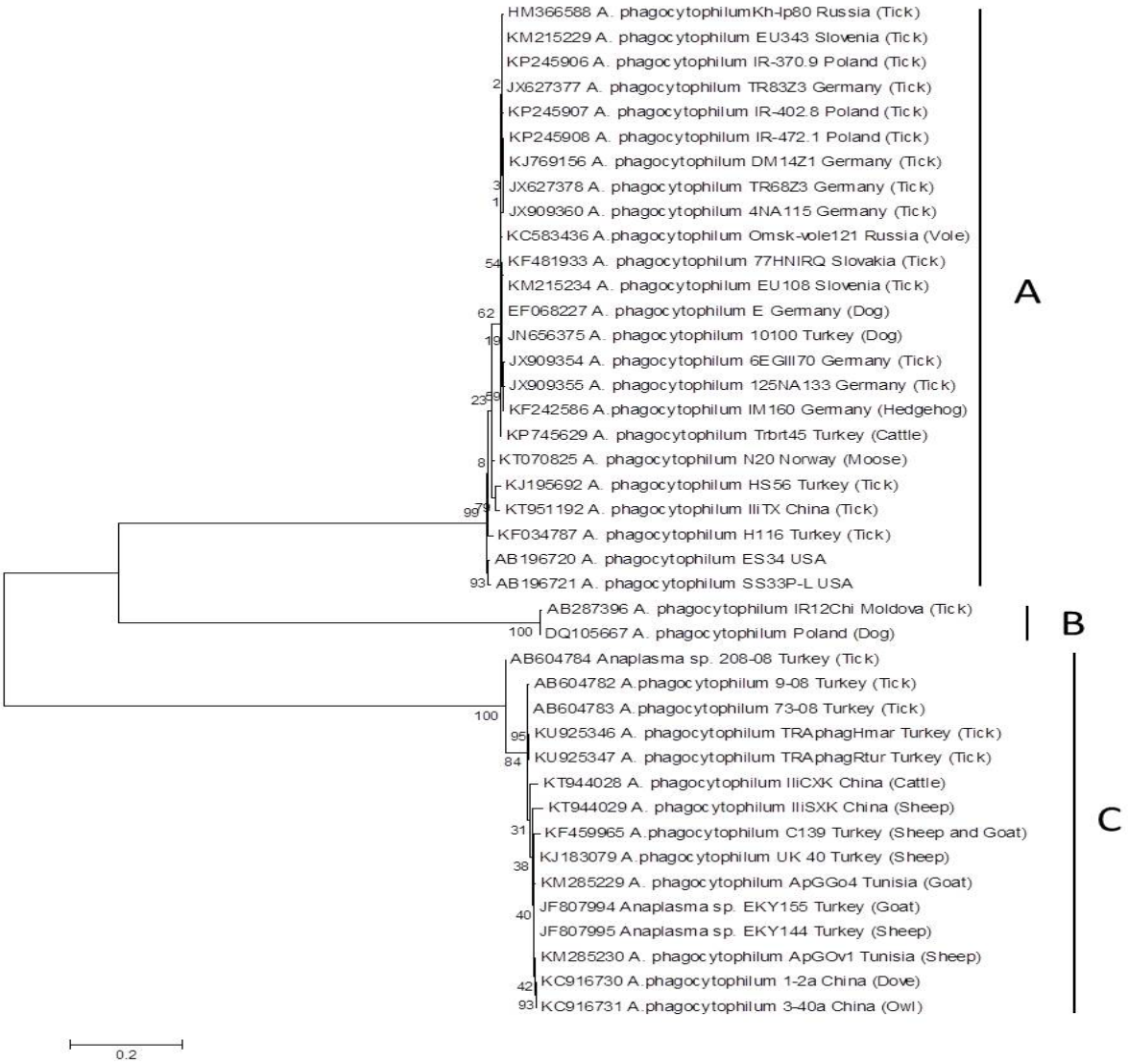

Figure II. Phylogenetic tree of the 16S rRNA gene sequences created using the maximum likelihood method (kimura 2 parameter) and bootstrap analysis of 1000 replicates. The scale bar indicates nucleotide substitutions per site. Isolation sources are shown in parentheses.

Our study found that the TRAphagHmar and TRAphagRtur isolates obtained from *H. marginatum* and *R. turanicus* ticks, respectively, belong to the *A. phagocytophilum* species based on pairwise sequence alignments of the partial 16S rRNA gene. In the phylogenetic analysis, our two strains (KU925346, KU925347) were placed in cluster C, which also contained strains from ticks, sheep, goats, cattle, dove, and owl in Turkey, China, and Tunisia. Cluster A mainly consisted of strains from ticks in different regions of the world, while cluster B contained strains from tick in Moldova and dog in Poland. The sequence lengths varied between 246-344 bp in cluster B, 575-642 bp in cluster C, and 405-1404 bp in cluster A. The similarity identities within clusters A, B, and C were 97.9-100%, 99.6%, and 94.5-100%, respectively. The genetic divergence was determined as 10.8%±2.0, 13.0%±2.7, and 13.8%±2.8 between cluster A-B, cluster A-C, and cluster B-C, respectively. The TRAphagHmar and TRAphagRtur isolates obtained in our study exhibited 100.0% homology within each other and shared 99.6-99.7% identity with strains from *I. ricinus* ticks in Turkey that were also in cluster C. Additionally, our strains had a sequence similarity of 98.2% with strains from sheep and goats in Turkey and 86.7-86.3% with strains from cattle, dog, and *Hae. sulcata* ticks in Turkey (Figure III).

DISCUSSION

Anaplasma phagocytophilum is an emerging tick-borne zoonotic agent of public health significance. The ecology of *A. phagocytophilum* is complex because of its reservoir hosts and several tick vectors. In the past few decades, increasing the movements of humans and animals,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1. AB196720 A. phagocytophilum ES34 USA		0.00	0.19	0.30	0.30	0.34	0.17	0.00	0.00	0.29	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
2. AB196721 A. phagocytophilum SS33P4 USA	0.00		0.19	0.30	0.30	0.34	0.17	0.00	0.00	0.29	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
3. AB287396 A. phagocytophilum IR12Chi Moldova (Tick)	1.08	1.08		0.38	0.38	0.25	0.00	0.19	0.19	0.35	0.35	0.19	0.19	0.19	0.21	0.21	0.19	0.19	0.35	0.35	0.21	0.21	0.35	0.19	0.35	0.19	0.19	0.19	0.19	0.35	0.40	0.19	0.19	0.19	0.19	0.19	0.38	0.36	0.19	0.36	0.36
4. AB604782 A.phagocytophilum 9-08 Turkey (Tick)	1.30	1.30	1.42		0.00	0.02	0.34	0.30	0.30	0.01	0.01	0.30	0.30	0.30	0.33	0.33	0.30	0.30	0.01	0.01	0.30	0.33	0.01	0.30	0.01	0.35	0.30	0.30	0.30	0.01	0.01	0.30	0.30	0.30	0.30	0.30	0.01	0.01	0.30	0.01	0.01
5. AB604783 A.phagocytophilum 73-08 Turkey (Tick)	1.30	1.30	1.42	0.00		0.02	0.34	0.30	0.30	0.00	0.00	0.30	0.30	0.30	0.33	0.33	0.30	0.30	0.01	0.01	0.30	0.33	0.00	0.30	0.00	0.35	0.30	0.30	0.30	0.00	0.01	0.30	0.30	0.30	0.30	0.30	0.01	0.01	0.30	0.00	0.00
6. AB604784 Anaplasma sp. 208-08 Turkey (Tick)	1.34	1.34	1.28	0.06	0.05		0.23	0.34	0.34	0.02	0.02	0.34	0.34	0.34	0.39	0.39	0.34	0.34	0.02	0.02	0.34	0.39	0.02	0.34	0.02	0.39	0.34	0.34	0.34	0.02	0.02	0.34	0.34	0.34	0.34	0.34	0.02	0.02	0.34	0.02	0.02
7. DQ105667 A. phagocytophilum Poland (Dog)	1.06	1.06	0.00	1.38	1.38	1.24		0.17	0.17	0.32	0.32	0.17	0.17	0.17	0.19	0.19	0.17	0.17	0.32	0.32	0.19	0.19	0.32	0.17	0.32	0.17	0.17	0.17	0.17	0.32	0.35	0.17	0.17	0.17	0.17	0.17	0.35	0.32	0.17	0.32	0.32
8. EF068227 A. phagocytophilum E Germany (Dog)	0.00	0.00	1.08	1.30	1.30	1.34	1.06		0.00	0.29	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
9. HM366588 A. phagocytophilumKh-1p80 Russia (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00		0.29	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
10. JF807994 Anaplasma sp. EKY155 Turkey (Goat)	1.28	1.28	1.40	0.01	0.00	0.05	1.36	1.28	1.28		0.00	0.29	0.29	0.29	0.32	0.32	0.29	0.29	0.01	0.01	0.29	0.32	0.00	0.29	0.00	0.32	0.29	0.29	0.29	0.00	0.00	0.29	0.29	0.29	0.29	0.29	0.01	0.01	0.29	0.01	0.01
11. JF807995 Anaplasma sp. EKY144 Turkey (Sheep)	1.28	1.28	1.40	0.01	0.00	0.05	1.36	1.28	1.28	0.00		0.29	0.29	0.29	0.32	0.32	0.29	0.29	0.01	0.01	0.29	0.32	0.00	0.29	0.00	0.32	0.29	0.29	0.29	0.00	0.00	0.29	0.29	0.29	0.29	0.29	0.01	0.01	0.29	0.01	0.01
12. JN656375 A. phagocytophilum 10100 Turkey (Dog)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28		0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
13. JX627377 A. phagocytophilum TR823 Germany (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00		0.00	0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
14. JX627378 A. phagocytophilum TR6823 Germany (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00		0.00	0.00	0.00	0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
15. JX909354 A. phagocytophilum 6EGII170 Germany (Tick)	0.00	0.00	1.11	1.34	1.34	1.38	1.08	0.00	0.00	1.31	1.31	0.00	0.00	0.00		0.00	0.00	0.00	0.29	0.29	0.01	0.00	0.32	0.00	0.32	0.01	0.00	0.00	0.00	0.32	0.29	0.00	0.00	0.00	0.00	0.00	0.38	0.36	0.00	0.35	0.35
16. JX909355 A. phagocytophilum 125NA133 Germany (Tick)	0.00	0.00	1.11	1.34	1.34	1.38	1.08	0.00	0.00	1.31	1.31	0.00	0.00	0.00	0.00		0.00	0.00	0.29	0.29	0.01	0.00	0.32	0.00	0.32	0.01	0.00	0.00	0.00	0.32	0.29	0.00	0.00	0.00	0.00	0.00	0.38	0.36	0.00	0.35	0.35
17. JX909360 A. phagocytophilum 4NA115 Germany (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00		0.00	0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
18. KC583436 A.phagocytophilum Omsk-vole121 Russia (Vole)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00		0.27	0.27	0.01	0.00	0.29	0.00	0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
19. KC916730 A.phagocytophilum 1-2a China (Dove)	1.24	1.24	1.40	0.02	0.01	0.05	1.36	1.24	1.24	0.01	0.01	1.24	1.24	1.24	1.28	1.28	1.24	1.24		0.00	0.26	0.29	0.01	0.27	0.01	0.29	0.27	0.27	0.27	0.01	0.00	0.27	0.27	0.27	0.27	0.27	0.01	0.01	0.27	0.01	0.01
20. KC916731 A.phagocytophilum 3-40a China (Owl)	1.24	1.24	1.40	0.02	0.01	0.05	1.36	1.24	1.24	0.01	0.01	1.24	1.24	1.24	1.28	1.28	1.24	1.24	0.00		0.26	0.29	0.01	0.27	0.01	0.29	0.27	0.27	0.27	0.01	0.00	0.27	0.27	0.27	0.27	0.27	0.01	0.01	0.27	0.01	0.01
21. KF034787 A. phagocytophilum H116 Turkey (Tick)	0.01	0.01	1.11	1.30	1.30	1.34	1.08	0.01	0.01	1.28	1.28	0.01	0.01	0.01	0.01	0.01	0.01	0.01	1.24	1.24		0.01	0.29	0.01	0.29	0.01	0.01	0.01	0.01	0.29	0.26	0.01	0.01	0.01	0.01	0.01	0.34	0.32	0.01	0.32	0.32
22. KF242586 A.phagocytophilum IM160 Germany (Hedgehog)	0.00	0.00	1.11	1.34	1.34	1.38	1.08	0.00	0.00	1.31	1.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.28	1.28	0.01		0.32	0.00	0.32	0.01	0.00	0.00	0.00	0.32	0.29	0.00	0.00	0.00	0.00	0.00	0.38	0.36	0.00	0.35	0.35
23. KF459965 A.phagocytophilum C139 Turkey (Sheep and Goat)	1.28	1.28	1.40	0.01	0.00	0.05	1.36	1.28	1.28	0.00	0.00	1.28	1.28	1.28	1.31	1.31	1.28	1.28	0.01	0.01	1.28	1.31		0.29	0.00	0.32	0.29	0.29	0.29	0.00	0.00	0.29	0.29	0.29	0.29	0.29	0.01	0.01	0.29	0.01	0.01
24. KF481933 A. phagocytophilum 77HNIRQ Slovakia (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28		0.29	0.00	0.00	0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
25. KJ183079 A.phagocytophilum UK 40 Turkey (Sheep)	1.28	1.28	1.40	0.01	0.00	0.05	1.36	1.28	1.28	0.00	0.00	1.28	1.28	1.28	1.31	1.31	1.28	1.28	0.01	0.01	1.28	1.31	0.00	1.28		0.32	0.29	0.29	0.29	0.00	0.00	0.29	0.29	0.29	0.29	0.29	0.01	0.01	0.29	0.01	0.01
26. KJ195692 A. phagocytophilum HS56 Turkey (Tick)	0.00	0.00	1.08	1.34	1.34	1.38	1.06	0.00	0.00	1.31	1.31	0.00	0.00	0.00	0.01	0.01	0.00	0.00	1.28	1.28	0.01	0.01	1.31	0.00	1.31		0.00	0.00	0.00	0.32	0.29	0.00	0.00	0.00	0.00	0.00	0.39	0.36	0.00	0.37	0.37
27. KJ769156 A. phagocytophilum DM1421 Germany (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00		0.00	0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
28. KM215229 A. phagocytophilum EU343 Slovenia (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00		0.00	0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
29. KM215234 A. phagocytophilum EU108 Slovenia (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00		0.29	0.27	0.00	0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
30. KM285229 A. phagocytophilum ApGGo4 Tunisia (Goat)	1.28	1.28	1.40	0.01	0.00	0.05	1.36	1.28	1.28	0.00	0.00	1.28	1.28	1.28	1.31	1.31	1.28	1.28	0.01	0.01	1.28	1.31	0.00	1.28	0.00	1.31	1.28	1.28	1.28		0.00	0.29	0.29	0.29	0.29	0.29	0.01	0.01	0.29	0.01	0.01
31. KM285230 A. phagocytophilum ApGOv1 Tunisia (Sheep)	1.24	1.24	1.44	0.01	0.01	0.05	1.40	1.24	1.24	0.00	0.00	1.24	1.24	1.24	1.28	1.28	1.24	1.24	0.00	0.00	1.24	1.28	0.00	1.24	0.00	1.28	1.24	1.24	1.24	0.00		0.27	0.27	0.27	0.27	0.27	0.01	0.01	0.27	0.01	0.01
32. KP245906 A. phagocytophilum IR-370.9 Poland (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24		0.00	0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
33. KP245907 A. phagocytophilum IR-402.8 Poland (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24	0.00		0.00	0.00	0.00	0.35	0.32	0.00	0.32	0.32
34. KP245908 A. phagocytophilum IR-472.1 Poland (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24	0.00	0.00		0.00	0.00	0.35	0.32	0.00	0.32	0.32
35. KP745629 A. phagocytophilum Trbnt45 Turkey (Cattle)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24	0.00	0.00	0.00		0.00	0.35	0.32	0.00	0.32	0.32
36. KT070825 A. phagocytophilum N20 Norway (Moose)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24	0.00	0.00	0.00	0.00		0.35	0.32	0.00	0.32	0.32
37. KT944028 A. phagocytophilum IIICKK China (Cattle)	1.34	1.34	1.42	0.03	0.02	0.07	1.38	1.34	1.34	0.03	0.03	1.34	1.34	1.34	1.38	1.38	1.34	1.34	0.03	0.03	1.34	1.38	0.03	1.34	0.03	1.38	1.34	1.34	1.34	0.03	0.03	1.34	1.34	1.34	1.34	1.34		0.02	0.35	0.01	0.01
38. KT944029 A. phagocytophilum IISKK China (Sheep)	1.31	1.31	1.40	0.03	0.02	0.06	1.36	1.31	1.31	0.02	0.02	1.31	1.31	1.31	1.35	1.35	1.31	1.31	0.02	0.02	1.31	1.35	0.02	1.31	0.02	1.35	1.31	1.31	1.31	0.02	0.01	1.31	1.31	1.31	1.31	1.31	0.05		0.32	0.01	0.01
39. KT951192 A. phagocytophilum IITX China (Tick)	0.00	0.00	1.08	1.30	1.30	1.34	1.06	0.00	0.00	1.28	1.28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.24	1.24	0.01	0.00	1.28	0.00	1.28	0.00	0.00	0.00	0.00	1.28	1.24	0.00	0.00	0.00	0.00	0.00	1.34	1.31		0.32	0.32
40. TRAphagHmar A. phagocytophilum TRAphagHmar Turkey (Tick)	1.33	1.33	1.41	0.01	0.00	0.06	1.37	1.33	1.33	0.01	0.01	1.33	1.33	1.33	1.37	1.37	1.33	1.33	0.02	0.02	1.33	1.37	0.01	1.33	0.01	1.37	1.33	1.33	1.33	0.01	0.01	1.33	1.33	1.33	1.33	1.33	0.03	0.03	1.33		0.00
41. TRAphagRtur A. phagocytophilum TRAphagRtur Turkey (Tick)	1.33	1.33	1.41	0.01	0.00	0.06	1.37	1.33	1.33	0.01	0.01	1.33	1.33	1.33	1.37	1.37	1.33	1.33	0.02	0.02	1.33	1.37	0.01	1.33	0.01	1.37	1.33	1.33	1.33	0.01	0.01	1.33	1.33	1.33	1.33	1.33	0.03	0.03	1.33	0.00	

Figure III. The distance matrix among the isolates in Genbank

socio-economic and environmental changes, and especially global warming induced the increase of the tick numbers belonging to different species. This situation caused that emerging/reemerging tick borne infections became a global threatening in all over the world. Investigations on ticks and tick borne pathogens could be useful in the evaluation of the risk factors for the latest tick borne diseases in the regions (11). The main vectors of *A. phagocytophilum* are *I. ricinus*, *I. persulcatus*, *I. scapularis,* and *I. pacificus*. Among these species, *I. ricinus* ticks are prevalent in especially Marmara, Black Sea, Aegean, and Mediterranean regions in Turkey (12-15). However, the reports of *A. phagocytophilum* from different ixodid tick species in Turkey and the other countries could probably cause a questing mark about the vector competence of the other ticks species except the main vectors (2, 16). This study was planned to exhibit the risk factors about the transmission of this tick-borne pathogen with the other possible ixodid ticks in the Central Anatolia region of Turkey, where the other ixodid ticks species are more prevalent than the *Ixodes* spp. *A. phagocytophilum* infection has been limited reported in ticks and domestic animals in Turkey. Most of these studies are based on microscopic and serological techniques. A few molecular studies have been reported about the prevalence of *A. phagocytophilum*. In these PCR based studies, the prevalence rates of *A. phagocytophilum* infection were found as 1% in cattle and 2.7-17.5% in ticks. In addition, all these findings about *A. phagocytophilum* in Turkey were reported from the regions which have the suitable habitat conditions for *I. ricinus*. There is no report about this pathogen from unsuitable habitats for the main vector tick. In this study, we firstly used TaqMan real time PCR for the investigation of *A. phagocytophilum* in ticks from the Central Anatolia region in Turkey, and we found the positivity as 0.8% in ixodid ticks. This prevalence finding exhibits highly similar results with a few previous studies from Turkey (17, 18), but lower than the other reports (19-21).

PCR-based methods are valuable tools and play a critical role in detecting *A. phagocytophilum* infections in samples. Numerous PCR-based assays and primer sets have been developed for identifying *A. phagocytophilum* to date. However, real-time PCR methods have gained prominence due to their higher sensitivity and specificity compared to other PCR assays (5, 22, 23). In this study, we used real-time PCR assays to investigate *A. phagocytophilum* in several ixodid ticks and found infections in *H. marginatum* and *R. turanicus* ticks. As the habitat suitability for *I. ricinus* was relatively low in the study area, it may serve as a source of infected ticks for transmitting *A. phagocytophilum* to animals and humans in the region. *H. marginatum* and *R. turanicus* ticks may also be involved in the transmission of *A. phagocytophilum*, and their higher habitat suitability in other regions of Turkey may explain differences in pathogen prevalence between these regions and the Central Anatolia region. However, we cannot claim that *H. marginatum* and *R. turanicus* ticks have vector competence for *A. phagocytophilum* based on the agent detected in our study, and further investigations are needed to determine the role of other tick species in transmitting this pathogen. Similar results have been reported in previous studies on *A. phagocytophilum* infection in *Hae. sul-*

cata, *H. marginatum*, and *H. excavatum* ticks (2, 19, 21) These findings provide evidence for the possibility of *A. phagocytophilum* existing in other tick species.

We performed sequencing and characterization of two isolates using phylogenetic analysis of the 16S rRNA gene region. Fragments of this gene are commonly used for detecting bacterial DNA in vectors, reservoirs, and in medical and veterinary diagnostics. The efficiency of this marker can vary between studies, depending on the amplified region. This highly conserved gene region has been utilized in several studies for genotyping *A. phagocytophilum*. In GenBank database, lots of different *A. phagocytophilum* strains from various localities have been published from ticks, cattle, vole, dog, sheep, goat, owl, dove, moose, human, etc. Most of these strains were used in our study for phylogenetic analyses and comparisons. The 16S rRNA gene region was preferred in the genetic analyses for *A. phagocytophilum* despite its high-level similarities among the human and animal hosts. Because this gene region was found to be linked to host tropisms (24-28).

In our study, various nucleotide variations were identified in *A. phagocytophilum* generations determined in animals such as cattle, sheep, goats, dogs, hedgehogs, and deer, and in ixodid ticks, according to the pairwise comparisons of *A. phagocytophilum* isolates obtained in our work with previously entered *A. phagocytophilum* isolates from the world and Turkey based on the 16S rRNA gene region. In the phylogenetic tree constructed using the maximum likelihood method (Kimura 2 Parameter model), it was determined that the *A. phagocytophilum* isolates determined in *H. marginatum* and *R. turanicus* samples collected from cattle in the Kayseri region were clustered in 3 main branches (A, B, and C groups) with the isolates previously entered GenBank from the world and Turkey. The isolates clustered in group A were generally obtained from ticks, and it was determined that isolates obtained from hedgehogs, rodents, dogs, cattle, and deer were also included in this group. It was found that one of the two isolates clustered in group B was obtained from a tick and the other was obtained from a dog. The C group, which also includes TRAphagHmar and TRAphagRtur isolates obtained from ticks collected from cattle in the Kayseri region, generally includes *A. phagocytophilum* isolates previously entered from Turkey and obtained from ticks, sheep, and goats, as well as isolates obtained from pigeons, owls, and cattle. It was determined that the isolates in groups A and B were homologous, and the isolates in group C had an average genetic difference of 0.2%. When the genetic differences between the groups were examined, it was found that the isolates in group A had an average genetic difference of 10.8±2.0% with those in group B and 13.0±2.7% with those in group C. The average genetic difference between the isolates in group B and those in group C was determined as 13.8±2.8%. It was determined that TRAphagHmar and TRAphagRtur isolates were 100% identical to each other. The genetic differences between TRAphagHmar and TRAphagRtur isolates and 9-08 (AB604782), 73-08 (AB604783), and 208-08 (AB604784) isolates obtained previously from Turkey and from Ixodes ricinus ticks (in group C) were determined to be 0.09%, 0.05%, and 0.58%, respectively. The genetic difference between TRAphagHmar and TRAphagRtur isolates and UK 40 (KJ183079), C139 (KF459965), EKY 144 (JF807995), and EKY 155 (JF807994) isolates obtained previously from Turkey from sheep and goats (in group C) was determined as 0.09%. The genetic differences between the Trbrt45 (KP745629), 10100 (JN656375), H116 (KF034787), and HS56 (KJ195692) isolates from Turkey, which were entered from cattle, dogs, and unspecified ticks, and the TRAphagHmar and TRAphagRtur isolates in group A were also determined. For the first three isolates, the genetic difference was determined to be 13.26%, and for the last HS56 isolate, it was determined to be 13.66%. The majority of isolates in Group A are obtained from ticks from different geographic regions of the world, while the isolates obtained in our study are in Group C, which includes isolates previously reported from Turkey and mainly obtained from animals such as sheep, goats, and cattle. This supports the possibility that the positivity determined in *H. marginatum* and *R. turanicus* species may have been transmitted to these ticks during feeding on infected cattle.

CONCLUSION

In conclusion, the presence of *A. phagocytophilum* was investigated molecularly in tick samples collected from cattle in the Kayseri region with respect to the 16S rRNA gene region, and their genetic characteristics were revealed. Considering the findings reported previously in Turkey and worldwide, the hypothesis that *A. phagocytophilum* species can be carried not only by *Ixodes* species, where vector competence has been demonstrated, but also by other ixodid tick species from different genera, is supported. However, further research is needed to confirm the validity of this hypothesis and to demonstrate the vector competence of other ixodid tick species from different genera.

Conflict of interest

No competing financial or non-financial interests exist.

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