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Molecular survey of *Anaplasma* and *Ehrlichia* species in cattle from Karaman of Turkey, including a novel tandem report of *Anaplasma marginale* msp1a gene

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Abstract: Tick-borne pathogens cause serious health problems and loss of productivity in domesticated and wild animals. A molecular study was performed to detect the frequency of infection with *Anaplasma*/*Ehrlichia* (A/E) in cattle from Karaman province of Turkey. Venous blood samples were taken from 150 apparently healthy cattle in 2016. After amplification the hypervariable V1 region of the 16S rRNA gene of A/E species, a reverse line blot (RLB) assay was performed using species-specific probes. Since some samples gave signal only to A/E catch-all probe, the samples analyzed in terms of major surface proteins (MSPs) of *Anaplasma marginale*. Genetic diversity and tandem repeat analysis were made for msp1α gene sequences of *A. marginale*. *Anaplasma*-like bodies were detected in four (2.66%) animals via microscopic examination. *Anaplasma centrale* was detected in eight (5.33%) animals via RLB. When the samples were examined in terms of *A. marginale* msp1a gene with semi-nested PCR, a total of nine (6.00%) animals [six of them (4.00%) were positive for *A. centrale* with RLB] were found to be infected with *A. marginale*. In addition, the sequences of MSP1a amplicons revealed one new tandem repeat (Tr70). According to these results, it was determined that *A. marginale* and *A. centrale* were found in cattle in Karaman province and this study provided the first evidence of genetic diversity of *A. marginale* with one new tandem repeat in cattle in the region.
Keywords: *A. centrale, A. marginale,* cattle, Karaman, tandem repeat.

Anaplasma marginale msp1a geninin yeni bir tandem raporunu da içeren, Türkiye'nin Karaman yöresindeki sığırlarda *Anaplasma* ve *Ehrlichia* türlerinin moleküler araştırması

Özet: Kene kaynaklı patojenler evcil ve yabani hayvanlarda ciddi sağlık problemlerine ve verim kaybına neden olur. Karaman ilindeki sığırlarda *Anaplasma* / *Ehrlichia* (A/E) ile enfeksiyon sıklığını saptamak için moleküler bir çalışma yapıldı. Venöz kan numuneleri görünüşte sağlıklı olan 150 sığırdan 2016 yılında alınmıştır. A/E türlerinin 16S rRNA geninin değişken V1 bölgesi amplifiye edildikten sonra tür spesifik problar kullanılarak reverse line blot (RLB) deneyi gerçekleştirilmiştir. Bazı örnekler sadece A/E probuna sinyal verdiğinden, örnekler *Anaplasma marginale*'nin major surface proteinleri (MSPs) açısından analiz edilmiştir. *A. marginale*'nin msp1α gen dizileri için genetik çeşitlilik ve tandem tekrar analizi yapıldı. Mikroskobik inceleme ile dört (%2.66) hayvanda *Anaplasma* benzeri cisimler tespit edildi. *Anaplasma centrale* RLB ile sekiz (%5.33) hayvanda tespit edildi. Örnekler semi-nested PZR ile *A. marginale* msp1a geni açısından incelediğinde, toplam dokuz (%6.00) hayvanın [6'sı (%4.00) RLB ile *A. centrale* açısından pozitif olan] *A. marginale* ile enfekte olduğu bulunmuştur. Ek olarak, MSP1a amplikonlarının dizileri bir tane yeni tandem tekrarı (Tr70) ortaya çıkardı. Bu sonuçlara göre Karaman ilindeki sığırlarda *A. marginale* ve *A. centrale*'nin bulunduğu tespit edilmiş ve bu çalışma bölgedeki sığırlarda bir yeni tandem tekrarı ile *A. marginale*'nin genetik çeşitliliğinin ilk kanıtını sağlamıştır.
Anahtar sözcükler: *A. centrale, A. marginale,* Karaman, sığır, tandem tekrarı.

Introduction

Tick-borne diseases (TBDs) poses a great impact for animal and human health in tropical and subtropical climatic regions including Turkey. Turkey has a grand potential for animal breeding and livestock population comprises 14 million cattle, 29 million sheep and 9 million goats. Since TBDs (e.g. theileriosis, babesiosis and anaplasmosis) cause management problems due to significant economic losses, accepted as pre-eminent health trouble worldwide (21) and it is important that a real diagnosis and an effective treatment should be performed.

Anaplasma spp. are significant tick-borne bacteria because of medical and veterinary significance (16). Most common agent for cattle anaplasmosis is *Anaplasma marginale*. Biological transmission of *A. marginale* is associated with ticks mainly genera of *Rhipicephalus* and *Dermacentor* (22). *Anaplasma marginale* is highly pathogenic for cattle and the major signs are anemia, fever, icterus, weight loss and death (6). Until today, a large number of tandem repeats and genotypes have been identified based on the variability of tandem amino acid sequences in the msp1a gene region of *A. marginale* (13). More than two hundred and fifty tandem repeats have been reported in various parts of the world (11). It has been reported new tandem repeats in China, Turkey and Russia with recent studies (4, 18, 28).

Bovine anaplasmosis can be diagnosed on the basis of clinical symptoms and microscopic examination of Giemsa-stained blood smears (20, 29). It is adequate for the detection of acute infection, but not possible for detection of carrier animals. Serologic tests have been employed in diagnosing subclinical infections in epidemiological studies (9), but cross-reactions between species and false-negative results are potential restrictions. It is possible to eliminate these disadvantages with molecular techniques provide improved sensitivity and specificity than microscopy and serology. In addition to these, veterinary practitioners have a limited laboratory facility in field conditions, also early treatment is very important for TBDs.

Although clinical and subclinical infections have been reported data concerning genetic variants of these pathogens is scarce. This study provides information about the distribution and frequency of *Anaplasma*/*Ehrlichia* (A/E) species in cattle from Karaman province of Turkey with a novel genetic variant of *A. marginale*.

Materials and Methods

Study area and sample collection: This study was carried out in Karaman province (37º 11' N, 33º 15' E) located in the south of the Central Anatolia Region of Turkey (Figure 1) and it was conducted in compliance with the regulation issued by Karamanoğlu Mehmetbey University Animal Experiments Local Ethics Committee (2016/01). Sampling was performed in 2016. The Karaman province is 1033 meters above sea level and has an area of 8869 km^2. It has a continental climate with hot summers and cold winters. The mean annual rainfall and temperature are 336.3 kg/m^2 and 12 °C, respectively. Agriculture/animal husbandry and related industrial sector activities have an important place in the Karaman economy. One hundred fifty clinically healthy cattle from 21 different locations throughout Karaman were examined for clinical findings of anaplasmosis (body temperature, mucous membrane color and size of subcutaneous lymph nodes) between April and September 2016. Age, gender and breed of animals were saved. Five ml of blood sample were taken from the *vena jugularis* into tubes containing K_3EDTA-anticoagulant from each animal.

Preparation of blood smears and DNA isolation: Thin blood smears prepared from animals were fixed with absolute methanol for five minutes and stained with 5% Giemsa stain for 30 minutes. The slides were rinsed with water, and after drying in the air they were screened under oil immersion (×100 magnification) for the presence of *Anaplasma*-like bodies. At least 100 microscope fields have been examined and even if an agent has been found, the sample has been evaluated as positive.

Blood samples were defrosted at room temperature and vortexed for 15 seconds to homogenize. A commercial kit (QIAamp DNA Mini Kit, 51306) was used to isolate total genomic DNA. The DNA extraction was performed as described in the kit protocol using 200 µl blood sample. Genomic DNAs were stored at -20 °C until used as a template in the PCR.

Polymerase chain reaction and reverse line blot hybridization assay: Nested PCR was performed using two universal primers. EC9 (5′-TACCTTGTTACG ACTT-3′) and EC12A (5′-TGATCCTGGCTCAGAACG AACG-3′) which amplify 1450 bp fragment in the hypervariable V1 region of the 16S rRNA gene of A/E was used for the first amplification (12). For the second amplification, one µl of first round PCR products were used as a template DNA. To amplify 492-498 bp in the hypervariable V1 region in 16S rRNA gene of A/E, 16S8FE (5'-GGAATTCAGAGTTGGATCMTGGYT CAG-3') and BGA1B-new (Biotin-5'- CGGGATCCC GAGTTTGCCGGGACTTYTTCT-3') primers (8, 26) were used. To reduce non-specific amplification, a touchdown program was performed. DNA from positive control and distilled water were used. The PCR was performed in a final volume of 25 µl, containing PCR buffer [750 mM Tris–HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20], 5 mM $MgCl_2$, 125 µM deoxynucleotide triphosphates, 1.25 U Taq DNA polymerase, forward and reverse primers (10 pmol/µl), and template DNA. Five microliters of PCR product were visualized using UV transillumination in a 1.6% agarose gel stained with ethidium bromide and the remaining amplicons were stored for RLB until hybridization. Probes containing N-terminal N-(trifluoracetamidohexyl-cyanoethyl,N,N-diisopropylphosphoramidite [TFA])-C6 amino linker were synthesized by "Midland Certified Reagent Company, Inc." and used with a range of 200-900 pmol/150 µl concentration (Table 1). Preparation of biodyne C membrane, hybridization and rinsing were as previously described (7). Black spots in rows were evaluated by ChemiDoc™ MP System (Bio-Rad, UK) can make chemiluminescence detection.

Table 1. Sequences of oligonucleotides used in RLB.
Tablo 1. RLB'de kullanılan oligonükleotidlerin dizilimleri.

Oligonucleotide probe	Sequence (5'–′3)	Reference
Anaplasma/*Ehrlichia*	AmMC6-TTATCGCTATTAGATGAGCC	26
Ehrlichia/*Anaplasma* catch-all	AmMC6-GGGGGAAAGATTTATCGCTA	8
Anaplasma marginale	AmMC6-GACCGTATACGCAGCTTG	8
Anaplasma centrale	AmMC6-TCGAACGGACCATACGC	8
Anaplasma bovis	AmMC6-GTAGCTTGCTATGAGAACA	8
Ehtlichia sp. strain Omatjenne	AmMC6-CGGGTTTTTATCATAGCTTGC	8
Anaplasma phagocytophilum 1	AmMC6-TTGCTATAAAGAATAATTAGTGG	26
Anaplasma phagocytophilum 3	AmMC6-TTGCTATGAAGAATAATTAGTGG	26
Anaplasma phagocytophilum 5	AmMC6-TTGCTATAAAGAATAGTTAGTGG	26
Anaplasma phagocytophilum 7	AmMC6-TTGCTATAGAGAATAGTTAGTGG	26
Anaplasma phagocytophilum A-HGE	AmMC6-GCTATAAAGAATAGTTAGTGG	26
Anaplasma phagocytophilum A-D-HGE	AmMC6-GCTATGAAGAATAGTTAGTG	26

***Anaplasma marginale specific semi-nested PCR*:** A semi-nested PCR protocol for msp1α gene was conducted as described by Lew et al. (23). The primers 1733F (5'-TGTGCTTATGGCAGACATTTCC-3') and 3134R (5'-TCACGGTCAAAACCTTTGCTTACC-3') were used in the first PCR, and the primer pair 1733F and 2957R (5'-AAACCTTGTAGCCCCAACTTATCC-3') was used in the second reaction. For the second PCR amplification, one µl of the first product was used as a template. Amplification was carried out under conditions previously reported. PCR amplicons were separated by electrophoresis on 1.6% agarose gel (40 min, 100 V), stained with ethidium bromide, and visualized under ultraviolet light. *Anaplasma marginale* control DNA was previously isolated from a cow (GenBank accession no. GU201518).

DNA sequencing, MSP1a microsatellite and tandem repeat analysis: Five amplified fragments containing variable regions of *A. marginale* msp1α gene were purified from the agarose gel using a commercial PCR Clean up System (MinElute Gel Extraction Kit, 28604) and directly sequenced. The MAFFT (https://mafft.cbrc.jp/) and Emboss Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) programs were used to conduct multiple alignments and translate nucleotide to an amino acid of MSP1a sequences respectively. Sequencing results submitted to GenBank after comparing with other sequences available in the NCB database (http://www.ncbi.nlm.nih.gov/nuccore). The isolates were identified according to the nomenclature as previously reported (10, 15). The nature of the microsatellite structure was GTAGG **(G/A TTT)** m **(GT)** n**T** ATG (17). Calculation of the SD-ATG distance was performed as (4 x m) + (2 x n) + 1. The microsatellite analysis was conducted using the RepeatAnalyzer (11), and the nature of tandem repeats was indicated as previously proposed (15).

Results

Microscopic examination of blood smears: *Anaplasma*-like bodies were detected in four (2.66%) Giemsa-stained blood smears. All of the animals evaluated as positive in the microscopic examination are from Karaman center.

Detection of Anaplasma spp. by RLB: One hundred and fifty blood samples were screened for the presence of bovine A/E species. According to RLB results, eight out of 150 cattle (5.33%) were found to be infected with *A. centrale*. Three samples gave positive signals to A/E catch-all probes and no species-specific probe signal received. No animals were infected with *Ehrlichia* spp. (Table 2).

Anaplasma marginale specific semi-nested PCR amplification: Nine samples (6.00%) [three of them gave positive signals to A/E catch-all probes and eight of them were found to be positive in terms of *A. centrale* via RLB] were positive in terms of *A. marginale* according to the semi-nested PCR with primers (1733F and 3134R - 1733F and 2957R) amplifying the msp1a gene.

DNA sequencing, MSP1a microsatellite and tandem repeat analysis: Five *A. marginale* positive samples were sent to sequence analysis and obtained sequences of msp1α gene were submitted to GenBank (accession numbers MG983513 to MG983517). Tandem repeat sequences and structure of the msp1α gene were analyzed to discover differences. Three different types of MSP1a tandem repeats with 23 to 29 amino acids for *A. marginale* strains were identified (Figure 2a). The MSP1a microsatellite analysis revealed that E genotype was detected in the analyzed sequences and microsatellite sequences produced SD-ATG distances 23 nucleotide. One new microsatellite structure designated as Tr70 [(m = 2, n = 7, SD-ATG distance = 23), (ADSSSAGGVLSQS GQASTSSQLG)] was described (Figure 2b). It was determined that *A. marginale* strains had 2 and 4 MSP1a repeat sequences in the studied area (Figure 2c).

Table 2. Distribution of *Anaplasma* species detected by microscopy, PCR and RLB.
Tablo 2. Mikroskop, PZR ve RLB ile tesbit edilen *Anaplasma* türlerinin dağılımı.

Province	n	Test			Overall results		
		Microscopy	RLB	*A. marginale* nPCR	*A. marginale*	*A. centrale*	*A. marginale* + *A. centrale*
		Anaplasma spp.	*A. centrale*	*A. marginale*			
Karaman center	42	4	2	3	2	1	1
Kazımkarabekir	16	-	3	3	-	-	3
Ayrancı	56	-	1	2	1	-	1
Ermenek	36	-	2	1	-	1	1
Total	150	4 (2.66%)	8 (5.33%)	9 (6.00%)	3 (2.00%)	2 (1.33%)	6 (4.00%)

Figure 1. Turkey map showing the study area.
Şekil 1. Çalışma alanını gösteren Türkiye haritası.

a)

Repeat form	Encoded sequence	Number of amino acid
Tr1	ADSSSAGDQQQESSVLSQSDQASTSSQLG	29
Tr70*	ADSSSAG------GVLSQSGQASTSSQLG	23
Is1, 73	TDSSSAGDQQQESGVSSQSGQASTSSQLG	29

b)

Isolates	Accesion number	Genotype	m	n	SD-ATG distance (nucleotide)
Kr3	MG983513	E	2	7	23
Kr7	MG983514	E	2	7	23
Kr37	MG983515	E	2	7	23
Kr58	MG983516	E	2	7	23
Kr78	MG983517	E	2	7	23

c)

Isolates	Structure of MSP1a tandem repeats			No. of repeats
Kr3	Tr1	Is1,73		2
Kr7	Tr8	Is1,73	Is1,73	4
Kr37	Tr1	Is1,73		2
Kr58	Tr8	Is1,73	Is1,73	4
Kr78	Tr8	Is1,73	Is1,73	4

Figure 2a. New repeat forms of *Anaplasma marginale* MSP1a (Tr70*) identified. The one letter amino acid code was used to depict the differences found in MSP1a repeats. 2b. The msp1α microsatellite and tandem repeat sequences in *Anaplasma marginale* isolates. The microsatellite (sequence in bold) was located between the Shine-Dalgarno and the translation initiation codon (ATG) with the structure: GTAGG (G/ATTT)m (GT)n T ATG. 2c. The structure of the MSP1a repeat regions, according to the nomenclature previously proposed (11, 15).
Şekil 2a. *Anaplasma marginale* MSP1a'nın (Tr70*) yeni tekrar formları belirlenmiştir. MSP1a tekrarlarında bulunan farklılıkları tasvir etmek için bir harfli amino asit kodu kullanılmıştır. 2b. *Anaplasma marginale* izolatlarında msp1α mikrosatellit ve tandem tekrar dizileri. Mikrosatellit (koyu sıralı) Shine-Dalgarno ile translasyon başlatma kodonu (ATG) arasında, yapı ile birlikte: GTAGG (G / ATTT)m (GT)n T ATG. 2c. MSP1a'nın tekrarlanan bölgelerinin yapısı, daha önce önerilen terminolojiye göre (11, 15).

Discussion and Conclusion

In recent years, studies related to ticks and TBDs has increased and TBDs cause a major health problem and loss of production in cattle in Turkey. *Anaplasma phagocytophilum*, *A. marginale*, *A. centrale*, *A. bovis* and *Ehrlichia* sp. strain Omatjenne have been reported in cattle from Turkey (1-3, 5, 19). Among *Anaplasma* species, *A. marginale* is the most pathogenic species causing infections in cattle and it is known to cause clinical infections resulting in death (22), while *A. centrale* causes milder infections (25).

Major surface protein 1a, an important protein to determine genetic diversity of *A. marginale* strains, interacts with the vertebrate and invertebrate host cells of the bacterium (14). Cattle movement is a prominent factor for the MSP1a genetic diversity in *A. marginale* worldwide (15, 27). Supported by the finding of one MSP1a genotype in Australia, where cattle entry is limited (23). To date, eleven different genotypes (A-K) of *A. marginale* msp1a gene in worldwide were described (10, 17). In this study, we present the genotypic variant E of *A. marginale* based on msp1a gene sequences. C, E, and G genotypes were previously reported in dairy cattle from Turkey (4). More than two hundred fifty *A. marginale* tandem repeats have been reported in various parts of the world (4, 13, 15, 18, 28). In studies on *A. marginale* msp1a gene conducted in China and Turkey, 21 and 3 new tandem repeats have been reported respectively. A computer program is prepared to prevent the confusion in tandem repeats (11). Tr1, Tr2, Tr3, 73, 74 and 76 tandem repeats have been reported in dairy cattle in Turkey (4). The one tandem repeat designated as ‘‘Tr70’’ in this study were not previously reported anywhere (11). Also, ‘‘Tr1’’ and ‘‘73’’ tandem repeats were reported with this present study.

The length of the microsatellite is related to the expression of the msp1a gene and affects the transmission of *A. marginale* and its infection (17). In this study, SD-ATG distances of 23 nucleotides is a higher expression level. This finding suggests a big capacity for infection and transmission of the *A. marginale* strains.

Studies conducted with RLB do not result in species level from time to time due to different genotypes among species. In a study conducted on *Theileria equi*, the sample was not signaled at the species level when signaling against catch-all probe due to different genotypes (24). Similarly, in this study, no signal was detected at the species level when three samples were signaled for A/E catch-all probe. As a result, msp1a gene of *A. marginale* was amplified and catch-all samples were confirmed as *A. marginale*.

In conclusion, the presence and distribution of A/E infections in cattle in Karaman province were investigated using microscopy, PZR and RLB methods in this study. *Anaplasma marginale* and *A. centrale* were detected in cattle. Three different types of MSP1a tandem repeats with one new microsatellite structures designated as Tr70 for *A. marginale* strains were identified. It is important to remember that there can be different genotypes and strains of *A. marginale* when A/E catch-all signal is received in similar studies. It is also expected that the diversity of msp1a genotypes can increase related to animal movements and animal imports from abroad.

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