Ankara Üniv Vet Fak Derg, **62**, 183-187, 2015

Bartonella species in wild small mammals in Western Black Sea Region of Turkey

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Summary: The species within the genus *Bartonella* are intracellular bacteria causing long-lasting bacteremia in humans and animals. In this study, *Bartonella* spp. in 173 small mammals, which were *Apodemus flavicollis*, *A. witherbyi*, *A. uralensis*, *A. mystacinus*, *Myodes glareolus*, C*rocidura suaveolens*, *Rattus rattus* and *Rattus norvegicus* species captured from Western Black Sea Region of Turkey, were investigated by blood culture and molecular methods. The positivity of *Bartonella* was 63.6% (110/173) by blood culture of small mammalian. The *gltA* gene regions for the isolated strains were identified by DNA sequencing analysis. Isolates were identified as *Bartonella taylorii*, *B. birtlesii*, *B. coopersplainsensis* and a zoonotic *B. grahamii*.

Key words: *Bartonella*, blood culture, *gltA*, PCR, rodent.

Batı Karadeniz Bölgesinde yabani küçük memelilerde *Bartonella* türleri

Özet: *Bartonella* genusundaki türler insanlarda ve hayvanlarda uzun süreli bakteriyemiye neden olan hücre içi bakterilerdir. Bu çalışmada Batı Karadeniz Bölgesi'nde yakalanan *Apodemus flavicollis*, *A. witherbyi*, *A. uralensis*, *A. mystacinus*, *Myodes glareolus*, C*rocidura suaveolens*, *Rattus rattus* ve *Rattus norvegicus* türlerinin dahil olduğu 173 küçük memelide, kan kültürü ve moleküler yöntemlerle *Bartonella* türleri araştırıldı. *Bartonella* pozitifliği küçük memelilerde kan kültürü ile %63.6 (110/173) bulundu. Kültürde izole edilen *Bartonella* suşlarının, sitrat sentez gen bölgesi (*gltA*) DNA dizi analizi ile tür tanımlamaları yapıldı. İzolatlar *Bartonella taylorii*, *B. birtlesii*, *B. coopersplainsensis* ve zoonotik bir tür olan *B. grahamii* olarak tanımlandı.

Anahtar sözcükler: *Bartonella*, *gltA*, kan kültürü, PCR, rodent.

Introduction

The genus *Bartonella* is a Gram-negative, slow growing, facultative intracellular bacteria that cause sustained bacteremia in humans and numerous types of animals. The members of this genus are transmitted among their sensitive hosts through blood-sucking arthropod vectors (5). *Bartonella taylorii, B. birtlesii, B. grahamii, B. dohiae, B. elizabethae, B. phoceensis, B. rattimassiliensis, B. tribocorum, B. vinsonii* subsp. *arupensis, B. vinsonii* subsp. *vinsonii, B. rattaustraliani, B. queenslandensis, B. coopersplainsensis* and *B. washoensis* have been isolated from diverse wild rodent populations (1,2,3,27)

Among the genus *Bartonella, B. grahamii, B. elizabethae, B. washoensis* and *B. vinsonii* subsp. *arupensis* are zoonotic species isolated from wild rodents. *B. grahamii* causes neuroretinitis (18), *B. vinsonii* subsp. *arupensis* and *B. elizabethae* cause fever and endocarditis (9,11,28) and *B. washoensis* causes myocarditis (21).

Molecular methods are used for the identification of *Bartonella* spp. The most frequently used genes are the intergenic transcribed spacer (*ITS*) gene placed between the 16S and 23S rRNA gene, citrate synthase gene (*gltA*), 60-kDa heat shock protein (*ftsZ*) gene, and the RNA polymerase β subunit (*rpoB*) gene (20). La Scola *et al.* suggested that *Bartonella* spp. could be described on the basis of DNA sequences from housekeeping genes, such as RNA polymerase (*rpoB*) and citrate synthase (*gltA*) (25).

Bartonella henselae, B. clarridgeiea and *B. vinsonii* subsp. *berkhoffii* in cats and dogs in Turkey have been identified by cultural, and molecular methods (6,7). The presence in wild mice for *B. taylorii,* and *B. grahamii* was firstly reported in Turkey by Karagoz *et al.* (17). A number of studies have shown that *Bartonella* spp. are widely distributed in wild rodents in many European countries, such as the United Kingdom (3), Slovenia (20), Sweden (14), Spain (12), Greece (26), Denmark (10) and Poland (29). This study identified and described

the presence of *Bartonella* spp. by blood culture and molecular techniques in wild rodent and insectivores captured from the city of Bartin and Zonguldak in Western Black Sea Region, Turkey.

Material and Method

In the field studies in Bartın (41°41'8"N, 32°13'49"E) & Zonguldak (41°27'20"N, 31°45'50"E) provinces at Western Black Sea region, 171 wild rodents and 2 insectivores were caught. Both provinces are placed in Northwest of Turkey and the land size distribution is 46% forest, 35% agricultural, 7% grassland & pasture. For the region, average temperature is 12.5°C, humidity 79% and rainfall 871mm.

A total of 159 small mammalian were trapped using Sherman live-capture traps in 8 different rural localizations of Bartın. Fourteen *Rattus* spp. were captured urban area of Zonguldak. Traps containing captured rodents were collected each morning and transported to a biosafety level-2 mobile laboratory where they were necropsied on the same day of collection. Tissues and samples of blood, lungs, kidneys, liver and heart of the rodents were collected and stored at -80°C until analysis. To reduce the chance of human infections with highly virulent rodent-borne agents, investigators wore proper personal protective equipment.

The rodents investigated for *Bartonella* spp., were identified at species level according to their external morphological size and phenotypic features. Morphological species identification was based on the size of body weight, head, body, tail, hind-foot and ear. The identified distribution of 159 caught rodents and insectivores in Bartin province are *Apodemus flavicollis* (n=47), *A. witherbyi* (n=44), *A. uralensis* (n=17), *A. mystacinus* (n=7), *Myodes glareolus* (n=42) and C*rocidura suaveolens* (n=2). The remaining 14 rat caught in Zonguldak province are *Rattus rattus* (n=8) and *R. norvegicus* (n=6) (Table1).

Isolation of Bartonella spp. *from animal blood*: Blood samples were aseptically collected from each animal. The blood samples were kept at –20ºC and transferred to –80ºC until analysis. Freeze-thawing method was used to isolate *Bartonella* spp. in animal blood. The blood sample were thawed at room temperature, a 100 µL sample was plated on brain heart infusion agar enriched with 5% horse blood. The inoculated plates were incubated for 21 days at 36°C in an incubator with 5% CO_2. The agar plate was examined for colony formation daily. The colonies were evaluated in terms of time of growth and colony morphology. Cauliflower-like, Gram-negative, catalase and oxidase-negative, R-type colonies, which leave a trace on the plate when removed, were accepted as *Bartonella* spp. (6).

PCR and sequencing study: DNA of the isolates were analyzed by Polymerase Chain Reaction (PCR) based on the amplification of (*gltA)* fragment. *B. henselae* DNA (ATCC 49882) was used as the positive control. DNA extraction of the isolates was performed using the boiling method (6). The amplification of the 380 bp location of the *gltA* gene was performed using BhCS.781 (5'-GGG GAC CAG CTC ATG GTG G-3') and BhCS.1137n (5'-AAT CGA AAA AGA ACA GTA AAC A-3') primers, as *Norman et al.* reported (22).

The mixture of PCR included 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, and 10 pmol from each primer (Iontek, Istanbul, Turkey), and 1 U *Taq* DNA polymerase for each sample. All reagents were provided by Fermantas (Vilnius, Lithuania). The PCR reaction was set with 45 µl of the mixture and 5 µl DNA template, and the PCR was performed according to *Norman et al.* (22). The cycles are initial denaturation 95°C for 3 min, 35 cycles of 95°C for 20 sec, annealing at 51°C for 30 sec, extension 72°C for 1 min and final extension 72°C for 5 min. The amplified products were run and viewed in a 1.5% agarose gel (Sigma, St Louis, MO, USA), and the visualization of 380 bp PCR product was accepted as positive for *Bartonella* spp.

DNA sequence analyses were performed to *gltA* amplification products using Agencourt Ampure purification kit (Beckman Coulter, Beverly, USA). Sequence reaction was performed using a Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter). The PCR products were purified using a Dye-Terminator removal kit (Agencourt CleanSEQ; Beckman Coulter). DNA sequences of the purified products were determined using Beckman Coulter 8000 equipment. The DNA sequences of isolates were identified comparing the DNA sequences of reference isolates with data stored in the GenBank using the Basic Local Alignment Search Tool (Blast version 2.0) program. A phylogenetic tree analysis was created using Clustal W using MegAlign.

Results

One hundred seventy three blood samples were analyzed and presumptive *Bartonella* spp. growth was observed in 110 (63.6%) samples within 4-7 days of blood culture. All the presumptive positive cultures were also found as positive for *Bartonella gltA* in the PCR analysis.

Isolates were further molecularly identified by DNA sequence analysis. The phylogenetic tree based on DNA sequence homology is shown figure 1. Of the 110 isolates, 77 are similar to *B. taylorii* at 96-99% (GenBank Number AY584852 and AF191502), 16 are *B. grahamii* at 99% (GenBank Number AY584857), 15 are *B. birtlesii* 96-98% (GenBank Number AF204272), one is *B. coopersplainsensis* at 99% (GenBank Number EU11803).

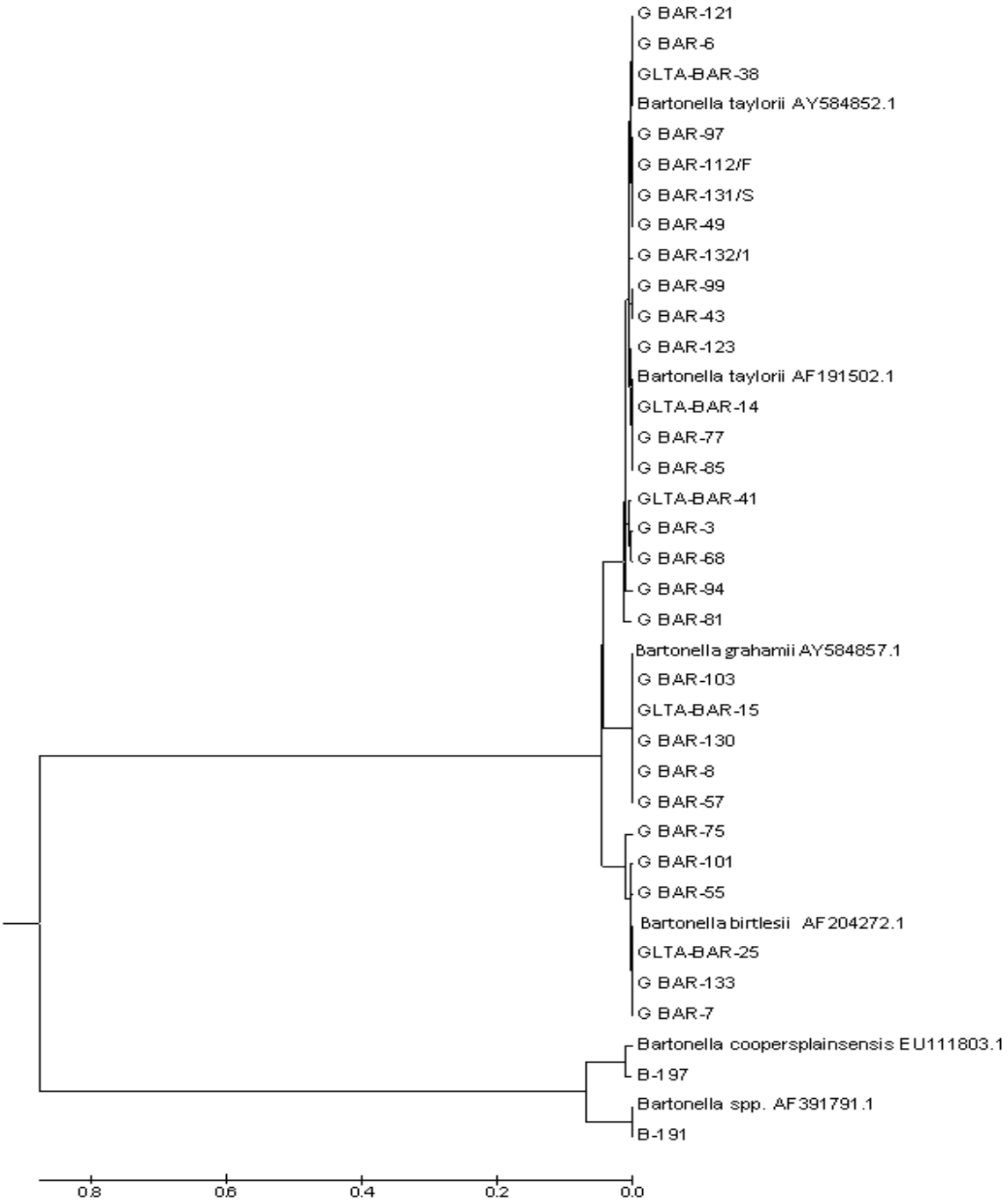

Figure1. Phylogenetic relations among the citrate synthase sequences (*gltA*) of *Bartonella* spp. genotypes detected in small mammalians (rodents and insectivores) from Turkey and previously described *Bartonella* spp. The phylogenetic tree was constructed by the UPGMA method.

Şekil 1. Genotipik olarak Türkiye'de ve daha önceki çalışmalarda küçük memelilerde (rodent ve böcekçiller) tanımlanmış olan Bartonella türlerinde Sitrat sentez gen bölgesi (*gltA*) filogenetik ilişkinin gösterilmesi. Filogenetik ağaç UPGMA metoduna göre gerçekleştirilmiştir.

One isolate isolated from insectivore C*rocidura suaveolens* showed complete similarity to *Bartonella* spp. (GenBank Number AF391791). Thirteen and 64 *B. taylorii* isolates show similarity to far-east Asia (east Siberia/Russia) (Genbank Number AY584852) and European genotypes (Genbank Number AF191502) at 99%, respectively.

Table 1 shows the *Bartonella* spp. isolated from rodents and positivity ratio. *Bartonella* spp. was isolated from *A. uralensis*, *A. flavicollis* and *A. witherbyi* at 88%, 85% and 72%, respectively. A zoonotic *B. grahamii* was isolated from wild rodents, *A. flovicollis, A. witherbyi* and *M. glareolus*. Interestingly, this is the first report for *B. birtlesii* and *B. coopersplainsensis* in Turkey.

Table 1. Distribution of 110 *Bartonella* spp. isolates in small mammalians (Rodents and Insectivores)
Tablo 1. Küçük memelilerden (Rodentler ve Böcekciller) izole edilen 110 *Bartonella* türünün dağılımı.

Animal Species (n)	*Bartonella* species				*Bartonella* spp. %
	B. taylorii	*B. grahamii*	*B. birtlesii*	*B. coopersplainsensis*	
Apodemus flavicollis (47)	28	5	7		40/47(85%)
Apodemus uralensis (17)	14		1		15/17(88%)
Apodemus witherbyi (44)	15	10	7		32/44(72%)
Apodemus mystacinus (7)					0/7(0%)
Myodes glareolus (42)	20	1			21/42(50%)
Rattus rattus (8)				1	1/8(12.5%)
Rattus norvegicus (6)					0/6(0%)
Crocidura suaveolens(2)	One isolate *Bartonella* spp				1/2 (50%)

Discussion and Conclusion

The prevalence of *Bartonella* infection in wild rodent in European countries has been reported as 40.4% in Slovenia (20), 26.8% in Spain (12), 30.6% in Poland (29) by direct PCR of splenic tissue, and 16.5% in Sweden (15), 27.5% in Denmark (11), 62% in England (3), and 31.3% in Greece (26) by blood culture. Karagoz *et al.* found 57.1% *Bartonella* positivity by PCR in liver tissue samples of field mice (*Microtus socialis*) in Central Anatolia Region of Turkey (17). The Bartonella infection rate within wild rodent population was determined as 63.6% in this study. Cultural and molecular analysis subtyped those as *B. taylorii*, *B. birtlesii*, *B. coopersplainsensis* and zoonotic *B. grahamii*. This suggests us that the positivity of *Bartonella* spp. in wild rodent is high in Turkey.

Bartonella positivity by Karagöz et al., (17) in field mice (*Microtus socialis*) population was reported 57.1% in liver by PCR, however liver culture for the same samples produced 16.6%. In this study, blood cultures showed 63.6% *Bartonella* positivity in wild rodent population. Our results show that why blood culture studies are preferred isolation method for intraerythrocytic agent like *Bartonella* spp.

Karagoz *et al.* (17) reported, four of the *B. taylorii* isolates had a 99% genotypic similarity to Far East Asian (East Siberia/Russia) isolates with Genbank accession number AY584852, and only one of them had a 99% similarity to European isolates (England, Greece) with Genbank accession number AF191502. In this study, the isolates 13 and 64 of *B. taylorii* were 99% similar to Far-East Asia (East Siberia, AY584852 Genbank number) and European genotype (AF191502 Genbank number)

Arthropods such as lice, fleas, ticks, and biting flies are effective in transporting the *Bartonella* species among animals. Transmission to humans is accomplished through scratches or bites by infected animals, and through ticks that have sucked blood from infected animals (4,5,8). Wild rodents are a possible source of potential Bartonella infection in humans based on findings of this and previous studies performed in other countries. Those studies determined high rates of *Bartonella* spp. positivity in mice and the role of ticks as a transmitting agent (24). Iralu *et al.* reported rodent-borne Bartonella infection in 9 (12%) of 76 patients with fever of unknown origin, diagnosed serologically with seroconversion and high antibody titers (15). According to the available data in Turkey related to tick-borne infections with fever of unknown reason encourages us to study further.

This study shows the isolation of *B. coopersplainsensis* which have been previously reported in Australia and Southeastern Asia (13, 16). Kılıc *et al.* also reported rodent-borne *Francisella tularensis* subsp. *holarctica* biovar *japonica* which was not isolated other than Japan (19). Recently, Oktem et al., (23) isolated first rodent-borne Hantavirus in Turkey. Increasing available data lead us to investigate further zoonotic infections transmitted by rodents which are not typically observed in Turkey.

Bartonella spp. isolated from insectivore subjected to this study have been found 100% similar to Sweden, England and Spain isolates (AF391791, EF031549, HM596457 Genbank numbers) respectively. This may indicate that they are possibly insectivore spesific *Bartonella* spp. isolates. Scola *et al.*, proposed that newly encountered *Bartonella* isolates should be considered new species if they show 96.0% sequence similarity in 327 bp gltA fragment (25). Therefore, we suspect that insectivore related to *Bartonella* spp. is new genotypes. Further genotyping studies are required to confirm.

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Geliş tarihi: 05.05.2014/ Kabul tarihi: 10.09.2014

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