Ankara Üniv Vet Fak Derg, **60**, 235-239, 2013

Detection of *Bartonella* spp. in field mice (*Microtus socialis*) by culture and PCR

Alper KARAGÖZ[1], Bekir ÇELEBİ[1], Hülya ŞİMŞEK[1], Müge TANER[1], Selçuk KILIÇ[1], Rıza DURMAZ[1], Mustafa ERTEK[2]

[1] Public Health Institution of Turkey (PHIT), Microbiology Reference Laboratories Department, Ankara, Turkey; [2] Dr. Abdurrahman Yurtaslan Ankara Onkology Hospital, Ankara, Turkey.

Summary: The species within the genus *Bartonella* are intracellular bacteria causing long-lasting bacteremia in humans and animals. Eleven *Bartonella* species isolated from animals have zoonotic characteristics. The four of zoonotic *Bartonella* species have been reported in rodents. In this study, *Bartonella* spp. in 42 field mice, (*Microtus socialis)*, captured from one spot in Central Anatolia Region were investigated by culture and molecular methods. The positivity of *Bartonella* was 16.6% (7/42) by culture, and 57.1% (24/42) by PCR (Polymerase Chain Reaction) from liver tissues of field mice. The citrate synthesis gene regions (*gltA*) of the isolated seven strains were identified by DNA sequencing analysis. It was determined that five isolates were *Bartonella taylorii*, and two isolates were *Bartonella grahamii* which are zoonotic. This study is the first report for the presence of *Bartonella* spp. in rodents in Turkey.

Key words: *Bartonella*, *Microtus socialis*, PCR.

Tarla farelerinde (*Microtus socialis) Bartonella* Spp. varlığının kültür ve PCR yöntemleriyle belirlenmesi

Özet: *Bartonella* genusundaki türler insanlarda ve hayvanlarda uzun süreli bakteriyemiye neden olan hücre içi bakterilerdir. Hayvanlardan izole edilen onbir *Bartonella* türü zoonoz karakterdedir. Zoonoz *Bartonella* türlerinden dördü rodentlerde bildirilmiştir. Bu çalışmada Orta Anadolu Bölgesinde bir odaktan yakalanan 42 *Microtus socialis* türü tarla faresinde kültür ve moleküler yöntemlerle *Bartonella* spp. araştırıldı. Tarla farelerinde, karaciğer dokusundan kültür ile % 16.6 (7/42), PCR (Polimeraz Zincir Reaksiyonu) ile % 57,1 (24/42) *Bartonella* pozitifliği tespit edildi. Kültürde izole edilen yedi izolatın, sitrat sentez gen bölgesi (*glt*A) DNA dizi analizi ile tür tanımlamaları yapıldı. İzolatlardan beşi *Bartonella taylorii*, ikisi zoonotik bir tür olan *Bartonella grahamii* olarak belirlendi. Bu çalışma ile ülkemizde rodentlerde ilk defa *Bartonella* türlerinin varlığı bildirilmektedir.

Anahtar sözcükler: *Bartonella*, *Microtus socialis*, PCR.

Introduction

The genus *Bartonella* are Gram-negative, slow growing, facultative intracellular bacteria that cause sustained bacteremia in humans and numerous types of animals. These bacteria are carried among their sensitive hosts through blood-sucking arthropod vectors (6). Until the 1990s, the agents causing trench fever and Oroya fever, which are specific to humans among the genus *Bartonella,* were known as *Bartonella quintana* and *Bartonella basilliformis*, respectively. With each passing day, with the improvements in molecular techniques, new species have been introduced and more than 30 species and sub-species have been reported in humans and animals. Eleven of these species are known to have a zoonotic character for humans. Among the genus *Bartonella, B. grahamii* and *Bartonella vinsonii* ssp. *arupensis* are zoonotic species isolated from wild mice (5, 13). *B. grahamii* causes neuroretinitis and the *B. vinsonii* ssp. *arupensis* causes fever and endocarditis (17, 22, 26). Other *Bartonella* species isolated from wild mice are *Bartonella birtlesii, Bartonella doshiae, B. taylorii* and *Bartonella vinsonii* ssp. v*insonii* (2, 3, 24). Since the species in this genus are negative in most of the biochemical tests, species identification by biochemical methods are not possible. Therefore, molecular methods are used in species and sub-species identification. The most frequently used gene locations in molecular techniques are the intergenic transcribed spacer (*ITS*) placed between the 16S and 23S rRNA gene locations, citrate synthase gene (*gltA*), 60-kDa heat shock protein (*ftsZ*) gene, and the RNA polymerase β subunit (*rpoB*) gene (18). The aim of this study was to detect *Bartonella* spp. in field mice, known as *Microtus socialis* in the city of Kırşehir, and to identify it in subspecies level by using culture and molecular techniques.

Material and Method

Forty-two *Microtus socialis* mice were caught in the rural town of Hamit, in the district of Kaman, in the city of Kirsehir in February 2010. The mice were necropsied and their tissues were separated on the same day in a biosafety level 2 mobile laboratory. The personnel took the necessary biosafety measures to avoid infections originating from rodents. The tissues were stored at -20 ^{o}C until used. Because the *Bartonella* species are intra-erythrocytic bacteria, blood is generally used in the cultures (4, 12, 15, 23). Liver tissue samples were used in this study as a blood-rich tissue, due to the unavailability of mouse blood. Because the *Bartonella* genus is bacteria with delayed and difficult growing, the direct PCR method was also used on liver tissue samples as an alternative to cultures.

Isolation of the genus Bartonella spp**.:** Liver tissue sample (200 mg) was placed in 200 µl PBS (pH 7.2) and was homogenized in a Roche Manga Lyser homogenizator (Roche, Rotkreuz, Switzerland). Homogenized tissue sample (100 µl) was inoculated into 5% horse blood BHI (brain heart infusion) agar. Inoculated plates were incubated for 21 days at 36 ^{o}C in an incubator with 5% CO_2. The colonies were evaluated in terms of time of growth, colony morphology and Gram characteristics. 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. (7).

PCR and sequence study: DNA extraction was performed on 100 µl homogenized tissue with a Qiagen (Qiagen, Hilder, Germany) mini tissue extraction kit. DNA extraction of the isolates, which were accepted as possible *Bartonella spp*., was performed with the boiling method (7). DNA samples obtained from the liver tissue and isolates were used for PCR.

Specific primers to the genus *Bartonella* were used in the identification of the *Bartonella* species. Amplification of the citrate synthase gene (*gltA*) location for isolates and the *Bartonella* 16S-23S intergenic transcribed spacer (*ITS*) location for liver tissue samples were performed.

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 (1995) (20). 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, excluding the primers, were provided by Fermantas (Vilnius, Lithuania). Amplification products were run and viewed in a 1.5% agarose gel and 380 bp PCR products were accepted as positive for *Bartonella* spp.

The amplification of the ITS target site was applied using 321s: 5'-AGA TGA TGA TCC CAA GCC TTC TGG-3' and 983s: 5'-TGT TCT YAC AAC AAT GAT GAT G-3' primers, as identified by Maggi et al. (2006) (19). Amplification products were run and viewed in a 1.5% agarose gel. According to the differences between the species, PCR products between 600 bp and 700 bp were accepted as positive for *Bartonella* spp. These differences cannot be used to differ the species (18).

DNA sequence analysis of the PCR products is the most effective method to identify the *Bartonella* species in rodents. DNA sequence analyses of the isolated species were performed. *GltA* amplification products were purified using Agencourt Ampure purification kit (Beckman Coulter, Beverly, USA). Sequence reaction was performed using a Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter). Sequence PCR products were purified using a Dye-Terminator removal kit (Agencourt CleanSEQ; Beckman Coulter). DNA sequences of the purified products were identified using Beckman Coulter 8000 equipment. The isolates were identified comparing the DNA 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 with Clustal W using MegAlign.

Results

Using the direct PCR technique targeting (*ITS*) on the liver tissue of 42 *Microtus socialis* mice, 24 of the 42 samples were found positive for *Bartonella* spp. (Figure 1). The growth was positive in the cultures of seven samples. These isolates were identified as *Bartonella* spp. by PCR (*gltA*) (Figure 2).

The species identification of the seven isolates, which were culture-positive and defined as *Bartonella* spp. using PCR (*gltA*), was performed using a DNA sequence analysis. The phylogenetic tree, created using DNA sequence homology, is shown in Figure 3. Five of these isolates were defined as *B. taylorii*, while the other two were defined as *B. grahamii*. Four of the *B. taylorii* isolates had a 99% genotypic resemblance to Far East Asian (East Siberia/Russia) isolates with accession number AY584852, and one had a 99% resemblance to European isolates with accession number AF191502. Two of the *B. grahamii* isolates were 99% compatible with the DNA strand with accession number CP001562.1, defined as *B. grahamii* in the GenBank.

16.6% and 57.1% liver tissue of the *Microtus socialis* mice caught from one source in Central Anatolia were found to be positive for *Bartonella* by culture method and by PCR respectively. Two of the seven isolates were identified as *B.grahamii,* which is reported as a zoonotic species.

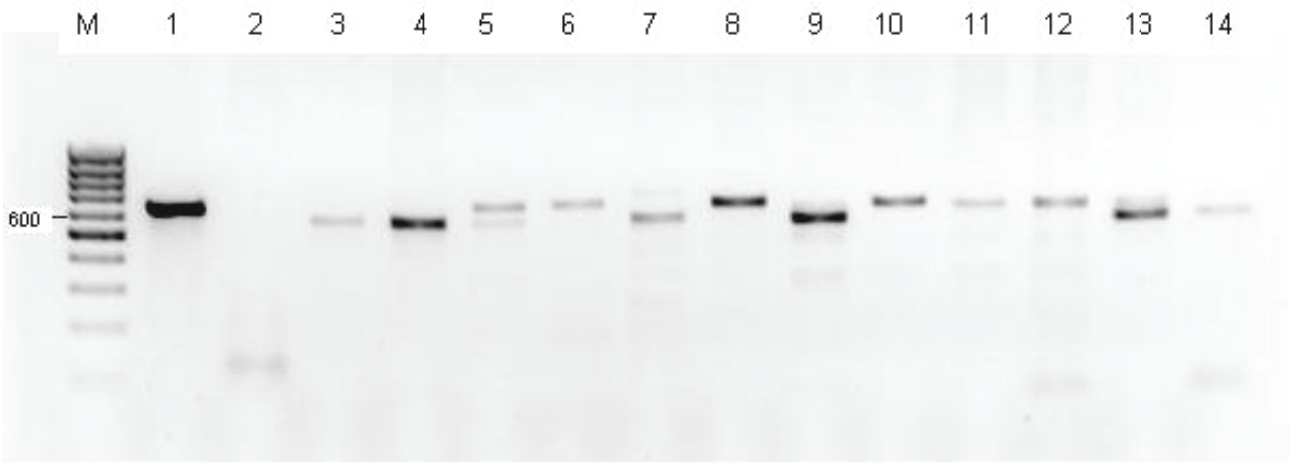

Figure 1. *Bartonella* PCR targeting (*ITS*) amplification agarose gel image of mice liver tissue. M; Marker line 1; Positive control *B. henselae* ATCC 49882, line 2; Negative control, line 3-14; *Bartonella* spp. isolates
Şekil1. Karaciğer dokusudan *Bartonella* PCR (*ITS*) amplifikasyonu agaroz jel görüntüsü. M;Marker 1; Pozitif kontrol *B. henselae* ATCC 49882, 2; Negatif kontrol, 3-14; *Bartonella* spp

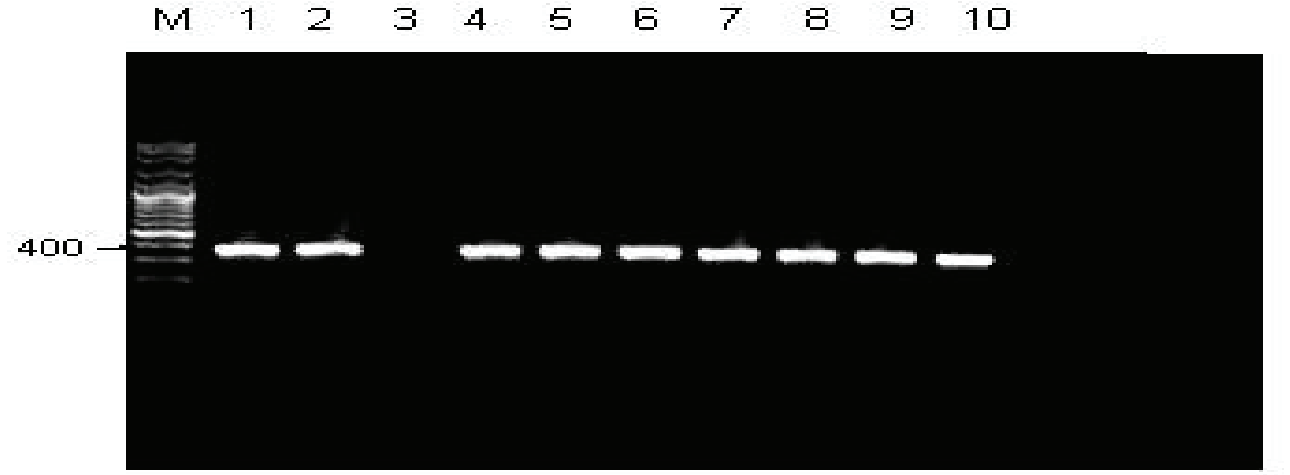

Figure 2. Isolates' *Bartonella* PCR targeting(*gltA*) amplification agarose gel image.M: marker, line 1-2 Positive control *B. henselae* ATCC 49882, *B. clarridgeiae* Ankara cat isolate, line 3: Negative control line 4-10 mice *Bartonella* spp. isolates
Şekil 2. İzolatların *Bartonella* PCR (*gltA*) amplifikasyonu agaroz jel görüntüsü.M: marker, 1-2 Pozitif kontrol *B. henselae* ATCC 49882, *B.claridgeiae* Ankara kedi izolatı, 3: Negatif kontrol. 4-10 fare *Bartonella* spp. izolatları

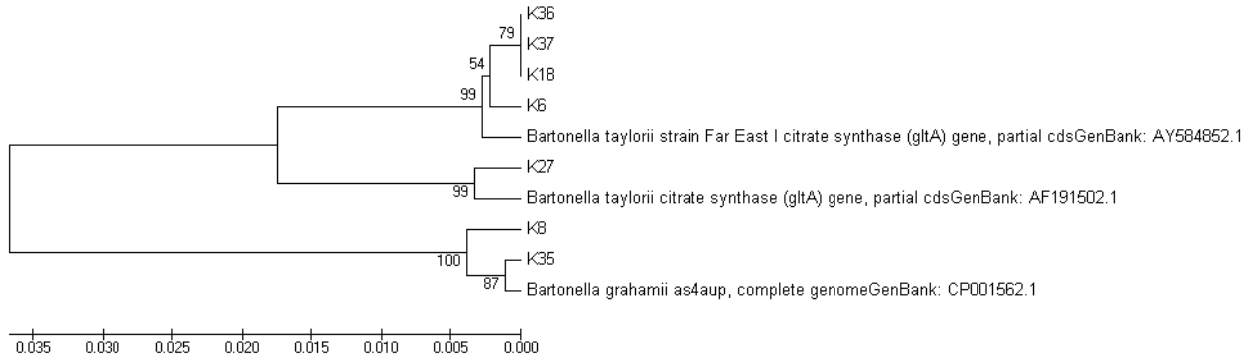

Figure 3**:** Phylogenetic tree of the seven *Bartonella* strains examined here and known *Bartonella* species constructed by the neighbor-joining method based on sequence analysis of the partial citrate synthase (*gltA*) gene.
Şekil 3**.** Genbank'ta bildirilmiş *Bartonella* türleri ve izole edilen yedi *Bartonella* suşunun, sitrat sentez gen (*gltA*) sekans analizi temel alınarak neighbor-joining metodu ile oluşturulan filogenetik ağaç

Discussion and Conclusion

Eleven zoonotic species: *Bartonella henselae, Bartonella elizabethae, B. grahamii, B. vinsonii* ssp. *arupensis, Bartonella vinsonii* ssp. *berkhoffii, B. washoensis, Bartonella koehlerae, Bartonella clarridgeiae, Bartonella rochalimae, Bartonella melophagi,* and *Bartonella tamiae* were reported in the genus *Bartonella* (1,11,13).

The presence of *B. henselae*, *B. claridgeiea,* and *B. vinsonii* ssp. *berkhoffii* in cats and dogs in Turkey has been identified by serological, cultural, and molecular methods (7,8,9,14). *B. henselae*, known to be the cause of cat scratch disease, on the other hand, was presented in humans by serological methods (10,26). In this study, for the first time in Turkey, a high rate of *Bartonella* infection in wild mice was presented and *B. grahamii,* which is a zoonotic species, and *B. taylorii* were defined by culture and molecular methods.

The prevalence of *Bartonella* infection in wild mice in European countries has been reported as 40.4% in Slovenia (18), 26.8% in Spain (13), 30.6% in Poland (25) by direct PCR of splenic tissue, and 16.5% in Sweden (15), 27.5% in Denmark (12), 62% in England (4), and 31.3% in Greece (23) by blood culture. Since *Bartonella* spp. was found at a rate as high as 57.1% by PCR in liver tissue samples of field mice in this study, and even at a higher rate than those of blood culture study (B. Celebi, unpublished data), it is suggested that the positivity of *Bartonella* spp. in wild mice in our country is high.

The low rate of the positivity (16.6%) obtained from the culture of the liver tissue, compared to the PCR results of this study and the data of European countries suggests that the *Bartonella* species cannot be grown in cultures due to their delayed and difficult growth type in culture. In addition, because the *Bartonella* species are generally intra-erythrocytic agent, blood is used as a culture material (4,12,15,23). Liver tissue was used as blood supplement in this study due to the unavailability of mice blood samples. Liver tissue may not be the best culture material for the isolation of *Bartonella* spp. The tissues of mice were stored after tissue dissection in -20 °C until they were used. These circumstances might negatively affect the isolation of bacteria. The recommended storage conditions for tissue are a temperature of -70 °C or in liquid nitrogen (4,12,15,23).

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 (5,6,11). Wild rodents are a possible source of potential *Bartonella* infection in humans, based on findings of this and some previous studies performed in other countries, which determined high rates of *Bartonella* spp. positivity in mice and the role of ticks in the transmission of these agents (21). Iralu et al. (2006) reported rodent-originated *Bartonella* infection in 9 of 76 patients (12%) with fever of unknown origin, diagnosed serologically with seroconversion and antibody presence in high titers (16). According to the present data, *Bartonella* infections should be among the differential diagnosis of cases with fever of unknown origin, which may be due to tick bites, and further studies should be performed accordingly.

Four of the B. *taylorii* isolates had a 99% genotypic resemblance to Far East Asian (East Siberia/Russia) isolates with accession number AY584852, and one of them had a 99% resemblance to European isolates (England, Greece) with accession number AF191502. These data suggest that these rodents are potentially moving around the world. These facts demonstrate the importance of the investigation of zoonotic infections, transmitted by rodents that are not typically seen in Turkey.

References

1. **Avidor, B., M. Graidy, G. Efrat, C. Leibowitz, G. Shapira, A.Schattner, O. Zimhony, and M. Giladi,** (2004): *Bartonella koehlerae, a new cat-associated agent of culturenegative human endocarditis*. J. Clin. Microbiol. **42**, 3462–3468.
2. **Bermond D, Heller R, Barrat F, Delacour G, Dehio C, Alliot A, Monteil H, Chomel B, Boulouis HJ, Piémont Y**. (2000): *Bartonella birtlesii sp. nov., isolated from small mammals (Apodemus spp.)*. Int J Syst Evol Microbiol. **6**: 1973-9.
3. **Birtles RJ, Harrison TG, Saunders NA, Molyneux DH**. (1995): *Proposals to unify the genera Grahamella and Bartonella, with descriptions of Bartonella talpae comb. nov., Bartonella peromysci comb. nov., and three new species, Bartonella grahamii sp. nov., Bartonella taylorii sp. nov., and Bartonella doshiae sp. nov.* Int J Syst Bacteriol. **45**:1-8.
4. **Birtles RJ, Harrison TG, Molyneux DH**. (1994): *Grahamella in small woodland mammals in the U.K.: isolation, prevalence and host specificity*. Ann Trop Med Parasitol. **88**:317-27.
5. **Boulouis HJ, Chang CC, Henn JB, Kasten RW, Chomel BB**. (2005): *Factors associated with the rapid emergence of zoonotic Bartonella infections*. Vet Res. 36:383-410.
6. **Çelebi B**. (2008): *Bartonella henselae and its infections*. Mikrobiyol Bul. **42**:163-75.
7. **Celebi B, Kilic S, Aydin N, Tarhan G, Carhan A, Babur C**. (2009): *Investigation of Bartonella henselae in cats in Ankara, Turkey*. Zoonoses Public Health. **56**:169-75.
8. **Celebi B, Carhan A, Kilic S, Babur C.** (2010): *Detection and genetic diversity of Bartonella vinsonii subsp. berkhoffii strains isolated from dogs in Ankara, Turkey.* J Vet Med Sci. **72**:969-73.

9. **Celebi B, Taylan Ozkan A, Kilic S, Akca A, Koenhemsi L, Pasa S, Yildiz K, Mamak N, Guzel M**. (2010): *Seroprevalence of Bartonella vinsonii subsp. berkhoffii in urban and rural dogs in Turkey.* J Vet Med Sci. **72**:1491-4.
10. **Çelebi B, Yalçın E, Babür C**. (2010): *Fever of unknown origin and detection of Bartonella henselae IgG seropositivity: a case report*. Mikrobiyol Bul. **44**:489-94.
11. **Chomel, B. B., H. J. Boulouis, and E. B. Breitschwerdt**,(2004): *Cat scratch disease and other zoonotic Bartonella infections.* J. Am. Vet. Med. Assoc. **224**, 1270–1279.
12. **Engbaek K, Lawson PA**.(2004): *Identification of Bartonella species in rodents, shrews and cats in Denmark: detection of two B. henselae variants, one in cats and the other in the long-tailed field mouse*. APMIS. **112**:336-41.
13. **Gil H, García-Esteban C, Barandika JF, Peig J, Toledo A, Escudero R, Jado I, Rodríguez-Vargas M, García-Amil C, Lobo B, Roales P, Rodríguez-Moreno I, Olmeda AS, García-Pérez AL, Anda. P. (**2010): *Variability of Bartonella genotypes among small mammals in Spain.* Appl Environ Microbiol. **76**:8062-70.
14. **Guzel M, Celebi B, Yalcin E, Koenhemsi L, Mamak N, Pasa S, Aslan O**. (2011): *A serological investigation of Bartonella henselae infection in cats in Turkey.* J Vet Med Sci. **73**:1513-6.
15. **Holmberg M, Mills JN, McGill S, Benjamin G, Ellis BA**.(2003): *Bartonella infection in sylvatic small mammals of central Sweden.* Epidemiol Infect. **130**:149-57.
16. **Iralu J, Bai Y, Crook L, Tempest B, Simpson G, Mckenzie T, Koster F**. (2006): *Rodent-associated Bartonella febrile illness, Southwestern United States*. Emerg Infect Dis. **12**:1081-6.
17. **Kerkhoff FT, Bergmans AM, van Der Zee A, Rothova A.** (1999): *Demonstration of Bartonella grahamii DNA in ocular fluids of a patient with neuroretinitis*. J Clin Microbiol. **37**: 4034-8.
18. **Knap N, Duh D, Birtles R, Trilar T, Petrovec M, Avsic-Zupanc T.** (2007): *Molecular detection of Bartonella species infecting rodents in Slovenia*. FEMS Immunol Med Microbiol. **50**: 45-50.
19. **Maggi RG, Chomel B, Hegarty BC, Henn J, Breitschwerdt EB.** (2006): *A Bartonella vinsonii berkhoffii typing scheme based upon 16S-23S ITS and Pap31 sequences from dog, coyote, gray fox, and human isolates*.Mol Cell Probes. **20**:128-34.
20. **Norman AF, Regnery R, Jameson P, Greene C, Krause DC**. (1995): *Differentiation of Bartonella-like isolates at the species level by PCR-restriction fragment length polymorphism in the citrate synthase gene*. J Clin Microbiol.**33**:1797-803.
21. **Reis C, Cote M, Le Rhun D, Lecuelle B, Levin ML, Vayssier-Taussat M, Bonnet SI.** (2011): *Vector competence of the tick Ixodes ricinus for transmission of Bartonella birtlesii.* PLoS Negl Trop Dis.**5**:1186.
22. **Serratrice J, Rolain JM, Granel B, Ene N, Conrath J, Avierinos JF, Disdier P, Raoult D, Weiller PJ**.(2003): *Bilateral retinal artery branch occlusions revealing Bartonella grahamii infection*. Rev Med Interne. **24**:629-30.
23. **Tea A, Alexiou-Daniel S, Papoutsi A, Papa A, Antoniadis A.** (2004): *Bartonella species isolated from rodents, Greece*. Emerg Infect Dis. **10**:963-4
24. **Welch DF, Carroll KC, Hofmeister EK, Persing DH, Robison DA, Steigerwalt AG, Brenner DJ**. (1999): *Isolation of a new subspecies, Bartonella vinsonii subsp. arupensis, from a cattle rancher: identity with isolates found in conjunction with Borrelia burgdorferi and Babesia microti among naturally infected mice*. J Clin Microbiol.**37**:2598-601.
25. **Welc-Faleciak R, Paziewska A, Bajer A, Behnke JM, Siński E.** (2008): *Bartonella spp. infection in rodents from different habitats in the Mazury Lake District, Northeast Poland.* Vector Borne Zoonotic Dis. **8**:467-74.
26. **Yılmaz C, Ergin C, Kaleli I**. *(2009): Investigation of Bartonella henselae seroprevalence and related risk factors in blood donors admitted to Pamukkale University Blood Center* Mikrobiyol Bul. **43**:391-401.

Geliş tarihi: 07.01.2013 / Kabul tarihi: 02.04.2013

Address for correspondence:
DVM, PhD. Bekir Çelebi,
Public Health Institution of Turkey (PHIT),
Microbiology Reference Laboratories Department,
Bacterial Zoonoses Reference Laboratories F Blok,
Sıhhiye Ankara,
e-mail: vetbekir@yahoo.com