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Research Article

Detection of the Presence of *Bartonella henselae* in Cats in Istanbul[#]

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ABSTRACT

In the present study, the determination of the presence of *Bartonella henselae* in Istanbul in 96 pet, shelter and stray cats were aimed. The samples from the cats were examined by two different bacteriologic culture methods. The blood samples collected into pediatric lysis isolator tubes were spun and the pellet inoculated onto Heart Infusion Agar supplemented with 5% rabbit blood. BACTEC Peds Plus/F were inoculated with samples and incubated in the BACTEC 9050 automated blood culture systems. After the incubation time, growth were evaluated according to the colony morphology, growth time, gram properties and biochemical properties, and it was determined that 27 isolates by solid medium, 10 isolates by BACTEC were *Bartonella* spp. Isolates were determined as *Bartonella henselae* by PCR amplification using specific primers of a portion of 16S-23S rRNA intergenic region. In conclusion, the prevalence of *Bartonella henselae* bacteremia was found 28.1% of cats in Istanbul.

Key Words: *Bartonella henselae*, cat, isolation, BACTEC, PCR

ÖZET

İSTANBUL'DA KEDİLERDE BARTONELLA HENSELAE VARLIĞININ SAPTANMASI

Bu çalışmada, İstanbul ilinde evlerde, barınakta beslenen ve sokakta bulunan 96 adet kedide *Bartonella henselae* varlığının saptanması amaçlandı. Kedilerden alınan kan örnekleri iki farklı bakteriyolojik kültür yöntemi kullanılarak incelendi. Pediatrik lizis izolatör tüplere alınan kanlar santrifüje edildikten sonra %5 tavşan kanı katkılı Heart Infusion Agarlara ekildi. BACTEC peds/plus F şişelerine alınan kanlar BACTEC 9050 otomatik üreme kontrollü kan kültür sistemi ile inkube edildi. İnkubasyon sonrası üremeler koloni morfolojisi, inkübasyon süresi, Gram özelliği ve biyokimyasal özelliklerine göre değerlendirildi ve izolatör tüplere alınan kanlardan Heart Infusion Agar'da üreyen 27 izolatın, BACTEC şişelerindeki kültürlerde üreyen 10 izolatın *Bartonella* spp. olduğu belirlendi. İzolatlar 16S-23S rRNA interjenik bölge sekanslarından elde edilen spesifik primerler kullanılarak tür düzeyinde identifiye edildi ve tüm izolatlar *Bartonella henselae* olarak belirlendi. Bu sonuçlar ile İstanbul yöresindeki kedilerde *Bartonella henselae* bakteriyemi prevalansı %28,1 olarak ortaya konulmuştur.

Anahtar Kelimeler: *Bartonella henselae*, kedi, izolasyon, BACTEC, PCR

[#] This study was summarized from the PhD thesis of first author.

Introduction

Cat scratch disease is a worldwide zoonosis which can be found in many species including humans. Agent of the disease, *Bartonella henselae* which is classified in the family *Bartonellaceae*, is an intraerythrocytic, slightly curved Gram negative bacterium (Euzéby, 2000; Kordick and Breitschwerdt, 1995). It can be more easily isolated from blood, subsequent to eritrocytic and leukocytic lysis (Paracıkoğlu, 2006). For identification, because of their slow growth, standard biochemical methods are not convenient (Sander et al., 1997) and cannot be used in differentiation of species (Paracıkoğlu, 2006), thus molecular methods are used (Kordick et al., 1995; Regnery et al., 1992; Zanutto et al., 2001).

The transmission of *B. henselae* from cat to cat occurs by the the cat fleas (Chomel et al., 1996; Foil et al., 1998; Zangwill et al., 1993). The cats which carry the agent can stay bacteremic for many years (Chomel et al., 1996; Greene et al., 1996; Kordick et al., 1995). The transmission of *B. henselae* from cats to humans usually occurs via scratches or bites, or rarely indirectly by cat flea (Brunt et al., 2006).

The agent can stay as a part of intravascular microbial flora for many months and years in healthy cats (Breitschwerdt and Kordick, 2000). In experimentally infected cats; pyrexia (Euzéby, 2002; Melter et al., 2003), moderate transient anemia (CSD, 2004), lymphadenopathy (Brunt et al., 2006; Francesco et al., 2007), neurologic dysfunctions (Brunt et al., 2006; Guptill et al., 1998), heart and kidney lesions (Chomel et al., 2003; Guptill et al., 1998; Kordick et al., 1999) and uveitis can be observed. In naturally infected cats no clinical signs were reported (Chomel et al., 1996; Greene et al., 1996).

In many countries the presence of Bartonella infections has been reported. Although prevalence varies depending on geographic location, cat population, age and flea infestations, over 20% of the tested cats were detected as bacteremic (Rolain et al., 2004).

Because Istanbul is located in a mild territory it is considered to have dense flea population and it carries a considerable risk of cat scratch disease. In this study the detection of presence of *Bartonella henselae* in cats in Istanbul region was aimed using by comparison of two different bacteriologic methods.

Materials and Methods

Samples

Blood samples from 96 cats, which stay or visit private clinics, veterinary faculty, shelter and streets of Istanbul, were collected between the months of July and December in 2007. From those cats; 15 of were limited inside the house (not allowed to go out), 35 of were house cats with street interaction (allowed to go out), 46 of were stray cats. Forty-five were female while 51 of were male. Their ages ranged between 2 and 16 years. Fifty of those cats had flea while 46 had no flea.

Prior to blood collection jugular area of the cats were shaved and disinfected with (70 % ethanol) and iodine. One and half ml of blood was collected into each pediatric isolator tubes (Wampole Isolator 1.5, Oxoid BC505C) and BACTEC Peds Plus/F blood culture vials. Samples were brought to the laboratory within 4 hours.

Isolation

In order to induce lysis in erythrocytes to expose agents, isolator tubes were centrifuged at 1800xg, for 75 minutes. The supernatant was discharged and the pellet was resuspended in 125 µl Medium 199 (Koehler et al., 1992). 250µl from the acquired suspensions were inoculated onto Heart Infusion Agar (HIA) supplemented with 5% defibrinated rabbit blood and the suspension in the medium were diffused on the surface of the medium using their own viscosity. The plates were incubated at 35°C with 5% CO_2 in a humid incubator, after 24 hours the plates were wrapped with parafilm and left to incubation for 5 weeks (Jacomo et al., 2002; Koehler et al., 1992).

BACTEC Peds Plus/F vials were tested for CO_2 production single day for fortnight by a BACTEC 9050 apparatus. The positive BACTEC Peds Plus/F vials were tested for the presence of bacteria by light microscopic

examination, and were subcultured on to Heart Infusion Agar (HIA) medium (Tsuneoka et al., 2004).

Identification

After six days of incubations, rough, dry, cauliflower-like, adhered to the agar colonies were observed. Gram staining was performed from the cultures to detect their individual morphologies.

Gram staining was performed from the blood culture flasks which started to give audial signal on second day. The culture with and without growth were passaged on to Heart Infusion Agar (HIA) medium. Gram staining was performed to determine the individual morphology of the S type observed from 3rd day (Tsuneoka et al., 2004). To identify the isolates oxidase, catalase, urease and nitrate reduction tests were performed (Chomel et al., 1996). *Bartonella* spp. isolates were stored in -20°C until the PCR assays (Jacomo et al., 2002).

PCR

DNA was extracted by using UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA) according to the manufacturer's instructions.

The PCR assay was performed as described by Jensen et al. (2000). From each sample, 5 µl of the template was added to 45 µl of the PCR mixture, containing 10 mM Tris (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 200 mM each dATP, dCTP, and dGTP, 400 mM dUTP, 1 mM each primer, and 2.5 U of Amplitaq Gold DNA polymerase (PE Applied Biosystems, Foster City, Calif.) Amplification buffer was optimized with dUTP for use with uracil glycosylase to prevent PCR amplification product carryover. Amplifications were performed in Uno-thermoblock Thermocycler (Biometra) by a PCR protocol, as follows: 10 min of incubation at 20°C, followed by 2 min of denaturation at 95°C and then 45 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 60°C, and 30 s of extension at 72°C.

Nine microliters of the PCR products of each sample were mixed with 1 µl of sample buffer 10X (MOBIO 15009-5) and were electrophoresed through an agarose gel containing 3% Agarose (Pronadisa 8012). As molecular size marker, 20 bp DNA ladder (Jena Bioscience M-212) was used. Electrophoresis was implemented at a constant voltage of 100 V in 1 x TBE buffer during 75 min. After staining with ethidium bromide, the gels were visualized using UV Transmilator.

Statistical analyses

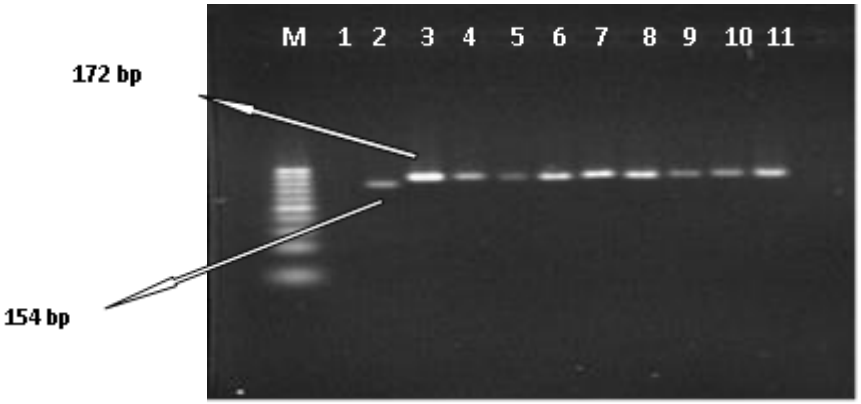
The differences between cat groups were analyzed with Statistical Package for Social Sciences (SPSS) software using chi-square (Özdamar, 1999a). The differences were categorized based on lifestyle (stray cats, cats limited to household and cats associated with street), gender, flea infestation and age. The sensitivity, specificity, positive and negative predictive values and accuracy values for BACTEC were determined using "Methods Used to Investigate the Reliability of Medical Tests" (Özdamar, 1999b).

Results

Twenty seven of the 96 (28.1%) blood samples yielded a positive culture result on blood agar and 10 of 96 (10.4%) yielded a positive culture in BACTEC medium. All of the 27 isolates detected as *Bartonella spp*. in blood agar and all of the 10 isolates in BACTEC were positive in PCR analyses and 172 bp *Bartonella henselae* specific bands were determined (Figure 1). *Bartonella clarridgeiae* bacteremia did not be detected in cats. *Bartonella henselae* bacteremia rate was 39.1% (18 of 96) for the stray cats. The *Bartonella henselae* bacteremia rate was 20% and 17.1% in cats limited to household and cats associated with street, respectively. In one year old and younger stray cats, the rate of bacteremia was 66.7% (8 of 13) and in adult stray cats it was 43.5% (10 of 33). In one year old and younger cats associated with street, the bacteremia ratios were 25% (4 of 16) and 37.5% (3 of 8) respectively and in adult cats associated with street were 10.5% (2 of 19), while none of the adult cats limited to household are bacteremic.

There was no statistically significant difference between the sex of the cats and the prevalence of bacteremia. However, age and the flea infestation were the statistically significant risk factors for Bartonella infection, because 36 (37.5%) of the cats were 1 year old or younger, and 15 (41.7%) of these young cats were bacteremic, but 12 (20%) of the 60 adult cats (>1 year old) were bacteremic (P=0.022). 48 of the cats had the flea infestation and 18 (37.5%) of these cats were bacteremic but 9 (18.8%) of the 48 cats without flea were bacteremic (P=0.041).

Using the method to investigate the reliability of medical tests, the sensitivity, specifity, positive predictive value, negative predictive value and accuracy rate of BACTEC according to the solid medium results were found as 37%, 100%, 100%, 19.8%, 82.3%, respectively.



M = Marker; 1 = Negative control ; 2 = Positive control of *B. clarridgeiae;* 3 = Positive control of *B. henselae;* 4– 11 = *B.henselae* positive samples

Figure 1. PCR results of culture positive samples.

Şekil 1. Kültür pozitif örneklerin PCR sonuçları.

Discussion

Epidemiologic studies, which have revealed worldwide distribution of *Bartonella henselae* infection in cats, have indicated the prevalence of bacteremia as from 15% to 55% in Australia and many other countries in America, Europe, Asia, and Africa (Chomel et al., 2004). In cats the various prevalence rates among those studies change depending on geographic localization, cat populations, flea infestations and ages (Rolain et al., 2004). The geographic spread of *Bartonella henselae* was associated with the geographic spread of the flea and it is reported that climate changes affects the positivity of the agent because the fleas' proliferation abilities are affected by climate changes (Schmidt, 1998). Moderate and humid climate areas are the areas with dense flea infestation of cats. Because of that, even the different climatic areas in a country can cause prevalence differences (Guptill et al., 2004; Maruyama et al., 2000).

Heller et al. (1997) found *Bartonella henselae* prevalence of bacteremia as 34% in France. In Italy the prevalence of bacteremia were found as 9.7% (Cabassi et al., 2002) and 18% (Fabbi et al., 2004). Pons et al. (2005) determined prevalence of bacteremia as 7% in Spain. In Turkey, Ankara, Çelebi et al. (2009) determined the prevalence of bacteremia as 8.2%. In this study prevalence of bacteremia of cats in Istanbul were determined as 28.1%. As Çelebi et al. (2009) reported; the difference between prevalence values is due to the climatic

differences, while Ankara has a continental climate, Istanbul has a temperate climate showing the transitions between the Black Sea climate and Mediterranean climate.

Bergmans et al. (1997) reported that identification of *Bartonella* species from blood cultures is more successful then determination of DNA with PCR and considered the blood culture as a gold standard for the identification of *Bartonella* species (Fabbi et al., 2004). Isolation of organisms from blood cultures were performed using isolator blood-lysis tubes or tubes containing EDTA. Brenner et al. (1997) reported that agent isolation using isolator tubes is much more specific rather than EDTA tubes and collecting and refrigerating blood samples in EDTA tubes can prevent the specificity of determining *Bartonella henselae*. Collecting blood samples and storing them is a delicate process to sustain the liveliness of blood cells. In this study blood samples were collected into isolator blood-lysis tubes as previously described by Brenner et al. (1997). After that, erythrocytes were lyzed by 75 minute centrifuge as reported by, Chomel et al. (1999).

Various media and methods were tried for the isolation of *Bartonella* spp. and it is reported that *Bartonella* spp. which need heamin, were growth best in agar supplemented with blood, humidity atmosphere, 5-10% CO_2 and 35°C (Kordick and Breitschwerdt, 1995). In this study as performed by Koehler et al. (1992) pellet at the bottom of the isolator tubes was diluted with inoculation media and cultured in HIA (Heart Infusion Agar) supplemented with 5% rabbit blood as Koehler et al. (1997).

Besides inoculating blood samples to solid medium in order to increase the chance of detection Heller et al. (1997) also had inoculation to liquid medium (BACTEC medium). While 50 of 94 samples (53%) inoculated to blood agar were isolated as *Bartonella* spp., 18 strains (19%) were isolated from BACTEC medium. 4 strains (11%) of *Bartonella henselae* were only isolated from solid medium as 3 of them (9%) were only isolated in liquid medium. Besides because BACTEC blood medium was used *B.clarridgeiae* was isolated from 15 samples. Tsuneoka et al. (2004) determined that six of 60 (10%) samples inoculated as *Bartonella henselae* in solid medium and one of 10 samples (10%) in BACTEC medium. In this study to increase the detection possibility of bacteria and to determine the sensitivity of BACTEC, blood samples collected to BACTEC Peds Plus/F vials and inoculated using BACTEC automated proliferation blood culture system. In solid medium 27 of 96 samples and 10 of 96 samples cultured in BACTEC were identified as *Bartonella henselae.*

As *B.clarridgeiae* could not be isolated from this study, the idea which was determinated by Heller et al. (1997), "BACTEC enabling growth to the species which cannot be proliferated in its first isolation in solid medium," could not be supported. The sensibility of BACTEC concerning solid medium was determined of 37%. This low sensibility value is because of the detection of *Bartonella* species in conventional automated blood culture systems is less reliable, as the organisms produce little or no CO_2 to indicate bacterial growth. Thus, blood cultures, which are accepted as gold standard, should be performed on solid medium along with liquid medium.

When the prevalence of bacteremia of cats is evaluated according to their living conditions, it is reported that stray cats have higher probability than domestic cats (Arvand et al., 2001; Chang et al., 2002; Gurfield et al., 1997). Çelebi et al. (2009) determined that in Ankara the prevalence of bacteremia was very close to each other in stray and outdoor domestic cats. In this study while the prevalence of bacteremia of *B henselae* were detected as 20% for domestic cats limited inside the house, 17.1% for domestic cats with street interaction and 39.1% for stray cats, the differences between them were no statistically significant (P=0.069). For all that, the higher prevalence of stray cats could explain with the fact that stray cats could have higher cat flea exposure.

The cats were evaluated with regard to their genders, and some researchers determinated that male cats had a higher prevalence then

female cats (Bergmans et al., 1997; Maruyama et al., 1998; Zangwill et al., 1993) while some other researchers suggest female cats had higher prevalence (Sander et al., 1997). In this study bacteremia positivity was determined as 24.4% (11 of 15) for female cats and 31.4% (16 of 51) for male cats, the statistical difference between them was insignificant (P=0.451). These results suggest that there is no significant difference between the two genders. This idea supports the reports determinated that gender was not a risk factor related with bacteremia (Fabbi et al., 2004; Glaus et al., 1997; Gurfield et al., 2001; Haimerl et al., 1999).

When the cat's ages effect was determined, higher ratios of positivity were found in cats younger than 1 year old (Cabassi et al., 2002; Piemont and Heller, 1998). Bergmans et al. (1997) reported that 30% of cats under a year old, 22.4% of cats between ages 1 to 4 and 16% of cats older than 4 years old were bacteremic. Sander et al. (1997) reported that 18% of cats under 2 years old and 5% of cats older than 2 years of age were bacteremic. Melter et al. (2003) reported the mean age of bacteremic animals as 12 months and determinated that the prevalence of bacteremia under 1 year old is found to be 80%. Çelebi et al. (2009) is found the prevalence of bacteremia as 12.3% for cats under 1 year old, 14.3% for cats 2 years old, 0% for cats 3 years old and older. In this study 51.9% of cats under 1 year old, 29.6% of cats between 1-3 years and 18.5% of cat older than 4 years old were found to be bacteremic. When bacteremia ratios of cats under 1 year old (41.7%) and older than 1 year old (20%) are evaluated the difference between them were statistically significant (P=0.022). These result confirmed the other research (Chomel et al., 1995; Guptill et al., 2004; Heller et al., 1997; Koehler et al., 1994) reporting that the Bartonella infections were observed more commonly in young cats than the older cats.

When the relation between flea infestations and *Bartonella henselae* bacteremia was evaluated it was observed that cats been infested with flea at any age of their lives have an increased risk of having *Bartonella henselae* bacteremia (Fabbi et al., 2004; Maruyama et al., 2001). In this study while the bacteremia positivity was 37.5% (18 of 48) in flea infested cats and 18.8% (9 of 48) in non-infested cats the difference between them was found to be significantly significant (p=0.041). While 67% of bacteremic cats have fleas, 33.3% of them were without fleas. Because samples were collected in summer and autumn, when flea and flea infestations can be found denser, we determined positive relation between flea infestations and bacteremia compatible with Chomel et al. (1995).

In this study in 96 cats while we isolated *Bartonella henselae* from 27 of them (28.1%), we could not isolate *B.clarridgeiae* which was compatible with other reports and while this finding demonstrated the extensity of *Bartonella henselae* it indicated the minority of *B.clarridgeiae* in cat populations and because of its high variation levels, it could not be isolated.

In conclusion, with these results the prevalence of *Bartonella henselae* bacteremia was found 28.1% of cats in Istanbul. In humans and cats, the best method of prevention of disease is regularly the control of fleas. If family members is immunocompresed and have a new cat, they should be preferred over the age of a healthy cat without flea. *Bartonella henselae* is a significant zoonotic disease, and all clinicians should focus on cats and all should focus on preventing zoonotic diseases. Before cats are owned, *Bartonella henselae* serological investigations will be effective in protecting against CSD.

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