

Investigation of *Bartonella henselae* and *Bartonella clarridgeiae* Prevalance in Cats



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Abstract

In this study, 50 blood samples taken from urban and stray cats which were examined in a private veterinary clinic in İzmir province were investigated by PCR method. PCR studies revealed that *Bartonella spp.* was identified from a total of % 12 of the samples. PCR identification results were 8 % for *B. henselae* and 4 % for *B. clarridgeiae* for species-based distribution. In conclusion, Bartonella species which have zoonotic importance were detected. Especially, it is confirmed the necessity of the distribution of *B. henselae* which transmit to human from cat fleas and importance of preventive flea medication to pet owners for feedback.

Keywords: *Bartonella spp.*; *B. henselae*; *B. clarridgeiae*; Cat Scratch Disease; PCR

Abbreviations: PCR: Polymerase Chain Reaction; CSD: Cat Scratch Disease; RFLP: Restriction Fragment Length Polymorphism; BA: bacillary angiomatosis

Introduction

Bartonella species are common zoonotic pathogens associated with direct contact to animals in the world. They are small, pleomorphic, Gram-negative, weakly stained bacilli or coccobacilli, oxidase and catalase negative microorganisms Chmielewski [1]. The *Bartonella* species are highly dependent on the haemin and are able to grow in the medium containing blood. They are fastidious and require a long incubation period at 35 °C in 5-10 % CO₂ concentration. They can be isolated from blood more easily following erythrocyte and leukocyte lysis. Since *Bartonella* species are fastidious microorganisms, standard biochemical methods for identification are not feasible and are not used in species identification, so molecular genetic methods are used for differential diagnosis. In recent years, the development of molecular techniques has conducted to identify many new *Bartonella* species. Polymerase Chain Reaction (PCR), which gains practical importance with the introduction of biomolecular methods, can directly indicate the nucleic acid of the infectious agent in the case of infectious diseases. At the same time, different genotypes can be identified by restriction fragment length polymorphism (RFLP) analysis of DNA amplified by PCR Anderson & Neuman [2]. With these methods, more than 22 known species of this bacterium, which is a sub-member of the α -Proteobacteria branch, have been identified Guptill [3], Jacomo [4].

Of these, there are three important species of pathogens for humans. These include *B. bacilliformis*, Carrion disease (Oroya fever and verruga peruana); *B. quintana* causes trench fever; *B.*

henselae causes neurological syndromes, infectious diseases of immunodeficient and immunosuppressed individuals, cat scratch disease (CSD) in immunocompromised individuals, bacillary angiomatosis and peliosis especially in HIV positive cases Zangwill [5]. Cat fleas (*Ctenocephalides felis*) are natural vectors of *B. henselae* in cats. Mites, midges, fleas and lice play an important role on the transmission of *Bartonella* species Anderson & Neuman [2], Guptill [3], Angelakis [6]. A species of mosquito for *B. bacilliformis* (*Lutzomyia verrucorum*); the body louse end (*Pediculus humanis corporis*) for *B. quintana*, are identified as vectors Akkaya [7]. It is known that *B. henselae*, *B. bacilliformis* and *B. quintana* are pathogenic for humans for a long time. However, *B. clarridgeiae*, *B. elizabethae*, *B. grahamii* have been accepted as pathogens Jacomo [4], Chomel [8].

Seroepidemiological and bacteriological studies suggest that *B. henselae* is widespread throughout the world in cats. Depending on the geographical location and the life conditions of the cats, the seroprevalence varies between 5-80 %. The prevalence of bacteremia may be at a very low rate, or even at a rate determined by more than half of the cat population Boulouis [9]. Cats are reservoirs for the transmission of *B. henselae* to humans Chomel [8], Regnery [10], Koehler [11], Maruyama [12]. The transmission of *B. henselae* to humans is generally possible with cat scratching and bite, possibly also indirectly by cat fleas Zangwill [5], Koehler [13]. Cat fleas harbor *B. henselae* in their intestines and spread infection with their faeces and transmit infection among the pets. Fleas and

contaminating nails increase the likelihood of infecting humans during biting by placing flea excreta between teeth during rubbing or while licking themselves Mehock [14].

Material and Methods

Sample Collection

The whole blood samples were collected from 50 cats. The neck parts of the cats were first shaved and cleaned with alcohol and iodine before sample collection. Approximately 2 mL of blood taken from the jugular vein of the cat using a vacuum needle was transferred to EDTA tubes. The samples were brought to the Routine Diagnostic Laboratory Adnan Menderes University Faculty of Veterinary Medicine Department of Microbiology under cold chain. The experimental protocol was approved by the Local Animal Ethics Committee of Adnan Menderes University (2014/094).

Bacterioscopy with Giemsa Staining

The slide froth is prepared as indicated in the scouring preparation procedure. Prepared froth was first fixed in methyl alcohol for 5 minutes. The slides were dyed in a 5 % Giemsa solution for 40 minutes at room temperature. After that, the slide was washed with water and dried. Immersion oil was dropped on the preparations and examined under microscope.

DNA Isolation

DNA isolation was performed according to the procedure with the genomic DNA extraction kit (Fermentas®). The isolated DNAs were stored in cryo-tubes at -20 °C in freezing until PCR step.

PCR

Master Mix Preparation: PCR amplification for a sample in the PCR reactions for the identification of *Bartonella henselae* and *Bartonella clarridgeiae* species in this study was performed by mixture consisting of 5 µl PCR Buffer, 5 µl from 25 mM MgCl₂, 10 mM deoxynucleotide triphosphate (dNTP) 1 µl, 25 pmol primers which were prepared by 1 µl of BARTON-1 and BARTON-2, 0.5 µl of Taq polymerase (5 µl), 10 µl of template DNA and 26.5 µl of distilled water (ddH₂O) Johnson [15]. Once the mastermix was prepared, 0.2 µL of the tubes were added to the mastermix, numbered up to the sample number, and 40 µl of which was prepared. Then, 10 µl of the extracted template DNA was taken, inserted into the relevant tube, and the mouths tightly closed. The prepared tubes are then loaded into the thermal cyclor and programmed. The PCR conditions were initial denaturation at 95 °C for 5 min, 37 cycles of denaturation at 96 °C for 20 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 3 min.

Gel Electrophoresis

10 µl of the PCR products were mixed with 3 µl of 6x loading dye solution. The whole mixture was taken, and the 2% agarose gel was loaded into the appropriate position of the gel. The electrophoresis was run for 15 minutes at 80V 500A current and then for 60 minutes at 40V 500A. The gel was placed in the chamber of the transilluminator device connected to the computer. After

visualized under UV light, the fragment lengths were assessed separately for each PCR. The assessment was made as previously reported. In the PCR analysis, fragment length of 588 bp for *B. henselae* and 585 bp for *B. clarridgeiae* were investigated.

Results

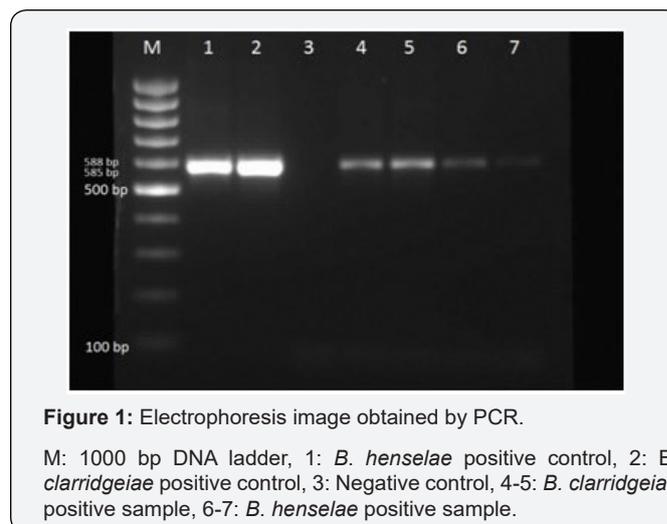
Assessment of the Cats

In our research, blood samples taken from 50 stray and pet cats were brought to the routine diagnosis laboratory of the Department of Microbiology, Faculty of Veterinary Medicine, Adnan Menderes University. The age range of the cats was between 7 months and 19 years. Twenty-seven (54%) of the cats were female and 23 (46%) were male. Twenty-seven of (54%) cats were kept as pets and 23 (46%) of them were stray cats.

Giemsa Staining Results

Fifty blood samples were examined under microscope after preparation of froths by Giemsa staining method. Intra-erythrocytic blood cells suggesting the presence of *Bartonellosis* infection was detected in 6 specimens.

PCR Results



A total of 6 (12%) samples of *Bartonella spp.* positivity was obtained after PCR analyses. Four (8%) of *B. henselae* and 2 (4%) of PCR positive specimens were identified as *B. Clarridgeiae* (Figure 1). The presence of intra-erythrocytic blood cells were also detected in the Giemsa staining of the PCR positive samples in the obtained results, thus further strengthened our research findings. As a result of the PCR run, 12% of the samples contained *Bartonella spp.* positivity. As a result of the PCR positivity of 50 samples, the percentage distribution by species was determined as 8% *B. henselae* and 4% *B. clarridgeiae* (Figure 1). Gender-based distributions of *Bartonella* species in cats with PCR positivity showed that 2 *B. henselae* and 1 *B. clarridgeiae* were identified from 27 female cats and 2 *B. henselae* and 1 *B. clarridgeiae* were identified from 23 male cats. As a result, it was seen that 7.4% *B. henselae* and 3.7% *B. clarridgeiae* were isolated in female cats. In male cat, 8.6% of *B. henselae* and 4.3% of *B. clarridgeiae* were isolated. Two *B. hense-*

lae and 1 *B. clarridgeiae* were identified from 27 pet cats. Two *B. henselae* and 1 *B. clarridgeiae* were identified from 23 stray cats, 2 *B. henselae* and 1 *B. clarridgeiae*. It is seen that 7.4% *B. henselae* and 3.7% *B. clarridgeiae* were identified from pet cats. 8.6% of *B. henselae* and 4.3% of *B. clarridgeiae* were isolated from stray cats. When age-based distributions of PCR-positive *Bartonella* spp. are examined, it is seen that the age distributions of *B. henselae* in cats are 1, 3, 5 and 6 years of age. It is seen that age distribution is 1 and 2 in *B. clarridgeiae* positive cats.

Discussion

Cats are considered to be the main reservoir of *B. henselae* and *B. clarridgeiae* species Anderson & Neuman [2], Baneth [16]. The seroepidemiological and bacteriological suggest that *B. henselae* is common across the world in cats also. According to geographical localization and lifestyle conditions of the cats, seroprevalence is seen between 5-80%, and the prevalence of bacteriemia varies from the very low rates up to 50% [9]. Different prevalence rates between studies changes depending on geographical location, population, age, and flea infestation level of the cats Rolain et al. [17]. The main vector that carries *B. henselae* in cat populations is the fleas. The prevalence of these fleas is the most important factor affecting the seroprevalence and bacteriemia of *B. henselae* in cats. Fleas are significantly affected by climatic characteristics in terms of reproduction and development. The flea infestation is common in temperate and humid climatic regions Jameson et al. [18]. Thus, even regional climate changes within countries cause differences in the prevalence of *B. henselae* seroprevalence and bacteriemia in cats Maruyama [12], Jameson et al. [18]. In a previous study Gup-till [19] a 33% bactericidal rate was found in the United States, 28% in Northern California, 12% in Washington and 6% in Chicago. In other data confirming that *B. henselae* infections differ from cold climates to high temperature and humid climates, 68% were found in the Philippines despite the presence of 0% *B. henselae* in Norway Boulouis [9].

In another study on the prevalence and seroprevalence of *B. henselae* bacteremia, a 16.5% seroprevalence of *B. henselae* bacteriemia was reported in pet cats by PCR Gurfield et al. [20], while the prevalence of bacteriemia in stray cats was found as 34% in another study Heller et al. [21]. Rolain et al. [17], reported 8.1% *B. henselae* positivity in pet cats. In Italy, Fabbi et al. [22], found that the prevalence of bacteraemia in stray cats was 18% and seroprevalence was 38% by PCR and in another study Fabbi et al. [23] the prevalence of bacteriemia was 23% and seroprevalence was 39%. Cabassi et al. [24] reported a bacterial prevalence of 9.7%. In Spain, Pons et al. [25] reported a bacterial prevalence of 7%. In addition, Baneth [16] reported the seroprevalence of 39.5% *B. henselae* in pet cats. The results in other regions of the world using PCR showed that the prevalence of *B. henselae* was 16.3% in Thailand Ioue et al. [26] 33.3% in South Korea Kim et al. [27] 90% in Brazil Souza [28], 22% in the Netherlands Bergmans [29] 61% in the Philippines (61%) Chomel [30], 17% in Brazil Staggemeier et al. [31] and 12.7% in China Yuan [32].

The prevalence of bacteraemia was reported as 8.2% In Ankara from a study conducted by Celebi et al. [33]. Aydin et al. [34] reported the seroprevalence of *B. henselae* IgG seroprevalence in risk group was found to be 11.5% in humans (cat/dog owner), 26.47% for pet dogs, 22.8% for pet cats, and 52% for house cats in a total of 281 samples including 131 cats and dogs, 105 cats and 45 dogs. Sigirci [35] reported the prevalence of bacteraemia was 28.1% in the study conducted by cats in Istanbul region. In this study, the prevalence of bacteraemia was found to be 12%. The reason for the different prevalence value is due to regional climate differences as reported by Celebi [33]. Despite the continental climate in Ankara, the fact that Istanbul has a temperate climate with transition characteristics between the Black Sea climate and the Mediterranean climate and the fact that Izmir is under the influence of the Mediterranean climate are leading to changes in prevalence values. It is therefore expected that these rates will be higher in the cat populations in coastal areas of Turkey, which are under the influence of the Mediterranean climate in our country where urbanization and population density are higher.

There are various methods in the laboratory diagnosis of *Bartonella* infections. Serological methods have been found to be more practical since the culture and other isolation techniques are time-consuming and difficult to perform, and the weeks are required to obtain results. However, when serological tests are used, it is reported that cross-reactions may occur between *Bartonella* spp. and other pathogenic bacteria such as *C. burnetti* and *Chlamydia* spp. Rolain [17], Foucault [36]. Bergmans [29] reported that identification of *Bartonella* spp. by culture from cat blood was a more successful way of isolating and identifying DNA by PCR. Heller [27] used culture media and broth (BACTEC medium) to increase the possibility of bacterial isolation. Regnery [10] reported that *Bartonella* species can be identified only at the genus level due to their negative results with biochemical tests and that species identification should be done with molecular methods. Engvall [37] found that *Bartonella* spp. after culture in blood from healthy cats in Sweden by the one-step PCR method and isolated *B. henselae* from 2 of the 91 cats. In this study, *Bartonella* spp. were detected as 12% as a result of PCR which was made specifically for BARTON-1 and BARTON-2 primers targeting riboflavin synthase gene.

When the prevalence of bacteraemia was assessed according to lifestyle conditions, it was reported that stray cats were more likely to be bacteriemic than pet cats. The rate of bacteraemia in Berlin was found as 1% for pet cats and 18.7% for stray cats Arvand [38]. Gurfield [20] reported that 23.6% of stray cats and 14.2% of pet cats were bacteriemic detected in the study conducted in France. Chomel [8] reported 24% bacteriemia ratio in pet cats and 61% in stray cats. Childs [39] found the seroprevalence of *B. henselae* as 12% in pet cats and 44% in stray cats. Barnes [40] reported that seroprevalence of *B. henselae* was 40.6% in pet cats and 41.8% in stray cats, but no significant difference between streets and house cats in terms of positivity. Celebi [33] found that

the bacteremia rate of cats in Ankara was 10.7% for pet cats and 2.9% for stray cats and was not statistically significant. The prevalence of bacteremia in this study was 6% in pet cats and 6% in stray cats and was not found significant.

Higher prevalence was found in the female cats Sander [41] while in some studies it was reported that male cats had a higher prevalence Zangwill [5], Maruyama [12], Bergmans [29]. In Celebi's study [42], 13.8% of *B. henselae* positivity in female stray cats and 2.7% *B. henselae* positivity were observed in the male cats. In the study of bacteremia positivity of female cats was determined as 24.4% and male cats as 31.4%. In this study, the rate of bacterial infection in female cats was 6% and the rate of bacteremia in male cats was 6%. The results were not found significant. This study agrees with other studies Fabbi [22], Gurfield et al. [43] since there is no significant difference in the positive rate between the two genders and that gender is not a risk factor associated with bacteremia.

When the prevalence of *B. henselae*, *B. clarridgeiae*, and *B. koehlerae* is assessed, *B. henselae* is the majority of the Bartonella species isolated from cats. In some cats both *B. henselae* and *B. clarridgeiae* bacteremia can be seen together Celebi [42]. Forty-eight (83%) of the 48 *Bartonella spp.* isolated from the cats in Israel were found to be *B. henselae*, 7 (15%) were *B. clarridgeiae* and 1 (2%) were *B. koehlerae* Boulouis [9]. Of the 19 Bartonella species isolated in the Philippines, 13 (68.4%) were identified as *B. henselae*, 2 (10.5%) were *B. clarridgeiae* and 4 (21%) were *B. henselae* and *B. clarridgeiae* Chomel [30]. Gurfield [43] found that *B. henselae* and *B. clarridgeiae* together accounted for 1.1% of 436 cats, 50% *B. henselae*, 21% *B. clarridgeiae*, 11% *B. henselae* and *B. clarridgeiae* together. Heller [21] identified 70% *B. henselae* and 30% *B. clarridgeiae* in 94 cats and Maruyama [44] identified 82.9% *B. henselae*, 11.8% *B. clarridgeiae*, 5.3% *B. henselae* and *B. clarridgeiae* in 275 cats. Marston [45] reported 43 % *B. henselae* and 21 % *B. clarridgeiae* in 54 cats and Celebi [33] identified 77% *B. henselae*, and 23% *B. clarridgeiae* in 256 cats. In addition, Celebi [33] identified 10 (% 77) *B. henselae* and 3 (% 23) *B. clarridgeiae* from 102 stray cats, 11 (77%) of *B. henselae* from pet cats.

Guptil [19] have not found identification of *B. clarridgeiae* while noted 24% *B. henselae* from 231 cats, Chomel [46] identified 22.6% *B. henselae* of 93 cats, and Fabbi [22] identified 18% *B. henselae* of 769 cats. In the study of Sigirci [35], *B. clarridgeiae* was not identified while *B. henselae* was isolated from 27 (28.1%) of 96 cats. In this study, *B. henselae* was isolated in 4 (8%) of 50 cases and *B. clarridgeiae* was isolated in 2 (4%) cases. These results indicate that *B. clarridgeiae* cannot be isolated at high levels of variation and in the frequency of appearance in cat populations, while supporting other studies showing *B. henselae* prevalence. *Bartonella species* are a common zoonotic pathogen in the world associated with contact with animals Chmielewski [1]. They are small, pleomorphic, gram negative, weakly stained bacilli or *cocobacilli*, oxidase and catalase negative microorganisms Guptill [3], Jacomo [4].

Due to slow growing period, standard biochemical methods for diagnosis are not suitable and are not used for species discrimination, so molecular genetic methods are used for species identification. Of these, there are three important species of pathogens for humans. These include *B. bacilliformis*, Carrion disease (Oroya fever and verruga peruana); *B. quintana* causes trench fires; *B. henselae* causes neurological syndromes Kordick & Breitshwerdt [47], infecting humans from natural reservoir catheters to immunodeficient and immunocompetent patients with cat scratch disease (CTS), immunocompromised individuals with bacillary angiomatosis (BA), bacillary peliosis and especially HIV. It is known that *B. henselae*, *B. bacilliformis* and *B. quintana* are pathogenic for humans for a long time. Recently, however, *B. clarridgeiae*, *B. elizabethae*, *B. grahamii* have been accepted as pathogens Jacomo [4], Chomel [48]. In this study, the presence of *B. henselae* and *B. clarridgeiae* in stray and pet cats visiting the private veterinary clinic determined in İzmir was revealed by PCR after Giemsa staining. As a result, Bartonella species, which are important for the people of existence, have been identified since they carry a zoonotic character in the Izmir region.

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