PREVALENCE OF BARTONELLA HENSELAE IN CATS IN CATALONIA, SPAIN

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Abstract. Bartonella henselae, an emerging pathogen bacterium, is the main causative agent of the cat scratch disease. While the first clinical descriptions were associated with immunosuppressed patients, it is now more frequently observed in patients with normal immune status (endocarditis and bacteremia). Cats were found to be the only known reservoir of B. henselae. In this paper, we report the results obtained in the first study made to investigate the prevalence of B. henselae bacteremia and antibodies in domestic cats in Catalonia, Spain. Serum samples from 115 cats were tested for antibodies to B. henselae by immunofluorescent antibody testing, and 29.6% had a titer ≥ 1:64. Seven B. henselae strains were isolated using standard culture techniques and amplification by a polymerase chain reaction and subsequent sequencing was performed on the intergenic spacer region between the 16 and 23S ribosomal RNA genes. Of all factors concerning the studied bacteremia rate (age, sex, habitat, presence of antibodies, contact with animals, parasites), only the presence of antibodies to B. henselae was statistically significant.

INTRODUCTION

In 1993, Brenner and others proposed to unify all the different species of Rochalimaea under the genus Bartonella, and they agreed to rename them B. quintana, B. vinsonii, B. henselae, and B. elizabethae, and they were added to the single previous species Bartonella bacilliformis. In 1995, Birles and others proposed to include the genus Grahamella species within Bartonella, and five more species were added. In 1997, B. claridgeiae was included, in 1998, B. tribocorum and B. washoensis were included, and from 1999 to 2002, B. koehlerae, B. alsatica, B. birtlesi, B. schoenbuchensis, B. capreoli, and B. bovis were included. Of the 19 species named, only 9 were acknowledged as human pathogen species: B. bacilliformis, B. quintana, and B. henselae are the most frequently described species, while B. elizabethae, B. vinsonii, B. washoensis, B. grahamii, B. claridgeiae, and B. koehlerae were recently identified as responsible for a few cases of human infections. Bartonella henselae, B. claridgeiae, B. koehlerae, and B. elizabethae are the main species isolated from cats, while the rest were isolated from other animals ( coyotes, cattle, rodent s, etc.).

Although the first clinical descriptions of Bartonella were associated with immunocompromised patients with bacillary angiomatosis, we currently know that this organism is directly involved in diseases affecting a large number of patients, regardless of their immune status. Cat scratch disease, hepatic peliosis, and some cases of bacteremia and endocarditis are directly caused by some species of the genus Bartonella. When studying the different diseases associated with B. henselae, contact with animals, especially cats, was found to be a common factor among many of the infected patients. The natural reservoir of this bacteria is the cat, and one of the main vectors of its transmission are fleas. The prevalence of Bartonella infection in cats determined by blood culture may vary from 4% to 70%. Seroprevalence varies according to the different studies conducted: from 4% to 95% in the United States, from 6% to 45% in Singapore; 8% in Switzerland, and 0% in eastern Canada and Norway.

Isolation of B. henselae from domestic cats has been reported from various parts of the world. However, no information is available from Spain. This is, to our knowledge, the first study that provides data about the prevalence of B. henselae bacteremia and antibodies to this organism in cats in Catalonia, Spain.

In this study, we determined the prevalence of Bartonella bacteremia and of B. henselae seroprevalence in 115 Catalan cats. We analyzed the Bartonella strains previously isolated from cats by Bartonella-specific polymerase chain reaction (PCR) and sequencing the 16S–23S ribosomal RNA (rRNA) gene fragment, and then correlated B. henselae seropositivity and bacteremia with factors such as flea infestation, contact with animals, age, and sex.

MATERIALS AND METHODS

Animals. From January 2001 to May 2002, 115 cats visiting a veterinary clinic in Vallès Occidental, a region near Barcelona in Catalonia, Spain, were chosen for our study. Thirty-eight cats (33%) were ≤ 1 year of age, 62 (54%) were females, and 87% were domestic cats. Ninety-nine cats (86%) were healthy animals visiting the clinic to be neutered or for an annual check-up, while 14% had some type of pathology (urinary tract infection, worms, and uterus infection). A total of 76.5% of the cats had contact with other animals (67% with only cats, 12% with dogs, and 21% with both) and 20 cats (17%) were infested with fleas.

Approximately 2.5 mL of blood was aseptically collected from the external jugular vein of each cat after receiving permission of the owners. One milliliter of blood was introduced in a serum-separating tube for serologic analyses; the remaining was placed into a pediatric isolator 1.5 tube (Isolator™, Oxoid, Ogdensburg, NY).

Bartonella isolation. Two hundred fifty microliters of blood were streaked onto Columbia agar plates supplemented with 5% sheep blood (BioMérieux, Marcy l’Étoile, France) in triplicate. The plates were incubated at 35°C in a moist atmosphere containing 5% CO2 for nearly two months. The plates were checked every week and gram staining was done on suspicious colonies. All isolates were then frozen at −80°C in Brucella broth supplemented with 10% glycerol (v/v).

Bacterial strain identification. Suspicious colonies, based on their morphology and gram staining, were also identified with the Rapid ID 32A identification panel (BioMérieux and by amplification of the intergenic spacer (ITS) region be-
between the 16S and 23S rRNA genes with *B. henselae* forward (nucleotides 302 to 321) and reverse (nucleotides 473 to 454) (Invitrogen, Carlsbad, CA) complimentary primers, as previously described by Jensen and others. The positivity of the amplification was confirmed by electrophoresis on 1.8% and 3% agarose gels. The sizes of the ITS PCR amplification products were determined by comparison with standard molecular mass marker VIII (Boehringer Mannheim, Mannheim, Germany). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), as reported by the manufacturer, and were sequenced with an automated sequencer ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Their sequences were compared with the corresponding sequences for *Bartonella* species available in the DNA analysis computer program NCBI Blast (National Center for Biotechnology Information, Bethesda, MD).

**Serologic analysis.** Sera were obtained by centrifugation of 1-mL samples of blood at 1,500 rpm for 10 minutes, and the samples were frozen at −80°C until used. Antibody titers against *B. henselae* were determined in 115 serum samples by an indirect immunofluorescence antibody (IFA) test. Commercial slides (*Bartonella* IFA IgG; Focus Technologies, Inc., Herndon, VA) were used. The IFA technique was standardized using two cat sera with known titers (kindly provided by Dr. Bruno B. Chomel, School of Veterinary Medicine, University of California, Davis, CA). These sera were also used as positive controls. A 1:128 dilution of a fluorescein isothiocyanate–conjugated goat anti-cyclopolynonal antibody (IgG heavy plus light chains; ICN Biomedicals, Irvine, CA) was used as a conjugate. Two-fold serial dilutions of the sera (from 1:64 to 1:1,024) were made in phosphate-buffered saline with 3% nonfat powdered milk and applied to the antigens, and the mixtures were incubated in a moist chamber for 30 minutes at 37°C. We considered specimens showing no fluorescence at IgG titers of 1:64 as negative. The intensity of each specific fluorescence was subjectively evaluated and independently graded by two of the authors.

**Statistical analysis.** The sample size was calculated taking into account an expected prevalence of 40%, with a precision of ± 10%, and losses ≤ 10%. Qualitative variables were studied descriptively with 2 × 2 tables and chi-square tests were used to check statistical significance. When applicability conditions were not met, Fisher’s exact test was used. A *P* value < 0.05 was considered significant.

**RESULTS**

**Isolation of *Bartonella* organisms.** Of the 115 cultures performed, 15 were rejected due to contamination (all three plates were contaminated before one month). *Escherichia coli*, *Staphylococcus aureus*, and *Stenotrophomonas* spp. were found in three cats (they showed clinical symptoms of uterine infection, pyodermatitis, and a poor general condition, respectively). *Bartonella* spp. were isolated from the blood of 7 of the 100 cats tested.

The characteristics of the cats with *B. henselae* bacteremia are shown in Table 1. When the data were related to the prevalence of bacteremia versus age group, we obtained positive cultures in four cats ≤ 1 year of age (12.1%) and in three in cats > 1 year of age (4.5%). The prevalence of bacteremia was higher in male cats (9.3%) than in females (5.3%); however, this difference was not statistically significant. Two cats (28.5%) with bacteremia had parasites and 19.3% of the non-bacteremic cats had fleas. Most (71.4%) bacteremic cats were in contact with other cats or dogs. All bacteremic cats also had IgG antibodies to *B. henselae* with titers > 1:128. Of all the factors considered (age, sex, habitat, serology, parasites, and contact with other animals), only the presence of antibodies against *B. henselae* in bacteremic cats showed statistical significance (*P* = 0.005).

**Identification of *Bartonella*.** The growth of colonies was not visible until after 10–15 days of incubation. Gram staining of the isolates showed small, slightly curved, gram-negative rods. The isolates were catalase and oxidase negative. Biochemical reaction profiles of the isolates were obtained with the Rapid ID 32A System (BioMérieux). Identification of the species was not possible by biochemical methods. A fragment of the 16S–23S rRNA gene was amplified from the DNA extracted from the isolates from cats. Sequencing of the 172-basepair fragment showed that all cats had bacteremia with *B. henselae*; the two macroscopically different types of colonies isolated from the same cat were *B. henselae*. The DNA amplified from the control strain of *B. clarridgeiae* had the expected 154-basepair fragment. In this study, no cats bacteremic with *B. clarridgeiae* were found. The results of the PCR analysis are shown in Figure 1.

**Serologic data.** Thirty-four (29.6%) of the 115 cats studied had antibodies to *B. henselae*. Of the 38 cats < 1 year of age, 10 (26.3%) had IgG antibodies against *B. henselae*; however, in older cats this value increased up to 31%. Seven (20.5%) of 34 cats positive for IgG antibodies also had fleas, while 16% of the cats with negative serologic results had fleas (this difference was not statistically significant). The relationship between immunofluorescence antibody titers to *B. henselae* and age and sex in 115 cats is summarized in Table 2.

**DISCUSSION**

Diseases associated with *B. henselae* have recently showed a gradual increase, and studies conducted in human and animal populations have provided more knowledge on the different epidemiologic aspects of *Bartonella* species. Cats are the main reservoir of *B. henselae*. Different studies have shown that there is a high seroprevalence and a relatively high rate of asymptomatic bacteremia in young cats. Seven percent of domestic cats in our study had *B. henselae* bacteremia. If we consider only cats < 1 year of age, this bacteremia rate increases up to 12.1%. This prevalence is slightly lower than those reported by other investigators (13% by Sander
growth and isolation. In our study, we used commercial (not a supplement in the culture medium) would have favored its isolation of Bartonella species isolated from cat blood. Shown is an ethidium bromide–stained 1.8% agarose gel demonstrating amplified 16S–23S ribosomal intragenic region products from template DNAs derived from Bartonella species. Lane M, DNA molecular mass marker VIII (Boehringer Mannheim); lane 2, negative control, lanes 3–9, isolates from cats; lane 10, B. clarridgeiae; lane 11, B. henselae. Lanes 10 and 11 (controls) both showed the expected products of 154 and 172 basepairs, respectively. The arrow indicates the specific 172-basepair (bp) band.

FIGURE 1. Polymerase chain reaction–based identification of Bartonella species isolated from cat blood. Shown is an ethidium bromide–stained 1.8% agarose gel demonstrating amplified 16S–23S ribosomal intragenic region products from template DNAs derived from Bartonella species. Lane M, DNA molecular mass marker VIII (Boehringer Mannheim); lane 2, negative control, lanes 3–9, isolates from cats; lane 10, B. clarridgeiae; lane 11, B. henselae. Lanes 10 and 11 (controls) both showed the expected products of 154 and 172 basepairs, respectively. The arrow indicates the specific 172-basepair (bp) band.

and others in Germany and 53% by Heller and others in France.

The culture medium used may have directly influenced the isolation of Bartonella because the use of fresh rabbit blood as a supplement in the culture medium would have favored its growth and isolation. In our study, we used commercial (not fresh) media supplemented with 5% sheep blood. In most studies, bacteremia is produced by B. clarridgeiae. Although B. clarridgeiae has been isolated as the single causative agent of infection in some studies, co-infections with both species have also been reported. To identify the isolated bacteria, we used a PCR technique with primers that best suited our study. The primers chosen allowed us to identify not only genus, but also the species of these bacteria. All strain isolated in our study were B. henselae.

Some investigators have pointed out that differences in bacteremia rates may be influenced by important climatic factors (high temperatures and humidity would favor infection). These climatic factors would also be directly related to an increase in flea infestations, since their biologic cycle is favored in countries with these conditions.

There are different and extensively described vectors for the different species of the genus Bartonella. Infection with B. bacilliformis is related to Lutzomyia spp. sand flies, which are also a vector of Leishmania. Humans are the only known reservoir of B. quintana, which has Pediculus humanus corporis (lice) as vector. As for B. henselae, whose main reservoir is domestic cats (especially young ones with asymptomatic bacteremia), its known vector for its transmission among felines is Ctenocephalides felis (the cat flea). Although this transmission mechanism is extensively described in felines, it has not been documented in humans. Also, horizontal transmission should not be overlooked since it can occur in absence of these vectors.

In this study, we believe that the two factors that can exert the greatest influence on the low prevalence of bacteremia are 1) the low number (33%) of cats less than one year of age in our sample, and 2) the low (17.4%) incidence of flea infestation. We also assume that the fact that animals came from only one clinic could also affect the results. Infestations, contact with other animals, or sex were not statistically significant risk factors in our study.

The single factor in this study with statistical significance (P = 0.005) was serologic results positive for infection with B. henselae. All bacteremic cats showed antibody titers ≥ 1:128 against B. henselae. Some investigators have reported the existence of bacteremic animals with negative serologic results; this may have occurred because detection of antibodies was conducted in an early stage of the infection, or because the animals had some immune disorder that altered their immune responses.

The seroprevalence value for B. henselae in our study was 29.6%. In the available literature, there is a broad variability of seroprevalence values: in some studies, the seroprevalence against B. henselae reaches 38–80% while in others, some selection of a feline population (cats with high rates of flea infestation or populations with a bacteremia rate of 70%). Conversely, some studies conducted in Scandinavian countries show a prevalence of B. henselae that is close to 0%. Another study reported an antibody positivity rate against B. elizabethae of 14%, whereas seroprevalence against B. henselae was only 1.5%.

In cats with antibody titers of 1:512, cultures were positive in 37.5%. In our study, the serologic test had a positive predictive value of 20.6%, which limited its utility to differentiate between bacteremic and non-bacteremic cats, whereas the

<table>
<thead>
<tr>
<th>Age (years)/sex</th>
<th>IgG (Neg)</th>
<th>IgG (1:164)</th>
<th>IgG (1:128)</th>
<th>IgG (1:256)</th>
<th>IgG (1:512)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1 Females</td>
<td>22/26 (84.6)</td>
<td>1/26 (3.8)</td>
<td>1/26 (3.8)</td>
<td>–</td>
<td>2/26 (7.7)</td>
</tr>
<tr>
<td></td>
<td>6/12 (50)</td>
<td>–</td>
<td>3/12 (25)</td>
<td>2/12 (16.6)</td>
<td>1/12 (8.3)</td>
</tr>
<tr>
<td>&gt; 1 Females</td>
<td>25/37 (67.5)</td>
<td>1/37 (2.7)</td>
<td>7/37 (18.9)</td>
<td>2/37 (5.4)</td>
<td>2/37 (5.4)</td>
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<td></td>
<td>28/40 (70)</td>
<td>2/40 (5)</td>
<td>5/40 (12.5)</td>
<td>2/40 (5)</td>
<td>3/40 (7.5)</td>
</tr>
<tr>
<td>Total</td>
<td>81/115 (70.4)</td>
<td>4/115 (3.5)</td>
<td>16/115 (13.9)</td>
<td>6/115 (5.2)</td>
<td>8/115 (6.9)</td>
</tr>
</tbody>
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*IFA = indirect immunofluorescence assay.
absence of antibodies against \textit{B. henselae} was highly predictive of the absence of bacteremia (negative predictive value = 100%).

Some studies reported bacteremia caused by co-infection of two different species of \textit{Bartonella}\textsuperscript{2,8,32}. In our study, all colonies, except one, had a similar morphology. Nevertheless, with the identification obtained by sequencing the 16S–23S rRNA gene fragment, it was determined that all colonies were \textit{B. henselae}.

Determining the presence or absence of bacteremia in cats is crucial in assessing the actual risk of transmission to humans. Culturing of cat blood samples still remains the only technique for identifying bacteremic cats. However, this procedure does not provide conclusive results because cats can be intermittently bacteremic. Serologic testing appears to be of limited value in predicting bacteremia. However, the inability to detect antibodies to \textit{B. henselae} appears to be predictive of the absence of bacteremia.

Despite some limitations in the cat population selected for our study, the data indicate that infection with \textit{B. henselae} in cats is present in the urban center of Catalonia, Spain. This is the first report to document the distribution of \textit{B. henselae} among cats in this country. Experimental studies are necessary to determine the kinetics and duration of bacteremia in cats. Similarly, studies are needed to better understand the method of transmission of \textit{B. henselae} from cat to cat and from cats to humans to develop strategies to prevent \textit{B. henselae} infection.

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