BARTONELLA HENSELAE AND BARTONELLA CLARRIDGEIAE INFECTION IN DOMESTIC CATS FROM THE PHILIPPINES

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Abstract. One hundred seven domestic cats from The Philippines were serologically tested to establish the prevalence of Bartonella infection. A subset of 31 of these cats also had whole blood collected to tentatively isolate Bartonella strains. Bartonella henselae and B. clarridgeiae were isolated from 19 (61%) of these cats. Bartonella henselae type I was isolated from 17 (89%) of the 19 culture-positive cats. Six cats (31%) were infected with B. clarridgeiae, of which four were coinfected with B. henselae. Sixty-eight percent (73 of 107) and 65% (70 of 107) of the cats had antibodies to B. henselae and B. clarridgeiae, respectively, detected by an immunofluorescence antibody (IFA) test at a titer $\geq 1:64$. When tested by enzyme immunoassay (EIA), 67 cats (62.6%) had antibodies to B. henselae and 71 cats (66.4%) had antibodies to B. clarridgeiae. Compared with the IFA test, the B. henselae EIA had a sensitivity of 90.4% and a specificity of 97%, with positive and negative predictive values of 98.5% and 82.5%, respectively. Similarly, the B. clarridgeiae EIA had a sensitivity of 97% and a specificity of 92% specificity, with positive and negative predictive values of 95.8% and 94.4%, respectively. The presence of antibodies to Bartonella was strongly associated with flea infestation. Domestic cats represent a large reservoir of Bartonella infection in the Philippines.

The genus Bartonella is presently composed of 11 species, of which at least four are known to be human pathogens: B. bacilliformis, the agent of Carrión's disease; B. quintana, the agent of trench fever and bacillary angiomatosis (BA); B. henselae, the agent of cat scratch disease (CSD) and BA; and B. elizabethae, which can cause endocarditis. Bartonella vinsonii var. berkhoffii has been found in a case of canine endocarditis as well. The other species (B. clarridgeiae, B. doshiae, B. grahamii, B. peromysci, B. talpae, and B. taylorii) have been isolated from the blood of various mammals but are not known to induce disease in the infected animal.

Two Bartonella species (B. henselae and B. clarridgeiae) have been isolated from the blood of domestic cats. Epidemiologic studies have implicated cats as a major reservoir of B. henselae, and it has been demonstrated that cats can remain asymptomatic and bacteremic for several months to several years. Bartonella henselae DNA has been amplified from fleas found on bacteremic cats and transmission of B. henselae by the cat flea Ctenocephalides felis has been demonstrated. Bartonella clarridgeiae was first isolated from the bloodstream of a healthy cat involved in a human case of CSD caused by B. henselae. However, only serologic evidence of a human case of CSD caused by this organism has been documented.

Isolation of B. henselae and/or B. clarridgeiae from domestic cats has been reported from various parts of the world, including North America, Europe, Japan, and Australia. However, no information is available for most of the Pacific Rim countries.

No human case of CSD from The Philippines has been officially recognized or published in the scientific literature. However, animal bites and scratches are certainly common events in this part of the world, where rabies is endemic and where an estimated 60,000 persons receive rabies postexposure treatment (PET) every year, and approximately 280,000 more Filipinos are bitten by dogs under circumstances for which PET would strictly be indicated. Among the 9,495 persons seen in 1996 at the Research Institute for Tropical Medicine in Manila for animal bites, cats accounted for 3.3% of the exposures (Carlos E, unpublished data). Furthermore, in this tropical setting, ectoparasite infestation, especially fleas, is common in pets. It was therefore of interest to establish if the feline reservoir of Bartonella was present in the Philippines. A serosurvey of domestic cats from the Philippines, mainly from Metro Manila and suburbs on Luzon Island, and from Cebu City on Cebu Island was conducted in February 1997 to establish the prevalence of Bartonella infection. A subset of 31 of these cats also had whole blood collected to tentatively isolate Bartonella strains.

MATERIALS AND METHODS

Animals. Serum samples were conveniently collected at specific veterinary clinics from 107 domestic cats from Metro Manila and suburbs (101 cats) and Cebu City (six cats), The Philippines. Most cats were healthy animals presented at the clinics for vaccination or convenience surgery. For 31 of these cats (25 from Metro Manila and suburbs and six from Cebu City), 1.5 ml of blood was also collected in a pediatric lysis-centrifugation tube (Isolator®, Wampole Laboratories, Cranbury, NJ). Most of the cats (87%) were from a domestic breed. Thirteen percent of the cats were pure breed (nine Persian, four Siamese, and one Tonkinese). Fifty-four percent of the cats were female, with an age range of two months to 12 years (mean = 2.5 years, mode = 2 years). Most of the cats (78%) were pet cats that were adopted as kittens after being found in the neighborhood, and most were free to roam from their household. More specifically, among the 31 cats for which a blood culture was performed, two were reported as being stray and all other were reported...
as pets found in the neighborhood. Flea infestation was common since 54% (58 of 107) of the cats were harboring fleas when examined at time of blood collection. For the 101 cats from Metro Manila, 35 were from Manila, 32 from Makati, 15 from Malabon, 11 from Parañaque, and eight from Quezon City. All cats from Makati were owned cats that were individually identified with a name.

**Isolation of Bartonella from cat blood.** The isolator tubes were centrifuged at 1,800 \( \times g \) for 75 min at ambient temperature and the supernatant was removed. The pellet was then resuspended in 125 \( \mu \)l of inoculation media and the volume was recorded. Two hundred fifty microliters of the suspension were inoculated onto heart infusion agar (Difco Laboratories, Detroit, MI) supplemented with 5% defibrinated rabbit blood and the remaining suspension was inoculated onto a second plate. The plates were incubated for one month at 35°C in 5% CO\(_2\) in a humid incubator and checked regularly for bacterial growth. After gram stain and were compared with type strains of *B. henselae* and *B. clarridgeiae* type II (strain U4, University of California, Davis) slides containing diluted in phosphate-buffered saline (PBS) and incubated on the citrate synthase gene was amplified using previously described primers and methods. The amplified products were verified using polymerase chain reaction/restriction fragment length polymorphism analysis (PCR-RFLP) of a fragment of the citrate synthase gene and a fragment of the 16S rRNA gene.

An approximately 400-basepair (bp) fragment of the citrate synthase gene was amplified using previously described primers and methods. The amplified products were verified by gel electrophoresis and then enzymatically digested using Taq I and Hha I restriction endonucleases. Banding patterns were compared with type strains of *B. henselae* Houston-1 (ATCC 49882; American Type Culture Collection, Rockville, MD) and *B. clarridgeiae* (ATCC 51734).

An approximately 1,500-bp fragment of the 16S rRNA gene was amplified using previously described primers and methods. The amplified products were verified by gel electrophoresis and then enzymatically digested using Dde I restriction endonuclease to determine the *B. henselae* type (type I or type II) (Kasten RW and others, unpublished data).

**Serologic analyses.** *Bartonella henselae* and *B. clarridgeiae* were determined using both an IFA technique as previously described, and an enzyme immunoassay (EIA) using the outer membrane proteins (OMPs) of *B. henselae* and *B. clarridgeiae*. For the IFA test, serum was serially diluted in phosphate-buffered saline (PBS) and incubated on slides containing *Felix catus* whole feta cells infected with *B. henselae* type II (strain U4, University of California, Davis) and *B. clarridgeiae* (ATCC 51734). The slides were washed and probed with fluorescein isothiocyanate goat anti-cat IgG (heavy plus light chain) conjugate (Cappel, Organon Teknika Corp., Durham, NC), and the fluorescence was graded independently by the same two persons. Any serum with a titer \(\geq 64\) was considered positive.

**Enzyme immunoassay (EIA).** Purification of the OMPs of *Bartonella*. The OMPs of two *Bartonella* strains (*B. henselae* and *B. clarridgeiae*) were prepared as previously described. Each *Bartonella* strain was grown on 5% rabbit blood agar in 5% CO\(_2\) at 35°C for 3–4 days. Each *Bartonella* strain was harvested from 50 plates and washed by centrifugation three times (5 min each) in sterile PBS, pH 7.4. The pellet was resuspended in 10 mM HEPES, pH 7.4 (Gibco BRL, Life Technologies Inc., Gaithersburg, MD) buffer solution. The whole cell bacterium suspension was then sonicated for 1 min five times with a 1-min pause between each sonication with a Sonifer-2 Cell Distributors (Baxter Diagnostics Inc., McGaw Park, IL) at 4°C. The sonicated organisms were then centrifuged at 1,700 \(\times g\) (4,000 rpm in an SS-4 superspeed centrifuge: Sorvall, Newtown, CT) for 20 min to eliminate the coarse debris pellet. The supernatant was then centrifuged by ultracentrifugation at 100,000 \(\times g\) (32,700 rpm in a type 70-Ti rotor; Beckman, Palo Alto, CA) for 60 min at 4°C. The pellet was resuspended in 10 mM HEPES buffer, pH 7.4, containing 1% sodium lauryl sarcosine (Sigma Chemical Co., St. Louis, MO) and incubated for 30 min at room temperature. The ultracentrifugation process was then repeated three times. Finally, the pellet was resuspended in distilled water. The protein concentrations of the final suspension were determined by a colorimetric technique using the Bio-Rad (Hercules, CA) protein assay kit. The OMPs were then stored at \(-20°C\) until used as an antigen for the EIA.

**Enzyme immunoassay.** *Bartonella henselae* and *B. clarridgeiae* OMPs used as antigens for the EIA were diluted in 0.1M sodium carbonate buffer, pH 9.6, to a protein concentration of 3.0 \(\mu\)g/ml. The EIA microtiter plates (Immulon® I; Dynatech Laboratories, Chantilly, VA) were coated with 100 \(\mu\)l of *B. henselae* or *B. clarridgeiae* OMP antigens on half of the plates. After overnight incubation at room temperature, the plates were washed five times with saline containing 0.05%. Tween 20 (Sigma Chemical Co.) and then blocked with 250 \(\mu\)l of Super Block® (Pierce, Rockford, IL) for 1 hr at 37°C. Serum samples (50 \(\mu\)l) to be tested and positive (IFA test titer \(\geq 1:1,024\)) and negative (specific pathogen-free cat serum) control sera diluted at 1:100 in EIA dilution buffer (0.15 M NaCl, 1 mM EDTA [Fisher Scientific, Fair Lawn, NJ], pH 7.4, 50 mM Tris, 0.05% Tween 20, 5% skim milk [Becton Dickinson, Cockeysville, MD], and 0.1% bovine serum albumin [Sigma Chemical Co.]) were then added in duplicate in both antigen-coated and noncoated test wells and incubated at 37°C for 1 hr. The plates were then washed five times with the same wash buffer and anti-cat IgG (heavy plus light chain) peroxidase-labeled conjugate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a 1:3,000 dilution was added to all wells and incubated for 1 hr at 37°C. The plates were then washed five with the wash buffer and 100 \(\mu\)l per well of the substrate solution (0.005% 3,3′,5′-tetramethyl benzidine [Sigma Chemical Co.] and 0.015% \(\text{H}_2\)\(\text{O}_2\) diluted in 0.05 M citric acid, pH 4) was added. Color development was allowed to proceed for 5–10 min at room temperature before being stopped by the addition of 50 \(\mu\)l/well of 1 N \(\text{H}_2\)\(\text{SO}_4\). The optical density (OD) of each well was read with dual filters (450 nm and 570 nm) using a microplate reader (Dynatech MR 5000; Dynatech Laboratories). The final OD value for each sample was determined as the average value of the antigen-coated well OD minus the noncoated well OD. Any serum sample with an OD \(\geq 0.3\) was considered positive (based on the mean plus three standard deviations of serum samples [n = 47] with negative results in the IFA test).
RESULTS

*Bartonella henselae* and *Bartonella clarridgeiae* were isolated from 19 (61%) of the 31 cats tested. However, for one of the cats, bacterial contamination did not allow identification of *Bartonella* infection. *Bartonella henselae* was isolated from 17 (89%) of the 19 culture-positive cats. All *B. henselae*-bacteremic cats were infected with *B. henselae* type I. Six cats (31%) were infected with *B. clarridgeiae*, of which four where coinfected with *B. henselae*. Coinfection was therefore observed in 21% of the bacteremic cats.

Only one of the 31 cats that had a blood culture obtained was free of fleas at the time of blood collection. All bacteremic cats were flea-infested and were outdoor cats.

Sixty-eight percent (73 of 107) and 65% (70 of 107) of the cats had antibodies to *B. henselae* and *B. clarridgeiae*, respectively, detected by the IFA test at a titer ≥ 1:64. Sixty-two cats had antibodies against both organisms, 11 cats were *B. henselae* antibody positive and *B. clarridgeiae* antibody negative, whereas, eight cats had antibodies only against *B. clarridgeiae*. The IFA test antibody titers against *B. clarridgeiae* were lower than those for *B. henselae* since as none had a titer > 1:256 (Table 1). When tested by EIA, 67 cats (62.6%) had antibodies to *B. henselae* and 71 cats (66.4%) had antibodies to *B. clarridgeiae*. Fifty-seven cats were positive for both species, 10 cats were positive only for *B. henselae*, and 14 cats were positive only for *B. clarridgeiae*. The OMP EIA for *B. henselae* antibody performed well compared with the IFA test since it had an agreement of 93.4%, a sensitivity of 90.4%, and a specificity of 97%. The positive predictive value was 98.5% and the negative predictive value was 82.5%. Similarly, when compared with the IFA test, the *B. clarridgeiae* EIA had an agreement of 92.5%, a sensitivity of 97%, and a specificity of 92%. The positive and negative predictive values were 95.8% and 94.4%, respectively.

The prevalence of antibodies to *B. henselae* and *B. clarridgeiae* increased with age and ranged from 48% to 52% in cats < 1 year of age, from 67% to 78% in one-year-old cats, from 73% to 80% in young adults (2–3 years old), and then moderately decreased (from 62% to 69%) in cats greater than or equal to four years of age (Table 2).

The presence of antibodies to *Bartonella* detected by the IFA test was strongly associated with flea infestation, breed of cat, and city of origin (Table 3). Domestic cats were more likely than pure breed cats to be seropositive and cats from Makati, a wealthy district of Metro Manila, were less likely than the cats from other cities to be seropositive. Similar results were observed for antibodies detected by the EIA. However, when statistically controlled for the presence of fleas, breed of cat and city of origin were no longer significant risk factors.

**DISCUSSION**

This is the first report of isolation of the agent of CSD and bacillary angiomatosis from its animal reservoir in the Philippines. A high percentage of the cats analyzed by blood cultures were bacteremic, which may be explained by the fact that all but one of the cats that had a blood culture obtained were flea-infested. A positive association between both seropositivity and bacteremia and flea infestation has been previously reported. It is the first time that flea infestation was found to be associated with seropositivity for *B. clarridgeiae*, suggesting a similar mode of transmission from cat to cat. The percentage of bacteremic cats harboring *B. clarridgeiae* is very similar to what has been observed in France by Heller and others or in The Netherlands by Bergmans and others. It is much higher than the 10% (7 of 70) reported in the United States or the lack of isolation of *B. clarridgeiae* in Japanese cats, despite the isolation of many *B. henselae* strains (Maruyama S, unpublished data). We found several cats coinfected with the two feline *Bartonella* species, confirming that coinfection occurs also in The Philippines, as previously demonstrated in Dutch and French cats. However, the most striking finding was the absence of any *B. henselae* type II isolates, whereas it is the most common isolate from European cats, and is also common in the United States (Chomel BB, unpublished data). Distribution of the various species and types of *Bartonella* isolated from cats appears to vary widely. Knowledge of the respective distribution of these types or species is of major importance since it appears that humans may be more likely to be infected by one type or species than another, as shown by Bergmans and others in The Netherlands. Such observations still need to be validated with human isolates from other parts of the world. Similarly, if a vaccine strategy is to be developed in domestic cats to prevent human infection, it appears essential to adapt the composition of the vaccines to the local strain characteristics. It has been recently shown that under experimental conditions, there is very limited cross-protection against reinfection in cats infected by heterologous types or species.

Several studies have determined the sensitivity and spec-
Risk factors associated with *Bartonella* immunofluorescence antibody (IFA) seropositivity in 107 Filipino cats

<table>
<thead>
<tr>
<th>Risk factors</th>
<th><em>B. henselae</em> IA+</th>
<th><em>B. henselae</em> IA−</th>
<th>Odds ratio</th>
<th>95% CI*</th>
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<tr>
<td>Fleas</td>
<td>Flea +</td>
<td>Flea −</td>
<td>52</td>
<td>21</td>
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<td></td>
<td><em>B. clarridgeiae</em> IA+</td>
<td><em>B. clarridgeiae</em> IA−</td>
<td>6</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Flea +</td>
<td>Flea −</td>
<td>46</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td><em>B. henselae</em> IA+</td>
<td><em>B. henselae</em> IA−</td>
<td>68</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><em>B. clarridgeiae</em> IA+</td>
<td><em>B. clarridgeiae</em> IA−</td>
<td>25</td>
<td>9</td>
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<tr>
<td>Breed</td>
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<td>Pure breed</td>
<td>66</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td><em>B. henselae</em> IA+</td>
<td><em>B. henselae</em> IA−</td>
<td>68</td>
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<tr>
<td></td>
<td><em>B. clarridgeiae</em> IA+</td>
<td><em>B. clarridgeiae</em> IA−</td>
<td>25</td>
<td>9</td>
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<tr>
<td>City</td>
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<td>Other</td>
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<td>62</td>
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<td><em>B. henselae</em> IA−</td>
<td>12</td>
<td>58</td>
</tr>
</tbody>
</table>

* CI = confidence interval.

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