Identification of Bartonella-Specific Immunodominant Antigens Recognized by the Feline Humoral Immune System

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The seroreactivities of both naturally and experimentally infected cats to Bartonella henselae was examined. Serum samples collected weekly from nine cats experimentally infected with B. henselae LSU16 were tested by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. The magnitude and isotype of the antibody response were investigated by ELISA. Western blot analysis allowed the identification of at least 24 Bartonella-specific antigens recognized by the cats during infection. Antibody titers to specific antigens, as determined by Western blot analysis, ranged from 10 to 640 and varied among the different antibody-antigen interactions. Absorption of sera from an experimentally infected cat, using whole cells and cell lysates of various Bartonella species and other bacteria that commonly colonize cats, supported the identification of those Bartonella-specific antigens recognized by the experimentally infected cats. Furthermore, a number of possible species- and type-specific antigens were identified. Finally, sera obtained from cats at local animal shelters were screened for the presence of antibodies directed against the Bartonella-specific bands identified in the experimentally infected cats. A number of Bartonella-specific antigens have been identified to which strong antibody responses are generated in both experimentally and naturally infected cats, some of which may be useful in diagnosing species- and/or type-specific infections. In addition, the results from these experiments will lead to the development of monoclonal antibodies targeted against those genus-, species-, and type-specific antigens.

Bartonella henselae is the causative agent of human cat scratch disease (CSD) (2, 13, 31, 35) and has been associated with bacillary angiomatosis, bacillary peliosis, recurrent bacteremia (26), and endocarditis (14). Epidemiological evidence indicates that cats serve as vectors for the transmission of B. henselae to people (27, 40, 42, 43). The cat flea (Ctenocephalides felis) may also be involved in transmission of this organism; B. henselae was found in fleas from an infected cat (26), and it was reported that fleas (12) and flea feces (19) transferred from B. henselae-bacteremic cats to specific-pathogen-free cats were capable of transferring the infection. Although cats are the natural reservoir for B. henselae, they appear to be asymptomatic even during bacteremic episodes (29, 35). There is, however, recent evidence to indicate that experimentally infected cats do present with mild clinical signs, such as fever, anorexia, lethargy, and peripheral lymphadenopathy (1, 22), which dissipate within a short time. Naturally infected cats may develop similar clinical signs, but these signs may not be noticed by cat owners.

A strong humoral response to infection with B. henselae has been seen; whether this immune response is protective against future infection is debated (12, 20, 36, 37). Furthermore, some cats with B. henselae bacteremia have high levels of circulating antibodies, and thus the role of the humoral immune response in Bartonella infections is not clear (12). Seroprevalence studies suggest that 3.7 to 65.4% of cats within the United States are positive for antibodies to B. henselae (9, 10, 25). Serologically positive cats are often also bacteremic (7, 11, 29). The mechanism by which this organism is able to survive and replicate within the cat and not cause overt symptoms is unclear. Similarly, the immunological response of the cat to this pathogen is not understood.

At this time, pet cats are not routinely screened for Bartonella infections; screening, however, could be of particular benefit to those owners who are immunocompromised. The most widely used serodiagnostic tool for Bartonella infections in cats is immunofluorescence assays (IFA) (35). The IFA, however, although specific and sensitive, has a number of drawbacks. This assay lends itself poorly to large numbers of samples and is time-consuming and costly. Furthermore, quantitation of IFA requires that titrations be performed, which increases the cost of the test. More recently enzyme-linked immunoabsorbent assays (ELISAs) have become available for diagnosis of Bartonella infections in humans (30) and in cats (21). While the ELISA is similar to the IFA in regard to reported sensitivity (86.2 versus 88%) and specificity (95.9 versus 94%) (21, 36), use of an ELISA has some inherent advantages. For instance, these assays are particularly useful because large numbers of samples can be screened at one time, and the tests are relatively inexpensive.

If the risk of contracting CSD from a pet cat is to be reduced, a better understanding of the history of the feline Bartonella infection must be obtained. This understanding will further aid in the improvement of diagnostic tools used in screening for Bartonella infections. The purpose of this study was to define the feline humoral immunological response to infection with Bartonella species. The hypothesis of this research was that characterization of the feline humoral immune response to infection with B. henselae will lead to the identification of possible genus-, species-, and type-specific antigens recognized by the cat’s immune system. These antigens could serve as targets for the generation of monoclonal antibodies.
that could be used to improve the sensitivities and specificities of currently available serodiagnostic tools.

The objectives of this research were (i) to utilize an ELISA to quantify the magnitude of antibody responses in nine cats experimentally infected with *B. henselae* LSU16 and to examine the isotype of these responses; (ii) to perform Western blot analysis on both unabsorbed and absorbed sera from experimentally infected cats in order to identify immunodominant proteins recognized by these cats and to identify those antigens which are possibly *Bartonella* genus, species, and type specific; and (iii) to examine, by Western blot analysis, sera obtained from cats at local animal shelters for seroreactivity to *B. henselae* species-specific antigens and to compare the reactivity patterns of naturally infected cats to those of the experimentally infected cats. These results will assist in the identification of those antigens that would be the most valid targets in the development of *Bartonella*-specific diagnostic screening tests and in determining the usefulness of Western blot analysis as a confirmatory test.

**MATERIALS AND METHODS**

**Bacteria and culture conditions.** *B. henselae* Houston-1 (ATCC 49882), *B. quintana* (ATCC VR-358), and *B. claridgeiae* (ATCC 700095) were obtained from the American Type Culture Collection (Rockville, Md.). *B. henselae* LSU16 was isolated at Louisiana State University from a naturally infected cat. *B. quintana* 127g was obtained from M.S. Laboratory Animal Medicine, Louisiana State University. *B. henselae* was cultivated in Luria-Bertani (39) broth at 37°C for 24 h. *B. henselae* agar plate at 35°C under 5% CO₂. The bacteria were scraped from the plates after 5 to 7 days of culture, suspended in infusion broth with 25% glycerol, and stored at −70°C until they were used.

**B. henselae** culture and serology. The cats were maintained at the Division of Laboratory Animal Medicine, Louisiana State University. All cats were allowed to acclimate to the facilities for 2 weeks before the commencement of the study. All nine cats were 6 to 8 months old and negative for *B. henselae* by culture and serology. The cats were maintained at the Division of Laboratory Animal Medicine, Louisiana State University. All cats were allowed to water and food ad libitum and were housed in individual cages according to the policies and guidelines of the Institutional of Laboratory Animal Care.

**Sera for the *Bartonella* seroreivalence study were obtained from cats at animal shelters in East Baton Rouge, West Baton Rouge, Plaquemine, and Lafayette parishes from May through August 1996.

**Experimental infection.** All cats were sedated with Telazol (Tiletamine HCL and Zolazepam HCL [Fort Dodge Animal Health, Fort Dodge, Iowa]; 7 mg/kg) prior to venous puncture and experimental infection. Nine cats were infected intravenously with *B. henselae* LSU16 in phosphate-buffered saline (1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄ [pH 7.2], 154 mM NaCl). All cats were kept under observation. Six 12- to 16-week-old kittens (cats 37, 40, 58, 39, and 54) were obtained from Harlan-Sprague-Dawley, Inc. (Indianapolis, Ind.). At the commencement of the study all nine cats were 6 to 8 months old and negative for *B. henselae* by culture and serology. The cats were maintained at the Division of Laboratory Animal Medicine, Louisiana State University. All cats were allowed to water and food ad libitum and were housed in individual cages according to the policies and guidelines of the Institutional of Laboratory Animal Care.

**RESULTS**

**Absorptions.** Cells from *B. henselae* Houston-1 and LSU16, *B. claridgeiae*, and *B. quintana* were washed five times with ELISA wash buffer. One hundred microliters of a 1:3,500 dilution of peroxidase-conjugated, affinity-purified goat anti-cat immunoglobulin G (IgG) (Fc fragment; Bethyl Laboratories, Inc., Montgomery, Tex.) was used for IgG testing. One hundred microliters of a 1:6,500 dilution of peroxidase-conjugated, affinity-purified goat anti-IgM (μ chain specific; Bethyl Laboratories, Inc.) was used for IgM testing. The conjugate was incubated in the wells for 30 min and washed five times as described above. Twenty microliters of TMB (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) mixed according to the manufacturer's instructions was added to the wells and incubated for 10 min, and the incubation was stopped by addition of 100 μl of 1 M H₂SO₄. The plates were read at 410 nm with an ELISA plate reader (Dynatech Laboratories, Inc., Chantilly, Va.). The optical densities (OD) of the triplicate wells were averaged in order to obtain an average OD for each test serum.

**Western blot analysis.** *Bartonella* species cultured as described above were adjusted to a final OD of 0.6 at 500 nm, and a bacterial lysate for each species was prepared. The cells were then subjected to centrifugation at 12,000 × g for 10 min, and then the pellets were resuspended in a half volume of sample buffer (62.5 mM Tris, 10% [vol/vol] glycerol, 0.5% [vol/vol] 2-mercaptoethanol, 2.3% [wt/vol] SDS), vortexed for 3 min, and boiled for 10 min. Western blot analysis was performed with modifications as described for other bacteria (38). Briefly, 75 μl of prepared lysate were electrophoresed on a 12% (wt/vol) polyacrylamide two-step preparation minigel (Bio-Rad Laboratories, Richmond, Calif.) at 100 V for 60 to 75 min. Electrophoretically separated antigens were transferred to pure nitrocellulose protein transfer membrane (Schleicher & Schuell, Keene, N.H.) at 100 V for 90 min with an electrophoretic transfer cell (Mini-Trans-Blot; Bio-Rad Laboratories). The blotted membranes were blocked overnight with 10% (wt/vol) nonfat dry milk in 100 mM NaCl, 10 mM Tris-HCl (pH 7.4) (milk-NET) and washed in NET with 0.05% (vol/vol) Tween 20 (NET-T) for 20 min. Feline sera diluted 1:10 in milk-NET were applied to the blots with a 28-cm miniblotter (MiniBlotter 28; Immunetics, Cambridge, Mass.) and incubated at room temperature for 1 h. The blots were then washed with NET-T and incubated with peroxidase-conjugated, affinity-purified F(ab')₂ fragment goat anti-IgG (H + L) (Jackson Immunoresearch Labs, Inc., West Grove, Pa.) diluted 1:1,000 in milk-NET for 1 h. Antibody-bound conjugate was detected by enhanced chemiluminescence (ECL; Western blot detection system; Amersham Life Science, Inc., Arlington Heights, Ill.). The enhanced chemiluminescence reagents were diluted 1:2 in distilled water.

**Antibody titers for three cats (cats 38, 59, and 182) were examined by Western blot analysis.** Serum samples were processed in the manner described above.

**ELISA analysis was performed in order to examine the magnitude of antibody response elicited in cats experimentally infected with *B. henselae* LSU16 and to examine the isotype of the antibody response.** *B. henselae*-specific IgM and IgG responses, as well as decreasing primary bacteremia with concur-
rent increasing IgG levels, were seen in all experimentally infected cats. The IgG response in these experimentally infected cats did not fall over time. While the magnitude of the antibody response varied among the cats, a similar pattern, in which peak IgM levels were followed by a rise in IgG antibodies, was observed. Three types of antibody responses were seen in these cats: one in which a strong peak in IgM antibodies occurred within 3 to 5 weeks and was closely followed and surpassed by a strong IgG antibody response (cats 37, 39, 50, and 184); another in which the IgM peak did not occur until between 6 and 8 weeks and was lower than that seen in cats 37, 39, 50, and 184 but was closely followed with a peak in IgG levels (cats 40, 54, and 182); and finally, an IgM peak that occurred at 3 weeks but was much lower than that in the other cats with IgM peaks at 3 weeks (cats 58 and 223). Interestingly, cat 58 underwent a secondary bacteremia that occurred without an additional peak in IgM levels or a rise in IgG levels as seen during primary bacteremia. Furthermore, the magnitude of the IgG response was lower in cat 58 and developed much more slowly than those in the other cats. Bacteremias lasted for 5 to 7 weeks in cats 37, 40, 50, and 184 and for 9 to 13 weeks in cats 39, 54, 182, and 223; bacteremia was recurrent in cat 58 (Fig. 1).

**Western blot analysis for the identification of immunodominant antigens recognized by experimentally infected cats.** Serum samples collected on a weekly basis were examined by Western blot analysis to identify immunodominant antigens recognized by the experimentally infected cats and to determine if there was a predominant pattern of seroreactivity in these cats (Fig. 2). Sera from all nine experimentally-infected cats exhibited similar patterns of immunoreactivity, the exception being the kinetics of the appearance of antibody to some of the **Bartonella**-specific antigens recognized by the cats' immune systems (Fig. 3). At least 24 unique antigens that were recognized by the immune system of each experimentally infected cat were identified. A representative blot is shown in Fig. 2 (cat 40). A strong response was elicited to the following antigens: 97.0, 76.0, 69.0, 65.0, 57.0, 54.0, 45.0, 16.9, 13.3, and 11.3 kDa. This response was evident by no later than four weeks postinfection and was maintained throughout the course of observation for each cat. A strong response to the 7.0-, 8.0-, 18.0-, 19.0-, 21.7-, and 51.0-kDa antigens was also elicited in each cat, but antibodies to these antigens were not detected in all cats until between 6 and 8 weeks postinfection. Antibodies to these antigens were also maintained throughout the course of infection. Antibody responses to the 15.0-kDa band were relatively weak in cats 39, 54, 182, and 223. Responses to the 15.0-, 28.2-, 30.0-, 33.7-, 36.0-, 48.0-, 60.5-, and 81.0-kDa antigens were weak, and they waxed and waned throughout the course of infection.

In order to further examine the magnitude of the feline immune response to infection with **B. henselae**, antibody titers to the 97.0-, 76.0-, 69.0-, 65.0-, 45.0-, 16.9-, 15.0-, 13.3-, and 11.3-kDa antigens were examined by Western blot analysis at 0, 4, 8, 12 (cats 39, 58, and 182), 16 (cats 39 and 58), and 20 (cat 58) weeks postinfection (Table 1). One cat from each pattern of bacteremia was chosen for titer determination. The peak magnitude of antibody titers to the 97.0-, 76.0/69.0-, and the 65-kDa antigens reached 640 for cats 39 and 182 and 160 for cat 58. Peak titers of antibody to the 16.9-, 13.3-, and 11.3-kDa antigens reached 160 for cats 39 and 182 and 40 for cat 58.
Quintana absorption did not remove antibodies to the 76.0-, \textit{B. henselae} antibody-antigen interactions; some extent support these results. Species of commonly colonize cats. Absorptions performed with other that seen with \textit{B. henselae} Houston-1, and \textit{B. henselae} cell absorption with \textit{B. henselae} LSU16 removed a majority of, as well as various species of \textit{Bartonella}, including \textit{B. clarridgeiae}, \textit{B. quintana}, \textit{B. henselae} Houston-1, and \textit{B. henselae} LSU16, were performed. Whole-cell absorption with \textit{B. henselae} LSU16 removed a majority of the seroreactivity, indicating that in these experimentally infected cats, most of the antibodies generated were against external antigens; absorption with bacterial cell lysate removed the remaining reactive antibodies (Fig. 4, lanes 1 to 3). Whole-cell and cell lysate absorption with \textit{B. bronchiseptica}, \textit{P. multocida}, and \textit{E. coli}, as well as various species of \textit{Bartonella}, including \textit{B. clarridgeiae}, \textit{B. quintana}, \textit{B. henselae} Houston-1, and \textit{B. henselae} LSU16, were performed. Whole-cell absorption with \textit{B. henselae} LSU16 removed a majority of the seroreactivity, indicating that in these experimentally infected cats, most of the antibodies generated were against external antigens; absorption with bacterial cell lysate removed the remaining reactive antibodies (Fig. 4, lanes 1 to 3). Whole-cell and cell lysate absorption with \textit{B. bronchiseptica}, \textit{P. multocida}, and \textit{E. coli} did not appear to have any effect on the Western blot antibody-antigen profile (Fig. 4, lanes 13 to 15). These results suggest that most of the seroreactive antigens identified by Western blot analysis are likely \textit{Bartonella}-specific, since they do not cross-react with the other bacteria that commonly colonize cats. Absorptions performed with other species of \textit{Bartonella} that are known to be cross-reactive to some extent support these results. \textit{B. henselae} Houston-1 absorption (Fig. 4, lanes 4 to 6) and \textit{B. quintana} absorption (Fig. 4, lanes 10 to 12) presented with antibody profiles similar to that seen with \textit{B. henselae} LSU16 with the exception of three antibody-antigen interactions; \textit{B. henselae} Houston-1 and \textit{B. quintana} absorption did not remove antibodies to the 76.0-, 69.0-, and 65.0-kDa antigens. These antibodies were also not removed by absorption with \textit{B. clarridgeiae} (Fig. 4, lanes 7 to 9). Although absorption with \textit{B. clarridgeiae} did remove most seroreactivity to the 13.3- and 21.7-kDa antigens, \textit{B. clarridgeiae} absorption had little effect on the overall antibody-antigen profile.

\textit{Bartonella} antigen-specific seroreactivity in cats from local animal shelters. Sera from 601 cats at local animal shelters were screened for \textit{B. henselae} Houston-1 seroreactivity by Western blot analysis. These blots were examined for the predominance of those \textit{Bartonella} immunoreactive antigens recog-

\begin{table}[h]
\centering
\caption{Titers in three experimentally-infected cats of antibody to eight \textit{B. henselae} LSU16 antigens}
\begin{tabular}{llllllll}
\hline
Molecular mass band (kDa) & Cat no. & 0 & 4 & 8 & 12 & 16 & 20 \\
\hline
97.0 & 58 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 58 & 10 & 10 & 40 & 40 & 160 & 160 \\
76.0 & 69.0 & 39 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 58 & 10 & 10 & 40 & 40 & 160 & 160 \\
65.0 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 58 & 10 & 10 & 40 & 40 & 160 & 160 \\
45.0 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
16.9 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
15.0 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
13.3 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
11.3 & 39 & 10 & 10 & 40 & 40 & 160 & 160 \\
 & 182 & 10 & 10 & 40 & 40 & 160 & 160 \\
\hline
\end{tabular}
\end{table}

\* Week postinfection.
TABLE 2. Summary of Bartonella antibody reactivity in 601 shelter cats

| Molecular mass (kDa) | No. reactive |
|----------------------|-------------|
| 7.0                  | 303         |
| 8.0                  | 350         |
| 11.3                 | 462         |
| 13.3                 | 463         |
| 15.0                 | 255         |
| 16.9/18.0            | 452         |
| 19.0                 | 331         |
| 21.7                 | 290         |
| 28.2                 | 173         |
| 30.0/36.0            | 269         |
| 45.0                 | 388         |
| 48.0                 | 327         |
| 57.0                 | 461         |
| 60.5/65.0            | 467         |
| 69.0/81.0            | 515         |
| 97.0                 | 470         |

was observed most often with the 11.3-, 13.3-, 16.9/18.0-, 51.0-/57.0-, 60.5/65.0-, 69.0-/81.0-, and 97.0-kDa antigens. There were 279 different banding patterns observed, most representing one or two animals; however, 21 patterns which were each found in at least four cats accounted for 43% of the cats. Sera from 72 cats were reactive with all 16 bands. Forty cats showed no reactivity to the 16 bands. The most common patterns of Western blot reactivity are shown in Table 3.

The goal of this experiment was to compare the patterns of reactivity seen in the naturally infected cats with that seen in the experimentally infected cats. Because the cats from the animal shelters could have been at any week postinfection when serum was collected and because they could have been infected with one or more species or types of Bartonella, and considering the kinetics of antibody appearance and antibody persistence in the experimentally infected cats, six antigens were chosen for use in defining a positive cat: 7.0, 8.0, 11.3, 13.3, 15.0, and 16.9/18.0 kDa. Further, to include the detection of early infections, the requisite that the serum only needed to react with two of the six molecular mass antigens was established. Therefore, in this study, a cat was considered Bartonella-seropositive if it reacted with two of the following six antigens: 7.0, 8.0, 11.3, 13.3, 15.0, and 16.9/18.0 kDa.

Using the above definition, the prevalence of Bartonella seroreactivity in this population of cats from the local animal shelters was determined to be 80% (482 of 601). As would be expected, the naturally infected cats had a wider variability of banding patterns than did the experimentally infected cats. More variable patterns were seen in the positive cats than in the negative cats (Table 3). The 16 most common positive patterns represent 32.8% of all the cats (197 of 601) and 40.9% (197 of 482) of the positive cats. The five most common negative patterns represent 10.5% of all the cats (63 of 601) and 53.9% (63 of 119) of the negative cats. The six defining bands are absent (0 of 63) in the five most common negative patterns. Table 4 summarizes the most common banding patterns with the selected antigens. There were 234 different banding patterns observed among the 482 seropositive cats, most representing one or two animals; however, 13 patterns which were each found in at least five cats accounted for 92.7% (447 of 482) of the positive cats. There were 45 different banding patterns observed among the 119 seronegative cats, with the 4 most common negative patterns accounting for 94.1% (112 of 119) of all the negative cats. Thirty-eight percent (182 of 482) of the positive cats had antibodies to all six bands, and 83%

![FIG. 4. Immunoblot after absorption. Cat serum (cat 40) that was previously absorbed with whole cells (W) and cell lysates (C) of B. henselae LSU16 (lanes 2 and 3), B. henselae Houston-1 (lanes 5 and 6), B. claridgeae (lanes 8 and 9), B. quintana (lanes 11 and 12), or B. bronchiseptica, P. multocida, and E. coli (lanes 14 and 15) is shown. Unabsorbed serum (U) (lanes 1, 4, 7, 10, and 13) from cat 40 (B. henselae LSU16 infected) was treated in the same manner as the absorbed sera. The sera were also examined for antibodies to the following antigens which were focused upon in this experiment are indicated on the left (in kilodaltons). Molecular mass markers are shown on the right (in kilodaltons).](image)

![FIG. 5. Representative immunoblot used in the determination of the prevalence in 601 cats of Bartonella-specific antibodies to B. henselae Houston-1 antigen. Sera which reacted to two of the six molecular mass bands indicated on the right (in kilodaltons) were considered seropositive (+) for Bartonella-specific antibodies. Whether a cat was considered seropositive or seronegative (−) is shown at the top. The sera were also examined for antibodies to the following Bartonella-specific bands: 19.0, 21.7, 28.2, 30.0 to 36.0, 45.0, 48.0, 51.0 to 57.0, 60.5/65.0, 69.0 to 81.0, and 97.0 kDa.](image)
of the positive cats had antibodies to the 11.3-, 13.3-, and 16.9/18.0-kDa bands.

**DISCUSSION**

Immunofluorescent-antibody tests are currently the most widely used serological tests for the diagnosis of CSD in humans. Regnery et al. (35) and Zangwill et al. (44) report an 88% sensitivity and a 94% specificity for the IFA. ELISAs, which are inherently less subjective, less expensive, and less time-consuming than the IFA, are also being developed. Litwin et al. (30) reported on an enzyme immunoassay (EIA) to detect IgM and IgG antibodies to *B. henselae* based on an outer membrane preparation of *B. henselae*; the sensitivity and specificity of this test were indicated to be 94.1 and 99.2%, respectively, for IgM and 86.2 and 95.9%, respectively, for IgG. Western blot analysis of sera from patients with CSD has also been performed in order to identify critical antigens involved in eliciting an immune response in humans. A 17-kDa antigen has been identified that, when used as an EIA test antigen for *Bartonella* seroreactivity, showed an agreement of 92% with IFA-positive serum samples and 88% with IFA-negative serum samples (3). The outer membrane preparation used by Litwin et al. (30) consisted of several antigens that reacted with sera from patients with CSD: 209-, 208.5-, 208-, 116-, 80.0-, 30.0-, 23.0-, 10.0-, and 8.0-kDa bands, along with a cluster of proteins with molecular masses ranging from 35.0 to 49.0 kDa. A strong correlation between reactivity to the 8.0-kDa band and a positive result by IFA and EIA was reported (30). The approximate molecular masses of other *Bartonella*-immunoreactive antigens that have been reported include 97.0-, 69.0-, 45.0-, and 23.0-kDa (41) and 48.5-kDa (32). Identification of these immunodominant antigens is leading to the development of monoclonal antibodies targeted to those specific antigens. For instance, it has recently been reported that *B. henselae* species-specific monoclonal antibodies to a 40.0- and a 30.0-kDa antigen have been generated (33). Identification of immunodominant antigens and the generation of monoclonal antibodies to these antigens should lead to a greater understanding of the human immune response to CSD and to improved diagnostic tests.

While a great deal of progress has been made in studying the human humoral immune response to CSD, less is known about the feline humoral immune response to *Bartonella* infections. Furthermore, the role of antibody in the pathogenesis of *Bar-
Bartonella infections in both humans and cats has not been determined. Feline serological surveys to date have been done by IFA (10, 11, 23, 25, 35). EIAs with outer membrane preparations for detecting serum antibodies in experimentally infected cats have also been utilized (21). Currently, there are no reports on Western blot analysis of feline sera for the purpose of screening for Bartonella-reactive antibodies. Thus, the aims of this study were (i) to examine the feline humoral immune response to infection with B. henselae by ELISA; (ii) to identify, using Western blot analysis and absorption assays, those antigens which are recognized by the cat’s immune system and possible species- and type-specific immunoreactive antigens; and (iii) to determine the seroprevalence of Bartonella in cats from local animal shelters based on the presence of antibodies to those Bartonella-specific antigens identified in step 2 and to examine the sensitivities and specificities of individual bands against an internal standard.

Using nine cats experimen tally infected with B. henselae, we were able to follow the development of the feline humoral immune response to B. henselae. All nine cats developed IgM and IgG responses that, although variable in magnitude between cats, were characterized by a peak in IgM levels followed by a rise in IgG levels. In each cat, primary bacteremia decreased as IgG levels increased. Decreasing bacteremia with increasing IgG levels has been reported in other studies (5). Three patterns of bacteremia were seen in the nine experimentally infected cats: one pattern in which cats became abacteremic by week 7 (cats 37, 40, 50, and 184), a second pattern in which cats became abacteremic between weeks 9 and 13 (cats 39, 54, 182, and 223), and a third pattern in which the cat underwent a secondary bacteremia (cat 58). Interestingly, in the one cat (58) that underwent a secondary bacteremia the IgM levels remained low throughout the course of infection, the IgG response developed much more slowly than those in the other eight cats, and virtually no change in the IgM levels was seen from the end of the primary bacteremia to the commencement of the secondary bacteremia. The apparent abacteremic state of cat 37 in week 4, cat 54 in week 8, and cat 58 in weeks 7 to 9 may represent bacteremias below the level of detection (<35 CFU/ml of blood) in our assay rather than true abacteremia.

Western blot analysis of the sera from the experimentally infected cats identified at least 24 Bartonella-specific bands that were immunogenic in all nine cats. We observed detectable levels of antibodies to some of these antigens (11.3-, 13.3-, 16.0/18.0-, 45.0-, 65.0-, 69.0-, and 76.0-kDa bands) by 3 weeks postinfection, while others did not appear until 4 to 8 weeks postinfection (7.0-, 8.0-, 19.0-, 21.7-, 30.0-, 33.3-, 36.0-, 48.0-, 51.0-, 54.0-, 57.0-, 60.5-, 81.0-, and 97.0-kDa bands). The different kinetics for antigen-specific antibody responses seen with each cat suggest that the antigenicity of B. henselae LSU16 is dependent upon the individual cat’s immune system.

While it is difficult to compare bands of reactivity in Western blots from one study to another because of variations in protein preparations and electrophoretic conditions, many of the same molecular mass antigens reported in this study have been found in other studies with human sera. However, it has not been determined whether any of the antigens identified in this study are equivalent to those identified in the human serum studies. Those antigens identified in this study that have not been found in other Western blotting studies may reflect differences between the immune responses of humans and cats. In addition, these differences could be a result of antigenic variability among B. henselae strains.

The titer in serum from one cat with each pattern of bacteremia was determined in order to examine the magnitude of the antibody responses to particular antigens and to determine if there was any relationship between the titer of the antibody response and the bacteremic state of the cat. Cat 58, which underwent a secondary bacteremia, generated an antibody titer lower than that generated by cats 39 and 182 to all of the bands examined. Furthermore, the titer of the antibody response generated by cat 182 increased much more slowly than that generated by cat 39, even though the levels of bacteremia were approximately the same throughout the course of infection (except that the bacteremia in cat 182 persisted for 3 weeks longer than that in cat 39). Thus, it appears that the duration of bacteremia may be related to the magnitude of the antibody response (titer) generated. Chomel et al. (11) reported a similar association, in which titers were higher in bacteremic cats than in nonbacteremic cats. Together, these results may suggest a role for antibodies in the clearance of B. henselae. It has not been determined if antibodies are protective; however, passive-transfer studies could be useful in addressing these questions. Although the titer numbers for the low-molecular-mass bands are generally lower than those for the higher-molecular-mass bands, this may not truly represent a difference in immunoreactivity. The difference could be a result of more epitopes being available on higher-molecular-mass antigens for interaction with specific antibodies. These results warrant further investigation.

DNA hybridization studies comparing the interrelatedness of various species of Bartonella indicate that B. henselae Houston-1 and B. quintana are 71% related (8) while B. henselae Houston-1 and B. clarridgeiae are only 47% related (28). DNA hybridization studies have not been performed for B. henselae LSU16. Comparative seroreactive studies of cats, rabbits, and humans have been described in the literature (4, 19, 34). It has been shown by IFA that absorption of cat serum with B. henselae is able to distinguish species-specific reactivity to B. henselae from cross-reactivity to B. quintana antigen (4). Immunoelectrophoretic studies with rabbit sera and antigen preparations of B. henselae and B. quintana indicate that rabbit antibodies produce antibodies to seven unique antigenic proteins when immunized with B. henselae compared to immunization with B. quintana (17).

In our study B. henselae Houston-1 absorption and B. quintana absorption of serum from a cat experimentally infected with B. henselae LSU16 yielded antibody profiles that were indistinguishable from each other. However, these two absorption antibody profiles were different from that of the absorption performed with B. henselae LSU16 in that antibodies to the 76.0-, 69.0-, and 65.0-kDa antigens were not removed. Two variants (type 1 and type 2) of B. henselae have been identified based on sequence differences in the 16S rRNA (6). B. henselae LSU16 has not been typed at this time, and it is possible that B. henselae LSU16 could represent a type 2 variant, whereas B. henselae Houston-1 has been identified as a type 1 variant. Alternatively, the difference in absorption antibody profiles may represent the loss of these immunodominant antigens through multiple passage of the Houston-1 strain; B. henselae LSU16 is a new isolate that has not been extensively passaged. Cross-reactivity with the 13.3-kDa antigen was found for each of the Bartonella species examined. While some antibody profiles did not appear to be affected by absorption, antigen-specific titers could have decreased; this experiment does not directly address this question.

The high degree of diversity among Bartonella species sheds some doubt on the sensitivities of various diagnostic procedures. A prevalence study of B. clarridgeiae based on blood culture estimates a 16% prevalence in stray cats (24). Considering the relatively low DNA relatedness and the lack of cross-
reactivity between \textit{B. henselae} Houston-1 and \textit{B. claridgeiae}, it is likely that IFA seroprevalence studies utilizing a \textit{B. henselae} antigen preparation would underestimate the number of cats with antibodies to \textit{Bartonella}. Evidence for this supposition is provided in a study in which a patient with CSD was seronegative for \textit{B. henselae}, \textit{B. quintana}, and \textit{Bartonella elizabethae} but was seropositive for \textit{B. claridgeiae} (28). Furthermore, a number of patients with CSD who tested negative by IFA for \textit{B. henselae} Houston-1 were determined to be seropositive for \textit{B. henselae} Marseille (15). Some studies have indicated that 60% of patients with CSD test negative in standard IFA (16, 42). Development of a test that includes antigens specific to the various species and types of \textit{Bartonella} is critical in improving the sensitivities of the IFA and other diagnostic tests.

In examining the sera from the 601 cats at local animal shelters for \textit{Bartonella} seroreactivity, a number of antigens were identified that could aid in the improvement of \textit{Bartonella} serology tests. The Western blotting and titration results indicated that a strong antibody response to the 13.3-kDa antigen was generated and that the response persisted throughout the course of infection. In addition, this antigen was present in a high percentage of shelter cats in association with other bands which appear to be \textit{Bartonella} specific. Together, these results suggest that the utilization of this antigen as a test antigen may be beneficial in improving serological tests. The use of the 11.3- or 16.9/18.0-kDa antigens as test antigens could help to increase the sensitivity of serological tests; antibodies to these antigens and to the 13.3-kDa antigen were not detected in the most common negative patterns of seroreactivity (Table 3). On the other hand, the 76.0-, 69.0-, and 65.0-kDa antigens may be extremely useful in distinguishing between seroreactivity with \textit{B. henselae} LSU16 and cross-reactivity with \textit{B. henselae} Houston-1. The 97.0-kDa protein may also serve as a useful test antigen. However, these antigens were not present in as many animals and thus the number of false negatives with these antigens may be higher. Grouping of some of the higher-molecular-mass antigens may have affected the usefulness of each individual antigen. In the prevalence determination, the higher-molecular-mass antigens were not initially considered because of the difficulty in determining the molecular mass of each antigen from gel to gel (due probably to slight variations in protein preparation and electrophoretic conditions). Future studies utilizing a decreased percentage of polyacrylamide will result in greater separation of the higher-molecular-mass antigens. Greater separation of these antigens should yield a better picture of the immune response to them. In a situation where a highly specific test is desired, the use of the 21.7- and 28.0-kDa antigens as test antigens would be beneficial. The 7.0-, 8.0-, and 15.0-kDa antigens were used as screen test antigens because, although these antigens were not detected in as many positive cats, the antibody responses to the antigens when seen produced a signal that was easy to detect by Western blot analysis. We would expect that the sensitivities associated with these antigens will be lower, given that the antibody responses to the antigens waxed and waned throughout the course of infection. While these three antigens may not be useful in improving the sensitivities of \textit{Bartonella} serology tests, they may be used to differentiate between an acute infection and a chronic infection. However, this has not been determined and warrants further investigation.

In designing an antibody-based \textit{Bartonella} screening test, a number of factors, such as the stage of infection, the degree of infection, the immunological state and response of the infected individual, and the serotype of the infecting organism, must be considered; all of these factors can influence the results of the test. An ideal test is one that can handle all the variabilities associated with these factors; however, it is unlikely that a test with only one antigen could meet this criterion. Based on the results of this study, a test with some combination of the 11.3-, 13.3-, and 16.9/18.0-kDa antigens may be prudent.

Absorption assays and Western blot analysis have proven to be useful tools in identifying a number of \textit{Bartonella}-specific immunodominant antigens recognized by the feline immune system during experimental infection with \textit{B. henselae} LSU16. The generation and use of multiple monoclonal antibodies against these antigens will aid in improving the sensitivities and specificities of various serological diagnostic tests. Additional studies of the feline humoral immune response to infection with \textit{Bartonella} should help to identify the roles of these antigens in the immune response. Furthermore, these studies should lead to the advancement of our understanding of the pathogenesis of infections due to this microorganism.

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