

Prevalence of *Bartonella henselae* and *Bartonella clarridgeiae* in Stray Cats

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The aim of the present work was to determine by blood culture the prevalence of blood infection with *Bartonella* species in a well-defined, European, urban stray cat population. Therefore, 94 stray cats were trapped from 10 cat colonies. Blood samples of these cats were cultured on both blood agar and liquid medium in order to raise the likelihood of bacterial detection. Fifty blood samples (53%) gave a positive culture result for *Bartonella* species. Isolate identification was performed by sequencing the first 430 bases of the 16S ribosomal DNA. Three types of sequences were thus obtained. The first type (17 isolates; 34%) was identical to that of *B. henselae* Houston-1 and the corresponding strains were referred as *B. henselae* type I. The second sequence type (18 isolates; 36%) was identical to that initially described as "BA-TF," and the corresponding strains were referred to as *B. henselae* type II. The third sequence type (15 isolates; 30%) was identical to that of the *Bartonella clarridgeiae* type strain (ATCC 51734). Our study points out the major role of stray cats as a reservoir of *Bartonella* spp. which can be transmitted to pet cats and, consequently, to humans. The study also highlights the high prevalence of *B. clarridgeiae* (16%) in the blood of stray cats.

Bartonella henselae is a bacterium responsible for several human infections: cat scratch disease in immunocompetent patients and bacillary angiomatosis and hepatic peliosis in immunocompromised patients (1). Other pathological manifestations can also be associated with this bacterial infection, such as endocarditis, bacteremia, osteolytic lesions, pulmonary nodules, neuroretinitis, and neurologic diseases (1). The natural host of this bacterium is the cat, from the blood of which it can be recovered. Nevertheless, no pathologic process has ever been identified in bacteremic cats. The prevalence of this bacterial infection, as determined by culture of cat blood, ranges from 4 to 70% according to the study and the type of cat population studied: pet, impounded, or former stray cats (6, 15). However, these studies provided evidence of only *B. henselae* isolates, not the newly described species *Bartonella clarridgeiae* (20), and did not look at stray cat populations. This study was performed in the context of a program aimed at evaluating the prevalence and circulation of infections in free-ranging feline populations. Our work consisted more specifically of the determination by blood culture of the prevalence of infection with *Bartonella* spp. in a well-defined urban stray cat population.

MATERIALS AND METHODS

Bacterial strains. *B. clarridgeiae* ATCC 51734^T was kindly provided by J. Clarridge III (Houston, Tex.), and *Bartonella doshiae* NCTC 12862^T and *Bartonella vinsonii* ATCC VR-152^T were kindly provided by R. Birtles (London, United Kingdom). *B. henselae* ATCC 49882^T was purchased from the American Type Culture Collection (ATCC; Rockville, Md.).

Cat population. The study was performed between May and December 1995 in the town of Nancy, which is in the eastern part of France. The cats enrolled in the study were living in 10 different cat colonies, labeled A to J (Fig. 1). These cat colonies were located in interior courtyards of urban blocks, as well as in the vicinity of collective or individual housing. One colony was located in a public

garden (colony D). The majority of the cats in colony G consisted of a permanent core group in association with some looser members. The 10 colonies studied were separated from each other by at least 350 m (Fig. 1). The territorial surface area of each of the colonies was an average (\pm standard deviation) of 23,000 m² (\pm 14,000 m²). The territorial surface area of each of the colonies was estimated by the investigators according to the location where they saw the cats and information given by the people feeding the cats. Each cat colony contained an average of 18 cats (\pm 7 cats). The number of cats in each colony was estimated by the individuals feeding each cat population.

The cats were caught by using an automatic box trap. All the cats were marked with an electronic identification system (Trovan; EuroID, Paris, France). At the time of blood sampling, sex and weight were recorded, and age was estimated by the investigators according to the level of erosion of the permanent teeth. The genetic structure of the fur was determined directly along with the phenotypic characteristics, in accordance with the criteria of Robinson (29), and the data were confirmed by another investigator with the help of pictures which were taken of each cat.

Isolation of strains. Approximately 2.5 ml of cat blood was collected aseptically with a syringe from the external jugular vein of each cat. One milliliter of blood was put into a serum-separating tube for serological analyses; the remainder (1.5 ml) was placed into a Pediatric Isolator 1.5 tube (Wampole Laboratories, Cranbury, N.J.) for hemolysis and was streaked without prior centrifugation onto two blood agar plates (200 μ l per plate). The medium used for primary culture and for subcultures was a Columbia agar base (Difco, Detroit, Mich.) containing 5% defibrinated rabbit blood hemolyzed with 1% saponin (Sigma, St. Louis, Mo.). Rabbit blood was collected aseptically by intracardiac puncture. Incubation of the plates was performed at 35°C in a moist atmosphere containing 5% CO₂ for up to 2 months. Enumeration of bacterial colonies was performed for each plate in order to obtain the numbers of CFU per milliliter of blood for all cats. Three randomly selected plates without any sample were incubated in parallel with the inoculated blood agar plates of the same batch as a sterility test. The blood remaining in the Pediatric Isolator 1.5 tube (about 1 ml) was injected into a BACTEC Peds Plus vial (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) containing 20 ml of broth supplemented with 500 μ l of Bacto Fildes enrichment (Difco Laboratories). The BACTEC Peds Plus vials were tested for CO₂ production each week for up to 6 months by a BACTEC NR 660 apparatus, the detection threshold being fixed at 20 arbitrary units according to the manufacturer's recommendations. The positive BACTEC Peds Plus vials were tested for the presence of bacteria by light microscopic examination, and their contents were subcultured on blood agar medium.

Light microscopic examination. The cultured microorganisms were evidenced by acridine orange staining and with the enhanced Gram stain (Carr Scarborough Microbiologicals, Inc., Stone Mountain, Ga.) by using a counterstain of Kinyoun carbol fuchsin at a 1/20 dilution (19).

Electron microscopy. Bacteria were suspended in phosphate-buffered saline and added to a 1% (wt/vol) solution of phosphotungstic acid at pH 7.5 (Prolabo,

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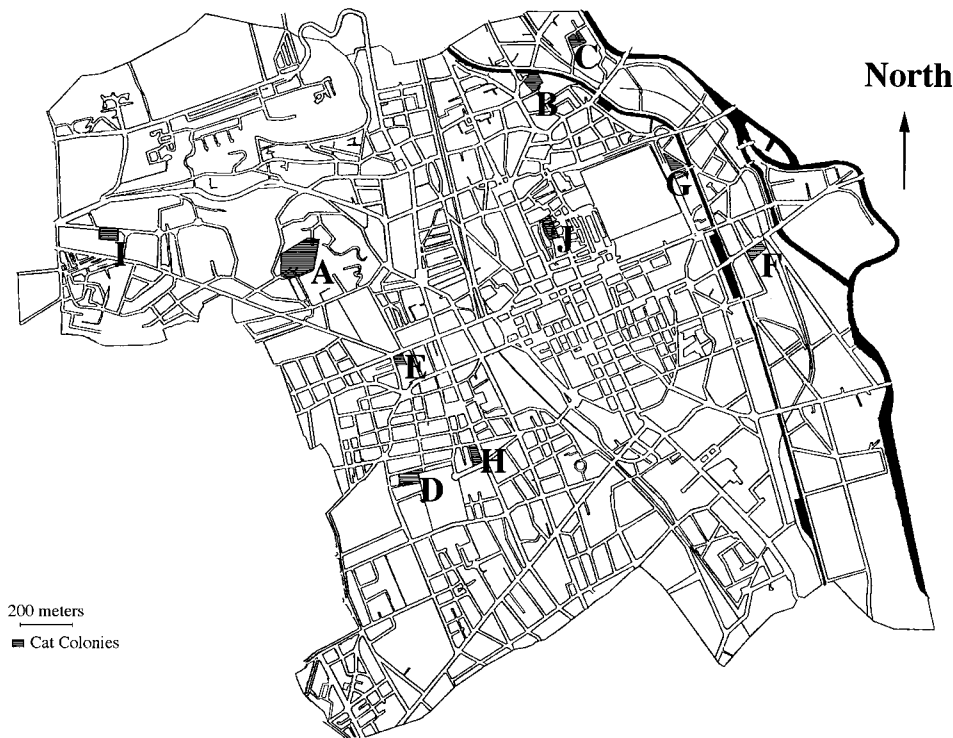


FIG. 1. Map of the urban area of Nancy, France, and locations of territories of cat colonies A to J.

Fontenay-sous-Bois, France). Negatively stained bacteria were observed with a Philips EM 410 electron microscope (Philips, Eindhoven, The Netherlands).

Isolation and amplification of the 16S ribosomal DNA. Chromosomal DNA was extracted from several colonies (3 to 10 colonies) obtained on blood agar plates by the method described by De Lamballerie et al. (10). DNA amplification in vitro was performed with *Taq* DNA polymerase (Gibco BRL, Cergy-Pontoise, France) and two 16S ribosomal DNA eubacterial universal primers, primers P8 (5'-AGAGTTTGATCCTGGCTCAG-3') and Pc1544 (5'-AAGGAGGTGATC CAGCCGCA-3'). Thermal cycling consisted of denaturation at 93°C for 5 min, followed by 30 cycles each of denaturation at 93°C for 30 s, primer annealing at 52°C for 1 min, and primer extension at 72°C for 1 min. After the last cycle, a final extension was performed at 72°C for 8 min, and the mixture was then cooled to 5°C. Thirty amplification cycles were performed in order to limit the amount of sequence errors generated by the *Taq* DNA polymerase. Standard procedures to prevent sample DNA contamination were taken (18). Particular attention was given to sample preparation, PCR amplification, and electrophoresis, which were performed with separate sets of pipettes, protective blouses, and caps in three separate and closed rooms where *Bartonella* spp. had never been cultured. Gloves were changed between handling of each sample, and all solutions were handled with pipettes with hydrophobic filter tips (Molecular BioProducts, San Diego, Calif.). Negative controls were included in each experiment to verify the absence of cross-contamination between samples and between previously amplified products and field samples.

Purification of the amplified product and DNA sequencing. The amplified DNA fragment was purified by phenol extraction and isopropanol precipitation (5). Sequencing was performed by using internal primer Pc535 (5'-GTATTAC CGCGGCTGCTGGCAC-3') 5' labeled with fluorescein isothiocyanate and with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, Buckinghamshire, England) according to the manufacturer's instructions. The sequence was obtained with an A.L.F. DNA Sequencer (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Sequencing was performed only once because all the sequences obtained corresponded exactly to 16S ribosomal DNA sequences known previously.

16S ribosomal DNA sequence analysis. The amplified sequence was compared to the sequences of other bacteria including *Bartonella* spp. contained in the EMBL-GenBank database. The sequences were aligned by using the clustal method (12) on DNASTar software (DNASTar Inc., Madison, Wis.).

Amplification of the *htrA*-like gene. Amplification and hybridization were done as described previously (2).

Serological analysis. Detection of feline immunodeficiency virus (FIV) antibodies and feline leukemia virus (FeLV) antigens by an enzyme-linked immu-

nosorbent assay method was performed as described previously (21, 27) by using the Petchek-FIV-antibody and the Petchek-FeLV tests (Idexx Laboratories Inc., Westbrook, Maine), respectively.

Statistical analysis. Association between qualitative factors and infection status was assessed by the chi-square test. Chi-square values and standard deviations were calculated by using Stat View IV software (Abacus Concept Inc., 1992).

Nucleotide sequence accession numbers. The EMBL-GenBank accession numbers for the 16S ribosomal DNAs are as follows: *B. henselae* Houston-1, M73229 (26); *B. henselae* BA-TF, Z11684 (28); *B. clarridgeiae*, X89208 (20); strain 73 (CIP104772, Collection de l'Institut Pasteur, Paris, France), X97822; *B. vinsonii*, LO1259; and *B. doshiae*, Z31351.

RESULTS

Cat population. A total of 94 cats were sampled. Samples from a minimum of six cats from each colony were tested. The sample size corresponded to 55% ($\pm 15\%$) of the cats in each colony (Table 1). Of the 94 cats studied, 50 (53%) were female and only 4 were spayed or neutered. The average estimated age was 2.9 years (± 1.7 years); only 11 cats (12%) were under 1 year of age. The average weight was 3.5 kg (± 0.9 kg).

Culture and identification. Fifty of the 94 (53%) blood samples obtained from the 10 cat colonies yielded a positive culture result for *Bartonella* spp. Sequencing of the first 430 bases of the 16S ribosomal DNAs from these bacteria generated three types of sequences. The first type (17 isolates; 34%) was identical to that of *B. henselae* Houston-1. The second type (18 isolates; 36%) was identical to the sequence initially described as "BA-TF," and the corresponding strains were referred as "*B. henselae* type II." The third type of sequence (15 isolates; 30%) was identical to that of the 16S ribosomal DNA of strain 73 that we previously isolated from a cat and was also identical to the sequence of the *B. clarridgeiae* type strain (ATCC 51734), as determined by us.

Strains with a 16S ribosomal DNA sequence identical to the *B. henselae* Houston-1 sequence were further characterized

TABLE 1. Isolation of *Bartonella* spp. from blood of stray cats

Colony	Total no. of cats (estimated)	No. (%) of cats sampled	No. of isolates recovered			Total no. (%) of cats positive
			<i>B. henselae</i> type I	<i>B. henselae</i> type II	<i>B. clarridgeiae</i>	
A	21	10 (48)	0	0	1	1
B	14	8 (57)	2	0	0	2
C	18	8 (44)	0	2	0	2
D	17	7 (41)	0	5	0	5
E	11	7 (64)	0	2	0	2
F	24	8 (44)	5	0	0	5
G	18	16 (76)	7	2	4	13
H	21	7 (39)	3	1	0	4
I	7	6 (86)	0	3	1	4
J	34	17 (50)	0	3	9	12
Total	185	94 (53)	17	18	15	50 (53)

more precisely since *B. doshiae*, *B. vinsonii*, and *B. henselae* Houston-1 have the same 16S ribosomal DNA sequence for the 430 first bases. Differentiation between these three species was determined by controlling for the presence or the absence of *htrA*-like sequences by in vitro amplification followed by hybridization with a labeled probe. We evidenced that amplicons obtained from the type strains of *B. doshiae* and *B. vinsonii* did not hybridize with the specific probe for the *htrA*-like sequences, whereas that of *B. henselae* Houston-1 did. All our strains with 16S ribosomal DNA sequences identical to that of *B. henselae* Houston-1 possessed *htrA*-like gene sequences and hybridized with the probe; they were therefore referred as "*B. henselae* type I."

Isolation of *Bartonella* spp. was dependent on the culture medium and on the *Bartonella* species. Most of the *B. henselae* isolates (80%) grew on blood agar and in BACTEC medium, whereas all isolates of *B. clarridgeiae* were obtained only from BACTEC medium. For *B. henselae*, four strains (11%) were isolated only on solid medium and three strains (9%) were isolated only in liquid medium. In fact, use of BACTEC medium permitted the isolation of more than one-third of the strains (18 strains). On primary culture, *B. henselae* was obtained within 2 to 6 weeks on blood agar medium (average, 2.8 ± 1.1 weeks) and within 2 to 19 weeks in BACTEC vials (average, 7.1 ± 4.2 weeks). The 15 *B. clarridgeiae* isolates were obtained from BACTEC vials within 2 to 7 weeks (average, 3.7 ± 1.4 weeks). The numbers of *B. henselae* grown on blood agar ranged from 4 to 4,000 CFU/ml (average, 424 ± 384 CFU/ml).

Epidemiology of *Bartonella* sp. infection according to cats and viral infection. No correlation between the prevalence of bacteremia and the sex of the cats was found. Age was a significant risk factor for *Bartonella* infection, because 26 (28%) of the cats were 1 year old or younger, but 18 (69%) of these young cats were bacteremic ($P = 0.055$), whereas 32 (47%) of the 68 adult cats (>1 year old) were bacteremic. From cats in six colonies (colonies A to F), accounting for 48 sampled cats, a single strain type was recovered: *B. henselae* type I (colonies B and F), *B. henselae* type II (colonies C, D, and E), or *B. clarridgeiae* (colony A). Two strain types were present in cats in three colonies (colonies H, I, and J). All three strain types were recovered from only one cat colony (colony G) (Table 1). Nine of the 94 (10%) cats (1 female and 8 males) were found to be seropositive for FIV, and no cat was found to be infected with FeLV. No correlation were found between bacteremia and FIV infection. The only correlation observed

was between sex (male) and seropositivity for FIV ($P < 0.05$), a relationship formerly described in other studies (9).

DISCUSSION

This is the first study of infection with *Bartonella* spp. in stray cat colonies. Stray cats living in towns are known to group themselves in structured colonies (23, 24), and intercolony exchange of individuals is very rare (13). These cats are not tame, but are completely dependent on humans for their food, which is given deliberately by cat lovers or obtained by searching in garbage (11). More than half (53%) of the stray cats were bacteremic for *Bartonella* spp. This infection rate is in accordance with that found in a study of former stray cats, in which 70% of the cats sampled (31 of 44 cats) were bacteremic for *B. henselae* (6).

We identified *Bartonella* species by sequencing the first 430 bases of the 16S ribosomal DNA. This region allows for a distinction between most *Bartonella* species to be made, in particular between the different species isolated from cat blood. This particular method of identification was selected because (i) the number of phenotypic tests available for identifying *Bartonella* spp. is limited, (ii) these phenotypic tests are often described only for a few isolates of a single species and thus their discriminative value for identification is often questionable, (iii) the genus *Bartonella* has not been extensively studied, so unknown species for which no phenotypic data for identification are available may be isolated, and (iv) this method classifies the isolates in an evolutionary tree, as shown previously by Woese (31). The other methods described for the identification of *Bartonella* spp. (8, 14, 22, 30) need to be updated to include the new species.

Although the DNA that was amplified was extracted from several colonies of the primary cultures for *B. henselae*, no ambiguity in the 16S ribosomal DNA of each of the sequences obtained was found. Therefore, no simultaneous infection with different *Bartonella* types was evidenced. These data do not exclude the possibility of infection with multiple *Bartonella* types, because (i) *B. clarridgeiae* isolates never grew on primary isolation on our solid medium; (ii) DNA from only 3 to 10 colonies was tested by extraction, amplification, and sequencing; and (iii) if a small minority of the sampled colonies had another type of sequence, they would not give a signal big enough on the chromatographic profile to interfere with the interpretation of the major signal.

Our study indicates the high prevalence of the recently de-

scribed species *B. clarridgeiae* in French stray cats. This species was evidenced in the blood of one of six cats and in nearly one-third of the *Bartonella* sp.-positive blood samples. In previous studies on the prevalence of *Bartonella* spp. in cat blood, blood agar was used as the growth medium and none of those studies reported isolation of *B. clarridgeiae* but reported the isolation only of *B. henselae*. In our study, detection of *B. clarridgeiae* was made possible because of the use of BACTEC vials. No isolate of this species was able to grow on our blood agar medium in primary culture. This underlines the need to use various and sensitive culture methods for the optimal recovery of these bacteria. The 15 *B. clarridgeiae* isolates grew weakly and inconsistently in subcultures on solid media. By contrast, the *B. clarridgeiae* type strain (8) grew in primary culture on brain heart infusion agar with 10% sheep blood. This was due either to the nutritional requirement of this isolate or to the precise chemical composition of the blood agar used. These two factors (type of strain and culture conditions) may explain why this species remained unrecognized for several years. Our results showing a significant prevalence of *B. clarridgeiae* infection in cats are not due to a geographic artifact or to particular epidemiological circumstances since this bacterium was also isolated with the same frequency (one-third of the isolates) from farm cats and pound cats living 100 and 50 km, respectively, from the town of Nancy during the same period; none of these isolates grew on blood agar in primary culture either (unpublished data). The 16S ribosomal DNA sequence that we obtained from the *B. clarridgeiae* type strain was exactly the same as the one that we determined for strain 73 and for the 15 isolates from stray cats. The sequence of *B. clarridgeiae* (type strain) as it appears in the EMBL-GenBank data base (accession no. X89208) differs by two bases from the sequence that we obtained. This two-base difference is located in a nonvariable region for all the other *Bartonella* spp. and could be a mistake. Both the type strain of *B. clarridgeiae* and strain 73 have polar flagella when observed by electron microscopy. This feature is consistent with the classification of both isolates in the same species, because *B. clarridgeiae* is the only known *Bartonella* species which has flagella and which can grow at 37°C.

The role of this new bacterial species in human pathology is not defined yet. A *Bartonella* sp. with flagella, therefore resembling *B. clarridgeiae*, was incriminated in a unique case of cat scratch disease after it was isolated from the patient's cat. However, although the patient responded serologically to this particular isolate, no direct detection of the bacterium was obtained from the patient's samples (16). By contrast, the two types of *B. henselae* that have been referred as *B. henselae* types I and II according to Bergmans et al. (3, 4) and that we isolated from cat blood have been demonstrated to be responsible for human pathology (3, 4).

The risk of human *Bartonella* infection from stray cats can be direct and indirect. Stray cats do not allow themselves to be stroked, and hence, their contacts with humans (scratching and biting) are relatively limited and the risk of direct infection should be low. The risk of indirect infection is greater, because pet cats, which are occasionally outside, can acquire *Bartonella* spp. either if they are scratched or bitten by stray cats or, more likely, if they are bitten by fleas (*Ctenocephalides felis*). The role of these arthropods as the vectors of *Bartonella* between cats has been demonstrated previously (7). These pet cats can later infect humans. Therefore, stray cats play a role as a reservoir for these bacteria.

B. henselae infection has been demonstrated more frequently in young cats than in older ones (6, 15, 32); our results are consistent with this observation. This may result from

growth not repressed by the immune system of animals in contact for the first time with *Bartonella* spp. By contrast, older stray cats are more likely to have been in contact with *Bartonella* spp. since most of them have specific antibodies, as demonstrated previously by Chomel et al. (6). These older animals often have chronic infection (15, 17) with low concentrations of bacteria. Therefore, detection of bacteremia in these older animals may be less successful.

In this work, no cat had evidence of FeLV infection and FIV infection was not correlated to infection with *Bartonella* species; this latter point is in accordance with the results formerly published by Chomel et al. (6). In fact, stray cats usually die before becoming immunocompromised because their life span is about 4 to 5 years and the asymptomatic period of FIV infection is about 3 to 5 years (25). In contrast to FIV infection, which is more frequently observed in males because it is preferentially transmitted by bites or scratches during fights (9), *Bartonella* sp. infection did not appear to be associated with the sex of the cats. This suggests that *Bartonella* spp. and FIV have distinct transmission routes and that scratches and bites are not the preferential means of transmission of *Bartonella* infection. Intercat transmission of *B. henselae* is therefore more likely to occur by another route, such as via cat fleas (7).

In certain cat colonies (e.g., colonies D and F), only bacteria with one type of 16S ribosomal DNA were recovered, suggesting intracolony and not intercolony transmission of the bacterium. This hypothesis is confirmed by the fact that intercolony exchange of individual cats, even if they are nearby, is known to be extremely rare, as described by Izawa et al. (13). This was also evidenced by the genetic structure of the fur of 94 cats that we sampled, which was very different among the 10 colonies (data not shown).

New procedures are now required for more refined epidemiological studies and also for the precise detection of *Bartonella* spp., particularly for the detection of *B. clarridgeiae*, which has become a new candidate for possible human bartonellosis.

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