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Detection and Identification of Two *Bartonella henselae* Variants in Domestic Cats in Germany

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To determine the prevalence of bacteremia caused by *Bartonella henselae* in domestic cats in the region of Freiburg, Germany, we investigated cultures of blood from 100 cats from 89 different households over a 12-month period. *B. henselae* could be isolated from 13% (13 of 100) of these cats. In eight households with two cats each and in one household with three cats, *B. henselae* bacteremia was found either in all of the animals or in none of the animals. Positive cultures were more likely to be found for female, young (24 months of age or younger) cats than for male or older cats. Identification of the *Bartonella* isolates was made by colony morphology, by Gram staining, biochemically by RapID ANA II or Rapid ID 32 A systems, and by whole-cell fatty acid analysis. Differentiation between *B. henselae* and *Bartonella quintana* was only possible by 16S rRNA sequencing, enterobacterial repetitive intergenic consensus (ERIC)-PCR, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Genomic fingerprinting of the *B. henselae* isolates by ERIC-PCR yielded two different patterns based on three distinct bands.

Bartonella (previously *Rochalimaea*) *henselae* was recognized for the first time in the United States in 1990 as the causative agent of fever, bacteremia, bacillary angiomatosis, and peliosis hepatis (7, 9, 11). Subsequently, Regnery et al. (8) and Zangwill et al. (17) demonstrated the association of *Bartonella henselae* with cat-scratch disease (CSD), an inflammatory infection of the lymph nodes. CSD is quite common and affects approximately 22,000 people in the United States annually (2). In contrast to CSD, which develops in immunologically healthy people, bacillary angiomatosis and peliosis hepatis are most common in individuals infected with the human immunodeficiency virus (10).

In 1994 Koehler et al. (3) demonstrated that 41% (25 of 61) of domestic cats in the Greater San Francisco Bay Region of Northern California had long-lasting bacteremia with *B. henselae*. The numbers of CFU per milliliter of blood varied from 3 to more than 1,000. This bacterium was also detected in fleas taken from infected cats.

To obtain comparable data for the region of Freiburg, Germany, we investigated blood samples from 100 cats. Our study was initiated to determine the role of our domestic cats as a possible reservoir for *B. henselae* in the etiology of CSD.

This is, to our knowledge, the first study which provides data on the prevalence of *Bartonella* species in domestic cats in Europe.

MATERIALS AND METHODS

Isolation of *Bartonella* organisms. During a 12-month period, the blood of 100 unselected domestic cats from 89 different households was obtained from a veterinary clinic. A physical examination of the animals was performed by an experienced veterinarian at the time that the blood for culture was drawn. The majority of these animals were brought to the veterinary clinic for castration or ovariohysterectomy. One lysis-centrifugation tube (Isolator system; Wampole Laboratories, Cranbury, N.J.) was filled with 1 ml of blood from each cat under aseptic conditions. Six chocolate agar plates incorporating 10% defibrinated sheep blood were each inoculated with 0.1 ml of the lysis-centrifugation sediment. The plates were incubated at 37°C in a 5% carbon dioxide atmosphere for 8 weeks.

From each of 60 different cats, two swabs (Culture Swab; Difco Laboratories, Detroit, Mich.) from the vestibular area of the teeth and gingiva were taken. One swab of each pair was suspended in 1 ml of nutrient broth, and the broth was diluted by a factor of up to 10^{-9}. From each dilution ($\geq 10^{-4}$), 0.1 ml was inoculated onto a chocolate agar plate and the plate was incubated as reported above. The other swab was suspended in 1 ml of phosphate-buffered saline, and DNA was extracted and used for the PCR amplification as described below.

Type strains. *B. henselae* and *Bartonella quintana* were obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. (*B. henselae* G5436), and from the Collection de l'Institut Pasteur (CIP), Paris, France (*B. henselae* 103737 and *B. quintana* 103739), and were used as controls for further testing.

Biochemical analysis. Biochemical tests were performed by using the Rapid ID 32 A (BioMerieux) and the RapID ANA II (Innovative Diagnostic Systems Inc.) systems.

Susceptibility testing. For susceptibility testing of various antibiotics, E-test strips (AB Biodisk, Solna, Sweden) were used to determine the MICs. The E-test was used as suggested by the manufacturer, except that we used chocolate agar plates and a longer incubation time (5 days, until bacterial growth became visible).

DNA extraction. The strains were grown on chocolate agar plates at 37°C in 5% CO_2 for 4 to 5 days. Cultures from each plate were harvested in 1 ml of 0.01 M phosphate-buffered saline and were centrifuged in 1.5-ml microcentrifuge tubes. The pellet was suspended in 100 μl of lysis buffer (10 mM Tris, 10 mM EDTA), and 10 μl of 10% Nonidet P 40 solution was added. The mixture was cooled on ice for 5 min and was subsequently heated for 4 min at 95°C. After centrifugation for 3 min, the supernatant was mixed with 200 μl of chloroform-isoamyl alcohol (24:1) and was centrifuged again. The upper phase was removed and added to 10 μl of 3 M sodium acetate and 275 μl of ice-cold ethanol, and the mixture was stored at −70°C for 30 min. After centrifugation the sediment was dried, suspended in 100 μl of distilled water, and used as a template for the PCR.

PCR amplification of 16S ribosomal DNA and sequencing. Primers p24E and p12B, previously described by Relman et al. (9), were used to amplify a *Bartonella* 16S rRNA gene fragment by PCR. The reaction mixture consisted of bovine serum albumin (8 ng/μl), deoxynucleoside triphosphates (200 μM each), primers (117 nM each), *Taq* polymerase (2 U; Pharmacia Biotech), and 2.5 μl of extracted DNA in 50.0 μl of TBE (Tris borate-EDTA) buffer. The mixture was overlaid with two drops of light mineral oil. PCR amplifications were performed in an automated thermal cycler (Robocycler 40; Stratagene) with initial denaturation (95°C, 5 min), followed by 30 cycles of denaturation (90°C, 1 min), annealing (57°C, 1 min), and extension (72°C, 90 s), with a single final extension (72°C, 3 min). After the reaction, 20 μl of the product was separated on a 1.5% agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid 667 film. The DNA molecular weight marker was a 1-kb ladder (ϕX174 replicative-form DNA *Hin*cII Digest; Pharmacia Biotech). All amplified products were sequenced in duplicate with an automated sequencer (373-A; Applied Biosystems), and their sequences were compared

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TABLE 1. Characteristics of the cats with *B. henselae* bacteremia

Cat no.[a]	Age (yr)	Gender[b]	No. of CFU/ml of blood	Growth time (days)
3	1	M	200	10
4	3	F	30	10
8	2	F	120	12
18	12	F	20	10
20	1	F	20	8
26	1	M	1,200	9
26a[c]	2	M	30	6
36	1	M	100	7
36a[c]	2	M	100	8
38	1	F	600	6
74	1	F	200	6
75	1	F	120	8
75a[d]	1	F	100	8
77	2	F	400	7
78	2	F	240	7
79	1	F	800	7

[a] Cats in the same household: cats 3 and 4, cats 36 and 38, cats 74 and 75, and cats 77, 78, and 79.
[b] M, male; F, female.
[c] A second positive blood culture was found 12 months later.
[d] A second positive blood culture was found 5 months later.

with the corresponding sequences for *Bartonella* species available in the EMBL database, Heidelberg (European Molecular Biology Laboratory, Heidelberg, Germany).

Enterobacterial repetitive intergenic consensus (ERIC)-PCR. The primers (primers ERIC1R and ERIC2) used in this study have previously been described by Versalovic et al. (14). The setup of PCRs was the same as that described above. The amplification cycles were as follows: 1 cycle at 94°C for 6 min; 30 cycles at 94°C for 1 min, 40°C for 1 min, and 68°C for 8 min; and 1 cycle at 68°C for 16 min. A 20-μl aliquot of the products was separated on a 1.0% agarose gel, stained with ethidium bromide, visualized on a UV transilluminator, and photographed with Polaroid 667 film. For analysis, the band patterns were compared with the patterns for DNA molecular weight marker VI (Boehringer Mannheim GmbH, Mannheim, Germany).

Whole-cell fatty acid (CFA) analysis. Cultures were grown on chocolate agar plates (37°C in 5% CO_2) and were harvested after 4 to 6 days. Fatty acid methyl esters were chromatographed on a Hewlett-Packard series II 5890 gas chromatograph as described previously (6). The molecules were identified by using a computer-assisted comparison of their retention times with a standard mixture (Microbial-Identification System, Newark, Del.).

Electrophoresis. One-dimensional analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (5) with a 10% separating gel and a 4.5% stacking gel. Low-molecular-weight standards (Bio-Rad) were used to estimate the molecular weights of the separated proteins. The gel was stained with Coomassie blue R-250 (Serva).

RESULTS

Isolation of *Bartonella* organisms. Bacteremia with *B. henselae* was found in the blood of 13 of the 100 cats investigated. Despite bacteremia, all cats were asymptomatic. We obtained blood from two cats each in nine households and from three cats in one household. Among the cats in these 10 households, bacteremia caused by *B. henselae* was found either in all animals (three households with two cats each and one household with three cats) or in none of them (six households with two cats each). Eleven of the 13 cats with a *B. henselae* bacteremia were 2 years of age or younger, and 10 of 13 were female (Table 1). In contrast, of all 100 cats investigated, only 47 were female and only 60 were 2 years of age or younger. On agar, the first growth of the colonies was visible after 6 to 12 days. Highly adherent, deeply embedded, small rough colonies with an average size of 0.3 to 2 mm appeared on the chocolate agar. The best growth occurred on fresh agar plates (less than 5 days old), and with several specimens growth was seen around contaminating colonies of a coagulase-negative staphylococcus strain from a cat. *B. henselae* formed satellite colonies around the staphylococcus species, and this strain was cocultivated in all cultures. After the fifth subculture, the time necessary for visible growth decreased from 2 weeks to 3 days. Due to the long incubation periods, problems with contaminations with molds or a swarming, spore-forming bacillus, which rapidly grew over the plates, were encountered. We lost the strains from cats 4, 18, and 20. The numbers of CFU of *B. henselae* per milliliter of blood varied from 20 to 1,200. A second blood sample for culture was obtained from four cats. Three of them revealed persistent or recurring *Bartonella* bacteremia (Table 1) after 5 (cat 75a) or 12 (cat 26a and cat 36a) months. All gingival swabs taken from the other 60 cats of a different population were negative for *Bartonella* species in culture and by PCR.

Morphology and biochemical characteristics. Gram stains of the isolates revealed small, slightly curved gram-negative rods. Biochemically, the isolates were catalase, oxidase, and indole negative. The reaction profiles of the isolates and the controls in the RapID ANA II system and in the Rapid ID 32 A system were 000 671 and 0000 0737 05, respectively. The results obtained with the latter system have not been reported before. Identification to the species level was not possible by biochemical methods. Furthermore, strains of *Corynebacterium* species isolated from cultures of blood from two cats had identical reaction profiles in both systems.

Antibiotic susceptibilities. All isolates were susceptible to the antibiotics tested. The MICs of erythromycin were ≤0.016 μg/ml for all strains tested. The MICs ranged from 0.25 to 4 μg/ml for gentamicin, from 0.016 to 1.00 μg/ml for tetracycline, from 0.25 to 1.50 μg/ml for ciprofloxacin, and from 1.00 to 4.00 μg/ml for vancomycin.

PCR amplification of 16S ribosomal DNA and sequencing. A fragment of the 16S rRNA gene was amplified from the DNA extracted from the type strains and the isolates from cats. Sequencing of this 241-bp fragment revealed that all cats had bacteremia with *B. henselae.*

ERIC-PCR. All isolates were identified as *B. henselae* on the basis of the ERIC-PCR fingerprint patterns. Two different patterns (pattern 1, lanes F, G, L, M, and N of Fig. 1; pattern 2, lanes H, I, J, and K of Fig. 1) were observed, with each pattern having three distinct bands (Fig. 1). Therefore, subtyping of our isolates revealed two variants of *B. henselae* in our

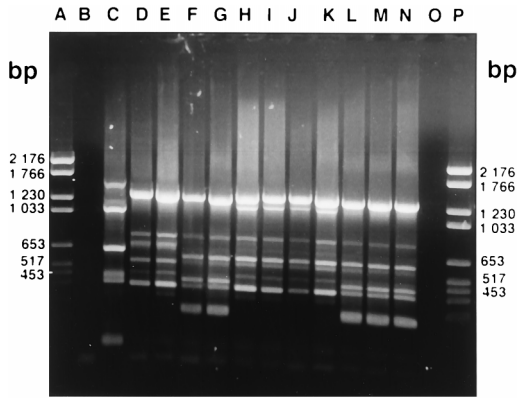

FIG. 1. ERIC-PCR of *Bartonella* species. Lanes A and P, DNA molecular size markers; lanes B and O, negative controls; lane C, *B. quintana* CIP; lane D, *B. henselae* CIP; lane E, *B. henselae* CDC; lane F, isolate from cat 3; lane G, isolate from cat 8; lane H, isolate from cat 36; lane I, isolate from cat 38; lane J, isolate from cat 74; lane K, isolate from cat 75; lane L, isolate from cat 77; lane M, isolate from cat 78; lane N, isolate from cat 79.

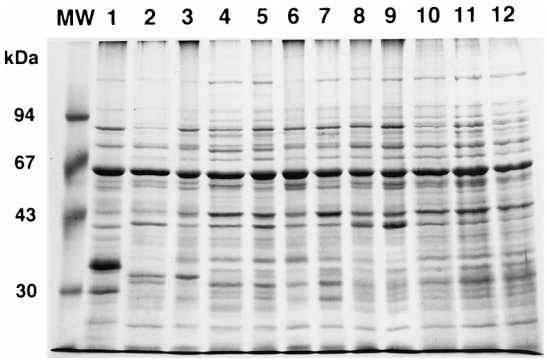

FIG. 2. SDS-PAGE profiles of the *Bartonella* strains. Lane MW, molecular mass standard; lane 1, *B. quintana* CIP 103739; lane 2, *B. henselae* CDC G5436; lane 3, *B. henselae* CIP 103737; lanes 4 to 12, *B. henselae* from cats 3, 8, 26, 36, 38, 74, 75, 77, and 78, respectively.

cats. The patterns of the type strains of *B. henselae* (the CDC and CIP strains) were different from those of our isolates.

CFA analysis. The major fatty acids observed for *B. henselae* and *B. quintana* are octadecanoic acid (18:1ω7; 50 to 58%), octadecanoic acid (18:0; 24 to 35%), and hexadecanoic acid (16:0; 9 to 20%). The results indicate that the cat isolates cannot be distinguished from the *B. henselae* or *B. quintana* type strains. Differentiation between *B. henselae* and *B. quintana* was not possible by CFA analysis.

SDS-PAGE analysis. SDS-PAGE analysis of Coomassie blue-stained gels showed distinct protein profiles for *B. henselae* and *B. quintana*. All of our isolates had protein profiles similar to those for the *B. henselae* type strain cultures (Fig. 2).

DISCUSSION

Thirteen percent (13 of 100) of healthy domestic cats in our study were found to have *B. henselae* bacteremia. Furthermore, our results indicated that cats in the same household were likely to be uniformly positive or negative. This suggests that *B. henselae* can easily be transmitted from cat to cat either by direct contact or by cat fleas, which are potential vectors (3, 17).

Koehler et al. (3) found in 41% (25 of 61) of their cats a *B. henselae* bacteremia. However, 37 of those cats came from only 13 different households, 22 cats came from a pound, and 2 were strays. Therefore, many of these animals had had close contact to each other, and this could be a reason for the high prevalence of *B. henselae* in that study.

Our results suggest that younger (24 months old or younger) and female cats are more likely than older animals to have a *B. henselae* bacteremia. *B. henselae* bacteremia was found in 18% (11 of 60) of all cats younger than 2 years but only in 5% (2 of 40) of the animals older than 2 years. Furthermore, we found that 21% (10 of 47) of all female and only 6% (3 of 53) of all male cats were infected. This supports the association between the development of bacillary angiomatosis or CSD and the exposure of these patients to young, female cats, which has been reported previously (2, 3, 13, 17). In three cases we could demonstrate persistent *B. henselae* bacteremia for at least 5 months (cat 26) or 12 months (cats 36 and 75). Consequently, it might be possible that the cats were already infected during the first months of life. The ability of *B. henselae* to persist in the bloodstreams of healthy cats is remarkable and has also been found in other cat populations (3, 4). However, we could not demonstrate *Bartonella* species from gingival swabs, and it is unlikely that *B. henselae* is part of the normal buccal flora. Domestic cats form a large reservoir for *B. henselae*, but the route of transmission to humans remains unknown.

Cat scratches or bites are possible modes of transmission of *B. henselae* to humans, but cat nails or cat saliva probably would have to be contaminated with infected blood. Recently, Kordick and Breitschwerdt (4) demonstrated the intraerythrocytic presence of *B. henselae* in feline blood by transmission electron microscopy. The examination of erythrocytes from two cats with a persistent *B. henselae* bacteremia for approximately 1 year identified 2.9 or 6.2% of infected cells, respectively. No epicellular or extracellular organisms were observed. Since *B. henselae* is intraerythrocytic, the blood specimen must be lysed in order to isolate this bacterium.

Biochemical tests have been reported to be nondiscriminatory for *Bartonella* species (1, 15). The results obtained with the RapID ANA II system differed from those of Welch et al. (15) and agreed with those of Clarridge et al. (1), who found identical profiles for *B. henselae* and *B. quintana*. We isolated a *Corynebacterium* species from two cats. These strains had the same profile in the RapID ANA II system as the *Bartonella* species. In the Rapid ID 32 A system *Corynebacterium xerosis* showed the same profile as *B. henselae* or *B. quintana.*

CFA analysis is useful in identifying members of the *Bartonella* genus, but it could not discriminate between *B. henselae* and *B. quintana*. The CFA patterns found in the present study are similar to those published previously (1, 12, 15, 16). Identification of the *Bartonella* species was done by 16S rRNA gene sequencing, SDS-PAGE, and ERIC-PCR. Genomic fingerprinting of the *B. henselae* isolates by ERIC-PCR yielded two different patterns based on three distinct bands. Isolates from three other cats in households with children with CSD showed patterns like those for the second variant (unpublished data). However, our isolates were different from the type strains (the CDC and CIP strains) by ERIC-PCR. Our results suggest the prevalence of various variants of *B. henselae* in different geographic regions (type strains versus isolates) as well as in cat populations. Further investigations should provide more information about the epidemiologies and pathogenicities of the different types of variants.

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