

## *Bartonella koehlerae* sp. nov., Isolated from Cats

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Two of the 25 *Bartonella* isolates recovered during a prevalence study of *Bartonella henselae* bacteremia in domestic cats from the greater San Francisco Bay region were found to differ phenotypically and genotypically from all prior *B. henselae* isolates. These isolates, C-29 and C-30, which were recovered from the blood of two pet cats belonging to the same household, grew on chocolate agar as pinpoint colonies following 14 days of incubation at 35°C in a candle jar but failed to grow on heart infusion agar supplemented with 5% rabbit blood. Additional phenotypic characteristics distinguished the isolates C-29 and C-30 from other feline *B. henselae* isolates. The restriction patterns obtained for C-29 and C-30 by citrate synthase PCR-restriction fragment length polymorphism (RFLP) analysis as well as by genomic RFLP could not be distinguished from each other but were distinctly different from that of the *B. henselae* type strain. In reciprocal reactions, DNAs from strains C-29 and C-30 were 97 to 100% related under optimal and stringent DNA reassociation conditions, with 0 to 0.5% divergence within related sequences. Labeled DNA from the type strain of *B. henselae* was 61 to 65% related to unlabeled DNAs from strains C-29 and C-30 in 55°C reactions, with 5.0 to 5.5% divergence within the related sequences, and 31 to 41% related in stringent, 70°C reactions. In reciprocal reactions, labeled DNAs from strains C-29 and C-30 were 68 to 92% related to those of the *B. henselae* type strain and other *B. henselae* strains, with 5 to 7% divergence. The 16S rRNA gene sequence of strain C-29 was 99.54% homologous to that of the type strain of *B. henselae*. On the basis of these findings, the two isolates C-29 and C-30 are designated a new species of *Bartonella*, for which we propose the name *Bartonella koehlerae*. The type strain of *Bartonella koehlerae* is strain C-29 (ATCC 700693).

*Bartonella* species cause a wide spectrum of human diseases. The principal causative agent of cat scratch disease in immunocompetent people is *Bartonella henselae* (9, 22). This bacterium is associated with bacillary angiomatosis and other clinical syndromes in immunocompromised humans (15, 21, 23, 27, 28, 32). Isolation of *B. henselae* from the blood of naturally infected cats, as well as the demonstration that cats remain highly bacteremic for several months, implicates cats as the major reservoir for this bacterium; the prevalence of *B. henselae* bacteremia in cats tested in northern California was 40% (6, 14). The prevalence of *B. henselae* antibodies in cats in the United States varies according to geographic region and ranges from 3.7% for Alaska to 54.6% for the southeastern states (12). The prevalence of another species, *B. clarridgeiae*, in a European urban stray cat population recently was demonstrated to be 16% (11), greater than that in the United States. The transmission of *B. henselae* from cats to humans usually occurs via scratches, and transmission from cat to cat readily occurs via the cat flea, *Ctenocephalides felis* (7). In the initial study identifying the prevalence of *B. henselae* bacteremia in domestic cats from the greater San Francisco Bay region, isolates were obtained from 41% (25 of 61) of the cats tested (14). Two of the 25 feline *Bartonella* isolates recovered during that study were phenotypically and genotypically characterized and found to be genetically closely related to, but distinct from, the type strain of *B. henselae* and, in addition, to differ phenotyp-

ically and genotypically from all other *B. henselae* isolates and all reported *Bartonella* species. These two feline isolates, C-29 and C-30, are proposed as a new *Bartonella* species, *Bartonella koehlerae*.

### MATERIALS AND METHODS

**Strains.** *B. henselae* type strain ATCC 49882 was provided by Russell Regnery, Viral and Rickettsial Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Ga. *Bartonella quintana* type strain ATCC VR-358 was obtained from the American Type Culture Collection (ATCC). The two *Bartonella* strains C-29 and C-30 were isolated from the blood of two cats belonging to the same household (14). *Bartonella* strains compared to C-29 by DNA hybridization analysis included *B. quintana* type strain ATCC VR-358, *B. henselae* ATCC 49882, *B. henselae* 88-712 (32), *B. henselae* G6486, *B. henselae* G6529, *B. henselae* G8378, *B. henselae* G5691, *B. elizabethae* ATCC 49927<sup>T</sup> (8), *B. vinsonii* ATCC VR-152<sup>T</sup>, *B. vinsonii* subsp. *berkhoffii* ATCC 51672 (17), *B. clarridgeiae* ATCC 51734<sup>T</sup>, *B. bacilliformis* ATCC 35685<sup>T</sup> (4), *B. grahamii* NCTC 12860<sup>T</sup> (2), and *B. doshiae* NCTC 12862<sup>T</sup> (2).

**Primary isolation of C-29 and C-30.** Blood was obtained from two cats during a prevalence study of *B. henselae* in domestic cats in the greater San Francisco Bay area of northern California (14). Blood (1.5 ml) was collected after thorough cleansing of the skin with 70% alcohol, using an aseptic technique similar to that used for humans, by jugular or saphenous venipuncture of each cat. Blood was transferred into a pediatric lysis-centrifugation tube (Wampole Laboratories, Cranbury, N.J.) and centrifuged, and the pellet was resuspended in inoculation medium (15). The resuspended blood pellet was plated onto BBL heart infusion agar (Becton Dickinson, Cockeysville, Md.) containing 5% fresh defibrinated rabbit blood, as well as onto chocolate agar. The chocolate agar (19) was prepared with hemoglobin powder and GC agar base (both from Acumedia Manufacturers, Inc., Baltimore, Md.) and was supplemented with 10 ml of IsoVitalax (Becton Dickinson). The plates were incubated at 35°C in a candle extinction jar for 3 weeks. Cultures were examined every 5 days for bacterial growth.

**Biochemical tests.** For the initial biochemical tests, standard methods were used; Gram staining and biochemical testing of the two isolates were performed as described previously (20). The inocula used for biochemical evaluations were obtained by growing both isolates, as well as the type strains *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358, on chocolate agar for 7 days at 35°C in a candle extinction jar. Preformed-enzyme tests were performed by using Mi-

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croScan Rapid Anaerobe Panels (31) (Baxter Diagnostics, Deerfield, Ill.) in accordance with the manufacturer's instructions regarding preparation, incubation, and biochemical interpretation. The reactions and identification codes obtained for the cat isolates C-29 and C-30 were compared to those of the *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358 type strains.

**PCR amplification and sequence analysis of the 16S rDNA.** Bacterial DNA was extracted from both *Bartonella* isolates by a standard protocol (33). The extracted DNA was then used as a template for amplification of 1,414 nucleotides of the gene encoding the 16S rRNA (16S rDNA). The 16S rDNA was amplified by using synthetic oligonucleotide primers (University of California, San Francisco, Biomolecular Resource Center) adapted from the primers P0mod and PC3mod (for the 5' half), P3mod and PC5 (for the 3' half) (34), and rD1 (30). In addition, two oligonucleotide primers corresponding to p24E and p12B (23) were utilized. The following restriction endonuclease sites were included in the oligonucleotide primers and then used to clone the PCR products into pUC18: for P0mod and P3mod, *Sall*; for PC3mod and PC5, *Hind*III; for rD1 and p12B, *Bam*HI; and for p24E, *Eco*RI. We synthesized three internal 16S primers (5'-GGTTGCCCCATTGTCC-3', 5'-CATTCAGTTGGGCACTC-3', and 5'-CCTTCCTCTCGGCTTAT-3') and primer BA-9-E (5'-GGAATTCCG GAGATGGATGAGCC-3'), the latter being located approximately 180 bp from the 5' end of the 16S rDNA, to complete sequencing of both strands of this rDNA. All PCR runs included negative controls (containing no DNA template) and positive controls (with *Bartonella* DNA).

The products of DNA amplification were digested with the appropriate restriction endonucleases, ligated into the vector pUC18 (Pharmacia, Piscataway, N.J.), and used to transform *Escherichia coli*. The sequences of the double-stranded DNAs of recombinants were obtained by the dideoxy sequencing method (26), using a *Taq* Dye Primer Cycle Sequencing kit (Applied Biosystems, Inc., Foster City, Calif.), followed by analysis on an automated sequencer (Applied Biosystems). Both DNA strands of a minimum of two clones containing overlapping fragments of the 1,414 nucleotides of the 16S rDNA were sequenced. Discrepancies were resolved when necessary by sequencing additional clones. The 16S sequence from strain C-29 was aligned with the 16S sequences, retrieved from GenBank, of 10 other strains, including *Bartonella* species, *Agrobacterium tumefaciens*, and, as the outgroup, *Ehrlichia canis*, using the Wisconsin Sequence Analysis Package program PILEUP (Wisconsin Package version 9.0; Genetics Computer Group, Madison, Wis.). The multiple-sequence alignment was edited to remove the 5' and 3' hypervariable regions (10). The resulting alignment was 1,330 bases long and corresponded to bases 106 to 1457 of *E. coli* (5). The edited alignment was used as input to the software program PHYLIP (version 3.5; J. Felsenstein, University of Washington, Seattle) to derive a phylogenetic dendrogram via the nucleotide substitution model of Jukes and Cantor (13) and the neighbor-joining method of Saitou and Nei (25). For determination of tree robustness, the multiple-sequence alignment was used as input to the PHYLIP SEQBOOT program and a majority-rule strict consensus tree was generated by the CONSENSE program. The plot file from PHYLIP 3.5 was reformatted for the Hewlett-Packard Laserjet 4 printer by using the PRINTGL software (Ravitz Software, Inc., Lexington, Ky.).

**Citrate synthase PCR-RFLP typing.** For PCR-restriction fragment length polymorphism (RFLP) analysis, primers (21) were employed to amplify a fragment of approximately 400 nucleotides from the citrate synthase gene of *Bartonella* species, with the following cycling program being used: 94°C for 1 min, 47°C for 1 min, and 72°C for 1 min for a total of 35 cycles. Chromosomal DNAs from *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358 type strains were used as positive controls for the PCR. Each set of reactions also included a negative control (a tube containing all of the reagents but no template DNA) and a positive control (a tube with *Bartonella* DNA). Undigested amplicons and amplicons digested with the enzymes *Hha*I, *Mse*I, and *Taq*I were separated electrophoretically on an 8% acrylamide gel, and the resulting patterns compared with the RFLP patterns obtained for the *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358 type strains (21).

**Genomic RFLP typing.** Genomic DNA also was subjected to RFLP analysis. The 13-day-old cultures of C-29 and C-30 were recovered by flooding the agar plates with sterile filtered phosphate-buffered saline and scraping their surfaces with a cell scraper. DNA extraction was performed by the standard cetyltrimethylammonium bromide (Sigma, St. Louis, Mo.) technique (33). *Nhe*I-digested genomic DNA fragments were separated electrophoretically on a 0.7% agarose gel, and the resulting pattern was compared with the fragment patterns of similarly digested genomic DNAs from the *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358 type strains.

**DNA hybridization methods.** C-29 and C-30 cells were grown on chocolate agar at 35°C in an atmosphere of 5% CO<sub>2</sub> for 7 days, scraped from the agar surface, and washed twice with sterile 0.1 M NaCl. The methods used for DNA extraction and purification as well as the hydroxyapatite method have been described previously (3). DNA was labeled enzymatically in vitro with [ $\alpha$ -<sup>32</sup>P]dCTP by use of a nick translation reagent kit (GIBCO BRL, Gaithersburg, Md.) according to the manufacturer's instructions. Divergence of related sequences was estimated to be approximately 1% for each degree decrease in thermal stability of a heterologous reassociated DNA duplex compared with that of the homologous reassociated DNA duplex (3). Divergence was calculated to the nearest 0.5%.

**Nucleotide sequence accession numbers.** The sequence for the C-29 16S rDNA has been assigned accession no. AF076237 in GenBank.

## RESULTS

**Recovery of *Bartonella* isolates C-29 and C-30.** The two asymptomatic cats, C-29 and C-30, were 5 and 6 months old, respectively, lived indoors and outdoors on a farm, and were infested with cat fleas (*C. felis*). The isolates obtained from cats C-29 and C-30 were visible on chocolate agar following incubation at 35°C in a candle jar for 14 days, much longer than for all other *B. henselae* feline isolates (usually 3 to 5 days).

**Phenotypic characteristics.** Both strains grew poorly and appeared as minute pinpoint colonies after 14 days of incubation on chocolate agar. In contrast to *B. henselae*, which grows preferentially on heart infusion agar supplemented with 5% rabbit blood rather than on chocolate agar, C-29 and C-30 grew preferentially on chocolate agar and usually failed to produce any growth on heart infusion agar supplemented with 5% rabbit blood. On subsequent passage, we also observed a much longer doubling time for C-29 and C-30 than for other *B. henselae* isolates. Gram staining revealed tiny, short, straight or slightly curved gram-negative bacilli.

Tests for production of catalase and oxidase were negative. Using a MicroScan Rapid Anaerobe Panel to test for preformed enzymes, our isolates C-29 and C-30 were negative for L-proline- $\beta$ -naphthylamide (PRO) as well as for L-lysine- $\beta$ -naphthylamide (acid) (LYA). In contrast, *B. henselae* ATCC 49882 and *B. quintana* ATCC VR-358 were positive for PRO. *B. henselae* ATCC 49882 was positive and *B. quintana* ATCC VR-358 was negative for LYA. In summary, *B. henselae* and the new species differed with regard to the preformed enzymes PRO and LYA whereas *B. quintana* and the new species differed only with regard to the preformed enzyme PRO. No other differences between our two strains and the reference strains were observed, resulting in the code 10073240 for C-29 and C-30. The code obtained for *B. henselae* ATCC 49882 was 10077640, and the one obtained for *B. quintana* ATCC VR-358 was 10073640. The codes for our reference strains were consistent with the ones previously reported for *B. henselae* and *B. quintana* (31). The biochemical characteristics of our isolates C-29 and C-30 were most similar to, but distinct from, those of the *B. henselae* type strain.

**Citrate synthase PCR-RFLP analysis.** Citrate synthase PCR-RFLP analysis was used to compare isolates C-29 and C-30. In contrast to the *B. henselae* and *B. quintana* type strains, two PCR products were observed for C-29 and C-30. The smaller amplicon was the same size as the single amplicons of the *B. henselae* and *B. quintana* type strains (approximately 400 bp), but the second one was larger (approximately 550 bp) and was not seen for *B. henselae* and *B. quintana* (Fig. 1). The patterns produced by digestion of the amplicons of both C-29 and C-30 were identical, but the sizes and number of digestion products differed from those of the *B. henselae* and *B. quintana* type strains (Fig. 1).

**Genomic RFLP analysis.** Genomic RFLP analysis was used to compare isolates C-29 and C-30 with the *B. henselae* and *B. quintana* type strains. The patterns observed for the C-29 and C-30 isolates when the enzyme *Nhe*I was used were identical to each other but were distinctly different from those of the *B. henselae* and *B. quintana* type strains (Fig. 2).

**16S rDNA sequence analysis.** The 16S rDNA sequences of cat isolates C-29 and C-30 were identical. The C-29 sequence was compared with the 16S rDNA sequences of 10 other *Bartonella* species or subspecies, obtained from the GenBank database. A total of 1,414 nucleotide positions, corresponding to

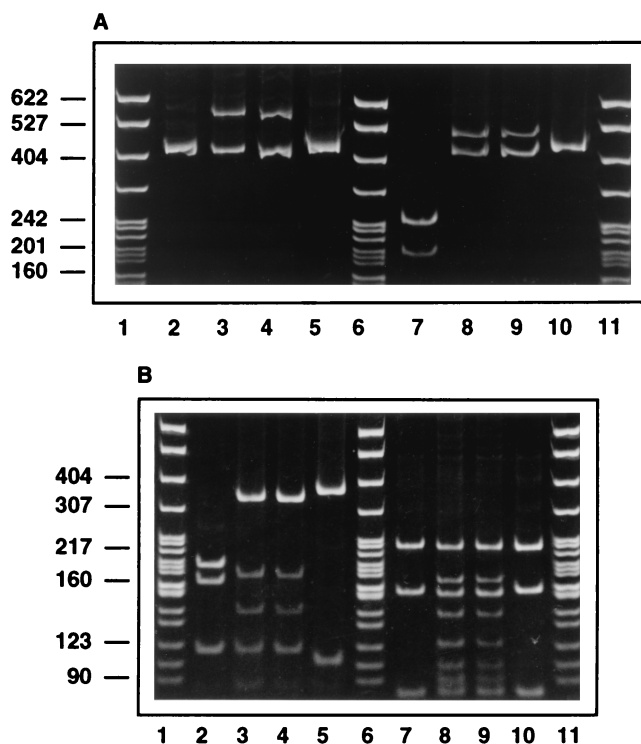


FIG. 1. Citrate synthase gene PCR-RFLP analysis of DNA from feline isolates C-29 and C-30, electrophoresed on 8% acrylamide gels and stained with ethidium bromide. (A) Undigested (lanes 2 to 5) or *HhaI*-digested (lanes 7 to 10) PCR-amplified citrate synthase gene fragments. (B) *TaqI*-digested (lanes 2 to 5) or *MseI*-digested (lanes 7 to 10) PCR-amplified citrate synthase gene fragments. Lanes 1, 6, and 11, size standards (in base pairs); lanes 2 and 7, *B. henselae* ATCC 49882; lanes 3 and 8, feline isolate C-29; lanes 4 and 9, feline isolate C-30; lanes 5 and 10, *B. quintana* ATCC VR-358.

*E. coli* sequence positions 23 to 1490 (5), were determined for both cat isolates. The 16S rDNA sequence obtained for C-29 was most similar to that of the *B. henselae* type strain. Comparing an overlapping region of 1,414 nucleotides of the C-29 and the *B. henselae* type strain 16S rDNAs, six differences were noted: at position 267, C-29 had a substitution of a cytosine residue for the thymine residue of the *B. henselae* type strain; at positions 860 and 861, there was an inversion of a guanosine and a cytosine residue; at position 940, there was an insertion of a guanosine residue; at position 1136, there was substitution of a cytosine residue for a thymine residue; and at position 1137, as well as at position 1455, there was a substitution of an adenine residue for a guanosine residue.

The 16S rDNA sequence of strain C-29 was most similar to that of *B. henselae* (99.54% similarity). The levels of similarity determined between the C-29 16S rDNA sequence and those of other *Bartonella* species (Table 1) were as follows: for *B. doshiae*, 99.39%; for *B. vinsonii*, 99.39%; for *B. vinsonii* subsp. *berkhoffii*, 99.16%; for *B. taylorii*, 99.09%; and for *B. grahamii*, 99.02%. The C-29 16S rDNA sequence showed less similarity to the sequences of *B. quintana* (98.86%), *B. elizabethae* (98.78%), *B. bacilliformis* (98.33%), and *B. clarridgeiae* (98.10%). The percentages of similarity between a number of other *Bartonella* species were identical to or greater than the 99.54% similarity observed between *B. henselae* and C-29 (e.g., 99.54% between *B. vinsonii* and *B. taylorii*, as well as between *B. grahamii* and *B. taylorii*, and even higher percentages of similarity between *B. grahamii* and *B. vinsonii* [99.62%], *B. taylorii* and *B. doshiae* [99.7%], and *B. vinsonii* and *B. doshiae* [99.85%]). C-29 was

94.89% similar to *Brucella abortus* and 93.99% similar to *A. tumefaciens*.

The phylogenetic dendrogram generated by the comparison of C-29 with 10 named *Bartonella* species and subspecies and *B. abortus* is shown in Fig. 3. In this figure, as well as in a dendrogram including 10 unnamed *Bartonella* strains, six brucellae, *A. tumefaciens*, and *E. canis* (data not shown), the bartonellae formed a monophyletic group, with *B. clarridgeiae* and *B. bacilliformis* exhibiting the highest degrees of divergence from the group. When the majority-rule consensus tree, derived from 100 trees constructed from the same input file, was used, C-29 was grouped with *B. henselae* in 86% of the trees; in the remaining 14 trees, C-29 was grouped with other bartonellae, but none of the groupings occurred more than 4% of the time.

**DNA hybridization studies.** Levels of DNA relatedness were determined by hybridizing labeled DNA from the cat isolates C-29 and C-30, the type strain of *B. henselae* (ATCC 49882), and an additional strain of *B. henselae* (G6486) with unlabeled DNA from C-29, C-30, and 14 *Bartonella* species or subspecies (Table 2). (Not all combinations of reciprocal hybridization were performed.) The cat strains C-29 and C-30 showed average levels of relatedness of 97 and 100%, with 0.0 and 0.5% divergence, in optimal DNA reassociation reactions at 55°C. In stringent DNA reassociation reactions at 70°C, they were 98 and 100% related. Labeled DNA from the type strain of *B. henselae* ATCC 49882 showed levels of relatedness to the cat *Bartonella* isolate C-29 of 65% at 55°C, with 5.5% divergence, and 41% at 70°C, and its levels of relatedness to the cat *Bartonella* isolate C-30 were 61% at 55°C, with 5.0% divergence, and 31% at 70°C. Labeled DNA from *B. henselae* G6486 showed levels of relatedness to the cat *Bartonella* isolate C-29 of 79%, with 6.0% divergence at 55°C, and 61% at 70°C, and its levels of relatedness to the cat *Bartonella* isolate C-30 were 73%, with 5.5% divergence, at 55°C and 54% at 70°C. Using labeled C-29 DNA hybridized with unlabeled DNA from the type strain of *B. henselae*, C-29 showed levels of relatedness of 84% at 55°C, with 6.5% divergence, and 51% at 70°C. For C-30, the levels of relatedness to the type strain of *B. henselae* were 92%, with 6.0% divergence, at 55°C and 69% at 70°C. Based on DNA

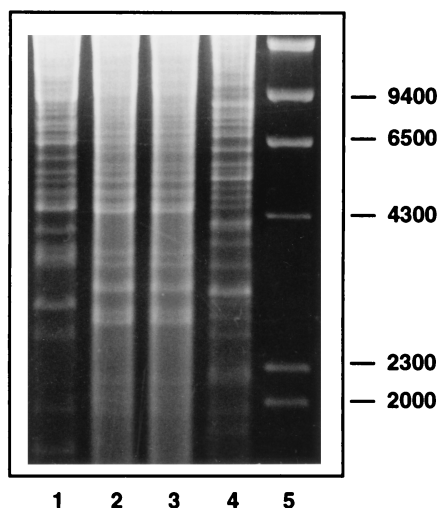


FIG. 2. Genomic RFLP analysis of feline *Bartonella* isolates C-29 and C-30. Genomic DNA was extracted and digested with *NheI*, and the restriction fragments were electrophoresed on a 0.7% agarose gel and then stained with ethidium bromide. Lane 1, *B. henselae* ATCC 49882; lanes 2 and 3, feline blood isolates C-29 and C-30, respectively; lane 4, *B. quintana* ATCC VR-358; lane 5, size standards (in base pairs).

TABLE 1. 16S rDNA similarity values

Organism	% Similarity													
	C-29	<i>B. henselae</i>	<i>B. elizabethae</i>	<i>B. doshiae</i>	<i>B. bacilliformis</i>	<i>B. clarridgeiae</i>	<i>B. quintana</i>	<i>B. vinsonii</i>	<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	<i>B. taylorii</i>	<i>B. grahamii</i>	<i>Brucella abortus</i>	<i>A. tumefaciens</i>	<i>E. canis</i>
C-29	100.00													
<i>B. henselae</i>	99.54	100.00												
<i>B. elizabethae</i>	98.78	98.86	100.00											
<i>B. doshiae</i>	99.39	99.39	99.24	100.00										
<i>B. bacilliformis</i>	98.33	98.17	98.25	98.33	100.00									
<i>B. clarridgeiae</i>	98.10	98.10	98.25	98.41	97.49	100.00								
<i>B. quintana</i>	98.86	98.63	98.41	98.71	97.73	97.79	100.00							
<i>B. vinsonii</i>	99.39	99.39	99.24	99.85	98.33	98.25	98.55	100.00						
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	99.16	99.16	99.31	99.62	98.17	98.41	98.71	99.62	100.00					
<i>B. taylorii</i>	99.09	99.09	99.02	99.70	98.25	98.25	98.79	99.54	99.39	100.00				
<i>B. grahamii</i>	99.02	99.16	99.39	99.62	98.17	98.25	98.55	99.62	99.54	99.54	100.00			
<i>Brucella abortus</i>	94.89	94.89	95.58	95.50	94.74	95.20	94.74	95.50	95.58	95.50	95.50	100.00		
<i>A. tumefaciens</i>	93.99	93.69	93.99	93.99	93.61	93.84	93.46	94.14	94.08	93.84	93.99	93.60	100.00	
<i>E. canis</i>	82.84	82.68	82.68	82.68	82.76	82.46	82.76	82.76	82.70	82.61	82.53	82.34	82.24	100.00

hybridization studies, our isolate C-29 was most closely related to *B. henselae* (range of levels of relatedness to the type strain and other strains, 65 to 84%, with 5 to 7% divergence, at 55°C). The levels of relatedness of C-29 and C-30 to *B. quintana* ATCC VR-358, *B. vinsonii* ATCC VR-152, and *B. vinsonii* subsp. *berkhoffii* ATCC 51672 ranged from 46 to 68% at 55°C. DNA from C-29 was least closely related (28 to 38%) to the type strains of *B. grahamii*, *B. clarridgeiae*, *B. bacilliformis*, and *B. doshiae*, as determined by DNA-DNA hybridization studies. Since no strains of *B. taylorii*, *B. talpae*, or *B. peromysci* are available (11a), it was not possible to include these species in the DNA hybridization studies.

## DISCUSSION

The recovery of these two new, distinct *Bartonella* isolates from two pet cats provides further evidence of the diversity of *Bartonella* species that infect the domestic cat, *Felis domesticus*. To date, two species, *B. henselae* and *B. clarridgeiae*, have been isolated from domestic cats, and apparently *B. clarridgeiae* is cultivable from a higher percentage of European cats than cats in the United States (1, 11). Repetitive-element PCR on *B. henselae* isolates from cats also demonstrated diversity among *B. henselae* isolates (24), but all *B. henselae* isolates obtained to date have been closely related both phenotypically and genotypically. Recent data suggest that an even greater diversity of *Bartonella* species infect small mammals such as mice, moles, and voles in the United States and the United Kingdom (2, 18).

Different phenotypic characteristics distinguish isolates C-29 and C-30 from *B. henselae* feline isolates. *B. henselae* and *B. clarridgeiae* are readily isolated from feline blood cultures, and colonies are usually apparent only 3 days after plating on agar, in contrast to isolates from human blood and tissue, whose colonies do not appear until after a minimum of 8 days of incubation (16). We observed only tiny colonies after 14 days of incubation of primary cultures, and these initially were ascribed to cellular debris from lysed erythrocytes and leukocytes present after processing of the blood in lysis-centrifugation tubes. Our isolates were slow growers, appeared only as minute pinpoint colonies, and, in contrast to other *B. henselae* strains, produced only slightly larger colonies on subsequent passaging. Drawing on our extensive experience in isolation and cultivation of *Bartonella* species from cat blood, as well as from human blood and tissues, and of *Bartonella* type strains,

C-29 apparently has the most-fastidious growth characteristics of any *Bartonella* species cultured to date.

*Bartonella* species have distinctive preferences for growth on different agar media, with *B. henselae* growing preferentially on heart infusion agar supplemented with 5% rabbit blood and *B. quintana* growing preferentially on chocolate agar (16). The C-29 and C-30 isolates were unique among our cat isolates because they did not grow on heart infusion agar supplemented with 5% rabbit blood but rather grew only on chocolate agar. They also failed to grow on Columbia or sheep blood agar. Although we found that these isolates represented 2 of the 25 *Bartonella* isolates (8%) recovered during documentation of *B. henselae* bacteremia in cats from the greater San Francisco Bay region, the prevalence of this new species remains unknown and will be difficult to quantitate because of the chocolate agar preference and extremely fastidious growth characteristics. Additionally, it is not known whether this new *Bartonella* species can infect humans.

The two isolates C-29 and C-30 were recovered from the blood of two kittens that had lived both outdoors and indoors on a farm with seven other cats; *B. henselae* was isolated from

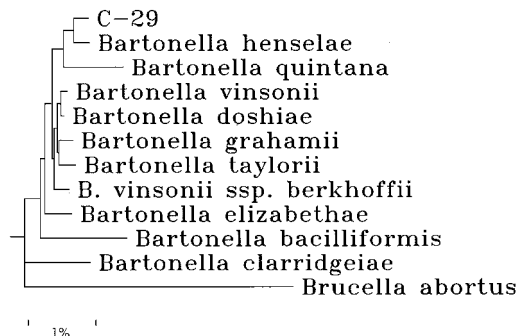


FIG. 3. Phylogenetic dendrogram based on a neighbor-joining comparison of 1,330-nucleotide sequences of 16S rRNA genes. The scale bar at the bottom of the dendrogram represents a 1% difference. The strain designations and accession numbers for the sequences retrieved from GenBank are as follows: *B. clarridgeiae* CIP 104772, accession no. X97822; *B. bacilliformis*, accession no. M65249; *B. henselae*, accession no. M73229; *B. quintana*, accession no. U28268; *B. grahamii* V2, accession no. Z31349; *B. elizabethae* ATCC 49927, accession no. L01260; *B. vinsonii* subsp. *berkhoffii* G7464, accession no. U26258; *B. taylorii* M6, accession no. Z31350; *B. doshiae* R18, accession no. Z31351; *B. vinsonii* ATCC VR-152, accession no. L01259; and *B. abortus* 11-19, accession no. X13695.

TABLE 2. DNA relatedness of C-29 and C-30 to other *Bartonella* species

Source of unlabeled DNA	% Relatedness to labeled DNA from:											
	C-29			C-30			<i>B. henselae</i> ATCC 49882 <sup>T</sup>			<i>B. henselae</i> G6486		
	55°C	D <sup>a</sup>	70°C	55°C	D	70°C	55°C	D	70°C	55°C	D	70°C
C-29	100	0.0	100	100	0.5	100	65	5.5	41	79	6.0	61
C-30	97	0.0	98	100	0.0	100	61	5.0	31	73	5.5	54
<i>B. henselae</i> ATCC 49882 <sup>T</sup>	84	6.5	51	92	6.0	69	100	0.0	100	100	0.5	100
<i>B. henselae</i> G6486	78	6.0	54	— <sup>b</sup>	—	—	83	0.5	90	100	0.0	100
<i>B. henselae</i> G6529	68	5.5	54	—	—	—	85	0.5	88	—	—	—
<i>B. henselae</i> G8378	79	6.5	60	—	—	—	92	0.0	94	—	—	—
<i>B. henselae</i> 88-712	83	7.0	62	—	—	—	100	0.5	100	—	—	—
<i>B. henselae</i> G5691	84	5.0	48	—	—	—	95	0.5	95	—	—	—
<i>B. quintana</i> ATCC VR-358 <sup>T</sup>	56	9.5	31	68	9.5	40	46	8.5	20	57	9.0	34
<i>B. vinsonii</i> ATCC VR-152 <sup>T</sup>	55	10.5	29	67	10.0	36	50	8.0	26	63	8.5	43
<i>B. vinsonii</i> subsp. <i>berkhoffii</i> ATCC 51672 <sup>T</sup>	46	12.0	—	57	12.0	—	42	10.5	—	—	—	—
<i>B. elizabethae</i> ATCC 49927 <sup>T</sup>	53	13.0	21	63	12.5	—	46	11.0	21	—	—	—
<i>B. clarridgeiae</i> ATCC 51734 <sup>T</sup>	31	12.5	—	—	—	—	—	—	—	—	—	—
<i>B. bacilliformis</i> ATCC 35685 <sup>T</sup>	34	12.5	—	46	13.0	—	33	11.0	—	—	—	—
<i>B. grahamii</i> NCTC 12860 <sup>T</sup>	28	11.5	—	—	—	—	—	—	—	—	—	—
<i>B. doshiae</i> NCTC 12862 <sup>T</sup>	38	10.0	—	—	—	—	—	—	—	—	—	—

<sup>a</sup> D, percent divergence at 55°C within related sequences, calculated to the nearest 0.5%.

<sup>b</sup> —, not done.

the blood of two of these seven cats, and the remaining five animals were culture negative. The relationship between infection of these kittens with this new species and their association with farm animals (one kitten lived primarily in the barn) is notable; the remainder of the 61 cats cultured in this study were urban or suburban, and those that were infected with a *Bartonella* species had *B. henselae* (14).

Although biochemical profiles cannot be used routinely and reliably for differentiation of *Bartonella* species because of the relatively inert nature of bartonellae, we found that C-29 and C-30 yielded a reproducibly distinct code for preformed enzymes when tested with the MicroScan Rapid Anaerobe Panel. The obtained code was similar, but not identical, to the ones reported for other *Bartonella* species (31). This observation may be of practical diagnostic value for laboratories attempting to distinguish among different *Bartonella* species.

PCR-RFLP analysis of the citrate synthase gene is commonly used to identify *Bartonella* isolates to the species level (21). The distinctive citrate synthase PCR-RFLP product observed for C-29 and C-30 provides additional evidence that C-29 and C-30 represent a distinct species. The pattern obtained by genomic RFLP analysis for our two cat isolates also was clearly different from those of the *B. henselae* and *B. quintana* type strains. The 16S rDNA sequence analysis confirmed that cat isolate C-29 is a member of the genus *Bartonella*, is most closely related to *B. henselae*, and is most distantly related to *B. grahamii*.

DNA-DNA hybridization remains the method of choice for defining a species. It has been recommended that a species be considered to consist of strains whose DNAs are 70% or more related under optimal DNA reassociation conditions and 55% or more related under stringent DNA reassociation conditions, with 5% or less divergence within related sequences (3, 29). From the results of our DNA-DNA hybridization study, we conclude that C-29 and C-30 represent the same species (average levels of relatedness to each other of 97 and 100%, with 0.0 and 0.5% divergence under optimal DNA reassociation reactions, at 55°C and of 98 and 100% at 70°C).

C-29 showed levels of relatedness to the type strain of *B. henselae* of 84%, with 6.5% divergence, at 55°C and 51% at 70°C, and for C-30 the levels of relatedness to the type strain

of *B. henselae* were 92%, with 6.0% divergence, at 55°C and 69% at 70°C. Regardless of the high degree of relatedness to *B. henselae* determined at 55°C when using labeled DNA from C-29 and C-30, the degree of relatedness of C-29 and C-30 to *B. henselae* does not fulfill the species definition because the divergence is greater than 5%. The percent DNA relatedness was substantially lower in reciprocal reactions, in which labeled DNA from the type strain of *B. henselae* showed levels of relatedness to the cat *Bartonella* isolate C-29 of 65%, with 5.5% divergence, at 55°C and 41% at 70°C and to C-30 of 61%, with 5.0% divergence, at 55°C and 31% at 70°C. This type of non-reciprocity in relatedness values has been seen in strains from many genera (2a) and emphasizes the importance of performing reciprocal DNA relatedness determinations when studying strains that are close to the species definition. Nonreciprocity can result from differences in the genome sizes of the two species being compared.

In regard to divergence and relatedness under stringent conditions, our isolates C-29 and C-30 do not fulfill the strict criteria for belonging to the species *B. henselae* and thus represent a new species. This is even more evident when taking into consideration the relatedness and divergence in the reciprocal reactions. Also, the hybridization of labeled C-29 DNA to that of other *B. henselae* strains demonstrates that the species definition is not fulfilled with any of the five other *B. henselae* strains tested, again substantiating that C-29 represents a new species, distinct from *B. henselae*.

These data demonstrate that our two cat isolates represent a new species of *Bartonella* which is genotypically and phenotypically different from all other *Bartonella* isolates. The cat isolates C-29 and C-30 can be readily differentiated from the *B. henselae* type strain on the basis of their slower growth, smaller colony size, clear preference for growth on chocolate agar, unique code in the MicroScan Rapid Anaerobe Panel, distinct RFLP pattern for the citrate synthase gene as well as for genomic DNA, and the presence of six base pair changes in their 16S rDNA compared with that of the *B. henselae* type strain. C-29 thus fulfills the criteria for a new species: it is closely related to *B. henselae* but exhibits genetic and phenotypic divergence (29). A new species for these organisms is proposed and described below.

**Description of *Bartonella koehlerae* sp. nov.** *Bartonella koehlerae* (koeh' ler. ae. N. L. fem. adj. *koehlerae*), in honor of Jane E. Koehler, who was the first to isolate *Bartonella* species from bacillary angiomatosis lesions and whose studies of *B. quintana* and *B. henselae* isolates from human immunodeficiency virus-infected patients have contributed to an improved understanding of the molecular epidemiology, reservoirs, and vectors of *Bartonella*-associated disease in humans. Growth is optimal on chocolate agar, and primary colonies are observed after 14 days of incubation at 35°C in a CO<sub>2</sub>-enriched environment. The code for preformed enzymes obtained with a MicroScan Rapid Anaerobe Panel is 10073240. The new species shows patterns for citrate synthase PCR-RFLP and for genomic RFLP which are not previously described and differ from those of *B. henselae*. The 16S rRNA gene sequence of C-29 is distinguished from that of the *B. henselae* type strain by six base pair changes. The two strains thus far isolated were from healthy cats; to date, no feline or human pathogenicity has been attributed to this species. The type strain, C-29 (ATCC 700693), was recovered from the blood of a healthy kitten during a prevalence study of *B. henselae* in domestic cats in the greater San Francisco Bay area of northern California.

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