Bartonella vinsonii subsp. *berkhoffii* subsp. nov., Isolated from Dogs; *Bartonella vinsonii* subsp. *vinsonii*; and Emended Description of *Bartonella vinsonii*

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Two bacterial strains, one isolated from the blood of a dog with valvular endocarditis and one isolated from the blood of a healthy dog, were similar to *Bartonella* species, as determined by a number of phenotypic criteria, including growth characteristics, biochemical reactions, and cell wall fatty acid composition. The results of 16S rRNA gene sequence similarity studies confirmed that these strains are closely related and belong in the genus *Bartonella* and that *Bartonella vinsonii* is their closest relative (the 16S rRNA of isolate 93-C01^T [T = type strain] was 99.37% identical to the 16S rRNA of the type strain of *B. vinsonii*, the 16S rRNA of isolate G7464 was 99.61% identical to the 16S rRNA of the type strain, and the 16S rRNAs of the dog isolates were 99.77% identical to each other). The 16S rRNAs of both strains contained a 12-base insertion that was not present in the 16S rRNA of the type strain of any *Bartonella* species. DNA relatedness tests revealed that these strains were related at the species level to the type strain of *B. vinsonii*. They were, however, significantly more closely related to each other than to *B. vinsonii*. On the basis of their unique 16S rRNA sequence insertion, their preferentially high level of relatedness, and their similar origins (dogs), we believe that strains 93-C01^T and G7464 should be placed in a separate subspecies of *B. vinsonii*, for which we propose the name *B. vinsonii* subsp. *berkhoffii* subsp. nov. The type strain of *B. vinsonii* subsp. *berkhoffii* is strain 93-C01 (= ATCC 51672). The description of *B. vinsonii* is emended to accommodate the new subspecies, and *B. vinsonii* subsp. *vinsonii* is described.

The genus *Bartonella* (which includes species formerly classified in the genera *Rochalimaea* [5] and *Grahamella* [2]) contains 10 species, 4 of which have been implicated in human disease. *Bartonella quintana* is the etiologic agent of trench fever and has been implicated as a causative agent of endocarditis, bacillary angiomatosis, and bacillary peliosis (23, 25, 30). *Bartonella henselae* is the primary etiologic agent of bacillary angiomatosis, bacillary peliosis, and cat scratch disease (19, 28, 30, 35). *Bartonella elizabethae* has been isolated from an endocarditis patient (7), and *Bartonella bacilliformis* is the agent of classical bartonellosis which manifests as a progressive anemia (Oroya fever) or as a cutaneous disease (verruca peruana) (34). *Bartonella vinsonii* was isolated from voles in Grosse Isle, Quebec Province, Canada, in the 1940s (1); only one strain of *B. vinsonii* has been available previously. *Bartonella talpae* and *Bartonella peromysci*, which are found in small feral mammals, were recently transferred to the genus *Bartonella* from the genus *Grahamella* (2). *Bartonella grahamii*, *Bartonella taylorii*, and *Bartonella doshiae* are recently described species that are also associated with various small feral mammals (2).

In this paper we describe two additional isolates of *B. vinsonii*, one (strain 93-C01^T [T = type strain]) obtained from a

dog with endocarditis and one (strain G7464) obtained from an apparently healthy dog. These isolates are related to *B. vinsonii* at the species level, but are sufficiently distinct from the type strain of *B. vinsonii* that they are placed in a distinct subspecies, for which the name *B. vinsonii* subsp. *berkhoffii* subsp. nov. is proposed.

MATERIALS AND METHODS

Strains. *B. quintana* ATCC VR-358^T, *B. vinsonii* ATCC VR-152^T, *B. henselae* ATCC 49882^T, *B. elizabethae* ATCC 49927^T, and *B. bacilliformis* ATCC 35685^T have been described previously (5, 35). The two *Bartonella* strains obtained from dogs were isolated independently in different laboratories. The workers in each laboratory began preliminary characterization by performing a biochemical analysis, a 16S rRNA gene sequence analysis, a cellular fatty acid analysis, and a restriction fragment length polymorphism (RFLP) analysis. Later, DNA hybridization studies were performed with both strains to determine the degrees of relatedness of the two isolates and *B. vinsonii*, and the 16S rRNA sequences were compared. Dog 1 (the source of strain 93-C01^T [= ATCC 51672^T]) was a 3-year-old spayed Labrador retriever that was presented to the North Carolina State University Veterinary Teaching Hospital and was subsequently diagnosed with valvular endocarditis. Details of the case history of this dog have been reported elsewhere (3). Dog 2 (the source of strain G7464 [= ATCC 35685]) was a healthy Greyhound participating as a control blood donor in a *Hemobartonella canis* study at the University of Georgia College of Veterinary Medicine. No pathology was attributed to *Bartonella* infection in dog 2.

Isolation method. Blood was collected aseptically by jugular venipuncture from dog 1 and was cultured on Trypticase soy agar supplemented with 5% defibrinated rabbit blood (BBL Microbiology Systems, Becton Dickinson, Cockeysville, Md.) as described previously (3). Blood collected for culture from dog 2

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in an Isolator 1.5 Microbial Tube was mixed by inverting the tube four or five times to lyse the cells and was transported to the Centers for Disease Control and Prevention laboratory the next morning. The method of Welch et al. (35) was used for blood cultures. The contents of the Isolator 1.5 tube were vigorously vortexed for 10 s, the blood was withdrawn from the tube with a syringe, and 0.35-ml portions were dispensed onto Columbia agar (BBL Microbiology Systems) supplemented with 5% sheep blood and onto chocolate agar prepared from GC base (BBL Microbiology Systems). The resulting plates were streaked for isolation and incubated in a candle jar at 37°C.

Biochemical tests. A biochemical evaluation of isolate 93-C01^T (from dog 1) was performed with 6-day-old cultures harvested from solid media as described previously (3, 16). Gram staining, flagellum staining, and biochemical tests were performed with isolate G7464 (from dog 2) as described previously (6). The inocula used for biochemical evaluations were obtained by growing both isolates in heart infusion agar supplemented with 5% rabbit blood (BBL Microbiology Systems) for 2 to 3 days at 35°C in a candle extinction jar. The motility of cells suspended in heart infusion broth was determined with a 100× oil immersion objective. Preformed enzyme tests were performed by using the RapID ANA II system as recommended by the manufacturer (Innovative Diagnostic Systems, Inc., Norcross, Ga.).

Cellular fatty acid analysis. The *Bartonella* isolates obtained from the two dogs were grown on rabbit blood agar at 35°C for 10 days. The cells were saponified, and the liberated fatty acids were methylated and analyzed by capillary gas-liquid chromatography (Microbial ID, Inc., Newark, Del.) (22).

Electron microscopy. Transmission electron microscopy was used to compare the morphological features of *B. henselae* found in aortic valve tissue with the morphological features of the plate-grown blood isolate obtained from dog 1 (3, 5).

Extraction of *Bartonella* DNA for PCR amplification. Bacterial DNAs were extracted from isolate 93-C01^T and aortic valve tissue obtained from dog 1 by a phenol-chloroform technique, as described previously (3). *Bartonella* DNA was extracted from the blood of dog 2 by a modification of the procedure developed by Kim (15) to extract *Listeria* DNA from paraffin-embedded tissues. A 100-μl portion of dog blood was centrifuged at 15,600 × g for 10 min, and the supernatant was discarded. The pellet was washed twice with 500 μl of TE (0.01 M Tris, 0.001 M EDTA; pH 8.0) and suspended in 100 μl of 5 mM EDTA (pH 8.0); 100 μl of lysozyme (50 mg/ml) was added, and the mixture was incubated at 37°C for 30 min. Then 50 μl of 20% sodium dodecyl sulfate was added, and, after mixing, the preparation was incubated for 10 min at 55°C to lyse the cells and release the DNA. Then 250 μl microliters of endotoxin-free water was added to the lysate. The lysate was extracted once with phenol and once with chloroform. The aqueous phase was removed and placed in a new tube, and 1 μl of an oyster glycogen solution (20 mg/ml; Sigma Chemical Co., St. Louis, Mo.) was added. The DNA was precipitated by adding 0.1 volume of 3 M acetate and 0.6 volume of isopropanol and was pelleted by centrifugation at 12,000 × g for 15 min. The precipitated DNA was washed once with 70% ethanol, dried, and resuspended in 80 μl of endotoxin-free water. Template DNA for PCR amplification was obtained from cultures of isolate G7464 by the method of Heller et al. (12).

PCR amplification of a fragment of the gene coding for 16S rRNA. DNAs obtained from blood culture isolate 93-C01^T and aortic valve tissue from dog 1 were amplified by PCR by using a previously described method (3). The PCR in which DNA from the blood of dog 2 was used was performed essentially as described by Relman et al. (20) by using *Bartonella*-specific primers p12B and p24E and universal eubacterial primers p11E and p13B. A positive control (paraffin-embedded tissue prepared from tissue that had been artificially inoculated with *B. henselae*) and a negative control (endotoxin-free water extracted by the procedure that was used for the clinical specimens) were included in each set of reactions to monitor the efficacy of extraction and to check for cross-contamination of specimens during extraction. A reagent cocktail control (containing all of the components of the PCR mixture except target DNA) was included to check for contamination during PCR setup. A positive control (*Bartonella* DNA) was included to verify that amplification of the desired PCR product occurred. The PCR product (length, 282 bp) was detected by electrophoresis on a 1.5% agarose–1.0% NuSieve agarose (FMC, Rockland, Maine) gel in 1× Tris-borate buffer at 100 V for 1.25 h, followed by ethidium bromide staining of the gel.

PCR-RFLP subtyping. The spacer region between the genes coding for 16S and 23S rRNAs was amplified by PCR by using the procedure of Matar et al. (17).

DNA hybridization methods. Strains were grown on heart infusion agar supplemented with rabbit blood at 35°C for 3 to 4 days. The DNA extraction and purification procedures and the hydroxyapatite hybridization method which we used have been described previously (4). The level of divergence of related sequences was estimated to be approximately 1% for each 1°C decrease in the thermal stability of a heterologous reassociated DNA duplex compared with the thermal stability of the homologous reassociated DNA duplex (4). Levels of divergence were calculated to the nearest 0.5%. DNA was labeled enzymatically in vitro with [α -³²P]dCTP by using a nick translation kit (Gibco BRL, Gaithersburg, Md.).

PCR amplification of the gene coding for 16S rRNA and sequencing of the amplified product. PCR amplification of the 16S rRNA gene from 93-C01^T and the subsequent sequence reaction experiments were performed with a model Gene-Amp 9600 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using

a previously described protocol (3). The template DNA used for direct sequencing of isolate G7464 was produced by enzymatic amplification of the 16S rRNA gene, using primers rD1 and rD1, as described by Weisburg et al. (31), and methods described by Daly et al. (7). PCR products were purified by using Dynabead M280 streptavidin-coated magnetic beads (DynaL, Inc., Great Neck, N.Y.) and the modification of the method of Hultman et al. (13) described previously (7). Ten oligonucleotides described by Stackebrandt and Charfreitag (27) were used as sequencing primers for the eluted strand, and reverse complementary oligonucleotides to these 10 oligonucleotides were used to sequence the bound strand.

16S rRNA sequence analysis. All of the 16S rRNA sequences used in this study were retrieved electronically from the GenBank database (5, 8). In the case of 93-C01^T sequence alignment was performed by using the Genetic Data Environment (24) and a Sun Sparcstation II (Sun Microsystems, Mountain View, Calif.). Sequence data for G7464 were aligned by using the multisequence alignment program PILEUP, which is part of the Genetics Computer Group (Madison, Wis.) software package (8). The alignment was masked to remove variable regions where the nucleotide alignment could not be reliably determined. Phylogenetic relationships were inferred from the data by using version 3.5c of the PHYLIP software package (10). Evolutionary distance values determined by the method of Jukes and Cantor (14) were used to calculate rRNA similarity values and to construct a dendrogram by the neighbor-joining method (21). The reliability of the tree was analyzed by bootstrapping the data (9).

Nucleotide sequence accession numbers. The 16S rRNA gene sequence of strain 93-C01^T has been deposited in the GenBank, EMBL, and DDBJ databases under accession number L35052. The 16S rRNA sequence of strain G7464 has been deposited in the GenBank database under accession number U26258.

RESULTS

While researchers were attempting to isolate *Hemobartonella* species from cats and dogs in 1992, an organism that resembled *Bartonella* species was isolated serendipitously from an apparently healthy greyhound (dog 2) whose blood was used as a negative control. This dog was retired from racing and was used as a chronic blood donor in the Small Animal Clinic at the School of Veterinary Medicine at the University of Georgia. Dog 2 had no clinical abnormalities other than lacerations resulting from a dog fight.

In 1993, dog 1 was presented to the Veterinary Teaching Hospital at the North Carolina State University College of Veterinary Medicine in Raleigh, and endocarditis involving the mitral and aortic valves was diagnosed. The use of conventional blood culture techniques failed to result in bacterial growth; however, the results of lysis centrifugation cultures demonstrated that *Bartonella* bacteremia occurred.

Isolation of microorganisms. The isolate obtained from dog 1 (strain 93-C01^T) appeared following 8 days of incubation at 35°C in the presence of 5% CO₂ on Trypticase soy agar supplemented with 5% rabbit blood. Subcultures grew vigorously within 4 days. The isolate obtained from dog 2 (strain G7464) grew as a pure culture on Columbia agar supplemented with 5% sheep blood after 4 days of incubation at 37°C in a candle jar. Strain G7464 was very fastidious and grew best when it was subcultured on chocolate agar and incubated at 35°C in the presence of 5 to 10% CO₂. It did not grow at 42°C or anaerobically.

Microscopic, biochemical, and cellular fatty acid analysis. Gram staining of both isolates revealed short, slender, faintly staining, gram-negative bacilli that often occurred in masses. No motility or flagella were detected. Tests for catalase activity, urease activity, acid production from D-glucose, lactose, maltose, D-mannitol, D-xylose, and sucrose, and nitrate reduction and spot indole tests were negative. When the Rapid ANA II system was used, we observed hydrolysis of leucylglycine, glycine, proline (weak), phenylalanine, arginine, and serine. The phenotypic characteristics of strains 93-C01^T and G7464 were compared with those of five other *Bartonella* species (Table 1). All of the strains grew on heart infusion agar supplemented with rabbit blood. Other than hydrolysis of the same nine substrates (when they were tested), there were few other

TABLE 1. Biochemical characteristics of bartonellae

Characteristic	<i>B. bacilliformis</i> ATCC 35685 ^T	<i>B. henselae</i> ATCC 49882 ^T	<i>B. quintana</i> ATCC VR-358 ^T	<i>B. elizabethae</i> ATCC 49927 ^T	<i>B. vinsonii</i> ATCC VR-152 ^T	Strain 93-C01 ^T	Strain G7464
Oxidase activity	— ^a	—	v	—	— ^b	v	—
Peptidase activities							
Alanine	ND	+	+	+	ND	+	ND
Arginine	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+
Histidine	ND	+	+	+	ND	+	ND
Leucine	ND	+	+	+	ND	+	ND
Leucylglycine	ND	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+
Proline	+ ^w	+	+	+	+	+	+ ^w
Serine	ND	+	+	+	+	+	ND
Tyrosine	ND	v	—	+	ND	+	ND
Valine	ND	+	+ ^w	—	ND	+	ND
Esterase activity	ND	—	—	—	ND	+ ^w	ND
Phosphohydrolase activity	ND	+	v	+ ^w	ND	+ ^w	ND
Trypsin activity	ND	+	—	—	ND	v	ND

^a —, negative reaction after 7 days; +, positive reaction within 48 h; +^w, weak positive reaction; v, variable; ND, not done.

^b Negative as determined by the routine oxidase test and weakly positive as determined by the oxidase test performed with the Kovacs reagent.

positive characteristics. *B. quintana* and *B. vinsonii* were weakly oxidase positive (Kovacs); *B. quintana*, *B. vinsonii*, *B. elizabethae*, and some strains of *B. henselae* grew on heart infusion agar containing X factors; and *B. elizabethae* produced incomplete hemolysis. Only *B. bacilliformis* was motile and grew better at 25°C than at 35°C.

The results of our cellular fatty acid analysis confirmed that the type strain of *B. vinsonii* (strain ATCC VR-152) and dog strains 93-C01^T and G7464 had profiles that in general were similar to those of *Bartonella* species. These results suggested, however, that the cellular fatty acid profile of G7464 was more similar to the profile of the *B. vinsonii* type strain than to the strain 93-C01^T profile.

Transmission electron microscopy revealed that the dog 1 isolate and bacilli found in the aortic valve were similar in size and morphology. However, a thinner glycocalyx layer was observed in the cultured organism.

PCR-RFLP analysis. DNA fragments of the expected sizes were amplified from dog blood when we used both the universal 16S rRNA primers and the *Bartonella*-specific primers, indicating that *Bartonella* ribosomal DNA was present in the dog blood. When the ribosomal intergenic spacer region was subtyped by the PCR-RFLP technique by using *Hae*III to restrict the PCR-amplified fragment, the pattern obtained was very different from the pattern obtained for the type strain of *B. vinsonii* and similar, but not identical, to one of the patterns obtained for *B. henselae* (Fig. 1).

rRNA gene sequence analysis. The partial 16S rRNA gene sequences of both dog isolates were compared with each other and with the sequences of other *Bartonella* species obtained from the GenBank database. A total of 1,446 nucleotide positions were determined for the dog 1 isolate, and 1,478 positions were determined for the dog 2 isolate. The sequence data obtained for both dog isolates were most similar to the *B. vinsonii* data (levels of similarity to 93-C01^T and G7464, 99.37 and 99.61%, respectively). The level of 16S rRNA similarity between the dog isolates was 99.77% (Table 2). Figure 2 is a dendrogram which shows the relationships of the dog strains to

Bartonella species and to other closely related species. This dendrogram is the majority rule consensus tree based on 100 trees constructed from bootstrapped data. The bootstrap value (calculated from the number of times out of 100 trees that the species or isolates grouped together) for the group containing the eight *Bartonella* species and the two dog isolates was 100%.

A unique insertion sequence (5' CAGGGAGACCTT 3') involving the first stem and loop of the variable V2 region between corresponding *Escherichia coli* positions 183 and 188 was identified in both canine isolates. No other *Bartonella* species or other microorganism in the version of the Ribosomal Database Project data which we used had as long an insertion at this point.

DNA hybridization studies. DNA relatedness studies were performed by using labeled DNAs from both dog *Bartonella* isolates and from the type strain of *B. vinsonii* (Table 3). The two dog strains were 91% related with 0.5% divergence in optimal DNA reassociation reactions at 55°C. They were 93% related in stringent DNA reassociation reactions at 70°C. These strains exhibited average levels of relatedness to the type strain of *B. vinsonii* of 82% with 4.3% divergence at 55°C and 70% at 70°C. In reciprocal reactions, labeled DNA from the *B. vinsonii* type strain exhibited average levels of relatedness to the dog *Bartonella* isolates of 75% with 4.8% divergence at 55°C and 63% at 70°C. The levels of relatedness of the dog *Bartonella* strains and the type strain of *B. vinsonii* to other *Bartonella* species in reactions at 55°C ranged from 26 to 68% with 9 to 13% divergence.

DISCUSSION

Rochalimaea vinsonii was isolated from voles during the 1940s by James Baker, Veterinary Corps, U.S. Army, while he was stationed at the War Disease Control Station at Grosse Isle, Quebec Province, Canada (1). During autopsies of several captured voles, Baker noticed that some of the animals had enlarged spleens. He observed coccoid and bacillary forms in stained tissues from voles with normal and enlarged spleens, but was unable to culture any organism in or on bacteriologic media. Because Grosse Isle was used as a quarantine station for immigrants in the 19th century and several Irish immigrants that reached the island had died of typhus and were buried there, Baker suspected that the vole organisms could be rick-

FIG. 1. PCR-RFLP patterns of dog isolate G7467 and type strains of *Bartonella* species obtained by restriction with restriction endonuclease *Hae*III. Lane 1, size marker (ϕ X174 DNA restricted with *Hae*III); lane 2, *B. quintana* ATCC VR-358^T; lane 3, *B. vinsonii* ATCC VR-152^T; lane 4, *B. henselae* ATCC 49882^T; lane 5, dog isolate G7467.

TABLE 2. 16S ribosomal DNA similarity values obtained from a multiple-sequence alignment of 1,327 nucleotides

Organism	% 16S ribosomal DNA similarity ^a										
	Dog isolate G7464	Dog isolate 93-C01 ^T	<i>Bartonella vinsonii</i> ATCC VR-152 ^T	<i>Bartonella henselae</i> ATCC 49882 ^T	<i>Bartonella quintana</i> ATCC VR-358 ^T	<i>Bartonella elizabethae</i> ATCC 49927 ^T	<i>Bartonella bacilliformis</i> ATCC 35685 ^T	<i>Brucella abortus</i>	<i>Agrobacterium tumefaciens</i>	<i>Ehrlichia sennetsu</i>	<i>Anacystis nidulans</i>
Dog isolate G7464	100										
Dog isolate 93-C01 ^T	99.77	100									
<i>Bartonella vinsonii</i> ATCC VR-152 ^T	99.61	99.37	100								
<i>Bartonella henselae</i> ATCC 49882 ^T	99.21	98.98	99.45	100							
<i>Bartonella quintana</i> ATCC VR-358 ^T	98.99	98.75	98.83	98.98	100						
<i>Bartonella elizabethae</i> ATCC 49927 ^T	99.29	99.21	99.21	98.90	98.67	100					
<i>Bartonella bacilliformis</i> ATCC 35685 ^T	98.28	98.04	98.44	98.36	98.12	98.44	100				
<i>Brucella abortus</i>	95.83	95.67	95.75	95.20	95.13	95.75	95.05	100			
<i>Agrobacterium tumefaciens</i>	94.20	94.43	94.27	93.88	93.57	94.27	93.73	93.71	100		
<i>Ehrlichia sennetsu</i>	81.85	81.99	81.92	81.84	81.60	81.92	81.60	82.34	82.32	100	
<i>Anacystis nidulans</i>	76.02	76.15	76.58	76.65	76.76	76.58	77.13	76.90	76.42	74.37	100

^a The values were corrected for multiple base changes by the method of Jukes and Cantor (14).

ettsiae. He started culturing the organisms in the yolk sacs of developing eggs.

Weiss et al. (33) initially characterized the Baker isolate (strain VR-152) as a strain of *Rochalimaea quintana*, but later classified the organism as a new species, *R. vinsonii*, on the basis of the results of DNA-DNA hybridization tests (32). We are not aware of other isolates of *R. vinsonii* obtained after Baker's original isolates, and this organism has not been associated with any human or animal disease.

In this study we compared two *Bartonella* isolates, one obtained from a presumably healthy dog and one obtained from a dog with endocarditis. The biochemical profiles of both isolates were consistent with the profiles observed for *Bartonella* species. Conventional biochemical tests, however, cannot be used dependably for differentiation of *Bartonella* species because of the inert nature and growth requirements of these organisms. Similarly, cellular fatty acid analysis can be used to determine identity at the genus level, but cannot be used to reliably distinguish among the species.

RFLP analyses of PCR-amplified ribosomal DNA intergenic spacer regions have been shown to be useful for intraspecies subtyping of *B. henselae* and *B. quintana* (17, 26). The two dog isolates could not be distinguished from each other when they were analyzed by using the PCR-RFLP procedure with *AluI* and *HaeIII* for restriction of the amplified DNAs, but their profiles were completely different from the profile of the type strain of *B. vinsonii*. In contrast, the restriction patterns of the two dog isolates were very similar to the restriction pattern of the type strain of *B. henselae* (they differed by a single band). As additional dog isolates are obtained, it will be interesting to determine if the PCR-RFLP method can be used for strain differentiation.

The 16S rRNA gene sequences of the two dog isolates were almost identical, which is consistent with placement of these organisms in the same species. The results of our 16S rRNA gene sequence analysis confirmed that the dog strains are members of the genus *Bartonella* and that their closest relative is *B. vinsonii*. Our 16S rRNA sequence analysis also revealed that the two dog strains contain the same 12-base insertion, the microbiological significance of which is unknown.

DNA hybridization has been recommended in preference to

16S rRNA gene sequencing as the method of choice for differentiating species (11, 18, 29). The recommended phylogenetic definition of a species is a group of "strains with approximately 70% or greater DNA-DNA relatedness and with 5°C or less delta T_m . Both values must be considered" (29) (delta T_m is the difference between the melting temperature of a homologous hybrid and the melting temperature of a heterologous hybrid); 1°C of delta T_m is approximately equivalent to 1% divergence. It has also been demonstrated that the subspecies concept is phylogenetically valid, on the basis of the frequency distribution of levels of divergence within related sequences and preferentially high levels of intraspecies relatedness (29).

The results of the DNA relatedness tests showed that the two dog isolates were members of the same species and that both of these strains were related to the type (and only available) strain of *B. vinsonii* at the species level. The levels of relatedness between the dog isolates, however, were substantially higher (91% with 0.5% divergence at 55°C; 93% at 70°C) than the levels of relatedness between either dog strain and the type strain of *B. vinsonii* (the average values in reciprocal reactions were 78% with 4.5% divergence at 55°C and 67% at 70°C). The levels of relatedness of the dog isolates to *B. gra-*

FIG. 2. Phylogenetic dendrogram based on an alignment of 1,327 nucleotides of the 16S ribosomal DNAs of organisms belonging to the alpha subdivision of the Proteobacteria. *Escherichia coli* and *Anacystis nidulans* were included as outgroups. Scale bar = 5% nucleotide difference.

TABLE 3. Levels of DNA relatedness for *Bartonella* dog isolates and other bartonellae

Source of unlabeled DNA	Source of labeled DNA								
	<i>Bartonella</i> sp. strain G7464			<i>Bartonella</i> sp. strain 93-C01 ^T			<i>B. vinsonii</i> ATCC VR-152 ^T		
	% Relatedness at 55°C	% Divergence at 55°C ^a	% Relatedness at 70°C	% Relatedness at 55°C	% Divergence at 55°C ^a	% Relatedness at 70°C	% Relatedness at 55°C	% Divergence at 55°C ^a	% Relatedness at 70°C
<i>Bartonella</i> sp. strain G7464	100	0.0	100	ND ^b	ND	ND	77	5.0	59
<i>Bartonella</i> sp. strain 93-C01 ^T	91	0.5	94	100	0.0	100	72	4.5	67
<i>B. vinsonii</i> ATCC VR-152 ^T	82	3.5	70	81	5.0	70	100	0.0	100
<i>B. henselae</i> G5436	68	9.0	38	ND	ND	ND	ND	ND	ND
<i>B. henselae</i> G1061	64	12.0	29	ND	ND	ND	ND	ND	ND
<i>B. henselae</i> ATCC 49882 ^T	62	10.0	39	55	10.5	ND	55	11.0	16
<i>B. henselae</i> G5829	62	11.0	30	ND	ND	ND	ND	ND	ND
<i>B. henselae</i> G1054	58	12.0	20	ND	ND	ND	ND	ND	ND
<i>B. quintana</i> ATCC VR-358 ^T	58	10.0	31	47	10.5	ND	49	10.0	20
<i>B. elizabethae</i> ATCC 49927 ^T	57	12.0	26	52	13.0	ND	54	12.5	17
<i>B. bacilliformis</i> ATCC 35685 ^T	26	11.0	ND	33	13.0	ND	33	10.5	ND

^a Level of divergence for related sequences, calculated to the nearest 0.5%.

^b ND, not done.

hamii, *B. taylorii*, and *B. doshiae* were not determined since the type strains of these recently described species were not available. Birtles et al. showed previously that these species were less than 40% related to *B. vinsonii* (2). *B. talpae* and *B. peromysci* were not tested as there are no known strains of these species (2). In view of these results, there seems to be no doubt that the dog isolates are *B. vinsonii* isolates and are the first isolates of this organism obtained in almost 50 years and the first isolates obtained from dogs; in addition, isolate 93-C01^T is the first member of this species that has been shown to be responsible for a disease. The dog isolates of *B. vinsonii* can be differentiated from the *B. vinsonii* type strain on the basis of their preferentially high levels of DNA relatedness and low levels of divergence in related sequences, by the presence of a unique insertion in their 16S rRNAs, by their different RFLP pattern, by their different source of isolation, and by their pathogenicity. Therefore, we feel that we are justified in creating a new subspecies for these organisms as proposed and described below.

Emendation of the description of *Bartonella vinsonii*. *Bartonella vinsonii* (Brenner, O'Connor, Winkler, and Steigerwalt 1993 [5]) (basonym, *Rochalimaea vinsonii* Weiss and Dasch 1982 [32]). The characteristics of this taxon are the same as those described for the genus (5) and those described by Weiss and Moulder for the species (34). Additional biochemical characteristics are shown in Table 1.

The species contains two subspecies, one of which has been isolated only from voles and one of which has been isolated only from dogs, including a dog with a case of canine endocarditis.

Description of *Bartonella vinsonii* subsp. *vinsonii* subsp. nov. *B. vinsonii* subsp. *vinsonii* (vin.so'ni.i. N. L. gen. n. *vinsonii*, of Vinson, in honor of J. William Vinson who, along with Henry S. Fuller, first demonstrated that *Bartonella* [formerly *Rochalimaea*] *quintana* could be grown on blood agar). The characteristics are the same as those of the species. Weakly oxidase positive as determined by the Kovacs method. Grows on heart infusion agar supplemented with X factor.

The type and only strain is strain ATCC VR-152, which was isolated from voles by Baker in 1946 (1). The nucleotide sequence of the 16S rRNA gene of the type strain has been deposited in the GenBank database under accession number M73230 (19).

Description of *Bartonella vinsonii* subsp. *berkhoffii* subsp. nov. *B. vinsonii* subsp. *berkhoffii* (berk.hof'fi.i. N. L. gen. n. *berkhoffii*, of Berkhoff, in honor of Herman A. Berkhoff, a veterinary microbiologist whose research encompasses a wide spectrum of veterinary pathogens, and who contributed to the study of *Bartonella* infection in domestic animals that led to the recognition of this subspecies). The characteristics are the same as those of the species. Oxidase negative as determined by the Kovacs method. Does not grow on heart infusion agar supplemented with X factor. Can be differentiated from *B. vinsonii* subsp. *vinsonii* by its RFLP pattern, by DNA hybridization values, and by the presence of a 12-base insertion in the V2 region of its 16S rRNA gene.

Isolated from a dog with endocarditis and a cause of this disease in dogs.

The type strain is strain 93-C01 (= ATCC 51672), which was isolated from a dog with endocarditis. The nucleotide sequence of the 16S rRNA gene of the type strain has been deposited in the GenBank database under accession number L35052.

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REFERENCES

1. Baker, J. A. 1946. A rickettsial infection in Canadian voles. *J. Exp. Med.* 84:37-51.
2. Birtles, R. J., T. G. Harrison, N. S. Saunders, and D. H. Molyneux. 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. *Int. J. Syst. Bacteriol.* 45:1-8.
3. Breitschwerdt, E. B., D. L. Kordick, D. E. Malarkey, B. Keene, T. L. Hadfield, and K. Wilson. 1995. Endocarditis in a dog due to infection with a novel *Bartonella* subspecies. *J. Clin. Microbiol.* 33:154-160.
4. Brenner, D. J., A. C. McWhorter, J. K. Leete Knutson, and A. G. Steigerwalt. 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J. Clin. Microbiol.* 15:1133-1140.
5. Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt. 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove

- the family *Bartonellaceae* from the order *Rickettsiales*. *Int. J. Syst. Bacteriol.* **43**:777–786.
6. Clark, W. A., D. G. Hollis, R. E. Weaver, and P. Riley. 1984. Identification of unusual pathogenic gram-negative aerobic and facultatively anaerobic bacteria. Centers for Disease Control, Atlanta.
 7. Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor. 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. *J. Clin. Microbiol.* **31**:872–881.
 8. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
 9. Felsenstein, J. 1985. Confidence limits on phylogenies. An approach using the bootstrap. *Evolution* **39**:783–791.
 10. Felsenstein, J. 1989. PHYLIP—phylogeny inference package (version 3.2). *Cladistics* **5**:164–166.
 11. Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk, Jr. 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**:166–170.
 12. Heller, M. J., L. J. Burgart, C. J. TenEyck, M. E. Anderson, T. C. Greiner, and R. A. Robinson. 1991. An efficient method for the extraction of DNA from formalin-fixed, paraffin-embedded tissue by sonication. *BioTechniques* **11**:372–377.
 13. Hultman, T., S. Stahl, E. Hornes, and M. Uhlen. 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res.* **17**:4937–4946.
 14. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*, vol. 3. Academic Press, Inc., New York.
 15. Kim, C., L. M. Graves, B. Swaminathan, L. W. Mayer, and R. E. Weaver. 1991. Evaluation of hybridization characteristics of a cloned pRF106 probe for *Listeria monocytogenes* detection and development of a nonisotopic colony hybridization assay. *Appl. Environ. Microbiol.* **57**:289–294.
 16. MacFaddin, J. F. 1980. Biochemical tests for identification of medical bacteria. The Williams and Wilkins Co., Baltimore.
 17. Matar, G. M., B. Swaminathan, S. B. Hunter, L. N. Slater, and D. F. Welch. 1993. Polymerase chain reaction-based restriction fragment length polymorphism analysis of a fragment of the ribosomal operon from *Rochalimaea* species for subtyping. *J. Clin. Microbiol.* **31**:1730–1734.
 18. Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. De Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, E. Stackebrandt, and G. A. Zaverzin. 1990. Report of the Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria*. *Int. J. Syst. Bacteriol.* **40**:213–215.
 19. Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr. 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. *J. Clin. Microbiol.* **30**:265–274.
 20. Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins. 1990. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. *N. Engl. J. Med.* **323**:1573–1580.
 21. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
 22. Sasser, M. 1990. Tracking a strain using the microbial identification system. MIDI Technical Note 102. Microbial ID, Inc., Newark, Del.
 23. Schwartzman, W. A. 1992. Infections due to *Rochalimaea*: the expanding clinical spectrum. *Clin. Infect. Dis.* **15**:893–902.
 24. Smith, W. W., C. Wang, P. M. Gillevet, and W. Gilbert. 1992. Genetic data environment and the Harvard genome database. Genome mapping and sequencing. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 25. Spach, D. H., K. P. Callis, D. S. Paauw, Y. B. Houze, F. D. Schoenknecht, D. F. Welch, H. Rosen, and D. J. Brenner. 1993. Endocarditis caused by *Rochalimaea quintana* in a patient infected with human immunodeficiency virus. *J. Clin. Microbiol.* **31**:692–694.
 26. Spach, D. H., A. S. Kanter, M. J. Dougherty, A. M. Larson, M. B. Coyle, D. J. Brenner, B. Swaminathan, G. M. Matar, D. F. Welch, R. K. Root, and W. E. Stamm. 1995. *Bartonella (Rochalimaea) quintana* bacteremia in inner-city patients with chronic alcoholism. *N. Engl. J. Med.* **332**:424–428.
 27. Stackebrandt, E., and O. Charfseit. 1990. Partial 16S rRNA primary structure of five *Actinomyces* species: phylogenetic implications and development of an *Actinomyces israelii* specific oligonucleotide probe. *J. Gen. Microbiol.* **136**:37–43.
 28. Tappero, J. W., J. Mohle-Boetani, J. E. Koehler, B. Swaminathan, T. G. Berger, P. E. LeBoit, L. L. Smith, J. D. Wenger, R. W. Pinner, C. A. Kemper, and A. L. Reingold. 1993. The epidemiology of bacillary angiomatosis and bacillary peliosis. *JAMA* **269**:770–775.
 29. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Trüper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* **37**:463–464.
 30. Webster, G. F., C. J. Cockerell, and A. E. Friedman-Kien. 1992. The clinical spectrum of bacillary angiomatosis. *Br. J. Dermatol.* **126**:535–541.
 31. Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
 32. Weiss, E., and G. A. Dasch. 1982. Differential characteristics of strains of *Rochalimaea*: *Rochalimaea vinsonii* sp. nov., the Canadian vole agent. *Int. J. Syst. Bacteriol.* **32**:305–314.
 33. Weiss, E., G. A. Dasch, D. R. Woodman, and J. C. Williams. 1978. Vole agent identified as a strain of the trench fever rickettsia, *Rochalimaea quintana*. *Infect. Immun.* **19**:1013–1020.
 34. Weiss, E., and J. W. Moulder. 1984. Order 1. *Rickettsiales* Gieszczykiewicz 1939, 25^{AL}, p. 687–701. In N. R. Krieg and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 1. Williams and Wilkins, Baltimore.
 35. Welch, D. F., D. A. Pickett, L. N. Slater, A. G. Steigerwalt, and D. J. Brenner. 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. *J. Clin. Microbiol.* **30**:275–280.