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Conservation of the 17-Kilodalton Antigen Gene within the Genus *Bartonella*

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The 17-kDa antigen of *Bartonella henselae* has previously been shown to elicit a strong humoral immune response in patients with cat scratch disease (CSD) and to be useful in screening human serum samples for CSD. In this study, PCR amplification of genes homologous to the 17-kDa antigen gene of *B. henselae* was performed using genomic DNAs from several species of *Bartonella*, including the currently recognized human pathogens. Amplicons of similar size were demonstrated using the following chromosomal DNA templates: *B. henselae* (two strains), *B. quintana* (two strains), *B. elizabethae*, *B. clarridgeiae*, *B. vinsonii* subsp. *vinsonii*, and *B. vinsonii* subsp. *berkhoffii*. No evidence of a *B. bacilliformis* homolog of the 17-kDa antigen gene was obtained using multiple primer pairs. DNA sequencing revealed open reading frames capable of coding for proteins with sizes similar to that of the 17-kDa antigen of *B. henselae* in all of the amplicons; however, extensive sequence divergence across the genus was noted. Cloning of the amplified products into pUC19 resulted in recombinants that directed synthesis of homologs of the 17-kDa protein. Immunoblot analysis using human sera from CSD cases demonstrated very little cross-reactivity among different species for this protein. In contrast, immunoblots using rabbit serum raised to the recombinant *B. henselae* antigen showed extensive cross-reactivity with the proteins of other *Bartonella* species. The data suggest that the use of the 17-kDa antigen as a serologic reagent may allow the development of more specific diagnostic assays. Furthermore, the nucleotide sequences from the various versions of the 17-kDa antigen gene should be useful for rapid identification of *Bartonella* at the species level.

The genus *Bartonella* consists of several recognized species that were reclassified by merging the genera *Rochalimaea* and *Grahamella* with *Bartonella* (11, 14). All species are oxidase-negative, fastidious gram-negative bacilli (14). Presently there are four species that are established human pathogens: *B. bacilliformis*, *B. henselae*, *B. quintana*, and *B. elizabethae* (6). Recently, a newly described species, *B. clarridgeiae*, has been associated with cat scratch disease (CSD) in humans (15, 28, 30). *Bartonella* species that have not yet been linked to human disease include, *B. vinsonii*, isolated from a Canadian vole (39); *B. vinsonii* subsp. *berkhoffii*, isolated from dogs (13, 29); and several species isolated from rodents (11, 20, 21). However, a new subspecies, *B. vinsonii* subsp. *arupensis*, was recently isolated from the blood of a single patient (40). Specific and practical diagnostic tests have not yet been developed for most of these species, and hence the tools necessary to associate them with human disease do not exist.

The disease spectrum among the human pathogens ranges from severe life-threatening infections such as the hemolytic anemia caused by *B. bacilliformis*, the agent of Carrion's disease and Oroya fever (1), to the relatively benign but common CSD caused by *B. henselae*. It is estimated that annually in the United States there are 22,000 cases of CSD contracted from domestic cats (26). In addition, both *B. henselae* and *B. quintana* have been shown to cause more severe disease syndromes, including fever with bacteremia, endocarditis, bacillary angiomatosis, and peliosis hepatis, in both immunocompromised and immunocompetent patients (6, 37). In one case a novel bacterium, *B. elizabethae*, was isolated from a 31-year-old man with aortic tissue vegetation (17); however, additional reports linking this organism to human disease have not been published.

Diagnosis of *Bartonella* infection is most frequently accomplished by serology. Isolation is possible but requires extended incubation periods far greater than are needed for most bacteria. Extended incubation times and fastidious growth requirements result in low sensitivity associated with isolation and problems with contamination of primary plating media (6). PCR is an option in laboratories with the proper equipment and expertise but has not yet gained widespread use in clinical diagnostic laboratories (7). For these reasons as well as convenience, serology remains the most frequently utilized means of diagnosis. Serologic assays are simple and easy to perform, but currently used methods only confirm exposure and do not conclusively indicate an acute infection (6). The indirect fluorescent-antibody assay (IFA) is the most common and thoroughly evaluated serologic test (2, 9, 16, 33, 35, 41). However, cross-reactivity among *Bartonella* species (23, 27) and variable sensitivities observed for the IFA in different laboratories have led some investigators to question the usefulness of this test (2, 9, 33). To address these concerns, we have focused on identifying and characterizing protein antigens of the various *Bartonella* species that may be of value as diagnostic reagents. One such protein, the 17-kDa antigen, was identified from *B. henselae* (5). The reactivity of a recombinant fusion protein derived from the 17-kDa antigen of *B. henselae* in a Western

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TABLE 1. Properties of the 17-kDa antigen homologs from different *Bartonella* spp.

Strain	ATCC no. (reference)	Species	Molecular mass (kDa)	No. of amino acid residues
Houston-1	49882 (34)	*B. henselae*	16.9	148
San Antonio-1	—[a] (31)	*B. henselae*	16.9	148
94-F40	700095 (30)	*B. clarridgeiae*	16.9	148
Fuller	VR-358	*B. quintana*	16.9	148
U.Mass.	—	*B. quintana*	16.9	148
F9251	49927 (17)	*B. elizabethae*	20.3	177
B. vinsonii subsp. *berkhoffii*	51672 (29)	*B. vinsonii*	17.8	155
Vole agent (*B. vinsonii* subsp. *vinsonii*)	VR-152 (39)	*B. vinsonii*	26.2	234
KC583	35685	*B. bacilliformis*	Not found	
KC584	35686	*B. bacilliformis*	Not found	

[a] —, no ATCC number.

blot format was shown to correlate well with IFA results and diagnosis of CSD (5). The focus of this study is to identify homologs of the 17-kDa protein in other species of *Bartonella*. Recombinant versions of these proteins should prove useful for serodiagnosis, and the corresponding genes may be of value as targets for species-specific amplification.

MATERIALS AND METHODS

Bacterial strains and preparation of genomic DNA. The sources and designations of the various isolates, representing six species of *Bartonella*, used in this study are summarized in Table 1. Bacteria were cultivated on heart infusion agar supplemented with 5% defibrinated rabbit blood at 37°C in 5% CO_2. Cultures were incubated for 3 to 5 days until growth was sufficient. *B. bacilliformis* was cultivated at 28°C without supplemental CO_2 for 7 to 10 days. Colony morphology and staining of bacterial cells by the Gimenez procedure (18) were used to monitor cell growth and purity. DNA was extracted using a procedure previously described (3). Briefly, cell growth was harvested into sterile TE buffer (10 mM Tris [pH 8.0] and 1 mM EDTA). Sodium lauryl sarcosinate was added to a final concentration of 1.0%, and proteinase K was added to a final concentration of 100 μg/ml. After 2 h of incubation at 65°C, the bacterial lysate was repeatedly extracted with an equal volume of buffer-saturated phenol and chloroform. DNA was precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of cold ethanol. The yield and size of the genomic DNA were assessed by agarose gel electrophoresis.

PCR amplification of the 17-kDa antigen gene homologs. Genomic DNAs from the species listed in Table 1 were used as a template for PCR. Multiple primer pair combinations were constructed from regions surrounding the *B. henselae* (Houston-1) 17-kDa antigen gene; however, four different primer pair combinations were shown to be optimal and were used to amplify each template as follows. *B. clarridgeiae* was amplified with primer pair 17KAF (5′ GGAATG AATGATGAGATCGC 3′) and 17KBR (5′ GTTGAGAAGACTATTCATCG-3′). *B. quintana* and *B. henselae*, were amplified with primer pair 240 (5′ GCT CTAGACAGGGACAAAGTTCCGTTGTTGC 3′) and 241 (5′-CGGGGTAC CGCCATTGTCGTCACAATGACG 3′). *B. elizabethae* and *B. vinsonii* subsp. *vinsonii* were amplified with primer pair 17KAF and R2 (5′ TGAAAAGAGG TCCAAGACCT 3′). *B. vinsonii* subsp. *berkhoffii* was amplified with primer pair 17KBR (5′ CTGAGCGAGAATTTGAGCTG 3′) and 17KAR (5′ CCAGAAA TGCTCTCAAACGG 3′). The positions of these primers are indicated in Fig. 1. An additional primer pair derived from highly conserved sequences internal to the 17-kDa antigen gene, consisting of IntF (5′ GAAAAAATATAGCTTAGT CAC 3′) and IntR (5′CTAAAGTCGGACATCAGATT 3′), was also used to confirm the presence of a 17-kDa antigen gene homolog in all of the *Bartonella* species that tested positive. Amplification was performed using the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 50°C for 2 min, and 70°C for 2 min. The last cycle was followed by incubation at 70°C for 7 min to ensure the adenylation of the 3′ end. PCR amplifications were performed in a DNA thermocycler (MJ Research, Watertown, Mass.) using EasyStart 100 prealiquoted tubes (Molecular BioProducts, Inc., San Diego, Calif.).

Cloning. Amplicons were cloned directly from the PCR mixture or following gel extraction into pCR2.1-TOPO according to the directions of the manufacturer (Invitrogen, Carlsbad, Calif.). The ligation junction of pCR2.1 is located between two *Eco*RI cleavage sites. The resulting ligation mixture was transformed into One Shot cells (Invitrogen) and plated on Luria-Bertani (LB) agar containing ampicillin (100 μg/ml) and 80 μl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 20 mg/ml. White colonies were selected and cultured overnight in LB broth with ampicillin. Plasmid DNA was isolated by alkaline lysis and cleaved with *Eco*RI, and the insert size was confirmed by agarose gel electrophoresis.

DNA sequencing. Plasmid DNA was isolated using the standard protocol from a QIAprep Spin Plasmid Kit (Qiagen Inc., Valencia, Calif.). Clones representing each strain were manually sequenced by a modification (42) of the dideoxy chain termination method of Sanger et al. (36). The resulting denatured double-stranded plasmid was sequenced using a Sequenase Quick-Denature Plasmid Sequencing Kit according to the directions of the manufacturer (Amersham Life Science, Cleveland, Ohio). ^{35}S-dATP-labeled sequencing reaction mixtures were electrophoresed on a 6% acrylamide gel. The dried gel was exposed to X-ray film, and the sequence was recorded. Analysis of DNA sequences was performed using DNAsis version 2.5 for Windows (Hitachi, San Bruno, Calif.).

PCR amplification for ligation into pUC19. Specific oligonucleotide pairs derived from the sequence obtained from each species were synthesized in order to amplify the entire 17-kDa gene with the putative ribosome binding site from each individual *Bartonella* strain. Each oligonucleotide primer derived from the 5′ end of the gene was designed with an *Xba*I site (-TCTAGA-) near the 5′ end, and the primer derived from the 3′ end of the gene contained a *Bam*HI site (-GGATCC-) near the 5′ end to allow directional cloning. Amplification was achieved through initial denaturation at 94°C for 4 min; 3 cycles of 94°C for 1 min, 42°C for 2 min, and 67°C for 2 min; and 30 cycles of 94°C for 1 min, 50°C for 2 min, and 70°C for 1 min 30 s. Each amplicon was digested with *Xba*I and *Bam*HI and ligated into pUC 19 cleaved with the same two enzymes so that the 17-kDa antigen gene homologs were immediately downstream of the inducible *lacZ* α-peptide promoter. The ligation mixtures were transformed into *E. coli* JM109 as previously described (19), and clones to be used for expression were identified by restriction endonuclease analysis and agarose gel electrophoresis. All recombinants were sequenced again using fluorescent-dye-labeled primers with a Thermosequenase cycle sequencing kit (Amersham) and an automated DNA sequencing and genetic analysis system (Li-Cor Inc., Lincoln, Nebr.).

SDS-PAGE and immunoblotting. Clones were grown to early log phase at 37°C in 5 ml of LB broth containing ampicillin (100 μg/ml) and induced with 1 mM isopropyl thio-β-D-galactopyranoside (IPTG) for an additional 3.5 h. Bacterial cells were harvested by centrifugation and resuspended in one-third of the original culture volume of 1× sample buffer (63 mM Tris [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 0.0025% bromophenol blue) (Novex, San Diego, Calif.), and β-mercaptoethanol was added to a final concentration of 1%. The samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4 to 20% gradient minigel (Novex). Mul-

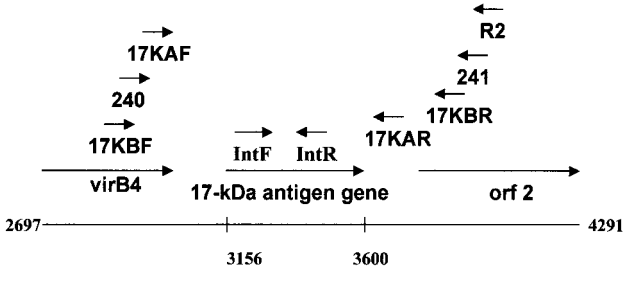

FIG. 1. Line diagram showing relative positions of oligonucleotide primers used for PCR amplification of the 17-kDa antigen gene from *Bartonella* species. The positions of the oligonucleotides are with respect to the sequence of the 17-kDa antigen gene and flanking sequences of *B. henselae* Houston-1 (accession number U23447). Nucleotide positions: 17KBF, 2921 to 2940; 240, 2986 to 3008; 17KAF, 3035 to 3054; 17KAR, 3609 to 3629; 17KBR, 3772 to 3791; 241, 3806 to 3826; R2, 4012 to 4031; Intf, 3158 to 3178; and IntR, 3583 to 3601.

tiMark multicolored standards were used to determine the approximate molecular weight (Novex). The proteins were transferred to nitrocellulose and blocked overnight in Tris-buffered saline (TBS) with 5% skim milk. The resulting membrane filter was incubated with serum diluted 1:200 (human sera) or 1:300 (rabbit serum) in TBS–5% skim milk for 2 h. The membrane filter was washed in TBS with 0.05% Tween 20 four times and exposed to either peroxidase-labeled goat anti-rabbit affinity-purified antibodies (Kirkegaard and Perry, Gaithersburg, Md.; diluted 1:7,000 in TBS with 5% skim milk) or peroxidase-labeled goat anti-human affinity purified antibodies (Kirkegaard and Perry; diluted 1:5,000 in TBS with 5% skim milk). The filter was then washed, and bound antibody was detected with TMB membrane substrate according to directions of the manufacturer (Kirkegaard and Perry).

The human sera used for immunoblots were collected from human immunodeficiency virus (HIV)-infected patients attending the HIV Clinic at the Bay Pines Veterans Affairs Medical Center, Bay Pines, Fla., after informed consent was obtained using a protocol approved by the Institutional Review Board. Serum samples were tested for antibodies to *Bartonella* by IFA as previously described (35). Only sera with IFA titers of 128 or greater were included, and these were tested individually (at a 1:200 dilution) or pooled (and then diluted 1:200). In another experiment, an individual serum sample obtained from a patient diagnosed with CSD with an IFA titer of 2,048 was provided by Patricia Emmanuel, Department of Pediatrics, University of South Florida College of Medicine. In other experiments, polyclonal hyperimmune rabbit serum raised to a fusion protein of the *B. henselae* (Houston-1) 17-kDa antigen was used at a 1:300 dilution. To produce this serum, a New Zealand White rabbit was immunized with 500 μg of the fusion protein, which has been previously described (5). The rabbit was boosted with an additional 500 μg after 4 weeks, and the animal was bled 1 week later and the serum was collected. The *B. henselae* (Houston-1) 17-kDa antigen expressed in *Escherichia coli* was used as a positive control, and *E. coli* JM109 harboring pUC19 with no insert was used as a negative control.

In vitro transcription-translation. Plasmid template was used in an in vitro transcription-translation reaction designed for circular prokaryotic templates (Promega, Madison, Wis.). Plasmid-encoded proteins were labeled with [^{35}S]methionine and resolved on a 4 to 20% gradient gel (Novex). The resulting gel was exposed to Enhance autoradiography enhancer (NEN Life Science Products, Boston, Mass.), dried, and exposed to X-ray film.

Nucleotide sequence accession numbers. The nucleotide sequence for the 17-kDa antigen gene of *B. henselae* has been previously published (5) and deposited in the GenBank database under accession number U23447. The accession numbers for the genes from other strains of *Bartonella* are as follows: *B. henselae* (San Antonio-1), AF199503; *B. quintana* (Fuller), AF199006; *B. quintana* (U.Mass), AF199007; *B. elizabethae*, AF195504; *B. clarridgeiae*, AF195506; *B. vinsonii* subsp. *vinsonii*, AF195505; and *B. vinsonii* subsp. *berkhoffii*, AF200337.

RESULTS

PCR amplification. PCR amplification of templates from various *Bartonella* species resulted in products of the approximate predicted size from *B. henselae* (San Antonio-1 strain), *B. clarridgeiae*, *B. quintana* (Fuller strain), *B. quintana* (U.Mass strain), *B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii*, and *B. elizabethae* with one or more primer pairs. The primer pairs which provided the best amplification for subsequent cloning and sequencing are described in Materials and Methods. *B. bacilliformis* produced small amounts of a PCR product that was much larger than predicted for a homolog of the 17-kDa antigen gene using primer pairs 17KAF-17KAR, 17KAF-17KBR, and 240-241. Sequencing of clones harboring these amplicons resulted in the identification of an open reading frame capable of coding for a protein of 18 kDa. However, despite the similarity in predicted size with the 17-kDa antigen, no obvious amino acid sequence identity was observed. In an additional experiment utilizing primers internal to the most highly conserved coding regions of the 17-kDa antigen gene (IntF and IntR), product was amplified from all *Bartonella* species tested except *B. bacilliformis* (data not shown). Thus, despite the use of multiple primer pairs for amplification, no evidence of a 17-kDa antigen gene was found for either strain of *B. bacilliformis*.

Sequence analysis. DNA sequencing revealed open reading frames capable of coding for proteins with deduced sizes similar to that previously described for the *B. henselae* 17-kDa antigen. Proteins of similar sizes were predicted from the sequence obtained from *B. henselae* (San Antonio-1 strain). *B. quintana* (Fuller strain and U.Mass strain), *B. clarridgeiae*, and *B. vinsonii* subsp. *berkhoffii* (Table 1). However, the *B. vinsonii* subsp. *vinsonii* and *B. elizabethae* versions of the gene were substantially larger (Table 1). Thus, there seems to be some discontinuity in predicted size among the various species, including both a human pathogen (*B. elizabethae*) and a strain currently thought to be nonpathogenic for humans (*B. vinsonii* subsp. *vinsonii*).

All homologs of the gene exhibited certain characteristics of prokaryotic gene structure. The antigen genes from *B. henselae* (San Antonio), *B. clarridgeiae*, *B. quintana* (Fuller), *B. quintana* (U.Mass.), *B. vinsonii* subsp. *vinsonii*, *B. vinsonii* subsp. *berkhoffii*, and *B. elizabethae* contained the identical polypurine-rich sequence (AGGAG) immediately upstream of the presumed ATG initiator methionine codon. These sequences presumably serve as ribosome binding sites for the antigen genes from the various species and strains. Similar sequences have been found immediately upstream of other *B. henselae* genes (4, 5, 12; A.W.O. Burgess, J.-Y. Paquet, and J.-J. Letesson, and B. Anderson, submitted for publication).

The putative initiator methionine is followed by a stretch of 18 to 25 residues that define a hydrophobic domain in all of the species that were analyzed using the algorithm of Hopp and Woods (24). In addition, two lysine codons follow the methionine start codon in all species. These properties are strongly predictive of bacterial signal peptides involved in targeting proteins for translocation across the cytoplasmic membrane (25). The deduced amino acid sequence alignment for *B. henselae* (San Antonio-1), *B. vinsonii* subsp. *berkhoffii*, and *B. clarridgeiae* indicates potential A-X-A peptidase cleavage sites (Fig. 2). Identical sequences have been shown on two other proteins of *B. henselae* that are processed and cleaved by signal peptidase before insertion into the outer membrane (12; Burgess and Anderson, submitted). Likewise, similar or identical peptide sequences have been shown to function as signal peptidase cleavage sites in *E. coli* (25). The other species revealed potential cleavage site variations that are similar to sites reported for *E. coli*, differing at one position from the consensus sequence of A-X-A (25): T-I-A, *B. quintana* (Fuller); T-I-A, *B. quintana* (U.Mass); A-F-S, *B. elizabethae*; and S-M-A, *B. vinsonii* subsp. *vinsonii*.

When the deduced amino acid sequence for the 17-kDa antigen gene is used to study phylogenetic relationships among the genus *Bartonella*, a number of interesting observations are noted (Fig. 3). First, no sequence variation was observed between the two different strains of *B. henselae*. Likewise, no amino acid variation was noted among the two strains of *B. quintana* sequenced, even though over 30 years separates their isolation. Second, *B. henselae* appears to be more closely related to *B. clarridgeiae* (approximately 15% amino acid sequence divergence) than to *B. quintana* (approximately 30% sequence divergence). Third, the remaining species, including *B. elizabethae*, *B. vinsonii* subsp. *vinsonii*, and *B. vinsonii* subsp. *berkhoffii*, appear to be only remotely related to the *B. henselae*, *B. quintana*, and *B. clarridgeiae* group. Finally, the two subspecies of *B. vinsonii* have homologs of the 17-kDa antigen that exhibit extensive sequence divergence (50%) (Fig. 3).

Expression and antigenicity of the 17-kDa antigen homologs. Immunoblot analysis with polyclonal anti-17-kDa rabbit serum demonstrated reactivity with clones of the antigen genes from *B. henselae* (Fig. 4, lanes B and C), *B. clarridgeiae* (lane D), and *B. quintana* (lanes E and F). These clones produce a doublet band with a size identical to that of the 17-kDa antigen from the Houston-1 strain of *B. henselae*. It is likely that the sources of the two bands seen in the doublet are proteins that are cleaved to various degrees by *E. coli* signal

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                10         20         30         40         50
B.h.(Hous) MKKYSLVTLL SLFCISHAKA QT--ATLTDE YYKKALENTQ KLDVAKSQTA
B.h.(S.a.) MKKYSLVTLL SLFCISHAKA QT--ATLTDE YYKKALENTQ KLDVAKSQTA
B.c.       MKKYSLVTLL SLFCISHAKA QT--APLADE YYKKALENKQ QLDVAKSQTA
B.q.(U.M.) MKKYGLVTLL SLSCVSHTIA ET--ALLADE YYKRALENTQ KLDAAKSETA
B.q.(Full) MKKYGLVTLL SLSCVSHTIA ET--ALLADE YYKRALENTQ KLDAAKSETA
B.v.b.     MKKYSLVTLL SSFFISHAVA NN--SKDVDE YYKQALESTQ KLNVVKSETA
B.e.       MKKYGLMTLL SFSFISHAFS QM--SPDADE YYKQALENTQ KLDTAKSETA
B.v.v.     MKKYSLITLL SLSFISQSMA ETVDAVDADE YYKKALESTQ ELNTVKSETA
CONS       ********** ** ***** *  *  * **** *** ****** *** ******

                60         70         80         90        100
B.h.(Hous) ESIYESATQT ANKIKDINNQ LANLKADTKT KPEQL----- ----------
B.h.(S.a.) ESIYESATQT ANKIKDINNQ LANLKADTKT KPEQL----- ----------
B.c.       ESIYESATQT ANKIKDINDQ LKTLKADTKT KPEQL----- ----------
B.q.(U.M.) ESIYASANEI TNKIKEINEK LRKAQAAEKT KPEEF----- ----------
B.q.(Full) ESIYASANEI TNKIKEINEK LRKAQAAEKT KPEEF----- ----------
B.v.b.     ETIFDHATQT AKKIQEISEQ LKQKQSQEKP DAEKL----- ----------
B.e.       ETIYKTATET AKKIKEINGQ LEQLKSQTET TATETTSDAD KNSNLEELQK
B.v.v.     ENILEQATKT AKKIQEISEK LTQAQAESQE KEKSDPEKLQ ALQIKLSVLQ
CONS       **** *** * ******** * *    *  **  ***
                110        120        130        140        150
B.h.(Hous) -----QALQI ELTLLQAQLQ ADTLKIQSLA MIQAKDTKTK EELREEQTQK
B.h.(S.a.) -----QALQI ELTLLQAQLQ ADTLKIQSLA MIQAKDTKTK EELREEQTQK
B.c.       -----QTLQI ELSLLQAQLQ ADTLKVQSLA MIQAKDTKTK EELREEQTQQ
B.q.(U.m.) -----QALQI ELSLLQAQLQ ADTLKIQFLS MIQAKNTKTK EDIREEQTQK
B.q.(Full) -----QALQI ELSLLQAQLQ ADTLKIQFLS MIQAKNTKTK EDIREEQTQK
B.v.b.     -----QTLQV ELSVLQAKLQ AEALKLQSL- MIQAKDSRTK EEIREEEIKK
B.e.       RQALRQELQV KVSLLQADLQ ANSLKLQALN MIQARDTKTK EELREEKEQQ
B.v.v.     ANLQADALKL QSLAMIQAKD SRTKEEVREE QIQKKHEEIA EKLKEKLGKS
CONS            ***** ********** ***** * *  ********** **********
                160        170        180        190        200
B.h.(Hous) KHEDLQKQLK EKLEKSDVRL *......... .......... ..........
B.h.(S.a.) KHEDLQKQLK EKLEKSDVRL *......... .......... ..........
B.c.       NHKKIEEKLK EKLGKSDVRL *......... .......... ..........
B.q.(U.M.) KHKDLQEKLK EKLGNSDVRL *......... .......... ..........
B.q.(Full) KHKDLQEKLK EKLGNSDVRL *......... .......... ..........
B.v.b.     QHEKIVQKLK EKLEKSNVPS TLGKLND*... .......... ..........
B.e.       KHKTLQAQLK EKEEKLKEKL ESTGADVRL* .......... ..........
B.v.v.     KSHQYLDRLM TDFSSSPFES VSGYILKPLN EAMDMTVENL SGALSAPLNL
CONS       *** *** ** *** ******
                210        220        230        240        250
B.h.(Hous) .......... .......... .......... .......... ..........
B.h.(S.a.) .......... .......... .......... .......... ..........
B.c.       .......... .......... .......... .......... ..........
B.q.(U.M.) .......... .......... .......... .......... ..........
B.q.(Full) .......... .......... .......... .......... ..........
B.v.b.     .......... .......... .......... .......... ..........
B.e.       .......... .......... .......... .......... ..........
B.v.v.     ASIIFIFLYG YNVMTGRVAL STHSLLNNVV KSLL*..... ..........
CONS
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FIG. 2. Multiple-sequence alignment of the deduced amino acid sequences of the 17-kDa antigen gene homologs from various *Bartonella* strains. The alignment was generated using the alignment routine of Higgins and Sharp (22) with a gap penalty of 5 and a window size of 5. The start codon (Met) is indicated at position 1, and stop codons are shown with asterisks. Putative signal sequences are underlined at residues 1 to 17, and potential signal peptidase cleavage sites are shown in boldface and underlined at positions 17 to 20. At positions where there is agreement between five or more of the different strains, a consensus sequence (CONS) is indicated by an asterisk. The sequences are identified on the left as follows. *B. henselae* Houston-1 strain, B.h. (Hous); *B. henselae* San Antonio-1 strain, B.h. (S.a.); *B. clarridgeiae*, B.c.; *B. quintana* U.Mass strain, B.q. (U.M.); *B. quintana* Fuller strain, B.q. (Full); *B. vinsonii* subsp. *berkhoffii*, B.v.b.; *B. elizabethae*, B.e.; and *B. vinsonii* subsp. *vinsonii*, B.v.v.

peptidase. An additional band is seen with *B. henselae* and *B. clarridgeiae* at approximately 28 kDa (Fig. 4, lanes B to D); it is possible that this protein is either incompletely solubilized (denatured) 17-kDa antigen or a dimer of the protein. It should be noted that only the *B. henselae* and *B. clarridgeiae* genes produced the 28-kDa band in *E. coli*, suggesting that some intrinsic variation of these versions of the protein results in the slower-migrating form of the antigen. Unlike those of the other *Bartonella* species, the *B. elizabethae* version of the antigen migrated at approximately 21 kDa (Fig. 4, lane H). The two subspecies of *B. vinsonii* failed to show any obvious immunoreactive bands at any size (Fig. 4, lanes G and I). When the DNAs from the recombinant *E. coli* strains harboring the genes derived from the two subspecies of *B. vinsonii* were used as templates in in vitro transcription-translation reactions, both plasmids directed synthesis of proteins of the predicted size

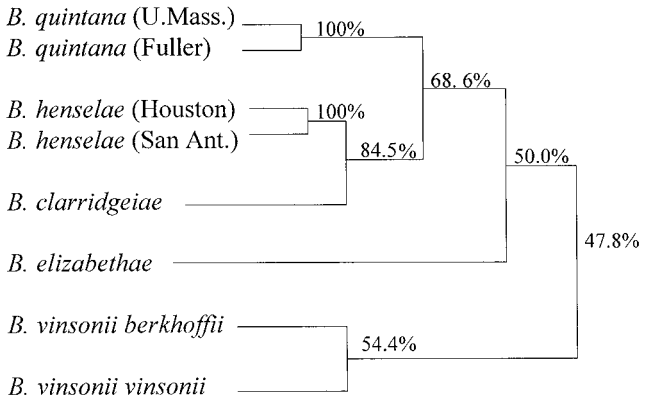

FIG. 3. Dendrogram of the deduced amino acid sequences from the 17-kDa antigen genes from each of the *Bartonella* species or subspecies. The multiple-sequence alignment was generated by the CLUSTAL package, with the branching order and matching percentages indicated at each branch point (22).

(data not shown). Thus, these proteins are expressed in *E. coli* but are not reactive with the rabbit anti-*B. henselae* 17-kDa antigen serum. Cross-reactive rabbit antibodies are observed to react with bands at other sizes but are also reactive with the *E. coli*-plus-vector control (Fig. 4, lane A), indicating they are *E. coli* proteins. These data indicate that epitope(s) recognized by the immunized rabbit is conserved in some but not all species of *Bartonella*.

The immunoblot shown in Fig. 5 was reacted with a representative human serum specimen from a patient diagnosed with CSD who was shown to have a positive IFA titer to *B. henselae*. Reactivity with a protein of approximately 17 kDa was noted only with the two strains of *B. henselae* (lanes B and C) and *B. clarridgeiae* (lanes D). However, unlike the immunoblot with the rabbit serum, there was no cross-reactivity with

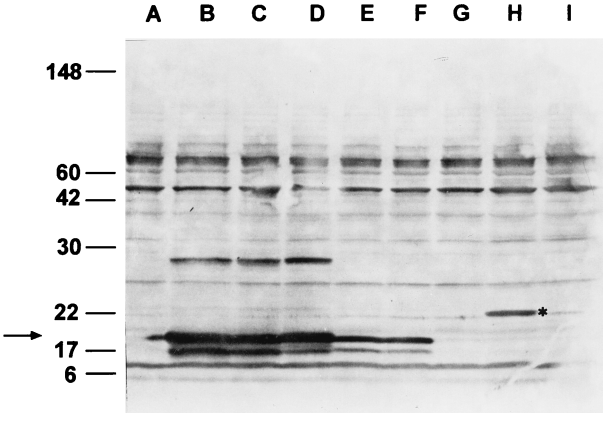

FIG. 4. Immunoblot of *E. coli* strains containing plasmids encoding homologs of the 17-kDa antigen gene from different *Bartonella* species reacted with polyclonal rabbit serum raised to the 17-kDa antigen of *B. henselae*. Total proteins from *E. coli* strains were resolved by SDS-PAGE and transferred to nitrocellulose. The serum sample used was raised to recombinant *B. henselae* 17-kDa antigen as described in the text. The sources of the antigen genes in the recombinants are as follows: lane B, *B. henselae* (Houston-1); lane C, *B. henselae* (San Antonio-1); lane D, *B. clarridgeiae*; lane E, *B. quintana* (Fuller); lane F, *B. quintana* (U.Mass); lane G, *B. vinsonii* subsp. *berkhoffii*; lane H, *B. elizabethae*; and lane I, *B. vinsonii* subsp. *vinsonii*. Lane A, *E. coli* host strain without cloned *Bartonella* DNA. The positions of molecular mass standards are indicated at the left in kilodaltons, and the position of the 17-kDa antigenic proteins is indicated with an arrow. The position of the larger *B. elizabethae* homolog is marked with an asterisk.

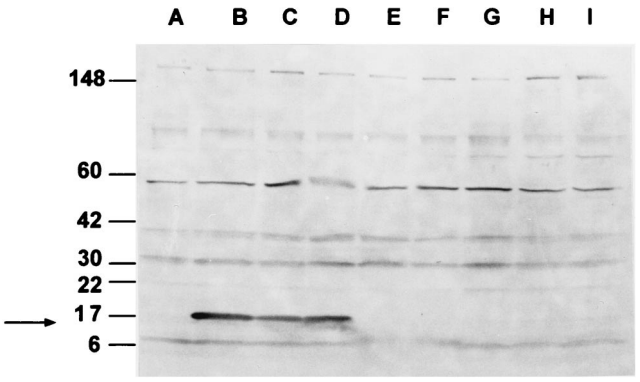

FIG. 5. Immunoblot of *E. coli* strains containing plasmids encoding homologs of the 17-kDa antigen gene from different *Bartonella* species reacted with human serum. Total proteins from *E. coli* strains were resolved by SDS-PAGE and transferred to nitrocellulose. The serum sample used was from a patient clinically diagnosed with CSD and having an IFA titer to *B. henselae* of 2,048. The sources of the antigen genes in the recombinants are as follows: lane B, *B. henselae* (Houston-1); lane C, *B. henselae* (San Antonio-1); lane D, *B. clarridgeiae*; lane E, *B. quintana* (Fuller); lane F, *B. quintana* (U.Mass); lane G, *B. vinsonii* subsp. *berkhoffii*; lane H, *B. elizabethae*; and lane I, *B. vinsonii* subsp. *vinsonii*. Lane A, *E. coli* host strain without cloned *Bartonella* DNA. The positions of molecular mass standards are indicated at the left in kilodaltons. The positions of the 17-kDa antigen homologs is indicated with an arrow.

either strain of *B. quintana* (lanes E and F), *B. elizabethae* (lane H), or the two strains of *B. vinsonii* (lanes G and I). A pool of three other human sera gave identical results, reacting only with the *B. henselae* and *B. clarridgeiae* clones (data not shown). Additional bands are observed at other sizes but are reactive with the *E. coli*-plus-vector control, indicating that they are *E. coli* proteins. These results suggest that infection in humans may elicit antibodies that are not broadly reactive across the genus.

DISCUSSION

Human infection by *B. henselae* and *B. quintana* results in a diverse array of clinical symptoms. Laboratory diagnosis of infections caused by these two agents requires isolation and identification, PCR amplification of bacterial DNA, or the presence of specific antibodies detected by serology. Only serologic testing is widely available, and it remains the most common means of diagnosing infections caused by *Bartonella* species. Several serologic assays have been described for detecting specific antibodies, including enzyme-linked immunosorbent assay (8, 23, 38) and IFA (35). Of these the IFA is the most widely used and thoroughly evaluated test (16). However, recent reports have indicated that the predictive value of the IFA may vary among different investigators (2, 9, 16, 33, 41). Variable results and sensitivities of this test have resulted in reports questioning the use of the IFA for diagnosis (9). The cause of such variability is not known, but it may be due to different methods of antigen preparation. Likewise, cross-reactivity between *Bartonella* and *Coxiella*, *Chlamydia*, and other bacteria has been well documented (23, 27). It is also known that the IFA is not specific for individual *Bartonella* species, with a patient's serum usually being reactive with antigens from one or more *Bartonella* species by IFA (6).

B. quintana appears to be a common pathogen both in the United States and abroad, and *B. elizabethae* and *B. clarridgeiae* have been associated with human disease (6). A number of other *Bartonella* species, subspecies, and strain variants have been associated with rodents, providing a common potential reservoir for transmission. In addition, *B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *berkhoffii* have been identified as being capable of causing diseases of veterinary importance. Standardized antigens for serologic testing to detect specific antibodies to many of these *Bartonella* species have not been described. By characterizing individual protein antigens of various *Bartonella* species, we may be able to identify antigens or epitopes specific for each of the *Bartonella* species that are more specific than current serologic tests.

To investigate the humoral immune response to *Bartonella* infection, we have focused on individual protein antigens. One such antigen, the 17-kDa antigen of *B. henselae*, has been expressed as a fusion protein and shown to be reactive with sera from 92% of the CSD cases tested, suggesting that it may be of value as a diagnostic reagent (5). A homologous version of this antigen was found in all species of *Bartonella* tested except *B. bacilliformis*. The nucleotide sequence and the corresponding deduced amino acid sequence shared various levels of homology among the species tested (Fig. 2). However, the first 4 amino acids (MKKY) were identical for all species, and the first 20 residues were similar with regard to overall hydrophobicity and charge. The hydrophobicity and presence of lysine residues at the immediate amino terminus are characteristic of translocated proteins of bacteria. The lysines are thought to interact with the phosphate groups of the membrane phospholipids, and the hydrophobic core domain is thought to interact with the lipid moieties (25). The hydrophobic core is followed by a potential signal peptidase cleavage site. Alanine is frequently found at the -3 and -1 positions upstream of the cleavage site in *E. coli* (25) and was found in the *B. henselae* and *B. elizabethae* antigens in this study. Other small neutral side chains, such as glycine, serine, and threonine, are often seen in these positions (25), which was consistent with findings for *B. quintana* (Fuller and U.Mass), *B. elizabethae*, and *B. vinsonii* subsp. *vinsonii*. The signal sequence of A-X-A has been shown by our laboratory to be a signal peptidase cleavage site for two other outer membrane proteins of *B. henselae* (12; Burgess and Anderson, submitted). The presence of doublet bands on the immunoblots is also consistent with the role of signal peptidase in processing this antigenic protein. Thus, it is likely that the proteins from each of the *Bartonella* species included in this study are translocated across the cytoplasmic membrane.

The deduced amino acid sequences were used to construct a dendrogram of phylogenetic relationships. Surprisingly, the two species that were the most closely related were *B. henselae* and *B. clarridgeiae* (84.5%). The *B. henselae* and *B. quintana* versions of the 17-kDa antigen showed extensive sequence divergence (>30%), and no variation for different strains within *B. henselae* (Houston-1 and San Antonio-1 strains) and *B. quintana* (Fuller and U.Mass strains) was noted. In contrast, the two subspecies of *B. vinsonii* produced vastly different deduced amino acid sequences with extensive sequence divergence for the antigen (45.6%). It is possible that these two subspecies are more remotely related than previously thought (29), or, alternatively, that genetic exchange involving the 17-kDa antigen gene has hastened the evolutionary process.

When the cloned versions of the 17-kDa antigen from each of the species were reacted with human serum samples from patients with *Bartonella* infections, only *B. henselae* and the *B. clarridgeiae* were reactive. These data are also consistent with the phylogenetic relationship showing that the antigen is more closely related between *B. henselae* and *B. clarridgeiae* than any two other species. These results also suggest that the epitope(s) recognized by human sera from patients infected with *B. henselae* is not cross-reactive with *B. quintana*; however, since

isolation from the patients whose sera were used in this study was not attempted, this observation cannot be confirmed. If this lack of cross-reactivity is confirmed upon further evaluation, recombinant 17-kDa antigens could be used to differentiate infections caused by *B. henselae* and *B. clarridgeiae* from those caused by *B. quintana*. Since *B. henselae* and *B. clarridgeiae* are associated with CSD and *B. quintana* is more frequently associated with severe systemic disease, identification of *B. quintana* as a causative agent may indicate the need for more aggressive treatment with antibiotics that are not always prescribed for CSD. The rabbit serum raised to purified recombinant *B. henselae* 17-kDa protein was broadly cross-reactive with all species except *B. vinsonii*, showing that at least one epitope recognized by the rabbit serum is well conserved.

The identification and sequencing of homologs of the 17-kDa antigen should facilitate the development of both serologic and genetic tools for the diagnosis of *Bartonella* infections. The use of recombinant protein derived from each of the pathogenic species of *Bartonella* as an antigen for enzyme-linked immunosorbent assay should permit rapid serologic testing that discriminates infections caused by *B. henselae* from those caused by *B. quintana*. If the need arises for serologic assays that are specific for other *Bartonella* species, then it should be possible to utilize the appropriate 17-kDa antigen homolog as an antigen. Additionally, the use of conserved antigen genes as targets for gene probes and PCR primers has proven useful for rapid detection and identification of bacteria directly in clinical specimens or clinical isolates. Currently, the species level identification of *Bartonella* isolates is often based on PCR amplification of conserved genes followed by the use of specific probes (7) or restriction fragment length polymorphism analysis (10, 32). The use of PCR primers derived from segments of the 17-kDa antigen gene which are unique to each species could easily shorten and simplify this process.

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