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Rapid Identification and Differentiation of *Bartonella* Species Using a Single-Step PCR Assay

WAYNE A. JENSEN,[1]* MAJILINDE Z. FALL,[1] JANE ROONEY,[1] DORSEY L. KORDICK,[2] AND EDWARD B. BREITSCHWERDT[2]

Heska Corporation, Fort Collins, Colorado 80525,[1] and Department of Companion Animal and Special Species Medicine, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606[2]

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Five species of *Bartonella* have been reported to infect humans and cause a variety of diseases that can be difficult to diagnose. Four species of *Bartonella* have been reported to infect cats and dogs, and two of these species are considered to be zoonotic pathogens. Diagnosis of *Bartonella* infections is hampered by the slow, fastidious growth characteristics of *Bartonella* species. We report on the development of a single-step PCR-based assay for the detection and differentiation of medically relevant *Bartonella* species. PCR-mediated amplification of the 16S-23S rRNA intergenic region resulted in a product of a unique size for each *Bartonella* species, thereby allowing differentiation without the necessity of restriction fragment length polymorphism analysis or sequencing of the amplified product. The ability of the single-step PCR assay to differentiate between *Bartonella* species was determined with characterized isolates and blood samples from animals known to be infected with either *Bartonella henselae*, *B. clarridgeiae*, or *B. vinsonii* subsp. *berkhoffii*. The sensitivity of the single-step PCR assay relative to that of in vitro culture was determined with blood samples from *B. henselae*-infected cats. *B. henselae* target DNA was amplified from 100% of samples with greater than 50 CFU/ml and 80% of samples with 10 to 30 CFU/ml. The single-step assay described in the report expedites PCR-based detection and differentiation of medically relevant *Bartonella* species.

Bartonella species are gram-negative, fastidious, aerobic, short rod-shaped bacteria. The genus *Bartonella* includes 13 described species, 3 of which have been isolated from humans only (*Bartonella bacilliformis*, *B. elizabethae*, and *B. quintana*), 2 of which have been isolated from cats and dogs only (*B. koehlerae* and *B. vinsonii* subsp. *berkhoffii*, respectively), and 2 of which have been reported to infect both humans and cats (*B. clarridgeiae* and *B. henselae*). The remaining *Bartonella* species (*B. doshiae*, *B. grahamii*, *B. peromysci*, *B. talpae*, *B. taylorii*, and *B. vinsonii* [Baker strain]) have been isolated from the blood of rodents and moles (for reviews, see references 1 and 2). *B. bacilliformis* is the etiologic agent of Oroya fever and verruga peruana in South America. *B. elizabethae* was isolated from the blood of a patient with endocarditis. *B. quintana* is the etiologic agent of trench fever and has been shown to cause bacillary angiomatosis and endocarditis in immunocompromised individuals. *B. henselae* is most frequently associated with cat-scratch disease (CSD) and has also been associated with bacillary angiomatosis and endocarditis in immunocompromised individuals. *B. clarridgeiae* was originally isolated from the blood of a cat (7) and was subsequently associated with CSD in humans (19, 23). *B. henselae* and *B. clarridgeiae* are frequently isolated from domestic cats and are considered to be zoonotic pathogens. Cats can be persistently infected with *B. henselae* or *B. clarridgeiae* without obvious signs of illness (17, 30), although histopathologic lesions (11, 18) and a pathogenic strain of *B. henselae* which causes swelling at the site of inoculation, fever, and lethargy have been described (29). Recently, *B. koehlerae*, a proposed new species belonging to the genus *Bartonella*, has also been isolated from cats (9). Infection of domestic dogs with *B. henselae* and the association of CSD in humans with scratches from dogs have been described (16, 35). *B. vinsonii* subsp. *berkhoffii* has been reported to cause endocarditis in dogs (6).

Bartonella species are associated with a variety of human diseases. Unfortunately, diagnosis of diseases caused by these species is hampered by their slow, fastidious, growth characteristics. A variety of rapid detection assays have been developed, including PCR amplification of the 16S-23S rRNA intergenic region with genus- and species-specific primer sets (26), species-specific amplification of *ftsZ* gene sequences (15), repetitive-element PCR (32), restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rRNA genes (4, 8), RFLP analysis of the PCR-amplified 16S-23S rRNA intergenic region (24, 34), RFLP analysis of the PCR-amplified citrate synthase gene (28), and sequence analysis of the PCR-amplified citrate synthase gene (5, 13). However, each of these methods requires multiple PCR amplifications or additional sample-processing steps beyond the primary PCR amplification. Additionally, assay performance was demonstrated only with cultured bacteria for all but one (8) of the assays described above.

We report on the development of a single-step PCR-based assay for the differentiation of *Bartonella* species from one another. PCR is used to amplify a fragment of the 16S-23S rRNA intergenic region with primers complementary to sequences conserved in all medically relevant *Bartonella* species. The 16S-23S rRNA intergenic region was chosen because of its uniqueness compared to the sequences of other bacteria (33). Identification of conserved 16S-23S rRNA intergenic sequences for relevant *Bartonella* species required sequencing of the 16S-23S rRNA intergenic region from *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii*. After amplification, PCR products are size fractionated by gel electrophoresis and *Bartonella* species are identified by the unique size of the amplified product without the necessity of further sample processing. The utility of this assay was tested by detection of *Bartonella* species di-

* Corresponding author. Mailing address: Heska Corporation, 1613 Prospect Parkway, Fort Collins, CO 80525. Phone: (970) 493-7272. Fax: (970) 493-7333. E-mail: jensenw@heska.com.

rectly from blood samples derived from bacteremic cats and dogs.

MATERIALS AND METHODS

Bacterial strains. *B. bacilliformis* ATCC 35685, *B. clarridgeiae* ATCC 51734 and ATCC 700095, *B. elizabethae* ATCC 49927, *B. quintana* ATCC 51694, *B. vinsonii* subsp. *berkhoffii* ATCC 51572, and *Brucella canis* ATCC 23365 were obtained from the American Type Culture Collection (Rockville, Md.). *B. henselae* isolates Houston-1 (ATCC 49882), Oklahoma (ATCC 49793), Marseilles, MO-2, SA-1, CA-4, Tiger-2, and Lassiter were kindly provided by Russell Regnery, Viral and Rickettsial Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Ga.

Clinical samples. Blood was obtained by aseptic procedures from the jugular veins of cats or dogs and placed in tubes that contained EDTA anticoagulant. Molecular characterization of the *B. henselae*, *B. clarridgeiae*, and *B. vinsonii* subsp. *berkhoffii* isolates from these naturally infected cats and dogs has been reported previously (17).

Determination of number of *Bartonella* species CFU per milliliter of blood. Blood from cats experimentally infected with the Houston-1 strain of *B. henselae* was collected and was placed in tubes with EDTA as an anticoagulant. The samples were stored at −70°C until they were processed for culture. A total of 100 μl of blood was serially diluted 10-fold three times in brain heart infusion broth. A total of 100 μl of blood and 100 μl of each serial dilution were plated onto heart infusion agar plates containing 5% rabbit blood (catalog no. 4321-356; BBL). Agar plates were placed inside plastic bags and were then incubated at 32°C with 5% CO_2 for 1 week. Cultures were read at 1 week; negative plates were incubated for 5 weeks before they were discarded as negative.

DNA extraction and PCR amplification of the 16S-23S rRNA intergenic region. DNA for PCR amplification was prepared from pure cultures of each bacterial strain by using the QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, Calif.) and from blood by using the QIAamp DNA Blood Mini Kit. PCR amplifications were performed in mixtures of 50 μl containing 10 mM Tris (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 200 μM each dATP, dCTP, and dGTP, 400 μM dUTP, 1 μM each primer, and 2.5 U of Amplitaq Gold DNA polymerase (PE Applied Biosystems, Foster City, Calif.). Amplification buffer was optimized with dUTP for use with uracil glycosylase to prevent PCR amplification product carryover. Optimum primer annealing temperatures were determined in a RoboCycler Gradient Temperature Cycler (Stratagene, La Jolla, Calif.). Amplifications were performed in a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems) by a timed-release PCR protocol (14), as follows: 10 min of incubation at 20°C, followed by 2 min of denaturation at 95°C and then 45 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 60°C, and 30 s of extension at 72°C. PCR amplification products were identified by ethidium bromide fluorescence after electrophoresis in 3% agarose gels.

DNA sequencing. PCR amplification of the entire 16S-23S rRNA intergenic region was accomplished with primers described by Matar et al. (24) and Roux and Raoult (33, 34). PCR products amplified from the 16S-23S rRNA intergenic regions of *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* were sequenced with an ABI PRISM model 377 with XL upgrade DNA Sequencer (PE Applied Biosystems) after product labeling with the PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) by the manufacturer's protocol. Sequence alignments and phylogenetic comparisons were done with the DNA analysis computer program DNAsis (Hitachi Software Engineering America Ltd., South San Francisco, Calif.).

Nucleotide sequence accession numbers. The 16S-23S rRNA intergenic sequences for *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* have been submitted to GenBank under accession nos. AF167989 and AF167988, respectively.

RESULTS

Comparison of 16S-23S rRNA intergenic sequences of *Bartonella* species. The 16S-23S rRNA intergenic sequences for *B. bacilliformis* (GenBank accession no. L26364 [27]), *B. elizabethae* (GenBank accession no. L35103 [33]), *B. henselae* (GenBank accession no. L35101 [33]), *B. quintana* (GenBank accession no. L35100 [33]), *B. vinsonii* (Baker strain) (GenBank accession no. L35102 [33]), and *B. abortus* (GenBank accession no. X95889 [31]) were aligned by using the DNA analysis computer program DNAsis (Hitachi Software Engineering America Ltd.). Figure 1 illustrates the alignment of approximately 200 nucleotides in the 5′ region of the 16S-23S intergenic sequences. In this region, a nonconserved area is bordered by two areas with high degrees of homology. The sequences of individual *Bartonella* species differ in the nonconserved region primarily due to sequence insertions and/or deletions. The extent of variation suggested that PCR primers designed to amplify across the nonconserved region would generate amplified products of different sizes for each species of *Bartonella*. A PCR assay was designed to amplify the region shown in Fig. 1. Template DNA was obtained from *B. bacilliformis*, *B. clarridgeiae*, *B. elizabethae*, *B. henselae*, *B. quintana*, and *B. vinsonii* subsp. *berkhoffii. B. koehlerae* was not available for analysis at the time that this work was performed. Analysis of *B. vinsonii* (Baker strain) was not included because this

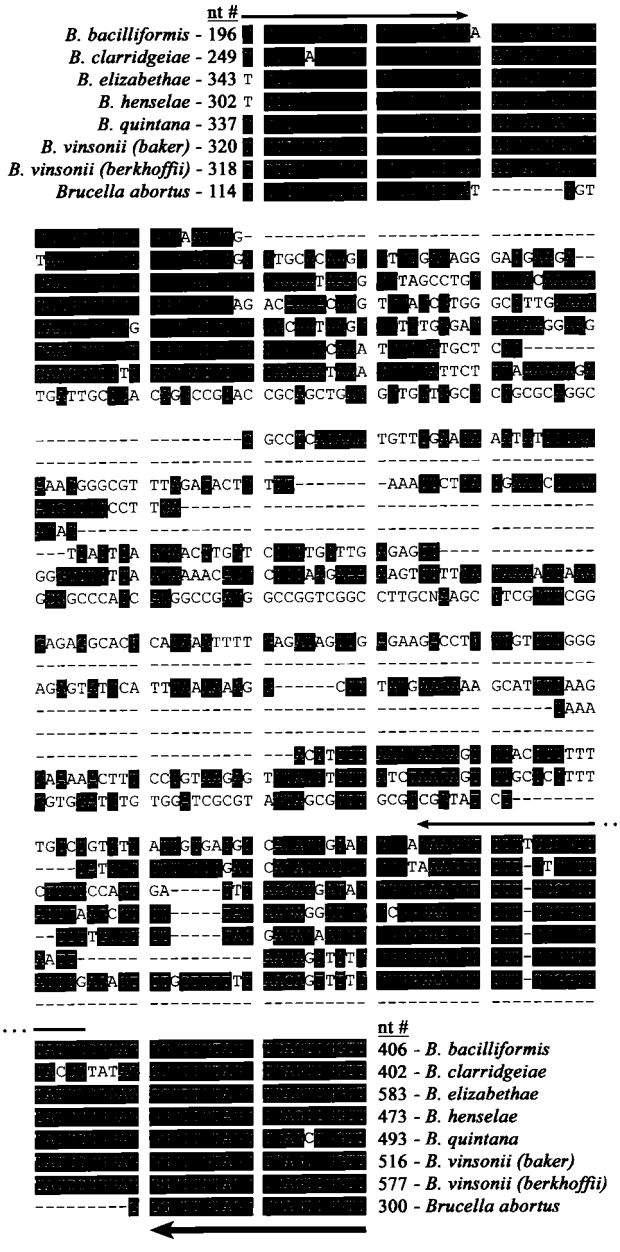

FIG. 1. Nucleotide sequence alignment of a portion of the 16S-23S rRNA intergenic region of *B. bacilliformis* (GenBank accession no. L26364), *B. clarridgeiae* (GenBank accession no. AF167989), *B. elizabethae* (GenBank accession no. L35103), *B. henselae* (GenBank accession no. L35101), *B. quintana* (GenBank accession no. L35100), *B. vinsonii* (Baker strain) (GenBank accession no. L35102), *B. vinsonii* subsp. *berkhoffii* (GenBank accession no. AF167988), and *B. abortus* (GenBank accession no. X95889). The corresponding GenBank nt numbers are indicated at the beginnings and ends of the sequences. The thin-line arrows designate the original PCR primer positions. The thick-line arrow designates the position of the revised reverse primer.

Bartonella species has not been associated with disease in either humans or domestic animals. Template DNA was amplified using 5′-(C/T)CTTCGTTTCTCTTTCTTCA-3′ (*B. henselae* nucleotides [nt] 302 to 321) and 5′-GGATAAACCGGAA AACCTTC-3′ (*B. henselae* nt 448 to 429) as forward and reverse primers, respectively. The 16S-23S rRNA intergenic sequences predict that these primers should amplify products of 186 bp (*B. bacilliformis*), 216 bp (*B. elizabethae*), 147 bp (*B. henselae*), and 132 bp (*B. quintana*). A predicted product size could not be determined for *B. clarridgeiae* or *B. vinsonii* subsp. *berkhoffii* because the sequence of the 16S-23S rRNA intergenic region for these *Bartonella* species had not been reported. After amplification, the PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and photographed. Products of the expected size were amplified from *B. bacilliformis*, *B. elizabethae*, *B. henselae*, and *B. quintana* template DNAs. The template DNA from *B. vinsonii* subsp. *berkhoffii* yielded a PCR product of approximately 235 bp rather than the 172-bp product predicted from the *B. vinsonii* (Baker strain) sequence. PCR amplification of the *B. clarridgeiae* template DNA yielded no product in reactions with these primers (data not shown).

Sequencing of the 16S-23S rRNA intergenic region for *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii*. To investigate the failure to amplify a product from *B. clarridgeiae* and the discrepancy between the amplification product from *B. vinsonii* subsp. *berkhoffii* and the size predicted from the *B. vinsonii* (Baker strain) sequence, the 16S-23S rRNA intergenic regions from *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* were sequenced (GenBank accession nos. AF167989 and AF167988, respectively). Alignment of the *B. vinsonii* subsp. *berkhoffii* 16S-23S rRNA intergenic region revealed 63 bp inserted in the target region relative to the *B. vinsonii* (Baker strain) sequence (Fig. 1). Analysis of the *B. clarridgeiae* 16S-23S rRNA intergenic region sequence revealed that the 3′ nucleotide of the reverse PCR primer sequence is not conserved in *B. clarridgeiae* (Fig. 1). Annealing of the 3′ nucleotide is critical for extension by *Taq* polymerase, thus explaining the inability to amplify a PCR product from *B. clarridgeiae* template DNA.

PCR-based differentiation of *Bartonella* species. To detect and differentiate medically relevant *Bartonella* species, a new reverse primer complementary to the 16S-23S rRNA intergenic region sequences shared by all of the *Bartonella* species was selected for PCR amplification (Fig. 1). Amplification of template DNA with 5′-(C/T)CTTCGTTTCTCTTTCTTCA-3′ (*B. henselae* nt 302 to 321) and 5′-AACCAACTGAGCTACA AGCC-3′ (*B. henselae* nt 473 to 454) as forward and reverse primers, respectively, resulted in amplified products corresponding to those of the predicted size, namely, 211 bp (*B. bacilliformis*), 154 bp (*B. clarridgeiae*), 241 bp (*B. elizabethae*), 172 bp (*B. henselae*), 157 bp (*B. quintana*), and 260 bp (*B. vinsonii* subsp. *berkhoffii*) (Fig. 2). PCR products from amplification of *B. clarridgeiae* (154 bp) and *B. quintana* (157 bp) DNAs are not readily differentiated by 3% agarose gel electrophoresis (Fig. 2) but can be differentiated by 4.5% agarose or 10% polyacrylamide gel electrophoresis (data not shown). Amplification of template DNAs derived from the CA-4, MO-2, SA-1, Houston, Lassiter, Marseilles, Oklahoma, and Tiger-2 isolates of *B. henselae* yielded amplification products of the same size, demonstrating conservation of this target region among different isolates of *B. henselae* (data not shown). Amplification of template DNAs derived from *Clostridium perfringens*, *Enterobacter cloacae*, *Escherichia coli*, *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, *Ehrlichia risticii*, *Fusobacterium necrophorum*, *Klebsiella pneumoniae*, *Salmonella choleraesuis*, and *Staphylococcus intermedius* did not result in product amplification (data not shown). Amplification of template DNA derived from *Brucella canis*, a member of the alpha-2 subdivision of the class *Proteobacteria* that is closely related to the genus *Bartonella* (10), yielded a product of 188 bp which was easily differentiated from the *B. henselae* (172-bp) and the *B. bacilliformis* (211-bp) products (data not shown). Amplification with forward and reverse primers that comprise sequences from adjacent regions, namely, 5′-CTCT TTCTTCAGATGATGATCC-3′ (*B. henselae* nt 311 to 332) and 5′-AACCAACTGAGCTACAAGCCCT-3′ (*B. henselae* nt 473 to 452) (Fig. 1), respectively, resulted in amplified products of approximately 202 bp (*B. bacilliformis*), 145 bp (*B. clarridgeiae*), 232 bp (*B. elizabethae*), 163 bp (*B. henselae*), 148 bp (*B. quintana*), and 251 bp (*B. vinsonii* subsp. *berkhoffii*) but failed to amplify a product from *Brucella canis* template DNA (data not shown).

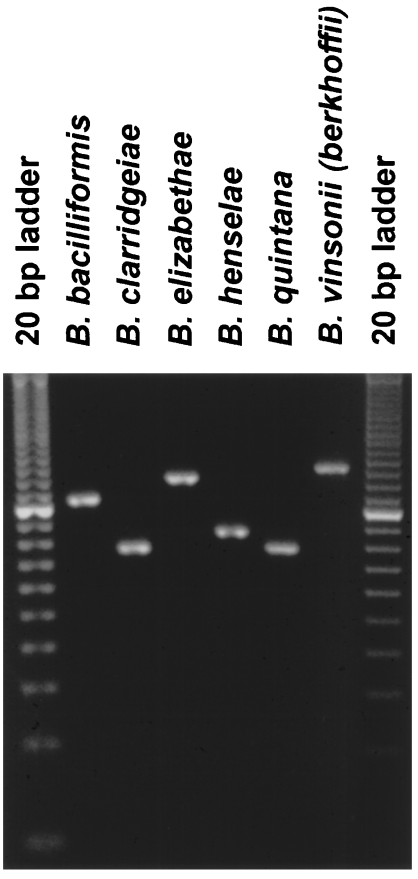

FIG. 2. PCR-based identification of *Bartonella* species. An ethidium bromide-stained agarose gel (3%) demonstrating amplified products from template DNAs derived from *Bartonella* species is shown. *B. bacilliformis*, *B. clarridgeiae*, *B. elizabethae*, *B. henselae*, *B. quintana*, and *B. vinsonii* subsp. *berkhoffii* yielded expected products of 211, 154, 241, 172, 157, and 260 bp, respectively. The first and last lanes each contain a 20-bp ladder.

Phylogenetic analysis of *Bartonella* species. The entire 16S-23S rRNA intergenic sequence of *B. clarridgeiae* and *B. vinsonii* subsp. *berkhoffii* were compared with the reported sequences of *B. bacilliformis*, *B. elizabethae*, *B. henselae*, *B. quintana*, and *B. vinsonii* (Baker strain) (Fig. 3). The 16S-23S rRNA intergenic regions of *Bartonella* species vary from 906 bp in length (*B. bacilliformis*) to 1,529 bp (*B. elizabethae*) (33) and have from 45 to 76% homology. As expected, *B. vinsonii* subsp. *berkhoffii* is most closely related to *B. vinsonii* (Baker strain).

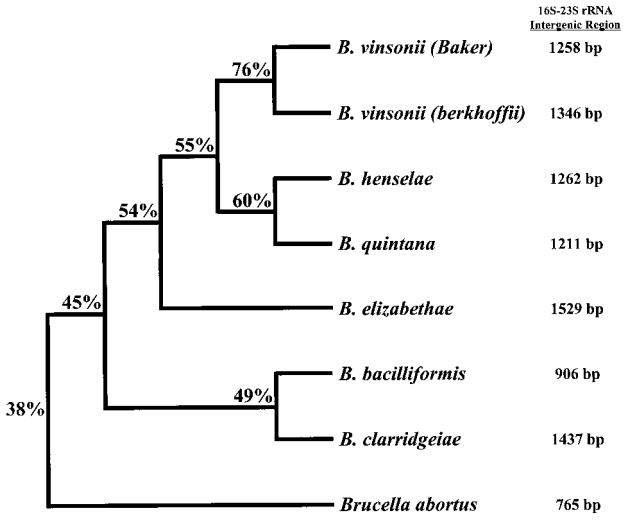

FIG. 3. Phylogenetic comparison of 16S-23S rRNA intergenic region sequences for *Bartonella* species and *B. abortus*. Calculated matching percentages are indicated at each branch point of the dendrogram. The lengths of the horizontal and vertical lines are not significant. The lengths of the 16S-23S rRNA intergenic region sequences (in base pairs) are located at the right.

On the basis of 16S-23S rRNA intergenic region sequences, *B. clarridgeiae* is most closely related to *B. bacilliformis* (Fig. 3). For comparison, Fig. 3 illustrates the relatedness of *B. abortus* to *Bartonella* species. The 16S-23S rRNA intergenic region of *Brucella* species are 765 bp in length, with approximately 99% homology among the six species (10, 31).

PCR detection of *Bartonella* species in clinical samples. To evaluate the utility of the PCR assay for detection of *Bartonella* species in clinical samples, DNA was prepared from the blood of animals known to be infected with either *B. henselae*, *B. clarridgeiae*, or *B. vinsonii* subsp. *berkhoffii*. Briefly, DNA was extracted from 200 μl of blood by using the QIAamp Blood Kit (QIAGEN Inc., Santa Clarita, Calif.) and was eluted in a final volume of 200 μl according to the manufacturer's protocol. Samples (5 μl of template DNA) were amplified with the primers described above. After amplification, the PCR products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and photographed. As illustrated in Fig. 4, the single-step PCR assay is capable of detecting and differentiating infections with *B. henselae*, *B. clarridgeiae*, and *B. vinsonii* subsp. *berkhoffii* in clinical samples derived from naturally infected animals.

Sensitivity of PCR versus blood culture for detection of *B. henselae*. To determine the sensitivity of the single-step PCR assay relative to that of blood culture, we purified template DNA from 200 μl of blood containing 10 to 100 CFU (per ml) of *B. henselae* derived from experimentally infected cats. DNA was eluted in 200 μl of buffer, and 5 μl was used as the template in the single-step PCR assay as described above. The single-step PCR assay detected *B. henselae* in 100% (12 of 12) of blood samples with 50 to 100 CFU/ml, 85% (6 of 7) of blood samples with 30 CFU/ml, and 75% (6 of 8) of blood samples with 10 to 20 CFU/ml.

DISCUSSION

Infections with *Bartonella* species have been associated with similar clinical signs. For example, *B. elizabethae*, *B. henselae*, and *B. quintana* have been isolated from people with endocarditis, CSD in humans can be caused by either *B. henselae* or *B. clarridgeiae*, and both *B. henselae* and *B. quintana* have been associated with bacillary angiomatosis in immunosuppressed individuals. Diagnosis of infections caused by *Bartonella* species by bacterial culture is difficult due to the fastidious, slow growth characteristics of *Bartonella* species. Serology is frequently used for the diagnosis of infections caused by *Bartonella* species. However, problems with both sensitivity (3, 21, 22) and specificity (20, 25) have been reported for serology-based diagnostic assays. Differences in in vitro susceptibilities to antimicrobial agents between various *Bartonella* species have been reported (12); however, in vitro activity is not necessarily predictive of treatment efficacy in people infected with *Bartonella* species (2). Identification of *Bartonella* species is important to clarify the epidemiology and clinical presentation of *Bartonella* species infections in humans and animals.

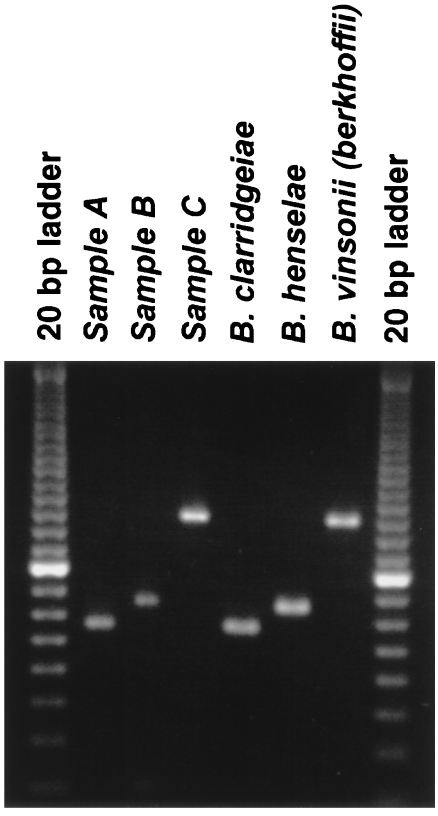

FIG. 4. PCR-based identification of *Bartonella* species from animals known to be infected with either *B. clarridgeiae* (sample A), *B. henselae* (sample B), or *B. vinsonii* subsp. *berkhoffii* (sample C). DNA was extracted from 200 μl of blood and was eluted in a final volume of 200 μl, and then 5 μl of template DNA was used in each PCR amplification. After amplification, the PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. Amplified control template DNA derived from isolated *B. clarridgeiae*, *B. henselae*, and *B. vinsonii* subsp. *berkhoffii* strains yielded expected products of 154, 172, and 260 bp, respectively. The first and last lanes each contain a 20-bp ladder.

To expedite the diagnosis of *Bartonella* infections and identification of the *Bartonella* species causing infections, a number of diagnostic assays including PCR with species-specific primers (15, 26), RFLP analysis of PCR products (4, 8, 24, 28, 34), and sequencing of PCR products (5, 13) have been proposed for the differentiation of *Bartonella* species. While these methods detect and differentiate *Bartonella* species, all require either multiple PCR assays or additional sample-handling steps to obtain results. The present study was undertaken to develop

a single-step PCR-based assay capable of detecting and differentiating *Bartonella* species. The 16S-23S rRNA intergenic region proved to be an appropriate target because it contains regions of sufficient sequence divergence to differentiate between *Bartonella* species as well as regions of adequate homology to enable the use of a single PCR primer pair (Fig. 1). A limitation of the single-step PCR assay described here is the inability to differentiate subspecies within different *Bartonella* species.

Phylogenetic analysis of *Bartonella* species has been based on the sequences of the 16S rRNA gene (5, 9); however, due to the highly conserved nature of this gene it is difficult to clearly distinguish relatedness. The sequence of the less conserved *gltA* gene has also been used for phylogenetic analysis of *Bartonella* species (5). On the basis of the *gltA* gene sequences, *B. bacilliformis* appears to be most closely related to *B. henselae* (5). Phylogenetic analysis based on sequences of the 16S-23S rRNA intergenic region suggests that *B. bacilliformis* is most closely related to *B. clarridgeiae* (Fig. 3). On the basis of both the *gltA* gene and the 16S-23S rRNA intergenic region sequences, *B. henselae* appears to be most closely related to *B. quintana* (5) (Fig. 3).

The single-step PCR assay described here can be used to directly screen samples from humans or animals, e.g., blood or tissue, for the presence of *Bartonella* species (Fig. 4). Using blood samples from *B. henselae*-infected cats, we compared the sensitivity of the single-step PCR assay to that of the culture assay. The theoretical sensitivity limit of the single-step PCR assay with 5 μl of template DNA, equivalent to 5 μl of blood, is 200 targets/ml. The observed sensitivity with 5 μl of template DNA was 100% for samples with 50 to 100 CFU/ml and 75% for samples with only 10 CFU/ml, or approximately 0.05 CFU. The ability to amplify *B. henselae* target DNA from template containing less than 1 CFU suggests amplification of target DNA derived from nonviable bacteria or, alternatively, inefficiency of the in vitro culture technique.

Although PCR-based assays for the detection and differentiation of *Bartonella* species have been described previously, a distinct advantage of the single-step PCR assay described here is that post-PCR sample handling is limited to gel electrophoresis. This technique dramatically decreases the cost and time required to obtain results as well as decreases the likelihood of sample contamination due to PCR product carryover. In conclusion, the single-step PCR assay described here provides a simple and rapid means of identifying pathogenic *Bartonella* species in humans and companion animals.

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