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Development of a Novel Genus-Specific Real-Time PCR Assay for Detection and Differentiation of *Bartonella* Species and Genotypes

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The genus *Bartonella* includes numerous species with varied host associations, including several that infect humans. Development of a molecular diagnostic method capable of detecting the diverse repertoire of *Bartonella* species while maintaining genus specificity has been a challenge. We developed a novel real-time PCR assay targeting a 301-bp region of the *ssrA* gene of *Bartonella* and demonstrated specific amplification in over 30 *Bartonella* species, subspecies, and strains. Subsequent analysis of *ssrA* sequences was sufficient to discriminate *Bartonella* species and provided phylogenetic data consistent with that of *gltA*, a commonly used gene for differentiating *Bartonella* genotypes. Using this assay, we identified *Bartonella* DNA in 29% and 47% of blood specimens from elk in Wyoming and cattle in the Republic of Georgia, respectively. Sequence analysis of a subset of genotypes from elk specimens revealed a cluster most closely related to *Bartonella capreoli*, and genotypes from cattle were identified as *Bartonella bovis*, both *Bartonella* species commonly found in wild and domestic ruminants. Considering the widespread geographic distribution and infectivity potential to a variety of hosts, this assay may be an effective diagnostic method for identification of *Bartonella* infections in humans and have utility in *Bartonella* surveillance studies.

The genus *Bartonella* includes over 30 different species and subspecies that infect a wide variety of mammalian hosts. *Bartonella* bacteria are transmitted by hematophagous insects such as ticks, fleas, and lice (4, 5, 8, 12, 15, 18), which can lead to widespread infection among localized populations of mammalian hosts. High prevalence of *Bartonella* bacteremia has been reported in populations of rodents, cats, and ruminants worldwide (3, 5, 6, 9, 13, 14, 26). Although members of the *Bartonella* genus infect a broad spectrum of mammalian hosts, most species exhibit restricted host specificity (25). Humans are also susceptible to *Bartonella* infection; to date, at least 10 *Bartonella* species have been implicated in human disease (2, 7, 16, 19, 21). The broad geographical distribution, wide spectrum of available reservoir hosts, and zoonotic potential of *Bartonella* species necessitate continued investigation of the biology and epidemiology of bacteria belonging to this genus.

Reliable detection methods are needed to facilitate ongoing epidemiological and ecological studies of *Bartonella*. Bacterial culture remains the preferred method for identification of *Bartonella* infections. However, culturing methods are laborious and time-consuming, and recovery of organisms is often low. Real-time PCR assays for detecting *Bartonella* have been reported previously (1, 10, 11, 24), but none of these are able to detect all species due to the significant genetic diversity within the genus. Furthermore, molecular detection methods must be applicable to a diverse array of specimen types, including insect and mammalian blood and tissue. In particular, amplification from whole blood remains a significant challenge to *Bartonella* detection (20).

Beyond detection, genetic targets that provide sufficient sequence diversity to allow identification to the species level are required to fully understand the distribution and host specificity of various *Bartonella* species and allow identification of the strains associated with human illness. The citrate synthase gene (*gltA*) is a common genetic target for *Bartonella* detection and is considered a reliable tool for distinguishing genotypes (22). One limitation of targeting this locus is its homology with sequences found in some host genomes, such as mouse, rat, and human, along with other human pathogens (11). This cross-reactivity creates a need for improved molecular diagnostics for *Bartonella*.

We developed a novel real-time PCR assay for detection of *Bartonella* spp. targeting the *ssrA* gene and demonstrated the ability to detect over 30 unique species, subspecies, and strains within this genus. SsrA RNA, also known as transfer-mRNA (tmRNA), is a single-copy prokaryotic-specific molecule involved in processing of incomplete peptides and resolution of stalled ribosomes during translation (17). This target has not been exploited previously for *Bartonella* detection. Furthermore, we demonstrate the utility of sequence-based species identification using the *ssrA* amplicon. Using this real-time PCR assay in combination with sequencing, we successfully amplified *Bartonella* DNA from ruminant blood specimens and identified the resulting genotypes.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. All bacterial strains were obtained from collections at the Centers for Disease Control and Prevention in Fort Collins, CO, and Atlanta, GA. Nucleic acid was extracted from 33 *Bartonella* strains, including 25 defined species or subspecies using a QIAamp DNA Minikit (Qiagen, Valencia, CA). *Bartonella* strains included in this study are the following: *B. alsatica* (IBS 382), *B. bacilliformis* (KC584), *B. birtlesii* (IBS 325), *B. bovis* (91-4), *B. capreoli* (WY-Elk), *B. chomelii* (A828), *B. clarridgeiae* (Houston-2), *B. doshiae* (R18), *B. elizabethae* (F9251), *B. henselae* (Houston-1), *B. grahamii* (V2), *B. japonica* (Fuji 18-1T), *B. koehlerae* (C-29), *B. melophagi* (K-2C), *B. phoceensis* (16120), *B. quintana* (Fuller), *B. rochalimae* (BMGH), *B. schoenbuchensis* (R1), *B.*

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silvatica (Fuji 23-1T), *B. tamiae* (Th307, Th239, and Th339), *B. taylorii* (M16), *B. tribocorum* (IBS 506), *B. vinsonii* subsp. *arupensis* (OK 94-513), *B. vinsonii* subsp. *vinsonii* (Baker), *B. washoensis* (Sb944nv), and *Bartonella* isolates (Sh6397ga, Sh6396ga, Sh6537ga, Sh8784ga, Sh8200ga, and Sh8776ga). Using a MagNA Pure Compact instrument with Total Nucleic Acid Isolation Kit I (Roche Applied Science, Indianapolis, IN), nucleic acid was extracted from 61 microorganisms that are closely related genetically to *Bartonella* or may occupy a similar ecological niche, including the following: *Afipia broomeae*, *Afipia clevelandensis*, *Afipia felis*, *Agrobacterium radiobacter*, *Agrobacterium tumefaciens*, *Babesia microti*, *Bordetella pertussis*, *Bordetella parapertussis*, *Bradyrhizobium*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella neotomae*, *Brucella ovis*, *Brucella suis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Erwinia*, *Escherichia albertii* (two strains), *Escherichia blattae*, *Escherichia coli* (four strains), *Escherichia fergusonii*, *Escherichia hermannii*, *Escherichia vulneris*, *Haemophilus influenzae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera intermedia*, *Legionella pneumophila*, *Methylobacterium organophilum*, *Ochrobactrum anthropi* (three strains), *Ochrobactrum intermedium*, *Oligella urethralis* (four strains), *Psychrobacter phenylpyruvicus* (2 strains), *Raoultella planticola*, *Salmonella bongori*, *Salmonella enterica* (*S. enterica* serovar Enteritidis, S. *enterica* serovar Typhi, and S. *enterica* serovar Typhimurium), *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Toxoplasma gondii*, and *Vibrio cholerae*. Human genomic DNA was also tested for cross-reactivity. All nucleic acid extracts were normalized to 1 ng/μl in Tris-EDTA buffer.

Real-time PCR. Sequences of the *ssrA* (tmRNA) gene of five representative *Bartonella* species were obtained from the tmRNA Website (http://www.indiana.edu/~tmrna/) and GenBank (accession numbers NC_005955.1, NC_005956.1, NC_010161.1, NC_012846.1, and NC_008783.1). Sequences were aligned using the Clustal W method (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Primers and probes were designed using Primer Express, version 3.0, software (Applied Biosystems, Foster City, CA) with some modification for amplification of a 301-bp region of *ssrA*. The reaction mixture (25 μl) contained the following components: 12.5 μl of 2× PerfeCta MultiPlex qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD), forward and reverse primers (ssrA-F, 5′-GCTATGGTAATAAATGGACAATGAAATAA-3′; ssrA-R, 5′-GCTTCTGTTGCCAGGTG-3′) at a final concentration of 500 nM, 6-carboxyfluorescein (FAM)-labeled probe (5′-FAM-ACCCCGCTTAAACCTGCGACG-3′-BHQ1, where BHQ is black hole quencher) at a final concentration of 100 nM, and 5 μl of extracted nucleic acid. Real-time PCR was performed on an Applied Biosystems 7500 real-time PCR instrument with the following thermocycling parameters: 1 cycle of 95°C for 2 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s, with data collection in the FAM channel. Primers and probe were tested using nuclease-free water (95 replicates of reactions) to ensure no signal in the absence of nucleic acid template.

The limit of detection was independently determined and verified for four species (*B. quintana*, *B. henselae*, *B. bovis*, and *B. elizabethae*) by testing 10 replicates each of 10-fold serial dilutions of genomic DNA ranging from 1 ng/μl to 0.1 fg/μl. The limit of detection was identified as the lowest dilution at which amplification was observed in at least 50% of replicates.

Specificity was assessed by performing the assay using 15 ng of nucleic acid from 61 different microorganisms representing 24 genera and 48 species.

Sequencing. Amplicons for sequencing were generated by conventional PCR with forward and reverse primers at 400 nM each using a Bio-Rad Dyad thermal cycler (Bio-Rad, Hercules, CA) and the following thermocycling conditions: 95° for 2 min, followed by 30 cycles of 95° for 15 s, 60° for 60 s, and 72° for 30 s, with a final step at 72° for 3 min. Amplicons were visualized by electrophoresis in a 1% agarose gel followed by staining with 0.05% methylene blue solution and purification using a Geneclean Turbo kit (MP Biomedicals, Solon, OH). Sequencing reactions were performed in both directions using a BigDye Terminator, version 3.1, kit (Applied Biosystems) according to the manufacturer's instructions with the same primers for the real-time PCR assay at a final concentration of 165 nM. Sequencing was performed on an Applied Biosystems 3130xl genetic analyzer.

Phylogenetic analysis. A 253-bp region of each amplified sequence (excluding forward and reverse primers) was used for alignment and phylogenetic comparison of *Bartonella* species and genotypes using a Lasergene, version 8, software suite (DNAStar, Madison, WI). All *ssrA* sequences were aligned using the Clustal V method. Phylogenetic trees were constructed using the neighbor-joining method and bootstrapping analysis with 1,000 replicates.

Testing of animal blood. Blood specimens collected from elk (*Cervus elaphus*) in Wyoming ($n = 55$) and cattle (*Bos primigenius*) in the country of Georgia ($n = 89$) between 2008 and 2009 were tested for *Bartonella* by bacterial culture using previously described methods (3). The culture results from this cohort of elk have been reported previously (3). All specimens were extracted using a DNeasy Blood and Tissue kit (Qiagen) or MagNA Pure Compact with Total Nucleic Acid Isolation Kit I (Roche Applied Science). Nucleic acid extract (5 or 10 μl) was used in each real-time PCR.

Nucleotide sequence accession numbers. Thirty-four unique *ssrA* sequences obtained from *Bartonella* strains and isolates were submitted to GenBank (http://www.ncbi.nlm.nih.gov/) and assigned the following accession numbers: JN029776 (*B. alsatica* IBS 382), JN029794 (*B. bacilliformis* KC584), JN029775 (*B. birtlesii* IBS325), JN029767 (*B. bovis* 91-4), JN029798 (*B. capreoli* WY-Elk), JN029773 (*B. chomelii* A828), JN029768 (*B. doshiae* R18), JN029774 (*B. elizabethae* F9251), JN029785 (*B. henselae* Houston-1), JN029795 (*B. grahamii* V2), JN029784 (*B. japonica* Fuji 18-1T), JN029769 (*B. koehlerae* C-29), JN029771 (*B. melophagi* K-2C), JN029770 (*B. phoceensis* 16120), JN029766 (*B. quintana* Fuller), JN029797 (*B. rochalimae* BMGH), JN029772 (*B. schoenbuchensis* R1), JN029782 (*B. silvatica* Fuji 23-1T), JN029778 (*B. tamiae* Th307), JN029779 (*B. tamiae* Th239), JN029780 (*B. tamiae* Th339), JN029781 (*B. taylorii* M16), JN029796 (*B. tribocorum* IBS 506), JN029783 (*B. vinsonii* subsp. *arupensis* OK 94-513), JN029777 (*B. vinsonii* subsp. *vinsonii* Baker), JN029786 (*B. washoensis* Sb944nv), JN029787 (*Bartonella* sp. Sh6397ga), JN029791 (*Bartonella* sp. strain Sh8200ga), JN029793 (*Bartonella* sp. strain Sh8776ga), JN029788 (*Bartonella* sp. strain Sh6396ga), JN029790 (*Bartonella* sp. strain Sh8784ga), JN029792 (*Bartonella* sp. strain Sh9282ga), JN029789 (*Bartonella* sp. strain Sh6537ga), JN982716 (*B. clarridgeiae* Houston-2). The *ssrA* sequence amplified from elk blood was assigned accession number JN982717, and the sequence identified in cattle blood was identical to that of *B. bovis* (JN029767).

RESULTS

Real-time PCR for detection of *Bartonella ssrA*. Amplification of the target sequence occurred with all *Bartonella* species ($n = 24$) and unclassified *Bartonella* strains ($n = 7$) tested (data not shown). Amplification curves demonstrated a sigmoidal shape and had crossing threshold (C_T) values between 15 and 21 with 5 ng of DNA per reaction. No amplification was observed in no-template control (NTC) reactions ($n = 95$) or with DNA from other microorganisms ($n = 61$; listed above in "Bacterial strains and DNA extraction") or with human genomic DNA (data not shown). The limit of detection was independently determined for four species (*B. quintana*, *B. henselae*, *B. bovis*, and *B. elizabethae*) and found to be ≤5 fg of nucleic acid per reaction (data not shown).

***Bartonella* phylogeny based on *ssrA* genotypes.** Phylogenetic analysis of *ssrA* sequences from each *Bartonella* strain or isolate showed that this region was sufficient to discriminate all *Bartonella* species and that separation of clades based on *ssrA* sequences was consistent with phylogeny based on *gltA*, which is considered a reliable tool for distinguishing closely related *Bartonella* geno-

TABLE 1 Detection of *Bartonella* in ruminant blood by real-time PCR and culture

Detection method	Elk specimen results ($n = 55$)		Cattle specimen results ($n = 89$)	
	No. (%) of *Bartonella*-positive specimens	Species identified by *ssrA* sequence	No. (%) of *Bartonella*-positive specimens	Species identified by *ssrA* sequence
Real-time PCR (*ssrA*)	16 (29.1)	*B. capreoli*	42 (47.2)	*B. bovis*
Culture	4 (7.3)[a,b]		34 (38.2)[b]	

[a] Culture results previously reported (3).
[b] All culture-positive specimens were also positive by real-time PCR.

types (22). First, the *ssrA* sequences from ruminant-associated *Bartonella*, including *B. chomelii*, *B. capreoli*, *B. bovis*, *B. melophagi*, and *B. schoenbuchensis*, formed an independent clade; sequence identity between these species was ≥94%. Further, both subspecies of *B. vinsonii* (*B. vinsonii* subsp. *vinsonii* and *B. vinsonii* subsp. *arupensis*) included in this study formed a separate grouping in the tree with 98% identity, as did three strains of the recently identified pathogenic *Bartonella* species *B. tamiae* (≥97.2% identity) (21). Among all *ssrA* sequences, the lowest percent identity (75.3 to 84.1%) was observed for strains of *B. tamiae* relative to other *Bartonella* species, thus supporting the separation of *B. tamiae* as a novel species (21). The division of two additional clades which are similarly separated by *gltA* comparison, one consisting of *B. elizabethae* and *B. tribocorum* and the other including *B. henselae* and *B. koehlerae*, was also supported by the phylogenetic analysis of *ssrA*. Overall, the separation of major *Bartonella* clades based on *ssrA* sequences was consistent with phylogeny based on *gltA* (20, 23).

Detection and identification of *Bartonella* in animal blood. Next, we utilized this assay to screen elk and cattle blood specimens for the presence of *Bartonella* and compared results to bacterial culture (Table 1). *Bartonella* DNA was detected in 16 of 55 (29.1%) and 42 of 89 (47.2%) specimens from elk and cattle, respectively. The appropriate amplicon size was confirmed for positive samples (data not shown). Using traditional culturing methods, *Bartonella* was recovered from only 4 of 55 (7.3%) elk and 34 of 89 (38.2%) cattle specimens (Table 1).

Since comparison of *ssrA* genotypes from *Bartonella* reference strains showed that this sequence provides sufficient information to discriminate *Bartonella* genotypes, we performed sequencing analysis of a subset of *ssrA* sequences amplified from elk ($n = 3$) and cattle ($n = 5$) specimens in order to identify the *Bartonella* species present. Analysis of *ssrA* sequences from elk blood revealed one genotype which clustered most closely with *B. capreoli* (Fig. 1), a *Bartonella* species found in wild and domestic ruminants (3). These results are consistent with previous identification of *B. capreoli* isolated from these samples using sequencing analysis of *gltA* (3). Similarly, a single *ssrA* genotype present in cattle blood was found to be identical to that of *B. bovis* (99.7% similarity) (Fig. 1). This result corroborates previous identification of *B. bovis* from these cattle specimens by analysis of *gltA* (data not shown).

DISCUSSION

Significant genetic diversity within the *Bartonella* genus presents a challenge for developing a molecular assay for detection of all species. The real-time PCR assay described here is able to detect all *Bartonella* species tested and demonstrated utility for screening primary specimens from animal hosts. Moreover, we established that this assay can also be used in combination with sequencing analysis to distinguish *Bartonella* species. Collectively, both of these accomplishments provide a significant advancement for studying *Bartonella*.

Unlike some previously reported PCR assays for *Bartonella* detection that display cross-reactivity with similar sequences present in host genomes (11), the genetic target of the current assay is a 301-bp region of the *ssrA* gene, which has not been identified in eukaryotic organisms to date, thereby reducing the potential for false-positive amplification in mammalian specimens. In addition, we performed extensive assessment of the specificity of this assay using a large collection of genetically or ecologically relevant organisms to ensure specific amplification of *Bartonella* species.

Identification of *Bartonella* by culture requires up to 4 weeks, whereas nucleic acid extraction and real-time PCR require only a few hours. Our results suggest that the *ssrA* real-time PCR assay is more sensitive than culture or other PCR assays and, therefore, represents an important advancement in *Bartonella* detection. Amplification of *Bartonella* DNA from whole-blood samples has been a challenge in some *Bartonella* studies. Using the new assay, we detected 100% of culture-positive ruminant blood specimens. Multiple factors, including extraction method, improved real-time PCR reagents, and oligonucleotide design, have likely contributed to the improvement in *Bartonella* detection from whole blood reported here. This assay is ideally suited for use as a screening tool to identify *Bartonella*-positive samples, which can then be subjected to the more laborious culture procedure in order to obtain an isolate.

Due to the improved sensitivity compared to culture, our data indicate a much higher prevalence of *Bartonella* bacteremia among elk species than previously reported (3). These results are more consistent with previous reports of high prevalence of *Bartonella* bacteremia among ruminant populations in other parts of the world (5, 6, 13). This assay may serve as an effective tool for investigating the dynamics of bacteremia in ruminants and other animals and for assessing the veterinary and medical importance of *Bartonella* infection in ruminants and potential zoonotic threat to humans.

Our analysis of *ssrA* genotypes showed that diversity within this region is sufficient to distinguish *Bartonella* species. Phylogenetic analysis revealed grouping of some *ssrA* genotypes obtained from *Bartonella* type strains according to host specificity. For example, *Bartonella* species associated with ruminants formed a single clade in the phylogenetic tree (Fig. 1). Also, separation of the two other clades of *Bartonella* including *B. elizabethae* with *B. tribocorum* and *B. henselae* with *B. koehlerae* was also supported by *ssrA* sequence analysis. In addition, three strains of *B. tamiae*, a recently identified pathogenic *Bartonella* species, formed an outlying group based on *ssrA* genotypes. These results are consistent with phylogenetic analysis of concatenated sequences from six

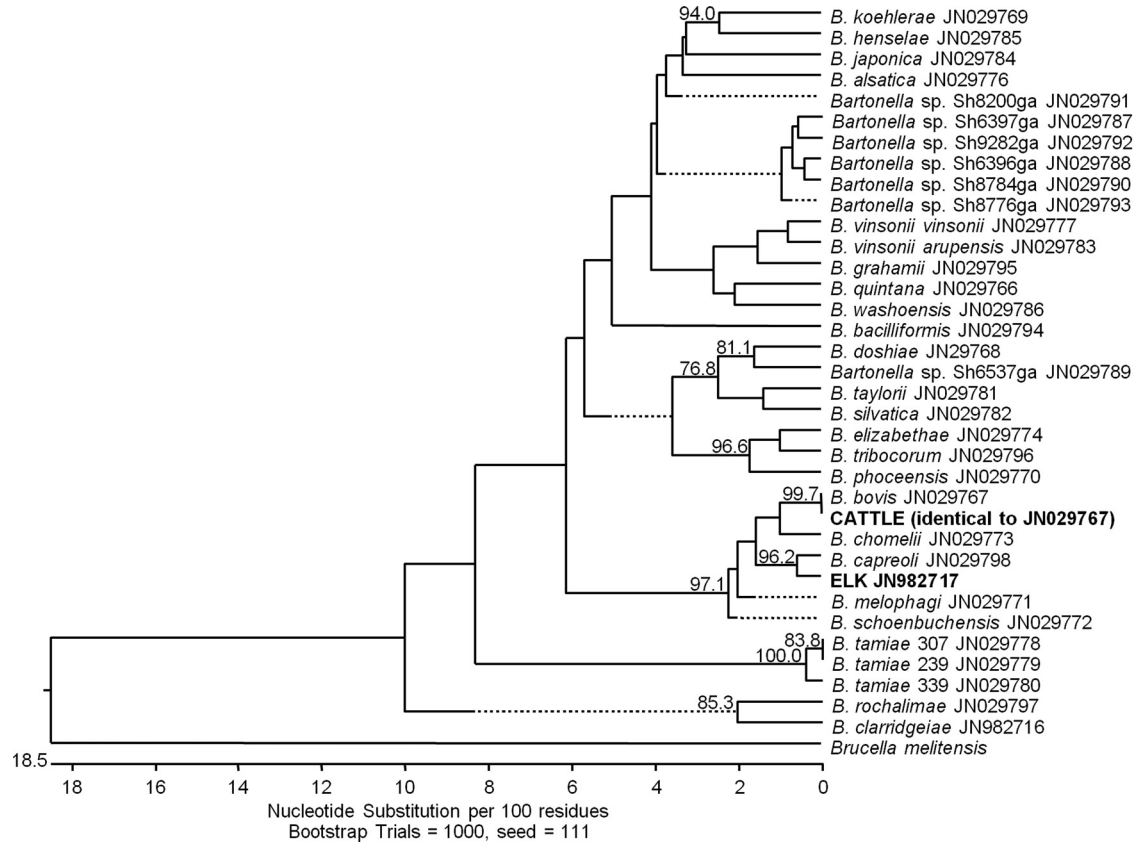

FIG 1 Phylogenetic relationships between *ssrA* sequences of all *Bartonella* species, subspecies, and isolates tested. GenBank accession numbers are shown for each genotype. Both *ssrA* genotypes obtained from ruminant blood were closely related to *Bartonella* species found in wild and domestic ruminants. The genotype identified from elk blood (JN982717) clustered closely with *B. capreoli* (96.2% similarity), and the single genotype identified in cattle blood was identical to *B. bovis* (99.7%). Only bootstrap replicates of >70% are noted.

genetic targets, including 16S rRNA, *gltA*, *rpoB*, *ftsZ*, *groEL*, and the 16S-23S rRNA intergenic spacer (ITS) previously reported by Kosoy and colleagues (21). According to La Scola et al., of the commonly used genetic targets for *Bartonella* identification, only *gltA* and *rpoB* sequences provide sufficient discriminatory power and interspecies diversity to allow discrimination of *Bartonella* species (22). Our data suggest that inclusion of *ssrA* sequences in phylogenetic analysis based on multiple genetic targets may provide additional supportive evidence for accurate identification of *Bartonella* isolates.

Considering the widespread geographic distribution and ability of *Bartonella* to infect a variety of animal species, this novel assay may be useful for determining the prevalence of *Bartonella* in large-scale surveillance studies. Furthermore, this assay may serve as an effective diagnostic method for identification of *Bartonella* infections in humans. Even within a given host organism, the types of specimens collected may vary based on different clinical manifestations; for instance, human clinical specimens may include whole blood, heart valve, or other tissue types. Therefore, PCR-based methods for *Bartonella* detection must be applicable to a variety of sample types. Additional studies are needed to assess the performance of this assay using human clinical specimens and other sample types.

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