Bartonella rochalimae Detection by a Sensitive and Specific PCR Platform

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Abstract. *Bartonella rochalimae* is an emerging zoonotic pathogen present in the United States, South America, and Europe. The molecular detection of *B. rochalimae* frequently relies on polymerase chain reaction (PCR) assays that target the genus *Bartonella* coupled with DNA sequencing for species determination. However, the presence of other *Bartonella* spp. in the sample being tested may result in false-negative results for *B. rochalimae*, especially when Sanger sequencing is used. We developed a sensitive and specific quantitative PCR platform for *B. rochalimae* by targeting the intergenic transcribed spacer, *gltA*, and *rpoB* genes, which are recommended for subtyping characterization. This PCR platform achieved the limit of detection between five and 10 genomic equivalents per reaction and did not amplify DNA from other *Bartonella* species or selected hosts. This PCR platform is a fast and cost-effective option to be used in epidemiological evaluations of reservoirs and vectors and in detecting and quantifying *B. rochalimae* infection in humans.

INTRODUCTION

Bartonella rochalimae are fastidious, pleomorphic, and hemotropic bacteria that can cause chronic intra-erythrocytic infections in mammals, including cats, dogs, guinea pigs, red foxes, raccoons, coyotes, and rats and can cause disease manifestations that include bacteremia, splenomegaly, fever, and myalgia in humans.^{1–3} Canids have been reported as the main reservoirs for *B. rochalimae*, supported by epidemiological and experimental data.^{1,4,5} This pathogen is believed to be transmitted by fleas and possibly ticks.^{6,7} In term of evolution, *B. rochalimae* is genetically related to *Bartonella clarridgeiae*, which has the cat as the mammalian reservoir host but can also infect and cause disease in humans. Based on deoxyribonucleic acid (DNA) sequencing of 478 genes, both *B. rochalimae* and *B. clarridgeiae* are clustered together under the Clade 3, separate from other *Bartonella* species frequently detected infecting dogs.⁸ Therefore, the discrimination between *B. rochalimae* and *B. clarridgeiae* by PCR is technically challenging because of limited genetic diversity.

Current, molecular detection of *B. rochalimae* is inefficient because it relies on DNA amplification using genus-level primers coupled with DNA sequencing. Such approach is prone to false-negative results if other species of *Bartonella* are present in larger quantity in the same sample. The detection of coinfections or multi-infections requires costly cloning and sequencing of multiple clones. Hence, a PCR assay with increased specificity for *B. rochalimae* would significantly reduce costs and increase turnover of results, which to these authors' knowledge, was not yet available. Following previously defined criteria for *Bartonella* species definition,⁹ we developed a cost-effective, sensitive, and specific PCR platform for the detection of *B. rochalimae* targeting the genes citrate synthase (*gltA*), β subunit of bacterial ribonucleic acid (RNA) polymerase (*rpoB*) genes, and the intergenic transcribed spacer (ITS). The PCR platform reported here is also capable of generating DNA fragments of these representative genes with an appropriate length for phylogenetic analyses.

METHODS

Whole genomic DNA (gDNA) from *B. rochalimae* (Human strain: ATCC #BAA-0498) was used as a positive control for all PCR assays. Genomic equivalents were calculated as previously described.¹⁰ This positive control was diluted 10-fold from 1,000,000 copies/ μ L to 0.02 copies/ μ L reaction in tris-ethylenediaminetetraacetic acid (TE) buffer and, in duplicate, in purified dog DNA and stored at -30°C . No samples were thawed and refrozen more than five times to prevent DNA degradation.

Twelve other species of *Bartonella* were used to determine the specificity of the PCR platform using extracted DNA from *Bartonella bovis*, *B. clarridgeiae*, *Bartonella durdenni*, *Bartonella elizabethae*, *Bartonella henselae*, *Bartonella melophagi*, *Bartonella monaxi*, *Bartonella quintana*, *Bartonella vinsonii* subsp. *berkoffii* genotype I, *B. vinsonii* subsp. *berkoffii* genotype II, *B. vinsonii* subsp. *berkoffii* genotype III, and *B. vinsonii* subsp. *vinsonii* strain Baker. Uninfected human, feline, and canine gDNA was also used to further test the specificity of primer sets.

All three set of primers were manually designed using Primer3 after alignment of *Bartonella* sequences available in National Center for Biotechnology Information GenBank and Integrated Microbial Genomes (IMG) and Microbiome Samples (Table 1).¹¹ Amplifications were performed on a StepOne Plus Real-Time PCR system, using a 25- μ L final volume reaction mixture containing 12.5 μ L of SYBR[®] Premix Ex Taq[™] with ROX Reference Dye (Takara Bio, Shiga, Japan) for all three assays. For the ITS- and *gltA*-PCR assays, 6.8 μ L of molecular grade water was used along with 10.5 pmols of the forward and reverse primers. For the *rpoB*-PCR assay, 6.5 μ L of molecular-grade water was used along with 15 pmols of the two primers. All three PCR assays used 5 μ L of template DNA. The thermocycler conditions for the ITS and *gltA*-PCR assays were 95°C for 1 minute, followed by 45 cycles at 95°C for 5 seconds, 66°C for 30 seconds, and 72°C for 45 seconds, followed by a melting cycle at 0.5-second intervals. The conditions of the *rpoB*-PCR assay differed only at the extension step, with 72°C interval for 60 seconds. All PCRs included a negative control as described previously.

Selected PCR products were analyzed using 1.5% agarose gel electrophoresis under ultraviolet exposure for confirmation of amplicon size and absence of primer dimer or nonspecific amplifications. Selected amplicons were purified (MiniElute

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FIGURE 1. Gel pictures of intergenic transcribed spacer (ITS) assay for positive *Bartonella rochalimae* serial dilutions, other *Bartonella* spp., and host genomic DNA (gDNA) samples. This figure appears in color at www.ajtmh.org.

kit; Qiagen, Valencia, CA) and sequenced using a fluorescence-based automated sequencing system (Eurofins MWG Operon, Huntsville, AL). *Bartonella* spp. and strains isolated were defined by comparing similarities with other sequences deposited in the GenBank database using Basic Local Alignment Search Tool (BLAST).¹²

To determine the analytical sensitivity, *B. rochalimae* genomic equivalents were tested from 1×10^6 to 0.1 genomic equivalent (GE) per PCR reaction diluted in canine gDNA. The limit of detection (LOD) was defined as the lowest dilution that was amplified 100% of the time and was determined based on 10 technical replicates for the following dilutions: 10, 5, 2, and 1 GE per PCR reaction. Specificity was determined using gDNA (10–30 ng/ μ L) from uninfected human, dog, and cat ethylenediaminetetraacetic acid (EDTA)-whole blood samples, as well as the other *Bartonella* spp. listed previously.

The genetic diversity of *gltA*, *rpoB*, 16S ribosomal ribonucleic acid (rRNA), and ITS was assessed by their Shannon's entropy profile. Multiple DNA sequences from the curated databases IMG¹¹ and Pathosystems Resource Integration Center¹³ were aligned using MUSCLE v3.8.31¹⁴ and trimmed for the described length of each gene based on the coverage of consensual sequence (Supplemental File 1). The overall Shannon's entropy score¹⁵ for each gene or region was calculated using the "diversity" function from the "vegan" R package.¹⁶ Overall Shannon's entropy was presented as violin plots using ggplot2 R library¹⁷ for *gltA*, *rpoB* genes, and the ITS region.

RESULTS

PCR assays targeting the ITS region, *gltA*, and *rpoB* genes produced an 864-bp, 425-bp, and 716-bp product, respectively, from the positive control for *B. rochalimae*. DNA sequencing confirmed the identity of *B. rochalimae* strain ATCC BAA-1498 (accession number FN645459) from all genes targeted. The melting temperature detected for the positive controls in the ITS, *gltA*, and *rpoB* reactions were 86.9°C, 81.9°C, and 81.6°C, respectively. No amplicons were generated from the 12 other *Bartonella* spp. tested nor from gDNA from dog, cat, or human for the ITS-, *gltA*-, and *rpoB*-PCR assays (Figure 1). Using the overall Shannon's entropy score, which

quantifies the amount of genetic information based on the DNA sequences of each gene, we also confirmed the higher diversity of sequences of *gltA*, *rpoB*, ITS when compared with 16S rRNA, a gene still widely used for phylotyping (Figure 2).

The LOD of the ITS- and *gltA*-PCR assays for templates with *B. rochalimae* diluted in TE buffer and in negative canine gDNA were 5 GE/reaction, whereas the LOD of the *rpoB*-PCR assay was 10 GE/reaction, although lower dilutions were also detected in a stochastic distribution (Figure 1). Historical gDNA samples from four dogs previously confirmed to be naturally

FIGURE 2. Violin plots depicting the overall Shannon's entropy score for all nucleotide sites on the multiple sequence alignments (MSAs) for each gene/spacer. Entropy of zero means no variability at a specific position in the MSA, whereas a high entropy score indicates better discriminatory power among DNA sequences. The width of each plot represents the full distribution of entropy for each nucleotide site of the gene/spacer. The overall mean is represented by filled circles, whereas the median is shown as open circles. Genes *gltA* and *rpoB*, as well as intergenic transcribed spacer (ITS) region demonstrated higher entropy scores than 16S rRNA gene ($P < 0.001$, Bonferroni-adjusted Mann-Whitney-Wilcoxon Test).

TABLE 1
Primers and corresponding annealing temperatures designed in this study

Gene targeted	Primer name	Sequence (5'–3')	Annealing temperature (°C)
<i>gltA</i>	<i>gltA</i> 180s	CGATGGTGACAAAGGAGTCCTAC	66°C
	<i>gltA</i> 604as	ACATATGAAGGAAGTTTGCAGCAT	–
<i>rpoB</i>	<i>rpoB</i> 38s	GCCGTAGACGTGTGCGTAAAT	66°C
	<i>rpoB</i> 753as	AGTACGCCACCCATTCTTAGTACG	–
ITS	ITS315s	GATTGAAGGTTTTTCAGTTTTCCTCG	66°C
	ITS1178as	GTTTCCTTGCACACATTATTCTACWC	–

ITS = intergenic transcribed spacer.

infected with *B. rochalimae*⁵ were also tested with the three assays: the ITS-PCR assay accurately detected infection in 3/4 of the samples tested, the *gltA*-PCR assay detected 1/4 of the samples tested, and the *rpoB* assay detected 2/3 of the samples tested. The discrepancy of results may be due to DNA degradation from multiple freeze–thaw cycles that these samples were subjected to for 4 years before their use in this study.

DISCUSSION

This report describes the development and validation of three sensitive and specific PCR assay for detection of *B. rochalimae*. Primers used in this study were designed to be selective only for *B. rochalimae* and to amplify a moderate to large DNA fragment that can be sequenced for further phylogenetic analysis. The three PCR assays described here can be performed with either conventional PCR or real-time PCR with Sybr chemistry, providing good flexibility to the user.

The *gltA* and *rpoB* genes were specifically targeted in our PCR platform based on the work of La Scola et al.,⁹ which recommended the use of both genes when characterizing *Bartonella* spp. based on DNA sequencing. Based on the overall Shannon's entropy score, we have confirmed that the three genes/spacer targeted in this study have a higher entropy when compared with 16S rRNA ($P < 0.001$). This score quantifies the amount of genetic information available in each gene; therefore, a higher entropy score indicates a higher discriminatory power to differentiate between DNA sequences available in the database, supporting better phylogenotyping of bacterial species/strains. Consequently, the three genes/spacer used in this study were indicated because they provide a better discriminatory power among *Bartonella* species, rather than the gold standard 16S rRNA gene used for identifying other bacteria.

The PCR assays also achieved an analytical sensitivity comparable with or better than previously published assays.^{18,19} The ITS- and *gltA*-PCR assays detected five copies of target DNA/reaction and the *rpoB*-PCR assay detected 10 copies of target DNA/reaction when mimicking real-life infections, by dilution-positive samples in gDNA from an uninfected dog. High analytical sensitivity is required for *Bartonella* detection because low levels of bacteremia have been reported in dogs and in humans.²⁰ Because *B. clarridgeiae* is genetically the closest species to *B. rochalimae*, we extensively tested our new sets of primers against *B. clarridgeiae*, with no nonspecific amplifications. Similarly, the lack of amplifications from other *Bartonella* spp. or host DNA (canine, feline, or human) further supports the high specificity obtained.

In summary, the PCR platform reported here can specifically confirm the presence of *B. rochalimae* in genomic DNA of dogs, cats, and humans with very good analytical sensitivity, generating DNA amplifications suitable for downstream phylogenotyping. This PCR platform may serve as a fast and economical option for large-scale screening of biological samples, vectors as well as a diagnostic tool in veterinary and human medicine.

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REFERENCES

- Chomel BB, Henn JB, Kasten RW, Nieto NC, Foley J, Papageorgiou S, Allen C, Koehler JE, 2009. Dogs are more permissive than cats or Guinea pigs to experimental infection with a human isolate of *Bartonella rochalimae*. *Vet Res* 40: 27.
- Eremeeva ME et al., 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. *N Engl J Med* 356: 2381–2387.
- Henn JB, Chomel BB, Boulouis HJ, Kasten RW, Murray WJ, Bar-Gal GK, King R, Courreau JF, Baneth G, 2009. *Bartonella rochalimae* in raccoons, coyotes, and red foxes. *Emerg Infect Dis* 15: 1984–1987.
- Diniz PPVP, Billeter SA, Otranto D, De Caprariis D, Petanides T, Mylonakis ME, Koutinas AF, Breitschwerdt EB, 2009. Molecular documentation of *Bartonella* infection in dogs in Greece and Italy. *J Clin Microbiol* 47: 1565–1567.
- Diniz PPVP et al., 2013. Infection of domestic dogs in Peru by zoonotic *Bartonella* species: a cross-sectional prevalence study of 219 asymptomatic dogs. *PLoS Negl Trop Dis* 7: e2393.
- Billeter SA, Gundi VA, Rood MP, Kosoy MY, 2011. Molecular detection and identification of *Bartonella* species in *Xenopsylla cheopis* fleas (Siphonaptera: Pulicidae) collected from *Rattus norvegicus* rats in Los Angeles, California. *Appl Environ Microbiol* 77: 7850–7852.

7. Billeter SA, Caceres AG, Gonzales-Hidalgo J, Luna-Caypo D, Kosoy MY, 2011. Molecular detection of *Bartonella* species in ticks from Peru. *J Med Entomol* 48: 1257–1260.
8. Pulliainen AT, Dehio C, 2012. Persistence of *Bartonella* spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation. *FEMS Microbiol Rev* 36: 563–599.
9. La Scola B, Zeaiter Z, Khamis A, Raoult D, 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. *Trends Microbiol* 11: 318–321.
10. Quorllo BA, Riggins D, Comyn A, Zewde MT, Breitschwerdt EB, 2014. Development and validation of a sensitive and specific sodB-based quantitative PCR assay for molecular detection of *Ehrlichia* species. *J Clin Microbiol* 52: 4030–4032.
11. U.S. Department of Energy Office of Biological and Environmental Research, 2016. *JGI/IMG—Integrated Microbial Genomes & Microbiome Samples*. Available at: <https://img.jgi.doe.gov/cgi-bin/m/main.cgi>. Accessed November 11, 2016.
12. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
13. Wattam AR et al., 2017. Improvements to PATRIC, the all-bacterial bioinformatics database and analysis resource center. *Nucleic Acids Res* 45: D535–D542.
14. Edgar RC, 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32: 1792–1797.
15. Shenkin PS, Erman B, Mastrandrea LD, 1991. Information-theoretical entropy as a measure of sequence variability. *Proteins* 11: 297–313.
16. Oksanen J et al., 2017. *Ordination Methods, Diversity Analysis and Other Functions for Community and Vegetation Ecologists*. Available at: <https://cran.r-project.org/web/packages/vegan/index.html>. Accessed June 1, 2017.
17. Wickham H, 2016. *Use R! ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer.
18. Cherry NA, Maggi RG, Cannedy AL, Breitschwerdt EB, 2009. PCR detection of *Bartonella bovis* and *Bartonella henselae* in the blood of beef cattle. *Vet Microbiol* 135: 308–312.
19. Diniz PPVP, Maggi RG, Schwartz DS, Cadenas MB, Bradley JM, Hegarty B, Breitschwerdt EB, 2007. Canine bartonellosis: serological and molecular prevalence in Brazil and evidence of co-infection with *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii*. *Vet Res* 38: 697–710.
20. Breitschwerdt EB, Maggi RG, Chomel BB, Lappin MR, 2010. Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. *J Vet Emerg Crit Care (San Antonio)* 20: 8–30.