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Improved Detection of *Bartonella* DNA in Mammalian Hosts and Arthropod Vectors by Real-Time PCR Using the NADH Dehydrogenase Gamma Subunit (*nuoG*)▽

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We used a whole-genome scanning technique to identify the NADH dehydrogenase gamma subunit (*nuoG*) primer set that is sensitive and specific enough to detect a diverse number of *Bartonella* species in a wide range of environmental samples yet maintains minimal cross-reactivity to mammalian host and arthropod vector organisms.

Bacteria in the genus *Bartonella* are found in a wide variety of mammalian hosts (2, 8, 18, 20, 21) and are thought to be transmitted by arthropod vectors, including fleas, ticks, and possibly mites (4, 7, 9, 14, 16); humans serve as accidental hosts. Of the at least 20 named *Bartonella* species, 10 have been shown to cause disease in humans, including Carrion's disease (13), cat scratch disease (7, 14, 25), endocarditis (6, 11), and recently a febrile illness in humans from Thailand (caused by *Bartonella tamiae*) (17). Because of their wide distribution and potential for frequent contact with humans, many *Bartonella* species are considered potential emerging pathogens (1, 26, 28).

Bartonella identification requires the ability to detect bacteria in both mammalian hosts and arthropod vectors. Although bacterial culture is considered ideal, the difficulty and time involved make it impractical for large-scale use. Additionally, nucleic acid-based detection techniques may be hindered by inhibitors in environmental and clinical samples, low sensitivity, and the absence of genus-specific primers (10, 27).

To address these issues, we used whole-genome scanning based on the complete genomes of *Bartonella bacilliformis*, *B. henselae*, and *B. quintana* to identify host- and vector-blind primer sets for real-time PCR detection of *Bartonella* in various field-collected samples. We identified a primer set based on the NADH dehydrogenase gamma subunit (*nuoG*) that is specific for *Bartonella* species and sensitive enough to detect *Bartonella* in both mammalian hosts and arthropod vectors.

Identification of host-blind primer sets. A whole-genome scan was performed on complete genomic sequences from *B. henselae* and *B. quintana* and shotgun sequences from *B. bacilliformis* available in GenBank. Each subsequence of 16, 17, 18, and 19 nucleotides present in published *Bartonella* genomes was compared with subsequences from other genomes present in GenBank, including genomes for bacteria that could infect human blood and tissues and potential mammalian hosts and arthropod vectors for bartonellae. The number of base changes necessary to convert each *Bartonella* subsequence to the closest subsequence in the background collection was calculated to identify potential primers with a reduced probability of hybridizing to and amplifying nontarget DNA.

In total, one ultraspecific, host-blind primer pair (the *nuoG* primer pair) was identified that met the following conditions: the pair (i) maintained at least a 2-base specificity among the complete GenBank sequence database, (ii) amplified fragments of identical sizes in the *B. henselae* and *B. quintana* genomes, (iii) had predicted amplicon sizes of less than 400 bp, and (iv) had primer melting temperatures (T_ms) within 2°C. Although they did not conform to all of these conditions, the *ftsZ* and *gltA* primer sets were included in further comparisons due to the large amount of sequence data available for these genes.

Primer pairs were tested in reaction with three *Bartonella* species (*B. henselae*, *B. quintana*, and *B. bacilliformis*) and then with the use of ~30-fold excess competitor DNA from J774 (murine) and THP1 (human) tissue culture cells over the template DNA from *B. henselae*. Interestingly, despite their common use, the *gltA* primer set demonstrated high cross-reactivity both to potential *Bartonella* hosts (*Rattus* spp., *Mus* spp., and *Homo sapiens*) and to bacterial species, such as *Ehrlichia* spp., that could inhabit similar ecological niches (Table 1).

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TABLE 1. Details of primers used in this study[a]

Gene and orientation	Nucleotide sequence	Primer T_m	Amplicon size (bp)	Expected amplification result	Other species carrying the gene
gltA				*B. henselae*, *B. quintana*	*Legionella pneumophila*, *Erlichia* spp., *Alkalilimnicola ehrlichei*, *Mus musculus*, *Rattus norvegicus*, *Homo sapiens*
F	GGGGACCAGCTCATGGTGG	57.58	340		
R	AATGCAAAAAGAACAGTAAACA	56.68			
nuoG				*B. henselae*, *B. quintana*, *B. bacilliformis*	None
F	GGCGTGATTGTTCTCGTTA	55.56	346		
R	CACGACCACGGCTATCAAT	56.68			
ftsZ				*B. henselae*, *B. quintana*	None
F	CGCATAGAAGTATCATCCA	50.72	753		
R	ACGATTAATCTGCATCGGC	53.99			

[a] Expected amplification results and occurrences in other genomes were determined by whole-genome scanning versus *B. henselae*, *B. bacilliformis*, and *B. quintana*. F, forward; R, reverse.

Amplification performance of the *nuoG*, *gltA*, and *ftsZ* primer sets against reference *Bartonella* DNA and environmental samples. The *nuoG*, *gltA*, and *ftsZ* primer sets were used to amplify reference DNAs from 11 *Bartonella* species, chosen for their distant phylogenetic relationships, under conditions optimized for each primer set. The amplification results differed considerably between primer sets and species of *Bartonella* being amplified (Table 2) and are as follows: the *nuoG* primer set performed best (amplifying first, with the lowest threshold cycle [C_T] value) on 3 of the 11 tested species, the *gltA* set performed best on 7 of the 11 species, and the *ftsZ* set performed best on 1 of the 11 species. Although the *gltA* primer set performed best for the highest number of reference species, only the *nuoG* set successfully amplified all 11 species.

The primer sets were next tested against a panel of DNA from field-collected samples (purified from liver samples from Nepalese rats and ticks from Colombia) to determine their efficacy in detecting *Bartonella* DNA in field-collected hosts and vectors. Consistent with the predicted specificities from the whole-genome scans, the *nuoG* primer set demonstrated significantly higher sensitivity and specificity for *Bartonella* than the other primer sets by consistently yielding more sequence-confirmed PCR-positive results (Table 3). For the 61 total ticks sampled, the *nuoG* primer set yielded 7 *Bartonella*-positive samples, compared to 1 and 0 for the *ftsZ* and *gltA* sets, respectively. Of 24 total rodent liver samples tested, 18 were found to be *Bartonella* positive by the *nuoG* primer set, compared to 10 and 2 for the *ftsZ* and *gltA* sets, respectively.

TABLE 2. C_T values for the three primer sets resulting from amplification of 11 reference DNAs derived from culture samples[a]

Species	C_T		
	nuoG1 primer set	*gltA* primer set	*ftsZ72* primer set
Cotton rat sp. A1	26.1	28.6	23.8
Cotton rat sp. C1	17.1	18.6	34.5
Bartonella grahamii	21.2	18.3	21.8
Bartonella phoceensis	25.5	28.2	29.8
B. rattimassiliensis	21.9	21.0	22.0
B. tamiae	37.7	**29.9**	NA
B. tribocorum	28.3	**11.8**	19.8
B. vinsonii subsp. *arupensis*	17.9	15.3	17.7
B. vinsonii subsp. *berkhoffii*	26.7	**14.9**	18.9
B. vinsonii subsp. *vinsonii*	22.4	22.0	21.9
Bartonella washoensis	16.9	NA	19.5

[a] Values for those cases with more than 3.3 cycles (1-log starting quantity) are in bold. C_T values higher than 35 are considered not valid (NA), due to the potential influence of primer dimers.

Phylogenetic analysis. Analysis of a *nuoG*-derived phylogeny showed strong statistical support for the following clades: *B. henselae* and *Bartonella koehlerae*; the species found in *Rattus* and related hosts, including *Bartonella elizabethae*, *B. rattimassiliensis*, and *B. tribocorum*; 3 *Bartonella vinsonii* subspecies (*Bartonella vinsonii* subsp. *arupensis*, *B. vinsonii* subsp. *vinsonii*, and *B. vinsonii* subsp. *berkhoffii*); and two strains of *B. tamiae*, described to occur in febrile Thai patients (type strain Th239 and strain Th307) (Fig. 1). All of these species groups share high genetic similarity within their respective clades, suggesting that the *nuoG* primer set provides better phylogenetic estimation with closely related species. *Bartonella bovis* was placed extremely distant to the other *Bartonella* species, with strong statistical support; conversely, *B. bacilliformis* was placed more centrally within the phylogeny than is seen with other genes, though this placement did not have strong statistical support. These placements, which are different from those generated with multiple concatenated *Bartonella* sequences (Fig. 1) (17), are likely due to the genetic rearrangements and horizontal gene transfer events that commonly occur in *Bartonella* (3, 12, 19, 23). Because of this, care should be taken when interpreting phylogenies based solely on *nuoG* sequences. A much more reliable approach is to include the *nuoG* sequence as one of many concatenated sequences to be used for phylogenetic analysis.

In summary, whole-genome scanning has allowed us to identify *nuoG* as a sensitive and specific target gene for use in

TABLE 3. *Bartonella*-positive samples, as verified by sequencing, based on primer set, for field-collected samples

Primer set	No. of Colombian ticks (%) (n = 61)	No. of Nepal rodent livers (%) (n = 24)
gltA set	0 (0)	2 (9)
ftsZ72 set	1 (2)	10 (42)
nuoG set	7 (11)	18 (75)

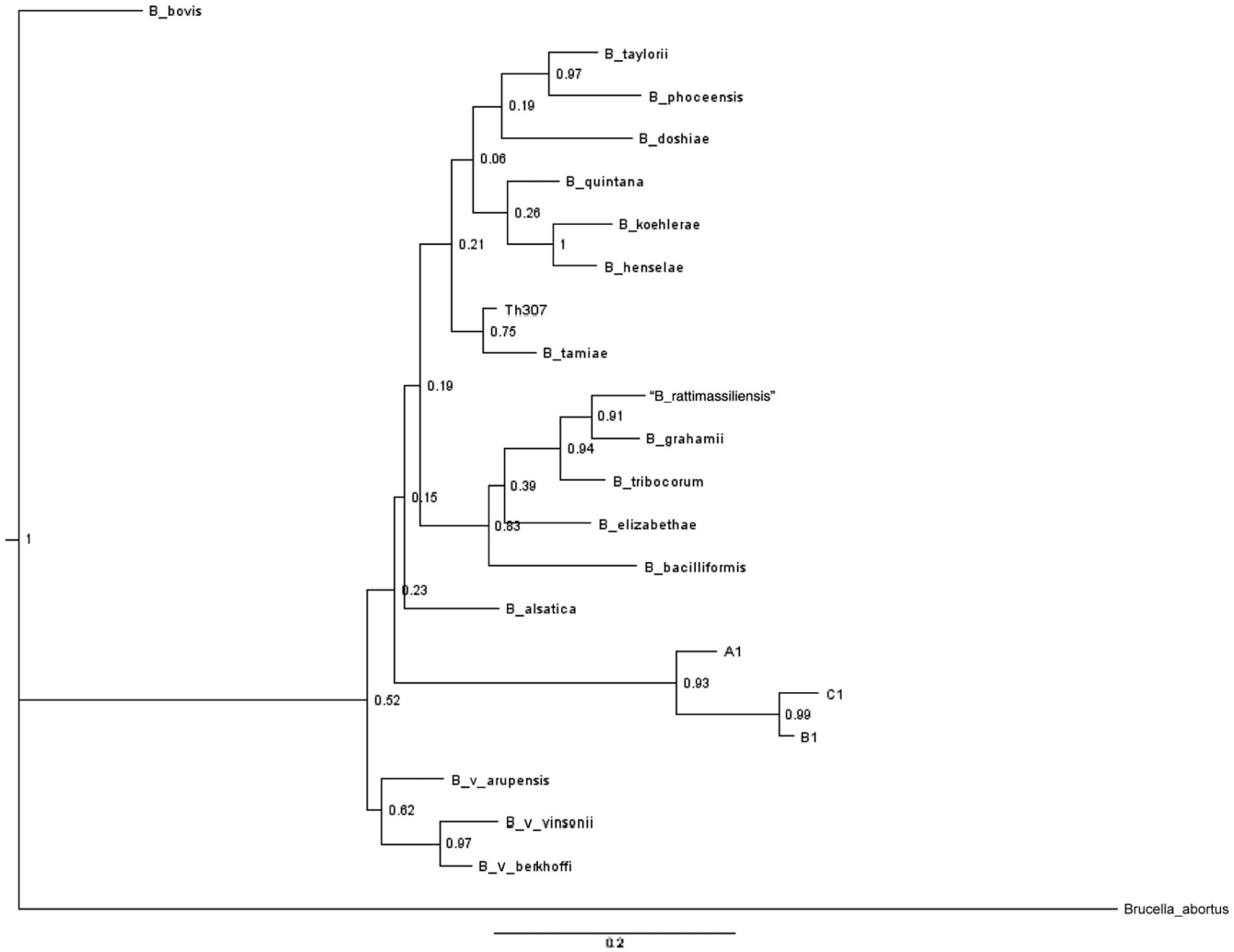

FIG. 1. Bayesian analysis of the *Bartonella* phylogeny based on the *nuoG* gene sequence. The sequences were obtained either from direct sequencing of PCR products or from GenBank. The listed node values represent posterior probabilities; *Brucella* was used as the outgroup.

detection of *Bartonella* species from various clinical and environmental specimens. *nuoG*'s superior performance in identifying *Bartonella* species in field-collected samples makes it an ideal candidate for complementing the use of *gltA* and *ftsZ* on culture samples.

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