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Serial Testing from a 3-Day Collection Period by Use of the *Bartonella Alphaproteobacteria* Growth Medium Platform May Enhance the Sensitivity of *Bartonella* Species Detection in Bacteremic Human Patients

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Patients with infection from bacteremic *Bartonella* spp., tested using *Bartonella Alphaproteobacteria* growth medium (BAPGM), were retrospectively categorized into one of two groups that included those whose blood was collected once (group 1; $n = 55$) or three times (group 2; $n = 36$) within a 1-week period. Overall, 19 patients (20.8%) were PCR positive for one or more *Bartonella* spp. using the BAPGM platform. Seven patients (12.7%) in group 1 tested positive, and 12 patients (33.3%) in group 2 tested positive. Detection was improved when the patients were tested three times within a 1-week period (odds ratio, 3.4 [95% confidence interval, 1.2 to 9.8]; $P = 0.02$). Obtaining three sequential blood samples during a 1-week period should be considered a diagnostic approach when bartonellosis is suspected.

The genus *Bartonella* comprises over 30 species of fastidious, Gram-negative, aerobic intracellular bacteria that induce chronic intravascular infection in a variety of hosts, including humans (1–4). At least 17 *Bartonella* species have been associated with an expanding spectrum of human diseases, ranging from acute self-limiting illness to more severe disease manifestations, including encephalopathy, endocarditis, neurologic dysfunction, pleural and pericardial effusion, pneumonia, and hemolytic anemia (2, 5–8). To enhance the diagnostic documentation of infection with *Bartonella* spp., our laboratory has combined enrichment cultures that utilize an insect cell culture liquid growth medium, *Bartonella Alphaproteobacteria* growth medium (BAPGM), with highly sensitive PCR assays (7, 9). When used to test blood, cerebrospinal and joint fluids, or pathological effusions from dogs or horses, PCR following enrichment culture has enhanced the diagnostic sensitivity over blood PCR testing alone (10–13). We refer to this diagnostic approach as the BAPGM enrichment culture platform.

Despite ongoing advances in diagnostic sensitivity when using whole-blood enrichment culture approaches compared to traditional agar culture isolation, the documentation of intravascular infection with *Bartonella* spp. using specimens obtained from a single point in time remains challenging (10, 14–16). Intravascular infection with *Bartonella* spp. can be associated with a relapsing pattern of bacteremia at 5-day intervals, as demonstrated in rodents and as observed in humans with *Bartonella quintana* infection (trench fever, historically referred to as 5-day fever) (17–21). Similarly, bartonellae often induce very low levels of bacteremia in nonreservoir-adapted hosts, resulting in diagnostically low levels of circulating bacteria in the bloodstream at a given point in time (2, 8, 10). Thus, screening enrichment blood cultures for documentation of infection with *Bartonella* spp. at a single time point might result in false-negative results. Serial testing is a widely accepted epidemiological approach designed to improve the diagnostic sensitivity of a laboratory test, thereby defining bacteremia positives as at least one positive out of multiple test results (22). For several reasons, including enhanced sensitivity, three blood cultures are typically recommended when bacterial sepsis is suspected (23). Using *Bartonella* testing and the BAPGM platform as an example, bacteremia would be confirmed if any of three serially obtained blood specimens taken over a 1-week span resulted in bacterial isolation or PCR amplification of *Bartonella* DNA from the patient's blood, serum, or enrichment blood culture. Bacteremia would not be confirmed if all BAPGM platform PCR results from the three specimen sets were negative. Historically, testing of blood specimens using the BAPGM platform has consisted of testing one blood specimen drawn from a patient at a single point in time. When some high-risk patients (veterinary professionals) who initially tested negative were subsequently found to be *Bartonella* bacteremic upon retesting, we began to consider the value of obtaining three blood culture sample sets within a 1-week period. Therefore, the objective of this retrospective study was to determine if the testing of specimens collected serially over a 1-week period significantly improved PCR documentation of *Bartonella* bacteremia in human patients compared to the testing of specimens from a single time point.

MATERIALS AND METHODS

From February through December 2010, 91 voluntary patients with extensive arthropod exposure and/or frequent animal contact were entered into the study. For the most part, the participants became aware of the study through lay or scientific publications or by attending lectures on bartonellosis at regional and national veterinary conferences. In most instances, the patients requested testing because of a history of chronic poorly defined illness, fatigue, joint pain, arthritis, and neurologic or neu-

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rocognitive abnormalities. The individuals were not recruited into a specific study but were aware that their test results could be used in one or multiple studies. Prior to 2010, each patient submitted one sample set (blood and serum) for BAPGM enrichment blood culture-PCR. Beginning in 2010, we requested submission of three sample sets, but for logistical reasons most individuals only submitted one sample set. Also, during 2010 there was consistency in the medium, inoculation methods, PCR primers, and amplification conditions. Therefore, the 91 patients used in this analysis reflect the individuals who were tested for *Bartonella* spp. with the BAPGM enrichment blood culture-PCR platform during the same period of time and with identical laboratory techniques. Demographic information, animal/arthropod exposure, history of visiting specialists, self-reported clinical symptoms, and comorbid conditions for each patient were collected using a standardized 5-page survey instrument. Questionnaires were mailed to each study participant for self-report, and upon return to the Intracellular Pathogens Research Laboratory (IPRL) at North Carolina State University, they were entered into an electronic database. This study was conducted in conjunction with North Carolina State University Institutional Review Board approval (IRB no. 164-08 and 1960-11).

Patients had blood samples drawn from one or three collection time points within 1 week (7 days). Following aseptic preparation of the venipuncture site, 91 blood specimens from 91 patients were collected into EDTA-anticoagulated blood and in serum separator tubes. Unopened collection tubes were transported directly or by overnight express carrier to the Intracellular Pathogens Research Laboratory (IPRL) for processing. A previously described approach that combines PCR amplification of *Bartonella* spp. DNA and enrichment culture of blood and serum in BAPGM was used to test whole-blood ($n = 91$) and centrifuged serum ($n = 91$) specimens (10, 24). The BAPGM platform incorporates 4 separate PCR testing time points, each representing a different component of the testing process for each patient sample: PCR amplification of *Bartonella* spp. following DNA extraction from (i) whole blood and (ii) serum, (iii) PCR following BAPGM enrichment of the whole-blood culture incubated for 7 and 14 days, and (iv) PCR from the subculture isolates if obtained after subinoculation from the BAPGM flask onto plates containing Trypticase soy agar with 10% sheep whole blood that are incubated for 4 weeks. PCR specimen preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms with unidirectional workflow to avoid DNA contamination. In addition, BAPGM cultures were processed in a biosafety cabinet with HEPA filtration in a limited-access biosafety level II laboratory. The methods used to amplify the *Bartonella* DNA from blood, serum, BAPGM liquid culture, and subculture isolates, if obtained, included conventional PCR with *Bartonella* genus primers targeting the 16S to 23S intergenic spacer (ITS) region and amplification using *Bartonella koehlerae* species-specific ITS primers as described previously (10, 15, 24, 25). Amplification of the *B. koehlerae* ITS region was performed using oligonucleotides Bkoehl-1s (5′-CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC-3′) and Bkoehl-1125as (5′-GCC TTT TTT GGT GAC AAG CAC TTT TCT TAA G-3′) as forward and reverse primers, respectively. Amplification was performed in a 25-μl final volume reaction mixture containing 12.5 μl of Tak-Ex Premix (Fisher Scientific, Rockford, IL), 0.1 μl of 100 μmol/liter of each forward and reverse primer (IDT DNA Technology, Coralville, IA), 7.3 μl of molecular-grade water, and 5 μl of DNA from each sample tested. Conventional PCR was performed in an Eppendorf Mastercycler EP gradient (Eppendorf, Hauppauge, NY) under the following conditions: a single cycle at 95°C for 2 s followed by 55 cycles with DNA denaturing at 94°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 18 s. The PCR was completed by a final cycle at 72°C for 30 s. As previously described for the ITS genus and *B. koehlerae* PCR assays, all products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under UV light, after which the amplicon products were sequenced to identify the species and ITS strain types. All positive test results were confirmed as *Bartonella* spp. through DNA sequencing.

PCR-negative controls were prepared using 5 μl of DNA from the blood of a healthy dog. *Bartonella henselae* (Houston 1 strain) at a concentration of 1 genome copy/μl was used as a PCR-positive control during the entire course of this study. In no instance was *B. henselae* or DNA of any other *Bartonella* spp. amplified in the negative control lane on any PCR gel. To assess potential contamination during blood sample processing into BAPGM, an uninoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient blood and serum samples tested. For all components of the BAPGM platform (PCR from blood, serum, enrichment cultures at 7 and 14 days, and subcultures), PCR-negative controls remained negative throughout the course of the study. In addition, subcultures of uninoculated BAPGM (culture control) at 7 and 14 days did not yield bacterial growth.

Patients were retrospectively divided into one of two groups: group 1 included individuals with blood specimens drawn from one collection day, and group 2 included individuals with blood specimens drawn from three collection days within a 1-week period. Statistical analysis was performed using SAS/STAT 9.2 for Windows (SAS Institute Inc., Cary, NC; 2008). The chi-squared test, Fisher's exact test, and the Mann-Whitney U test were used to detect differences in demographic characteristics by collection group. The chi-squared test and univariate logistic regression were used to assess differences in the proportions of positive patients by group to determine if serial testing within 1 week increased the likelihood of a positive test result using the BAPGM platform.

RESULTS

A total of 91 patients, for whom testing was performed in an identical manner, were included in the study population. Group 1 comprised 55 patients (60.5%), and group 2 comprised 36 patients (39.5%). Demographic characteristics of the study population, by collection group, are listed in Table 1. No significant differences were detected between collection groups for demographic information, risk factors, disease severity, prior antibiotic use, or self-reported symptoms, with the exception of self-reported weight loss ($P = 0.01$). Overall, 19 (20.8%) patients were PCR positive for one or more *Bartonella* spp. using the BAPGM platform. All positive test results were confirmed as *Bartonella* spp. through DNA sequencing. The numbers of patients who tested positive with the BAPGM platform, by group, are listed in Table 2. Overall, 13 patients (14.3%) tested positive following direct extraction of blood or serum, and 10 patients (10.9%) tested positive following liquid enrichment culture. No patients tested positive through subculture isolation. *B. henselae* and *B. koehlerae* were detected in 6 (6.6%) and 15 (16.5%) patients, respectively (Table 3). Overall, seven patients (12.7%) in group 1 tested positive, and 12 patients (33.3%) in group 2 tested positive ($P = 0.02$). Based upon the observed proportion of individuals who tested positive in each group, group 2 patients were more likely to test positive than group 1 patients (odds ratio [OR], 3.4 [95% confidence interval, 1.2 to 9.8]; $P = 0.02$) for the overall BAPGM platform and during the liquid enrichment culture stage of the BAPGM platform ($P = 0.04$) (Table 2). Of the *Bartonella* bacteremic patients in group 2, only 3 patients (23.1%) had positive specimens from more than 1 day. No patient had a positive result for all three specimen dates.

DISCUSSION

In order to enhance detection of *Bartonella* spp. in immunocompetent patients, serial testing of multiple specimens collected during a 1-week period should be considered. In this study, our analysis was restricted to a subpopulation of individuals who had been tested previously for *Bartonella* spp. through the IPRL (8); there-

TABLE 1 Demographic characteristics of 91 individuals tested for *Bartonella* spp. using the BAPGM platform, by collection group

Demographic characteristic	Group 1: 1 collection in 1 wk[a]	Group 2: 3 collections in 1 wk	P^b
Male	25 (45.5)	14 (38.9)	0.53
Female	30 (54.5)	22 (61.1)	
Age (yr)	42.0 (30–53)[c]	38.0 (28–50)[c]	0.40
Length of symptoms (mo)	24 (0–60)[c]	3 (0–42)[c]	0.48
Chronically ill (self-reported)	22 (40.0)	17 (47.2)	0.42
Animal contact	39 (70.9)	31 (86.1)	0.21
Tick exposure	38 (69.1)	29 (80.6)	0.41
Flea exposure	38 (69.1)	28 (77.8)	0.39
Prior antibiotic therapy	21 (38.2)	16 (44.4)	0.41
Balance problems	20 (36.4)	16 (44.4)	0.43
Shortness of breath	15 (27.3)	14 (38.9)	0.31
Blurred vision	19 (34.5)	13 (36.1)	0.32
Confusion	17 (30.9)	14 (38.9)	0.39
Depression	16 (29.1)	15 (41.7)	0.29
Bowel/bladder dysfunction	14 (25.5)	11 (30.5)	0.40
Difficulty remembering	22 (40.0)	18 (50.0)	0.38
Insomnia	20 (36.4)	16 (44.4)	0.40
Disorientation	8 (14.5)	7 (19.4)	0.39
Fatigue	32 (58.2)	23 (63.9)	0.38
Headaches	26 (47.3)	15 (41.7)	0.18
Irritability	26 (47.3)	17 (47.2)	0.31
Joint pain	28 (50.9)	20 (55.6)	0.39
Loss of sensation/numbness	23 (41.8)	17 (47.2)	0.41
Muscle pain	20 (36.4)	17 (47.2)	0.36
Muscle weakness	20 (36.4)	17 (47.2)	0.36
Paralysis	4 (7.3)	2 (5.6)	0.38
Sleeplessness	27 (49.1)	20 (55.6)	0.41
Syncope	8 (14.5)	6 (16.7)	0.41
Tremors/shakes	9 (16.4)	12 (33.3)	0.12
Weight loss	4 (7.3)	11 (30.6)	0.01[d]
Previous consultation with a:			
Neurologist	15 (27.3)	16 (44.4)	0.18
Infectious disease doctor	11 (20.0)	13 (36.1)	0.16
Rheumatologist	6 (10.9)	10 (27.8)	0.08
Endocrinologist	7 (12.7)	7 (19.4)	0.34
Psychiatrist	1 (1.8)	2 (5.6)	0.25

[a] Values shown are *n* (%) unless otherwise specified.
[b] The Fisher exact test was used when the cell size was <5. The nonparametric Mann-Whitney U test was used for nonnormally distributed continuous variables.
[c] Continuous variables are reported as medians (interquartile ranges).
[d] Statistically significant difference.

TABLE 2 *Bartonella* PCR amplification results from blood, serum, and enrichment blood culture processed using the BAPGM platform by collection group

	No. (%)		
	Group 1: 1 collection in 1 wk	Group 2: 3 collections in 1 wk	P^b
Blood			
Direct extraction	3 (5.4)	3 (8.3)	0.67
BAPGM enrichment	3 (5.4)	7 (19.4)	0.04[c]
Subculture isolates	0 (0.0)	0 (0.0)	—[d]
Total[a]	5 (9.1)	10 (27.8)	0.02[c]
Serum			
Direct extraction	4 (7.3)	4 (11.1)	0.71
BAPGM enrichment	0 (0.0)	0 (0.0)	—
Subculture Isolates	0 (0.0)	0 (0.0)	—
Total	4 (7.3)	4 (11.1)	0.71
Blood and serum combined			
Direct extraction	6 (10.9)	7 (19.4)	0.35
BAPGM enrichment	3 (5.4)	7 (19.4)	0.04[c]
Subculture isolates	0 (0.0)	0 (0.0)	—
Total	7 (12.7)	12 (33.3)	0.02[c]

[a] Numbers do not sum to the total because an individual could be positive at more than one stage of the BAPGM platform.
[b] The Fisher exact test was used when the cell size was <5.
[c] Statistically significant difference.
[d] —, no statistics were calculated for zero cell comparisons.

fore, individuals were not randomized into sampling groups prior to entry into the study, and our results might be subject to unintentional selection bias. For example, patients tested multiple times for *Bartonella* bacteremia might have been more likely to test positive due to more severe symptoms. Due to the limited sample size within our group 1 population, we were unable to control for potential confounders, such as a discrepancy of disease severity or exposure between groups that might have affected the observed association between bacteremia and serial specimen collection. While there was no difference between our two groups for self-reported demographics, disease severity, exposure, or disease symptom variables, as shown in Table 1, due to the univariate nature of our analysis, we acknowledge that unmeasured confounders might exist that could potentially distort our observed association. Additionally, our retrospective analysis relied on previously obtained data, and no projection can be accurately made as to the true proportion of positive versus negative individuals or the exact increase in sensitivity obtained within this overall study population. To confirm the association obtained from our observational data, experimental studies are needed to evaluate the effect of serial testing on the diagnostic sensitivity of *Bartonella* testing in individuals or animals with equal known infection rates. Diagnostic testing for *Bartonella* spp. using the BAPGM platform has been shown to be highly specific because amplicon identity is confirmed by DNA sequencing; however, sensitivity is currently unclear due to the low levels of bacteria found in human patient samples (2, 8, 10). *Bartonella* bacteremia is more readily documented in a primary reservoir species, such as cats or rodents, and might occur less frequently or to a much lower level in accidental

TABLE 3 Detected species of *Bartonella* following the BAPGM platform, by group

	No. (%)		
Species	Group 1: 1 collection in 1 wk	Group 2: 3 collections in 1 wk	P^b
B. henselae	2 (3.6)	4 (11.1)	0.21
B. koehlerae	5 (9.1)	10 (27.8)	0.02
B. vinsonii subsp. *berkhoffii*	0 (0.0)	0 (0.0)	—[c]
Total[a]	7 (12.7)	12 (33.3)	0.02

[a] Numbers do not sum to the total because an individual could be positive for more than one species of *Bartonella*.
[b] The Fisher exact test was used when the cell size was <5.
[c] —, no statistics were calculated for zero cell comparisons.

hosts, such as humans. For example, in humans, the average bacterial levels in blood are 1 to 10 genome copies/μl, compared to the 10^5 to 10^6 copies/μl often found in cats (2). In a recent study of 192 patients, the BAPGM enrichment blood culture-PCR detected *Bartonella* spp. in an additional 34.7% of patients compared to PCR on extracted blood alone (8). Overall, in that study, 46 patients (23.9%) were infected with *Bartonella*. Serial sampling over a 1-week period in the current study appears to have further increased the diagnostic sensitivity of the BAPGM platform within the study population.

Bartonella bacteremia appears to exist in a cyclical nature within its natural reservoir hosts (17, 20, 21, 26). In the *Bartonella tribicorum* rat model of *Bartonella* infection, high numbers of bacteria are detectable in the blood following the initial infection, until a level of 8 to 15 bacteria per erythrocyte is reached. Bacteremia then declines and drops below a detectable level (20, 21). While the location of *Bartonella* during this nonbacteremic phase of infection is currently unknown, endothelial cells and bone marrow have been hypothesized as primary niches in both incidental and reservoir hosts (17, 20, 27, 28). Bacteria have been observed to release from the primary niche and reinvade circulating erythrocytes to create peaks of bacteremia at intervals of 3 to 6 days (17, 20). Several other observations support the persistence of *Bartonella* in a cellular/acellular compartment, with subsequent seeding into circulation (17, 29, 30). In a cat naturally infected with *B. henselae*, high levels of bacteria initially demonstrated in the blood by culture gradually declined to undetectable levels over a 5-month period. Bacteremia was again documented 2 months later, and the cat became cyclically culture negative at 2-month intervals (29). Although the pattern of bacteremia was variable among individual animals, similar results were found during experimental *B. henselae* transmission studies involving specific-pathogen-free (SPF) cats (19, 30). Unfortunately, the unpredictable nature of bacteremia within the host might result in an inaccurate microbiological diagnosis even if the patient is tested three times during a 1-week period.

Bartonellosis is an emerging infectious disease, with 17 *Bartonella* spp. having been associated with an expanding spectrum of human pathology (2, 8). Due to the zoonotic potential of *Bartonella* spp. as human pathogens, the medical relevance of this genus is undergoing rapid redefinition (2). Although epidemiologic studies are needed to establish causation for many nonspecific *Bartonella*-associated symptoms, enhanced diagnostic detection of *Bartonella* is necessary to accurately generate data for epidemiological investigations and to properly define disease pathology, determine effective treatment options, and assess microbiological outcomes for patients. Obtaining three sequential specimens during a 1-week period appears to enhance detection of *Bartonella* bacteremia in human patients and should be considered as a diagnostic approach when bartonellosis is suspected.

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