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BARTONELLA HENSELAE IN CAPTIVE AND HUNTER-HARVESTED BELUGA (*DELPHINAPTERUS LEUCAS*)

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ABSTRACT: Previously, we reported the isolation of *Bartonella henselae* from the blood of harbor porpoises (*Phocoena phocoena*) and loggerhead sea turtles (*Caretta caretta*) from the North Carolina coast. Hematologic, pathologic, and microbiologic findings surrounding the death of a juvenile captive beluga in Vancouver initiated an outbreak investigation designed to define the molecular prevalence of *Bartonella* infection in belugas. Using polymerase chain reaction analyses targeting the intergenic spacer region (ITS), two *B. henselae* ITS strains were identified in 78% of captive and free-ranging hunter-harvested belugas. These findings may have public health implications and may influence aquarium management procedures for captive marine mammals.

Key words: *Bartonella*, beluga, captive, *Delphinapterus leucas*, free-ranging.

INTRODUCTION

Bartonella species comprise an important group of fastidious, intravascular, pathogenic bacteria that can be transmitted by numerous arthropod species, by animal scratches, and by blood transfusion (Kordick et al., 1999; Breitschwerdt and Kordick, 2000; Chomel et al., 2006). Previously, we reported the isolation of *Bartonella henselae* from the blood of two live stranded harbor porpoises (*Phocoena phocoena*) recovered along the northern North Carolina coast (Maggi et al., 2005b) and the presence of *B. henselae* DNA in blood samples obtained from wild loggerhead sea turtles (*Caretta caretta*; Valentine et al., 2007). The geographic distribution, mode(s) of transmission, pathogenicity, and reservoir potential of *Bartonella* species infecting marine mammals have not been established.

In July 2005, a previously healthy beluga died unexpectedly within one hour following routine blood sampling. Examination of the blood smear revealed basophilic stippling and intra-erythrocytic inclusions, potentially consistent with an infectious organism. Blood and necropsy tissue samples were sent to the Intracellular Pathogens Research Laboratory at North Carolina State University to determine if *B. henselae* or another erythrocyte-associated organism could be identified. This event initiated the testing of captive and hunter-harvested belugas described in this study.

MATERIALS AND METHODS

Case study

A 3-yr-old captive male beluga (*Delphinapterus leucas*) developed marked anemia with erythrocytic basophilic stippling and rare cytoplasmic inclusions following restraint for routine blood collection. The hematocrit decreased from 50% to 34% between the initial sample and just prior to death, one hour later. Blood lead levels were within normal reference ranges. Serum iron concentration and total iron-binding capacity (TIBC) were both elevated. No toxicologic abnormalities were found by water analyses. Gross necropsy lesions included mesenteric edema within the midlevel of the jejunum and ileal lymph nodes, an ulcer spanning the gastroesophageal junction, and multifocal epicardial

hemorrhages. Histopathologic lesions included nonsuppurative portal hepatitis, encephalitis, bronchopneumonia, interstitial myocarditis, and eosinophilic enteritis. There was no evidence of antemortem hemolysis within the blood sample or microscopic indication of hemoglobinuria within examined kidney sections; however, marked macrophage hemosiderosis was visible within the liver, spleen, lymph node, and bone marrow, indicative of extravascular hemolysis. Bone marrow histopathology supported an active regenerative response. Postmortem trace mineral and vitamin A analyses of the liver were within normal in-house reference limits. Aerobic culture of the lung, liver, and brain did not yield bacterial growth, and only 1–2 colonies of *Myroides* sp. were isolated from the lymph nodes and kidney. Polymerase chain reaction (PCR) analyses of pooled tissues were negative for *Brucella* spp., morbillivirus, *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, *Chlamydophila psittaci*, *Coxiella burnetii*, influenza, *Toxoplasma gondii*, *Leptospira* spp., and herpes virus. Inoculation of tissue homogenates onto Madin Darby and VERO cell lines did not induce cytopathic effect during a 2-wk incubation period. Pooled tissues were PCR positive for *Mollicutes* (*Mycoplasma* or *Ureaplasma* spp.), and an agonal blood sample contained *Bartonella* sp. DNA.

Animals and clinical signs

The beluga that died suddenly was one of six whales that originated or were descended from the Hudson Bay stock. These whales had been maintained in a very stable aquatic environment at the Vancouver Aquarium for up to 30 yr. The whale that died was born at the Vancouver Aquarium, where the whales are housed in an ~1,950,000-liter semiclosed seawater system. Annual water temperatures typically vary between 10 and 13 C. Filtration is primarily by diatomaceous earth filters, and disinfection accomplished through a combination of chlorination and ozonation. Exposure to other animals was controlled; however, birds and raccoons occasionally accessed the local environment in which the whales were maintained. The food source for adult whales consisted of frozen wild-caught fish and squid. Based on the initial observations from the captive beluga, and as part of a long-term health-monitoring study of wild animals, blood samples, lymph node, liver, and spleen were also collected from three hunter-harvested beluga in the Mackenzie Delta, Northwest Territories (NWT), Canada (69°N, 133°W).

PCR and blood culture

Tissue samples, including blood, lymph node, liver, and spleen, from free-ranging, hunter-harvested ($n=3$) beluga and EDTA anticoagulated blood samples from captive ($n=5$) beluga were analyzed for *Bartonella* species DNA using a PCR targeting the 16S–23S rRNA intergenic transcribed spacer (ITS) region (Maggi and Breitschwerdt, 2005). Blood samples from all captive beluga were cultured in *Bartonella* alpha Proteobacteria growth medium (BAPGM) and subinoculated onto blood agar plates to facilitate *Bartonella* detection and bacterial isolation as previously described (Daybell et al., 2004; Maggi et al., 2005a; Breitschwerdt et al., 2007; Duncan et al., 2007). All samples were obtained for testing between June 2005 and June 2006.

Pre-enrichment BAPGM culture

Blood samples were cultured in a 25-ml filter-cap cell-culture flask by inoculation of a 1-ml aliquot of each blood sample into 9 ml of BAPGM medium. Blood samples from all captive belugas were cultured at 35 C in a 5% CO_2 water–saturated atmosphere for 7 days with constant shaking. For each series of processed blood samples, an uninoculated control BAPGM culture ($n=8$) was processed at the same time and in an identical manner. For colony isolation, a 1-ml aliquot of pre-enriched sample or uninoculated BAPGM control sample was subinoculated onto 5% defribrinated rabbit blood tryptic soy agar (TSA) plates (BAP; Fischer Scientific, Pittsburg, Pennsylvania) and cultured for 7 days using the same culture conditions. Colony formation was followed for 21 days.

DNA extraction from tissues, blood, pre-enrichment culture, and blood agar plate isolates

DNA was prepared from 25–50 mg of wet tissue, 200 µl of each blood and pre-enrichment culture samples, or from colonies resuspended in sucrose phosphate glutamate (SPG) buffer by using Qiamp DNA Mini Kit (QIAGEN Inc., Valencia, California, USA). After extraction, DNA concentration and purity were measured using an absorbance ratio between 260/280 nm for PCR analysis.

PCR detection of *Bartonella* DNA

Identification of *Bartonella* species DNA in extracted tissue samples, blood, pre-enrichment culture, and plate isolates was performed using conventional PCR targeting the 16S–23S rRNA intergenic transcribed spacer region (ITS) by conventional PCR (Maggi et al., 2005a; Duncan

et al., 2007). The ITS PCR screening of the *Bartonella* ITS region was performed using oligonucleotides 325s: 5′ CTT CAG ATG ATG ATC CCA AGC CTT TTG GCG 3′ and 1100as: 5′ GAA CCG ACG ACC CCC TGC TTG CAA AGC A 3′ as forward and reverse primers, respectively. Amplification of DNA from blood, pre-enrichment cultures, and isolates was performed in a 25-µl final-volume reaction vessel containing 12.5 µl of Tak-Ex® Premix (Fisher Scientific), 0.25 µl of 30 µM of each forward and reverse primer (IDT®, Integrated DNA Technology, Park Coralville, Iowa), 7 µl of molecular-grade water, and 5 µl of DNA from each sample tested. The PCR negative controls were prepared using 5 µl of either deionized water (when testing isolates from plates), DNA from blood of a healthy dog (when testing blood extractions), or DNA extracted from uninoculated BAPGM controls (when testing BAPGM pre-enrichment media). Plasmid clones of ITS amplicons from *B. henselae* at a final concentration of 2.5 copies per reaction were used as positive controls for PCR reaction. Conventional PCR analysis was performed in an Eppendorf Mastercycler EPgradient® (Ependorf, Westbury, New York) under the following conditions: a single hot-start cycle at 95 C for 2 min followed by 55 cycles of denaturing at 94 C for 15 sec, annealing at 66 C for 15 sec, and extension at 72 C for 18 sec. Amplification was completed by an additional cycle at 72 C for 1 min, and products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light. Amplicon products were sequenced to establish the *Bartonella* species and strain.

Cloning and sequencing of ITS amplicons

The DNA sequencing was performed for *Bartonella* ITS positive amplicons to confirm the species and to establish strain identification. The amplified PCR products were cloned into plasmid pGEM-T Easy Vector System (Promega®, Madison, Wisconsin, USA). Recombinants with white colonies were selected on the basis of the right size of insert in the plasmid using Qiagen plasmid miniprep. Sequencing of plasmid inserts was provided by Davis Sequencing, Inc., Davis, California, USA. Sequence analysis and alignment with GenBank sequences were performed using AlignX software (Vector NTI Suite 6.0, Invitrogen Corp., Carlsbad, California).

RESULTS

Bartonella sp. DNA was amplified from one of three pre-enrichment blood samples from each of three captive beluga and from one of two pre-enrichment blood samples from a fourth captive beluga. *Bartonella* sp. DNA was not detected in a single blood sample from a fifth captive beluga. For all *Bartonella* PCR positive samples, DNA amplification was possible only after pre-enrichment blood culture in BAPGM. *Bartonella* PCR amplification was never positive when DNA was extracted directly from the blood sample (Fig. 1). All *Bartonella* ITS amplicons were of a size consistent with *B. henselae*, and all negative controls remained negative throughout the study period. Attempts to amplify the Pap31 bacteriophage associated heme-binding protein gene were not successful. Alignment analysis of ITS sequences for three captive beluga samples showed 100% homology (600/600 base pairs [bp]) with *B. henselae* strain Houston I (GenBank accession no. NC_005956). Sequences derived from the fourth captive beluga were 99.8% similar (629/630 bp) to *B. henselae* (GenBank accession no. DQ529247), which was amplified and sequenced from the blood of a stranded porpoise (Maggi et al., 2005), and were 99.7% similar (628/630 bp) to *B. henselae* strain SA2 (GenBank accession no. AF369529). Agar plate isolates were not obtained following subculture from any BAPGM pre-enrichment liquid culture.

Bartonella henselae DNA was detected in spleen, but not other tissues (blood, lymph node, and liver), from two of three hunter-harvested beluga whales (Fig. 1). Sequences derived from both splenic amplicons had a single base-pair difference (629/630 bp) when compared with *B. henselae* SA2 (GenBank accession no. DQ529247).

DISCUSSION

Hematologic, pathologic, and microbiologic findings surrounding the sudden, unexpected death of a juvenile captive beluga initiated an outbreak investigation to define the molecular prevalence of

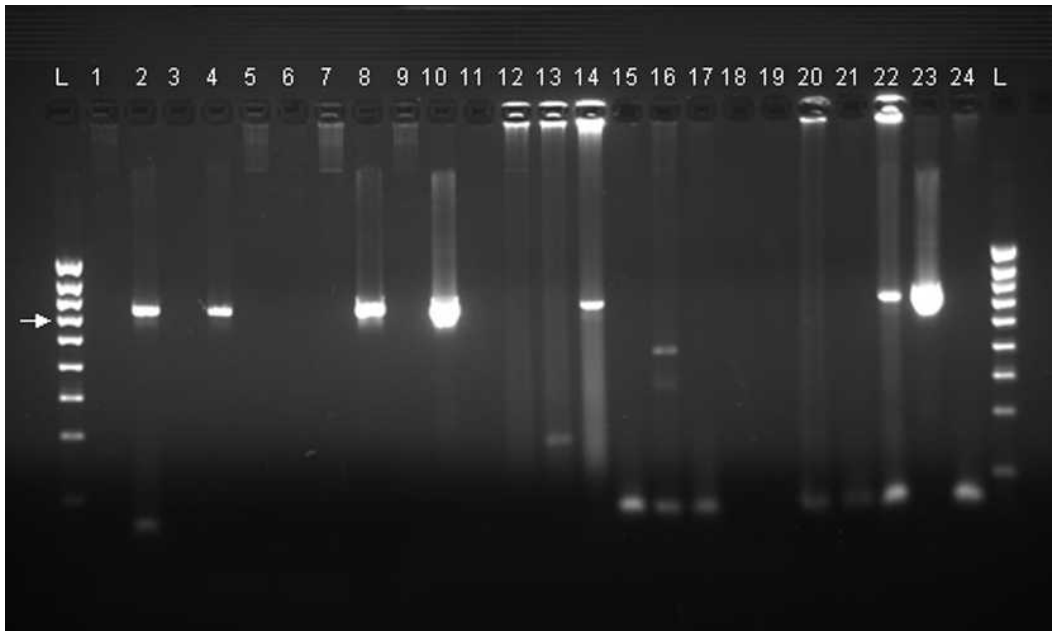

FIGURE 1. PCR amplification of *Bartonella* DNA from beluga blood or tissue samples using 16S–23S intergenic transcribed spacer (ITS) primers. L = DNA ladder (1 kbp). 1: beluga A blood sample; 2: beluga A pre-enrichment culture; 3: beluga B blood sample; 4: beluga B pre-enrichment culture, 5: beluga C blood sample; 6: beluga C pre-enrichment culture; 7: beluga D blood sample; 8: beluga D pre-enrichment culture; 9: beluga E blood sample, 10: beluga E pre-enrichment culture; 11–14: beluga 2006-02289 blood, lymph node, liver, and spleen tissues, respectively; 15–18: beluga 2006-02290 blood, lymph node, liver, and spleen tissues, respectively; 19–22: beluga 2006-02291 blood, lymph node, liver, and spleen tissues, respectively; 23: *Bartonella henselae* DNA (5 copies/reaction); 24: negative control. Arrow = 600 bp.

Bartonella species infection in captive and free-ranging beluga. Although the number of animals tested was low, the overall *B. henselae* prevalence based on PCR was 78%, and a similar prevalence of infection was found in captive and free-ranging beluga. This would seemingly suggest frequent *B. henselae* exposure, chronic intravascular infection, or both. Based upon DNA sequencing, two *B. henselae* ITS strains were found in beluga. Although the source, the mode of transmission, and the timing of infection for individual animals could not be established, the detection of *B. henselae* in both beluga populations suggests that these marine mammals may sustain chronic, low-grade, nonclinical infections, such as occurs in cats (Kordick et al., 1999; Breitschwerdt and Kordick, 2000; Chomel et al., 2006). For captive animals, chronic infection with *B. henselae* may have potential health implications, whereas from a public health perspective, occult infection in hunter-harvested beluga could represent an exposure risk to individuals rendering beluga carcasses. Both extensive animal contact and arthropod exposure may represent risk factors for human *B. henselae* infection (Maggi et al., 2005a; Chomel et al., 2006; Breitschwerdt et al., 2007).

In this study, pre-enrichment BAPGM blood culture was necessary to increase bacterial numbers to a level in which PCR detection was successful. This observation is similar to results obtained in our laboratory when testing blood samples from infected dogs and immunocompetent humans (Breitschwerdt et al., 2007; Duncan et al., 2007). Based upon previous work from our group and others, these bacteria will grow for a finite period in only a limited number of described enrichment media (Maggi et al., 2005a). Another recognized limitation of this

approach is the frequent failure to obtain stable agar plate isolates following subculture from the enriched *Bartonella*-infected media (Breitschwerdt et al., 2007; Duncan et al., 2007). Despite improvements in isolation and molecular detection, diagnostic confirmation of *Bartonella* sp. infection in clinical samples remains challenging. As used in this study, the *Bartonella* ITS-PCR will detect 2–5 genome equivalents (bacteria) when using 5 μl of template DNA. This level of PCR sensitivity requires approximately 400 *B. henselae* organisms per milliliter of blood or an equivalent number in the BAPGM pre-enrichment culture sample to achieve a PCR amplification product.

Bartonella henselae DNA was detected only in splenic tissues from hunter-harvested beluga, suggesting organism sequestration or immunologic clearance of *B. henselae* by this hematopoietic organ. Splenic rupture and granulomatous splenitis have been reported in humans infected with *B. henselae* (Kahr et al., 2000; Daybell et al., 2004). In addition, splenectomy, performed as an adjunctive therapy for idiopathic immune-mediated thrombocytopenia, resulted in detection of persistent *Bartonella bacilliformis* bacteremia (Henriquez et al., 2004). Future studies should consider the role of the spleen in the immunomodulation of *Bartonella* spp. infection and the relative distribution and cellular localization of *Bartonella* organisms in various organs, with specific emphasis on the role of the spleen in disease pathogenesis.

As discussed already, *Bartonella* species are a well-adapted cause of chronic intravascular infections in cats and potentially other animal species. Therefore, assuming chronic infection in the index beluga, death was most likely multifactorial and not solely related to infection with *B. henselae*. A universal PCR for mollicutes on pooled tissues from stranded whales has been a routine part of our necropsy protocol for several years, and, to date, lesions have not been reported in whales or porpoises in association with mycoplasma infection. In addition, this animal was culture positive for *Myroides* spp. Salt water contains *Myroides* spp., which could suggest that the occasional colonies obtained from lymph node or kidney cultures were a reflection of the marine environment. Alternatively, *Myroides* spp. have been infrequently associated with cellulitis and bacteremia in immunocompetent individuals, and it was isolated from people injured during a recent tsunami catastrophe (Green et al., 2001; Kallman et al., 2006). Therefore, the organism (myroides) may have been a commensal or may have represented a dual infection. One could speculate that occult *B. henselae* infection may have contributed to an acute hemolytic crisis in the index animal with subclinical encephalitis, hepatitis, and myocarditis. Immune status appears to greatly influence disease severity, the variation in clinical manifestations, the pattern of histopathology, and the relative ease of diagnostic detection of *B. henselae* in infected human patients (Kahr et al., 2000; Karem et al., 2000; Ben-Ami et al., 2005; Chomel et al., 2006). Hematologic abnormalities found in the index whale, including basophilic stippling, erythrocyte deformity, and intra-erythrocytic inclusions, were similar to observations in a cynomolgus research monkey (*Macaca fascicularis*), in which erythrocytic structural abnormalities facilitated the detection of *Bartonella quintana* (O'Rourke et al., 2005). In addition, *Bartonella bacilliformis* is a well-recognized cause of life-threatening, severe hemolytic anemia in people (Karem et al., 2000; Henriquez et al., 2004). There is also limited serologic or molecular evidence to support a role for *Bartonella vinsonii* subsp. *berkhoffii* as a cause or co-factor in the development of immune-mediated hemolytic anemia, myocarditis, and encephalitis in dogs (Breitschwerdt et al., 1999, 2004). Vascular infection with a *Bartonella* species appears to contribute to the development

of hepatitis, myocarditis, and encephalitis in dogs and people, similar to the microscopic lesions observed in the *B. henselae*–infected captive juvenile beluga that initiated this study (Breitschwerdt et al., 1999, 2004; Karem et al., 2000; Chomel et al., 2006). Future studies are needed to investigate the mode of transmission, the duration of infection, and pathogenicity of *Bartonella* species in marine mammals.

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