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**Original article**

# *Bartonella* species detection in captive, stranded and free-ranging cetaceans

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**Abstract –** We present prevalence of *Bartonella* spp. for multiple cohorts of wild and captive cetaceans. One hundred and six cetaceans including 86 bottlenose dolphins (71 free-ranging, 14 captive in a facility with a dolphin experiencing debility of unknown origin, 1 stranded), 11 striped dolphins, 4 harbor porpoises, 3 Risso's dolphins, 1 dwarf sperm whale and 1 pygmy sperm whale (all stranded) were sampled. Whole blood ($n = 95$ live animals) and tissues ($n = 15$ freshly dead animals) were screened by PCR ($n = 106$ animals), PCR of enrichment cultures ($n = 50$ animals), and subcultures ($n = 50$ animals). *Bartonella* spp. were detected from 17 cetaceans, including 12 by direct extraction PCR of blood or tissues, 6 by PCR of

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enrichment cultures, and 4 by subculture isolation. *Bartonella* spp. were more commonly detected from the captive (6/14, 43%) than from free-ranging (2/71, 2.8%) bottlenose dolphins, and were commonly detected from the stranded animals (9/21, 43%; 3/11 striped dolphins, 3/4 harbor porpoises, 2/3 Risso's dolphins, 1/1 pygmy sperm whale, 0/1 dwarf sperm whale, 0/1 bottlenose dolphin). Sequencing identified a *Bartonella* spp. most similar to *B. henselae* San Antonio 2 in eight cases (4 bottlenose dolphins, 2 striped dolphins, 2 harbor porpoises), *B. henselae* Houston 1 in three cases (2 Risso's dolphins, 1 harbor porpoise), and untyped in six cases (4 bottlenose dolphins, 1 striped dolphin, 1 pygmy sperm whale). Although disease causation has not been established, *Bartonella* species were detected more commonly from cetaceans that were overtly debilitated or were cohabiting in captivity with a debilitated animal than from free-ranging animals. The detection of *Bartonella* spp. from cetaceans may be of pathophysiological concern.

***Bartonella* / cetacean / dolphin / porpoise**

## 1. INTRODUCTION

Bartonellosis, a new emerging worldwide zoonotic disease [3, 7], can be caused by a spectrum of *Bartonella* spp. These microorganisms are Gram-negative aerobic bacilli, members of the alpha subdivision of the class Proteobacteria, comprised of at least 20 different species and subspecies. Best known of these are *B. quintana* (trench fever), and *B. henselae* (cat-scratch disease). Infection with *Bartonella* species is known to cause lymphadenopathy [17, 24, 31], neurological disorders [1, 22, 33], bacillary angiomatosis and bacillary peliosis [9, 23, 37], endocarditis [4, 5, 22, 24], hepatosplenic involvement, skin lesions, and vertebral osteomyelitis in domestic and wild animals and in humans [22, 23, 36].

*Bartonella* spp. have been isolated from wild and domestic animals including cats, dogs, deer, cattle, and rodent, among others [7, 11, 20, 24]. Because *Bartonella* spp. frequently induce persistent intravascular infections, attributing disease causation to *Bartonella* spp. infection in animal or in human patients has been difficult. Due to chronic *Bartonella* bacteremia, particularly in natural reservoir hosts, satisfying Koch's postulates remains difficult or impossible [21], requiring reliance on associational evidence.

Because conventional microbiological techniques lack sensitivity for *Bartonella* spp., bartonellosis is usually diagnosed by PCR amplification or serology [3, 6, 26, 29]. Recently, the development of a more sensitive isolation approach, using *Bartonella* Alpha-Proteobacteria growth medium (BAPGM) followed by real time PCR has facilitated the molecular detection or isolation of *Bartonella* spp. from the blood of sick and healthy animals [8, 13, 29]. These improved techniques led to the first recognition of blood-borne *Bartonella* spp. infections in marine animals: stranded harbor porpoises (*Phocoena phocoena*) [30] and clinically normal loggerhead sea turtles (*Caretta caretta*) [35]. Both of these reports from marine animals were based on molecular detection without positive culture of *Bartonella* spp., and neither included a comparison of debilitated and healthy animals. Here we present prevalence of *Bartonella* spp. for multiple cohorts of wild and captive cetaceans. We use a combination approach including PCR, PCR of enrichment cultures in BAPGM, and BAPGM subculture isolates [13].

## 2. MATERIALS AND METHODS

### 2.1. Captive case description

A six-year-old captive-born female bottlenose dolphin (*Tursiops truncatus*) housed with 13 other bottlenose dolphins in semiclosed pools at a facility in the Gulf Coast of the United States exhibited intermittent inappetance, recurrent leukocytosis, reticulocytosis, intermittent regenerative anemia and pneumonia, with a linear corneal opacity and a suspected mediastinal abscess. Treatment with antibiotics and antifungals would resolve the leukocytosis and anemia, but problems recurred following withdrawal of medications. Fungal serology (Cerodex Laboratories, Immuno-Mycologics, Inc., Norman, OK, USA) for *Candida*, *Aspergillus*,

*Histoplasma*, *Coccidioides* and *Blastomyces* were negative, except for a low *Candida* titer of 1:1. Serum heavy metal and toxicology screens were unremarkable (copper, iron, selenium, zinc, lead, and general pesticide screen, Okalahoma Animal Disease Diagnostic Laboratory, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, USA). *Chlamydophila* serology (Comparative Pathology Laboratory, Department of Pathology, University of Miami, Miami, FL, USA) was positive, at 1:10 on 24 January 2003, 1:50 on 16 April, and 1:25 on 12 December. *Chlamydophila* PCR of heparinized whole blood (University of Miami) on 5 March was negative. In August 2004, unidentified small extracellular bodies were observed on several blood smears. Serology for sarcosystis and toxoplasmosis (University of Miami) were negative. Whole blood in EDTA was submitted for vector-borne disease screening (Intracellular Pathogens Research Laboratory, North Carolina State University, College of Veterinary Medicine, Raleigh, NC, USA) by PCR for *Rickettsia*, *Babesia*, *Leishmania*, *Ehrlichia*, and *Bartonella*, of which all were negative except for *Bartonella*. Based on the positive PCR for *Bartonella*, the other 13 dolphins were sampled. No other captive *Bartonella*-positive dolphins exhibited clinical abnormalities. None of the captive *Bartonella*-positive dolphins subsequently expired, so there are no post-mortem findings for these cases.

### 2.2. Stranded and free-ranging animals

Following the detection of *Bartonella* in captive marine mammals, screening was expanded to stranded and free-ranging dolphins and porpoises. Stranded animals ($n = 21$) included 11 striped dolphins (*Stenella coeruleoalba*; WAM 612, 613, 614, 616, 617, 618, 620, 621, 622 and 623, all from a single mass stranding 22 August 2005, and BRF 141, 19 April 2007), four harbor porpoises (*Phocoena phocoena*, AAH 009, 23 March 2005 and MLC 001, 5 May 2005 [30], CALO 0601, 12 January 2006, and BRF 130, 17 March 2007), three Risso's dolphins (*Grampus griseus*, BRF 030, 23 June 2005, MML 0514A, 1 February 2006, and BRF 059, 23 March 2006), one bottlenose dolphin (BRF 028, 20 June 2005), one dwarf sperm whale (*Kogia sima*, BRF 108, 20 January 2007), and one pygmy sperm whale (*K. breviceps*, GNL 056, 22 March 2007). With the exception of one Risso's dolphin from Florida, all stranded animals were from North Carolina.

Free-ranging bottlenose dolphins ($n = 71$) were captured and released in the course of population biology and health assessment investigations by the National Marine Fisheries Service (NMFS), the National Ocean Service (NOS) and Chicago Zoological Society's Sarasota Dolphin Research Program (CZS) [38]. Bottlenose dolphins were sampled from waters near Charleston, South Carolina (NOS, August 2005, $n = 20$), Beaufort, North Carolina (NMFS, April 2006, $n = 14$), Sarasota, Florida (CZS, June 2006, $n = 19$), and Port St. Joe, Florida (NMFS, July 2006, $n = 18$).

### 2.3. Blood collection

EDTA-anticoagulated blood was aseptically collected from 71 free-ranging, 14 captive and 10 stranded animals, either from the periarterial venous rete of the flukes (free-ranging, captive, and some stranded animals) or from the heart (sedated stranded animals at the time of humane euthanasia). Blood was chilled on ice and shipped overnight to the laboratory for processing. Hematology and serum chemistry values of the two *Bartonella*-positive free-ranging dolphins were not outliers for any parameter and were within reference ranges for the species [18].

### 2.4. Tissue sampling

Tissues were collected from 15 live-stranded animals that subsequently died spontaneously or were euthanized. Animals were necropsied immediately post-mortem or were chilled and necropsied within 36 h. Tissues collected included brain, heart valves, lung, lymph nodes, spleen and bone marrow. Tissue samples were placed in cryogenic vials or plastic bags, chilled, and transported to the laboratory within 24 h of collection. Tissues were also saved in 10% neutral-buffered formalin for routine histological processing. Where available, postmortem findings of *Bartonella*-positive stranded animals included moderate, diffuse, nonsuppurative encephalitis in a Risso's dolphin, mild encephalitis, myocardial fibrosis and glomerulopathy in the three striped dolphins, and emaciation, lymphoid hyperplasia, and mild verminous pneumonia in one harbor porpoise.

### 2.5. Sample analysis

Molecular detection, cloning and bacterial isolation from blood and tissues were performed using

**Table I.** Summary of *Bartonella* positive results by species, group, sample and method of detection, out of 106 dolphins and porpoises (71 free-ranging, 14 captive, and 21 stranded). *Bartonella* genus and species determinations were performed by real-time PCR and sequencing (Bh SA2 = *Bartonella henselae* San Antonio 2, Bh H1 = *Bartonella henselae* Houston 1, Bh = *Bartonella henselae*, sequencing not done, *Bartonella* sp. = *Bartonella* species undetermined, ND = not done).

| Species | Group | Sample | Direct extraction PCR | Pre-enrichment PCR | Subculture isolation |
|---|---|---|---|---|---|
| *T. truncatus* | Free-ranging | Blood | *Bartonella* sp. | Negative | Negative |
| *T. truncatus* | Free-ranging | Blood | Negative | Negative | *Bartonella* sp. |
| *T. truncatus* | Captive | Blood | Negative | Bh SA2 | Negative |
| *T. truncatus* | Captive | Blood | Bh SA2 | Bh | Bh SA2 |
| *T. truncatus* | Captive | Blood | Negative | Bh SA2 | ND |
| *T. truncatus* | Captive | Blood | Bh | Negative | Negative |
| *T. truncatus* | Captive | Blood | *Bartonella* sp. | *Bartonella* sp. | Bh SA2 |
| *T. truncatus* | Captive | Blood | *Bartonella* sp. | ND | Negative |
| *S. coeruleoalba* | Stranded | Lung | *Bartonella* sp. | ND | ND |
| *S. coeruleoalba* | Stranded | Lung | Bh SA2 | ND | ND |
| *S. coeruleoalba* | Stranded | Brain | Bh SA2 | ND | ND |
| *P. phocoena* | Stranded | Blood | Negative | Bh SA2 | Negative |
| *P. phocoena* | Stranded | Blood | Bh SA2 | Negative | Negative |
| *P. phocoena* | Stranded | Serum | Negative | Bh H1 | Negative |
| *G. griseus* | Stranded | Brain | Bh H1 | ND | ND |
| *G. griseus* | Stranded | Blood | *Bartonella* sp. | Negative | Bh H1 |
| | | Cyamid | Bh H1 | ND | ND |
| *K. breviceps* | Stranded | Brain | *Bartonella* sp. | ND | ND |

previously-described methods [13, 26–29]. EDTA-anticoagulated blood samples ($n = 95$ animals) and tissues ($n = 15$ freshly dead animals) were analyzed. Following DNA extraction real-time PCR (RT-PCR) was used to screen for the presence of *Bartonella* 16S-23S intergenic spacer region (ITS) partial DNA sequences in each sample ($n = 106$ animals), with conventional PCR for the ITS region and phage-associated protein (Pap) 31 used to generate amplicons for sequencing to confirm RT-PCR results and typing [26, 27]. Tissue and ectoparasite samples were screened only by direct extraction PCR. For blood samples only, a pre-enrichment culture was established from the original sample using liquid BAPGM [29]; after a seven day incubation period a sample was removed for molecular screening using conventional and real-time PCR ($n = 50$ animals). Finally, a BAPGM blood agar plate was subinoculated using the liquid pre-enrichment blood culture, maintained for at least three weeks, at which time colony growth was again tested by conventional and real-time PCR ($n = 50$ animals). This combined approach has been shown to enhance the detection and isolation of *Bartonella* spp. in dog blood samples [13]. Negative PCR and un-inoculated pre-enrichment cultures were processed simultaneously to assess for laboratory contamination, and were routinely negative. Amplicons from conventional PCR were cloned using pGEM-T Easy Vector System (Promega®, Madison, WI, USA) for sequencing by Davis Sequencing, Inc. (Davis, CA, USA). Sequence analysis and alignment with GenBank sequences were performed using AlignX software (Vector NTI Suite 6.0, InforMax, Inc., Frederick, MD, USA).

### 2.6. Statistical analysis

Prevalence of *Bartonella* detection from blood between free-ranging and captive groups was

compared by Fisher's exact test (JMP 5.1.2, SAS Inc, Cary, NC, USA), considering (1) overall positive results from any laboratory method (direct extraction PCR, PCR of pre-enrichment cultures, and subculture isolation), and (2) by direct extraction PCR only.

## 3. RESULTS

A summary of positive results is presented in Table I. *Bartonella* spp. were detected from 17 cetaceans overall, including 12 detections by PCR following direct extraction of the blood sample, 6 by PCR of pre-enrichment cultures, and 4 by subculture isolation. Stranded animals included multiple species and both blood and tissue samples, whereas captive and free-ranging animals included only bottlenose dolphin blood samples, precluding statistical comparisons among all three groups. *Bartonella* spp. were more commonly detected by all methods combined from captive (6/14, 43%, 95% CI = 18–71%) than from free-ranging (2/71, 2.8%, 95% CI = 0.3–9.8%, both positives from North Carolina) bottlenose dolphins. Prevalence of detection from blood was significantly higher for captive versus free-ranging bottlenose dolphins by combined laboratory methods ($p = 0.0002$) and by only direct extraction PCR ($p = 0.0022$). *Bartonella* spp. were commonly detected from stranded cetaceans (9/21, 43%, 3/11 striped dolphins, 3/4 harbor porpoises, 2/3 Risso's dolphins and 1/1 pygmy sperm whale, 0/1 dwarf sperm whale, 0/1 bottlenose dolphin).

Real-time PCR and sequencing of the 16S-23S ITS region identified a *Bartonella* sp. most similar to *B. henselae* San Antonio 2 (SA2) in eight cases (4 bottlenose dolphins, 2 striped dolphins, and 2 harbor porpoises), *B. henselae* Houston 1 (H1) in 2 Risso's dolphin cases and 1 harbor porpoise case, and unidentified (unable to sequence) in 6 cases (4 bottlenose dolphins, 1 striped dolphin, and 1 pygmy sperm whale). The 16S-23S ITS region of the *B. henselae* SA2-like amplicons were 100% identical (679/679 bp) with *B. henselae* SA2 (Genbank accession no. AF369529) except for those from the 2 harbor porpoises which were 99.7% (675/677) and 99.8% (676/677) similar [30]. The 16S-23S ITS region of the *B. henselae* H1-like amplicons were likewise 100% identical (648/648 bp) with *B. henselae* H1 (Genbank accession no. BX897699), except for that from Risso's dolphin BRF 030 which was 99.5% similar (645/648 bp, Genbank accession No. FJ010195). When successfully sequenced, *Pap31* gene sequences (identical between H1 and SA2 strains) provided further confirmation of *B. henselae* detection from two bottlenose dolphins (subculture isolation) and two harbor porpoises (direct extraction PCR or pre-enrichment PCR detection), with 100% (544/544 bp) identity to sequence from *B. henselae* SA-2 phage 60457 (AF308168, DQ529248). Follow-up bartonellosis testing on the first captive bottlenose dolphin case was positive by PCR and subculture isolation 83 days after the initial sampling, and PCR-negative 20 months later.

## 4. DISCUSSION

In recent years, emerging and re-emerging infectious diseases with epizootic or zoonotic potential have been described in marine mammals [12, 16, 34]. These threats include morbilliviruses, brucellosis, toxoplasmosis, sarcocystosis, papillomavirus, and West Nile virus, some of which may be linked to anthropogenic factors [12, 19]. Like these diseases, bartonellosis may be a contributor to pathologies observed in marine mammals.

Difficulties associated with *Bartonella* spp. detection and isolation have compromised efforts to define their role in disease causation. Enhanced isolation efficiency through the use of an optimized medium such as BAPGM, aids in the evaluation of diagnostic assays and advances the understanding of the diversity, adaptation, and epidemiology of this genus [25, 29]. The combined approach employed here (direct extraction PCR, pre-enrichment PCR, and subculture isolation) has previously been shown to enhance the detection and isolation of *Bartonella* spp. from dog blood samples [13]. Results of the three methods do

not always coincide. Positive direct extraction PCR with negative pre-enrichment PCR and subculture suggests insufficient viable bacteria to grow in BAPGM. Negative direct extraction PCR with positive pre-enrichment PCR or subculture implies a low bacteremia. Positive subculture in the absence of positive pre-enrichment PCR may be explained by the larger volume of inoculated BAPGM subsampled. Based upon the recent use of BAPGM in our laboratory, it is the opinion of the authors that chronic infection with *Bartonella* spp. can contribute to very subtle clinical abnormalities or vague symptoms in companion and wild animals or in human patients. In 1999, angiomatosis, which is an important pathological manifestation of *Bartonella* infection in humans [17, 23, 31, 37], was newly described in bottlenose dolphins [34]. In addition, the involvement of *Bartonella* spp. in the development of neurologic disorders in animals and people [1,22,33], suggests that the association of this genus with stranding events should be investigated in the future. Of note in this regard is the encephalitis of unknown origin identified histologically in four of the stranded animals in the current study. Although current evidence suggests that *Bartonella* infection in the vasculature of reservoir hosts is a highly adaptive process that is generally not accompanied by pathology, severe stress, malnutrition, increased exposure to toxins and concurrent infection with other organisms may allow *Bartonella* spp. to become pathogenic.

This study was initiated by a request to use molecular diagnostic testing to evaluate blood from a single captive bottlenose dolphin experiencing a debility of unknown origin. Following detection and isolation of a *B. henselae* SA2-like strain from that animal, additional samples from that collection and from stranded and free-ranging wild dolphins and porpoises ensued. Detection of *Bartonella* spp. from five species of dolphins and porpoises under a range of circumstances (free-ranging, stranded, and captive), suggests that infection of odontocetes may occur commonly. Detection of *Bartonella* spp. DNA by PCR does not necessarily indicate an active infection, because the organisms detected may not be viable. Subculture isolation, however, verifies the presence of viable *Bartonella* spp. organisms and an active infection. The four subculture isolations reported here are the first from marine mammals.

This study demonstrated a higher prevalence of *Bartonella* spp. from bottlenose dolphins in the affected captive facility than from free-ranging wild bottlenose dolphins. The magnitude of differences in prevalence between groups and the common detection of *Bartonella* spp. in stranded cetaceans are compelling and suggest circumstances in which *Bartonella* spp. infection may become evident, and possibly pathogenic, in marine mammals: cohabitation with a *Bartonella*-positive animal (captive situation) and severe debility leading to or associated with stranding. Although not all free-ranging wild dolphins sampled were considered completely disease-free (12/20 of the free-ranging dolphins from near Charleston, South Carolina, were classified as unhealthy or possibly unhealthy [32], while the two *Bartonella*-positive dolphins from near Beaufort, North Carolina were considered healthy) they were considered healthier than the stranded near-dead animals. They also may not have experienced close contact with *Bartonella*-positive animals comparable to the captive animals.

In terrestrial animals, proven competent vectors of bartonellosis include lice, sand flies and fleas [2]. Interestingly, a cyamid amphipod ectoparasite, *Isocyamus delphinii*, from a healing skin wound of Risso's dolphin BRF 059 carried the same *B. henselae* H1-like strain as its host (unpublished data). Proving vector competence requires experimental demonstration of reliable transmission between the vector and the host, however, so while intriguing, this finding does not demonstrate a mode of transmission. *Bartonella* spp. can also be transmitted by animal bites and scratches, and *Bartonella* spp. DNA has been detected in dog saliva [14]. Raking (biting), such as occurs in conspecific and interspecific aggression in odontocetes, is therefore a possible mode of transmission. Transmission of *B. henselae* by cat bites and scratches, however, is thought most likely to occur as a

result of innoculating contaminated flea feces into the wound [15]. A marine analogue of this mechanism of transmission is not readily apparent.

Detection of *B. henselae*-like organisms in marine mammals may be of potential zoonotic concern for marine mammal handlers and pathologists. It is, however, but one of several pathogens of potential zoonotic concern found in marine mammals [10], and there is currently no indication that unusual precautions are warranted.

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