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Novel *Bartonella* infection in northern and southern sea otters (*Enhydra lutris kenyoni* and *Enhydra lutris nereis*)



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ARTICLE INFO

Article history:

Received 9 October 2013

Received in revised form 11 February 2014

Accepted 14 February 2014

Keywords:

Bartonella

Streptococcus bovis/equinus

Vegetative valvular endocarditis

Northern sea otter

Southern sea otter

Enhydra lutris

ABSTRACT

Since 2002, vegetative valvular endocarditis (VVE), septicemia and meningoencephalitis have contributed to an Unusual Mortality Event (UME) of northern sea otters in southcentral Alaska. Streptococcal organisms were commonly isolated from vegetative lesions and organs from these sea otters. *Bartonella* infection has also been associated with bacteremia and VVE in terrestrial mammals, but little is known regarding its pathogenic significance in marine mammals. Our study evaluated whether *Streptococcus bovis/equinus* (SB/E) and *Bartonella* infections were associated with UME-related disease characterized by VVE and septicemia in Alaskan sea otter carcasses recovered 2004–2008. These bacteria were also evaluated in southern sea otters in California. *Streptococcus bovis/equinus* were cultured from 45% (23/51) of northern sea otter heart valves, and biochemical testing and sequencing identified these isolates as *Streptococcus infantarius* subsp. *coli*. One-third of sea otter hearts were co-infected with *Bartonella* spp. Our analysis demonstrated that SB/E was strongly associated with UME-related disease in northern sea otters ($P < 0.001$). While *Bartonella* infection was also detected in 45% (23/51) and 10% (3/30) of heart valves of northern and southern sea otters examined, respectively, it was not associated with disease. Phylogenetic analysis of the *Bartonella* ITS region allowed detection of two *Bartonella* species, one novel species closely related to *Bartonella* spp. JM-1, *B. washoensis* and Candidatus *B. volans* and another molecularly identical to *B. henselae*. Our findings help to elucidate the role of pathogens in northern sea otter mortalities during this UME and suggested that *Bartonella* spp. is common in sea otters from Alaska and California.

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1. Introduction

Ranging from the Aleutian Islands southward through central California, sea otters (*Enhydra lutris*) live offshore throughout much of the Pacific coastal waters of the United States (U.S.) (USFWS, 2008). Northern sea otter populations (*Enhydra lutris kenyoni*) have declined up to 50% in the Southwest stock of Alaska since the mid-1980s and as much as 90% in the Aleutian Archipelago throughout the 1990s (Doroff et al., 2003; USFWS, 2008), prompting the U.S. Fish and Wildlife to list the Southwest stock as Threatened under the U.S. Endangered Species Act in 2005. The Threatened southern sea otter population (*Enhydra lutris nereis*) in California also declined during the mid-1970s and mid/late-1990s, and population recovery continues to falter despite more than 100 years of legal protection (USGS, 2010).

The reasons for these sea otter population declines are likely multi-factorial. While disease from infectious pathogens has been proposed as a cause for reduced growth and survival of southern sea otters (Kreuder et al., 2003), infectious diseases impacting the health and survival of northern sea otters are less well characterized. One reason is the difficulty of recovering carcasses from remote areas with rocky coastlines and harsh weather in Alaska. Some areas, such as the shorelines of Kachemak Bay in southcentral Alaska, are more accessible and allow monitoring for beach-cast sea otter carcasses, as is possible in the sea otter range along parts of the California coast. Although Kachemak Bay is occupied by a stable population of sea otters from the Southcentral stock, there has been a significant increase in the number of sea otter carcasses being recovered from Kachemak Bay since 2002, resulting in an Unusual Mortality Event (UME) being declared by the U.S. Working Group on Marine Mammal Unusual Mortality Events in 2006 (Gill, 2006).

Post-mortem examinations of Kachemak Bay sea otters between 2002 and 2006 indicated that 43% (63/147) of the carcasses had vegetative valvular endocarditis (VVE) as the predominant lesion (Gill, 2006), often accompanied by septicemia. The severity of VVE was unprecedented, based on prior records for which VVE was a sporadic finding in northern and southern sea otters (Joseph et al., 1990; Gill, 2006). Preliminary examinations identified *S. bovis/equinus* (SB/E) from many of the lesions in northern sea otters (Gill, 2006). Because many bacterial pathogens can cause endocarditis, and group D *Streptococcus* spp. (to which SB/E belongs) is estimated to be the causative agent in 10–15% of human cases with infective endocarditis (Gupta et al., 2010), we investigated whether this and other emerging marine mammal pathogens, such as *Bartonella* spp. and *Brucella* spp., known to cause valvular endocarditis in animals and humans (Chomel et al., 2009b), were present in the northern sea otter carcasses associated with the UME.

Bartonella spp. are facultative intracellular Gram-negative bacteria that cause hemotropic infections in mammals and are transmitted by blood-sucking arthropods. Of the 26 species that have been identified, at least 13 are known or suspected to be zoonotic pathogens (Chomel et al., 2009a). *Bartonella* spp. are commonly implicated as a

primary cause of multiple diseases, including chronic bacteremia and lymphadenopathy, bacillary angiomatosis–peliosis and VVE in humans and animals (Breitschwerdt et al., 2010). However, little is known about its pathogenic significance in marine animals. *Bartonella* spp. were recently detected by culture and PCR in loggerhead sea turtles (*Caretta caretta*), harbor seals (*Phoca vitulina*) and river otters (*Lontra canadensis*) (Morick et al., 2009; Chinnadurai et al., 2010). In addition, *Bartonella* spp. were detected by culture and PCR from blood and organs from sick cetaceans with post-mortem findings, suggesting that *Bartonella* infection may have been associated with the deaths of these animals (Maggi et al., 2005; Harms et al., 2008).

Since *Brucella* spp. are also associated with lesions in tissues, including heart valves in marine mammals (Gonzalez-Barrientos et al., 2010), we also assessed whether *Brucella* DNA was present in heart valves of northern and southern sea otters. However, the primary aim of this study was to examine any potential association between *Bartonella* spp. and SB/E infection with UME-related disease of northern sea otters, characterized by valvular endocarditis and septicemia.

2. Materials and methods

2.1. Study population and sample collection

Between 2004 and 2008, 51 heart valve and 37 spleen samples were collected from 51 northern sea otter (*Enhydra lutris kenyoni*) carcasses that were recovered on beaches from southcentral Alaska. Spleens were collected because *Bartonella* spp. can infect this organ in accidental or immune-compromised hosts after intra-erythrocytic bacteremia (Resto-Ruiz et al., 2003). Whole heart blood ($n = 9$) samples were additionally collected from a subset of animals. Northern sea otters that died with VVE or had evidence of VVE and septicemia as primary contributing factors were categorized in this study as having UME-related disease ($n = 33$), whereas otters that died from other causes were categorized as “comparisons” ($n = 18$). As a second comparison group, we analyzed 30 heart valves and 18 spleens from 30 southern sea otters (*Enhydra lutris nereis*) from central California that died with VVE, degenerative cardiomyopathy and other causes between 2007 and 2008. Post-mortem examinations were performed by veterinary pathologists or veterinarians and biologists with marine mammal necropsy experience at the U.S. Fish and Wildlife Service Marine Mammals Management, Anchorage Alaska or the Marine Wildlife Veterinary Care and Research Center, California Department of Fish and Wildlife (CDFW), Santa Cruz, CA, USA. Tissue samples were shipped on dry ice overnight and stored in sterile plastic vials or polyethylene bags at $-80\text{ }^{\circ}\text{C}$ prior to bacteriologic and molecular examination.

The age of each sea otter was determined at the time of necropsy based on tooth wear and dental measurements of premolar teeth (Von Biela et al., 2008). Age was categorized as immature (≤ 3 years) or mature (> 3 years). Body condition was determined by the amount of observed subcutaneous fat and palpation of ribs, backbone and other

bony protuberances (Doroff and Mulcahy, 1997); and categorized as (1) poor if thin or emaciated with scant subcutaneous fat/evidence of muscle loss or (2) good if moderate to abundant subcutaneous fat was present.

2.2. *Streptococcus bovis/equinus* complex culture and molecular characterization

Aerobic bacterial culture was performed on heart valves from northern ($n = 51$) and southern ($n = 30$) sea otters in order to detect SB/E organisms. Fragments of aortic and right or left atrio-ventricular valves were transported to the Microbiology Laboratory, Veterinary Medical Teaching Hospital, University of California, Davis (UC Davis), CA, USA. Samples were inoculated onto non-selective Columbia agar with 5% sheep blood (Hardy Diagnostics, Santa Maria, CA, USA). The plates were incubated at 35 °C in a 5% CO₂-enriched atmosphere for a minimum of five to seven days. Bacterial identification was accomplished using standard microbiological algorithms including Gram staining, tubed media and strip identification (Ruoff, 2003).

Following culture of SB/E organisms, small, alpha-hemolytic colonies of catalase negative, Gram-positive cocci were further screened for a positive reaction on bile esculin agar and failure to grow in broth containing 6.5% NaCl. Lancefield serotyping was performed using a Streptex kit (Remel Inc. Lenexa, KS, USA) by latex bead antibody agglutination. Further biochemical characterization was performed using an API 20 (analytical profile index; Biomerieux Inc., Hazelwood, MO, USA). Definitive characterization of the SB/E organisms was performed at the Centers for Disease Control and Prevention (CDC) using conventional biochemical testing, including the rapid ID32 STREP identification system (BioMerieux Inc.) and 16S gene sequencing as previously described (Carvalho Mda et al., 2004).

PCR analysis was performed on a subset of SB/E complex isolates from northern sea otters ($n = 9$) at UC Davis. The 16S rRNA gene of SB/E DNA was amplified using the Sp3F1 (forward 5'-CTTCCCAATCATCTATCC-3') and Sp3R1 (reverse 5'-GTGGGGAGCAAACAGGATTA-3'), as previously described (Whitehead and Cotta, 2000). Amplified PCR products (940 bp) were resolved by 2% agarose gel electrophoresis, purified with the QIAquick PCR purification kit (Qiagen Inc, Valencia, CA, USA) and submitted for direct sequencing of both DNA strands using a fluorescence-based automated sequencing system (^UC DNA Sequencing, Davis, CA, USA). Sequences from both strands were compared with nucleic acid sequence entries in the GenBank nucleotide database using Basic Local Alignment Search Tool (BLAST).

2.3. *Bartonella* spp. culture and PCR procedures

Cryopreserved heart blood samples collected from stranded northern sea otters ($n = 9$) were used in culture attempts for *Bartonella* spp. at UC Davis (Dr. Chomel's laboratory). Samples were cultured on heart infusion agar containing 5% rabbit blood and incubated in 5% CO₂ at 35 °C for up to 4 weeks.

DNA was extracted from approximately 25 mg pieces of aortic, right or left atrio-ventricular heart valves ($n = 81$ sea otters) and 10 mg of spleen ($n = 55$ sea otters) using the DNeasy Blood & Tissue Kit (Qiagen Inc). Genomic DNA was stored at -20 °C until analysis for the presence of *Bartonella* spp. DNA using conventional PCR targeting the 16S–23S rRNA intergenic region (ITS). PCR targeting the heme-binding protein gene (*Pap31*) and RNA polymerase beta-subunit gene (*rpoB*) of *Bartonella* spp. was also performed from DNA extracted from all heart valves. The ITS region was amplified using the ITS-P21 and P22 (Rolain et al., 2003) and ITS-F2 and R2, as forward (5'-CCCAGGCC-CACCAATAAC-3') and reverse (5'-CTGAGCTACGGCCCC-TAATAC-3') primers, respectively. Amplified PCR products were evaluated using 2% agarose gels. Expected ITS amplicons obtained from ITS P21/P22 primers and ITS species-specific set of primers (F2/R2) were approximately 585 bp and 238 bp fragments, respectively. Extracted DNA from heart valves was also submitted on dry ice to the Intracellular Pathogens Research Laboratory, College of Veterinary Medicine, North Carolina State University (NCSU-IPRL) for additional conventional PCR analysis and cloning. *Bartonella* ITS amplicons using two sets of primers, ITS-321/ITS-1000 and ITS-438/ITS-1100, were nearly 560 bp and 364 bp, respectively, at the NCSU-IPRL laboratory (Maggi et al., 2008). PCR amplification of the *Bartonella Pap31* gene and *rpoB* gene from heart valve samples ($n = 81$) was performed using *Pap31*-P50 (forward) and P51 (reverse) primers and *rpoB*-P19 (forward) and P20 (reverse) primers, respectively (Renesto et al., 2001; Maggi et al., 2005). Amplified PCR products were analyzed by electrophoresis on 2% agarose gels, yielding DNA fragments that ranged from 445 bp to 498 bp for *Pap31* and 661 bp for *rpoB*. Amplification of *Bartonella* DNA using *Pap31* conventional PCR was also performed at the NCSU-IPRL laboratory to confirm the UC Davis PCR findings (Maggi et al., 2005).

Amplified PCR products from the ITS region and *Pap31* and *rpoB* genes that were generated at UC Davis were sequenced by ^UC DNA Sequencing (Davis, CA, USA). Amplicons obtained at the NCSU-IPRL laboratory were cloned into the Plasmid pGEM-T Easy Vector System according to the manufacturer's instructions (Promega, Madison, WI, USA). Plasmid DNA inserts were sequenced by Davis Sequencing, Inc. (Davis, CA, USA). Sea otter *Bartonella* sequences were compared with *Bartonella* spp. DNA sequences in the GenBank nucleotide database using BLAST. Consensus sea otter *Bartonella* sequences for each separate gene were then aligned with known strains of *Bartonella* species, as well as with closely related *Bartonella* sequences using CLUSTALW program in MEGA version 5.1. Phylogenetic trees for consensus ITS regions ranging between 160 bp and 220 bp were constructed using the neighbor-joining method with the Kimura two-parameter model of nucleotide substitution in MEGA. A neighbor-joining tree was also constructed based on analysis of concatenated sequences for two genes (ITS region and *rpoB*) with the Kimura two-parameter model in MEGA. Consensus fragments ranging between 160 bp and 220 bp for ITS region and 652 bp and 675 bp for *rpoB* were used in this concatenated analysis. Bootstrapping was used to

estimate the reliability of phylogenetic reconstructions, with values obtained from 1000 randomly selected samples of the aligned sequences. Alignments were formatted using BOXSHADE version 3.21.

2.4. *Brucella* spp. PCR procedures

Extracted DNA from all heart valves ($n = 81$) was also tested via conventional PCR to detect the presence of *Brucella* spp. DNA, as it is also known to be associated with endocarditis in other species (Gonzalez-Barrimentos et al., 2010). Amplification of *Brucella* DNA was attempted using primers that target the 16S rRNA gene from *Brucella* spp. (Herman and De Ridder, 1992).

2.5. Statistical analysis

Chi-square analyses were used to evaluate associations between *Bartonella* and SB/E infection and age, sex, body condition and cause of death in northern sea otters (SPSSv11[®] statistical software, SPSS Inc., Chicago, IL, USA). Cause of death was divided in two categories: northern sea otters that died with UME-related disease and northern sea otters that died due to other causes. A P -value < 0.05 was considered statistically significant. When indicated, odds ratios and 95% confidence intervals were calculated to determine the strengths of associations.

3. Results

3.1. Bacteriology and *Streptococcus bovis/equinus* complex identification

Fifty-one bacterial isolates were obtained from heart valves collected from 37 of the 51 northern sea otters (Table 1). Several microorganisms grew on non-selective media when SB/E culture of heart valves of northern sea otters was performed; these organisms were identified at the bacterial order and family level (Table 1). SB/E organisms were cultured from 23 (45%; 23/51; Table 1) of the northern sea otter heart valves and were commonly cultured from the valves affected by UME-related disease (66%; 22/33). SB/E was less frequently isolated from heart valves of northern sea otters that had died from other causes (6%; 1/18; $P < 0.001$). Nine of these SB/E isolates

Table 1

Bacteria cultured from heart valves of northern sea otters ($n = 51$). Culture results were categorized by primary cause of death.

Bacteria	UME-related disease ($n = 33$)	Trauma ($n = 12$)	Other causes ($n = 6$)
<i>S. bovis/equinus</i>	22		1
<i>S. phocae</i> group F	4		2
Coagulase-positive staphylococci			1
<i>Enterococcus</i> spp.	1	1	
<i>Escherichia coli</i>	7		2
Hemolytic <i>E. coli</i>	1		1
<i>Psychrobacter phenylpyruvica</i>	2	1	1
<i>Lactococcus</i> spp.	1		
<i>Leuconostoc</i> spp.		1	
<i>Bacillus</i> spp.	1		
<i>Hafnia alvei</i>	1		

were further speciated by PCR and sequence analysis yielded 99% identity to *Streptococcus infantarius* strain 908 (GenBank accession number EU163504). Further sequencing and biochemical analyses of these isolates by the CDC identified them as *Streptococcus infantarius* subsp. *coli*.

Of the 33 northern sea otters that died with UME-related disease, 11 (33%) were culture-negative for SB/E organisms. *Streptococcus phocae* was cultured from three of these 11 northern sea otters. *Escherichia coli* was also isolated from three of these 11 northern sea otters with UME-related disease that did not have SB/E organisms. Four northern sea otters with UME-related disease were culture negative, and one sea otter heart valve had mixed bacterial growth.

Streptococcus bovis/equinus organisms were not cultured from the heart valves of southern sea otters ($n = 30$). Instead, 23 different bacteria were cultured, including *Psychrobacter* spp. ($n = 6$), *S. phocae* group F ($n = 4$), *Corynebacterium* spp. ($n = 3$), *Lactococcus* spp. ($n = 3$), *Enterococcus* spp. ($n = 2$), Beta-hemolytic streptococci ($n = 2$), *S. phocae* group G ($n = 1$), *S. viridians* ($n = 1$) and coagulase-positive staphylococci ($n = 1$). Of the 30 southern sea otters, five had either cardiomyopathy ($n = 4$) or septicemia and VVE ($n = 1$) as the primary cause of death. Of these five, one southern sea otter dying with cardiomyopathy was infected with *S. phocae* group G, and the one with septicemia and VVE tested positive to *S. phocae* group F.

3.2. *Bartonella* spp. and *Brucella* spp. culture and molecular detection

Bartonella spp. DNA was amplified from 23 (45%; 23/51) northern and three (10%; 3/30) southern sea otter heart valves. *Bartonella* spp. DNA was detected in 14 northern sea otters that died with UME-related disease and nine that died from other causes (Table 2). Amplified PCR products from heart valves were obtained from the 16S–23S ITS region ($n = 23$) and the *Pap31* ($n = 7$) and *rpoB* ($n = 2$) genes. Detection of *Bartonella* spp. DNA from heart valves was more successful using the ITS primers (both from UC Davis and NCSU) than the *Pap31* or *rpoB* primers (Table 3). We were not able to amplify *Bartonella* spp. DNA from heart valves of southern sea otters when targeting *Pap31* and *rpoB* genes. No *Bartonella* spp. DNA was detected using PCR amplification of the ITS region or the *Pap31* gene in spleen samples from northern or southern sea otters. *Brucella* spp. DNA was not amplified from any of the heart valve

Table 2

Cause of death of northern sea otters categorized by SB/E and *Bartonella* infections. Fifty-one northern sea otters were evaluated. Eleven individuals that died with UME-related disease were co-infected with both SB/E organisms (culture) and *Bartonella* spp. (PCR).

Bacteria	UME-related disease ($n = 33$)	Trauma ($n = 12$)	Other causes ($n = 6$)	Total ($n = 51$)
SB/E organisms	22	0	1	23
<i>Bartonella</i> spp.	14	7	2	23
Negative for both organisms	8	5	4	17

Table 3

Bartonella spp.-infected individuals by location of stranding, sequence identity and cause of death. *Bartonella* spp. DNA detected from heart valves of northern and southern sea otters by PCR targeting the 16S–23S ITS, *Pap31* and *rpoB* genes.

ID#	Host	Location of stranding (area, state ^b)	Primer type	Amplicon size (bp)	<i>Bartonella</i> DNA maximum % nucleotide identity (Accession number)	Cause of death
1	Northern sea otter	Homer, AK	ITS	421	100% <i>B. henselae</i> H-1 (BX897699)	Trauma
2	Northern sea otter	Homer, AK	ITS	423	100% <i>B. henselae</i> H-1 (BX897699)	Emaciation/starvation
3	Northern sea otter	Homer, AK	ITS	431	100% <i>B. henselae</i> H-1 (BX897699)	Gun shot
4	Northern sea otter	Homer, AK	ITS	577	98% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
5	Northern sea otter	Homer, AK	<i>Pap31</i>	497	98% <i>Bartonella</i> spp. JM-1 (JX896166)	VVE
			<i>rpoB</i>	661	98% <i>Bartonella</i> spp. JM-1 (AB611855)	
			ITS	551	98% <i>Bartonella</i> spp. JM-1 (AB674236)	
			<i>Pap31</i>	493	98% <i>Bartonella</i> spp. JM-1 (JX896166)	
6	Northern sea otter	Kodiak Island, AK	ITS	558	97% <i>Bartonella</i> spp. JM-1 (AB674236)	Boat strike-presumptive
			ITS	558	98% <i>Bartonella</i> spp. JM-1 (AB674236)	Trauma
7	Northern sea otter	Homer, AK	ITS	312	97% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
8	Northern sea otter	Homer, AK	ITS	313	97% <i>Bartonella</i> spp. JM-1 (AB674236)	Trauma
9	Northern sea otter	Homer, AK	ITS	313	98% <i>Bartonella</i> spp. JM-1 (AB674236)	Septicemia-presumptive ^a
10	Northern sea otter	Ninilchik, AK	ITS	313	97% <i>Bartonella</i> spp. JM-1 (AB674236)	Vascular congestion
11	Northern sea otter	Homer, AK	ITS	313	98% <i>Bartonella</i> spp. JM-1 (JX896166)	Trauma
12	Northern sea otter	Homer, AK	<i>Pap31</i>	475	97% <i>Bartonella</i> spp. JM-1 (AB674236)	Trauma
			ITS	313	98% <i>Bartonella</i> spp. JM-1 (JX896166)	
13	Northern sea otter	Homer, AK	ITS	207	96% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
14	Northern sea otter	Kodiak Island, AK	ITS	416	100% <i>Bartonella</i> spp. JM-1 (AB674236)	Boat strike
15	Northern sea otter	Homer, AK	ITS	416	100% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
16	Northern sea otter	Kamishak Bay, AK	ITS	285	97% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
17	Northern sea otter	Homer, AK	ITS	285	98% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
18	Northern sea otter	Homer, AK	ITS	313	97% <i>Bartonella</i> spp. JM-1 (AB674236)	VVE
19	Southern sea otter	Monterey Harbor, CA	ITS	208	96% <i>Bartonella</i> spp. JM-1 (AB674236)	Acanthocephalan peritonitis
20	Southern sea otter	Moss Landing, CA	ITS	416	100% <i>Bartonella</i> spp. JM-1 (AB674236)	Protozoal meningoencephalitis
21	Northern sea otter	Seldovia, AK	ITS	485	84% <i>Bartonella bacilliformis</i> (CP000524)	VVE
22	Northern sea otter	Homer, AK	ITS	443	84% <i>Bartonella bacilliformis</i> (CP000524)	VVE
23	Southern sea otter	North Monterey Bay, CA	ITS	426	84% <i>Bartonella bacilliformis</i> (CP000524)	Cardiomyopathy
24	Northern sea otter	Homer, AK	<i>Pap31</i>	302	83% <i>Bartonella</i> spp. JM-1 (JX896166)	Bacterial meningoencephalitis ^a
25	Northern sea otter	Homer, AK	<i>Pap31</i>	450	98% <i>Bartonella</i> spp. JM-1 (JX896166)	Splenic mass ^a
26	Northern sea otter	Kamishak Bay, AK	<i>Pap31</i>	445	98% <i>Bartonella</i> spp. JM-1 (JX896166)	Bacterial meningoencephalitis ^a

^a These northern sea otters were categorized as UME-related disease for our statistical analysis since VVE was a contributing factor to death.

^b Stranding locations listed by area and state.

samples. Culture of *Bartonella* spp. was not successful from heart whole blood ($n = 9$) from northern sea otters.

3.2.1. PCR amplification of 16S–23S ITS region

Bartonella spp. DNA was detected in 20 northern sea otter and three southern sea otter heart valves using the ITS primers (Table 3). Phylogenetic analysis of three ITS sequences (Table 3, ID# 1–3; northern sea otters group C in Fig. 1) revealed 100% identity to *B. henselae* strain Houston I. These three ITS sequences also shared 99% identity with *Bartonella* DNA detected in blood from a Risso's dolphin (*Grampus griseus*; GenBank accession number FJ010195), and 93% identity with *B. henselae* DNA detected in blood from a harbor porpoise (*Phocoena phocoena*; GenBank accession number DQ529247) (Maggi et al., 2005; Harms et al., 2008). The amplicon size and maximum percentage of DNA sequence identity to *Bartonella* spp. from each sea otter sequence is summarized in Table 3.

For 15 of the 20 northern sea otters (Table 3, ID# 4–18; represented by group A in Fig. 1) and two of three southern sea otters (Table 3, ID# 19–20; represented by group B in Fig. 1), ITS sequences were closely related to *B. washoensis* and a proposed new species, *Candidatus B. volans*. A BLAST

search of individual sequences derived from 15 northern and two southern sea otters showed sequence identities ranging from 96% to 100% (Table 3, ID# 4–20; GenBank accession number AB674236) with *Bartonella* spp. JM-1 isolated from blood from a Japanese marten (*Martes melampus*) (Sato et al., 2012). Sequences from two northern sea otters and one southern sea otter (Table 3, ID# 21–23) shared 84% identity (GenBank accession number CP000524) with *B. bacilliformis* KC583.

Alignments of representative northern and southern sea otter (ID# 4 and 19 in Table 3) ITS sequences with four closely-related *Bartonella* ITS sequences from a Japanese marten, North American river otters (*Lontra canadensis*), and Southern flying squirrels (*Glaucomys volans*) (GenBank accession numbers AB674236, GQ856648, EU294521 and AB674247, respectively) showed 94% (288/306 bp) identity between the northern (ID# 4) and southern (ID# 19) sea otter sequences (Fig. 1S, Additional file 1). The northern and southern sea otter sequences (ID# 4 and #9) shared 98% (299/306 bp) and 92% (283/306 bp) identities with the ITS sequence derived from *Bartonella* spp. JM-1 and 90% (282/314 bp) and 85% (266/314 bp) identities with the river sea otter sequence, respectively. Alignments of the

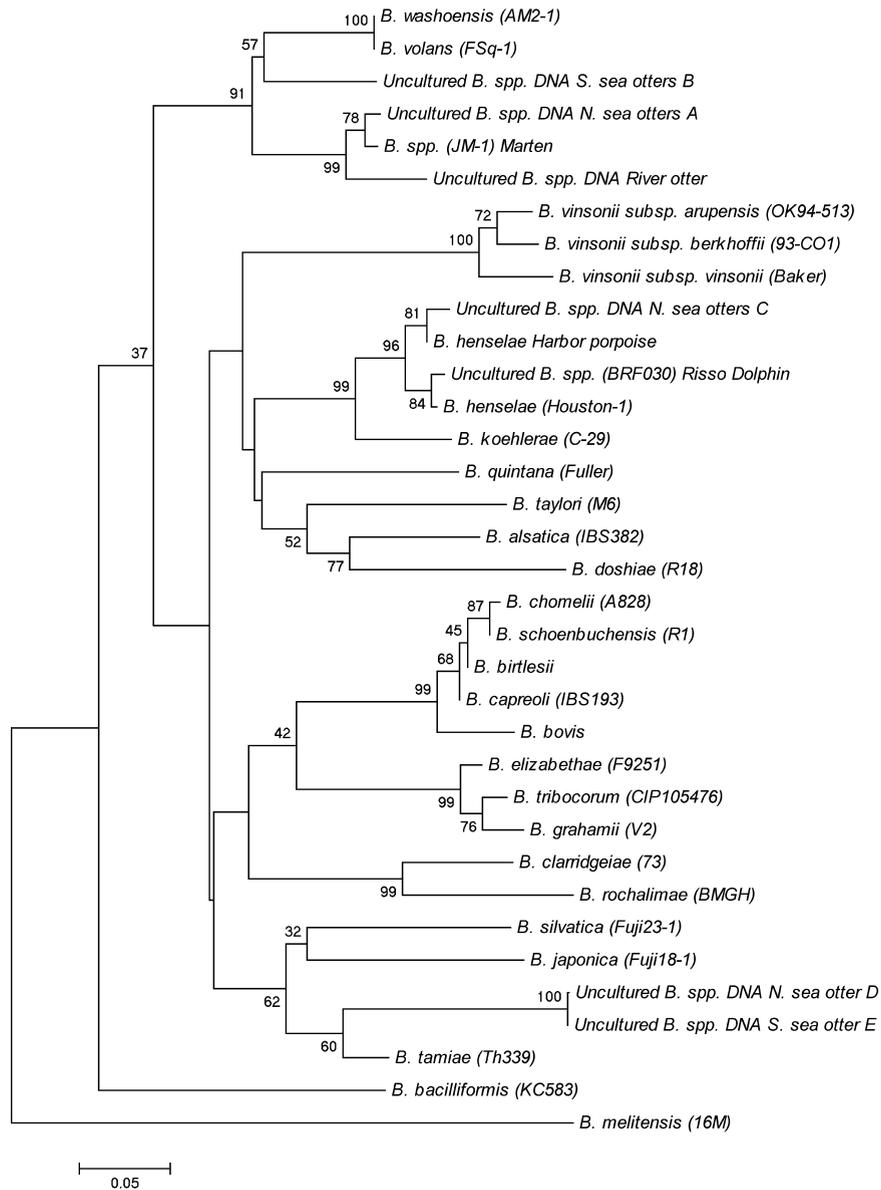


Fig. 1. Phylogenetic tree of a consensus fragment from the ITS region derived from *Bartonella* spp. Comparison of 16S–23S rRNA ITS region relationships from cultured and uncultured *Bartonella* species using the Neighbor-Joining method based on the Kimura two-parameter model of nucleotide substitution. A consensus fragment ranging between 160 bp and 220 bp was used in the ITS alignment to generate this phylogenetic tree. Eighteen northern sea otter sequences clustered into group A. Two southern sea otter sequences clustered into group B. Three northern sea otter sequences clustered into group C and shared 99–100% identity to *B. henselae* strains. Two northern sea otter and one southern sea otter clustered into group D and E, respectively. The tree was rooted by using *Brucella melitensis* strain 16M as the out-group. Bootstrap values are shown next to the tree nodes and were based on 1000 replicates. The bar indicates 0.05 estimated nucleotide substitutions per site.

northern and southern sea otter sequences shared 88% (269/307 bp) and 83% (254/307 bp) identities with candidate *B. volans* and *B. washoensis* ITS sequences derived from squirrels.

3.2.2. PCR amplification of *Pap31* gene

Using *Pap31* primers, *Bartonella* spp. DNA was amplified from seven northern sea otter heart valves. *Bartonella* spp. DNA was amplified using *Pap31* primers only in three

northern sea otter samples (Table 3, ID# 24–26). The other four northern sea otters were positive by PCR using both *Pap31* and ITS primers. *Pap31* sequences obtained from these sea otters shared 98% identities with *Bartonella* spp. JM-1 (GenBank accession number: JX896166) and 85% identities with *B. henselae* (GenBank accession number: DQ529248), which were amplified and sequenced from the blood of a Japanese marten and a harbor porpoise, respectively (Maggi et al., 2005; Sato et al., 2012).

3.2.3. PCR amplification of *rpoB* gene

Bartonella rpoB was amplified from only two northern sea otter heart valves (Table 3, ID# 4 and 5). The presence of *Bartonella* spp. DNA in these samples was confirmed using ITS primers, as reported above. Sequence analyses of the *rpoB* gene shared 98% identity with *Bartonella* spp. JM-1 and 95% identity with *B. washoensis* Sb1865nv detected in blood of a Japanese marten and a California ground squirrel (*Otospermophilus beecheyi*), respectively (GenBank accession numbers AB611855 and AB674242) (Sato et al., 2012). A phylogenetic analysis based on the concatenated sequences of *rpoB* and ITS genes provided further evidence that these two sea otter sequences (Table 3, ID# 4 and 5; represented by group A in Fig. 2S, Additional file 2) were closely related to *B. washoensis*, Candidatus *B. volans* and *Bartonella* spp. JM-1.

3.3. Risk factors and geographic distribution of *Bartonella* cases

No significant associations were found between SB/E infection and age, sex or body condition. Infection of heart valves with SB/E organisms was significantly associated with UME-related disease (chi-square $P < 0.001$). Northern sea otters infected with SB/E organisms were 34 times more likely to have UME-related disease as a primary cause of death when compared with other causes of death, including trauma cases (95% CI = 3.9–289.7).

No statistically significant associations were found between *Bartonella* infection and age, sex or body condition. *Bartonella* infection was found in immature (two pups and one sub-adult) and mature (19 adults, and one aged adult; >3 years) northern sea otters. Although we found a high prevalence of *Bartonella* infection in the heart valves tested (45%; 23/51), no significant association was found between *Bartonella* infection and UME-related disease in northern sea otters (chi-square $P = 0.60$). One-third (33%; 11/33) of the UME-related disease cases were co-infected with *Bartonella* spp. and SB/E organisms. Heart valves from two northern sea otters that had died with UME-related disease (2/33) were culture-positive for *S. phocae* and PCR-positive for *Bartonella* DNA. *Bartonella* infection was also found in immature and mature southern sea otters, including one juvenile otter that had died of acanthocephalan peritonitis, one adult that died of protozoal meningoencephalitis and one aged adult that had died of cardiomyopathy. The heart valve from this last animal was co-infected with *S. phocae* group G.

Bartonella infection was commonly detected in northern sea otters that stranded in Kachemak Bay (45%; 18/40) and also in animals that stranded in areas adjacent to Kachemak Bay, including in Ninilchik (100%; 1/1), Kodiak Island (67%; 2/3) and Kamishak Bay (67%; 2/3; Fig. 3S, Additional file 3). The three southern sea otters with PCR-confirmed *Bartonella* spp. infection stranded in three different locations within Monterey Bay, California: Monterey Harbor, Moss Landing Harbor and Manresa State Beach.

4. Discussion

These results provided confirmatory evidence that *Streptococcus infantarius* subsp. *coli* infection was strongly

associated with the UME-related disease in the northern sea otters that died in southcentral Alaska, supporting previous findings showing isolation of *Streptococcus infantarius* subsp. *coli* from lesions in UME sea otter carcasses (Gill, 2006). Although *Bartonella* spp. infection was not associated with valvular endocarditis in northern sea otters, this study confirmed that *Bartonella* spp. infection was prevalent in northern sea otters in Alaska (45%, 23/51) and provided evidence of *Bartonella* spp. in southern sea otters in California (10%, 3/30) for the first time. The high degree of sequence identity between the *Bartonella volans*-like sequences found in southern and northern sea otters suggests that this bacterium may be widely distributed among this species. Because *Bartonella* spp. are capable of infecting normal intact vascular endothelium and we found a high prevalence in northern sea otter heart valves, our findings suggest that sea otter heart may be an important site for maintaining *Bartonella* infection. Co-infection of heart valves with SB/E and *Bartonella* spp. was common in sea otters dying with UME-related disease (33%, 11/33), but infection with *Bartonella* was also common in animals without this condition (67%, 12/18). It may be possible that sea otters can carry this bacterium in their heart valves asymptotically and develop signs of disease, such as valvular insufficiency from endocarditis, when co-morbid infections are present or as a consequence of chronic infection, as shown for *Coxiella burnetii* infection in humans (Fournier et al., 2010). Examination of larger samples and broader lesion categories for *Bartonella*-infected and non-infected sea otters may provide additional insight on the pathogenicity of this organism for sea otters.

Streptococcus infantarius subsp. *coli* organisms have also been isolated from multiple organs from northern sea otters (Gill, 2006; Counihan-Edgar et al., 2012); however, little is known about the source and route of entry, how this type of streptococcus invades the bloodstream or virulence factors involved in adherence to and invasion of vascular endothelial cells. It is possible that this *S. infantarius* subsp. *coli* may gain access to the bloodstream from the gastrointestinal tract because *S. infantarius* subsp. *coli* is sometimes isolated from the intestine, and closely related isolates have been detected in sea otter prey items in Alaska and California (Counihan-Edgar et al., 2012). In humans, SB/E complex is also a major cause of valvular endocarditis and is commonly associated with pre-existing neoplastic lesions in the large intestine, hepatic disease (Gupta et al., 2010), underlying cardiac disease and immunosuppression (Mylonakis and Calderwood, 2001). Once this bacterium has entered the bloodstream, it may spread to different parts of the body and invade cardiac tissues of sick sea otters. It is well accepted that streptococcal organisms adhere to heart valves with pre-existing endothelial lesions (Moreillon et al., 2002). Because of the lack of heart valve defects seen in the northern sea otter carcasses (Goldstein et al., 2009) and the marked tropism of *Bartonella* for endothelial cells, we can speculate that sea otter *Bartonella* strains may act as a predisposing factor by subverting endothelial cell functions (Dehio, 2008). This change in the endothelium may facilitate streptococcal adhesion and invasion of cells in sea

otters co-infected by both *Bartonella* and *S. infantarius* subsp. *coli*. Further in-vitro studies are needed to confirm this possibility and evaluate the effects of both bacteria on endothelial integrity and endothelial cell function.

Although our study focused primarily on the detection of *Bartonella* spp. and isolation of SB/E organisms, a number of bacteria were isolated in pure culture, or as mixed bacterial growth from northern and southern sea otter heart valves. The most interesting finding regarding these other bacteria was the co-infection with *S. phocae* and *Bartonella* in three sea otters affected by UME-related disease, illustrating the potential for co-morbid infections with *Bartonella* spp. Since *Bartonella* spp. DNA was more frequently detected in northern sea otters than in southern sea otters, a pattern that is reflected in the different prevalence of VVE in general, it could be speculated that a primary infection in cardiac tissue with *Bartonella* initiates the development of VVE, regardless of the bacterial strains isolated from fully developed lesions. It is also possible that other underlying conditions are contributing to poor general health or immunosuppression, allowing multiple species to colonize the heart valves.

Analysis of DNA sequences derived from the *Bartonella* ITS region suggests sea otters may be infected by at least two different *Bartonella* spp., similar to cats and dogs that can be infected with more than *Bartonella* spp. (Diniz et al., 2007; Breitschwerdt et al., 2010). Three of the northern sea otter *Bartonella* sequences were 100% identical to *B. henselae*, whereas 17 sequences from northern and southern sea otters were 98% identical to *Bartonella* spp. JM-1, closely related to *B. washoensis* strains and the newly proposed *B. volans*. Other ITS sequences from northern (2) and southern (1) sea otters belonged to the *Bartonella* family; however, further characterization was not possible. Because sequence analyses of *Bartonella* DNA from cetaceans were 99–100% identical to *B. henselae* strains (Harms et al., 2008), it is possible that a *B. henselae*-like organism is circulating among sea otters and other marine mammals. Similarities of the ITS, *Pap31* and *rpoB* sequences from the northern sea otters and sequences derived from Japanese marten suggest that mustelids could be natural hosts for a novel group of *Bartonella* species. It is possible that these *Bartonella* species have co-evolved with mustelids, as described with *B. washoensis* and Candidatus *B. volans* in ground and flying squirrels (Breitschwerdt et al., 2010).

Although this investigation provides molecular evidence of *Bartonella* ITS, *Pap31* and *rpoB* in some samples, a limitation of our findings was the inability to confirm ITS PCR results with a second *Bartonella* gene in all PCR-positive samples. Four of 23 samples were PCR-positive for the ITS region and *Pap31* or *rpoB*. Differences in detection of *Bartonella* genes among human and animal samples have been previously reported (Zeaiter et al., 2002; Diniz et al., 2007; Beard et al., 2011). One possibility is the lack of hemin-binding protein genes, such as *Pap31* in some *Bartonella* species. It is possible that variations in *Pap31* also exist among *Bartonella* species infecting northern sea otter heart valves, contributing to the lower detection of the *Pap31* fragment in these samples. Another possibility was the presence of endogenous inhibitors in samples that

could affect detection of *Bartonella* DNA in tissues (Duncan et al., 2007). In fact, the presence of competing bacterial DNA in greater quantities than *Bartonella* genes in sea otter heart valves may have interfered with our ability to detect *rpoB* or *Pap31*. Therefore, targeting genes from possibly co-infecting bacteria in addition to *Bartonella* genes should be considered when assessing *Bartonella* infection and co-infections in tissues from sea otter carcasses.

There is limited information regarding the vectors and mode of transmission of *Bartonella* strains among marine animals. Recent studies found evidence of *B. henselae*-like and *B. grahamii*-like organisms in spleen and lice (*Echinophthirus horridus*) from harbor seals (Morick et al., 2009). In another study, *B. henselae* Houston-1-like organism from a cyamid amphipod ectoparasite (*Isoyamus delphinii*) was identified in a Risso's dolphin (Harms et al., 2008). These studies reported 100% identity between the *Bartonella* sequences found in both the lice and amphipod. These findings may suggest that sea otters could acquire *Bartonella* spp. from invertebrates or arthropods adapted to marine ecosystems. Transmission of a *B. henselae*-like organisms among sea otters could also occur through animal bites due to aggressive territorial behavior among males and aggressive mating of males causing facial wounds to females, as described in cats (Breitschwerdt et al., 2010).

In conclusion, this study revealed that northern and southern sea otters were infected by a potentially novel genus of bacteria that has been increasingly reported among aquatic mammals and chelonians in the United States. Amplification of *Bartonella* DNA by PCR in sea otter samples has the potential to be used as a diagnostic tool for marine mammal health and veterinary professionals to accurately detect *Bartonella* infections when sick otters are admitted to marine rehabilitation centers. We confirmed that *S. infantarius* subsp. *coli* is the primary infectious agent associated with mortalities with UME-related disease in northern sea otters during 2004–2008, complementing recent health assessments of Threatened populations of northern and southern sea otters (Brownstein et al., 2011; Goldstein et al., 2011).

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

We wish to acknowledge the personnel from the US Fish and Wildlife Service (USFWS), Anchorage, Alaska; Alaska SeaLife Center, Seward, Alaska; and the Marine Wildlife Veterinary Care and Research Center, California Department of Fish and Wildlife (CDFW), Santa Cruz, California for assistance with sample collection. Carcass collection and necropsies for northern sea otters were conducted by the USFWS under MMPA permit # MA041309-1. A special thanks also to Angela Doroff for helpful information on sea otter biology, Alison Kent (WHC-UC Davis) for her assistance on mapping locations

and Spencer Jang (VMTH-UC Davis) for microbiological testing and logistics. We also gratefully acknowledge support from the CDC for the identification of *Streptococcus infantarius* subsp. *coli* isolates. This study was supported under Grant No. NA06OAR4310119 (Training Tomorrow's Ecosystem and Public Health Leaders Using Marine Mammals as Sentinels of Oceanic Change) from the National Oceanic and Atmospheric Administration (NOAA), Grant No. D08ZO-046 from Morris Animal Foundation and the Karen C. Drayer Wildlife Health Center graduate student fellowship, University of California, Davis. Sebastian Carrasco was supported by Grant No. NA06OAR4310119 and NIH, NRSA T32 AI 060519, Immunology and Infectious Disease Program at IUSM. The findings and conclusions in this article are those of the authors(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service.

Appendix A

Additional file 1 – Fig. 1S: Alignment of the *Bartonella*-ITS region from a subset of northern and southern sea otters. Alignment of representative 16S–23S rRNA ITS region sequences derived from *Bartonella* DNA that was detected in northern and southern sea otters and compared with published *Bartonella*-ITS sequences. A consensus fragment ranging between 286 bp and 314 bp was used in this alignment. Northern sea otter A = *Bartonella* DNA from northern sea otters group A represented by ITS sequence from ID# 4 (Table 3); Southern sea otter B = *Bartonella* DNA from southern sea otters group B represented by ITS sequence from ID# 19 (Table 3); River otter = *Bartonella* DNA from a North American River otter (GQ856648); *Bartonella* spp. Strain JM-1 = *Bartonella* isolated from blood of a Japanese marten (AB674236); *Bartonella volans* FSq-1 = *Bartonella* isolated from a ground squirrel (EU294521); *Bartonella washoensis* AM2-1 = *Bartonella* isolated from blood of a Southern flying squirrel (AB674247). Black indicates identical nucleotides and grey indicates mismatches. Arrows indicate the position and direction of the primers. Extensions of primers are shaded in yellow. Star symbol (*) indicates point mutations, point deletions, or base substitutions from either northern or southern sea otter sequences.

Additional file 2 – Fig. 2S: Phylogenetic relationships of *Bartonella* species based on the combined ITS region and *rpoB* sequence alignments. The phylogenetic tree was constructed using the Neighbor-Joining method based on the Kimura two-parameter model of nucleotide substitution. Consensus fragments ranging between 160 bp and 220 bp for ITS region and 652–675 bp for *rpoB* were used in the alignment to generate this phylogenetic tree. *Bartonella* spp. strains are indicated in parentheses. Two northern sea otter ITS and *rpoB* sequences clustered into group A. The tree was rooted by using *Brucella melitensis* strain 16M as the out-group. Bootstrap consensus values (percentages of 1000 replicates) with over 70% confidence are indicated at the tree nodes.

Additional file 3 – Fig. 3S: Stranding locations of northern sea otters that were tested for *Bartonella* spp. Distribution of stranding locations of northern sea otters that died and were evaluated for *Bartonella* spp. infection 2004–2008. *Bartonella* spp. DNA was detected in northern sea otters stranded in

different parts of Kachemak Bay (18/40), including Homer Spit and Seldovia, as well as in other locations, including Kodiak Island (2/3), Ninilchik (1/1) and Kamishak Bay (2/3). *Bartonella* DNA was not detected from sea otters samples collected in Whittier (0/3) and Hallo Bay (0/1).

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