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BRUCELLA PINNIPEDIALIS INFECTIONS IN PACIFIC HARBOR SEALS (*PHOCA VITULINA RICHARDSI*) FROM WASHINGTON STATE, USA

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ABSTRACT: In 1994 a novel *Brucella* sp., later named *B. pinnipedialis*, was identified in stranded harbor seals (*Phoca vitulina*). This *Brucella* sp. is a potential zoonotic pathogen and is capable of causing disease in domestic animals. Serologic, microbiologic, and pathologic data collected from live captured and stranded harbor seals were used to better describe the epizootiology of *B. pinnipedialis* in harbor seals from Washington State, USA, in 1994 through 2006. We found no sex predilection in harbor seal exposure or infection with *B. pinnipedialis* but noted a significant difference in prevalence among age classes, with weaned pups, yearlings, and subadults having highest exposure and infection. The most common postmortem finding in 26 *Brucella*-positive animals (culture and/or PCR) was verminous pneumonia due to *Parafilaroides* spp. or *Otostrongulus circumlitus*. Our data are consistent with exposure to *B. pinnipedialis* post-weaning, and it is likely that fish or invertebrates and possibly lungworms are involved in the transmission to harbor seals. *Brucella pinnipedialis* was cultured or detected by PCR from seal salivary gland, lung, urinary bladder, and feces, suggesting that wildlife professionals working with live, infected seals could be exposed to the bacterium via exposure to oral secretions, urine, or feces. Endangered sympatric wildlife species could be exposed to *B. pinnipedialis* via predation on infected seals or through a common marine fish or invertebrate prey item involved in its transmission. More work is required to elucidate further potential fish or invertebrates that could be involved in the transmission of *B. pinnipedialis* to harbor seals and better understand the potential risk they could pose to humans or sympatric endangered species who also consume these prey items.

Key words: *Brucella pinnipedialis*, brucellosis, disease screening, harbor seal, marine, *Phoca vitulina richardsi*, zoonosis.

INTRODUCTION

In 1994 a novel *Brucella* was reported in harbor seals (*Phoca vitulina*), harbor porpoise (*Phocoena phocoena*), and a common dolphin (*Delphinus delphis*); (Ross et al., 1994) and from an aborted bottlenose dolphin (*Tursiops truncatus*) fetus (Ewalt et al., 1994). Subsequently antibodies to and isolates of this marine *Brucella* were detected in multiple marine mammal species (Foster et al., 1996; Nielson et al., 2001). Marine *Brucella* spp. have been classified as two species, *Brucella ceti* and *Brucella pinnipedialis*, for isolates from cetaceans and seals, respectively (Cloeckaert et al., 2001; Foster et al., 2007; Banai and Corbel, 2010) with subgroups identified within each (Maquart et al., 2009).

Like terrestrial isolates, *B. ceti* and potentially *B. pinnipedialis*, are zoonotic (Cloeckaert et al., 2011). In 1999 a case of laboratory-acquired brucellosis of marine origin occurred (Brew et al., 1999). Subsequently, three cases of naturally acquired brucellosis of marine origin were reported from people having no known

history of exposure to marine mammals (Sohn et al., 2003; McDonald et al., 2006). Consumption of raw seafood is a potential route of human exposure (Whatmore et al., 2008); however, the lack of understanding of the epizootiology of marine *Brucella* infection makes it difficult to assess how people were exposed in the cases of naturally acquired infections.

Marine *Brucella* spp. also can cause disease in domestic animals. Experiments using marine *Brucella* of different origins and different inoculation routes caused abortion and seroconversion in cattle (Rhyan et al., 2001), infection and seroconversion without abortion in sheep, and fulminant infection in guinea pigs (Perrett et al., 2003). Detailed information on how marine *Brucella* is transmitted is needed to assess the risk that it presents to domestic animals.

We used serologic, microbiologic, and pathologic data collected from live-captured, stranded, and rehabilitated animals to elucidate the epizootiology of *B. pinnipedialis* in harbor seals and hypothesized potential routes of infection for humans, domestic animals, and sympatric endangered species.

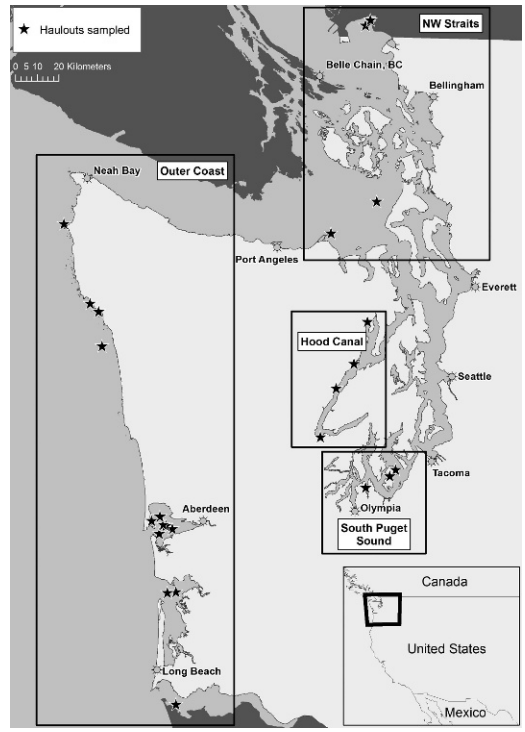

FIGURE 1. Map of 24 harbor seal haulout locations from four regions in Washington State, USA, where harbor seals (*Phoca vitulina*) were captured and sampled. Regional divisions are based on known harbor seal genetics and or contaminant levels and signatures.

MATERIALS AND METHODS

Harbor seal capture, handling, and sample collection

Harbor seals were captured at 24 sites in Washington State, USA (Fig. 1), using a beach seine (Jeffries et al., 1993) or beach rush. Blood was drawn from the extradural intravertebral vein using 18 gauge 3.81–8.89 cm needles (Bossart et al., 2001). Whole blood was stored chilled; serum was separated as soon as possible and frozen (−20 C). Blood collected in heparinized containers was frozen for culture.

Using known pupping dates from a well-studied harbor seal haul-out site (Gertrude Island, Washington) and weight/age correlations for harbor seals from British Columbia (Bigg, 1969, Cottrell et al., 2002), harbor seals were classified as pups (<2 mo), weaned pups (2–12 mo), yearlings (12–24 mo), subadults (24–48 mo), or adults (>48 mo).

Stranded harbor seal carcasses in good postmortem condition (carcass code 2 or 3; Geraci and Lounsbury, 2005) were necropsied, as were seals that died or were euthanized during rehabilitation. Fresh placentas were recovered at harbor seal haul-out sites. Tissues collected for histopathology were stored in 10% neutral buffered formalin. Tissues collected for bacterial isolation and other tests were stored frozen (−30 or −40 C). Using methods described above, blood also was collected from live and dead stranded animals.

Serology

Serum was tested at the Washington Department of Agriculture (Olympia, Washington) for antibodies to *Brucella* using a previously described method (Garner et al., 1997), identified as suitable for detecting antibodies to marine *Brucella* (Nielsen, 2002). Animals were considered suspect positive if the buffered plate agglutination test antigen (BAPA) or brucellosis card test using buffered *Brucella* antigen (BBA) detected

antibodies. They were considered positive if they were positive on BAPA or BBA and also positive on either or both the complement fixation (CF) and the Rivanol (RIV; +50 to 200) precipitation tests.

To evaluate serology by location, age-class-adjusted serologic results from all sites sampled were lumped as a reference population. Serologic results from harbor seal haul-out sites were grouped based on harbor seal genetics (Huber et al., 2010; Lamont et al., 1996) and contaminant levels (Ross et al., 2004). Samples were divided into four locations (Fig. 1): South Puget Sound (SPS; 3 sites), Hood Canal (HC; 4 sites), Washington Outer Coast (WOC; 13 sites), and the Northwest Straits (NWS; 4 sites).

Bacterial culture and identification

Bacterial isolation and identification were performed at the National Veterinary Services Laboratory (Ames, Iowa) with the addition of Columbia agar with 5% blood (Alton et al., 1988; Ewalt et al., 1983; Ewalt 1989). Tissues were dissected, mixed with approximately 2 mL of sterile phosphate buffered saline (pH 7.2), macerated, and inoculated onto tryptose agar with 5% bovine serum and antibiotics (7.5 U/mL bacitracin, 30 μg/mL cycloheximide, and 1.8 U/mL polymyxin B); tryptose agar with 5% bovine serum, antibiotics, and ethyl violet; Ewalt's media; Farrell's media; and Columbia agar with 5% blood. Plates were incubated for 14 days in 10% CO_2 at 37 C and observed for growth at 7 and 14 days. Bacterial isolates were circular, convex, smooth, translucent, off-white/honey colored. After 7 days colonies of average size consistent with *Brucella* (1.5–2 mm diameter) were counted and recorded, and representative colonies were transferred for identification (Mayfield et al., 1990).

Marine *Brucella* isolates were confirmed with the following tests: growth in the presence of basic fuchsin (1:25,000 and 1:100,000), thionin (1:25,000 and 1:100,000), and thionin blue (1:500,000); growth on medium containing penicillin (5 units/mL) or erythritol (1 mg/mL and 2 mg/mL plus 5% bovine serum); urease and catalase activity; H_2S production; and CO_2 dependence. Biotyping was conducted as previously described (Alton et al., 1988). The dominant antigen was determined with an agglutination test using A- and M- monospecific antisera (1:50-1:200) and R antiserum (1:25-1:100). Isolates were tested for susceptibility to lysis by the following phages: Tbilisi (Tb), Firenze (Fi), Weybridge (Wb), S708, Me/75, D, BK_2, R, R/C, and R/O.

Polymerase chain reaction

Polymerase chain reaction (PCR) was conducted at Animal Health Center (AHC) and Mystic Aquarium & Institute for Exploration (MAIE) using previously described PCR techniques for *Brucella* (AHC; Bricker et al., 2000) and real-time PCR (qPCR) analysis using primers, probe, and adapted protocols targeting the gene for a 31 kDa outer membrane protein *bcsp31* specific to the genus *Brucella* (MAIE; Probert et al., 2004, Sidor et al., 2013).

Histopathology and immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were stained with hematoxylin and eosin and select sections were also stained with Giemsa and with Brown and Brenn. Immunohistochemistry was performed on a subset of culture-positive cases. Tissue sections were mounted on charged slides, deparaffinized, hydrated with a buffer (PBS), treated with 3% H_2O_2 (5 min) to quench endogenous peroxidase, incubated for 5 min at 37 C with nonimmune goat serum, rinsed, and incubated for 30 min at 37 C with a polyclonal antibody (1:10,000) prepared against *B. abortus*. Amplification was conducted with biotinylated, goat origin, anti-rabbit immunoglobulin (Ig), and peroxidase-labeled streptavidin; the chromagen was 3-amino-9 ethylcarbazole in N, N-dimethylformamide. Sections were counterstained with Gill II hematoxylin. Nonimmunized rabbit Ig fraction was substituted for primary antibody as a negative control (Garner et al., 1997).

Statistical analysis

The chi-square test of independence was used to evaluate sex predilection for animals that were serologically positive for *Brucella* antibodies, and to evaluate sex predilection for animals that were culture-positive or negative for marine *Brucella*. Chi-square tests also were used to evaluate serologic and culture-positive animals by age class. For comparing serologic results among the four locations sampled (SPS, HC, WOC, and NWS), direct standardization was used to remove the biasing effect of age structure. Using chi-square tests, the number of serologically positive animals in each subpopulation was compared to the predicted number of positive animals based on the single standard population, which was developed by grouping the four populations (Jeckel et al., 2001).

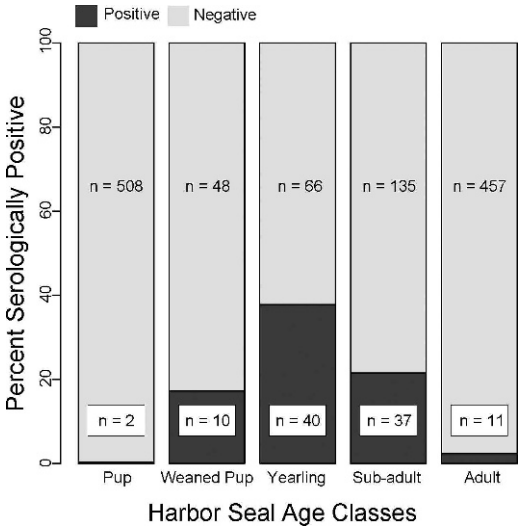

FIGURE 2. *Brucella* serology results as percentage positive by age class for 1,314 harbor seals (*Phoca vitulina*) live captured in Washington State, USA, between 1993 and 2007. Numbers correspond to the numbers of positive and negative animals by age class.

RESULTS

Serology

Between 1993 and 2006, 1,314 serum samples were collected from live healthy harbor seals captured at 24 haul-out sites from four discrete locations (Fig. 1): SPS (n=826), HC (n=212), WOC (n=116), and NWS (n=160). One hundred were antibody-positive, 101 suspect, and 1,113 negative animals. When suspects were lumped with negative animals, no sex predilection was detected with 7.4% of males (n=50) and 7.8% of females (n=50) positive (χ^2=0.0813, df=3, P=0.994). The distribution of animals with *Brucella* antibodies was significantly different among age classes (χ^2=229.078, df=4, P<0.001) with 0.4% of pups, 17.2% of weaned pups, 37.7% of yearlings, 21.5% of subadults, and 2.4% of adults positive (Fig. 2).

Compared to the overall reference population, antibody prevalence was different by subregion (χ^2=7.259, df=3, P=0.064; Table 1). The SPS had a higher number of positive animals than predicted, while HC, WOC, and NWS animals had fewer.

Of 1,314 harbor seals sampled, 79 samples came from 39 animals that were captured and sampled more than once. Twenty-eight (72%) of these animals were negative for antibodies to *Brucella* at first capture and again on subsequent captures. Two pups (5%; one newly weaned, SPENO904, the other nursing, SPENO1873) were negative on initial capture but then suspect 2 wks and 4 yr later, respectively. Six animals (15%) were suspect or positive initially and negative at a later capture. One animal (SPENO902) was suspect when captured as a subadult and again 4 yr later, and negative when recaptured the following year as an adult. A subadult (SPENO933) and adult (SPENO938) were suspect on initial capture and negative subsequently 3 and 1 yr later, respectively. Three animals were positive initially and then negative, including a weaned pup (SPENO987) recaptured as a subadult 2 yr later, another weaned pup (SPENO1630) recaptured as an adult 5 yr later, and an adult (SPENO1010) estimated to be 4 yr old at initial capture and recaptured 2 yr later. Three animals (8%) were suspect or positive initially and remained so at recapture including an adult (SPENO277) and yearling (SPENO899) with suspect titers initially, and then again when recaptured 4 yr and 2 wks later, respectively, and a yearling (SPENO854) that was positive when captured and again as an adult 4 yr later.

Bacterial culture

Between 1996 and 2004 *Brucella* was cultured from 18 of 102 harbor seals tested (Table 2; Fig. 3). All isolates were CO_2-required, H2S-negative, urease-positive, catalase-positive, basic fuchsin-positive, thionin blue-positive, thionin-positive, penicillin-positive, erythritol-positive, dominant antigen A, no to partial lysis of *Brucella* phage, rifampacin-negative, Wb-negative, Fi-negative, delta-negative, BK2-positive, R/O-negative, S708-negative, Me/75-negative, and R/C-negative. Based on

TABLE 1. Actual number of antibody-positive harbor seals (*Phoca vitulina*) by region (Washington State, USA) compared to the predicted number of positive animals based on the reference population and corrected for age class of animals sampled.

Location	Actual no. of antibody-positive animals	Predicted no. of antibody-positive animals
Hood Canal	8	16.2
NW Straits	6	7.1
Outer Coast	5	7.6
South Puget Sound	81	69.1

biochemical tests and origin (seals), the species was identified as *B. pinnipedialis* (Foster et al., 2007). Of the seals tested, 14% of males (8/57) and 24% of females (10/42) were positive. Both animals of unknown sex were negative. No sex predilection was detected ($\chi^2=3.948$, df=3, $P=0.267$). Distribution of culture-positive animals by age class was significantly different ($\chi^2=19.018$, df=3, no yearlings sampled, $P<0.001$; Fig. 3), with *B. pinnipedialis* cultured from 37% of weaned pups ($n=46$) and 25% of subadults ($n=4$). Culture attempts were negative for all placentas ($n=24$), fetuses/stillborn ($n=3$) pups ($n=26$), and adults ($n=23$) sampled. *B. pinnipedialis* was cultured from 36 of 45 different tissues, organs, and parasites. The highest number of colonies cultured and the highest percentage of positive samples were from lung, mediastinal and pulmonary lymph nodes, vitreous humor, and lungworms (Table 2).

Polymerase chain reaction

As tested at the AHC, four of the 336 harbor seals and none of 16 harbor seal placentas were positive for *Brucella* sp. by PCR on pooled tissues (typically brain, lung, liver, spleen and lymph node). At MAIE, four of 83 seals and none of eight placentas were positive for *Brucella* sp. by PCR. All four were weaned pups. Three of the four seals were confirmed positive by bacterial culture as well. The PCR-positive tissues included an abscess, brain, feces, kidney, liver, lung, lungworm (*Otostrongulus circumlitus*), lymph node (inguinal, mediastinal, mesenteric, and suprascapular), a reproductive tract, and spleen (Table 3; Fig. 4). The PCR failed to amplify *Brucella* nucleic acid from urinary bladder, vitreous humor, and whole blood (Table 3).

Pathology and immunohistochemistry

Between 1996 and 2007, necropsies were performed on 24 weaned pups and two subadult harbor seals (Table 4; Fig. 2) that were *Brucella*-positive by bacterial culture ($n=18$) or PCR ($n=8$). Common findings included verminous pneumonia (22/26) due to *Parafilaroides* spp., *O. circumlitus,* or a combination of the two, lymph node lymphoid hyperplasia (16/26), emaciation (12/26), enterocolitis (9/26), and gastritis (6/26) with gastrointestinal parasitism (acanthocephalans, unidentified nematodes, and trematodes; 5/26). Of the 21 culture- or PCR-positive animals that also were tested for *Brucella* antibodies, only 52% ($n=11$) were positive, 33% ($n=7$) were suspect positive, and 14% ($n=3$) were negative (Table 4).

Immunohistochemistry exhibited positive staining in four of seven culture-positive cases (Table 4). Tissues staining positive included lung, lungworm (*Parafilaroides* spp.), lung abscess, spleen, and lymph nodes.

Rehabilitation cases

Of the 205 harbor seals (177 pups and 28 weaned pups) admitted into rehabilitation in Washington State between 1996 and 2004 and tested for *Brucella* antibodies, five of 28 weaned pups (18%) were positive. Four of five animals (WDFW97-4

TABLE 2. Tissues cultured and bacteriologic results from *Brucella*-positive harbor seals (*Phoca vitulina*; $n=18$) from Washington State, USA. Tissue or sample cultured is listed in descending order from highest number of colonies cultured.

Tissue or sample cultured	No. positive	No. tested	% positive	No. of colonies cultured
Lymph nodes				
Mediastinal	9	14	64	1–>1,000
Pulmonary	12	15	80	2–1,000
Sublingual	7	14	50	1–750
Supra scapular	9	15	60	1–705
Inguinal	6	15	40	2–600
Pancreatic	3	4	75	3–420
Sub scapular	7	15	47	1–395
Submandibular	5	14	36	3–306
Mesenteric	10	16	63	1–147
Reproductive	3	4	75	1–72
Hepatic	5	7	71	1–67
Iliac	2	10	20	1–38
Cardiac	5	7	71	12–37
Splenic	4	6	67	1–23
Renal	4	9	44	1–16
Gastric	3	7	43	1–14
Ovarian	1	2	50	12
Pooled sample	1	1	100	1–10
Samples				
Lung	9	18	50	65–pure growth
Vitreous humor	1	1	100	>1,000
Eye	3	11	27	4–400
Feces	3	9	33	6–300
Pancreas	2	16	13	1–85
Reproductive tract	2	16	13	1–64
Urinary bladder	1	11	9	60
Salivary gland	4	13	31	1–58
Kidney	2	18	11	1–52
Spleen	3	17	18	2–11
Spinal cord	1	11	9	11
Brain	2	14	14	11
Tonsil	4	13	31	1–10
Liver	2	17	12	1–7
Adrenal gland	1	15	7	6
Synovial tissue	1	5	20	3
Thymus	1	10	10	1
Stomach	0	16	0	0
Heart	0	17	0	0
Blood	0	5	0	0
Urine	0	4	0	0
Abscess	0	1	0	0
Gall bladder	0	4	0	0
Parasites				
Lungworm	3	6	50	1–>1,000
Flipper lice	0	3	0	0
Heartworm	0	1	0	0
Stomach worms	0	3	0	0

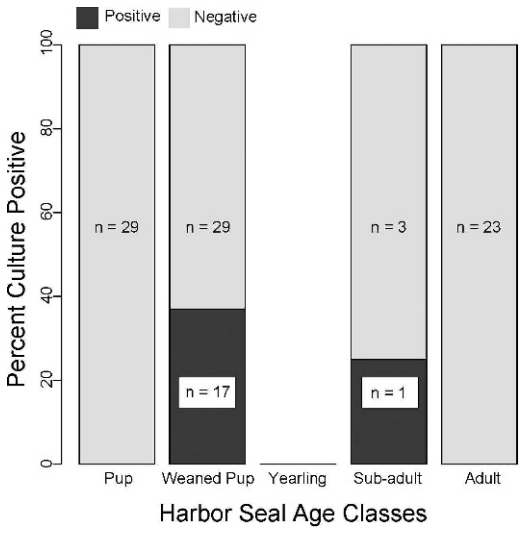

FIGURE 3. *Brucella* culture results as percent positive by age class for 102 harbor seals (*Phoca vitulina*) sampled in Washington State between 1996 and 2004. Numbers correspond to the numbers of positive and negative animals by age class.

reported in Garner et al., 1997, WDFW98-7, WDFW0404-08, and WH03-742; Table 4) were euthanized and necropsied. The fifth animal (WH02-687) was released after being surgically implanted with a VHF transmitter. Three of the four euthanized animals were positive for antibodies to *Brucella* on admission. The fourth (WH03-742) was negative for *Brucella* (BAPA and BBA) on 27 November 2003 at admission and seroconverted on 15 January 2004 (BAPA, RIV +50). It remained positive when retested on 5 February (BAPA, BBA, RIV and CF) and on four additional tests between February and April. Feces collected on 26 February 2004 were culture-positive for *B. pinnipedialis,* and the seal was euthanized (Table 4). The seal that was ultimately released (WH02-687) tested negative upon admission (BAPA negative) on 18 September 2002 and seroconverted on 5 December 2002 (BAPA, BBA, and RIV +200). It remained positive on five additional tests between

TABLE 3. Tissues or harbor seals (*Phoca vitulina*) from Washington State, USA, tested by PCR and results from *Brucella*-positive animals ($n=8$). Samples are listed in descending order from the highest percent positive.

Tissue or sample tested by PCR	No. positive	No. tested	% positive
Lymph nodes			
Mediastinal	3	3	100
Inguinal	2	4	50
Mesenteric	1	2	50
Suprascapular	1	3	30
Organ or sample			
Lung	3	3	100
Brain	2	2	100
Flipper (cutaneous) abscess	1	1	100
Spleen	3	4	80
Tonsil	1	2	50
Reproductive tract	1	2	50
Feces	1	3	30
Kidney	1	3	30
Liver	1	3	30
Whole blood	0	0	0
Vitreous humor	0	1	0
Urinary bladder	0	1	0
Pooled tissue	4	8	50
Parasites			
Lungworm	2	2	100

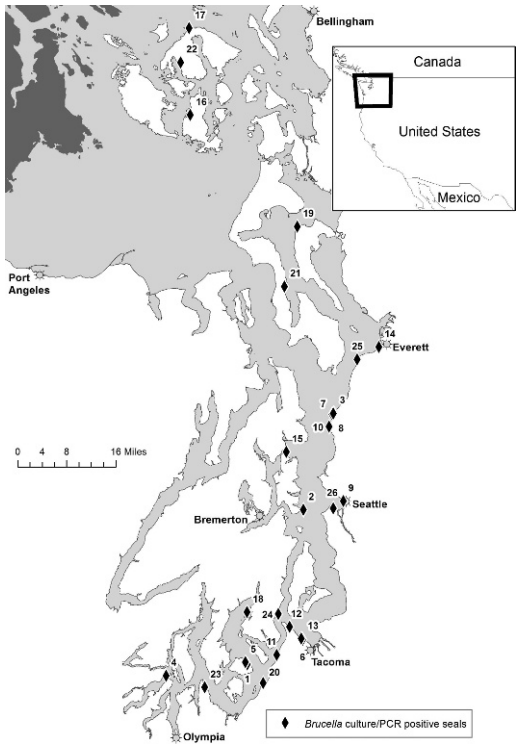

FIGURE 4. Locations of origin for harbor seals (*Phoca vitulina*) found positive for marine *Brucella* by culture or PCR. Numbers correspond to individual detail provided in Table 4.

January and May with no change in RIV titer. Attempts to culture *Brucella* from nasal and oral swabs were negative, and the seal was implanted with a subcutaneous VHF transmitter (Lander et al., 2005) and released. The seal's VHF radio signal was never detected post-release.

DISCUSSION

In Washington State, harbor seal exposure to *Brucella pinnipedialis* is widespread. Animals seroconvert after weaning and positive animals typically present with few pathologic lesions. Our data suggest exposure through foraging with the highest number of culture- and antibody-positive animals occurring after seals are weaned. The 0.4% of nursing pups with detectable *Brucella* antibodies likely represented passively acquired antibodies or exposure secondary to ingestion of infected prey items late in the preweaning stage. Low antibody prevalence in pups and lack of infection in placentas fail to support transplacental or transmammary transmission.

Adult harbor seals are primarily piscivorous, feeding on fish that are seasonally abundant (Olesiuk et al. 1990; Lance et al., 2012), suggesting fish as a possible route of *B. pinnipedialis* exposure. Weaned pups also consume shrimp (*Pandalus* spp.), crabs (*Cancer* spp.), and other invertebrates (Lambourn, unpubl. data), suggesting invertebrate consumption also might play a role in *B. pinnipedialis* transmission. Serologic data (Fig. 2) are consistent with *Brucella* seroconversion beginning at weaning, with a higher number of yearlings than weaned pups showing exposure due to an increased opportunity for encountering the bacterium through feeding over time. Using competitive enzyme-linked immunosorbent assays (cELISA), Zarnke et al. (2006) found similar serologic results with *Brucella* antibody prevalence in harbor seals from Alaska increasing from 11% in pups to 70% in yearlings, 68% for subadults, and dropping to 43% in adults. Our age-class–dependent culture results mimic our serologic results with the exception of culture data from yearling harbor seals due to lack of samples.

The marine origin competitive ELISA that is more sensitive than the serology used in this study (Meegan et al., 2010) was not available when this work commenced. Evaluating serology from culture- and PCR-positive animals necropsied in this study corroborates the work of Meegan et al. (2010) and suggests that the serology used for *B. abortus* underestimates the number of infected animals by about 50%. Consequently infection in weaned pups, yearlings, and subadults is likely higher than indicated by our serologic results (Fig. 2). Future efforts should use more specific independent competitive immunoassays such as the competitive and indirect

TABLE 4. Origin, serologic, culture, PCR, and immunohistochemistry (IHC) results for 26 *Brucella*-positive harbor seals (*Phoca vitulina*) from Washington State, USA, that received complete postmortem necropsies. Map ID corresponds to Figure 4.

Map ID	Identifier	Necropsy date	Location	Age[a]	Sex	Origin[b]	Serology[c]	Culture[c] (no. pos/no. sampled)	PCR[c] (no. pos/no. sampled)	IHC[c] (sample)
1	WDFW96-7	28 February 1996	Gertrude Island	SA	M	Dead, wild	Suspect[d]	Positive (pooled LNs)	Positive[f]	Negative
2	WDFW96-13	28 March 1996	Bainbridge Island	W	F	Dead, wild	Positive	Positive (6/13)	Positive[f]	Positive[g] (LW, LG)
3	WDFW97-4	27 January 1997	Edmonds	W	F	Euth., rehab.	Positive	Positive (24/29)	Positive[f]	Positive (LW, LN, abscess LG)
4	WDFW97-9	28 January 1997	Steamboat Island	W	F	Euth., wild	Positive	Positive (18/23)	N/D	Positive (LN)
5	WDFW98-4	28 January 1998	McNeil Island	W	M	Dead, wild	Negative[e]	Positive (5/13)	N/D	N/D
6	WDFW98-6	2 February 1998	Tacoma	W	M	Euth., wild	Positive	Positive (5/25)	Positive	Negative
7	WDFW98-7	6 March 1998	Edmonds	W	M	Euth., rehab.	Positive	Positive (8/27)	Positive[f]	Negative
8	WDFW00-2	25 January 2000	Edmonds	W	F	Dead, wild	Negative	Positive (15/27)	N/D	N/D
9	WDFW01-01	2 February 2001	Seattle	W	F	Dead, wild	Suspect	Positive (2/19)	N/D	N/D
10	WDFW1204-03	30 December 2003	Richmond Beach	W	M	Dead, wild	Negative	Positive (2/20)	N/D	N/D
11	WDFW0104-03	8 January 2004	Camano Island	W	M	Euth., wild	Positive	Positive (19/32)	N/D	N/D
12	WDFW0104-04	13 January 2004	Tacoma	W	M	Dead, wild	Positive	Positive (4/24)	N/D	N/D
13	WDFW0204-03	11 February 2004	Tacoma	W	M	Dead, wild	Suspect	Positive (8/24)	N/D	N/D
14	WDFW0404-08	28 April 2004	Everett	W	F	Euth., rehab.	Positive	Positive (6/31)	N/D	N/D
15	WDFW0504-01	1 May 2004	Bainbridge Island	W	F	Dead, wild	Suspect	Positive (12/32)	N/D	N/D
16	2004-WH782-03	12 May 2004	Lopez Island	W	F	Euth., rehab.	Positive	Positive (3/36)	Positive (1/4)	Positive (spleen)
17	2003-SJ012	27 May 2003	Orcas Island	W	F	Dead, wild	N/D	Positive (1/8)	N/D	N/D
18	WDFW0504-04	13 May 2004	Gig Harbor	W	F	Euth., wild	Positive	Positive (1/28)	N/D	N/D
19	CBW04-PV-15	28 November 2004	Camano Island	W	F	Dead, wild	Positive	N/D	Positive (pooled)	N/D
20	WDFW1204-05	17 December 2004	Steilacoom	SA	F	Dead, wild	N/D	N/D	Positive (pooled)	N/D
21	WID04121820SB	26 December 2004	Whidbey Island	W	F	Dead, wild	N/D	N/D	Positive (pooled)	N/D
22	2005-SJ012	5 June 2005	Orcas Island	W	M	Dead, wild	N/D	Negative	Positive (1/7)	N/D
23	WDFW1206-02	6 December 2006	Olympia	W	F	Dead, wild	Suspect[d]	Positive (1/2)	Positive (12/14)	N/D
24	WDFW1006-09	2 November 2006	Gig Harbor	W	M	Dead, wild	Suspect[d]	Positive	Positive (4/11)	N/D
25	WDFW0307-01	6 March 2007	Mukilteo	W	M	Dead, wild	Suspect[d]	Positive	Positive (4/13)	N/D
26	WDFW1007-16	31 October 2007	Seattle	W	M	Died, rehab.	N/D	N/D	Positive (pooled)	N/D

[a] Age class: W = weaned pup, SA = subadult.

[b] Origin: Euth.= euthanized, rehab.= taken into rehabilitation.

[c] Samples: LN = lymph node (s), LW = lungworm (*Parafilaroides* spp.), LG = lung, N/D = not done.

[d] Only brucellosis card test using buffered *Brucella* antigen (BBA).

[e] Only buffered plate agglutination test antigen (BAPA) conducted.

[f] PCR conducted on bacterial isolate, reported by Bricker et al. (2000).

[g] Reported by Garner et al. (1997).

ELISA used to test harbor seals from Alaska (Zarnke et al., 2006) and Hawaiian monk seals (*Monachus schauinslandi*) (Nielsen et al., 2005).

Two of five *Brucella*-infected harbor seals in rehabilitation seroconverted almost 7 wk post-admission. Because the serologic tests used underestimate positive animals by 50%, this could represent a type-2 testing error. Alternately, it could suggest that infection occurred very close to the time of admission and could not be detected for several months, that it was infected by a fish fed while in rehabilitation, or that alternate modes of transmission for *B. pinnipedialis* exist.

If harbor seals remain infected or produce anti-*Brucella* antibodies for life, we would have expected to see higher numbers of culture- and antibody-positive adults. Young harbor seals infected with *B. pinnipedialis* could have higher mortality rates than those not infected, reducing antibody prevalence in older animals. Alternately, it is possible that young infected animals cease to produce *Brucella* antibodies, or clear infection and become serologically negative. Data from one recaptured animal (SPENO854) that was positive as a yearling and again as an adult 4 yr later show that seals can maintain *Brucella* titers for at least 4 yr. However, in three other recaptured animals, *Brucella* antibodies had waned when the seals were retested 2 yr (SPENO987, SPENO1010) and 5 yr (SPENO1630) later. It is unknown if seals can clear infection and be reinfected and what the consequent antibody response could be.

Since harbor seals are exposed to *B. pinnipedialis* after they began to forage, fish or invertebrates could be involved in *B. pinnipedialis* transmission. There are no known sex-based dietary preferences in harbor seals. Our findings of no sex predilection for *Brucella* exposure or infection and similar findings by Nielsen et al. (2001) are consistent with this. The next step is to attempt to identify *B. pinnipedialis* in fish or invertebrate prey.

The lungworms *O. circumlitus* and *Parafilaroides* spp., which are transmitted to seals via fish, could also be responsible for *B. pinnipedialis* transmission. We identified *B. pinnipedialis* in three of six lungworms tested by culture (Table 2) and two of two tested by PCR (Table 3). Immunohistochemistry also exhibited staining in lung, lungworm (*Parafilaroides* spp.) and lung abscesses associated with *Parafilaroides* spp. Lungworm transmission of *Brucella* has been suggested for harbor seals (Garner et al., 1997) and for harbor porpoise (Dawson et al., 2008); however, more work is required to determine the role that lungworms play in *Brucella* transmission. Verminous pneumonia was identified as the most common pathologic finding in positive seals and is consistent with findings from harbor seals in Scotland (Foster et al., 2002); however, verminous pneumonia is a common finding in weaned and yearling stranded harbor seals, not all of which are infected with *B. pinnipedialis*. For example, between 2000 and 2006, lungworms were identified in nine of 90 harbor seals that stranded in San Juan County and were necropsied (Gaydos, unpubl. data). All seals were weaned pups or yearlings except for one adult. Species identified included *O. circumlitus* ($n=2$), *Parafilaroides gullundae* ($n=1$), *Parafilaroides gymnurus* ($n=1$), and unidentified *Parafilaroides* ($n=7$). One animal was coinfected with *O. circumlitus* and *P. gymnurus* and another with *O. circumlitus* and an unidentified *Parafilaroides*. Of nine animals infected with one or more species of lungworm, six were diagnosed with verminous pneumonia, but *Brucella* infection was identified by PCR or bacterial culture in only three animals, all of which had lungworms and were diagnosed with verminous pneumonia, suggesting a 50% coinfection rate for verminous pneumonia and *Brucella*. Testing known or putative intermediate fish hosts for *Parafilaroides* spp. and *O. circumlitus* (Bergeron et al., 1997) for *B. pinnipedialis* is a good

starting point for better understanding the relationship between the fish, lungworms, and transmission of *B. pinnipedialis*. The definitive life cycles for these metastrongyloids are not well known, and testing should include invertebrates, as they too could be intermediate or paratenic hosts (Bergeron et al., 1997).

If harbor seals are exposed to *Brucella* via ingestion of prey, humans also could potentially be exposed to marine *Brucella* by consuming uncooked marine fish or invertebrates. None of the three documented cases of community-acquired marine *Brucella* infection in humans had prior exposure to marine mammals (Sohn et al., 2003; McDonald et al., 2006). People also could be exposed to marine *Brucella* via the environment. We cultured *B. pinnipedialis* from the feces of 33% of infected harbor seals tested and isolated marine *Brucella* nucleic acid from the feces of 33% of infected harbor seals tested. Seals defecating on docks and beaches could expose humans to *B. pinnipedialis* without their being aware of the infection source. The potential for humans to be exposed to *B. pinnipedialis* without apparent exposure to harbor seals warrants additional research to uncover the history of infected humans and to better understand the putative role that marine fishes and invertebrates could play in the transmission of this potentially zoonotic bacterium to the public. Physicians should be made aware that, where clinical signs warrant, marine *Brucella* should be considered a differential diagnosis, even in patients from countries considered *Brucella*-free because of the absence of *Brucella* of livestock or domestic animal origin.

Based on our current understanding of *B. pinnipedialis* epizootiology, laboratory workers, biologists, veterinarians, and wildlife rehabilitators who work with harbor seals or seal samples likely have the highest risk for exposure, especially when working with weaned harbor seal pups and yearlings. We cultured or detected marine *Brucella* by PCR from tonsil, salivary gland, lung, urinary bladder, and feces of infected animals, suggesting that oral secretions, bites, urine, and feces are potential sources for human exposure from live seals.

South Puget Sound had a higher number of animals serologically positive for *Brucella* than predicted (Table 1). This warrants further investigation. Genetics and contaminant work suggest the four regions sampled represent discrete harbor seal subpopulations. One hypothesis for higher antibody prevalence in SPS could be the possibility of polychlorinated biphenyl (PCB) mediated immunotoxicity increasing *B. pinnipedialis* infection rates. Harbor seals in SPS are more heavily contaminated with high levels of PCBs than seals from NWS and WOC (Ross et al., 2004). Harbor seal PCB contaminant levels in SPS approach the threshold for PCB immunotoxicity, suggesting that young seals from this region could be more vulnerable to diseases, including infection with *B. pinnipedialis,* because of reduced immunocompetence (Ross et al., 2004).

It has been suggested that marine *Brucella* infection in endangered southern resident killer whales (*Orcinus orca*) could reduce their fecundity and ultimately the population's recovery (Gaydos et al., 2004). Killer whales could carry or be exposed to *B. ceti* but could also be exposed to *B. pinnipedialis* through occasional predation of young harbor seals (Gaydos et al., 2005 and unpubl. data) or more likely through consumption of fish (Ford et al., 1998), if fish are in fact involved in *B. pinnipedialis* transmission.

Harbor seals are exposed to and infected with *B. pinnipedialis* after being weaned when they begin foraging for fish and invertebrates, supporting the hypothesis that marine fish or invertebrates are responsible for transmitting this bacterium between seals. This bacterium is considered enzootic within the harbor seal population, yet seals are currently at carrying capacity (Jeffries et al., 2003), suggesting that *B. pinnipedialis* is likely not affecting seal populations in Washing-

ton State. The presence of this pathogen in harbor seals, the potential transmission through feces, urine, and oral secretions, and marine fish or invertebrates as vectors of *B. pinnipedialis* indicate the importance of understanding the epizootiology of this bacterium and of evaluation of the risk it poses to humans, domestic animals, and sympatric endangered species.

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