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ISOLATION AND CHARACTERIZATION OF A NOVEL MARINE *BRUCELLA* FROM A SOUTHERN SEA OTTER (*ENHYDRA LUTRIS NEREIS*), CALIFORNIA, USA

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ABSTRACT: We characterize *Brucella* infection in a wild southern sea otter (*Enhydra lutris nereis*) with osteolytic lesions similar to those reported in other marine mammals and humans. This otter stranded twice along the central California coast over a 1-yr period and was handled extensively at two wildlife rehabilitation facilities, undergoing multiple surgeries and months of postsurgical care. Ultimately the otter was euthanized due to severe, progressive neurologic disease. Necropsy and postmortem radiographs revealed chronic, severe osteoarthritis spanning the proximal interphalangeal joint of the left hind fifth digit. Numerous coccobacilli within the joint were strongly positive on *Brucella* immunohistochemical labelling, and *Brucella* sp. was isolated in pure culture from this lesion. Sparse *Brucella*-immunopositive bacteria were also observed in the cytoplasm of a pulmonary vascular monocyte, and multifocal granulomas were observed in the spinal cord and liver on histopathology. Findings from biochemical characterization, 16S rDNA, and *bp26* gene sequencing of the bacterial isolate were identical to those from marine-origin brucellae isolated from cetaceans and phocids. Although *omp2a* gene sequencing revealed 100% homology with marine *Brucella* spp. infecting pinnipeds, whales, and humans, *omp2b* gene sequences were identical only to pinniped-origin isolates. Multilocus sequence typing classified the sea otter isolate as ST26, a sequence type previously associated only with cetaceans. Our data suggest that the sea otter *Brucella* strain represents a novel marine lineage that is distinct from both *B. pinnipedialis* and *B. ceti*. Prior reports document the zoonotic potential of the marine brucellae. Isolation of *Brucella* sp. from a stranded sea otter highlights the importance of wearing personal protective equipment when handling sea otters and other marine mammals as part of wildlife conservation and rehabilitation efforts.

Key words: *bp26*, *Brucella*, *Enhydra lutris nereis*, multilocus sequence typing, *omp2*, osteoarthritis, southern sea otter.

INTRODUCTION

Most well-characterized *Brucella* strains are associated with livestock, pets, and terrestrial wildlife, but diverse marine-origin brucellae are increasingly recognized. Since the first report of an atypical *Brucella* from a bottlenose dolphin (*Tursiops truncatus*; Ewalt et al. 1994), the number of known-infected marine hosts has grown, especially among cetaceans (dolphins, porpoises, whales) and pinnipeds (seals, sea lions, walruses; Foster et al. 1996; Ross et al. 1996; Jahans et al. 1997). Two species of marine *Brucella* are known, based on host, biochemical, and molecular criteria: *B. ceti* (cetacean-origin strains) and *B. pinnipedialis* (seal-origin strains; Bricker et al. 2000; Foster et al. 2007). However, precise characterization of the taxonomy, host range, and pathophysiology of marine brucellae

awaits further study (Bricker et al. 2000; Moreno et al. 2002).

Marine brucellae are distinguished from terrestrial strains via an 870-base-pair (bp) insertion (IS*711*) downstream of the antigenic *bp26* locus (Bricker et al. 2000; Cloeckaert et al. 2000). Marine isolates are subdivided by phenotypic characteristics, host species, and characterization of *omp2* genes that code for surface porins. Cetacean-origin brucellae typically possess two *omp2b* genes, whereas isolates from domestic animals, rodents, pinnipeds, and river otters typically possess one copy each of *omp2a* and *omp2b* (excluding *B. ovis*, which has two copies of *omp2a*) (Ficht et al.1996; Ohishi et al. 2005). Recent reports reveal greater heterogeneity within the marine brucellae than was previously recognized, including pinniped-derived strains that possess cetacean *omp2* genotypes, and vice versa (Ohishi et al. 2004; Vizcaino et al. 2004). Chimeric strains containing *omp2a* and *2b* sequences from both marine and terrestrial brucellae are also reported (Ohishi et al. 2005).

Recently, multilocus sequence typing (MLST) has been used to characterize population structure and phylogenetic relationships for the brucellae (Whatmore et al. 2007). Initially 27 sequence types (STs) were described, including five (ST23-27) previously described marine (*B. ceti* and *B. pinnipedialis)* strains (Foster et al. 2007). Nine additional terrestrial STs (ST28-36) have since been described (Chen et al. 2013). Sequence types generally correspond to host taxa, although exceptions exist (Whatmore et al. 2007).

Serologic studies reveal low to moderate *Brucella* antibody prevalences in sea otters from California, Alaska (Hanni et al. 2003), and Russia (Goldstein et al. 2011). However, aside from a single report in a European river otter (*Lutra lutra*) (Foster et al. 1996), *Brucella* infection of estuarine or marine otters has not been confirmed, and associations with morbidity and mortality have not been described. Sea otters occupy a specialized niche in coastal ecosystems, acting as both keystone species and sentinels for anthropogenic pollution (Jessup et al. 2004). Infectious disease is a common cause of southern sea otter (*Enhydra lutris nereis*) mortality (Kreuder et al. 2003; Miller et al. 2010). Although shark-associated mortality and protozoal disease have received much attention in recent years (VanWormer et al. 2013; Tinker et al. 2015), few bacterial diseases have been well characterized. Here we describe the isolation and preliminary characterization of *Brucella* from a southern sea otter, and describe associated lesions and potential health risks for humans working in wildlife rehabilitation facilities.

MATERIALS AND METHODS

Clinical history

On 26 December 2001, a subadult female southern sea otter was found weak and emaciated (14 kg) on Pismo Beach, California and was transported to rehabilitation facility #1. Superficial lacerations were noted on the left flank and left hind flipper, with an exposed left hind fifth proximal interphalangeal joint. Swelling, crepitus, and reluctance to use the affected limb were suggestive of infection. A joint aspirate contained Gram-negative bacteria, but culture was not performed. A second subcutaneous abscess was identified and drained on the right hind limb. Based on a provisional diagnosis of shark bite with secondary infection, the wounds were repeatedly debrided, and the animal was treated with intramuscular enrofloxacin (Baytril, Bayer, Leverkuse, Germany) and penicillin (Dual-pen, AgriPharm, Westlake, Texas, USA) for 2 wk.

During hospitalization, occasional episodes of disorientation, tremors, unusual tameness and reduced reactivity to external stimuli were noted. Serodiagnostic tests revealed elevated titers to *Toxoplasma gondii* and *Sarcocystis neurona* (Table 1), so the otter was placed on oral antiprotozoal medication (Diclazuril, Virbac, Fort Worth, Texas, USA). From December 2001 through March 2002 progressive clinical improvement was noted. The left hind interphalangeal joint wound eventually closed, but remained swollen, firm, and mildly warm, and the otter was reluctant to use this foot. All other lesions healed satisfactorily and as clinical condition improved, stereotypic behavior suggestive of stress due to captivity increased. Following intraperitoneal implantation of a VHF transmitter, the animal was released at Shell Beach, California on 21 March 2002.

The otter stranded again on 22 December 2002 at Oceano Dunes, California (10 km from the original release site). Examination at rehabilitation

TABLE 1. Chronological results of protozoal (*Toxoplasma gondii* and *Sarcocystis neurona*) and *Brucella* spp. serology performed on serum collected from a southern sea otter (*Enhydra lutris nereis*) during two periods of captive care and necropsy. Dash indicates test not performed.

		IFAT[a]		cELISA[b]	FPA[c]
Otter location/event	Serum collected	*T. gondii*	*S. neurona*	*Brucella* spp.	*Brucella* spp.
First stranding episode	27 December 2001	—	—	Negative	Negative
Captive care	30 December 2001	1,280	160	—	—
Captive care	14 January 2002	1,280	320	—	—
Captive care	11 February 2002	—	—	Negative	Negative
Release	15 March 2002	—	—	Negative	Negative
Second stranding episode	22 December 2002	—	—	97	242
Captive care	15 January 2003	—	—	93	260
Euthanasia	13 February 03	—	—	96	263
Necropsy	14 February 03	10,240	2,560	95	267

[a] IFAT = Indirect fluorescent antibody test. Titers expressed as reciprocal of highest dilution with positive result.

[b] cELISA = Competitive enzyme-linked immunosorbent assay. Results for control and test sera were expressed as percent inhibition of antibody activity against *Brucella* antigen, as described by Lucero et al. (1999). In test serum with no anti-*Brucella* antibody, a mouse monoclonal antibody (MAb) binds, resulting in color development. In test serum containing anti-*Brucella* antibody, the test serum competes with the monoclonal antibody for epitope binding, and inhibition of MAb binding is inversely proportional to subsequent color development.

[c] FPA = fluorescence polarization assay. This assay utilized O-polysaccharide prepared from *B. abortus* lipopolysaccharide (mw 20–30 kDa) conjugated with fluorescein isothiocyanate and used as a tracer (Nielsen et al. 1996a). Fluorescence polarization was measured with an FPM-1 fluorescence polarization analyzer. Results are expressed in millipolarization units, and results from test samples are compared with those from known-negative samples.

facility #2 revealed emaciation and generalized paresis and asymmetrical (left>right) hind limb paraparesis. The otter was unable to leave the water unaided or flex her spine to groom her abdominal fur, necessitating periodic grooming by staff. As nutritional condition improved, self-grooming increased, but the otter avoided grooming her dorsal lumbar region and resisted staff attempts to brush this area. Minimal use of the left hind flipper was noted throughout hospitalization. Several weeks poststranding, multiple alopecic, pink, raised plaques were noted on the patient's head. These were attributed to poor grooming or stereotypic rubbing. Also noted were tremors, intermittent left front limb rigidity, and paresis that worsened with stimulation. Diclazuril therapy was resumed, but the tremors worsened, and progressive, severe stereotypic circling was noted. Due to a poor prognosis, euthanasia was performed 53 d poststranding.

Necropsy, histopathology, and protozoal immunohistochemistry, culture, serology, and PCR

Necropsy, including radiographs, bacterial culture, and cryo-archiving of tissues and serum, was performed the following day. Tissue samples were formalin-fixed, paraffin-embedded, and 5-μm sections were stained with hematoxylin and eosin stain (H&E) for histopathology. Skin was not collected for microscopic examination. Upon receiving bacterial culture results (described below), the frozen-thawed left hind fifth proximal interphalangeal joint was collected, formalin-fixed, decalcified, and processed for microscopic examination. Fite's acid fast and Gomori methenamine silver (GMS) stains were also performed on selected tissues using standard laboratory protocols.

During necropsy, brain tissue was collected aseptically to test for *T. gondii* and *S. neurona* infection via cell culture and PCR amplification of the *B1* gene as described previously (Miller et al. 2002). Postmortem serum was evaluated with an indirect fluorescent antibody test (IFAT) employing polyclonal antisera directed against *T. gondii* and *S. neurona*, and immunohistochemical stains for both parasites were prepared from paraffin-embedded brain, spinal cord, and skeletal muscle, as described (Miller et al. 2002, 2008).

Brucella culture, biochemical characterization, biotyping, serology, and immunohistochemistry

Swabs inoculated from heart blood, gallbladder, and the left hind fifth proximal interphalangeal joint were held in Amies transport media (Copan Diagnostics Inc., Murrieta, California, USA), then plated onto sheep blood and MacConkey agar (Hardy Diagnostics, Santa Maria, California,

USA) and streaked for isolation on the same day as the necropsy. Plates were incubated at 35 C in 5% CO_2 for 4 d, and any with visible bacterial growth were submitted to the University of California, Davis Veterinary Medical Teaching Hospital (VMTH) for identification. Bacterial colonies were subcultured, Gram stained, tested for urease activity and CO_2 dependence, and bacterial identity was confirmed with 16S rDNA sequence analysis (Murray and Stackebrandt 1995).

Serum collected throughout each period of captive care and at necropsy was submitted to Agriculture Canada (Ottawa, Canada) to assess the presence and concentration of *Brucella*-reactive antibodies. A competitive enzyme-linked immunosorbent assay (cELISA) and a fluorescence polarization assay (FPA) were used to screen sera for antibodies to *Brucella* spp., as previously described (Nielsen et al. 1996a; Lucero et al. 1999). The cELISA and FPA do not require species specificity. The cELISA measures antibody capable of competing with a mouse monoclonal antibody specific for *Brucella* O-polysaccharide (OPS) for antigen binding sites on the polystyrene plate. The amount of competition is measured using a goat antibody to Mouse IgG labelled with enzyme. Less conjugate binding indicates higher antibody activity in the test sample. The FPA measures any antibody of any species capable of binding to a labelled antigen and thereby reducing the rotational rate of the labelled antigen. In both cases, the serologic tests were set up according to standards described by the World Organisation for Animal Health (OIE) and were performed by the OIE Regional Reference Laboratory at the Canadian Food Inspection Agency, Nepean, Canada. Because species-specific reference control sera were not available, assay validity was established using bovine standard reference sera.

To screen for systemic brucellosis and clarify associations between *Brucella* sp. detection and observed lesions, major tissues including formalin-fixed brain, spinal cord, and the decalcified left interphalangeal joint were immunostained with antibodies directed against *B. abortus* at the Veterinary Services Laboratory (Fort Collins, Colorado). This assay and antibody has been demonstrated to label brucellae in several pinnipeds (Garner et al. 1997; J.C.R. unpubl.). Tissues (5 μm) were stained using a labeled streptavidin–biotin system employing polyclonal *B. abortus* antibody, as described by Rhyan et al. (1997), and were examined on a compound microscope.

Molecular characterization for *Brucella*

Selected frozen and formalin-fixed samples were assessed using real-time PCR (qPCR) for the gene coding for a 31 kDa outer membrane protein (*bcsp31*), which is specific to the genus *Brucella*. Briefly, DNA was extracted from fresh or frozen tissues using the Qiagen DNeasy kit. A multiplex Taqman qPCR assay incorporated primers, probe, and adapted protocols to target the *bcsp31* gene (Sidor et al. 2013). Two internal controls assessed DNA quality/quantity and presence of PCR inhibitors, and all samples were run in duplicate.

Molecular characterization of the *Brucella* isolate was performed at University of California, Davis using published protocols for amplification and sequencing of a 775-bp section of the 16S ribosomal DNA (16S rDNA), the genes coding for the *Brucella* 26 kDa protein (BP26) (Cloeckaert et al. 2000), and outer membrane protein (OMP) 2a and 2b (Ficht et al. 1996). The DNA sequences were analyzed using Chromas (Technelysium Pty Ltd., Tewantin, Queensland, Australia), GeneDoc (Nicholas et al. 1997), and Geneious 5.3.6 (Biomatters, Auckland, New Zealand) software.

The MLST was performed as described by Whatmore et al. (2007). Briefly, sequences from nine loci derived from 160 isolates (accessions AM694191–AM695630) were downloaded from the European Molecular Biology Laboratory–European Bioinformatics Institute. Each distinct allele was numbered according the original schema, and new alleles were defined if the sequence differed from previously described strains. Each combination of alleles was identified as an ST, and a new ST was identified if the combination of alleles differed from any previously described.

RESULTS

Necropsy, histopathology, and protozoal immunohistochemistry, culture, serology, and PCR

The otter was in excellent nutritional condition (20.2 kg) following 8 wk of rehabilitation. Musculature was symmetrical and adequately developed, with no gross abnormalities of the vertebrae, intervertebral discs, brain, or spinal cord.

Postmortem radiographs and gross necropsy revealed a severe osteolytic lesion spanning the left hind fifth proximal interphalangeal joint, corresponding with a region of grossly apparent soft tissue swelling (Fig. 1A, B). Articular surfaces were irregular and roughened with erosion of hyaline cartilage, periarticular fibrosis, and minimal opaque tan joint

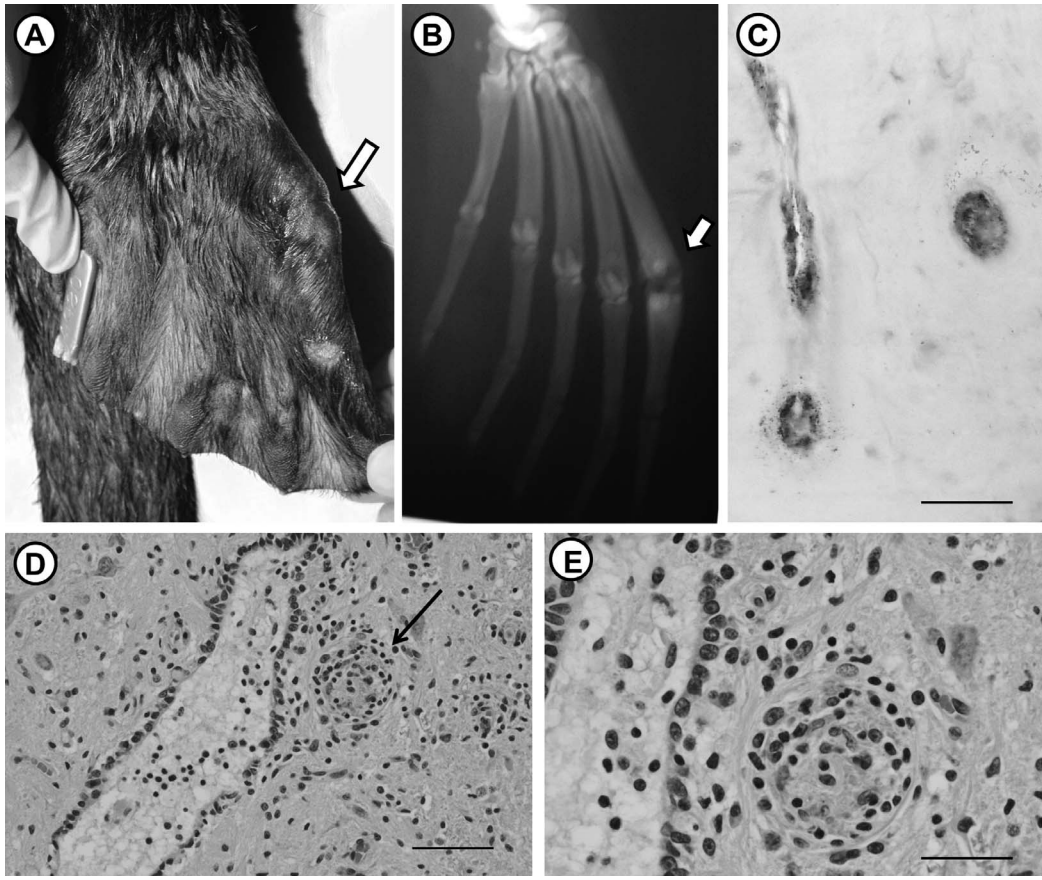

FIGURE 1. Lesions suggestive of chronic systemic brucellosis in a *Brucella*-infected southern sea otter (*Enhydra lutris nereis*). (A) Left rear flipper showing a markedly swollen proximal (first) interphalangeal joint on the fifth digit. (B) Radiograph of same flipper, showing marked osteolysis and scant periosteal bone formation surrounding the affected joint. A marine-origin *Brucella* sp. was obtained in pure culture from this joint following necropsy. (C) Immunohistochemical preparation of decalcified bone from the above flipper lesion, prepared utilizing polyclonal antiserum to *Brucella abortus*. Areas of osteonecrosis and osteolysis are filled with small, strongly *Brucella*-immunopositive coccobacilli (Bar=20 μm). (D) H&E-stained spinal cord from the same otter, showing a large granuloma near the central canal with perilesional mononuclear inflammation and vascular congestion (Bar=70 μm). (E) Higher magnification view of the same granuloma (Bar=35 μm). Granulomas were found throughout the spinal cord and the hepatic parenchyma.

fluid. Moderate diffuse lymphadenopathy was noted; affected lymph nodes were solid and tan with prominent cortical thickening.

Minimal orange-white mottling of the myocardium and mild hepatosplenomegaly was observed (possible euthanasia artifact). Approximately 100 acanthocephalan parasites (*Profilicollis* spp.) were deeply embedded in the wall of the distal duodenum and jejunum. Points of acanthocephalan attachment corresponded to 1–2 mm diameter, raised, yellow serosal nodules indicative of transmural parasite migration. The omentum was slightly thickened, red, and opaque, but no peritoneal fluid was observed.

Microscopic examination of the decalcified left hind fifth proximal interphalangeal joint revealed chronic granulomatous osteomyelitis and arthritis, with erosion of articular cartilage, exposure of underlying trabecular bone, and extensive periarticular fibrosis. Sparse inflammation was admixed with bacteria along articular surfaces, underlying marrow spaces, and Haversian canals.

Striking multifocal nodular granulomatous myelitis was also noted (Fig. 1D, E), although there was no gross or microscopic evidence of spinal compression or pressure necrosis. Spinal granulomas were sparsely distributed, well demarcated, large (50–100 μm), and were composed of dense aggregates of epithelioid macrophages and monocytes, with adjacent areas of moderate perilesional congestion and inflammation. Smaller granulomas were observed throughout the hepatic parenchyma. No gross or microscopic evidence of oophoritis or endometritis was noted in this immature female.

Also noted was mild multifocal nonsuppurative meningoencephalitis dominated by small lymphocytes, and rare large ($\geq$100 μm^2) cavitated lesions with scant perilesional nonsuppurative inflammation. Scant, amorphous basophilic crystalline material was scattered along the edge of some cavitated lesions (dystrophic mineralization), along with gitter cells containing granular blue-grey pigment (lipofuscin) and sparse glial cells. Rare thin-walled protozoal tissue cysts compatible with *T. gondii* were observed in brain tissue with no adjacent inflammation. Although immunohistochemistry was attempted, no parasite profiles were present in the recuts. No bacteria were observed in the brain, spinal cord or liver on H&E, acid fast or GMS stains.

Several 50–100 μm-long, thick-walled intracytoplasmic protozoal sarcocysts with prominent surface projections and fine internal septations encompassing thousands of tiny banana-shaped zoites (*Sarcocystis* spp.) were observed in skeletal myofibers. These were associated with mild myositis. These sarcocysts (an incidental finding) showed weakly positive labelling for *S. neurona* on immunohistochemistry.

Lymph nodes exhibited moderate follicular and paracortical lymphoid hyperplasia. Mild lymphocytic inflammation was observed in the myocardium, adrenal cortex, and renal cortices. Mild multifocal granulomatous omentitis, including rare foreign body giant cells surrounding mineralized debris, was attributed to degenerating peritoneal acanthocephalans.

Serum collected 4 d after the first stranding was weakly positive for *T. gondii* and *S. neurona* antibodies (Table 1). Testing of postmortem serum samples revealed increased reactivity to *T. gondii* and *S. neurona*. Tachyzoites were visible in cell monolayers following exposure to brain tissue collected at necropsy. The zoites were morphologically consistent with *T. gondii* and the cells were *T. gondii*-positive via *B1* gene PCR. Brain and spinal cord were negative for *S. neurona* by histopathology, immunohistochemistry, cell culture, and PCR.

Brucella culture, biochemical characterization, biotyping, serology, and immunohistochemistry

All agar plates inoculated with heart blood and gallbladder samples were negative for bacteria 4 d postinoculation. No growth was apparent on a MacConkey plate inoculated with the left fifth interphalangeal joint swab after 4 d, so the plate was discarded. After 4 d of incubation, the interphalangeal joint sample that was plated on blood agar yielded numerous tiny monomorphic, nonhemolytic, pale grey to nonpigmented colonies of Gram-negative coccobacilli. This plate was submitted to the VMTH where subculture confirmed growth of Gram-negative, urease-positive coccobacilli that required CO_2, and 16S rDNA sequence analysis confirmed the presence of *Brucella* sp. The isolate (hereafter denoted as SSO-1) was submitted to the National Veterinary Services Laboratory (NVSL). Cryopreserved lung and multiple lymph nodes were culture-negative for *Brucella* at the NVSL.

Serum samples from both stranding episodes and from necropsy were assessed for *Brucella* antibodies using cELISA and FPA. Results from both tests indicated that *Brucella* seroconversion occurred between release from rehabilitation facility #1 and the second stranding (Table 1). *Brucella* titers did not vary appreciably throughout the second period of hospitalization, suggestive of chronic infection.

All tissues tested except for lung and the left hind interphalangeal joint samples were *Brucella*-immunonegative. In the lung, one

mononuclear cell within a pulmonary vein contained a cytoplasmic cluster of positive-staining coccobacilli. The interphalangeal bones contained large numbers of *B. abortus*-immunopositive bacterial coccobacilli along the joint surface, in necrotic Haversian canals and in a small marrow space (Fig. 1C).

Molecular characterization of Brucella

All multiplex Taqman qPCR testing of cryopreserved (spleen, liver, brain, cerebrospinal fluid, lung, and hilar, retropharyngeal, and mesenteric lymph node) and formalin-fixed, paraffin-embedded tissues (heart, lung, brain, spinal cord, spleen, liver, tongue, tonsil, adrenal, stomach, intestine, and mesenteric lymph node) was negative for the *bcsp31* gene.

The 775-bp partial 16S rDNA gene sequence from bacteria isolated from the left hind interphalangeal joint (GenBank accession DQ295026) was identical to all GenBank *Brucella* spp. sequences, confirming this isolate as *Brucella* sp. A 1,900-bp *bp26* amplicon from SSO-1 contained an IS*711* insertion downstream of the *bp26* gene, consistent with marine *Brucella* strains (Cloeckaert et al. 2000). The SSO-1 isolate possessed both *omp2a* and *omp2b* genes. The *omp2a* gene sequence (Fig. 2 and Supplementary Table S1) was identical to *B. pinnipedialis* strains from harbor seals (*Phoca vitulina;* B2/94, M2533/93/1, and 6/566), 17a-1 from a hooded seal (*Cystophora cristata*), two pacific cetacean-origin strains (F599 from a bottlenose dolphin, and JM13/00 from a pacific minke whale; *Balaenoptera acutorostrata scammoni*), and the human-derived, marine-type ST27 strain 02611. The SSO-1 *omp2b* sequence was identical to two pinniped-associated strains, M2533/93/1 (ST 24, *P. vitulina*, Scotland) and 6/566 (ST25 *P. vitulina*, Washington, USA). The MLST (Whatmore et al. 2007) classified SSO-1 as an ST26 strain (KU057692-KU057700).

DISCUSSION

Well documented as a cause of disease in terrestrial animals and humans (Morgan and Corbel 1984), bacteria of the genus *Brucella* also infect marine mammals, causing disease of varying severity. To our knowledge, this is the first report *Brucella* infection in a sea otter, and the first report of a *Brucella* ST26 strain from any host in the Pacific Ocean. Our data suggest that SSO-1 represents a marine lineage that is distinct from both *B. pinnipedialis* and *B. ceti*. The current *Brucella* classification system, based primarily on pathogenicity and host preference includes eight terrestrial species, each associated with particular hosts: *B. abortus* (cattle; *Bos taurus*), *B. melitensis* (sheep; *Ovis* spp. and goats; *Capra* spp.), *B. suis* (swine; *Sus scrofa*), *B. ovis* (sheep), *B. canis* (Canidae), *B. neotomae* (wood rats; *Neotoma* spp.; Morgan and Corbel 1984), *B. microti* (voles: Scholz et al. 2008), and *B. inopinata* (tree frogs: Fischer et al. 2012). Furthermore, *Brucella* infection appears to have caused, at a minimum, chronic granulomatous arthritis in this sea otter. Except for *B. neotomae,* all brucellae can exhibit significant host pathogenicity, causing placentitis, metritis, abortion, epididymitis, orchitis, discospondylitis, and myeloencephalitis (Morgan and Corbel 1984). Interspecies transmission is recognized, such as sharing of *B. melitensis* infection across livestock species (Kahler 2000). Marine-origin brucellae are more recent discoveries (Bricker et al. 2000). Two species are currently recognized: *B. pinnipedialis* (associated with seals, *Phocidae*; sea lions, *Otaridae*; and walruses, *Odobenidae*) and *B. ceti* (associated with *Cetacea*; porpoises, dolphins, and whales). Although an understanding of their evolutionary origins and host range is incomplete, the potential pathogenicity of marine brucellae for animals and humans is well recognized (Miller et al. 1999; Sohn et al. 2003; McDonald et al. 2006; Hernández-Mora et al. 2008).

The SSO-1 *Brucella* strain was isolated from a chronically infected joint, which was positive for *Brucella* on immunohistochemistry (Fig. 1C). Necropsy revealed chronic granulomatous osteoarthritis (Fig. 1A, B) and myelitis (Fig. 1D, E) that was distinct from other known sea otter inflammatory diseases, including coccidioidomycosis (Huckabone et

al. 2015) and toxoplasmosis (Miller 2008). Disseminated granulomas are common in *Brucella*-infected animals and humans (Ceviker et al. 1989; Bingöl et al. 1999). Due to sparse bacterial loading, these lesions are commonly negative on culture and special stains (Gonzalez et al. 2002; Sohn et al. 2003). Immune-associated disease might contribute to lesion development and severity (Krishnan et al. 2005).

The relative contributions of concurrent brucellosis and toxoplasmosis with respect to observed progressive neurologic disease are unknown. Although the cavitated and partially mineralized brain lesions were typical of chronic toxoplasmosis in sea otters (Miller 2008), humans, and experimentally-exposed rodents (Stahl et al. 2004), and *T. gondii*-like tissue cysts were observed nearby, due to lesion chronicity the underlying cause could not be confirmed. Based on the severity of the spinal granulomas and their similarity to lesions in *Brucella*-infected animals and humans (Gonzalez et al. 2002; Sohn et al. 2003; Gonzalez-Barrientos et al. 2010), it is possible that both *T. gondii* and *Brucella* contributed to morbidity.

Brucella has not previously been isolated from sea otters, but prior serologic surveys have revealed low to moderate antibody prevalence in otters from California (6%), Alaska (3–8%), and Russia (28%) (Hanni et al. 2003; Goldstein et al. 2011). *Brucella* antibodies have also been detected in sympatric harbor seals, Steller sea lions (*Eumetopias jubatus*), bottlenose dolphins, and walruses (*Odobenus rosmarus*) (Nielsen et al. 1996b; Burek et al. 2005; Lambourn et al. 2013). Despite serologic evidence of exposure, infection was not previously confirmed in sea otters. This could be because infection is often transient, subclinical, and characterized by low bacterial burdens, as for many other host species (Moreno et al. 2002). Infection might also be underdiagnosed because brucellae are fastidious, slow-growing, and easily obscured by other bacteria (De Miguel et al. 2011). Systemic protozoal infections or other common infectious diseases of southern sea otters could also inhibit detection due to lesion overlap. Finally, sea otter spinal cords are not routinely examined microscopically, so the unique granulomas that appear to be associated with *Brucella* infection in this case could be under-recognized.

Isolate SSO-1 was confirmed as *Brucella* sp. based on 16S rDNA sequence and molecular and phenotypic features consistent with marine-origin brucellae, including presence of an extra copy of the IS*711* gene at the *bp26* locus (Cloeckaert et al. 2000). In common with *B. pinnipedialis* from pinnipeds and a river otter, growth of SSO-1 was CO_2-dependent. Carbon dioxide dependence was the most accurate predictor of host origin among 102 pinniped and cetacean isolates. Although some exceptions have been noted, pinniped strains are generally CO_2-dependent, and cetacean strains CO_2-independent (Dawson et al. 2008).

OMP analysis was in agreement with phenotypic characteristics, suggesting that SSO-1 is most closely related to pinniped strains. As with most *Brucella* spp., SSO-1 has one copy of each *omp2* gene (*2a* and *2b*). The *omp2a* sequence is 100% identical to refer-

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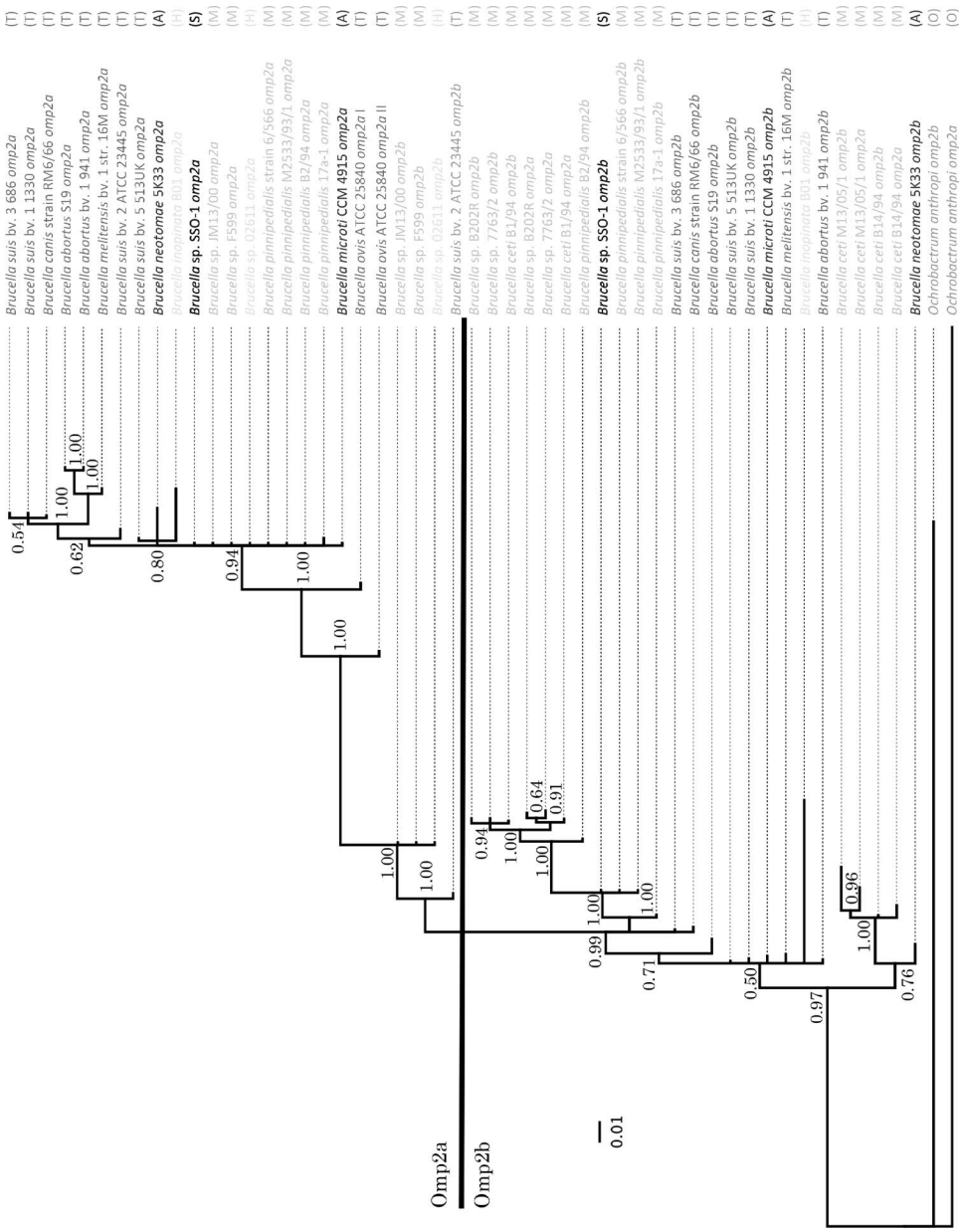
FIGURE 2. Markov Chain–Monte Carlo (MCMC) phylogenetic analysis of *Brucella omp2a* and *omp2b* gene sequences. Node labels denote Bayesian posterior probability. The Markov chain was simulated for 1,100,000 cycles under a HKY85 model. The first 100,000 cycles were discarded as burn-in and the chain was sampled every 500 updates thereafter. Scale bar indicates the number of substitutions per site. SSO-1 has one *omp2a* and one *omp2b* gene copy, in common with pinniped-origin isolates. Cetacean-origin isolates generally have two *omp2b* gene copies. Both copies are identical to sequences found in pinniped-origin isolates. The SSO-1 *omp2a* sequence is also identical to the atypical strains JM13/00 (minke whale) and 02611 (human, suspected marine origin). Constructed using MrBayes and Geneious 5.3.6. Genbank accession numbers are included in Supplementary Table S1. Legend: O (Grey)=*Ochrobactrum anthropi* (outgroup); T (green)=terrestrial classical *Brucella* strains; M (blue)=marine strains; A (brown)=terrestrial atypical strains; H (purple)=human-origin isolates; S (red)=sea otter isolate SSO-1.


Brucella suis bv. 3 686 omp2a (T)
Brucella suis bv. 1 1330 omp2a (T)
Brucella canis strain RM6/66 omp2a (T)
Brucella abortus S19 omp2a (T)
Brucella abortus bv. 1 941 omp2a (T)
Brucella melitensis bv. 1 str. 16M omp2a (T)
Brucella suis bv. 2 ATCC 23445 omp2a (T)
Brucella suis bv. 5 513UK omp2a (T)
Brucella neotomae 5K33 omp2a (A)
Brucella inopinata BO1 omp2a (H)
Brucella sp. SSO-1 omp2a (S)
Brucella sp. JM13/00 omp2a (M)
Brucella sp. F599 omp2a (M)
Brucella sp. 02611 omp2a (H)
Brucella pinnipedialis strain 6/566 omp2a (M)
Brucella pinnipedialis M2533/93/1 omp2a (M)
Brucella pinnipedialis B2/94 omp2a (M)
Brucella pinnipedialis 17a-1 omp2a (M)
Brucella microti CCM 4915 omp2a (A)
Brucella ovis ATCC 25840 omp2a I (T)
Brucella ovis ATCC 25840 omp2a II (T)
Brucella sp. JM13/00 omp2b (M)
Brucella sp. F599 omp2b (M)
Brucella sp. 02611 omp2b (H)
Brucella suis bv. 2 ATCC 23445 omp2b (T)
Brucella sp. B202R omp2b (M)
Brucella sp. 7763/2 omp2b (M)
Brucella ceti B1/94 omp2b (M)
Brucella sp. B202R omp2a (M)
Brucella sp. 7763/2 omp2a (M)
Brucella ceti B1/94 omp2a (M)
Brucella pinnipedialis B2/94 omp2b (M)
Brucella sp. SSO-1 omp2b (S)
Brucella pinnipedialis strain 6/566 omp2b (M)
Brucella pinnipedialis M2533/93/1 omp2b (M)
Brucella pinnipedialis 17a-1 omp2b (M)
Brucella suis bv. 3 686 omp2b (T)
Brucella canis strain RM6/66 omp2b (T)
Brucella abortus S19 omp2b (T)
Brucella suis bv. 5 513UK omp2b (T)
Brucella suis bv. 1 1330 omp2b (T)
Brucella microti CCM 4915 omp2b (A)
Brucella melitensis bv. 1 str. 16M omp2b (T)
Brucella inopinata BO1 omp2b (H)
Brucella abortus bv. 1 941 omp2b (T)
Brucella ceti M13/05/1 omp2b (M)
Brucella ceti M13/05/1 omp2a (M)
Brucella ceti B14/94 omp2b (M)
Brucella ceti B14/94 omp2a (M)
Brucella neotomae 5K33 omp2b (A)
Ochrobactrum anthropi omp2b (O)
Ochrobactrum anthropi omp2a (O)
0.54
1.00
1.00
1.00
0.62
0.80
0.94
1.00
1.00
1.00
1.00
0.94
1.00
0.64
0.91
1.00
1.00
1.00
0.99
0.71
0.50
0.97
0.96
1.00
0.76
Omp2a
Omp2b
0.01

ence *B. pinnipedialis* (B2/94, M2533/93/1, and 6/566), marine *Brucella* spp. isolates from a dolphin (F599) and a minke whale (JM13/00), and *B. microti* from a vole (CCM4915) and a human with spinal osteomyelitis (02611). The above cetacean- and human-origin marine strains are members of an atypical ST27 group that possess one *omp2a* gene copy and one *omp2b* copy, whereas members of the classical *B. ceti* ST23 group possesses two *omp2b* gene copies. The SSO-1 *omp2b* gene is also 100% identical to reference *B. pinnipedialis* isolates from harbor seals (M2533/93/1 and 6/566). Thus, *omp2* gene sequences suggest that SSO-1 is more closely related to *B. pinnipedialis* than *B. ceti*.

In contrast to the phenotyping results and OMP analysis, MLST places SSO-1 within sequence type 26. Previous ST26 isolates are generally associated with cetaceans and have previously been recovered only from common (*Delphinus delphis*), bottlenose, and striped dolphins (*Stenella coeruleoalba*) from UK or Mediterranean waters (Whatmore et al. 2007; Alba et al. 2013). Two single nucleotide substitutions in different genes separate ST26 (composed solely of cetacean strains), from ST25 (comprising European river otter and most pinniped strains). Although MLST sequence types largely correlate with host of origin, this is not the first time that a marine *Brucella* isolate was assigned a MLST sequence type that is associated with another marine mammal taxon (Whatmore et al. 2007). Our molecular and biochemical data, although preliminary, suggest that the sea otter *Brucella* strain SSO-1 represents a novel marine *Brucella* lineage that is distinct from both *B. pinnipedialis* and *B. ceti*. However, additional molecular testing such as MLVA-16 typing (Al Dahouk et al. 2007) should be performed to clarify these distinctions.

Routes of *Brucella* exposure in humans and animals include ingestion, inhalation, conjunctival infection, transplacental transfer, and transcutaneous spread through traumatized skin (Carvalho Neta et al. 2010). Southern sea otters consume filter-feeding invertebrates that can concentrate fecal bacteria (Miller et al. 2010), raft in groups, groom extensively, can haul out on rough surfaces, and often wound conspecifics during territorial and breeding interactions. Thus, all known routes for *Brucella* infection are possible, with perhaps the exception of lungworms (Garner et al. 1997), given that pinniped lungworms are not known to parasitize sea otters.

Although marine brucellae often exhibit mild pathogenicity in host species, highly variable (and sometimes severe) pathogenicity is reported across hosts (Perrett et al. 2004). For example, experimentally infected cattle aborted (Rhyan et al. 2001), although sheep remained asymptomatic and lesion-free (Perrett et al. 2004). In humans, spinal and intracranial granulomas have been associated with seizures, progressive paresis, and paralysis (Ceviker et al. 1989; Bingöl et al. 1999; Sohn et al. 2003; Krishnan et al. 2005).

This report broadens the Brucella host range, expands our understanding of the heterogeneity of marine-origin brucellae, and describes associated lesions in sea otters. Multifocal granulomas were similar to those described from other Brucella-infected animals and humans, but distinct from classical protozoal and fungal-associated inflammatory lesions of sea otters (Miller 2008; Huckabone et al. 2015). Our report also highlights potential health risks for persons rehabilitating or consuming infected marine animals. Three cases of brucellosis associated with infection by marine brucellae have been reported in humans; two Peruvian patients presented with severe, progressive neurologic disease (Sohn et al. 2003), and a New Zealand man suffered vertebral osteomyelitis (McDonald et al. 2006). Marine-origin brucellosis might be underdiagnosed in humans, because these bacteria are difficult to detect and are unlikely to be considered, except following laboratory exposure. At-risk human populations often receive minimal medical surveillance, and clinical signs can be nonspecific and easily confused with other infectious or immune-mediated diseases. Persons with higher occupational risk for marine brucellae exposure include veterinarians, animal rehabilitation personnel, laboratory technicians, and those engaged in subsistence harvest

(Sohn et al. 2003; MacDonald et al. 2006; Sears et al. 2012). However, only one case of laboratory-acquired marine brucellosis has been reported in humans (Brew et al. 1999). All three naturally-acquired cases of marine-origin brucellosis in humans were speculatively associated with consumption of raw fish, not marine mammal contact, suggesting that consumption of undercooked seafood, especially fish, could pose a higher risk for human infection.

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SUPPLEMENTARY MATERIAL

Supplementary material (Table S1) for this article is online at http://doi:10.7589/2015-12-326.

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