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Brucella Infection in Asian Sea Otters (*Enhydra lutris lutris*) on Bering Island, Russia

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ABSTRACT: Infection with *Brucella* spp., long known as a cause of abortion, infertility, and reproductive loss in domestic livestock, has increasingly been documented in marine mammals over the past two decades. We report molecular evidence of *Brucella* infection in Asian sea otters (*Enhydra lutris lutris*). *Brucella* DNA was detected in 3 of 78 (4%) rectal swab samples collected between 2004 and 2006 on Bering Island, Russia. These 78 animals had previously been documented to have a *Brucella* seroprevalence of 28%, markedly higher than the prevalence documented in sea otters (*Enhydra lutris*) in North America. All of the DNA sequences amplified were identical to one or more previously isolated *Brucella* spp. including strains from both terrestrial and marine hosts. Phylogenetic analysis of this sequence suggested that one animal was shedding *Brucella* spp. DNA with a sequence matching a *Brucella abortus* strain, whereas two animals yielded a sequence matching a group of strains including isolates classified as *Brucella pinnipedialis* and *Brucella melitensis*. Our results highlight the diversity of *Brucella* spp. within a single sea otter population.

Key words: Asian sea otter, Bering Island, *Brucella*, *Enhydra lutris*, IS*711*, marine, PCR, Russia.

Bacteria of the genus *Brucella* are among the most common causes of zoonotic disease worldwide (Pappas et al. 2006), and they cause serious livestock losses (6–10% of income) in endemic areas (McDermott et al. 2013). Isolation of *Brucella* spp. from a marine mammal was first reported in 1994 (Ross et al 1994). Since then, serologic evidence of infection has been detected in many marine species. A previous review documented serologic evidence from 53 species (including 35 cetaceans and 14 pinnipeds), but active infection in only 11 cetacean and 6 pinniped species (Hernández-Mora et al. 2013), and the known host range continues to expand. Clinical illness has been observed in cetaceans, typically as reproductive and neurologic disease (Miller et al. 1999; González-Barrientos et al. 2010). Isolation of *Brucella* spp. from sea otters (*Enhydra lutris*) has not been reported, but one isolate was recovered from a European otter (*Lutra lutra*), although it is not known whether this animal had lesions (Foster et al. 1996). Serologic evidence of exposure has been reported previously in sea otters. Seroprevalence estimates using diagnostic tests developed for livestock have been low: Hanni et al. (2003) report values of 8% among wild northern sea otters (*Enhydra lutris kenyoni*) from Alaska in 1997 and 6% in Southern sea otters (*Enhydra lutris nereis*) from California between 1995 and 2000 by using the Rose Bengal test.

To address uncertain performance of serologic tests that were developed for livestock but used in marine mammals, a competitive enzyme-linked immunosorbent assay (cELISA; Meegan et al. 2010) was optimized specifically for detecting antibodies to marine *Brucella* spp. By using *Brucella pinnipedialis* antigen and was validated in bottlenose dolphins (*Tursiops truncatus*) and Hawaiian monk seals (*Neomonachus schauinslandi*). Using this test, a seroprevalence of 28% was estimated in Asian sea otters (*Enhydra lutris lutris*) on Bering Island, Russia, sampled between 2004 and 2006 (Goldstein et al. 2011). Northern sea otters from Kodiak Island, Alaska, sampled in summers 2004

and 2005 and tested with the cELISA yielded a much lower seroprevalence (2.7%; Goldstein et al. 2011). The reason for the comparatively high *Brucella* prevalence at Bering Island is unknown. Unlike other *Brucella* serologic tests, the cELISA does not seem to cross-react with *Yersinia enterocolitica* (Meegan et al. 2010). The cELISA test has not been validated in sea otters, but in cetaceans, a sensitivity and specificity of 100% and 73%, respectively, was determined, whereas in pinnipeds, it was 67% and 77%, respectively. Although this test will detect antibodies to any *Brucella* species, Meegan et al. (2010) concluded that it is more sensitive in marine species than the terrestrial antigen-based tests. Retesting Hawaiian monk seal samples previously analyzed with the terrestrial *Brucella* serologic tests yielded two additional positives using the cELISA test (Nielsen et al. 2005).

We examined 89 rectal swab samples from the same live-captured Asian sea otters from Bering Island (55°11′N, 166°8′E) collected by Goldstein et al. (2011) between 2004 and 2006 and stored at –70 C. We extracted DNA (DNeasy Blood and Tissue Kit, QIAGEN, Venlo, the Netherlands), and 78 of 89 samples contained amplifiable DNA based on mammalian ferritin PCR (Goldstein et al. 2004). Samples were tested by PCR, targeting a 150-base pair (bp) fragment of the gene encoding the insertion sequence IS*711*, by using primers IS*711*F (5′-TACCGCTGCGAA TAAAGCCAAC-3′) and IS*711*R (5′-TGAGAT TGCTGGCAATGAAGGC-3′) described by Wu et al. (2014). Samples testing negative with this primer set were retested with the same forward primer and an alternative reverse primer IS*711*R2 (5′-GCATCATAGGCTGCAT CAGCAA-3′), which yields a shorter (118-bp) product. The PCR products of the expected size were purified (QIAquick Gel Extraction Kit, QIAGEN), cloned (pCR 2.1 TOPO TA Vector, One-Shot® TOP10 *Escherichia coli*; Invitrogen, Carlsbad, California, USA), and sequenced (BigDye® Terminator v3.1 chemistry, Invitrogen). Sequences were analyzed in Geneious Pro 5.3.6 (Biomatters, Auckland, New Zealand) and phylogenetic analysis was conducted using MRBAYES 2.03 (Huelsenbeck and Ronquist 2001).

Three Asian sea otter rectal swab samples (from one adult male, one adult female, and one juvenile male) tested positive by PCR. Of these animals, only the adult female was seropositive to *Brucella* on the cELISA assay (Goldstein et al. 2011). Such imperfect agreement is expected, as infected animals may not yet have seroconverted, whereas many seropositive animals may no longer be shedding *Brucella* DNA. Sequencing produced four sequences of 115 bp (KU666447–KU666450; see Supplementary Material Table S1), each 100% identical to IS*711* gene sequences from one or more previously characterized *Brucella* spp. (Fig. 1). The sample SO-024 yielded two IS*711* sequences, SO-024 1/2 (GenBank accession no. KU666447) matching only *Brucella abortus* strain 86/8/59 and SO-024 2/2 (GenBank accession no. KU666448) that was identical to a different IS*711* gene copy from *B. abortus* strain 86/8/59, as well as several *Brucella suis*, *Brucella ovis*, and *Brucella melitensis* sequences. The sequence from SO-025 (GenBank accession no. KU666450) was identical to *Brucella melitensis* (bv. 2 strain 63/9) only. The sequence from SO-123 (GenBank accession no. KU666449) was identical to two *B. melitensis* strains (bv. 2 63/9 and 16M); many *B. pinnipedialis* strains from pinnipeds; and the cetacean-origin strains B1/94, B14/94, and JM13/00.

The PCR test used in this study was modified from the method of Wu et al. (2014), who reported a 100% sensitivity at 0.27 fg per reaction volume, due in part to the presence of multiple copies of the IS*711* gene in the *Brucella* genome. Unfortunately, the amplicon is a short fragment that is conserved and does not allow for speciation of *Brucella*. Furthermore, not all copies of the IS*711* gene in a *Brucella* genome are identical, complicating phylogenetic inference. We attempted to characterize the sequences detected, targeting the *bp26* gene (i.e., *omp28*), with several assays (Cloeckaert et al. 2000) and two multilocus sequence typing assays for *omp25* and glucokinase genes (Whatmore et

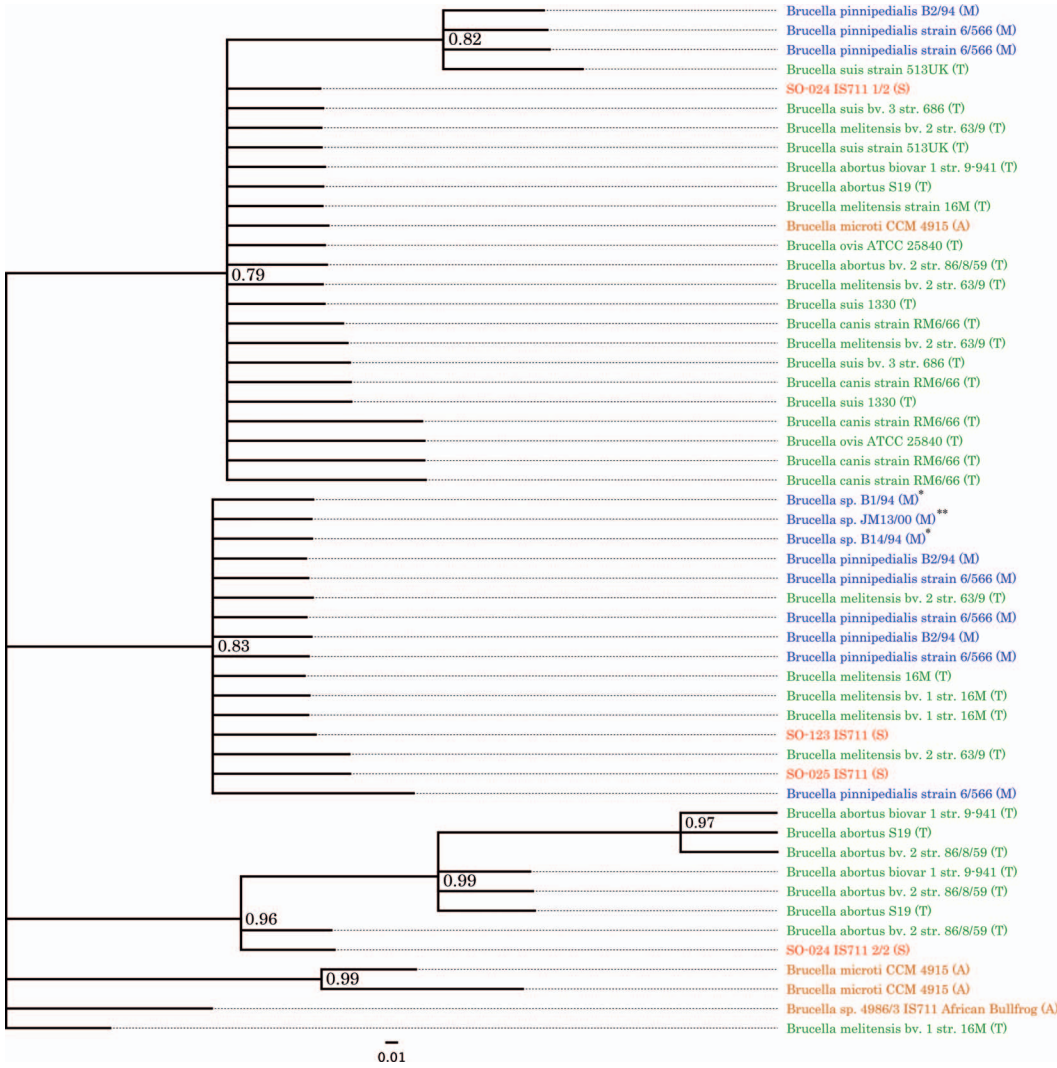

FIGURE 1. Markov Chain-Monte Carlo phylogenetic analysis of 58 partial *Brucella* IS*711* sequences (118 base pair) obtained from sea otters (*Enhydra lutris*) captured on Bering Island, Russia, between 2004 and 2006. The tree was constructed using MRBAYES (Huelsenbeck and Ronquist 2001) and Geneious Pro 5.3.6 (Biomatters) with the African bullfrog isolate (*Brucella* spp. 4986/3) as outgroup. Node labels denote Bayesian posterior probability. The Markov chain was simulated for 2,100,000 cycles under an HKY85 model. The first 100,000 cycles were discarded as burn-in, and the chain was sampled every 500 updates thereafter. The scale bar indicates the number of substitutions per site. Sequences are labeled with the following letter codes (and colors): sea otter sequences from the present study are labeled S (red), sequences from terrestrial species are T (green), atypical terrestrial species are A (orange), and previously described marine-origin species are M (blue). A full list of sequences in this tree, with GenBank accessions, is given in Supplementary Material Table S1. Single asterisk (*) indicates isolates have since been classified as *B. ceti*. Double asterisk (**) indicates cetacean-origin isolate not classified to species level.

al. 2007), but all were unsuccessful. The additional protocols were developed by others to characterize cultured isolates with abundant genetic material, rather than the low DNA concentrations found in swab samples from apparently healthy animals. Thus, we were unable to confirm that the *Brucella* spp. detected was of marine or terrestrial origin, and further work is required to determine the species of *Brucella* resulting in seroconversion

of almost 30% of Bering Island sea otters tested. Culture was not attempted on these samples as only DNA was available, but isolates in pure culture would allow more thorough characterization.

Exposure to *Brucella* in Asian sea otters on Bering Island may occur via land-sea spillover of the infectious agent, either from introduced reindeer (*Rangifer tarandus*) or domestic cattle (*Bos taurus*) grazing along the beaches in coastal areas of Bering Island. Information on the infection status of ungulates on Bering Island was not available, but *B. abortus* and *B. suis* biovar 4 are endemic (in cattle and reindeer, respectively) in mainland Russia (Meyer and Morgan 1973). Sea otters are reported to spend long periods hauled out on land Bering Island, potentially providing opportunities for interspecies transmission of *Brucella* spp. from terrestrial species or other marine mammal species sharing haulout sites, including harbor seals (*Phoca vitulina stejnegeri*), northern fur seals (*Callorhinus ursinus*), and Steller sea lions (*Eumatopius jubatus jubatus*). Alternatively, it has been proposed that fish are involved in *Brucella* transmission in marine environments. *Brucella* infection has been detected in lung worms that use a fish intermediate host (Garner et al. 1997) and in some fish species (El-Tras et al. 2010). Sea otters from Bering Island prey on benthic fish (Kornev and Korneva 2006), unlike southern sea otters that eat almost exclusively invertebrates. However, fish foraging is also observed in Northern sea otters in Alaska (Estes 1990), which had a much lower *Brucella* seroprevalence (Goldstein et al. 2011).

Our report of *Brucella* in sea otters in Russia adds to the already broad recognized host range of the genus *Brucella*. Our data provide evidence that *Brucella* spp. infect Asian sea otters, but information on the pathogenicity and transmission pathway is still limited. Evidence that *Brucella* can infect sea otters emphasizes that this pathogen should be included on the list of potential zoonotic risks to those handling sea otters.

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SUPPLEMENTARY MATERIAL

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