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Brucellosis in Endangered Hector's Dolphins *(Cephalorhynchus hectori)*

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Abstract

Brucella spp infections of marine mammals are often asymptomatic but have been associated with reproductive losses and deaths. Zoonotic infections originating from marine isolates have also been described. Hector's dolphins *(Cephalorhynchus hectori)* are an endangered species with a declining population, and the role of infectious disease in population dynamics is not fully understood. In this study, 27 Hector's dolphins found dead around the New Zealand coastline between November 2006 and October 2010 were evaluated for lesions previously associated with cetacean brucellosis. Tissues were examined using histological, immunohistochemical, and molecular (polymerase chain reaction [PCR]) techniques. Seven of 27 dolphins (26%) had at least 1 tissue that was positive on PCR for *Brucella spp.* Lesions consistent with brucellosis were present in 10 of 27 (37%) dolphins, but in 8 of these dolphins *Brucella* infection could not be demonstrated in lesional tissues. Two dolphins (7%) were diagnosed with active brucellosis: 1 female with placentitis and metritis, and 1 stillborn male fetus. *Brucella* identified in these 2 dolphins had genetic similarity (99%) to *Brucella pinnipedialis*. The *omp2a* gene amplicon from the uterus of the female had 100% homology with ST27 genotype isolates from a human in New Zealand and a bottlenose dolphin of Pacific origin. The remaining 5 PCR-positive dolphins were assessed as having asymptomatic or latent infection. While most *Brucella* infections identified in this study appeared to be subclinical, the finding of 2 dolphins with reproductive disease due to *Brucella* infection suggests that this disease has the potential to affect reproductive success in this species.

Keywords

cetacean, brucellosis, *Brucella*, stillbirth, zoonosis, reproductive loss, immunohistochemistry, PCR

Since discovery of the genus in 1887, *Brucella* species have become well-known pathogens in terrestrial mammals and more recently have been isolated from marine mammals, with the first cases reported in 1994.[23] Two new marine mammal species of *Brucella* have been characterized based on their host association: *Brucella ceti,* found predominantly in cetaceans, and *Brucella pinnipedialis,* predominantly isolated from pinnipeds.[8] While many dolphin species carry marine *Brucella* species without apparent disease,[6,29] a variety of lesions have been attributed to marine *Brucella* infection in cetaceans, including blubber abscesses, meningoencephalitis, hepatic necrosis, lymphoid necrosis, myocarditis, osteoarthritis, orchitis, mastitis, fetal distress, pneumonia, endometritis, placentitis, and abortion.[4,14,19,30] Fatal disseminated infections have been described in Atlantic white-sided dolphins *(Lagenorhynchus acutus)*.[5,7]

The Hector's dolphin *(Cephalorhynchus hectori)* is a small coastal marine dolphin endemic to New Zealand. The species as a whole is considered endangered due to a decreasing population size and a limited natural range.[13] A subspecies, the Maui dolphin *(C. hectori spp maui)* is classified as critically endangered. [13] Similar to other small odontocetes, Hector's dolphins have a low population growth potential, with late-onset female breeding (7–9 years of age) and calving every 3 years subsequently until an estimated maximum age of 20 years.[25] The Hector's dolphin population as a whole is estimated to be approximately 14,900 individuals, while only 55 Maui dolphins over 1 year of age are estimated to remain.[12,15] The population decrease has coincided with the rise of near-shore commercial and recreational fisheries, where dolphins are incidental bycatch.[26] Data on the importance of infectious disease in Hector's dolphins are sparse, although *Toxoplasma gondii* has been recently identified as an important pathogen. [22] Members of the *Brucella* genus have long been known to cause

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Table 1. PCR Primers Used for Sequencing of the Omp25 and Omp2a *Brucella* Genes, and the *Stenella spp* GAPDH Gene From Hector's Dolphin Tissues.

Gene Target	Reference Sequence	Primer Name	Nucleotide Position	Primer sequence (3′-5′)	Expected Product Size (bp)
Omp25[a]	GenBank BMU33003	BC1	109	GTTGAAGTAGCTCCCCAGTA	441
		BC2	550	ACTGGGTGTAACGGTACTCA	
		Bcp1a	247	AACTTCCAGAAGGACCAGATCGTA	271
		Bcp3	518	ATGTTGTCCGTCAGCTTGGCTTC	
Omp2a[a]	GenBank BMU26440	Omp2aA	2013	GGCTATTCAAAATTCTGGCG	1232
		Omp2aB	3245	ATCGATTCTCACGCTTTCGT	
GAPDH[b]	GenBank DQ404538	GAPDH-F	396 (7th exon)	CAAGGCTGTGGGCAAGGTCATC	111
		GAPDH-R	497 (7th exon)	TTCTCCAGGCGGCAGGTCAG	

[a]McDonald et al 2006.[17]
[b]Spinsanti et al 2006.[28]

reproductive losses in a range of terrestrial species,[19] and *Brucella*-associated reproductive lesions including abortion, placentitis, metritis, and fetal distress/pneumonia[7,9,18] have been reported in dolphins.

Duignan and coworkers (unpublished) found that 1 of 3 healthy Hector's dolphins sampled during live-capture in 2004 was seropositive for *Brucella abortus* on competitive enzyme-linked immunosorbent assay (ELISA), but it is unknown whether *Brucella* infection causes clinical disease or mortality in Hector's dolphins. The aims of this study were to investigate the prevalence of *Brucella spp* infection in a group of stranded Hector's dolphins; to determine whether *Brucella spp* play a role in morbidity and mortality in this species; and to characterize any *Brucella spp* detected using molecular techniques.

Materials and Methods

Study Population and Samples

Twenty-seven Hector's dolphins, including 2 individuals of the Maui subspecies, were examined. All were found dead around the New Zealand coastline and were submitted for postmortem examination between November 2006 and October 2010. Dolphin tissues were collected under permit from the New Zealand Department of Conservation (Permit Nos. Rnw/HO/2008/03, 35561-MAR and Rnw/22/2003/182). Submission reports and gross necropsy reports were reviewed for all study individuals. The cause of death and concurrent diseases present in 20 of these dolphins were previously published.[22] For the remaining dolphins, the most likely cause of death was determined for each dolphin based on the history, postmortem findings, and histological lesions. Formalin-fixed archived tissues were processed routinely for histological examination, mounted on glass slides, and stained with hematoxylin and eosin (HE). Gross and histological lesions were noted, with particular attention paid to lesions known to be associated with *Brucella spp* infection in other cetacean species, as reviewed by Olsen and Palmer.[19]

Brucella Immunohistochemistry

Where formalin-fixed tissues were available, immunohistochemistry directed against *Brucella spp* antigen was conducted on tissues from dolphins with lesions consistent with *Brucella spp* infection, as follows. Paraffin-embedded tissues were cut at 5 μm, mounted on positively charged glass slides, dewaxed, and hydrated. Antigen retrieval was performed in citrate buffer (pH 6) using a decloaker held at 125°C under pressure for 10 minutes. Endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxide for 30 minutes at room temperature, followed by washing in 0.05 M Tris-buffered saline (TBS; BioRad Laboratories, Hercules, California). Slides were incubated for 60 minutes at room temperature in polyclonal rabbit anti–*B. abortus* antibody (240934-BD Brucella Positive Control antiserum; Becton-Dickinson, Sparks, Maryland) diluted to 1:1000 in TBS. Antigen detection was achieved using the Vectastain Universal Elite kit (Vector Laboratories, Burlingame, California) as per the manufacturer's instructions. The resulting complex was visualized using 3,3′-diaminobenzidine (DAB) diluted in TBS (Liquid DAB Substrate Chromogen System; Dako, Victoria, Australia). Sections were counterstained in hematoxylin and mounted on glass slides. Positive (known *Brucella spp*–positive lesions from harbor seal lung and bison testis; kindly supplied by J. Rhyan, USDA) and negative (omission of primary antibody) control tissues were processed with study tissues.

DNA Extraction and Polymerase Chain Reaction

DNA was recovered from frozen archived samples using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions for fresh tissue. Extracted DNA was stored at –20°C until use. The primers used in subsequent steps are shown in Table 1. The conserved *Brucella spp omp25* gene fragment was amplified by nested polymerase chain reaction (PCR) as follows. A 50-μl PCR mixture containing 1× PCR buffer, 1 U of HotStar Taq Polymerase (Qiagen), 0.2 mM each dNTP (Invitrogen, Carlsbad, California), 1.5 mM $MgCl_2$, 1 μM of each primer (BC1 and BC2,

Table 2. Lesions Suggestive of Brucellosis and Detection of Brucella in Tissues of Hector's and Maui Dolphins.

Dolphin No.	Subspecies	Sex	Age Class	*Brucella*-Like Lesions	One or More Tissues PCR Positive	*Brucella*-Like Lesion PCR Positive	*Brucella*-Like Lesion IHC Positive	Tissues Tested by *Brucella* PCR[a]	Cause of Lesion	Diagnostic Method
1	Hector's	Female	A	Metritis; placentitis	+	+	+	Lu, Li, S, K, **tLN**, aLN, **U**, M	*Brucella spp*	PCR, IHC
2	Hector's	Female	A	Metritis; lymphadenitis; hepatic necrosis	–	–	–	Lu, Li, S, K aLN, U	*Toxoplasma gondii*	PCR, IHC
3	Hector's	Female	A	Metritis	+	–	–	**Li**, S, K, uLN, U	No diagnosis	Lesion negative for *Brucella spp* and *T. gondii* (PCR and IHC); negative aerobic culture and Gram's stain
4	Hector's	Female	A	Lymphadenitis	+	–	–	Li, **S**, K, uLN, **A**	*T. gondii*	PCR, IHC
5	Hector's	Female	A	Meningoencephalitis; myocarditis	+	–	–	Li, S, **K**, tLN, aLN	*Trueperella pyogenes*	Aerobic culture; Gram's stain
6	Hector's	Female	A	Lymphadenitis	–	–	–	Li, aLN, U	*T. gondii*	PCR, IHC
7	Hector's	Female	A	Meningoencephalitis	–	–	–	Lu, Li, K, uLN, U	*Aspergillus fumigatus*	Fungal culture; PCR
8	Hector's	Male	S	Lymphadenitis	–	–	–	Li, S, K uLN	*T. gondii*	PCR, IHC
9	Hector's	Female	A	Blubber abscess	–	–	n/d	Li, S, K, uLN, U, M, BA	Likely parasite granuloma	Lesion negative for *Brucella spp* (PCR)
10	Maui	Male	F	Stillbirth with possible fetal pneumonia	+	+	+	**Li/K**	*Brucella spp*	PCR, IHC
11	Hector's	Male	A	None	+	n/a	n/a	**Li**, S, K, uLN, T	n/a	n/a
12	Hector's	Male	N	None	+	n/a	n/a	Li, S, K, **uLN**, T	n/a	n/a

Abbreviations: A, adult; aLN, abdominal lymph node; F, female; IHC, immunohistochemistry; K, kidney; Li, liver; Lu, lung; M, mammary gland; n/a, not applicable; n/d, not done; PCR, polymerase chain reaction; S, spleen; T, testis; tLN, thoracic lymph node; U, uterus; uLN, unknown lymph node; +, positive; –, negative.
[a]Boldface indicates that tissues were positive on Brucella PCR.

Table 1), and 5 µl of extracted DNA was denatured at 95°C for 15 minutes followed by 30 cycles of 96°C for 40 seconds, 60°C for 60 seconds, and 72°C for 60 seconds, with a final extension step of 72°C for 5 minutes. Nested PCR was conducted using a 50-µl PCR mixture containing 1× PCR buffer, 2 U of HotStar Taq Polymerase (Qiagen), 0.2 mM each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 1 µM of each primer (Bcp1a and Bcp3, Table 1), and 1 µl of DNA from the initial PCR. The nested PCR was conducted with a denaturation step of 95°C for 15 minutes, followed by 30 cycles of 96°C for 40 seconds, 70°C for 60 seconds, and 72°C for 60 seconds, with a final extension step of 72°C for 5 minutes. DNA from a known positive marine-origin *Brucella spp* culture (isolate 02/611 from a human case of osteomyelitis[17]) was used as a positive control, and water blanks were included as negative controls. To confirm successful amplification, 10 µl of each final PCR product was run on a 1.5% agarose gel containing ethidium bromide at 100 V for 45 minutes.

To determine the phylogenetic relationships of the *Brucella spp* detected, attempts were made to amplify the *omp2a* gene as previously described by Cloeckaert et al.[3] Briefly, a 50-µl initial PCR mixture contained 1× PCR buffer (Qiagen), 1 U of HotStar Taq Polymerase (Qiagen), 0.2 mM each dNTP (Invitrogen), 0.75 mM $MgCl_2$, 0.4 µM of each primer (omp2aA and omp2aB, Table 2), and 5 µl of extracted DNA. The PCR was conducted with a denaturation step of 95°C for 15 minutes, followed by 35 cycles of 95°C for 1 minutes, 58°C for 1 minute, and 72°C for 2 minutes, with a final extension step of 72°C for 10 minutes. DNA from the positive marine *Brucella* culture sample 02/611 was used as a positive control, and water blanks were included as negative controls.

To confirm DNA quality and successful extraction from tissue samples, the dolphin species specific housekeeping gene (GAPDH) was amplified from all samples using a technique adapted from that described by Spinsanti et al.[28] Briefly, a 20-µl PCR mixture contained 1× PCR buffer, 1 U of HotStar Taq Polymerase (Qiagen), 0.2 mM each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 0.4 µM of forward primer GAPDH-F, 0.4 µM of reverse primer GAPDH-R (Table 1), and 1 µl of DNA. The PCR was conducted as above, with minor modifications:

Denaturation was for 1 minute, followed by 35 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 15 seconds, with a final extension step of 72°C for 5 minutes. The GAPDH-positive control used was DNA from a well-preserved Hector's dolphin.

Sequencing

Brucella-positive *omp25* PCR amplicon samples ($n = 3$), *omp2a* amplicons ($n = 1$), and a GAPDH-positive Hector's dolphin sample were purified using a PureLink PCR purification kit (Invitrogen) and subjected to automatic dye-terminator cycle sequencing with BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, Carlsbad, California) to confirm genomic sequence using both the forward and reverse primers. The GAPDH and *Brucella* isolate sequences obtained were compared with other published sequences available from GenBank. The GAPDH sequences were confirmed of dolphin origin with a 95% maximum identity to other cetacean species including *Stenella coeruleoalba* (striped dolphin) (GenBank DQ404538.1) and *Neophocaena phocaenoides* (finless porpoise) (GenBank FJ176353.1). *Brucella spp* sequences from the uterus of dolphin No. 1 were submitted to the GenBank database (KX664689 [*omp25*], KX664690 [*omp2A*]).

Culture

Where frozen tissues were available and histological examination demonstrated a bacterial or fungal agent, routine aerobic bacteriological and fungal cultures were performed.

Brucella spp culture was attempted for the following *Brucella* PCR-positive tissues: dolphin No. 1 uterus, dolphin No. 3 liver, dolphin No. 5 kidney, dolphin No. 10 pooled liver and kidney, dolphin No. 11 liver, and dolphin No. 12 lymph node. Approximately 0.1–0.3 g of each tissue was minced and inoculated into *Brucella* enrichment broth (BEB), which was prepared as follows: 15 g *Brucella* broth (Becton, Dickinson and Company) and 490 mL distilled water were combined, mixed, autoclaved, and cooled to 55°C. *Brucella* selective supplement (Oxoid Ltd, Basingstoke, UK) was added and mixed as per the manufacturer's instructions. Finally, 10% sterile inactivated horse serum and 5% sterile dextrose solution (10%) were added to the base broth. As well as BEB, samples were inoculated onto the following solid agars—sheep blood agar (SBA), supplemented chocolate agar, MacConkey agar, and Thayer Marin agar (Fort Richard Laboratories, Auckland, New Zealand)—and incubated at 37°C in CO_2. Solid media were examined after 3 days of incubation with the final read at day 7. Subculture from BEB onto the 4 solid media was performed after 3–7 days depending on turbidity, followed by subculture from BEB to solid media weekly for 6 weeks. Any suspect colonies were subcultured onto SBA incubated at 37°C in CO_2, and basic biochemical testing including Gram's staining, oxidase, indole, catalase (Becton-Dickinson), and urea (Fort Richard Laboratories) was carried out to distinguish between *Brucella* and other species.

Results

Twenty-seven Hector's dolphins were examined, comprising 1 stillborn fetus, 4 neonates, 5 subadults, and 17 adults, including 12 males and 15 females (2 of which were pregnant). Two dolphins belonged to the Maui subspecies (1 stillborn male and 1 pregnant adult female). Details are shown in Table 2 for all individuals that either had lesions that have been associated with brucellosis in other cetacean species ($n = 10$) and/or had at least 1 tissue that tested positive for *Brucella spp* DNA by PCR assay ($n = 7$). Histological details are reported below for the 2 dolphins identified as having active brucellosis. For dolphins that had lesions suggesting brucellosis that were subsequently determined to be of another cause, relevant findings (including histological lesions) are included as Supplemental Material.

Dolphin No. 1 was an adult female pregnant with a 40.5-cm-long, 4.8-kg female fetus in the right uterine horn. The uterus and placental membranes were grossly normal, and the fetus was moderately autolyzed. Histological features of the uterus and allantochorion are shown in Figures 1 and 2. Within the uterus there was stromal edema with infiltration of the uterine glands and stroma by large numbers of neutrophils with fewer plasma cells, macrophages, and lymphocytes. Occasional vascular thrombi were present. A thick layer of exudate and necrotic cellular debris coated the endometrial surface, with multiple small (<2 μm) gram-negative bacilli embedded within the exudate. Similar lesions were present within the allantochorion, being most severe in the fetal villi. Villi were coated with a mat of necrotic exudate and degenerate neutrophils, with rare gram-negative coccobacilli. Sections of allantochorion showed strong multifocal immunolabeling for *Brucella spp* within the suppurative exudate with focally extensive immunopositivity within the stroma of the chorionic plate (Fig. 3). The uterus had multifocal clusters of immunopositive bacteria within exudate (Fig. 4) and occasional immunopositive bacteria and macrophages in the uterine stroma. The liver and kidney from the fetus of dolphin No. 1 were moderately to severely autolyzed, with large numbers of gram-positive bacilli (most likely representing postmortem invasion by *Clostridium spp*) present. Within the lung of the fetus there was increased cellularity of the pulmonary interstitium. Alveoli and bronchioles frequently contained fibrin, large numbers of squames, and low numbers of macrophages (fetal interstitial pneumonia and aspiration) (Fig. 5). No histological abnormalities were detected in other fetal tissues. *Brucella spp*–immunopositive bacteria were present within macrophages in occasional bronchioles and alveoli (Fig. 6).

Tissues from the stillborn male Maui dolphin, dolphin No. 10, were severely autolyzed, obscuring cellular and architectural detail on examination of HE-stained sections (Fig. 7). *Brucella spp*–immunopositive bacteria were present within pulmonary airways, but the degree of autolysis meant that the

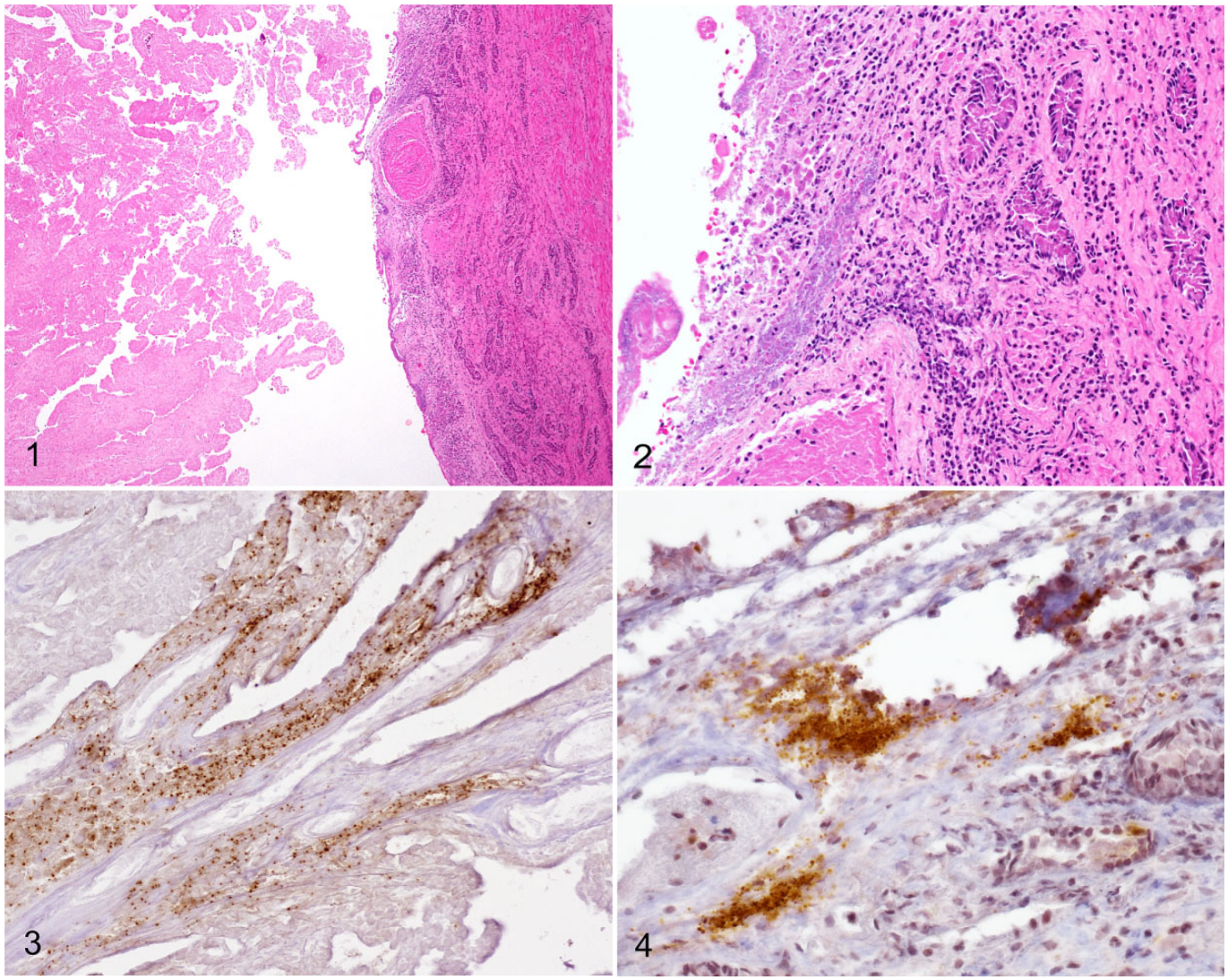

Figure 1. Allantochorion and uterus, Hector's dolphin, dolphin No. 1. The endometrial surface of the uterus (right) is hypercellular, with a mat of exudate coating the surface and a large thrombus. Hematoxylin and eosin (HE). **Figure 2.** Uterus, Hector's dolphin, dolphin No. 1. Necrotic debris, bacteria, and inflammatory cells cover the mucosal surface, and inflammatory cells are present between mucosal glands. HE. **Figure 3.** Chorionic villi, Hector's dolphin, dolphin No. 1. Bacteria within the chorionic stroma are immunopositive (brown staining) for *Brucella abortus*. Immunohistochemistry with diaminobenzidine chromogen. **Figure 4.** Uterus, Hector's dolphin, dolphin No. 1. Bacteria within the exudate are immunopositive for *Brucella abortus*. Immunohistochemistry with diaminobenzidine chromogen.

specific location (intracellular vs extracellular) could not be determined (Fig. 8).

Overall, *omp25* PCR assays were conducted on a total of 124 tissues from 27 individuals. Five of the 10 dolphins with *Brucella*-type lesions had at least 1 tissue test positive for *Brucella* on PCR, but in only 1 dolphin was the relevant tissue positive: the uterus of dolphin No. 1, a pregnant female Hector's dolphin with metritis and placentitis. In dolphin No. 10, a stillborn Maui dolphin fetus, pooled liver and kidney were positive for *Brucella* on PCR, although the degree of autolysis prevented meaningful histological interpretation. In both of these dolphins, the presence of *Brucella spp* antigen was confirmed using immunohistochemistry. Other causal agents were confirmed in the remaining 8 dolphins with *Brucella*-like lesions (see Table 2 and Supplemental Material).

Three *Brucella spp* PCR amplicons (dolphin No. 1 uterus, dolphin No. 3 liver, and dolphin No. 4 spleen) were sequenced and showed 99% sequence homology to GenBank submissions from *omp25* genes from multiple *Brucella* species including *B. pinnipedialis* B2/94 (CP002078.1), *B. canis* (EU001647.1), *B. suis* (EU001656.1), and *B. microti* (CP001578.1). Only 1 product (dolphin No. 1 uterus) was amplified using the *omp2a* gene. This amplicon had 100% homology with the NZ human isolate 02/611 (GenBank DQ865280) and the bottlenose dolphin F5/99 (DQ865282).

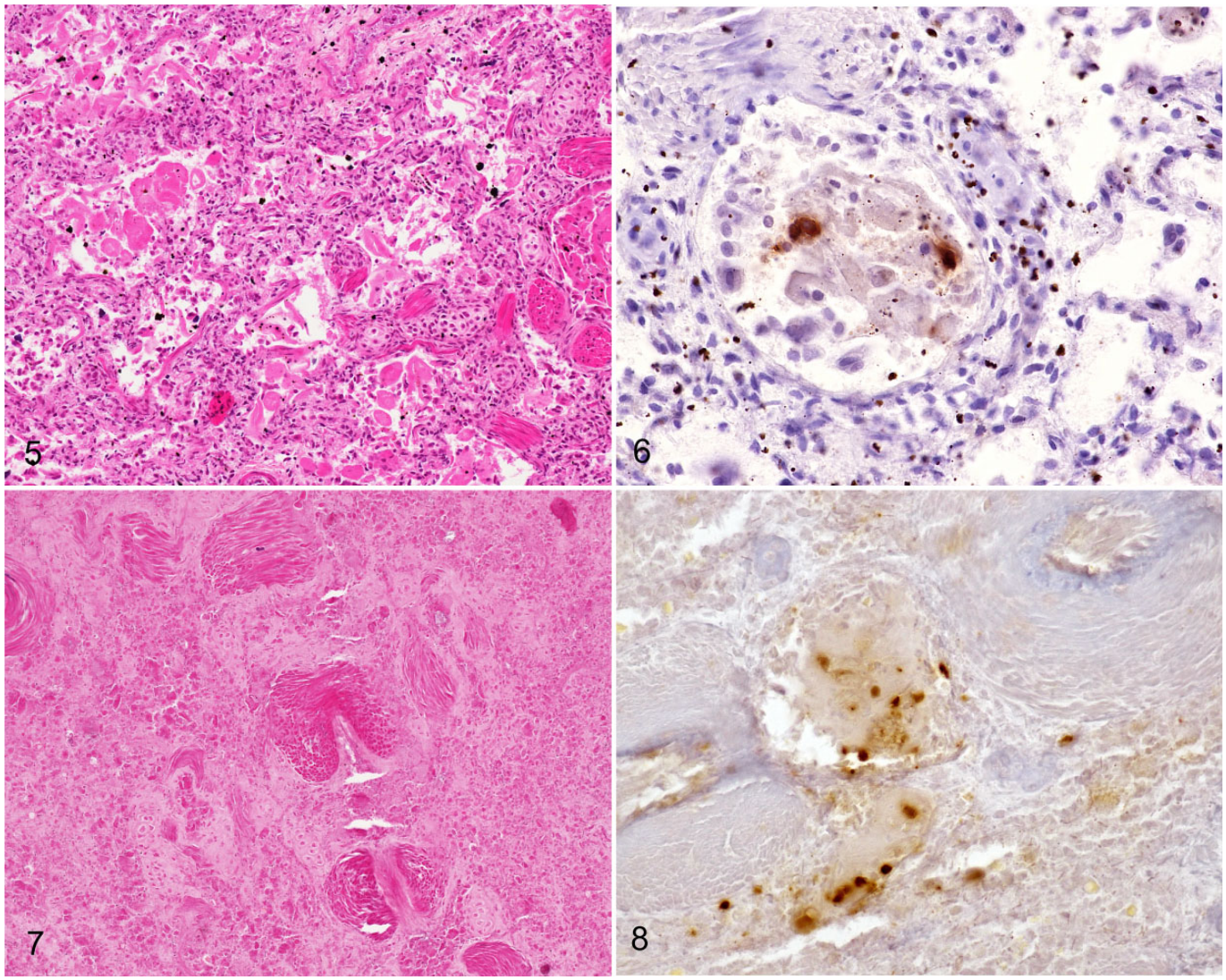

Figure 5. Fetal pneumonia and aspiration, lung, Hector's dolphin, fetus of dolphin No. 1. Squames and fibrin are present within alveoli, and the interstitium is hypercellular. Hematoxylin and eosin (HE). **Figure 6.** Fetal pneumonia and aspiration, lung, Hector's dolphin, fetus of dolphin No. 1. Bacteria within airways are immunopositive for *Brucella abortus*. Immunohistochemistry with diaminobenzidine chromogen. **Figure 7.** Lung, stillborn Maui dolphin, dolphin No. 10. There is increased eosinophilia and loss of cellular detail, reflecting autolysis and obscuring detailed histological interpretation. HE. **Figure 8.** Lung, stillborn Maui dolphin, dolphin No. 10. Despite autolysis, *Brucella*-immunopositive bacteria are visible within airways and vessels. Immunohistochemistry with diaminobenzidine chromogen.

No *Brucella spp* were cultured from any of the 6 dolphins tested. Results for other bacterial and fungal cultures are shown in Table 2.

Discussion

Seven of 27 (26%) dolphins examined in this study had 1 or more tissues that were PCR-positive for *Brucella spp.* Two dolphins (7%), 1 female with metritis and placentitis and 1 stillborn Maui dolphin fetus, were diagnosed as having active brucellosis, based on the presence of *Brucella spp* antigen (demonstrated by immunohistochemistry) and DNA (demonstrated by PCR assay) within relevant organs. Brucellosis has been well described as a cause of placentitis and abortion in terrestrial species, but only 3 cetacean cases were found in the published literature, including 2 captive bottlenose dolphins[18] and a striped dolphin.[9] Lesions of *B. abortus* abortions in domestic cattle[32] and bison[21] involve necrotizing and suppurative placentitis and endometritis with immunopositive bacteria in placental and uterine exudate. Strong immunopositivity of placental and uterine epithelial cells is a common feature and is believed to be associated with specific tissue tropism of the causal agent.[21,32] Necrotizing and suppurative placentitis was present in the previously published cetacean cases[9,18] as well as in dolphin No. 1 in the current study. Strong immunostaining of epithelial cells was not detected in the cetacean cases.

Characterization of fetal lesions associated with *Brucella* abortions is frequently hampered by autolysis. Bronchointerstitial

pneumonia, fibrinous pleuritis, and fibrinous pericarditis are the most frequently reported histological lesions in aborted domestic cattle[32] and bison[21] fetuses. Immunolabeled bacteria can be seen in fetal lungs, but most fetuses have no gross lesions.[21,32] Of the 3 published cetacean fetal deaths in early studies,[9,18] as well as in the 2 Hector's dolphins reported here, no gross fetal lesions were present. Histological examination of 2 captive bottlenose dolphin fetuses was not attempted due to autolysis,[18] and no histological lesions were present in a striped dolphin fetus.[9] A more recent study[4] found evidence of fetal distress and pneumonia due to in utero *Brucella spp* infection in 5 perinatal bottlenose dolphins. Histologically, these animals had aspiration of meconium and squames, infiltration of inflammatory cells into alveolar spaces and septa, and follicle-like aggregates of lymphocytes. *Brucella*-immunopositive bacteria were present in inflammatory cells and within alveoli.[4] In the current study, *Brucella*-immunopositive bacteria were present within airways of both the fetus (dolphin No. 1) and the stillborn dolphin (dolphin No. 10). In dolphin No. 1, these bacteria were associated with pneumonia and fetal distress, characterized by alveolar septal inflammation, accumulation of inflammatory cells and fibrin in the alveolar spaces, and aspiration of squames. Lymphoid follicles were not a feature in this dolphin. While autolysis prevented histological interpretation for dolphin No. 10, demonstration of the presence of *Brucella* in this stillborn fetus using both PCR and IHC was considered to justify a diagnosis of brucellosis.

In 5 of the 7 (71%) PCR-positive dolphins in this study, demonstration of *Brucella spp* DNA within a tissue was not associated with histological evidence of active disease, suggesting that these represent subclinical infections or carrier status, as appears to be common in cetaceans.[11] Infected Hector's dolphins had a small proportion of positive tissues, similar to findings from some culture-based studies in other marine mammals[6,20] but in contrast to findings from a subadult female Hector's dolphin, where all 6 tissues tested were PCR positive (Duignan, unpublished). Although subcutaneous (blubber) abscesses from several species have yielded *Brucella* organisms in culture,[6,10,24] the 1 blubber abscess in this study was PCR-negative.

For both dolphins presented here, *omp25* gene sequencing showed that the marine mammal *Brucella* species showing closest similarity to the causal isolate was *B. pinnipedialis*. This is uncommon for cetacean isolates, where the most extensive study published to date found that 100% of 102 isolates from cetaceans clustered with *B. ceti.*[16] All of these latter isolates, however, were from Atlantic-origin animals, and recent studies suggest the possible existence of a third species of marine-origin *Brucella spp*, which may be confined to the Pacific region.[2,16,31] Few Pacific origin marine *Brucella spp* isolates have been well characterized, but typing of 3 naturally acquired human cases and 1 common dolphin isolate found a unique IS711 chromosomal location in all 4, corresponding with the multilocus variable-number tandem-repeat analysis (MLVA) ST27 genotype.[2] An isolate from an aborted Pacific-origin bottlenose dolphin fetus (strain F5/99) was also identified as ST27 genotype.[6,29] Two of 4 reported human cases of marine mammal origin brucellosis were diagnosed in Peruvian nationals with neurological lesions,[27] while a third case was from a New Zealand man with spinal osteomyelitis.[17] None of these people had direct contact with marine mammals, but all 3 regularly consumed raw seafood, suggesting carriage by a nonmammalian marine vector species.[17,25,31] *Omp2a* sequencing of the isolate from the uterus in dolphin No. 1 presented here showed 100% homology with the bottlenose dolphin isolate and the New Zealand human isolate (02/611), consistent with the hypothesis that a distinct species may be present in the Pacific region. The only other published case of human brucellosis due to a marine-mammal origin strain was a laboratory-acquired infection by an ST23 isolate[1] and was mild, suggesting increased pathogenicity of the ST27 type in humans.[31]

Full genetic characterization of Hector's dolphin *Brucella spp* has been hampered by a failure to culture these bacteria. This could be in part be due to postmortem degeneration of tissues and overgrowth by postmortem contaminants that occurs because of delays in processing imposed by the logistics of recovery and transport. Colegrove et al[4] identified similar issues associated with failure to culture *Brucella spp* in cetaceans diagnosed with brucellosis via histology, immunohistochemistry, and PCR. Future studies in Hector's dolphins should focus on improving these processes, where possible, to optimize the chance of obtaining good quality DNA and enabling MLVA typing.

While it appears from the findings presented here that the majority of *Brucella spp* infections in Hector's dolphins are subclinical, the true prevalence in this species remains unknown. Furthermore, the finding of reproductive brucellosis in 1 Hector's and 1 Maui dolphin may have implications for the species in general, and more specifically for recovery of the critically endangered Maui dolphin subspecies, since infected animals would be less likely to successfully reproduce.

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Declaration of Conflicting Interests

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