NOTE

Coinfection by *Streptococcus phocae* and cetacean morbillivirus in a short-beaked common dolphin *Delphinus delphis*

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ABSTRACT: We describe gross, histopathological, and immunohistochemical features of *Streptococcus phocae* and cetacean morbillivirus coinfection in a short-beaked common dolphin *Delphinus delphis*. Major gross findings were cutaneous purulent nodules in the tail fluke, vegetative mitral valve endocarditis, and presumed postpartum pyometra. Histologic examination revealed bacterial septicemia characterized by widespread intravascular coccoid bacterial emboli. These were associated with fibrinonecrotizing to pyogranulomatous dermatitis and panniculitis, embolic pneumonia, neutrophilic and lymphoplasmacytic meningochoroiditis, random neutrophilic hepatitis, lymphoplasmacytic myocarditis and epicarditis, necrotizing adrenalitis, suppurative endometritis, and multicentric reactive lymphadenopathy. Bacteriology and molecular analysis with sequencing of the 16S rRNA gene identified *S. phocae* from lung, brain, and adrenal gland tissue. Immunohistochemical analysis for morbillivirus detection revealed positive immunolabeling in the epithelium of the choroid plexus of the fourth ventricle. Published reports on *S. phocae* infection in cetaceans are rare, and pathological details are limited. The present case indicates that *S. phocae* has potential pathogenic capacity in common dolphins. The pathogenesis is proposed to have involved cutaneous penetration after a skin trauma, leading to initial cutaneous disease and eventual systemic infection.

KEY WORDS: Bacteremia · Cetacean pathology · Coinfection · Immunohistochemistry · Morbillivirus · *Streptococcus phocae* · Zoonosis

INTRODUCTION

Bacterial disease is a major ailment in marine mammals, causing worldwide morbidity and mortality (Dunn et al. 2001). Members of *Streptococcus* are among the most commonly reported pathogens in pinnipeds and cetaceans (Dunn et al. 2001). These bacteria have been isolated from apparently healthy individuals and are therefore considered commensal or non-pathogenic, although in other instances, they have been associated with significant pathological changes, e.g. *S. agalactiae* (Evans et al. 2006), and

may even show zoonotic potential, e.g. *S. iniae* (Agnew & Barnes 2007) and *S. agalactiae* (Evans et al. 2008). Although *S. phocae* is recognized as an important pathogen for different pinniped, mustelid, and aquaculture fish species (Bartlett et al. 2016), scarce pathological data on cetacean species exist (Raverty et al. 2004, Taurisano et al. 2015, Lair et al. 2016).

Cetacean morbillivirus (CeMV) is considered one of the most important viral pathogens in cetaceans. Currently, there are 3 well-characterized strains (porpoise morbillivirus, dolphin morbillivirus, pilot whale morbillivirus), and 3 new strains from Hawaii, USA (West et al. 2013), Brazil (Groch et al. 2014), and Australia (Stephens et al. 2014). Studies suggest different species-specific susceptibility to morbillivirus infection in cetaceans. Bottlenose dolphins *Tursiops truncatus*, harbor porpoises *Phocoena phocoena*, long-finned pilot whales *Globicephala melas*, short-beaked common dolphins *Delphinus delphis*, and striped dolphins *Stenella coeruleoalba* are susceptible to epizootics of lethal disease (Van Bressem et al. 2014). Furthermore, serological studies lend support for endemic disease in certain species in the Atlantic and Pacific Oceans, which may act as reservoirs and vectors of infection to susceptible species (Duignan et al. 1995, Van Bressem et al. 2014, van Elk et al. 2014).

CeMV may cause severe respiratory, lymphoid, and central nervous system (CNS) disease in susceptible species, leading to strandings and death. One of the major morbilliviral pathogenic mechanisms is immunosuppression, which allows preexisting infections to be exacerbated and secondary infections to spread, often with fatal consequences (Domingo et al. 1992, Van Bressem et al. 2014).

This report describes the gross, histopathological, and immunohistochemical features of *Streptococcus phocae* and CeMV coinfection in a short-beaked common dolphin. To our knowledge, no previous record of coinfection between these 2 agents has been reported in a cetacean species.

MATERIALS AND METHODS

An 88 kg, 198 cm long, adult female short-beaked common dolphin in fair body condition stranded alive in La Graciosa, Canary Islands (29° 8′ 24″ N, 13° 28′ 48″ W) on 26 November 2007. Though initially sighted with a calf, the adult died shortly after stranding alone and was submitted for necropsy within 24 h postmortem.

A complete and standardized necropsy was performed. Representative samples of skin, longissimus dorsi and rectus abdominis muscles, peritoneum, diaphragm, brain, pterygoid sacs, tympanoperiotic complexes, tongue, oral mucosa, pharyngeal and laryngeal tonsils, esophagus, stomach, intestine, liver, spleen, pancreas, trachea, lungs, heart, aorta, adrenal glands, kidneys, ureters, urinary bladder, lymph nodes, ovaries, uterus, vagina, and vulva were collected and fixed in 4% neutral buffered formalin. All tissues were processed routinely and embedded in paraffin, and 5 µm sections were stained with hematoxylin and eosin (H&E) for histologic analysis. Selected sections were also stained with Gram/Twort stain.

Immunohistochemical (IHC) analysis for morbillivirus was carried out on formalin-fixed, paraffin-embedded (FFPE) lung, kidney, and brain tissue sections. The primary morbilliviral antibody (a mouse monoclonal IgG2B [kappa light chain] against the nucleoprotein [N] antigen of CDV [canine distemper virus]; Veterinary Medical Research & Development) was used, as previously described (Stone et al. 2011). Positive tissue controls consisted of FFPE sections of canine brain with CDV-inclusion bodies. As a negative control, the primary antibody was eliminated and substituted with normal mouse serum. Molecular detection of CeMV (muscle, liver, kidney, brain) employed a nested-PCR method (Sierra et al. 2014). CeMV IHC and molecular analysis results on this individual have already been published (Case No. 6 in Sierra et al. 2014).

For bacteriology analysis, fresh lung, brain, and adrenal gland tissues were cultured on Columbia 5% sheep blood agar and Columbia naladixic acid agar plates (bioMérieux) that were incubated at 37 ± 5°C for 24 h under aerobic and anaerobic (with 4−10% CO_2 using a BBL GasPak Plus system (BD Diagnostics) conditions. Identification was achieved by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using the BioTyper system (software version 3.1; Bruker-Daltonics) according to the manufacturer's instructions, and identification was further molecularly confirmed by sequencing of the 16S rRNA gene of clinical isolates. The 16S rRNA gene of each isolate was amplified by PCR and further sequenced, as described previously (Vela et al. 2011). The determined sequences consisted of about 700 nucleotides and were compared with the sequences of other Gram-negative, catalase-negative, and oxidase-positive species available in GenBank, using the FASTA program.

RESULTS

On necropsy examination, major gross findings included multifocal 3 to 8 cm diameter, firm, raised, and occasionally ulcerated purulent subcutaneous nodules along the ventral, dorsal, and cranial edge of the caudal fluke, bilaterally, and more prominently at its insertion with the peduncle (Fig. 1A). These foci of inflammation multifocally involved the primary and secondary arterial branches of the fluke and their respective associated periarterial venous rete. We also noted vegetative endocarditis of the mitral valve with multifocal hemorrhage in the papillary muscles (Fig. 1B), and postpartum pyometra and vaginitis (Fig. 1C) were observed. Additional findings included pulmonary edema, generalized lymphadenomegaly, and splenomegaly and ulcers in the keratinized and pyloric gastric compartments. Parasitosis included severe subcutaneous infestation by merocercoids of *Phyllobotrium delphini* in the anogenital and peduncle area, and severe biliary tree infestation by trematodes (Brachycladiidae sp.).

Histologically, the main lesions comprised multifocal fibrinonecrotizing to pyogranulomatous dermatitis and panniculitis with leukocytoclastic vasculitis, vascular fibrinoid degeneration and necrosis, thrombosis, perivascular hemorrhage, and numerous intralesional and intravascular Gram-positive coccoid bacteria, typically forming clusters (Fig. 1D); multifocal neutrophilic embolic pneumonia with coccoid bacterial emboli (CBE); focally extensive neutrophilic and lymphoplasmacytic meningochoroiditis (fourth ventricle) with CBE (Fig. 1E); multifocal random neutrophilic hepatitis with single hepatocellular necrosis; multifocal lymphoplasmacytic myocarditis and epicarditis with CBE; multifocal lymphoplasmacytic and necrotizing adrenalitis with vascular wall necrosis and coagulative parenchymal necrosis and CBE; multifocal suppurative endometritis (pyometra) with hemorrhage; multifocal lymphoplasmacytic vaginitis; and multicentric lymphoid hyperplasia with lymphocytolysis, sinus histiocytosis, erythrocytosis, and neutrophilia with occasional CBE. No viral inclusions were noted in examined tissue sections.

Lesions compatible with a live-stranding event included acute cardiomyocyte and skeletal myocyte degeneration and necrosis; acute tubular degeneration and necrosis; and hepatocellular cytoplasmic hyaline globules with 'pink points.' Histologically confirmed parasitosis included proliferative and necrotizing cholangitis and pancreatic ductitis with intralesional adults and trematode eggs (Brachycladiidae sp.), and pulmonary and mesenteric granu-lomatous lymphadenitis with intralesional (unidentified) nematode larvae.

Beta-hemolytic, catalase-negative, Gram-positive cocci were isolated in pure culture from the lung, brain, and adrenal gland tissues, and no other bacteria were isolated. The 3 isolates were identified by MALDI-TOF MS as *Streptococcus phocae* (score: >2.0). Definitive molecular identification was performed by sequencing the 16S rRNA gene (>700 nucleotides) of each isolate. The 16S rRNA gene analysis revealed that the 3 isolates were genotypically identical, displaying 99.9% similarity with that of the type strain of *S. phocae* CCUG 35103T (accession number AJ621053).

IHC revealed positive diffuse and finely granular immunolabeling in the cytoplasm of the epithelial cells of the choroid plexus of the fourth ventricle. No expression of morbilliviral antigen was detected anywhere else in the CNS, suggesting a hematogenous route (Sierra et al. 2014). Negative controls did not exhibit any detectable antigen. Molecular analysis for CeMV detection was negative. Based on the above analytical results, septicemia by *S. phocae* and concomitant morbilliviral infection confined to the brain was determined.

DISCUSSION

In the present case, *Streptococcus phocae* is considered the main cause of morbidity and mortality of this animal, and given the severity, extent, and chronicity of skin lesions, a cutaneous route of entry for this bacterium is favored. Endometritis and vaginitis were considered less likely sources in this case. First isolated in 1994 from seals, *S. phocae* has subsequently been isolated from a variety of pinnipeds and otters (Bartlett et al. 2016), and is currently recognized as an emergent pathogen in salmonid culture (Romalde et al. 2008). To our knowledge, published reports of *S. phocae* in cetaceans are rare and include 7 harbor porpoises (Raverty et al. 2004, Taurisano et al. 2015) and a beluga *Delphinapterus leucas* (Lair et al. 2016), and pathological descriptions are limited. Furthermore, other *Streptococcus* species have been associated with diverse pathology in cetaceans, e.g. bronchopneumonia, metritis, sepsis, and mortality after cutaneous wounding (Higgins et al. 1980, Dunn et al. 2001). Beta-hemolytic *S. iniae* has been isolated from cutaneous abscesses in Amazon River dolphins *Inia geoffrensis* (Bonar & Wagner 2003), where it is coined 'golf ball disease.' *S. agalactiae* has been associated with
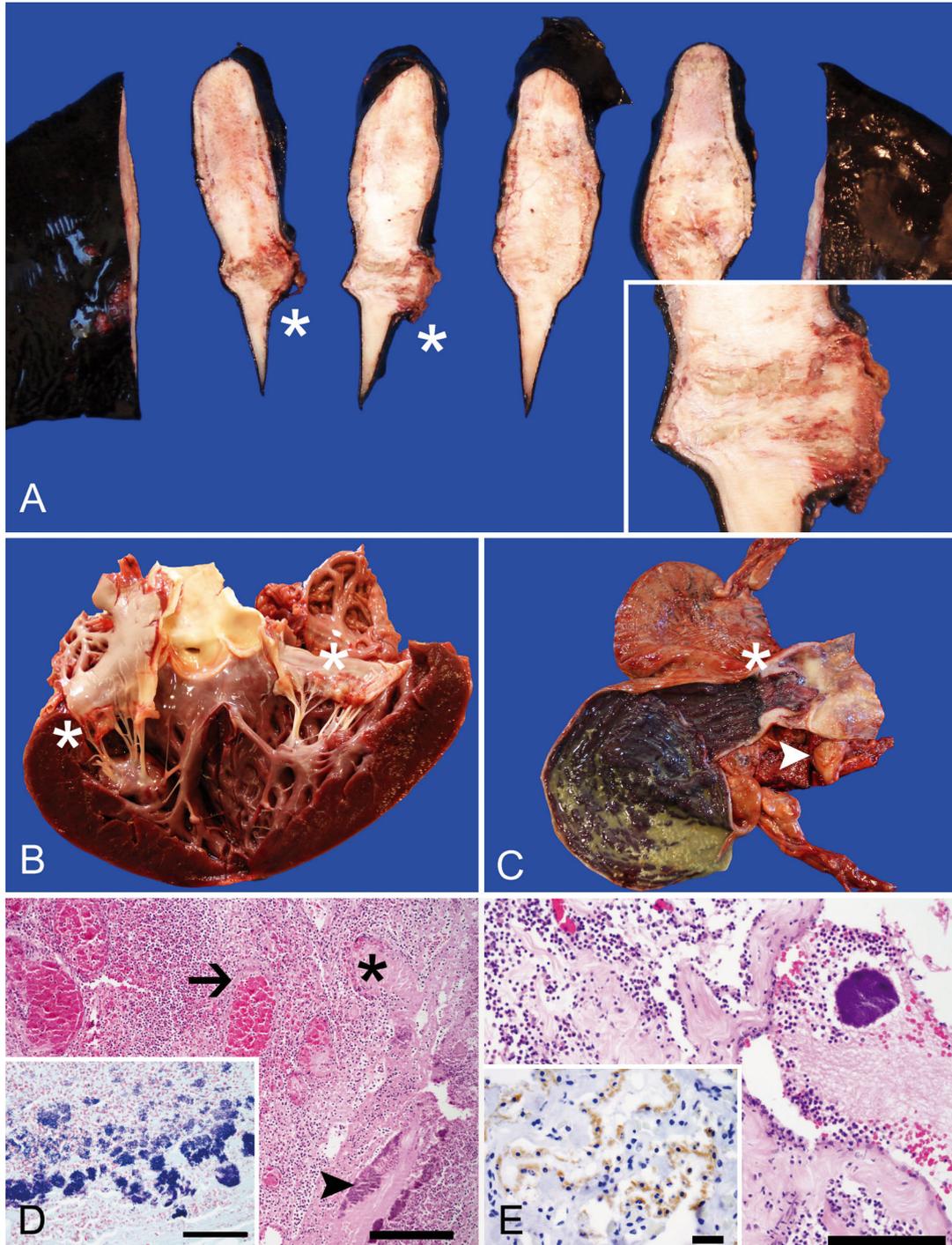
Fig. 1. (A,B,C) Macroscopic and (D,E) microscopic lesions found in a short-beaked common dolphin *Delphinus delphis*. (A) Transverse sections of the caudal fluke. Connective tissue and dermis are expanded by coalescing purulent nodules that occasionally contain draining tracts (fistula: asterisks; inset: close-up view of the process extending transmurally). (B) Mitral valve vegetative endocarditis (asterisks). (C) Uterus with dissection of the left uterine horn, cervix (asterisk), and proximal vagina showing pyometra; an enlarged para-salpingeal lymph node is visible (arrowhead). (D) Dermal focus of dense pleo-cellular inflammatory infiltrate largely composed of degenerate and viable neutrophils and macrophages, intermingled with abundant fibrin, karyorhectic cellular debris, and multifocal coccoid bacterial colonies (arrowhead; inset: detail of a bacterial cluster). Within inflamed areas, the microvasculature is frequently obliterated by fibrinocellular thrombi (asterisk) and there is fibrinoid degeneration and vascular wall necrosis (arrow). (E) Connective tissue of the cerebral leptomeninges is infiltrated by lymphocytes, plasma cells, and neutrophils. A small vein contains a luminal coccoid bacterial embolus. Inset: Epithelial cells lining the choroid plexus have moderate, finely granular, diffuse cytoplasmic positive immunolabeling for canine distemper virus-mAb. Scale bars = (D) 100 µm. Inset in (D) = 50 µm; (E) 50 µm. Inset in (E) = 20 µm

necrotizing fasciitis and myositis in a captive common bottlenose dolphin (Zappulli et al. 2005) with disseminated infection. Other examples include *S. equi* in a long-finned pilot whale with bronchopneumonia (Higgins et al. 1980) and *S. zooepidemicus* and several unspecified β-hemolytic streptococci in harbor porpoises and bottlenose dolphins variably associated with abscesses, bronchopneumonia, septicemia suppurative leptomeningitis, pyelonephritis, myocarditis, and osteomyelitis (Dunn et al. 2001).

Some streptococcal bacteria may be considered commensal with opportunistic pathogenic capacity in marine mammals. In cetaceans, different streptococci have been isolated from healthy and diseased organs, most commonly the skin, blowhole, trachea, lungs, pharynx, and uterus (Higgins et al. 1980, Bonar & Wagner 2003). These organs may provide clues as to the route of entry and transmission. In the present case, the pathogenesis is proposed to have involved cutaneous penetration after a skin trauma, as suggested by distribution, severity, extent, and chronicity of cutaneous lesions, and subsequent disseminated disease. These findings are in agreement with classic disease patterns observed in group B streptococci infections and recent observations in *S. phocae*-infected southern sea otters, wherein skin trauma was identified as the single greatest risk factor for infection (Bartlett et al. 2016). Two major initiating events are hypothesized: a direct transmission of *S. phocae* from an infected carrier and an opportunistic skin or genital infection (*S. phocae* as 'normal flora') on an immunocompromised host. In the present case, changes in the lymph nodes suggested appropriate reactive response to systemic inflammation. *S. phocae* has been recognized as a secondary and opportunistic pathogen in several marine mammal species (Johnson et al. 2006). Furthermore, studies suggest certain streptococcal infections in cetaceans occur after the ingestion of contaminated material, e.g. fish (Bromage & Owens 2002, Bonar & Wagner 2003, Evans et al. 2006). Disease outbreak in fish is epidemiologically significant with potential to affect other aquatic cohabitants (Bromage & Owens 2002). Additionally, an infected marine mammal could transmit bacteria to other susceptible marine mammal species or fish by shedding (Swenshon et al. 1998). Although potential horizontal intra- or interspecies transmission might not be a serious threat in free-ranging marine mammal species, the risk could be significant in captivity.

In the present case, histopathological, IHC, and molecular results diverge from any previously reported CeMV-associated disease (CeMVAD) presentation (Van Bressem et al. 2014). The virus might have reached the CNS hematogenously without spreading through the cerebrospinal fluid (Sierra et al. 2014). These features may suggest very early disease (diverging from traditional 'acute form'), subclinical disease, or an 'atypical infection.' However, the pathogenic participation of CeMV in this case remains obscure. Interestingly, the existence of potential subclinical CeMV infection and reduced neurovirulence in common dolphins and other species has been previously raised (Reidarson et al. 1998, Bossart et al. 2011, van Elk et al. 2014)

The pathogenesis of CeMVAD is not fully resolved. Cetaceans that survive the acute stage of CeMV infection may succumb to virus-associated sequelae or non-lethal opportunistic infections, including viruses (e.g. *Herpesvirus*, *Papillomavirus*), bacteria (e.g. *Photobacterium damselae, Brucella* sp.), parasites (e.g. *Toxoplasma gondii, Halocercus lagenorhynchi*), and fungi (e.g. *Aspergillus* sp., [*A. fumigatus*], *Lacazia loboi/Paracoccidioides brasiliensis*) (Bossart et al. 2011, Casalone et al. 2014, West et al. 2015, Cassle et al. 2016). *S. phocae* was reported as a concomitant pathogen in 3 Caspian seals *Pusa* [*Phoca*] *caspica* involved in the canine distemper epidemic in the Caspian Sea in 2000 (Kuiken et al. 2006). Such a role for this bacterium has not been identified in a cetacean species to date.

Our observations indicate that *S. phocae* has potential pathogenic capacity in common dolphins. Given the severity, extent, and chronicity of cutaneous lesions in this case, the pathogenesis is proposed to have involved cutaneous penetration after a skin trauma, leading to initial cutaneous disease and eventual systemic infection. *S. phocae* should be added to the growing list of pathogenic bacteria capable of localized or disseminated infection in cetacean species, and of coinfection with CeMV.

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