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NOTE

Pyogranulomatous obliterative laryngotracheitis by *Rhizopus arrhizus* (syn. *R. oryzae*) in a free-ranging Atlantic spotted dolphin *Stenella frontalis*

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ABSTRACT: We report the gross and microscopic findings and molecular identification of fungal hyphate infection in a juvenile female Atlantic spotted dolphin *Stenella frontalis* found dead off Arguineguin, Gran Canaria (Canary Islands, Spain). On necropsy examination, the animal had a large cranial intrathoracic mass and multiple variably-sized nodules throughout the larynx and trachea that obliterated the lumen. Microscopically, the masses were composed of abundant pyogranulomatous inflammation with numerous fungal hyphae. These were pauciseptate (coenocytic) and had non-parallel walls, non-dichotomous irregular to right angle branching, and bulbous dilations. PCR analysis from these inflammatory foci yielded *Rhizopus arrhizus* (syn. *R. oryzae*). This fungal pathogen is often ascribed to opportunistic infections in immunosuppressed humans and animals. In the present case, a potential cause for immunosuppression was not identified; PCR analysis for cetacean morbillivirus was negative. Herein, we report the first confirmed case of *R. arrhizus* infection in a free-living Atlantic cetacean. These findings add to the body of knowledge on fungal disease in cetaceans in general and, in particular, in odontocetes, where respiratory involvement is common.

KEY WORDS: Cetacean pathology · Fungal infection · Marine mammal · Mycosis · Zygomycetes

INTRODUCTION

The Atlantic spotted dolphin *Stenella frontalis* is an odontocete widely distributed along the tropical and warm-temperate Atlantic, from the Gulf of Mexico to Rio Grande do Sul (Brazil) to Senegal (Africa) (Perrin 2002). This species is listed as Data Deficient on the IUCN Red List of Threatened Species. Reported anthropogenic threats to *S. frontalis* mainly include bycatch (Perrin et al. 1994), entanglement, and direct killing by firearm or fishing artifacts (Mignucci-Giannoni et al. 1999). Furthermore, natural causes of disease in *S. frontalis* may include bacteria, e.g. *Erysipelothrix rhusiopathiae* (Díaz-Delgado et al. 2015a), parasites, e.g. *Toxoplasma gondii* (Arbelo et al. 2013), and fungi, e.g. *Aspergillus* spp. (Groch et

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al. 2018, Reidarson et al. 2018). There are also some reports of neoplastic disease processes (Díaz-Delgado et al. 2015b,c).

Respiratory disease of infectious origin is one of the most common ailments in both free-ranging and captive delphinids (Reidarson et al. 2018). Parasitic and bacterial infections are by far the most common reported causes, mainly represented by Halocercidae and Pseudaliidae nematodes and many different bacteria (Reidarson et al. 2018). Additionally, viral pathogens such as cetacean morbillivirus (CeMV) (Domingo et al. 1992), herpesvirus (Esperón et al. 2008), parainfluenza (Nollens et al. 2008), and polyomavirus (Anthony et al. 2013) have been linked to variable respiratory compromise in cetaceans. Furthermore, fungal infections of the upper and lower respiratory tract have increasingly been recognized (Delaney et al. 2013, Grattarola et al. 2018, Reidarson et al. 2018). Nonetheless, there is a dearth of knowledge on their etiopathogenetic and immunopathogenetic mechanisms. Very few reports of fungal disease in *S. frontalis* are available (Groch et al. 2018). The present study aimed to describe the gross and microscopic findings, and molecular identification of a poorly known fungal pathogen in cetaceans, *Rhizopus arrhizus* (syn. *R. oryzae*), in a free-ranging *S. frontalis.*

MATERIALS AND METHODS

A 30 kg, 1.46 m long, juvenile female *Stenella frontalis* in poor nutritional status and moderate autolysis was found by a scuba diver at 40 m depth off Arguineguin, Gran Canaria (Spain; 27°48′ 8″ N, 15°45′ 48″ W). The carcass was retrieved and submitted for necropsy at the School of Veterinary Medicine, University of Las Palmas of Gran Canaria. The age of the animal was determined based on morphometrics and gross and histological gonad development (Geraci & Lounsbury 2005). Representative tissue samples from major organs (aorta, diaphragm, esophagus, heart, keratinized, glandular, and pyloric stomachs, intestines, adrenal glands, liver, pituitary, tongue, preescapular, tracheobronchial, pulmonary, mesenteric, and mammary lymph nodes, spinal cord, skeletal muscle, middle and inner ears, eye, lungs, rete mirabile, ovaries, pancreas, skin, blubber, thymus, thyroid, tonsils, trachea, uterus, vagina, kidneys, urinary bladder, spleen, cerebrum, cerebellum, brainstem, and mammary gland) were collected and fixed in 10% neutral buffered formalin. All tissues were processed routinely and embedded in paraffin wax, and 5 µm sections were stained with hematoxylin and eosin for microscopic analysis. Selected tissue sections of larynx and tracheal masses were also stained with Grocott and Gomori methenamine silver (GMS), periodic acid-Schiff (PAS), and Gram/Twort stains to better characterize the microscopic findings.

For molecular analysis of fungi, formalin-fixed paraffin-embedded (FFPE) tissue sections from the tracheal masses were used for a pan-fungal PCR using published primers (Rakeman et al. 2005) targeting fungal ribosomal RNA genes. Additionally, samples were assessed using published broad-range fungal primers (Rakeman et al. 2005) targeting the ITS2 region of the ribosomal RNA genes followed by a nested PCR with a mixture of Zygomycetes-specific primer set (Sivagnanam et al. 2017). The amplified products were separated by electrophoresis, and those of expected molecular size were sequenced to identify the zygomycete species. The assembled sequence was compared with sequences in GenBank by performing a BLAST search.

Given the known immunosuppressive effects on the host and occasional reports linking CeMV with fungal coinfections (Domingo et al. 1992), a 1-step real-time RT-PCR method to detect a partial fragment of the fusion protein (F) gene of CeMV (Sacristán et al. 2015) was performed on FFPE tissue sections of brain, pulmonary lymph nodes, and lung. Additionally, CeMV immunohistochemical (IHC) investigation was performed on brain, lymph nodes, and lung tissue sections. A monoclonal antibody raised against the nucleoprotein of canine distemper virus (Veterinary Medical Research and Development) known to react with dolphin morbillivirus and pilot whale morbillivirus was used as primary antiserum (Sierra et al. 2016). Tissue sections in which the primary antibodies were replaced by nonimmune homologous serum served as negative controls.

RESULTS

Major gross pathologic findings were confined to the thoracic cavity. Within the cranial mediastinum, there was a 15.5 × 12.4 × 8.7 cm tan, lobulated, expansile, and slightly infiltrative mass. It adhered dorsally to the ventral aspect of the esophagus, encroached the trachea and the cranial portion of the right lung, and infiltrated and obliterated the right accessory bronchus (Fig. 1A). On cut surface, the mass had a nodular appearance with multifocal foci of necrosis. A cranial mediastinal lymph node was markedly enlarged (13 × 13 × 10 cm) and firm and

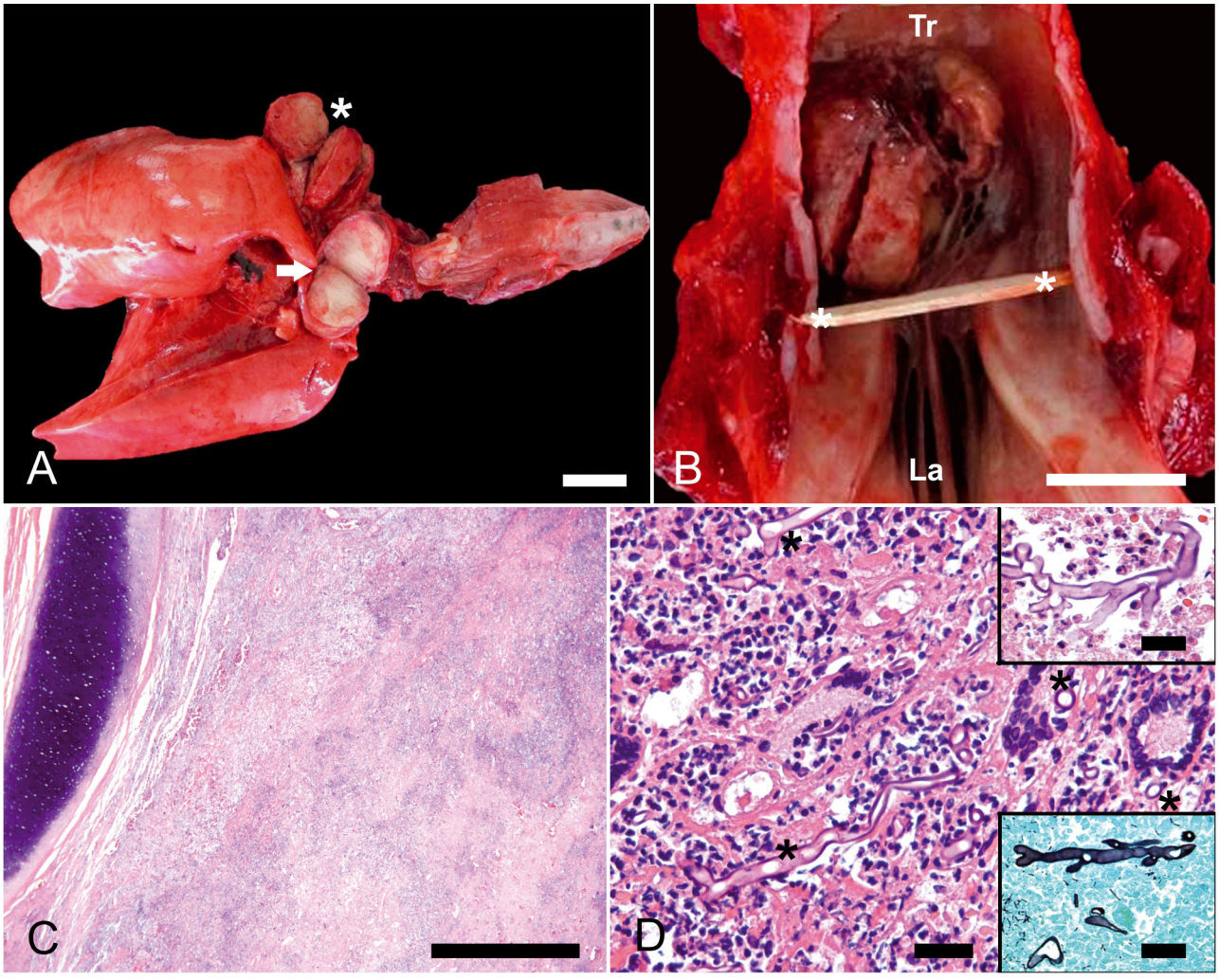

Fig. 1. (A,B) Macroscopic and (C,D) microscopic lesions found in an Atlantic spotted dolphin *Stenella frontalis*. (A) Dorsal view of the respiratory system (cranial is to the right). A large mass expands the cranial mediastinum (arrow) and compresses adjacent esophagus and mainstem bronchi. A cranial mediastinal lymph node is markedly enlarged (asterisk). Scale bar = 15 cm. (B) Dorsal view of larynx (La) and trachea (Tr) dissected and kept opened with a rod (asterisks). A 4.2 × 3.1 × 3 cm tan to grey, solid mass arising from the caudal laryngeal mucosa severely occludes the laryngotracheal lumen. Scale bar = 3 cm. (C) The tracheal lamina propria and submucosa are effaced and expanded by pleocellular pyogranulomatous inflammatory infiltrates. Scale bar = 500 µm. (D) Pyogranulomatous inflammatory infiltrates are centered on extracellular and phagocytosed non-pigmented hyphae (asterisks). There is abundant necrotic cell debris, intact and degenerated neutrophils, and fibrin. H&E. Scale bar = 50 µm. Right upper inset: hyphae are 15–20 µm wide, non-septate (coenocytic), non-parallel walled, non-dichotomous, with irregular or right angle branching, and bulbous dilation. Detail of growth pattern with non-dichotomous branching at acute angles. H&E. Scale bar = 50 µm. Right lower inset: Above hyphae features highlighted with Grocott and Gomori methenamine silver. Scale bar = 50 µm

lacked cortical-medullary demarcation. Within the larynx and throughout the mucosal surface of the trachea, there were multifocal to coalescing, variably sized, occasionally multinodular, exophytic, solid, firm, tan masses with areas of necrosis and loosely adhered fibrin strands. The largest mass (4.2 × 3.1 × 3 cm) was located in the larynx and severely occluded the lumen (Fig. 1B). The remaining tracheal mucosa was diffusely reddened and the lumen contained abundant malodorous, tan, suppurative material. The main and secondary bronchi contained copious amounts of the above exudate, as well as free-floating fibrin strands. Additional relevant gross findings included: moderate to severe systemic parasitism, i.e. dermatitis/panniculitis caused by *Phyllobothrium delphini* merocercoids, mastitis by

Crassicauda sp. nematodes, and pancreatitis and pancreatic ductitis by Brachicladiidae trematodes; bilateral pulmonary emphysema; generalized (superficial cervical, tracheobronchial, pulmonary, and mammary) lymphadenomegaly; and edema with serous atrophy of fat in the nuchal and thoracolumbar area.

Microscopically, the large tracheal exophytic mass consisted of abundant nodular pyogranulomatous inflammatory infiltrates centered on numerous hyphae and extensive areas of lytic and liquefactive necrosis, effacing and replacing the mucosal lining (Fig. 1C) and infiltrating the adjacent cartilage. There were large numbers of intact and degenerated neutrophils, reactive and epithelioid macrophages, lymphocytes, plasma cells, and frequent multinucleated giant cells (Langhan's and foreign body type macrophages), as well as hemorrhage, necrotic cell debris, and fibrin (Fig. 1D). The hyphae were 15–20 µm wide, pauciseptate (coenocytic), had non-parallel walls, non-dichotomous and irregular to right angle branching, and bulbous dilations that were highlighted by PAS and GMS stains (insets in Fig. 1D). Fibrinoid vascular wall necrosis and necrotizing vasculitis with obliterative thrombosis and fungal angioinvasion were common within inflamed foci. The adjacent lamina propria, submucosa, muscularis, and serosa were expanded by edema, hemorrhage, neutrophils, lymphocytes and plasma cells, and fibrosis. Similar histopathologic findings were seen in the examined mediastinal mass, additional nodules throughout the tracheal and main bronchi mucosae, and the mediastinal lymph node. Analogous localized inflammatory infiltrates with intralesional hyphae were seen in the lung along with marked alveolar edema.

Sequence analysis of the amplicon product had 100% (sequence match span 246 bp) identity to many *Rhizopus arrhizus* GenBank sequences (e.g. accession numbers KX957745, KU933760, KJ417552). CeMV nested PCR and IHC were negative. Based on the gross and histopathologic findings and the molecular results, the animal was diagnosed with severe fungal pyogranulomatous laryngotracheitis by Zygomycetes most consistent with *R. arrhizus* (syn. *R. oryzae*).

DISCUSSION

Herein, we report gross and microscopic findings, and molecular identification of *Rhizopus arrhizus* (phylum Zygomycota, Order Mucorales) laryngotracheitis with cranial mediastinal lymph node and pulmonary involvement in a *Stenella frontalis.* Respiratory disease of infectious origin is one of the most frequent ailments seen in free-ranging and captive cetaceans (Reidarson et al. 2018), often affecting the pulmonary parenchyma but sparing the large airways (Delaney et al. 2013). Primary or secondary respiratory fungal disease is common in cetacean species, and various pathogenic and opportunistic molds and yeasts have been reported (Reidarson et al. 2018). A recent large-scale survey indicated an increase of reported Mucorales cases over the last 2 decades, mainly involving animals in captivity in semi-tropical and tropical regions (Reidarson et al. 2018). Published reports of confirmed *Rhizopus* spp. infections are rare, although there are descriptions of *Rhizopus* spp. and *R. stolonifera* in few cetaceans (Reidarson et al. 2018). *R. arrhizus* was first identified from blowhole samples of a captive common bottlenose dolphin *Tursiops truncatus* (Palmero et al. 2014). In the present case, severe fungal disease presented as a large intrathoracic mass and laryngotracheal exophytic masses leading to luminal occlusion. Additionally, there were foci of analogous fungal-related inflammation in the lung and cranial mediastinal lymph node. Histomorphological features of the hyphae, highlighted by PAS and GMS, were consistent with previous *Rhizopus* sp. cases (Ciesla et al. 2000). The lesions in the present case recapitulate features observed in captive *T. truncatus* mortality with *Aspergillus* sp. and *A. fumigatus* tracheitis (Delaney et al. 2013) and tracheobronchitis by *A. fumigatus* in a free-living striped dolphin *S. coeruleoalba* (Grattarola et al. 2018). Intralumenal mass effect may have accounted for increased air resistance and respiratory distress and could explain bilateral pulmonary emphysema in the present case.

There was also moderate to marked systemic parasitism, suggesting immunological dyshomeostasis. Known immunosuppressive factors in free-ranging cetaceans may include biotoxins, chemical pollutants, and pathogens. Infection by CeMV, a known potent immunosuppressive virus, was investigated by PCR and IHC; however, tested organs were negative. As no toxicologic analyses were performed, a potential role for xenobiotics cannot be discarded. A cause for immunosuppression was not apparent in this case. Mucorales may have a predilection for immunocompromised hosts, such as those on steroid therapy, although it is also reported on healthy individuals (Reidarson et al. 2018). Infections due to these opportunistic molds are usually marked by poor responses to antifungal therapy, *in vitro* resist-

ance to most available antifungals, and an overall poor outcome with high fatality rates. Mucormycosis cases in particular are generally acute and rapidly progressive, with high mortality rates in humans and dolphins (Reidarson et al. 2018). The relatively low prevalence of mucormycosis compared to aspergillosis may indicate that these pathogens possess fewer (and/or milder) virulence factors (Spellberg et al. 2005). Common sites of infection include wounds, brain, and the respiratory system. Some studies have linked recurrent or prolonged voriconazole treatment to increased mucormycosis (Segal et al. 2007).

R. arrhizus is ubiquitous worldwide, being the most common environmental *Rhizopus* species, and is found in soil, decaying matter, volcanic mud, and a variety of crops, including barley, rice, and onions, among others (Ribes et al. 2000). *R. arrhizus* is an opportunistic pathogen in nature, as it is not considered a commensal organism of either humans or animals and is not shed during infection. Animals may come in contact with the fungal spores through inhalation from the environment, especially decaying matter (Pak et al. 2008). In humans, *R. arrhizus* is considered one of the most common life-threatening zygomycoses, typically associated with tissue angioinvasion, necrosis, dissemination, and death. Rhinocerebral infections are very common (Wickes 2013). Noteworthy, serum iron availability, which is often dependent on pH, plays a major role in the pathogenesis of *R. arrhizus* and other major pathogenic fungi. The pathogenesis relies on the ability of the fungus to synthetize and secrete a siderophore that binds iron from the host and utilizes it. Considering that metabolic acidotic status and hemochromatosis can aggravate this mechanism, the relatively high incidence of diabetes, renal disease, and hemochromatosis may render captive dolphins at risk to develop zygomycosis. Although this hypothesis requires further research to be proven, the above disease conditions parallel the rise of mucormycosis cases in dolphinaria worldwide (Reidarson et al. 2018). Further studies are required to assess whether any metabolic disease process comparable to the above could play a role in free-ranging cetacean mycosis cases.

In conclusion, we documented severe occlusive laryngotracheitis with cranial mediastinal lymph node and pulmonary involvement by *R. arrhizus* in an Atlantic spotted dolphin *S. frontalis.* A potential cause for immunosuppression was not apparent; PCR and IHC analyses for CeMV were negative. The route of entry was presumably the upper airways, but no evidence of predisposing trauma was observed. Intralumenal mass effect may have accounted for increased air resistance and respiratory distress. These results add to the body of knowledge on cetacean pathology by confirming *R. arrhizus* infection as a potential cause of severe upper airway respiratory disease and by broadening the range of susceptible cetacean species.

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