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Molecular detection of *Chlamydia abortus* in a stranded Mediterranean striped dolphin *Stenella coeruleoalba*

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ABSTRACT: This study reports gross, histopathological, and molecular features of a *Chlamydia abortus* infection in a stranded female striped dolphin *Stenella coeruleoalba* from the Tyrrhenian coast of southern Italy. Post-mortem examination revealed liver congestion, splenic lymphoid depletion with capsular petechiae, and pneumonia. Histology revealed disseminated intravascular coagulation with vasculitis and congestion. Hepatocellular and acute myocardial degeneration were also observed. Basophilic, coccobacillary inclusions consistent with *Chlamydia* spp. were observed histologically in the type II pneumocytes, myocardial fibers, and hepatocytes, and in macrophages and plasma cells of liver, spleen, and prescapular lymph nodes. Chlamydial antigen was detected by immunofluorescence assay using genus-specific anti-*Chlamydia* antibodies. PCR assay revealed *C. abortus* in spleen, liver, heart, and lungs. *C. abortus* was the only pathogen detected. The main pathological changes suggest that *Chlamydia* infection may have been the cause of stranding and death of the striped dolphin. This case represents the first molecular detection of a member of the *Chlamydiaceae* in a marine mammal.

KEY WORDS: *Chlamydia abortus* · *Chlamydiaceae* · Septicemia · Zoonosis · Cetacean pathology · Molecular assay

1. INTRODUCTION

The family *Chlamydiaceae* consists of obligate intracellular Gram-negative bacteria, containing 11 confirmed species within the genus *Chlamydia* (*C. trachomatis, C. suis, C. psittaci, C. pneumoniae, C. pecorum, C. muridarum, C. gallinacea, C. felis, C. caviae, C. avium*, and *C. abortus*) and 1 candidate species (*C. ibidis*). This genus includes human and animal pathogens. Those species with zoonotic potential include *C. psittaci*, *C. felis*, and *C. abortus* (all derived from the previously classified *C. psittaci*) (Vorimore et al. 2013, Sachse et al. 2015).

The striped dolphin *Stenella coeruleoalba* is a cosmopolitan species that occurs in tropical and warm temperate waters. It is the most common cetacean in the Mediterranean Sea; however, according to Aguilar & Gaspari (2012), the population has declined by more than 30% in size over the past 60 yr. The main causes of reduction of the Mediterranean striped dolphin population are linked to habitat deterioration, incidental mortality in fisheries, and the effects of pathogens and pollutants. In the last few years, Mediterranean striped dolphins have been investigated for the presence of a number of pathogens of medical and veterinary concern. Among these, dolphin morbillivirus (DMV) has emerged as the most significant infectious agent for cetacean strandings in the Mediterranean basin. In contrast, other pathogens including *Brucella* spp., *Erysipelothrix rhusiopathiae*, *Listeria monocytogenes*, and *Toxoplasma gondii* have been identified in a few stranded

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individuals, possibly related to spillovers between the regional terrestrial and the local aquatic ecosystem (Di Guardo et al. 2011, Alba et al. 2013, Grattarola et al. 2016, Centelleghe et al. 2017).

Here, we report a *C. abortus* infection in a Mediterranean striped dolphin stranded along the Tyrrhenian coast of Italy. Despite serologic evidence of exposure to *Chlamydiaceae* in pinnipeds (Sladen 1962, Meyer 1967, Moore & Cameron 1969, Burek et al. 2005, Aguirre et al. 2007) and dolphins (Schaefer et al. 2009, Bossart et al. 2014), this study provides the first molecular detection of a member of the *Chlamydiaceae* in a marine mammal.

2. MATERIALS AND METHODS

2.1. Animal

A 70 kg, 189 cm total length female striped dolphin, with moderate nutritional status and decomposition code (grade 2), stranded dead on the beach of Torre Annunziata (Naples) on 4 December 2017. The animal was submitted to the Istituto Zooprofilattico Sperimentale del Mezzogiorno in Portici for postmortem workup.

2.2. Microbiological, pathological, and parasitological examination

A thorough necropsy was performed by conventional techniques. Swabs were collected from brain, lymph nodes, lungs, liver, spleen, kidneys, and adrenal glands for conventional microbiology. Tissues were inoculated either into trypticase soy agar (TSA) supplemented with 5% sheep blood (Oxoid) and TSA supplemented with 2% NaCl (Oxoid) and then incubated aerobically for 24/72 h at 37°C, and into Sabouraud agar and incubated aerobically for 5 d at 25°C for fungi. Swab samples from brain, lymph nodes, liver, spleen, and uterus were also inoculated into *Brucella* agar and incubated at 37°C in air supplemented with 5–10% (v/v) CO_2 for 6 wk. Enrichment liquid media followed by subculture on solid media for *Listeria* spp. (Half-Fraser broth, Frazer broth, Oxford *Listeria* agar) and *Salmonella* spp. (Rappaport-Vassiliadis soya peptone broth, tetrathionate Müller-Kaufmann broth, Brilliance *Salmonella* agar, Xylose lysine deoxycholate agar) were also performed (OIE 2015). Fungi were identified to genus level according to morphological features (McClenny 2005).

For parasitological investigation, the skin, blubber, heart, trachea, lungs, urinary bladder, liver, gallbladder, kidneys, pancreas, uterus, peritoneum, esophagus, stomach chambers, and intestine were examined for helminths following an established protocol by Santoro et al. (2018). Helminths were collected and preserved in 70% alcohol before examination by light microscopy for morphological identification.

Samples for histopathology were collected from all organs and tissues, fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin and Giemsa according to routine methods.

2.3. Immunofluorescence assay

Tissues (including heart, lung, liver, and spleen) collected for histopathological examination that contained inclusions resembling *Chlamydia*-like elements were deparaffinized and examined for *Chlamydia* antigens using an immunofluorescence (IF) protocol based on a genus-specific anti-*Chlamydia* mouse monoclonal antibody (Progen Biotechnik) following the manufacturer's recommendations.

2.4. DNA extraction, PCR reactions, and sequencing analysis for *Chlamydia* sp. detection

Total DNA was extracted from the striped dolphin tissue samples (heart, liver, lung, and spleen) using the QIAamp DNA mini Kit (Qiagen) following the manufacturer's instructions. *Chlamydia* sp. DNA was amplified by real-time PCR with a StepOne Plus thermocycler (ThermoFisher Scientific) using the protocol described by Ehricht et al. (2006), which included the primers Ch23S-F (5′-CTG AAA CCA GTA GCT TAT AAG CGG T-3′) and Ch23S-R (5′-ACC TCG CCG TTT AAC TTA ACT CC-3′) and the probe Ch23S-p (FAM-CTC ATC ATG CAA AAG GCA CGC CG-TAMRA) targeting the 23S rRNA gene. The reaction was carried out in a final volume of 25 µl including 2.5 µl of total DNA, 1.5 µM of each primer, 0.1 µM of the specific probe, and 1× TaqMan Universal MasterMix (ThermoFisher Scientific). The thermal profile consisted of an initial step of 2 min at 50°C, followed by a denaturation cycle of 10 min at 95°C and 50 cycles of 15 s at 95°C and 1 min at 60°C.

Positive samples were further processed by end point PCR to amplify the 16S rRNA gene as described by Ossewaarde & Meijer (1999) for species identification. Briefly, the PCR assay was carried out in a final

volume of 25 µl including 5 µl of total DNA, 0.4 µM of each primer (forward: CHL16SFOR2 5′-CGT GGA TGA GGC ATG CAA GTC GA-3′ and reverse: CHL-16SREV4 5′-ATC TCT CAA TCC GCC TAG ACG TCA AAA-3′), and 1× HotStar *Taq* MasterMix (Qiagen). PCR reactions were performed with a MyCycler thermocycler (BioRad Laboratories) and consisted of an initial step at 95°C for 10 min, followed by 10 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, 35 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. PCR products were first resolved and verified by the automated electrophoresis system QiaXcel (Qiagen), and further processed for sequencing analysis. Amplicons were purified and sequenced using the same primers used for the PCR reaction and the Big Dye Terminator Cycle Sequencing Kit v1.1 (ThermoFisher Scientific) with an automated sequencer (ABI-PRISM 377). Sequences were aligned using the EMBOSS Needle Nucleotide Alignment program (European Bioinformatics Institute EMBL-EBI, https://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html) and compared with those available in GenBank (BLAST – http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Additional pathogen screening

Given that DMV represents the most common infectious agent linked to cetacean stranding along the Mediterranean coasts (Di Guardo et al. 2011, Centelleghe et al. 2017), a brain sample was examined for the presence of DMV genomic RNA by reverse transcription PCR RFLP using the methodology established by Verna et al. (2017). Brain and heart samples were also screened by conventional PCR to detect *Neospora caninum* and *T. gondii* (Hughes et al. 2008). Brain, lymph nodes, liver, spleen, uterus, and feces samples were tested by real-time PCR for *Brucella* spp. (Hinić et al. 2008), and feces for *Cryptosporidium* spp. and *Giardia* spp. (Guy et al. 2003, Haque et al. 2007). Primers and specific amplification reaction conditions are those reported in the referenced articles.

3. RESULTS

Post-mortem examination of the striped dolphin revealed liver congestion, splenic lymphoid depletion (hypoplasia) with capsular petechiae, and pulmonary congestion with irregular pleural surfaces, consolidation, and fibrosis (Fig. 1). We observed hyperemia of the cerebral leptomeninges. Parasites included *Pennella* sp. from the skin, merocercoids of *Phyllobothrium delphini* from the blubber, tetraphyllidean larvae from the liver, *Pholeter gastrophilus* from the stomach, *Tetrabothrius forsteri* from the intestine, *Crassicauda* sp. from the uterus, and merocercoids of *Monorygma grimaldi* from the peritoneum. *Aspergillus* sp. was cultured from the lungs.

Histologically, the main finding was disseminated intravascular coagulation with vasculitis in various organs including brain, liver, spleen, lungs, heart, and gastrointestinal tract associated with congestion and hemorrhage. Peribronchial (Fig. 2A) and perivascular cuffs (Fig. 2B) with lymphocytes and plasma cells along with a few eosinophils and neutrophils were observed in the alveolar interstitium and spaces in the lung. Similar infiltrates were also seen in the liver with hepatocyte degeneration, and in the heart associated with acute myocardial degeneration characterized by myofiber swelling and fragmentation. Reactive microglial cells were widespread in the brain.

Basophilic, coccobacillary cytoplasmic inclusions consistent with *Chlamydia* spp. were observed histologically in the type II pneumocytes, myocardial fibers, and hepatocytes and in macrophages and plasma cells of prescapular lymph nodes (Fig. 2C), liver (Fig. 2D), and spleen (Fig. 2E). Chlamydial antigen was demonstrated by IF with the most intense IF signal in the areas of severe inflammation.

PCR detected *C. abortus* in samples of spleen, liver, heart, and lungs. The 4 sequenced amplicons were identical (GenBank accession numbers MH842738 to MH842741) and showed a 100% nucleotide identity with *C. abortus* 16S rRNA available in GenBank (MG-554733.1, MG554732.1, MG554731.1, CP021996.1, EF486853.1). None of the other screened pathogens was detected.

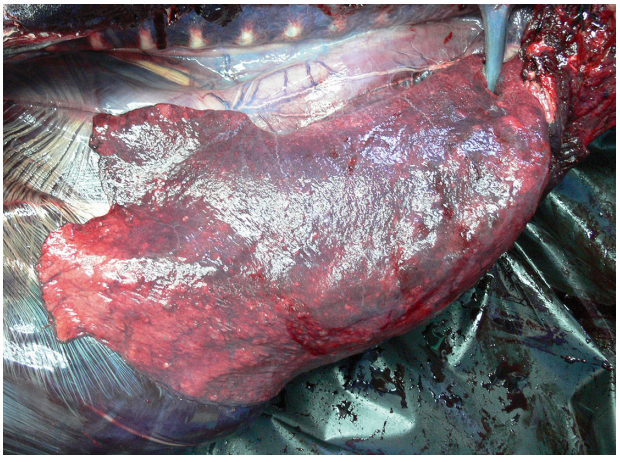

Fig. 1. Lung of the Mediterranean striped dolphin *Stenella coeruloalba* with mottled and irregular pleural surfaces

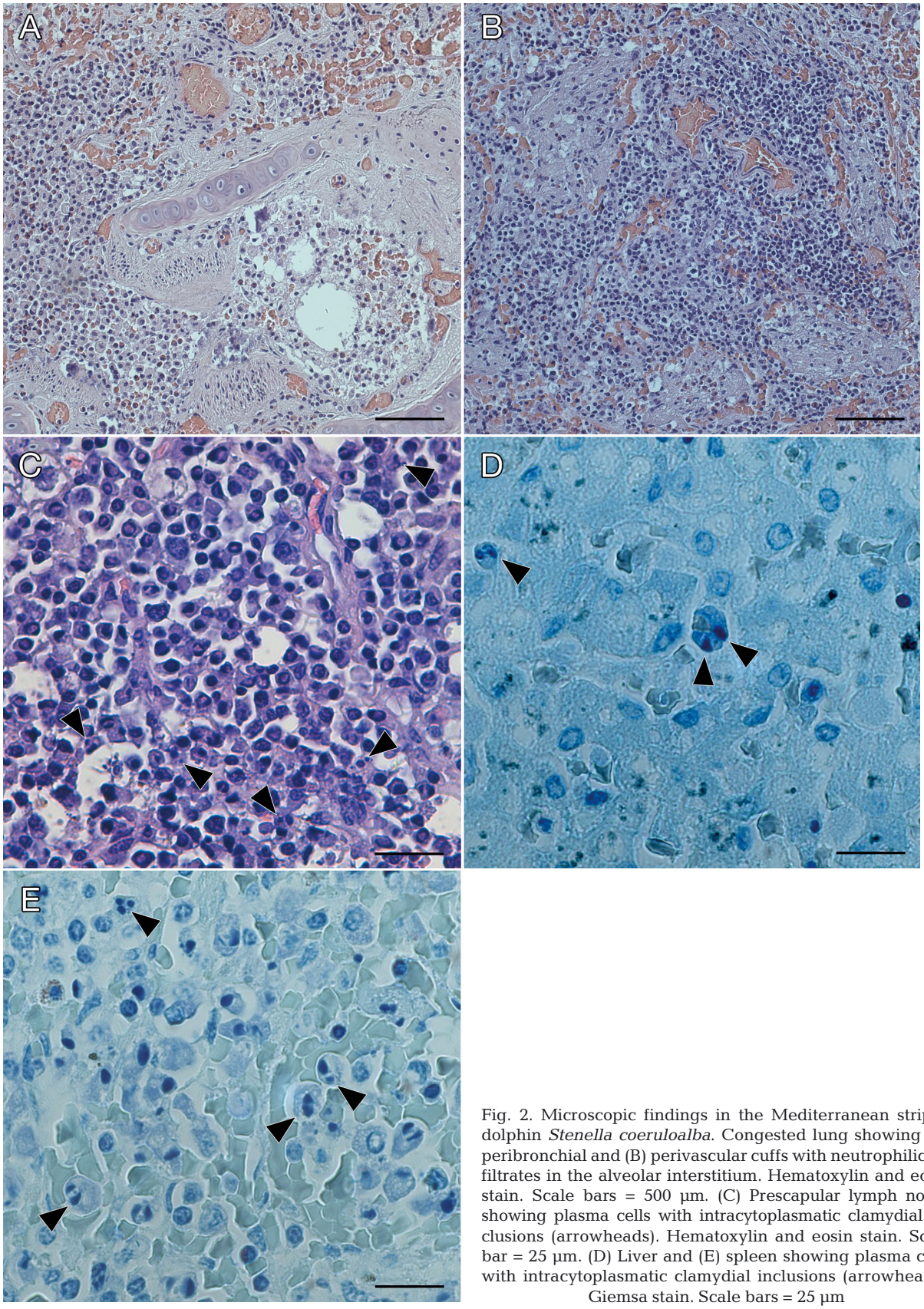

Fig. 2. Microscopic findings in the Mediterranean striped dolphin *Stenella coeruloalba*. Congested lung showing (A) peribronchial and (B) perivascular cuffs with neutrophilic infiltrates in the alveolar interstitium. Hematoxylin and eosin stain. Scale bars = 500 µm. (C) Prescapular lymph nodes showing plasma cells with intracytoplasmatic clamydial inclusions (arrowheads). Hematoxylin and eosin stain. Scale bar = 25 µm. (D) Liver and (E) spleen showing plasma cells with intracytoplasmatic clamydial inclusions (arrowheads). Giemsa stain. Scale bars = 25 µm

4. DISCUSSION

This report describes an unprecedented case of IF and molecular detection of *Chlamydia abortus* in a marine mammal. Among chlamydial species, *C. abortus* is principally a pathogen of ruminants, and is the most common organism responsible for abortion in sheep and goats in Europe. Furthermore, it can cause severe systemic infection both in animals and in humans (Rodolakis & Souriau 1989, Longbottom & Coulter 2003), leading to abortion and life-threatening illness with disseminated intravascular coagulation and multi-organ failure in pregnant women (Buxton 1986, Longbottom & Coulter 2003).

In the present study, *C. abortus* was the only pathogen detected, and the pathological changes were consistent with systemic infection, which eventually caused the stranding and death of the striped dolphin. The most severe lesions were observed in the heart, lungs, liver, and spleen, with involvement of the vascular system. This pattern of lesions is common in terrestrial mammals where interstitial pneumonia together with disseminated intravascular coagulation have been reported (Pienaar & Schutte 1975).

Wildlife hosts of *Chlamydiaceae* include birds, mammals, marsupials, amphibians, and reptiles (Burnard & Polkinghorne 2016). Data on the pathogenesis of *Chlamydiaceae* infection in aquatic mammals is lacking, although evidence of their exposure to these pathogens has been reported. Chlamydial antibodies have been reported in northern fur seals *Callorhinus ursinus* from the Arctic (Meyer 1967) and in a crabeater seal *Lobodon carcinophagus* (Sladen 1962) and a Weddell seal *Leptonychotes weddelli* (Moore & Cameron 1969) from Antarctica. More recently, antibodies to *C. psittaci* and *C. abortus* were reported respectively in Steller sea lions *Eumetopias jubatus* in the northern Pacific (Burek et al. 2005) and in Hawaiian monk seals *Monachus schauinslandi* in the northwestern Hawaiian Islands (Aguirre et al. 2007), both with a high seroprevalence in adults. Schaefer et al. (2009) reported the first evidence of exposure to *C. psittaci*, or a closely related *Chlamydiaceae*, in free-ranging Atlantic bottlenose dolphins *Tursiops truncatus* in Florida and South Carolina (USA). In that study, the high prevalence of the infection from both regions (>80%) was linked to the shedding of the pathogen by the local aquatic bird populations (Schaefer et al. 2009). Bossart et al. (2014) reported that the infection of free-ranging bottlenose dolphins with *Chlamydiaceae* suggests a variety of clinicopathologic and immunologic disturbances possibly impacting the dolphins subclinically. Accordingly, it is plausible that some stress in the marine ecosystem (i.e. ocean noise, pollution, and human interactions) in the case reported here may have contributed to predisposing or possibly reactivating a latent *Chlamydia* leading to primary pneumonia and multisystemic disease as previously observed in terrestrial mammals (Pienaar & Schutte 1975).

The route of infection remained undetermined; however, given that *C. abortus* is a widespread pathogen of farmed animals in the Mediterranean basin, the most likely source of the infection is spillover from regional terrestrial animals into the local aquatic ecosystem as suggested for other pathogens (i.e. *Toxoplasma gondii* and *Erysipelothrix rhusiopathiae*) (Bowater et al. 2003, Schaefer et al. 2009, Grattarola et al. 2016). Our results document that *C. abortus* occurs and can be associated with systemic disease in striped dolphins. We propose that *Chlamydiaceae* should be considered a differential diagnosis for pneumonia, septicemia, and stranding at least in this dolphin species. Finally, due to the zoonotic potential of these organisms, further epidemiological investigations are needed to identify a potential point source of environmental contamination and assess the risks of infection in humans from contact with live and stranded cetaceans.

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