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Pleuropneumonia associated with *Ureaplasma* spp. in a bottlenose dolphin (*Tursiops truncatus*) stranded along the coast of Abruzzo, Italy

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Introduction

In recent years, there has been growing interest on aquatic mammals' health, ecology and conservation. This has enabled to expand our knowledge on infectious diseases of cetaceans, some of which are also considered emerging public health issues. Within such context, a National Cetacean Stranding and Health Surveillance Network was created in Italy under the auspices of the Ministry for the Environment and of the Ministry of Health, in order to standardize the diagnostic protocols and increase data collection. Within this framework, histochemical evidence of fungal bodies was obtained from the lung of a bottlenose dolphin (*Tursiops truncatus*) found stranded in 2014 along the central Adriatic Sea coast of Italy (Abruzzo Region), with a strain of *Photobacterium damselae* subsp. *piscicida* and a *Mycoplasma/Ureaplasma*-like agent being also isolated from the same tissue. For the latter, by means of biomolecular investigations, a genome fragment of approximately 1,400 bp from the 16S rDNA was amplified and sequenced. BLAST analysis revealed 100% identity with an uncultured *Ureaplasma* spp. (JQ193826.1). Additional studies are currently underway on this as well as on additional *Mycoplasma* spp. and *Ureaplasma* spp. isolates obtained from other stranded cetaceans.

Materials and methods

An adult male bottlenose dolphin (*Tursiops truncatus*), found stranded in July 2014 along the central Adriatic Sea coast (Pineto, Abruzzo Region, Italy), was subjected to a detailed *post mortem* examination at Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (Teramo, Italy), with its body tissues being partly fixed in 10% neutral buffered formalin and partly frozen at -20°C for microbiological and ecotoxicological investigations. Furthermore, faecal samples were collected, with blood clots being also obtained from the heart chambers and centrifuged, and the resulting serum was stored at -20°C. Tissues were routinely processed for histopathology, with periodic acid Schiff (PAS), Grocott and Ziehl-Neelsen staining techniques being also applied on selected lung sections and Gram stain on fresh pulmonary tissue smears. Immunohistochemistry (IHC) against *Mycoplasma* spp. was additionally performed on some pulmonary tissue sections, utilizing a rabbit polyclonal antiserum. Biomolecular (PCR, RT-PCR) and IHC investigations against *Morbillivirus* and *Toxoplasma gondii* were also carried out from a range of tissues. Detailed microbiological analyses were performed from all major organs, with the dolphin's lung being cultured in PPLO-selective medium, along with the pulmonary parenchyma from 5 additional bottlenose dolphins found stranded along the coast of Abruzzo Region in 2014. Furthermore, biomolecular (PCR) investigations aimed at detecting *Mycoplasma* spp. DNA were performed from the pulmonary tissue. Once DNA was extracted from broth-derived pellets, the 16S rDNA was amplified by a modified PCR protocol for *Mycoplasma* spp. (Kumar *et al*., 2013), a fragment of gene coding for a penicillum-binding protein for *Photobacterium damselae* (Serracca *et al*., 2011), and a sequence of approximately 550 bp of ITS (internal transcribed spacer region) of the nrRNA for detecting fungi (Schoch *et al*., 2012) were amplified by PCR. PCR products were sequenced and submitted for a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Macroscopically, a diffuse and severe, bilateral pleuropneumonia was observed, with the lung tightly adhering to the coastal pleural surface and with pulmonary lesions being characterized by more or less extensive, multifocal and coalescing necrotic areas (**Figure 1**). Furthermore, a loco-regional lymphadenopathy involving pulmonary, mediastinal and tracheo-bronchial lymph nodes was apparent, with mesenteric lymph nodes also exhibiting a marked enlargement. Histologically, variably sized, multifocal and coalescing pyogranulomatous lesions were seen scattered throughout both lungs' parenchyma. The central area of the aforementioned pyogranulomas showed a more or less extensive necrosis, with lesions being consistent with Splendore-Hoeppli bodies (so-called "Splendore-Hoeppli phenomenon") (**Figure 2**, **a** and **b**). Bacterial colonies, associated with fungal organisms, were frequently observed either within or in proximity and at the periphery of the aforementioned lesions. These bacterial and fungal structures could be successfully stained by means of Gram and Grocott techniques (**Figure 3**, **a** and **b**), while no positive staining was observed by means of PAS and Ziehl-Neelsen techniques. Bacterial aggregates were also seen in proximity to slight, non-purulent inflammatory foci affecting the dolphin's myocardium (**Figure 4**, **a** and **b**), Biomolecular and IHC investigations against *Morbillivirus* and *T. gondii* yielded negative results, similarly to cultures for fungi, mycobacteria and *Brucella* spp. Microbiological analyses led to the simultaneous identification of *Photobacterium damselae* and of a *Mycoplasma*-like agent from the lung tissue. For the latter, a genome fragment of approximately 1,400 bp from the 16S rDNA was amplified and sequenced. BLAST analysis revealed 100% identity with an uncultured *Ureaplasma* spp. (JQ193826.1). Moreover, the BLAST analysis of a fragment of gene coding for a penicillin-binding protein of approximately 290 bp in lenght revealed 99% identify with the *Photobacterium damselae* subsp. *piscicida* (EU164926.1).
Finally and still of interest, *Mycoplasma* spp-specific genome sequenze were identified within the lung tissue from 2 out of the 5 additional bottlenose dolphins found stranded in 2014 along the coast of Abruzzo Region.

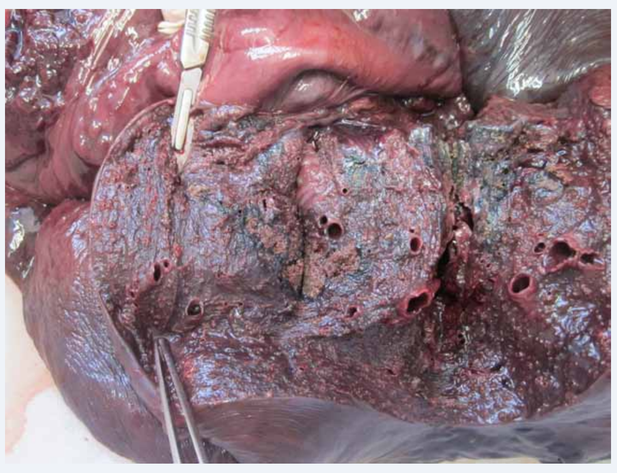


Figure 1. Bottlenose dolphin (*T. truncatus*). Lung.
Multifocal, more or less extensive and coalescing necrotic foci are found scattered throughout the parenchyma.

Figure 2. Bottlenose dolphin (*T. truncatus*). Lung.
Microscopic appearance of the pyogranulomatous lesions, centered by necrotic foci, scattered throughout both lungs' parenchyma. Haematoxylin and eosin (HE) stain (**a** = 5X objective); Splendore-Hoeppli bodies (so-called "Splendore-Hoeppli phenomenon") are evident within the pulmonary inflammatory lesions. HE stain (**b** = 40X objective).

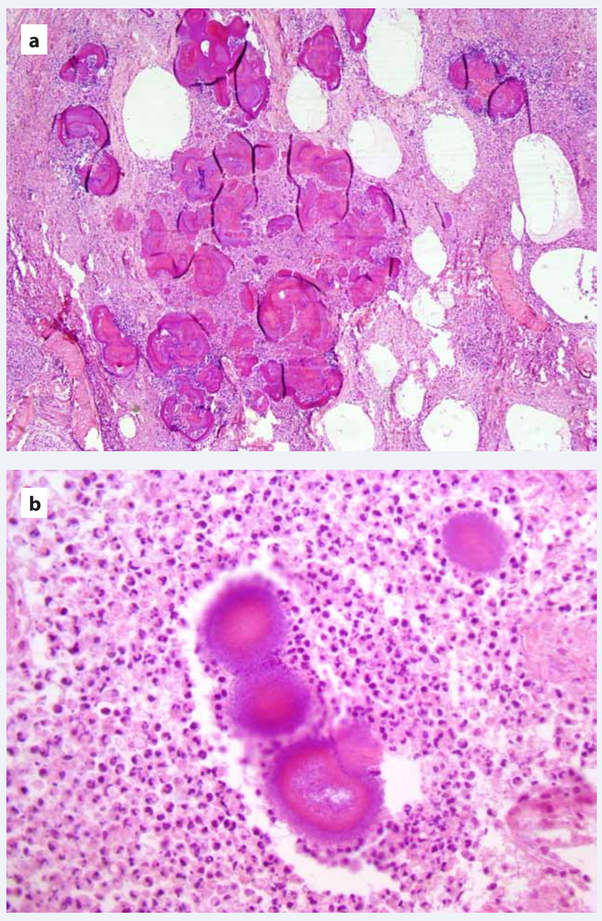


Figure 3. Bottlenose dolphin (*T. truncatus*). Lung.
Positive histochemical labeling with Gram (**a**) and Grocott (**b**) staining techniques is seen either within or in proximity and at the periphery of pyogranulomatous inflammatory lesions. Gram and Grocott stains (**a** = 100X objective; **b** = 40X objective).

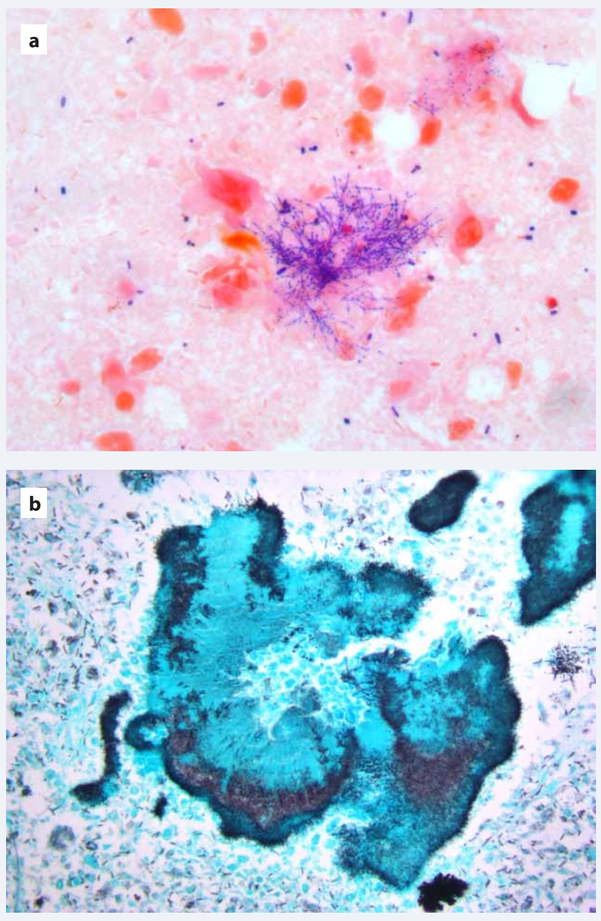
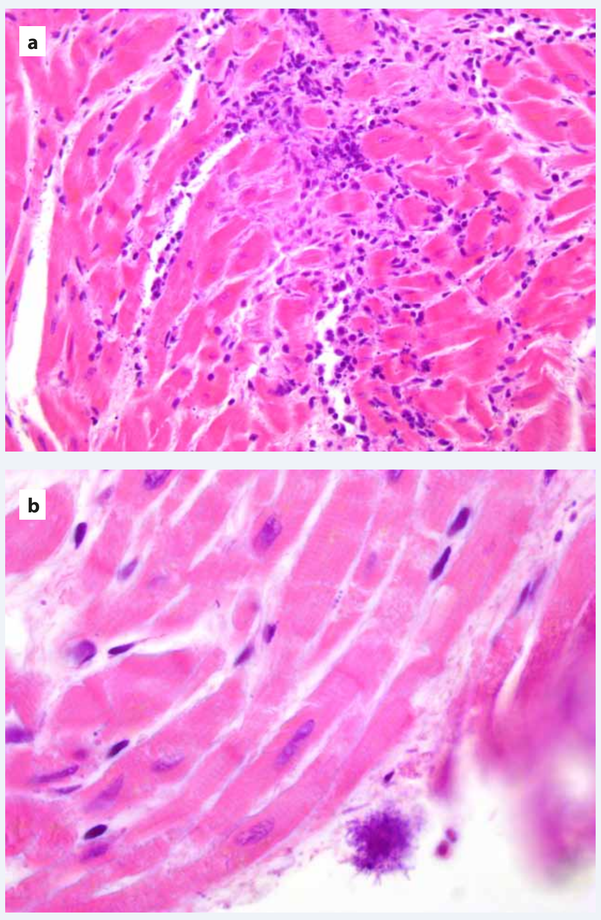


Figure 4. Bottlenose dolphin (*T. truncatus*). Heart.
Bacterial aggregates are seen in proximity to slight, non-purulent inflammatory foci affecting the animal's myocardium. HE stain (**a** = 40X objective; **b** = 100X objective).



Conclusions

Although no definitive conclusions can be drawn, more pathogens factors may have contributed the severe pneumonia including *Photobacterium damselae* subsp. *piscicida* or environmental factors may also have contributed to this recent mortality event. Additional investigations aimed at characterizing the fungal-like and the *Mycoplasma*-like organisms detected in this stranded dolphin, along with their pathogenic role, are warranted.

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