

Aeromonas dhakensis pneumonia and sepsis in a neonate Risso's dolphin *Grampus griseus* from the Mediterranean Sea

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ABSTRACT: A neonate Risso's dolphin *Grampus griseus* was found stranded alive on a beach in Catalonia, Spain. Rehabilitation attempts were unsuccessful and it died 2 d later, showing pneumonia and sepsis. A pure bacterial culture was obtained from all tissues and blood and identified as *Aeromonas hydrophila* using the API 20NE. However, sequencing the *rpoD* gene showed that the strain in fact belongs to *A. dhakensis*, making this the first report of fatal haemorrhagic-necrotizing pneumonia and sepsis due to this species in a marine mammal. The *A. dhakensis* strain GMV-704 produced β -haemolysis, possessed several virulence genes and showed sensitivity to several antimicrobials. This study provides a new potential host for *A. dhakensis*, and its potential virulence in dolphins and its presence in the marine environment may warrant considering this species a potential threat to marine mammals.

KEY WORDS: *Aeromonas dhakensis* · *rpoD* · Virulence · Pneumonia · Sepsis · Dolphin · *Grampus griseus*

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INTRODUCTION

Aeromonas are Gram-negative bacteria that are autochthonous in aquatic environments and are the causative agents of human and fish infections (Figueras et al. 2005, Beaz-Hidalgo et al. 2010, 2013, Janda & Abbott 2010, Parker & Shaw 2011, Figueras & Beaz-Hidalgo 2015). However, in contraposition to the extensive literature implicating *Aeromonas* in fish diseases, few cases of *Aeromonas* infections have been reported in marine mammals (Cusick & Bullock 1973, Krovacek et al. 1998, Thornton et al. 1998, Nielsen et al. 2013). Moreover, it has been shown that routine phenotypic identification of *Aeromonas* spp. tends to erroneously classify 70 to 80% of the

isolates as belonging to *A. hydrophila*, as demonstrated using reliable molecular identification methods, such as the sequences of housekeeping genes (Figueras et al. 2005, 2011, Beaz-Hidalgo et al. 2010, Figueras & Beaz-Hidalgo 2015). In this study, we present a case of pneumonia and sepsis in a neonatal Risso's dolphin *Grampus griseus* caused by *Aeromonas*. The fatal infection was first attributed to *A. hydrophila* on the basis of the API 20NE identification, but sequencing of the *rpoD* gene classified the isolate as *A. dhakensis*. This relatively unknown species has recently been discovered to be the second-most prevalent *Aeromonas* species in human infections in Asia and Australia (Figueras & Beaz-Hidalgo 2015), but it has never been described in cetacean

species. The clinicopathological presentation as well as the virulence characteristics of the isolated *A. dhakensis* strain (i.e. antimicrobial susceptibility and presence of virulence genes) is provided.

MATERIALS AND METHODS

Case history and pathological studies

A neonatal Risso's dolphin was found stranded alive on the beach of Canet de Mar, Catalonia, Spain (41° 35' 13" N, 2° 35' 03" E) on 18 July 2013, the day after a heavy storm. The specimen was a male, 143 cm in length, and still showed skin foetal folds. The calf was detected swimming close to the beach (about 10–15 m away from the coast), and finally stranded about 5 h after first sighting. Despite the very low probability of successful re-introduction of such a young calf, euthanasia was not applied, and after 2 h, the dolphin was transported to a marine water swimming pool at a rescue centre for rehabilitation. The animal died 2 d and 6 h after rescue, in spite of rehabilitation efforts.

A complete necropsy was performed 8 h after the calf died. A large set of organs and tissues were collected and fixed in 10% buffered formalin and processed for routine histopathology as previously described (Soto et al. 2011). Routine detection techniques (immunohistochemistry and reverse transcription PCR, RT-PCR) for cetacean morbillivirus (CeMV) were performed in lung, liver, spleen, kidney and blood as previously described (Soto et al. 2011). Detection of antibodies against *Brucella* spp. was performed in serum with the routine Bengal Rose test, following established procedures (OIE 2013).

Bacterial isolation and identification

Samples from lung, liver, spleen, kidney and blood were taken aseptically and submitted for bacteriological culture. Homogenates of tissue samples were inoculated onto Columbia agar with 5% sheep blood (Difco) and MacConkey agar (Oxoid) and incubated overnight at 37°C in 5% CO₂. A blood sample was enriched in thioglycolate broth at 37°C for 24 h before subculturing onto the above-mentioned culture media.

Isolates were phenotypically identified using the API 20NE identification system (bioMérieux). Molecular identification methods, i.e. DNA extraction, amplification, sequencing and analysis of the *rpoD*

gene, were performed as previously described (Soler et al. 2004).

Virulence gene and antimicrobial susceptibility analysis

Screening for the presence of several virulence genes by PCR was performed in parallel for the recovered *Aeromonas dhakensis* strain GMV-704 and for the type strain of this species, CECT 5744^T. The investigated genes using primers and conditions described previously (Beaz-Hidalgo et al. 2012) were those encoding toxins (*act*, *alt* and *ast*), enzymes (*aer*, *lip* and *ser*), effector and structural proteins of the type-III secretion system (*aexT*, *aopP*, *asc-V* and *asc-FG*) and a flagellar structural protein (*lafA*).

Antimicrobial susceptibility testing for several antibiotics was performed using the disk-diffusion method following the criteria of the Clinical and Laboratory Standards Institute (CLSI 2013).

RESULTS

Necropsy findings

At necropsy, the calf showed foetal folds and a small skin excoriation in the anterior maxillary and mandibular extremes, due to traumatism of stranding. The main lesions were observed in the lung, with a single, unilateral, not well-demarcated area of consolidation affecting the caudal zone of the left lung. The size of this lesion was approximately 6 × 8 × 6 cm, with a dark red colour and a harder consistency than the surrounding lung tissue (Fig. 1). The pleural surface over this area showed a thin filamentous fibrin layer, and pleural lymphatic vessels were distended and reddened. The lesion was classified as an acute, severe, focal, unilateral fibrinous-necrotizing pleuropneumonia. Affected lung portions sank completely in fixative, indicating obliteration of aerial spaces by exudate. The left diaphragmatic lymph node was slightly enlarged and had an increased consistency. The ductus arteriosus was still patent. The first (muscular) stomach was empty, and there was a small amount of milky fluid in the second and third gastric chambers. The intestinal lumen was almost empty. Meconium was not present. The liver was pale and of friable consistency. No external or internal parasites were found, and no other macroscopic lesions were recorded.



Fig. 1. Left lung lobe of a neonate Risso's dolphin *Grampus griseus*, showing haemorrhagic necrotizing pneumonia in the caudal portion

Microscopically, the grossly affected lung zones showed an acute inflammatory reaction, with exudation of fibrin, neutrophils and macrophages into the alveolar spaces, and into the bronchial and bronchiolar lumen. Lesions were acinar and confluent in distribution. Alveolar walls and cells in the exudate showed necrosis, with karyorrhectic debris in neutrophils and macrophages (Fig. 2A). Abundant small bacillary bacteria were evident in haematoxylin-eosin stained sections as well as in Grocott's silver methenamine impregnation, located in the bronchiolar lumen and also attached to the epithelial cells, as well as in alveolar spaces, especially evident in the necrotic areas (Fig. 2B). Numerous medium-size venules and lymphatic vessels showed thrombosis. Widespread haemorrhages and congestion in the affected lung parenchyma were observed. The pleural serosa was also infiltrated with neutrophils and macrophages, and lymphatic vessels were massively distended with fibrin and neutrophils. The left diaphragmatic lymph node showed lymphoid depletion in follicular and parafollicular zones and had distended subcapsular sinus filled with neutrophils, macrophages and numerous bacillary bacteria. The thymus showed lymphoid depletion, both in cortex and in medullary zones. In general, secondary lymphoid tissues, like mesenteric and prescapular lymph nodes, and spleen showed signs of immaturity, with a sparse development and lack of germinal centres. Additionally, the laryngeal tonsil (a lymphoglandular organ located at the basis of the larynx) had multiple necrotic foci arranged around cryptal structures and affecting surrounding lymphoid tissue, with abundant bacillary bacterial colonies. The liver showed a severe

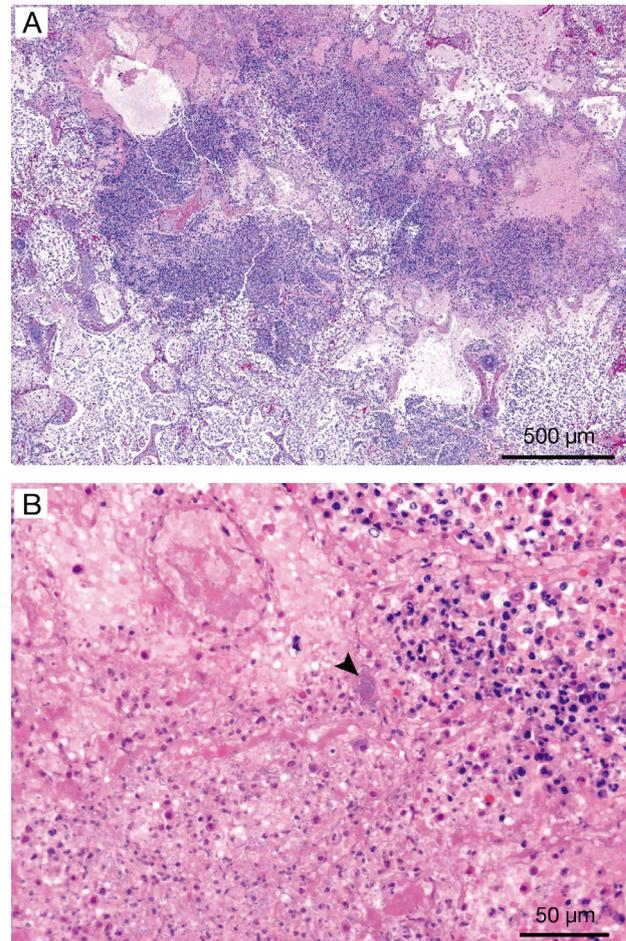


Fig. 2. Histologic lung section of a neonate Risso's dolphin *Grampus griseus* (haematoxylin and eosin stain). (A) Acute inflammatory reaction, with fibrin, neutrophils and macrophages extending into alveolar spaces and bronchiolar spaces, surrounding wide areas of necrosis of lung tissue. (B) Necrosis and inflammatory infiltration by neutrophils and macrophages. Groups of small bacillary bacteria (arrow-head) are evident in the necrotic zones

lipidosis and moderate congestion, and the kidney was congested, with vacuolization of proximal tubules. The central nervous system showed congestion and multifocal perivascular haemorrhages, especially numerous in thalamus and in cerebellar white substance. Routine tests for *Brucella* spp. and CeMV were negative.

Culture identification, virulence and antimicrobial susceptibility analysis

Pure cultures of Gram-negative rods in moderate to heavy growth were recovered from all tissues and

from a whole blood sample. Colonies were β -haemolytic on Columbia agar with 5% sheep blood, and non-lactose fermenter on MacConkey agar. The API 20NE identification profile obtained was 7576755 with 83.8% identity to *Aeromonas hydrophila*. One isolate recovered from the lung (GMV-704) was identified by sequencing the *rpoD* gene, and the highest similarity (98.9%) was found with the type strain of *A. dhakensis* (CECT 5744^T). The identity of the strain was also confirmed when constructing a phylogenetic tree as shown in Fig. 3.

Strain GMV-704 carried the *aer*, *alt*, *lip* and *ser* genes and did not possess the *act*, *ast*, *aexT*, *aopP*, *asc-V*, *asc-FG* and *lafA* genes. The strain showed resistance to the cephalosporins of the first and second generation, to most penicillins and to tetracycline. However, the strain was susceptible to the cephalosporins of the third and fourth generation, carbapenems, aminoglycosides, fluoroquinolones, aztreonam, trimethoprim-sulphamethoxazole, polymyxin B and chloramphenicol.

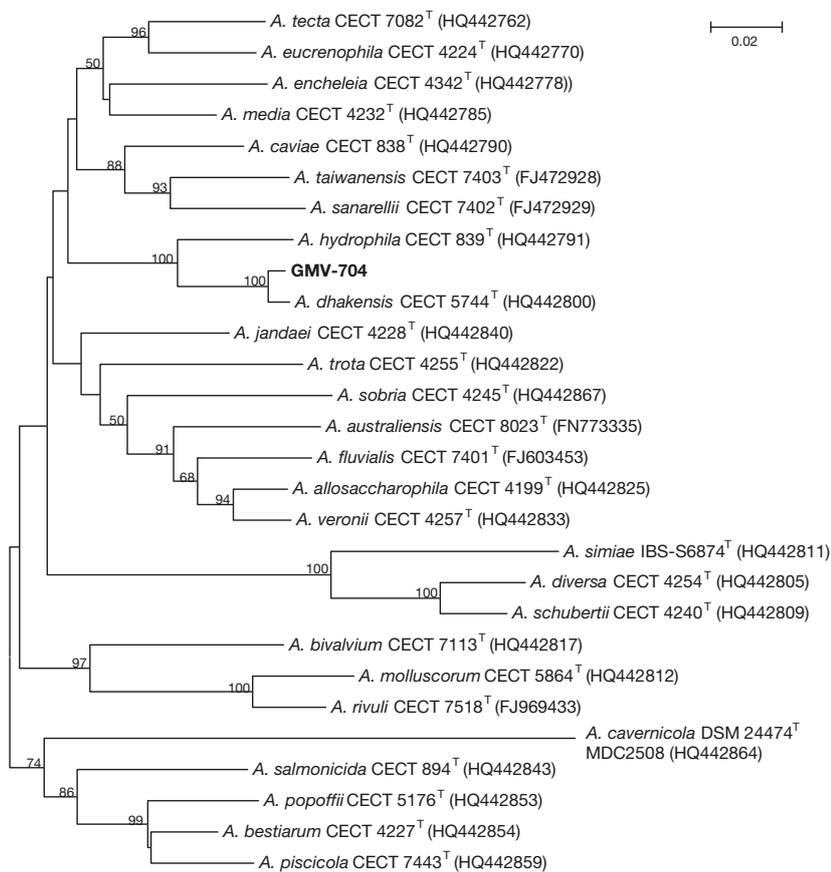


Fig. 3. Unrooted neighbour-joining phylogenetic tree derived from *rpoD* gene sequences (667 bp) showing the relationships of the *Aeromonas* GMV-704 strain (in bold) and all currently known *Aeromonas* species. Numbers at the nodes indicate bootstrap values (>50%)

DISCUSSION

This is the first report of fatal haemorrhagic-necrotizing pneumonia and sepsis due to *Aeromonas dhakensis* in a marine mammal. In cetaceans, *Aeromonas* spp. have been frequently recovered from blowhole and faecal swabs, from wild bottlenose dolphins *Tursiops truncatus* (Morris et al. 2011) and from several cetacean species in Brazilian coastal waters (Pereira et al. 2008), but their role as pathogenic organisms in these species remained unclear. *Aeromonas* spp. were the predominant Gram-negative bacteria found in post-mortem studies in dugongs *Dugong dugon*, contributing to pyogranulomatous pneumonia in this species, frequently as mixed infections (Nielsen et al. 2013). The only report relating *A. hydrophila* with disease in cetaceans was a case of multifocal necrotizing dermatitis and supportive bronchopneumonia described in a captive bottlenose dolphin in 1973 (Cusick & Bullock 1973). In pinnipeds, *A. hydrophila* was cultured from lung and spleen in a stranded grey seal *Halichoerus grypus* with pneumonia and septicaemia (Krovacek et al. 1998). This bacterium was also occasionally isolated from stranded elephant seals, California sea lions and harbour seals (Thornton et al. 1998). It is possible that the *A. hydrophila* identified phenotypically in those studies could belong, after a genetic identification, to *A. dhakensis* (synonym of *A. aquariorum*), as occurred in previous studies (Figueras et al. 2009, 2011, Aravena-Román et al. 2011, Morinaga et al. 2013). In our case, pathologic and bacteriologic examination revealed an acute haemorrhagic-necrotizing pneumonia and sepsis associated with *A. dhakensis*, which was recovered in pure culture from lung, liver, spleen, kidney and blood. Virulence genes detected in our strain (GMV-704), like the *alt*, *aer*, *lip* and *ser* genes, have been previously recorded with variable prevalence (27–100%) in *A. dhakensis* isolates (Figueras et al. 2009, 2011, Puthuchery et al. 2012, Chen et al. 2013, Morinaga et al. 2013). The fact that the *A. dhakensis* GMV-704 strain showed β -haemolysis on sheep blood agar and possessed the *aer* and *lip* genes implicated in the production of

haemolysis, as well as many other known virulence genes, means that these factors may play an important role in the development of the disease. The haemorrhagic-necrotizing character of the pneumonic lesions and the possession of well-known virulence genes by the isolated *A. dhakensis* strain highlight the primary pathogenic role of the bacteria in this case. However, the immaturity of the lymphoid system in a new-born calf and a possible lack or insufficient transfer of maternal immunity by colostrum to the calf could also have increased susceptibility to infection by this bacterium. Routine investigation of CeMV, a well-known immunosuppressive cetacean pathogen, was negative in this animal.

Differentiation of *Aeromonas* species based on phenotypic methods alone is difficult, and frequently, *A. hydrophila* strains have been reclassified into different species by the use of molecular methods (Soler et al. 2004, Figueras et al. 2005, 2011, Beaz-Hidalgo et al. 2010, Aravena-Roman et al. 2011, Figueras & Beaz-Hidalgo 2015). In line with this observation, the API 20NE identification profile of our strain provided 83.8% identity with *A. hydrophila*, because L-arabinose assimilation was negative. This and the production of urocanic acid are typical phenotypic characteristics for *A. dhakensis*, useful to differentiate it from *A. hydrophila* (Beaz-Hidalgo et al. 2013). The present study corroborates once more that API 20NE, as other phenotypic identification methods, is unreliable for the identification of *Aeromonas* spp. because it tends to identify the isolates as belonging to the species *A. hydrophila* (Figueras et al. 2005, 2011, Beaz-Hidalgo et al. 2010). Studies in marine mammals that use these phenotypic identification methods to characterize *Aeromonas* isolates will probably uncover the prevalence of *A. dhakensis* under *A. hydrophila*. However, *A. dhakensis* can be easily recognised using molecular identification with the sequences of housekeeping genes as shown in different studies (Figueras et al. 2009, 2011, Beaz-Hidalgo et al. 2010, 2013, Aravena-Román et al. 2011, Esteve et al. 2012, Puthucheary et al. 2012, Sedláček et al. 2012, Wu et al. 2012, Chen et al. 2013, Morinaga et al. 2013, Soto-Rodriguez et al. 2013). The housekeeping genes *rpoD* and *gyrB* were shown to be very useful (Beaz-Hidalgo et al. 2013). Sedláček et al. (2012) identified 1 *A. dhakensis* among the 21 phenotypically identified *A. hydrophila* isolates, using the sequences of the *cpn60* gene and also found that ribotyping with the *EcoRI* restriction enzyme was a suitable and efficient method for the identification of *A. dhakensis*. Other identification methods used in the recent literature for the identification of *A. dhakensis* include MALDI-TOF, which was able to cor-

rectly identify 29 of the 30 strains assayed (Chen et al. 2014). The expected genus-characteristic resistance against ampicillin was observed for our *A. dhakensis* strain. Also the results obtained for the susceptibility analysis of the rest of the antimicrobials tested agreed with those reported by other authors, showing that the cephalosporins (third and fourth generation), monobactams, carbapenems, aminoglycosides, fluoroquinolones, sulphonamides, polypeptides and amphenicols tested were highly effective against this microorganism (Figueras et al. 2009, Esteve et al. 2012, Wu et al. 2012, Chen et al. 2013, Morinaga et al. 2013).

So far, *A. dhakensis* has only been reported in freshwater habitats (fish, river water, pond, egg masses of the non-biting midge *Chironomus* sp.) in many parts of the world such as Israel, Spain, Australia, Portugal and Mexico (Beaz-Hidalgo et al. 2013, Soto-Rodriguez et al. 2013), and it has been associated with disease in freshwater fish, i.e. rainbow trout *Oncorhynchus mykiss*, European eel *Anguilla anguilla*, Nile tilapia *Oreochromis niloticus* and Mozambican tilapia *O. mossambicus* (Orozova et al. 2009, Esteve et al. 2012, Soto-Rodriguez et al. 2013).

Furthermore, *A. dhakensis* is considered a new emerging human pathogen, and it has been recognised as a prevalent species among *Aeromonas* spp. in human clinical and environmental isolates in Australia, Taiwan and Malaysia (Aravena-Roman et al. 2011, Puthucheary et al. 2012, Chen et al. 2013, Figueras & Beaz-Hidalgo 2015). This study indicates another potential host for *A. dhakensis*, now considered the second-most prevalent *Aeromonas* pathogenic species in humans after *A. caviae* and before *A. veronii* (Figueras & Beaz-Hidalgo 2015), and an emerging pathogen in freshwater fish (Orozova et al. 2009, Esteve et al. 2012, Soto-Rodriguez et al. 2013).

The virulence of *A. dhakensis* for cetaceans is a novel observation, and since this bacterium has been reported to be distributed worldwide, it should be considered a potential threat to marine mammals in fresh water or estuarine environments, particularly river dolphins, but also to marine mammals kept, even shortly, outside of their natural milieu.

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