Short communication

*Edwardsiella tarda* sepsis in a live-stranded sperm whale (*Physeter macrocephalus*)

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**A B S T R A C T**

Whale strandings remain poorly understood, although bacterial infections have been suggested to contribute. We isolated *Edwardsiella tarda* from the blood of a stranded sperm whale. The pathogen was identified with MALDI-TOF MS, confirmed by 16S rRNA gene sequencing and quantified in blood by qPCR. We report the first case of sepsis in a sperm whale. The zoonotic potential of *E. tarda* and the possible role of bacterial infections in the enigmatic strandings of cetaceans are discussed. © 2013 Elsevier B.V. All rights reserved.

1. Introduction

Sperm whales are inhabitants of oceans and deep seas. They only occur in the shallow southern North Sea as vagrants. Their occasional strandings on the shores of the North Sea are poorly understood, but are usually explained by a navigational error, followed by a debilitation due to starvation and eventually a stranding in shallow waters with strong tidal currents (Jacques and Lambertsen, 1997). Bacterial infection has been suggested to be an important and underestimated factor leading to strandings of marine mammals (Cowan et al., 2001). As for sperm whales, to our knowledge, no reports of infection leading to stranding and/or death have been published. Here we report the first case of sepsis in a live-stranded sperm whale, caused by the pathogen *Edwardsiella tarda*, and we discuss how this sepsis may be related to the stranding and death of the animal.

2. Materials and methods

2.1. Stranding event

On the 8th of February 2012 an adult, but not yet fully grown male sperm whale (*Physeter macrocephalus*) of 13.5 m length and an estimated weight of 30 tons live stranded at Knokke-Heist, Belgium. No attempts could be made to euthanize the animal, and it died approximately 8 h after its stranding.

2.2. Collection of samples

Six hours after the natural death of the animal, the necropsy was performed at the beach, tentatively following a standardized protocol (Jauniaux et al., 2002). Samples...
from stomach and duodenum were collected using sterile surgical scissors and forceps, and blood from an abdominal vein was collected with a syringe. Within an hour, all samples were transported in cooled boxes to the Laboratory Bacteriology Research (LBR, University of Ghent, Belgium) for further investigation. The stomach content was collected as completely as possible, and all cephalopod beaks and fish bones were further investigated to identify the species they had belonged to.

2.3. Microbiological culturing

Direct plating of blood on different media was carried out by inoculating 50 μL of blood on the top section of each plate, followed by streaking for isolation using sterile 1 μL inoculation loops. For the stomach and duodenum samples, a suspension was made by vortexing the tissues in tryptic soy broth, after which 50 μL of the suspension was plated out. The media used were tryptic soy agar + 5% sheep blood, Columbia CNA agar with 5% sheep blood, chocolate agar, McConkey agar, and Schaedler agar. All media were from Becton Dickinson (Erembodegem, Belgium). Culture plates were incubated at 37 °C aerobically and anaerobically (10% H₂, 10% CO₂, 80% N₂) (BugBox, LedTechno, Heusden-Zolder, Belgium). In total, 30 plates were inoculated.

2.4. Matrix-assisted laser desorption ionization – time of flight mass spectrometry (MALDI-TOF MS)

From each of the 30 plates, 5 overnight grown colonies were picked, using a 1 μL disposable loop, and spotted evenly over the wells of the MALDI-TOF target plate, without a preceding extraction. The preparations were covered with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and dried for 2 min at room temperature. A bacterial test standard (BTS 255343, Bruker Daltonics, Germany) was used as positive control and an empty well covered with matrix served as negative control.

Mass spectra were generated with a Microflex Biotyper™ spectrometer (Bruker Daltonics, Germany), using the manufacturer’s standard settings. For each sample, mass fingerprints were acquired, using Bruker Daltonics’ flexControl version 3.0 software, analyzed over a mass range of 2000–20,000 Da, and compared with the Bruker Daltonics’ database.

2.5. Extraction and purification of DNA from blood and pure cultures

For 16S rRNA gene amplification and sequencing, bacterial deoxyribonucleic acid (DNA) was obtained from pure cultures by alkaline lysis (Vaneechoutte et al., 2000). To quantify E. tarda in blood, DNA from blood was extracted and purified, using the High Pure PCR Template Preparation Kit (Roche Applied Science, Basel, Switzerland), according to the manufacturer’s instructions. Using this same kit, the E. tarda DNA for the standard dilution series was extracted from an overnight culture of the isolated strain.

2.6. 16S rRNA gene sequencing

To determine the sequence of the 16S rRNA gene, the 16S rRNA gene was amplified with the conserved primers αβNot and oMB (Vaneechoutte et al., 2000). The reactions were performed in a final reaction mixture of 20 μL containing 10 μL of FastStart PCR Master Mix (Roche Applied Science, Basel, Switzerland), 0.2 μmol/L of each primer, and 2 μL of DNA template. Using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, US), the following PCR program was run: 94 °C for 5 min, 3 cycles of 45 s at 94 °C, 2 min at 50 °C, 1 min at 72 °C, and 30 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. After confirming the presence of amplification products by electrophoresis on 2% agarose gels, stained with ethidium bromide, the amplicons were purified using the ExoSAP purification kit (Fermentas, Ontario, Canada), according to the manufacturer’s instructions.

Sequencing was done using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, US) with 1 μL BigDye v3.1 master mix, 3.5 μL BigDye v1.1 v3.1 buffer, and 2 μmol/L of the αβNot or the oMB primer per reaction. Electrophoresis was performed on an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Foster City, US). The obtained 16S rRNA gene sequence was compared with all known bacterial sequences in Genbank using the Basic Local Alignment Search Tool (BLAST) software (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST/).

2.7. E. tarda quantitative PCR (qPCR)

To quantify E. tarda in blood, an E. tarda specific qPCR was developed. Primers EDWTAR_Fw (5’-GGATCCTGTC-TGTGCCTGC-3’) and EDWTAR_Rv (5’-AGCAAAGCGT-CCGGTC-3’) (Savan et al., 2004) were checked for secondary structures using the mFOLD software (http://mfold.rna.albany.edu/?q=mfold) and checked for specificity using the Blast software (http://blast.ncbi.nlm.nih.gov/). For the construction of a standard curve, DNA was extracted from an overnight culture on TSA +5% sheep blood (Becton Dickinson, Erembodegem, Belgium) of the isolated E. tarda. After extraction, the DNA concentration was determined using the Qubit® Fluorometer (Invitrogen, Auckland, New Zealand) and the genomic concentration was calculated using the recently published genome of E. tarda (Wang et al., 2009). A tenfold dilution series was prepared by dilution of the DNA stock in HPLC grade water.

PCR reactions were performed in a final volume of 10 μL, containing 5 μL of LightCycler 480® SYBR Green 1 Master (Roche Applied Science), 3 μmol/L of each primer (Eurogentec, Liège, Belgium), and 2 μL of blood DNA extract or 2 μL of standard curve DNA or 2 μL of HPLC (as negative template control). Cycling conditions were as follows: 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min, followed by a dissociation curve analysis. The standard curve was run in duplicate, the blood DNA sample in triplicate. Amplification and quantification of E. tarda was carried out using the LightCycler480® platform and
the LightCycler® 480 Software Version 1.5 (Roche, Basel, Switzerland). The number of *E. tarda* was expressed as bacteria/mL blood.

3. Results

After overnight incubation, the inoculated blood samples yielded pure cultures as dense growth on all media, incubated aerobically and anaerobically. All isolates from each plate were identified by MALDI-TOF MS as *E. tarda*, with a maximum identification score of 2.323, regarded as ‘highly probable species identification’, according to the manufacturer. These identifications were confirmed by sequencing of the 16S rRNA gene of the isolates. The determined sequence was 100% identical to the 592 bases of the 16S rRNA gene sequence of *E. tarda* GenBank accession no. AB682263.1, as such confirming the MALDI-TOF identification. The *E. tarda* specific qPCR designed to quantify *E. tarda* in blood indicated the presence of $1.1 \times 10^{10}$ bacterial cells/mL in blood. Inoculation of the intestinal and stomach suspensions yielded a polymicrobial growth on all plates, aerobically and anaerobically, but from each plate, using MALDI-TOF MS, isolates could be identified as *E. tarda*.

All fish remains in the stomach had belonged to cod (*Gadus morhua*). Next to loose oololiths and other fish bones, two intact skulls of adult cod were present. The vast majority of the more than 260 cephalopod beaks had belonged to *Gonatus fabricii*, a species common in oceanic waters around the Arctic. In addition to animal remains, a piece of hard plastic was collected, originating from a jerrycan of 15–20L.

4. Discussion

We report the first case of sepsis in a live-stranded sperm whale, caused by the pathogen *E. tarda*, which we assume/propose to be the direct or indirect cause leading to the stranding, which in turn caused the death of the animal.

Male sperm whales in northwest Europe occur mainly in waters deeper than 200 m around the British Isles, Iceland and Norway, and in the Bay of Biscay. They are rarely observed in the North Sea, a habitat considered as not suitable and too shallow for these deep divers. When they do appear in the North Sea, they probably enter it during their southward migration (Smeenk, 1997). Strandings of sperm whales along the shores of the North Sea are uncommon events. Smeenk (1997) reported 168 strandings during the period 1560–1995. The causes of the strandings of these animals remain poorly understood.

On the 8th of February 2012, a young male sperm whale stranded on the beach of Knokke-Heist, Belgium. *E. tarda* was isolated in pure culture from the blood. The identification using MALDI-TOF MS was confirmed by sequencing the 16S rRNA gene of the bacterium. To exclude possibility of a contaminated blood sample, and to determine the severity of infection, we determined the *E. tarda* bacterial load in the blood, by development of an *E. tarda* specific qPCR. The quantitative results indicated that *E. tarda* was present in the blood at a concentration of $1.1 \times 10^{10}$ bacteria per mL. Several studies in humans have shown a very strong correlation between high bacterial loads in blood and increased risk of sepsis, septic shock, and mortality (Darton et al., 2009; Rello et al., 2009). Besides the detection of the pathogen in the blood, stomach and duodenum, *E. tarda* was also found in the liver (Jauniaux TP, personal communication). Also the detection of bacterial pathogens in the liver – the primary clearing house of blood circulating bacteria (Blatteis, 2007), where the febrile process is initiated by the arrival of bacterial lipopolysaccharides (Li and Blatteis, 2004) – is strongly suggestive of sepsis (Moeller, 2012).

*E. tarda* (Ewing et al., 1965) is a pathogen known for its ability to cause sepsis in fish (where the disease is known as edwardsiellosis or Edwardsiella septicaemia) (Park et al., 2012) and humans (Abbott and Janda, 2006). In captive marine mammals, Dunn et al. (2001) reported that most isolates from diagnosed cases of bacteremia and fatal septicemias were Gram-negative bacteria from the genera *Aeromonas*, *Edwardsiella*, *Klebsiella*, *Pasteurella*, *Pseudomonas* and *Vibrio*.

Combining the extremely high load of *E. tarda* in the blood of this animal, the known pathogenicity of this bacterium in piscine species, invertebrates, reptiles, amphibians, avians, and mammals (including humans) (Abbott and Janda, 2006; Wang et al., 2009), and the fact that it was also detected in the liver, strongly suggest – mainly based on microbiological findings – that this was a case of *E. tarda* sepsis.

Although the evidence that this sepsis eventually led to the stranding of this animal inevitably remains circumstantial, it is possible to speculate on the course of events, i.e. how *E. tarda* was acquired, how it disseminated into the bloodstream, and how this sepsis may have been at the origin of the stranding of this animal. As for the acquisition, *E. tarda* could be a commensal of the sperm whale, but no data on this commensalism have been published. On the other hand, the stomach content analysis of the sperm whale revealed the presence of cod (*G. morhua*), and therefore it is not unlikely that *E. tarda*, being primarily a fish pathogen, entered the digestive tract of the sperm whale through contaminated cod. In the stomach contents of stranded sperm whales in the North Sea, fish are rarely found (Santos et al., 2002), and the few gadoid remains that have been found, belonged to saithe (*Pollachius virens*).

There are several possibilities of how *E. tarda* could have disseminated into the bloodstream. First, the immune system of the sperm whale may have been compromised, because of several reasons. When sperm whales during their southward migration enter the North Sea – with an average depth of 93 m –, they will reach progressively shallower and more treacherous waters, only 25–35 m deep in the southern part. Being animals of the deep ocean, they find insufficient suitable food, which can lead to a progressive debilitation and loss of blubber. Loss of blubber may lead to insufficient protection from the cold and to a stressed, weakened and debilitated animal (Smeenk, 1997). However, this animal probably was not weakened, nor present for a long time in the North Sea,
based on the thickness of the blubber layer. Second, there are reports of exposure of marine mammals to high levels of anthropogenic substances such as organohalogens, causing a compromised immune system, leading to bacterial infection (Parsons and Jefferson, 2000). Third, injury of the digestive tract caused by the sharp beaks of squids consumed by sperm whales, marine debris like plastic containers, fish hooks, or severe stomach parasite infestation (Jacques and Lambertsen, 1997) may damage the immune barrier. Ingestion of debris has been related to the cause of death of a number of sperm whales (Lambertsen, 1990). However, although a piece of jerry can was found in the stomach in this case, the piece of plastic was relatively small, no stomach wall damage could be observed, and there was no sign of obstruction. While an immunocompromised animal may be at higher risk for developing sepsis, E. tarda may cause a sepsis in an animal without evidence of debilitation, because this bacterium possesses several virulence factors to adhere to and invade host cells, and to disseminate into the bloodstream (Leung et al., 2012).

This sepsis and the stranding may be strongly inter-twined. Considering the likelihood of fever, known to lead to disorientation, confusion and general illness (Cruz and Dellinger, 2003), it can be suggested that this also compromises the echo-navigational abilities, and therefore, this sepsis may have lead to the death of the animal, indirectly by causing its straying off into the North Sea, considered a sperm whale trap, and leading to a fatal stranding.

With regard to bacterial infection as a cause of live strandings of cetaceans, little information is available. It has been suggested that the high pathogenicity of many of the bacteria isolated from bacteremic stranded cetaceans contributes to the high mortality rate seen in these animals (Cowan et al., 1998), although we were unable to find published data supporting this suggestion. In humans, bacterial sepsis causes mortalities that are close to 50%, despite appropriate antibiotic therapy and optimum supportive care (Munford, 1994). For wild marine mammals, in the absence of antibacterial therapy, it might be expected that this percentage will be higher.

As such, bacterial infection may be a largely overlooked reason for the still enigmatic strandings of cetaceans, and it might be advisable to include standard microbiological documentation of stranded animals to further clarify the importance of infection in this phenomenon. In addition, prudence for workers handling stranded marine mammals may be advisable. Because of the zoonotic potential of E. tarda and the ability to cause gastro-enteritis and/or sepsis (Janda and Abbott, 1993; Mainous et al., 2010), the chance for transmission is nowhere more likely to happen than during the post-mortem examinations of large whales, where researchers sometimes find themselves literally inside of the animal.

In conclusion, we report the first case of sepsis in a sperm whale, document that it was caused by E. tarda, and provide indications that this sepsis may have led, directly or indirectly, to the stranding, which caused the death of the animal.

Conflict of interests

The authors declare they have no actual or potential competing financial interests.

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References


