HELICOBACTER CETORUM INFECTION IN STRIPED DOLPHIN (*STENELLA COERULEOALBA*), ATLANTIC WHITE-SIDED DOLPHIN, (*LAGENORHYNCHUS ACUTUS*), AND SHORT-BEAKED COMMON DOLPHIN (*DELPHINUS DELPHUS*) FROM THE SOUTHWEST COAST OF ENGLAND

Nicholas J. Davison[1,4,6], James E.F. Barnett [1, 5],

Mark Koylass [2], Adrian M. Whatmore[2],

Matthew W. Perkins[3], Robert C. Deaville [3], Paul D. Jepson[3]

[1]Animal Health and Veterinary Laboratories Agency, Polwhele, Truro, Cornwall TR4 9AD, United Kingdom.

[2]Department of Bacteriology, Animal Health and Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom. .

[3]Cetacean Strandings Investigation Programme (CSIP), Institute of Zoology, Zoological Society of London, Regents Park, London NW1 4RY, United Kingdom.

[4]Current address Scottish Marine Animal Strandings Scheme, SAC Veterinary Services, Stratherrick Road, Inverness IV2 4JZ, United Kingdom.

[5]Current address Environment and Sustainability Institute, University of Exeter Cornwall Campus, Penryn, Cornwall TR10 9EZ, United Kingdom.

[6] Corresponding author (email: nick.davison@sruc.ac.uk)

Key words: Delphinus delphis, Helicobacter cetorum, Lagenorhynchus acutus, Stenella coeruleoalba,

Abstract

Helicobacter infection in cetaceans was first reported from the USA in 2000 when the isolation of a novel *Helicobacter* species was described from two Atlantic white-sided dolphins (*Lagenorhynchus acutus).* Since then *Helicobacter* species have been demonstrated in both cetaceans and pinnipeds from around the world. Since 1990 the Animal Health and Veterinary Laboratories Agency Polwhele, Truro has been involved in the UK Cetacean Strandings Investigation Program to establish the cause of death of cetacean species stranded along the coast of Cornwall, England. Here we describe the first opportunistic isolation of *Helicobacter cetorum* in a striped dolphin (*Stenella coeruleoalba*), and the first evidence of *Helicobacter cetorum* infection in cetaceans from European waters.

Introduction

The genus *Helicobacter* has expanded over recent years and species have been isolated from a wide range of animals (Fox 2002). They have been implicated in a number of conditions including typhlitis or colitis and gastritis in several species of mammal, and specifically gastritis, peptic ulcer disease and cancer in humans (Marshall 2002). *Helicobacter* infection in cetaceans was first reported in 2000 when novel *Helicobacter* species were isolated from Atlantic white-sided dolphins and characterized by analysis of partial 16S rRNA gene sequence from a short-beaked common dolphin (*Delphinus delphis)* with multifocal lymphoplasmacytic gastritis

found stranded in Massachusetts USA (Harper et al.2000). In 2002 a similar organism was isolated from three species of cetacean kept in captivity, a bottlenose dolphin (*Tursiops truncatus*) from Hawaii USA, a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) from Illinois USA, and a beluga whale (*Delphinapterus leucas*) from Connecticut USA. These isolates and two from Atlantic white sided dolphins in 2000 were described, as a new species, *Helicobacter cetorum* in 2002 (Harper et al. 2002). Evidence of infection by *Helicobacter* in cetaceans from European waters has not been previously described.

Since the first isolation of *Helicobacter pylori* from the gastric mucosa of humans (Marshall & Warren 1984), there have been numerous species of this genus characterized from the gastrointestinal tract of both mammals and birds (Solnick & Schauer 2001). Since *H. cetorum* was first described, there have been several reports of *Helicobacter spp.* in cetaceans from application of PCR-based techniques. *Helicobacter* species DNA was detected in the dental plaque of two captive bottlenose dolphins with gastritis in Argentina, and suggested that the oral cavity may be a reservoir of the bacterium; however, cultures were not attempted, and the species was not identified (Goldman et al. 2002). A *Helicobacter* species was also demonstrated using 16S rRNA PCR and sequence analysis in the gastric fluid of six captive bottlenose dolphins in Australia (Oxley et al. 2005). The sequences of both of these suggest they may represent more novel species, but again, no cultures were attempted. The detection of *Helicobacter* species by 16S rRNA PCR and DNA sequence analysis with a 98-99% identity to sequences from *H. cetorum* was reported in the digestive tract of an Atlantic spotted dolphin (*Stenella frontalis*) that stranded on the Caribbean coast of Venezuela. The inflammation seen in the duodenal ampulla and pyloric stomach of this animal may have been associated with the presence of

Helicobacter in these tissues (Suárez et al. 2010). Molecular evidence of *Helicobacter spp.* has also been demonstrated in gastric fluids from a captive killer whale (*Orcinus orca*), a false killer whale (*Pseudorca crassidens*) and one free-living Franciscana (*Pontoporia blainvillei*) in Argentina using PCR and 16S rDNA sequence analysis and although cultures were attempted from these animals they were all negative (Goldman et al. 2011).

Materials and Methods

Four animals were submitted to the Animal Health and Veterinary Laboratory Agency Polwhele by the Cornwall Wildlife Trust Marine Strandings Network for routine post mortem examination using a standardized protocol for cetaceans (Jepson 2006) under the Cetacean Stranding Investigation Program. These were an Atlantic white-sided dolphin, two short-beaked common dolphins (1&2) and a striped dolphin (Table 1).

In addition to routine capnophilic bacterial cultures from tissue sections of heart blood, lung, liver, kidney & brain, distal small intestine from the Atlantic white-sided dolphin and proximal jejunum from common dolphin1 were inoculated directly onto 5% sheep blood agar (Oxoid Basingstoke UK) and, MacConkey agar (Oxoid); these were incubated at 37C in a capnophilic atmosphere (10% CO_2) and examined daily for 7 days.

For the striped dolphin only tissue sections from fundic stomach and duodenum were taken and these were inoculated directly onto 5% sheep blood agar and MacConkey agar, incubated at 37C in a capnophilic atmosphere and examined daily for 7 days. Additional tissue sections of fundic stomach and duodenum from the striped dolphin were inoculated directly onto 5% sheep blood agar (Oxoid), incubated

at 37C in a microaerophilic atmosphere (10% CO_2, 5% O_2, 5% H_2, & 80% N_2) in gas jars containing **Campy**Gen™ gas packs (Oxoid) and examined daily for 7 days. From common dolphin 2 in addition to routine cultures from tissue sections of the brain, cardiac ulcers tissue sections were inoculated onto 5% sheep blood agar and *Helicobacter* agar, modified (Becton Dickinson, Heidelberg Germany), incubated at 37C in a microaerophilic atmosphere in gas jars using **Campy**Gen™ gas packs and examined daily for 7 days.

Bacterial isolates were examined using standardized methods for Gram stain, oxidase and catalase production and other phenotypic tests as described Harper et al. 2002. Identification was confirmed by undertaking sequencing of virtually the entire 16S rRNA gene of the isolates following amplification of the gene with the primer pair 5' AGTTTGATCCTGGCTCAG 3' and 5'ACGGCTACCTTGTTACGACTT 3' directly from crude cell lysates.

Tissues including intestine (duodenum, , proximal jejunum and distal small intestine), fundic stomach and cardiac stomach ulcers adjacent to where cultures were taken were fixed in 10% neutral buffered formalin and then processed and stained with hematoxylin & eosin and Warthin & Starry silver stains prior to microscopic examination.

Results

At post mortem examination, all four animals were in poor body condition. Death in common dolphin 2 was due to meningoencepahlitis and arthritis of the atlanto–occipital joint associated with *Brucella ceti* infection (Davison et al. 2013). The cause of the death of the three remaining animals was starvation and hypothermia

(Table 1). All four animals had gross lesions within the gastrointestinal tract (Table 2)

Culture from the distal third (jejunum) of the small intestine of the Atlantic white-sided dolphin produced a profuse mixed growth of *Actinobacillus delphinicola,* an unidentified *Mycoplasma/Ureaplasma* species and a Gram negative, curved rod resembling a *Campylobacter* or *Helicobacter* species after 36 hr. Histological examination of the mucosa of the jejunum revealed no evidence of inflammation or infection but this may have been masked in the mucosal layer by autolysis. Warthin & Starry silver stained sections demonstrated foci of argyrophilic spiral bacteria in the mucosa.

Cultures from the proximal small intestine of common dolphin1 produced a scant growth of a Gram negative, curved rod resembling a *Campylobacter* or *Helicobacter* species after 72 hr. Histological sections of pyloric stomach showed chronic verminous hemorrhagic gastritis characterized by focal hemorrhage in the submucosa and cellular sub mucosal infiltration cuffing the necrotic remains of a trematode parasite possibly *Pholeter gastrophilus*. Warthin & Starry silver stain did not demonstrate the presence of argyrophilic spiral bacteria in this section. Sections of intestine showed early autolysis of the epithelium and there was no evidence of cellular or vascular changes consistent with infection or inflammation. Warthin & Starry silver stained sections did reveal some foci of argyrophilic spiral bacteria within the sections of jejeunm and duodenum.

Cultures of both fundic stomach and duodenum of the striped dolphin produced a light growth of *Vibrio alginolyticus* after 24 hr and a light growth of a Gram negative, curved rod resembling a *Campylobacter* or *Helicobacter* species after 4 days incubation. Histology sections of the fundic stomach lesion were diffusely

congested. Warthin & Starry silver stained sections revealed foci of argyrophilic spiral bacteria in the mucosal region including gastric pits (Figure 2). Both the jejunum and duodenum sections showed marked congestion of the mucosa and submucosa. The outer mucosal epithelium was missing possibly due to autolysis but there were areas of hemorrhage within the inner mucosa consistent with acute enteritis. Warthin & Starry silver stained sections did not demonstrate the presence of argyrophilic spiral bacteria in these sections

Cultures of the cardiac stomach ulcers from common dolphin 2 produced a gross mixture but with a predominant growth of *Actinobacillus delphinicola* and a moderate growth of a Gram negative, curved rod resembling a *Campylobacter* or *Helicobacter* species after 4 days incubation (Table 2). Examination of the cardiac stomach lesion (a mucosal ulcer) showed granulating tissue, exhibiting some superficial necrosis, hemorrhage, thrombosis and mixed (mainly neutrophilic) cellular infiltration. The intact cardiac stomach epithelium was mildly hyperplastic at the margin of the ulcer. Warthin & Starry silver stained sections of the cardiac stomach revealed numerous argyrophilic spiral bacteria located superficially within the ulcer consistent with focal, subacute ulcerative gastritis associated with *Helicobacter* sp.

On subculture, all the suspect *Helicobacter/ Campylobacter* isolates did not grow aerobically or anaerobically but good growth was achieved microaerophilically at both 37C and 42C but not 25C. All isolates produced catalase and oxidase, were motile and rapidly hydrolyzed urea. Tests for indoxyl acetate hydrolysis, nitrate reduction and alkaline phosphatase, hydrolysis were all negative. The isolates were resistant to nalidixic acid and sensitive to cephalothrin. The isolates were tentatively identified as a *Helicobacter* species and sent for 16S rRNA sequencing. Comparison of the 16S rRNA sequence with online databases (GeneBank) showed top matches

with entries for *Helicobacter cetorum*. Phylogenetic analysis was carried out following alignment of sequences with the top database matches consisting of a number of entries for *H. cetorum* from North American studies, including the type strain (Accession Number AF292378) and type strains of other closely related *Helicobacter* species such as *H. pylori* and *H. felis* (Figure 1). All four isolates described here clustered closely with *H. cetorum* entries differing by between 4 and 16 bp from the type strain sequence. The most divergent isolate from common dolphin 2 shared an identical sequence with an entry AF292377, previously described as divergent from other *H. cetorum* entries although found to be phenotypically identical (Harper et al, 2002). Sequences of 1441bp were obtained from each of the four isolates and have been deposited in the EMBL database with the following accession numbers: isolate 22/M98/7/08, Accession Number FN565162; isolate 22/M47/10/08, Accession Number FN565163; isolate 22/M111/7/09, Accession Number FN565164; isolate 22/M74/10/09, Accession Number FN565165.

Discussion

Evidence for pathogenicity of *H. cetorum* is so far limited to individuals of six species of cetacean; short-beaked common dolphin, bottlenose dolphin, beluga whale, Atlantic white-sided dolphin, Pacific white-sided dolphin and Atlantic spotted dolphin (Harper et al. 2000; Harper et al. 2002; Suárez et al. 2010) all of which had gastritis. Therefore it is possible that some of the more recently discovered species of *Helicobacter* not confirmed as *H.cetorum* are commensals or opportunistic pathogens, rather than primary pathogens. *Helicobacter* infections in humans are associated with sub clinical to clinically significant inflammation of the stomach with a risk of cancer in a smaller percentage of those infected. Development of disease is influenced by

many factors including the expression of *Helicobacter* virulence factors such as *H. pylori cagA* which is associated with a higher risk of atrophic gastritis and cancer in humans (Marshall 2002).

In terrestrial mammals, *H.mustelae* and *H. acinonyx* are reported as a cause of chronic gastritis in ferrets (*Mustela putorius furo*) and cheetahs (*Acinonyx jubilatus*) (Fox et al. 1990; Eaton et al. 1993) Similarly *H. cetorum* has been associated with gastric ulcers, lethargy, in-appetence and regurgitation in cetaceans (Harper *et al*. 2002) .With the obvious disadvantage of not being able to perform transmission studies, it is difficult to confirm that the *Helicobacter* species isolated from marine mammals are the primary cause of gastritis. However the fact that *H.cetorum* isolation in marine mammals is associated with gastritis would suggest that it may have a role to play, as is the case with *H. pylori* in humans

None of the animals in the present study had any evidence of recent feeding. All four animals had gross pathological lesions in the gastrointestinal tract. The Atlantic white-sided dolphin had multifocal coalescing hemorrhages present in the mucosa of the distal small intestine (jejunum) and both short-beaked common dolphins and the striped dolphin had mucosal hemorrhages in the pyloric stomach. Histological examination of these lesions confirmed ulcerative gastritis in common dolphin 2 consistent with those described in Atlantic white-sided dolphins, short beaked common dolphins, bottlenose dolphins, Pacific white-sided dolphins and a beluga whale (Harper et al. 2002). Lesions consistent with acute enteritis in the striped dolphin were similar to those described in Atlantic spotted dolphins (Suárez et al. 2010); however this may be due to the isolation of *Vibrio alginolyticus.* *Helicobacter*-type organisms were also demonstrated in Warthin & Starry silver stained sections from various gastrointestinal sites in all four animals. Unfortunately

performing electron microscopy on sections of the gastrointestinal tract on these animals to confirm features consistent with *Helicobacter* sp. as opposed to other spiral type bacteria was beyond the scope and finances of this study. Although autolysis was present in some histology sections this may reflect a delay in processing the tissues rather than autolysis at the time of sampling. Nevertheless *Helicobacter cetorum* was the only consistent isolate from the gastrointestinal tract in all four animals, from both stomach and intestine although isolation from the latter site would suggest possible passage from the stomach and may just be an incidental finding. Although *Helicobacter* infection can be excluded as the cause of death of all these animals, it may have contributed to the poor condition of common dolphin 2.

These four cases are the first confirmed evidence for *H. cetorum* infection in free-living cetaceans in European waters. This is also the first confirmed report of *H. cetorum* from a striped dolphin, increasing the host range of this organism. More work is necessary to determine the prevalence of *Helicobacter* sp., whether they are just commensal organisms, opportunistic pathogens or a primary pathogen and their role in gastrointestinal disease in marine mammals.

Acknowledgements;

Cornwall Wildlife Trust Marine Strandings Network volunteers and British Divers Marine Life Rescue volunteers for submission of carcasses. Postmortem investigation was carried out under the aegis of the UK Cetacean Strandings Investigation Programme, which is jointly funded by the Department for Environment Food and Rural Affairs (Defra) and the UK Devolved Administrations.

References

Davison NJ. Barnett JEF, Perrett LL, Dawson CE, Perkins MW, Deaville RC, Jepson PD. 2013. Meningoencephalitis and Arthritis Associated with Brucella ceti in a Short-beaked Common Dolphin (Delphinus delphis) J Wildl Dis. 49**:**632-636.

Eaton KA, Dewhirst FE, Radin MJ, Fox JG, Paster S, Krakowa S, Morgan DR. 1993. *Helicobacter acinonyx* sp.nov. isolated from cheetahs with gastritis. Int J Syst Bacteriol. 43**:**99-106.

Fox JG, Correa P, Taylor NS, Lee A, Otto G, Murphy JC, Rose R. 1990. *Helicobacter mustelae* associated gastritis in ferrets: an animal model of *Helicobacter pylori* gastritis in humans. J Gastro. 99**:**352-361

_____. 2002. The non-*H.pylori* helicobacters: their expanding role in gastrointestinal and systematic diseases. Gut.50:273-283

Goldman CG, Loureiro JD, Quse V, Corach D, Calderon E, Caro RA, Boccio J, Rodriguez-Herdia S, Di Carlo MB, & Zubillaga MB. 2002. Evidence of *Helicobacter* sp. in dental plaque of captive dolphins (*Tursiops gephyreus*). J Wildl Dis. 38**:**644-648.

_____, Matteo MJ, Loureiro JD, Almuzara M, Barberis C, Vay C, Catalano M, Rodriguez-Herdia S, Mantero P, Boccio, JR, Zubillaga MB, Cremaschi GA, Solnick JV, Perez-Perez GI, Blaser MJ. 2011. Novel gastric helicobacters and oral campylobacters are present in captive and wild cetaceans. Vet Microbiol. doi: 10.1016/j.vetmic.2011.04.023

Harper CMG, Dangler CA, Xu S, Feng Y, Shen Z, Sheppard B, Stamper A, Dewhirst FE, Paster B, Fox JG. 2000. Isolation and characterization of a *Helicobacter* sp. from the gastric mucosa of dolphins *Lagenorhynchus acutus* and *Delphinus delphis*. Appl Environ Microbiol. 66, 4751-4757

_____, Feng Y, Xu S, Taylor NS, Kinsel M, Dewhirst FE, Paster B, Greenwell M, Levine G, Rogers A, Fox JG. 2002 *Helicobacter cetorum* sp. nov., a Urease-positive *Helicobacter* Species isolated from Dolphins and Whales. J Clin Microbiol. 40:4536-4543

Jepson PD, editor 2006. Cetacean Strandings Investigation and Co-ordination in the UK 2000-2004. Final report to the Department for Food and Rural Affairs. pp 1-79. http://randd.defra.gov.uk/Document.aspx?Document=WP01011_8244_FRP.pdf Accessed July 2013.

Marshall B, Warren JR. 1984. Unidentified curved bacilli in the

stomach of patients with gastritis and peptic ulceration. Lancet i:1311-1314.

_____. 2002. *Helicobacter pylori*: 20 years on. Clin Med. 2**:**147-152.

Oxley APA, Argo JA, McKay DB. 2005. *Helicobacter* spp. from captive bottlenose dolphins (*Tursiops* spp.) and Polar bears (*Ursus maritimus*). Vet J. 170: 377-380.

Solnick JV, Schauer DB. 2001. Emergence of diverse *Helicobacter* species in the pathogenisis of gastric and enterohepatic. Clin Microbiol Rev. 14:59-97

Suárez PM, Contreras M, Fernández-Delgado V, Salazar R, Peña F, Michelangell, F, García-Amando MA. 2010. Detection of *Helicobacter* in the digestive tract of an Atlantic spotted dolphin (*Stenella frontalis*). J Wildl Dis. 46:622-626.

Figure legends

Fig 1 Phylogenetic relationships of isolates identified in this study with the closest database matches based on sequence of the virtually complete 16S rRNA gene (1441bp). Isolates cluster with a number of previously described isolates of *H. cetorum* from bottlenose dolphins, beluga whales, Atlantic white sided dolphins and Pacific white sided dolphins where equivalent sequences are available (Harper et al 2000, 2002). The 16S rRNA sequences used were downloaded from GenBank and were aligned in MEGA4 using CLUSTALW prior to construction of a phylogenetic tree using the neighbour-joining algorithm. Taxon labels include strain names and the accession numbers of sequences obtained from GenBank, (T) = type strain.

Fig. 2. Photomicrograph of striped dolphin (*Stenella coruleoalba*) stomach mucosa showing clumps of positive staining material (Warthin-Starry method) consistent with *Helicobacter*-type organisms. Bar=100