Veterinary Microbiology 152 (2011) 138–145

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Novel gastric helicobacters and oral campylobacters are present in captive and wild cetaceans

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ARTICLE INFO

Article history:
Received 4 January 2011
Received in revised form 4 March 2011
Accepted 14 April 2011

Keywords:
Helicobacter
Campylobacter
Marine mammals
Cetaceans
Gastritis

ABSTRACT

The mammalian gastric and oral mucosa may be colonized by mixed *Helicobacter* and *Campylobacter* species, respectively, in individual animals. To better characterize the presence and distribution of *Helicobacter* and *Campylobacter* among marine mammals, we used PCR and 16S rDNA sequence analysis to examine gastric and oral samples from ten dolphins (*Tursiops gephyreus*), one killer whale (*Orcinus orca*), one false killer whale (*Pseudorca crassidens*), and three wild La Plata river dolphins (*Pontoporia blainvillei*). *Helicobacter* spp. DNA was widely distributed in gastric and oral samples from both captive and wild cetaceans. Phylogenetic analysis demonstrated two *Helicobacter* sequence clusters, one closely related to *H. cetorum*, a species isolated from dolphins and whales in North America. The second related cluster was to sequences obtained from dolphins in Australia and to gastric non-*H. pylori* helicobacters, and may represent a novel taxonomic group. Dental plaque sequences from four dolphins formed a third cluster within the *Campylobacter* genus that likely represents a novel species isolated from marine mammals. Identification of identical *Helicobacter* spp. DNA sequences from dental plaque, saliva and gastric fluids from the same hosts, suggests that the oral cavity may be involved in transmission. These results demonstrate that *Helicobacter* and *Campylobacter* species are commonly distributed in marine mammals, and identify taxonomic clusters that may represent novel species.

1. Introduction

The mammalian stomach represents an ancient ecological niche that probably has evolved over >100 million years, and as with other luminal organs in the gastrointestinal tract, appears to have a residential microbiota (Atherton and Blaser, 2009). Several conditions have been linked with the development of gastric ulceration in marine mammals, including stress and the presence of

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doi:10.1016/j.vetmic.2011.04.023

parasites within the stomach (Sweeney and Ridgway, 1975). While its role in the etiology of gastric ulceration is not known, the isolation of *Helicobacter cetorum* from the stomach and fecal samples of dolphins and whales (Harper et al., 2000, 2002a, 2002b), suggests a potential infectious etiology for ulcer disease in marine mammals. A wide host range has been described for these bacteria in the marine environment after the finding of DNA highly homologous to that of *H. cetorum* in South American fur seals (Goldman et al., 2009a).

A variety of *Helicobacter* species have been characterized from the gastrointestinal tract of many vertebrate species. However, because of their fastidious conditions for growth, the isolation of these microorganisms continues to be challenging (Solnick and Schauer, 2001). Several *Helicobacter* species can co-colonize the same host, as is the case for cats and dogs, in which diverse helicobacters, including *H. felis*, *H. bizzozeronii*, *H. salomonis*, "*Candidatus Helicobacter heilmannii*", *H. cynogastricus*, and *H. baculiformis* are present in their gastric mucosa (Haesebrouck et al., 2009). Despite being highly prevalent in humans and other animals, the modes by which *Helicobacter* species are transmitted remains uncertain. Its isolation from dental plaque, saliva, and feces from humans, suggests both oral–oral and fecal–oral transmission for *H. pylori* (Li et al., 1996; Oshowo et al., 1998; Song et al., 1999). Although the oral cavity could act as a primary bacterial reservoir (Thomas et al., 1997), regurgitation of contaminated gastric juice also could be responsible for the local presence of helicobacter (Madinier et al., 1997). In marine mammals, similar routes of transmission could be present for the *Helicobacter* spp. that have been isolated from feces (Harper et al., 2002b) and detected in the dental plaque of captive dolphins (Goldman et al., 2002). We have previously reported detecting helicobacter DNA from regurgitated otoliths from marine mammals and from water from pools inhabited by captive cetaceans and pinnipeds (Goldman et al., 2009b); such findings suggest that contaminated water may be a reservoir for transmission among marine mammals. In the present study, we aimed to evaluate the presence of *Helicobacter* and *Campylobacter* spp. in gastric fluids, dental plaque, and saliva from several species of captive and wild cetaceans.

2. Materials and methods

2.1. Animals and sample collection

Samples from ten dolphins (*Tursiops gephyreus*), one killer whale (*Orcinus orca*) and one false killer whale (*Pseudorca crassidens*) living in the Mundo Marino Oceanarium (San Clemente del Tuyú, Argentina; 36°18′S, 56°46′W) were collected between 2000 and 2007. Gastric fluids, dental plaque, and saliva were obtained from each animal, after a fasting period of 8–14 h. Gastric fluids were aspirated from the stomach by gastric intubation. Dental plaque samples were removed from the tooth surfaces with a sterile periodontal curette. Saliva was collected from the oral cavity using sterile plastic pipettes. An aliquot of each sample obtained in 2007 was inoculated into a transport medium (Brucella broth with cysteine hydrochloride as a reducing agent, supplemented with 20% glycerol) and transported on dry ice for culture in the laboratory in Buenos Aires. DNA was extracted from all samples to identify the presence of *Helicobacter* spp. by polymerase chain reaction (PCR) of 16S rDNA and by DNA sequence analysis of the PCR product.

Gastric tissue also was obtained from three wild La Plata river dolphins (*Pontoporia blainvillei*) that were stranded in 2004 on the northern coast of the southwestern Atlantic Ocean, Buenos Aires Province, Argentina. These mammals were recovered by the Mundo Marino Foundation and were dead on arrival at the aquarium.

2.2. Culture conditions

Samples were plated on Blood Agar Base No. 2 (Oxoid Ltd., Basingstoke, UK) containing 7% (v/v) horse blood, pyruvic acid, and MEM vitamin supplement (Oxoid Ltd., Basingstoke, UK), vancomycin (10 mg/L), polymyxin B (2500 U/L), and amphotericin B (5 mg/L), to obtain an enriched, selective, and differential culture media. Plates were incubated at 37 °C under microaerobic conditions (CO_2 10%, O_2 5%, H_2 5% and N_2 80%) for up to 20 days. *H. pylori* ATCC 43504 was plated as a positive culture control.

2.3. DNA extraction

DNA was directly extracted from gastric fluids, gastric tissue, dental plaque and saliva samples, using the CTAB phenol/chloroform method (Wilson, 1994) as described (Goldman et al., 2009a).

2.4. PCR amplification

Two primer pairs were designed, one to amplify *Helicobacter* spp. and the other to amplify helicobacter and non-helicobacter bacteria within the order Campylobacterales. From GenBank, 29 16S rDNA sequences from *Helicobacter* spp. and 15 from *Campylobacter* spp. that had been isolated from different animals were retrieved for comparisons (Table 1) and aligned using ClustalX version 1.83 (Thompson et al., 1997). Conserved regions among the helicobacter species and among both genus were selected to synthesize primers with appropriate specificity. Two forward primers and one reverse primer were designed to provide two primer pair combinations. Primer F1 (5′-GTATCCGGCCTGAGA-3′) was chosen to amplify *Helicobacter* spp. because it showed 100% homology with all the helicobacter sequences, but differed by 4 bp from the campylobacter sequences. Primers F0 (5′-GAGTTTGATCCTGGCTCAGAG-3′) and R1 (5′-ATTTTACCCCTACACCAA-3′) had 100% homology with both helicobacter and campylobacter sequences. Primers F1 and R1 (pair CG1) were used to amplify a 387 bp fragment of the 16S rRNA gene of the *Helicobacter* genus (positions 271–657 of the 16S rDNA of *H. pylori* ATCC 26695 (Tomb et al., 1997). Primers F0 and R1 (pair CG2) were used to amplify a fragment of approximately 650 bp from the 16S rDNA of helicobacter and non-helicobacter bacteria within the order Campylobacterales (positions 9–657 of the 16S rDNA of *H. pylori* ATCC 26695 (Tomb et al., 1997). The Primer 3