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Short communication

Detection of *Helicobacter* and *Campylobacter* spp. from the aquatic environment of marine mammals

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ABSTRACT

The mechanism by which *Helicobacter* species are transmitted remains unclear. To examine the possible role of environmental transmission in marine mammals, we sought the presence of *Helicobacter* spp. and non-*Helicobacter* bacteria within the order Campylobacterales in water from the aquatic environment of marine mammals, and in fish otoliths regurgitated by dolphins. Water was collected from six pools, two inhabited by dolphins and four inhabited by seals. Regurgitated otoliths were collected from the bottom of dolphins' pools. Samples were evaluated by culture, PCR and DNA sequence analysis. Sequences from dolphins' water and from regurgitated otoliths clustered with 99.8–100% homology with sequences from gastric fluids, dental plaque and saliva from dolphins living in those pools, and with 99.5% homology with *H. cetorum*. Sequences from seals' water clustered with 99.5% homology with a sequence amplified from a Northern sea lion (AY203900). Control PCR on source water for the pools and from otoliths dissected from feeder fish were negative. The findings of *Helicobacter* spp. DNA in the aquatic environment suggests that contaminated water from regurgitated fish otoliths and perhaps other tissues may play a role in *Helicobacter* transmission among marine mammals.

1. Introduction

In spite of being a widespread infection in humans and animals, the mode by which *Helicobacter* species are transmitted remains unclear. Oral–oral and fecal–oral modes of transmission have been suggested for human *H. pylori* infection (Feldman et al., 1998). Contaminated water could be implicated in transmission of the infection (Hulten et al., 1996; Azevedo et al., 2008), although it does not seem to be the main route of transmission (Feldman et al., 1998). Waterborne environmental transmission of *H. pylori* infection might be related to a coccoid form, a viable but non-cultivable bacterial stage that has been widely described for other bacteria in aquatic environments (Byrd et al., 1991; Engstrand, 2001).

Oral–oral and fecal–oral routes may also be implicated in transmission of *Helicobacter* infection in marine mammals, since *Helicobacter* spp. have been isolated from the feces of dolphins (Harper et al., 2002), and detected in

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their dental plaque (Goldman et al., 2002) and in the feces of seals (Oxley and McKay, 2004). However, transmission via contaminated water is an alternative possibility that has not been investigated. Captive pinnipeds and cetaceans typically eat two fish species, corvina (*Micropogonias furnieri*) and small hake (*Macrodon ancylodon*), and then regurgitate fish otoliths, which are calcium carbonate crystals from the inner ear of the fish (Popper et al., 2005). These otoliths might contaminate the water environment and serve as a vehicle for transmission of *Helicobacter* or *Campylobacter* spp.

To address this hypothesis, we sought to identify *Helicobacter* spp. and non-*Helicobacter* bacteria within the order Campylobacterales, in water samples and in fish otoliths regurgitated by captive marine mammals.

2. Materials and methods

2.1. Sample collection

Water samples were collected from two connected pools that were inhabited by ten dolphins (*Tursiops gephyreus*) and a killer whale (*Orcinus orca*), one pool that was inhabited by two elephant seals (*Mirounga leonina*), and three pools that were inhabited by seven sea lions (*Otaria flavescens*) and one fur seal (*Arctocephalus australis*). Samples were designated as dolphin water 1 and 2, and seal water 1–4, respectively. Each dolphin pool contained 3×10^6 L of water, whereas each seal pool contained between 2×10^5 and 4×10^5 L of water. All the pools were filled from the same water source, which was sampled as a control.

Three samples of otoliths were also collected from the bottom of the two pools inhabited by the dolphins (designated otolith 1 and 2), and another one from a third pool that connects them (otolith 3). As a control, otoliths, gastrointestinal tract, muscle, and branchiae samples were dissected from forty specimens each of corvina (*M. furnieri*) and small hake (*M. ancylodon*). Samples from the same type and fish species were pooled and transported on dry ice for further analysis at the laboratory in Buenos Aires.

Five liters of water were collected from each pool in sterile plastic containers. Chlorine was inactivated by the addition of 5 mL of 10% (v/v) sodium thiosulfate in water. The containers were refrigerated at 4 °C for their transportation to the laboratory in Buenos Aires.

2.2. DNA extraction

Each water sample was filtered under vacuum with four 0.45 μm cellulose nitrate sterile membranes (Microclar®, Buenos Aires, Argentina). One half of each of the four membranes were combined in an Eppendorf tube, mixed with proteinase K and lysis buffer, incubated at 56 °C overnight, and then processed for DNA extraction using the QIAamp Mini Kit (QIAGEN, Inc., CA, USA) according to the manufacturer's directions. Approximately 25 mg of each fish tissue and otolith sample were mixed with the same reagents and processed in the same manner.

2.3. Culture conditions

For culture analysis, the other half of the four membranes from each sample were laid directly 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). The membranes were cultured no more than 24 h after the collection of water samples. Otoliths and tissue samples were mixed with 0.5 mL of sterile saline, ground under sterile conditions, and then plated as above. Plates were incubated at 37 °C under microaerophilic conditions (CO_2 10%, O_2 5%, H_2 5% and N_2 balance) for up to 20 days, as described for *H. cetorum* by Harper et al. (2002). *Helicobacter pylori* ATCC 43504 was plated as a positive culture control.

2.4. PCR amplification

Two PCR amplifications were performed. We first amplified with primer pair CG2 (F0 5′-GAGTTTGATCCTGGCTCAGAG-3′ and R1 5′-ATTTTACCCCTACACCAA-3′), which amplifies a fragment of approximately 650 bp of the 16S rRNA gene of *Helicobacter* and non-*Helicobacter* bacteria within the order Campylobacterales (positions 9-657 of the 16S rRNA gene of *H. pylori* ATCC 26695, Tomb et al., 1997). Amplification was carried out in a total volume of 50 μL containing 1× *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleotide, 1.0 U of Platinum® *Taq* DNA Polymerase (Invitrogen Argentina, Buenos Aires, Argentina), 0.1 μg each oligonucleotide primer, and 10 μL of DNA template. PCR (94 °C for 1 min; 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; 72 °C for 5 min) was performed with an automatic thermocycler (MyCycler, BioRad, CA, USA) and a 10 μL aliquot was analyzed by electrophoresis through a 1.5% (wt/vol) agarose gel stained with ethidium bromide. PCR products were visualized by excitation under UV light. Because some reactions appeared to yield product that was of the correct size, but not sufficient for DNA sequencing, we reamplified the material from each reaction (positive and negative) as before, using 10 μL of amplicon from the original PCR.

A second PCR was performed using primer pair CG1 (F1 5′-GTATCCGGCCTGAGA-3′ and R1), which amplifies a 387 bp fragment of the 16S rRNA gene of the *Helicobacter* genus, and corresponds to positions 271–657 of the 16S rRNA gene of *H. pylori* ATCC 26695 (Tomb et al., 1997). PCR amplification was carried out with the same conditions described for primer pair CG2, but using an annealing temperature of 51 °C. Material was examined by gel electrophoresis and reamplified as described for primer CG2. Positive PCR results with primer pairs CG1 and CG2 indicate the presence of *Helicobacter* spp. DNA or *Helicobacter* and non-*Helicobacter* Campylobacterales, while a negative PCR result with CG1 and a positive result with CG2 indicates the presence of non-*Helicobacter* bacteria within the order Campylobacterales. Positive results with CG1 and negative results with CG2 would be unexpected and was never observed. All PCR assays