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Temporal study of *Helicobacter pylori* presence in coastal freshwater, estuary and marine waters

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ABSTRACT

Helicobacter pylori, a gastric pathogen, is believed to be transmitted via the fecal-oral route as well as the oral-oral route. Its presence and viability in environmental waters is not well characterized. The goals of this study were to test *H. pylori* presence via molecular methods in freshwater, estuarine and beach sites in Delaware over both short and long time scales and to establish whether fecal indicator bacteria, including total *Enterococcus* and human-specific *Bacteroidetes* species, are predictive of the pathogen in these waters. The presence of *Helicobacter pylori* was initially tested by PCR with newly designed 23S rRNA gene primers against *Helicobacter* spp. and confirmed by sequencing. Two coastal beach sites were repeatedly positive in 2007. Clone library analysis indicated the persistence of one operational taxonomic unit (OTU) over time at one site. Detection of *H. pylori* was also determined by PCR assays from DNA and RNA for the 16S rRNA gene, as well as DNA for the *ureA* and *cagA* genes. Approximately 21% of the samples were positive for *H. pylori* 16S rRNA gene and 80% of those were also positive for *H. pylori* 16S rRNA, indicating that this potential pathogen is not only present in natural waters, but also probably viable. There was no correlation between the occurrence of *H. pylori* and fecal indicator bacteria, suggesting that standard water quality tests are ineffective in predicting the presence of this pathogen in natural waters. These results demonstrate the widespread presence of potentially viable *H. pylori* in coastal marine and estuarine waters. Additionally, the repeatedly positive samples indicate either a continual contamination source or persistence of *H. pylori* in marine waters.

1. Introduction

Helicobacter pylori asymptomatically colonize the stomachs of 20–50% of the human population in developed countries (Atherton and Blaser, 2009). In some cases, however, it can cause gastritis, peptic ulcers, and gastric cancer depending on the presence or absence of genetic virulence factors (Hocker and Hohenberger, 2003). Transmission of this bacterium is thought to be by several routes, including fecal-oral (Bellack et al., 2006; Herrera et al., 2008; Delport et al., 2006). Potential reservoirs for *H. pylori* in aquatic environments are not well known, mainly because of the difficulty in detecting this pathogen by standard methods (Azevedo et al., 2008; Queralt and Araujo, 2007; Adams et al., 2003). Because *H. pylori* is one of the EPA drinking water contaminant candidates (http://www.epa.gov/ogwdw000/ccl/ccl1.html), further research is needed on the presence and persistence of this bacterium in natural environments.

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H. pylori and other *Helicobacter* species have been detected in natural environmental waters, including groundwater, freshwater streams, and off-shore marine waters, generally by PCR-based methods from environmental DNA (Hegarty et al., 1999; Voytek et al., 2005; Carbone et al., 2005; Cellini et al., 2004). *H. pylori* has been found associated with feces and appears to be correlated with the extent of fecal contamination (Queralt et al., 2005). However, *H. pylori* is also present in aquatic samples that test negative for fecal contamination (Hegarty et al., 1999; Voytek et al., 2005; Carbone et al., 2005; Cellini et al., 2004). Two seasonal studies of marine water demonstrated a high level of positive samples (up to 42%), with a greater number of positive samples during the summer months (Carbone et al., 2005; Cellini et al., 2004). Both of these longitudinal studies were from only two sites, one 50 m and one 500 m off the Italian coast. It is still unclear how prevalent *H. pylori* are in coastal and estuarine waters over longer time periods and across a gradient of salinity and what their relationship with fecal indicator bacteria (FIB) is in such environments.

The goals of this study were to examine *Helicobacter* species and *H. pylori* incidence over two years in freshwater, estuaries and marine waters of southern Delaware using PCR-based assays. The occurrence and phylogenetic affiliation of members of the *Helicobacter* genus was determined in these waters using newly designed PCR primers targeting the 23S rRNA gene. *H. pylori* presence was also identified by PCR primers targeting the 16S rRNA, *ureA*, and *cagA* genes. Additionally, we determined if the samples contained just rRNA genes (DNA) or rRNA from intact cells in a subset of the samples that were *H. pylori* positive by PCR. Finally, we determined whether the use of the conventional culture-based FIB test, total *Enterococcus*, or the use of molecular techniques targeting human-specific fecal *Bacteroides* species are more predictive of presence of these enteric pathogens in Delaware recreational waters.

2. Materials and methods

2.1. Site selection and sample collection

During this two year study, 137 samples were collected from a total of 23 sites from the Atlantic coast (Rehoboth Beach, Fenwick Island Beach, North Inlet Beach), Delaware Bay, Inland Bays (Rehoboth, Indian River, and Little Assawoman Bays; Tower Road and Holt's Landing) and Broadkill River (Fig. 1). Two of the beach sites, Rehoboth and Fenwick Island, have storm-water drainage pipes that discharge 5 m from shore. The Rehoboth drainage pipes also contain overflow from two lakes, Silver Lake and Lake Gerar. One of these sites, Lewes Canal, is adjacent to the community marina and less than 2 miles from the wastewater discharge site. The North Inlet site is immediately adjacent to the only Inland Bays outlet to the Atlantic Ocean and is within 0.5 km of a large public marina.

The 2007 sampling season lasted from June 26 through October 16 and included five coastal and bay sites sampled weekly, five Inland Bay sites sampled monthly and six sites sampled only once. During the 2008 season, which lasted from February 26 through September 19, four beach and bay sites were sampled bi-weekly, two freshwater sites were sampled monthly and four Inland Bay sites were sampled bi-monthly. Additionally, water samples were collected from two public marinas and one wastewater treatment facility once each during this study. Surface water samples were collected in black 2 L Nalgene bottles that were cleaned in the laboratory then triple rinsed with sample water immediately prior to collection. All beach sites (Tower Road, Holt's Landing, Rehoboth, Fenwick Island, North Inlet) were sampled approximately 5 m from shore, the rest of the samples were collected via boat or bridge access. Samples were kept on ice until processing in the laboratory within 4 h of collection.

2.2. *Environmental and* Enterococcus *measurements*

A handheld YSI 650 MDS was used to measure water temperature, salinity, pH, and dissolved oxygen at each sampling site at the time of sample collection. Chlorophyll *a* concentrations were measured on 25 mm GF/F (Whatman) filters in triplicate. The filters were frozen at −80 °C until processed. The filters were extracted in 90% acetone and chlorophyll *a* was measured using a fluorometer (Fluorometer 10-AU from Turner Designs, Sunnyvale, CA) (Parsons et al., 1984). Water for nutrient analysis was filtered through 25 mm GF/F (Whatman) filters and frozen at −80 °C until processed. Nitrate-nitrite, ammonium, and phosphate were measured by standard methods (Parsons et al., 1984) by an automated, segmented flow colorimetric analysis using an O/I Analytical Flow-Solution Analyzer (College Station, Texas).

To determine if *Enterococcus* spp. were present, water samples were tested using the most probable number method of the Enterolert Test Kit from IDEXX (Westbrook, ME) (Budnick et al., 1996).

2.3. Sample filtration and nucleic acid extraction

Water samples (400 mL) were filtered through 25 mm 0.22 μm GV Durapore filters. Samples were placed in 1 mL of 2% CTAB buffer and stored at −80 °C until nucleic acid extraction. We extracted the nucleic acids from the filters using the acetyltrimethylammonium bromide/polyvinylpyrrolidone/mercaptoethanol (CTAB/PVP/-ME) method (Dempster et al., 1999), which involves high-salt precipitation, chloroform extraction, and ethanol precipitation. The nucleic acid concentrations were determined from the UV-absorbance at 260 and 280 nm using a BioPhotometer spectrophotometer (Eppendorf, Westbury, NY).

2.4. PCR primer design and PCR analysis

Genus-specific PCR primers were designed to target the 23S rRNA gene of *Helicobacter* species (Table 1). Primers were designed in ARB using the Probe-Design tool with the LSU-rRNA Silva 93 database (Ludwig et al., 2004). Primer specificities were then analyzed using an NCBI-BLAST (www.ncbi.nlm.nih.gov/BLAST) search for a best hit match. PCR reactions with these primers and with the modified *H. pylori* 16S rRNA gene primers were tested with known DNA samples to test specificity. Isolates tested include: 12 representatives of the *Epsilonproteobacteria* (*Sulfurimonas autotrophica*, *Hydrogenimonas*