Detection of *Helicobacter pylori* and fecal indicator bacteria in five North American rivers

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ABSTRACT

This study examines the use of fecal indicator bacteria (FIB) as a predictor of the presence of *Helicobacter* spp. A combination of standard culture and molecular techniques were used to detect and quantify FIB, *Helicobacter* spp. and *H. pylori* from five North American rivers of different size and with different land use characteristics. Primers designed to amplify genes specific to *Helicobacter* spp. and *H. pylori* were evaluated for their efficacy in detection and quantification in environmental samples. *Helicobacter* spp. were detected in 18/33 (55%) of river samples. *H. pylori* was detected in 11/33 (33%) of river samples. FIB were found in 32/33 (96%) of river samples. When FIB abundance exceeded USEPA water quality standards for single samples, *Helicobacter* or *H. pylori* were detected in 7/15 (47%) cases. No numerical correlation was found between the presence of FIB and either *Helicobacter* spp. or *H. pylori*. This suggests that the presence of FIB will be of limited use for detection of *Helicobacter* spp. or *H. pylori* by public health agencies.

Key words | fecal coliforms, *Helicobacter pylori*, indicator, molecular detection, polymerase chain reaction, water quality

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INTRODUCTION

Traditional assessments of microbiological water quality have focused on the presence of fecal indicator bacteria (FIB). Although FIB are not necessarily pathogenic, they are abundant in human and other animal waste products where pathogenic organisms are found (Noble *et al.* 2003). High concentrations of FIB can be associated with agricultural operations (*e.g.*, feedlots, cattle pastures, manure spreading on crops), as well as human habitation. Understanding the effects of changing land usage and population density on river microbiology is important for managers and public health officials seeking to predict the presence of microbial pathogens in water resources.

The gram negative enteric bacterium, *Helicobacter pylori* is a frequent colonizer of the human stomach and is recognized as the causative agent of chronic gastritis, and peptic and duodenal ulcers, and the etiologic agent in gastric cancers (Dubois 1995; Dunn *et al.* 1997; Velazquez & Feirtag 1999). In 1996, the World Health Organization designated *H. pylori* as a Class I carcinogen and has issued a gastric cancer warning (World Health Organization 1996). The prevalence of *H. pylori* infection in human populations worldwide is believed to be >50% (Dunn *et al.* 1997). In developing countries about 70–90% of adults show serological evidence of current or past infection with *H. pylori* (Nurgalieva *et al.* 2002; Parkinson *et al.* 2000; Parkinson & Butler 2001). Infection rates are considerably lower in the developed world; *e.g.*, the rate of infection in the U.S. is estimated at 10–25% of the general adult population (Velazquez & Feirtag 1999). The principal mode of *H. pylori* transmission in human populations remains unproven although evidence that supports fecal–oral, oral–oral and waterborne transmission has been presented

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(Velazquez & Feirtag 1999). Recent investigations have focused on the potential for waterborne transmission based on the identification of *H. pylori* from both treated and untreated water sources (Nurgalieva *et al.* 2002; Engstrand 2001; Hegarty *et al.* 1999; Sasaki *et al.* 1999; Krumbiegel *et al.* 2004). Given the widespread occurrence of *H. pylori* infection in human populations, and current uncertainties concerning mode of infection and transmission, determining the potential for rivers to act as an environmental source is important to understanding the biology, etiology and epidemiology of this pathogen.

In this study, we examined the relationships between land use, water quality, FIB concentrations and the presence of *H. pylori* in five geographically distinct North American rivers. We compared samples from forested, sparsely populated areas of the Yukon River system in central Alaska, from agricultural regions of the Upper Illinois drainage basin in N.W. Indiana and N.E. Illinois, and from an urban-suburban region near the Washington, DC metropolitan area. The decision to focus sampling of the Alaskan rivers near settlements was based on epidemiological data showing infection rates similar to those observed in developing countries. Eddy (2004a, b) has described the rudimentary sewage handling practices that persist in some of these villages. In natives from rural Alaskan villages, 86% of the population (age 20 years and above) shows serological evidence of current or past *H. pylori* infection (Parkinson & Butler 2001). Samples collected from Indiana and Illinois were taken with the expectation that the impact of surrounding agricultural activities would be reflected by high nutrient concentrations (*e.g.*, nitrate) and high numbers of FIB (US Geological Survey National Water Quality Assessment Program 2002). Samples collected from the Potomac River near the Washington, DC metropolitan area allowed examination of water that was heavily impacted by human activity (Ator *et al.* 1998). These samples were expected to contain both high concentrations of nutrients as well as high numbers of FIB (Interlandi & Crockett 2003).

A specific aim of this investigation was to determine what predictive value the presence of FIB would have for a potential waterborne pathogen, *H. pylori*. We examined the co-occurrence of FIB, *Helicobacter* spp. and *H. pylori* through a combination of traditional microbiological analyses (FIB) and molecular detection (*Helicobacter* spp. and *H. pylori*) using the polymerase chain reaction (PCR). A culture-independent method of identifying *Helicobacter* spp. and *H. pylori* in the environment was chosen for several reasons. Previous work has indicated that *H. pylori*, when confronted with unfavorable environmental conditions (*e.g.*, a non-enteric environment), will enter into a viable but non-culturable stage (VnC) that may or may not maintain virulence (West *et al.* 1992; Beneduce *et al.* 2003; Krumbiegel *et al.* 2004). *H. pylori,* if present in rivers, has probably entered into a VnC stage, precluding the use of culture techniques to accurately determine presence and abundance. Our choice of molecular technique allows detection of non-culturable cells in the environment and takes advantage of the high degree of specificity and sensitivity that can be achieved using a PCR-based assay. In addition, PCR was used to detect other members of the genus *Helicobacter*, several of which are known human pathogens (Solnick & Schauer 2001).

MATERIALS AND METHODS

Study areas

The Yukon River is one of the largest rivers in North America. An estimated 130,000 people live in the drainage basin of 855,000 km^2 (about 65% of these live in Fairbanks) with an average population density of about 0.15 people/km^2 (Brabets *et al.* 2000). Sample collection was targeted to sites that were representative of both local (*e.g.,* from towns or villages) and overall watershed inputs. Two sampling locations in the Yukon drainage basin were chosen based on their proximity to relatively large human population centers. We sampled at the city of Eagle (population 150), located about 150 km downstream from Dawson, Yukon Territory, Canada (Table 1). The permanent population in Dawson is about 2000 and increases to 40,000 in the summer. Similarly, a tributary of the Yukon River, the Tanana River was sampled at the town of Nenana (population 500), about 72 km downstream of Fairbanks (population 84,000) (Table 1). We also sampled two locations in the Yukon drainage basin that were distant from large human population centers. Collections on the Yukon River were made at Fort Yukon (population 581) and at Stevens Village (population 85) (Table 1).

Table 1 | Physical, chemical and biological measures of water quality

	Date	SS mg/L	Discharge cfs	Temp. deg. C	pH	SC uS/cm	DOC mg C/L	DO mg/L	N-NO_3 mg N/L	N-NH_4 mg N/L	P-PO_4 mg P/L	F.C. cfu/L	*E. coli* cfu/L	Ent. cfu/L	PCR* *Helicobacter* spp. (16S rDNA) cells/L	PCR** *H. pylori* (glm M) cells/L	qPCR *Helicobacter* spp. cells/L	qPCR *H. pylori* cells/L
Yukon River																		
City of Eagle[a]	8/10/01	730	188,000	14.6	7.9	216	3.5	9.5	0.02	0.002	0.006	430	430	40	–	–	100	0
City of Eagle - bank[a]	8/10/01	730	188,000	14.6	7.9	189.5	3.5	9.5	0.01	0.2	0.01	240	230	30	–	–	0	0
City of Eagle - bank[b]	5/29/02	603	177,000	8	8.1	154	13.6	11.7	0.01	<.015	<.007	0	0	ND	–	–	0	0
Fort Yukon - bank[a]	8/14/01	ND	ND	ND	ND	181	3.27	ND	0.14	0.02	0.065	120	130	230	–	–	4	70
Fort Yukon - swimming bole[b]	8/14/01	ND	ND	ND	ND	368	ND	ND	0.25	ND	0.02	0	0	0	–	–	ND	ND
Fort Yukon - slough[b]	8/14/01	ND	ND	ND	ND	199	ND	ND	0.02	ND	0.01	130	140	130	–	–	5	0
Fort Yukon - bank[b]	5/31/02	ND	ND	11	ND	175	ND	ND	0.08	ND	0.05	0	0	ND	–	–	0	0
Fort Yukon - slough[b]	5/31/02	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	80	80	ND	–	–	ND	ND
Stevens Village[a]	8/15/01	507	215,000	14	7.7	227	3.5	9.5	0.028	0.012	0.006	150	180	500	–	–	0	0
Stevens Village - bank[a]	8/15/01	507	215,000	14	7.7	227	3.5	9.5	0.028	0.012	0.006	270	270	**4,980**	–	–	0	0
Stevens Village - bank[b]	5/30/02	388	253,000	9	8	145.5	7.9	9	0.105	<.015	0.05	10	20	ND	–	–	150	0
Porcupine River																		
Porcupine River @ Ft. Yukon[b]	8/9/01	ND	16,700	ND	ND	200	ND	ND	1.44	ND	0.23	20	20	60	–	–	0	10

Table 1 | (*continued*)

	Date	SS mg/L	Discharge cfs	Temp. deg. C	pH	SC uS/cm	DOC mg C/L	DO mg/L	N-NO_3 mg N/L	N-NH_4 mg N/L	P-PO_4 mg P/L	F.C. cfu/L	*E. coli* cfu/L	Ent. cfu/L	PCR* *Helicobacter* spp. (16S rDNA) cells/L	PCR** *H. pylori* (glm M) cells/L	qPCR *Helicobacter* spp. cells/L	qPCR *H. pylori* cells/L
Porcupine River[a,f]	8/9/01	15	16,700	13.1	7.9	244	6.8	10.5	0.012	0.002	0.006	50	50	100	–	+	10	110
Tenana River																		
City of Nenana[a]	8/8/01	3,452	98,800	13.5	7.7	187	3.9	10	0.06	0.002	0.004	320	300	**850**	–	–	10	80
City of Nenana - bank[a]	8/8/01	3,452	98,800	13.5	7.7	188	3.9	10	0.05	0.065	0.004	210	210	560	–	–	650	0
City of Nenana - bank[b]	6/1/02	1,204	51,000	12.1	8	205	3.7	9	0.78	<.015	0.05	160	160	ND	–	–	0	0
Sugar Creek																		
Site 1	9/13/01	4.3	1.77	23.6	8.04	656	4.2	9.6	0.30	0.063	0.02	**14,500**	**12,700**	**5,700**	+	+	247,000	594,000
Site 8	9/13/01	15.4	30.5	22.5	8.00	660	2.9	8.6	3.4	0.03	0.02	**14,600**	**13,000**	**9,600**	–	+	8,200	120
Site 9	9/13/01	16.2	ND	20.3	8.00	663	3.4	7.8	3.5	0.098	0.054	**17,400**	**12,100**	**10,500**	–	+	0	389,000
Site 1	6/10/03	17	13.6	21.4	8.27	600	2.8	10.9	11.0	0.016	<0.006	1,460	1,310	ND	–	–	700	0
Site 8	6/10/03	31	72.8	20.3	8.09	630	3.3	6.9	9.81	0.040	<0.006	**2,840**	**2,480**	ND	–	–	1,000	0
Site 9	6/10/03	44	68.8	20.8	8.10	633	3.2	7.0	9.68	0.041	<0.006	**2,840**	1,960	ND	–	–	0	0
Iroquois River																		
Site 4	9/14/01	22.7	47.9	21.1	8.00	654	6.4	6.2	1.1	0.10	0.048	**5,000**	**4,200**	**800**	+	+	10,100	0
Site 5	9/14/01	36.5	43	19.9	8.10	659	6.6	5.4	1.0	0.067	0.048	**7,300**	**3,900**	**1,500**	+	+	8,600	0
Site 4	6/10/03	56	689	17.3	7.83	657	ND	6.62	11.2	0.173	0.035	1,980	1,320	ND	–	–	0	0
Site 5	6/10/03	66	613	17.6	7.8	663	5.48	6.77	13.7	0.153	0.042	1,910	1,020	ND	–	–	0	0
Potomac River																		
Algonkian Park	7/11/01	10[d]	1,890[c]	28.1[d]	7.8[d]	363[d]	NA[d]	5.0[d]	0.78[d]	0.008[d]	0.03[d]	**219,000**	**68,000**	ND	–	+	219,000	24,900

Table 1 | (*continued*)

	Date	SS mg/L	Discharge cfs	Temp. deg. C	pH	SC uS/cm	DOC mg C/L	DO mg/L	N-NO_3 mg N/L	N-NH_4 mg N/L	P-PO_4 mg P/L	F.C. cfu/L	*E. coli* cfu/L	Ent. cfu/L	PCR* *Helicobacter* spp. (16S rDNA) cells/L	PCR** *H. pylori* (glm M) cells/L	qPCR *Helicobacter* spp. cells/L	qPCR *H. pylori* cells/L
Algonkian Park	10/10/01	4[d]	937[c]	14.6[d]	8.6[d]	443[d]	NA[d]	9.6[d]	1.03[d]	0.002[d]	0.013[d]	**2,100**	1,000	140	–	+	1410	910
Algonkian Park	10/16/02	8.8[d]	3510[d]	16.9[d]	7.7[d]	320[d]	3.1[d]	10.2[d]	0.71[d]	0.02[d]	0.03[d]	**2,300**[c]	250[c]	ND	–	–	0	0
Algonkian Park	11/25/02	11.6[d]	15,191[d]	7.3[d]	7.6[d]	175[d]	2.9[d]	12.3[d]	1.93[d]	0.02[d]	0.04[d]	300[c]	250[c]	ND	–	–	0	0
Algonkian Park	5/27/03	41[d]	26,526[d]	16.3[d]	7.5[d]	203[d]	3.1[d]	10.8[d]	1.68[d]	0.07[d]	0.03[d]	**50,000**[c]	2,000[c]	ND	–	+	180	0
Algonkian Park	6/3/03	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	**28,000**[c]	**12,000**[c]	ND	–	+	350	0
Algonkian Park	6/10/03	60[d]	48,640[d]	19.3[d]	7.5[d]	210[d]	3.0[d]	9.5[d]	1.52[d]	1.52[d]	0.04[d]	**17,000**[c]	**14,000**[c]	ND	–	–	0	0
Wastewater																		
Blue Plains[g]	4/19/02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	TNTC	TNTC	ND	+	+	161,000	12,300
Blue Plains[h]	4/19/02	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	TNTC	TNTC	ND	+	–	82,000	0
USEPA FIB Standards																		
Drinking water												0	0	0				
Recreational water[i]												2000	2350	610				

* " + " indicates > 1 pg *Helicobacter* spp. DNA/reaction (ca. 500 cells/reaction).
** " + " indicates > 10 pg *H. pylori* DNA/reaction (ca. 500 cells/reaction).
[a]Sample average reflects watershed characteristics
[b]Sample value reflects local site characteristics
[c]Washington Suburban Sanitary Commission data
[d]Metropolitan Washington Council of Governments Data
[e]US Geological Survey Data
[f]US Geological Survey site 15389000 is about 160 km upstream from the confluence of the Porcupine River and the Yukon River.
[g]0.22 um filter capsule.
[h]0.45 um filter capsule
[i]Single sample limit expressed per/L.
"Bold" indicates FIB exceedence of USEPA standard
BD = below detection; NA = not available; ND = not determined, TNTC = too numerous to count
SS = Suspended sediment; cfs = cubic feet/second; SC = Specific conductance, DOC = dissolved organic carbon, DO = dissolved oxygen, F.C. = total fecal coliforms, *E. coli* = *Escherichia coli*, Ent. = *Enterococci*
PCR = Standard polymerase chain reaction, qPCR = quantative PCR, cfu = colony forming units

The Porcupine River, another tributary of the Yukon, was also sampled at two sites. One collection was made above the confluence of the Porcupine and Yukon Rivers, adjacent to a sewage lagoon near the town of Fort Yukon and a second was taken at a remote, uninhabited site about 160 km upstream (Table 1). Sampling in Alaska occurred in August 2001 and May 2002. The discharge of the Yukon River follows a general seasonal pattern with peak flows occurring one to two months after the spring break-up. The Yukon River system sampling in August 2001 occurred about 6-8 weeks after peak high water and the second sampling of the Yukon River system in May 2002 occurred within two weeks of the spring ice break-up.

As a comparison to the relatively pristine rivers in Alaska, we sampled Sugar Creek and the Iroquois River in the Upper Illinois basin, and the Potomac River near the Washington, DC metropolitan area (Table 1). Sugar Creek drains an area of about 422 km^2 and the Iroquois River drains an area of about 1,532 km^2. Both watersheds are predominantly agricultural, with >90% of each drainage basin being farmland used primarily for the cultivation of corn and soybeans. Human populations in Sugar Creek (Newton County, IN) and the Iroquois River (Iroquois County, IL) watersheds are dispersed in small towns and isolated farms with an average population density of about 12 people/km^2 (US Census Bureau 2000; Indiana Business Research Center 2000). Sugar Creek and Iroquois River samples were taken from a series of locations along a 10–26 km reach of each river. Runoff to these rivers is the highest in late spring, with a significant reduction in flow occurring typically by early fall. These rivers were sampled during a period of low water in September 2001, and during a period of high water in June 2003. The land surrounding Sugar Creek site #1 was predominantly corn and soybean fields, site #8 was near a cattle pasture with visible wildlife activity (raccoon, fox, and waterfowl tracks), and site #9 was in a wooded area adjacent to the town of Milford, IN. On the Iroquois River, site #4 was located downstream of a small wastewater treatment plant, while site #5 was located in an agricultural area near a cattle pasture.

The Potomac River was sampled during July and September 2001, October and November 2002 and May and June 2003 at Algonkian Regional Park near Sterling, VA (about 30 km upstream from the District of Columbia border) (Table 1). This sampling location is located within the Middle Potomac drainage (4,753 km^2; population 3,722,000; or 783 people/km^2) in a rapidly developing suburban area of northern Virginia (17% developed land, 36% agriculture, 41% forested) (Chesapeake Bay Program 2000). This site is part of a large recreational area/complex commonly used for picnics, swimming and boating. In addition to river collections, we obtained a sample of raw sewage from the District of Columbia Water and Sewer Authority's Blue Plains (DCWASA) wastewater treatment plant. This plant treats all the sewage from the Washington, DC metropolitan area and was sampled as a positive control based on the expectation of significant microbial contamination.

Sample collection and environmental characterization

Physical, chemical and biological measurements of water quality from rivers in Alaska were carried out using standard methodologies. Phosphorus and nitrate concentrations were determined by ion chromatography using a DIONEX DX 120 ion chromatograph equipped with an AS14 analytical column and a conductivity detector (DIONEX, Sunnyvale, CA). Ammonium concentrations were determined by colorimetric assay using a Hach kit (method 8038, Hach Co., Loveland, CO). Dissolved organic carbon and dissolved oxygen concentrations were determined as part of the U.S. Geological Survey (USGS) National Stream Quality Accounting Network (NASQAN) Yukon Basin Project using standard National Water Quality Laboratory methods (US Geological Survey 2003a). Temperature, pH and specific conductance were measured on site at the time of collection. Collections were made as surface grab samples from near shore to reflect local inputs. Additional surface grab samples at selected sites in Alaska were taken from the center of the main river channel and were chosen to reflect average watershed characteristics. To evaluate each populated site in Alaska as a potential source of microbial contamination, samples were taken upstream and downstream of each location.

Physical and chemical measurements of water quality from Sugar Creek and the Iroquois River were taken as part of an ongoing USGS program (US Geological Survey

2003b). The overall water quality in the Potomac is measured routinely by several private and government agencies including the USGS, the Chesapeake Bay Program (CBP) and the Washington Suburban Sanitary Commission (WSSC) (Chesapeake Bay Program 2000, Washington, DC Suburban Sanitary Commission 2003). No significant site differences in water quality data were observed between the sampling location at Algonkian Park, the WSSC Potomac Water Filtration Plant, and the USGS monitoring station at Chain Bridge (currently monitored by the Metropolitan Washington Council of Governments (MWCOG)) (Metropolitan Washington Council of Governments 2003). We utilized a combination of water quality and FIB data collected by the WSSC, the MWCOG and the USGS in our comparative analyses (Table 1). When no significant differences were observed between samples from a single site (e.g., upstream, downstream), results were reported as averages (Table 1).

Microbial biomass for total DNA extraction was collected using inline filtration capsules (SpiralCap® PF Capsule filters, Pall Gelman Laboratory Co., Ann Arbor, MI.) containing a dual pre-filter/filter (0.8 μm/0.2 μm pore sizes). Post-filtration, 150 μl of sterile filtered EDTA (0.5 M) was added to each capsule to chelate metals and inhibit the activity of nucleic acid degrading enzymes. Capsules were stored on dry ice for return to the laboratory and maintained at −80°C until processing. Suspended sediments in both the Yukon and Tanana Rivers were extremely high. This presented significant difficulties for the collection of microbiological samples and precluded the use of immunomagnetic separation and concentration. Our samples were concentrated by filtration and, as a consequence, the total volume of water that could be passed through the filter varied with the sediment load at each location. The volume of water filtered for each sample ranged from 1 litre to 20 litres. Exact volumes were recorded and taken into consideration in setting up the PCR reactions and calculating concentrations. Riverbed sediments were sampled by coring the surface with sterile polypropylene sleeves (Table 2). Sediment cores used for DNA analysis were divided into slices by depth (1-2 cm intervals), stored on dry ice for return to the laboratory and maintained at −80°C until processing.

Quantification of fecal indicator bacteria

Fecal indicator bacteria from river water were quantified using standard membrane filtration techniques (US Environmental Protection Agency 2000; *Standard Methods* 1998). Water was filtered in 100 ml, 10 ml, and 1 ml volumes. Sediment was mixed with phosphate buffered saline and processed as 10 ml, 1 ml and 0.1 ml volumes. Fecal coliform bacteria were enumerated after a 24 hour incubation on m-FC/Rosolic acid media at 44.5°C (Hach, Loveland, CO). *E. coli* was then distinguished from other fecal coliform bacteria by transfer to Na-MUG agar (Difco, Sparks, MD) followed by a 4 hour incubation at 37°C and enumeration of colonies with blue fluorescent halos. A second membrane filter for each volume of filtered water was incubated on mEI agar at 41.5°C for 24 hours. Enterococci colonies were identified by formation of a blue halo and counted.

DNA extraction

Total DNA was extracted from SpiralCap® PF filter capsules as follows. A 1:1 solution of 10 mM Tris - 0.1 mM EDTA (TE):1% sodium dodecyl sulfate (12 ml) was added to each capsule. Capsules were sealed and incubated for 20 min. at 100°C. Lysates were collected and total nucleic acids purified and concentrated by standard phenol:chloroform extraction and sodium chloride/ethanol precipitation (Sambrook *et al.* 1989). Precipitated nucleic acids were washed once with 70% ethanol, air-dried and resuspended in a final volume of sterile TE (40 μl – 200 μl). Total DNA from sediment samples was extracted from the top 1–2 cm of each core using the UltraClean™ Soil DNA isolation kit following the recommendations of the manufacturer (MoBio Laboratories, Solana Beach, CA.). Based on the results of PCR amplification using 16S rDNA primers (see below) some samples were purified further using the UltraClean™ 15 DNA purification Kit (MoBio Laboratories, Solana Beach, CA.).

To evaluate the efficiency of our extraction protocol for the recovery and detection of *H. pylori* from the environment, a series of *H. pylori*-river water artificial environmental samples were prepared. A pure culture of actively growing *H. pylori* was quantified by direct microscopic count and four replicate samples of Potomac

Table 2 | Detection and quantification of fecal indicator bacteria and *Helicobacter* DNA sequence from sediments

Location	Data	F.C. cfu/g wet wt.	*E.coli* cfu/g wet wt.	Ent. Cfu/g wet wt.	PCR* *Helicobacter* spp. (16S rDNA) cells/g	PCR** *H. pylori* (*glm* M) cell/g	qPCR *Helicobacter* spp. (16S rDNA) cells/g	qPCR *H. pylori* (*glm* M) cells/g
Yukon River								
Eagle - downstream	8/10/01	4	4	0	–	–	0	0
Eagle - upstream	8/10/01	3	3	0	–	–	460	0
Eagle - downstream	5/29/02	1	1	ND	–	–	0	0
Fort Yukon - slough	8/14/01	2	0	3	+	–	1050	0
Stevens Village - upstream	8/15/01	13	13	4	–	–	270	200
Stevens village - central	8/15/01	4	4	1	+	–	2240	1170
Stevens Village - downstream	5/30/02	0	1	ND	+	–	900	0
Tanana River								
Nenana - upstream	8/8/01	15	11	26	+	–	11,100	0
Sugar Creek								
Site 1	6/9/03	270	270	ND	–	–	0	0
Site 8	6/9/03	9	9	ND	–	–	0	240
Site 9	6/9/03	6	5	ND	–	–	640	0
Iroquois River								
Site 4	6/9/03	320	150	ND	–	–	0	0
Site 5	6/9/03	210	120	ND	–	–	0	0
Potomac River								
Algonkian Park	6/10/03	ND	ND	ND	–	–	0	0

*" + " indicates > 1 pg *Helicobacter* spp. DNA/reaction (ca. 500 cells/reaction)
** " + " indicates > 10 pg H. *pylori* DNA/reaction (ca. 500 cells/reaction)
F.C. = total fecal coliforms, *E. coli* = *Escherichia coli*, Ent. = enterococci, ND = not determined
PCR = standard polymerase chain reaction, qPCR = quantitative PCR, cfu = colony forming units

River water were inoculated with 10^5–10^8 cells. These samples and an uninoculated blank sample were filtered, and the total DNA extracted as described above. Based on previous work, we anticipated that *H. pylori* in rivers would have entered into a VnC cell stage (Velazquez & Feirtag 1999). To control for the recovery of *H. pylori* DNA from VnC cells in the environment, we prepared two additional *H. pylori* - river water artificial samples. The first 10 litre sample (Potomac River water) was inoculated with 10^7 cells of actively growing *H. pylori* and held at room temperature (25°C) for 48 hours before processing. This allowed sufficient time for the added cells to enter

into the VnC stage (West *et al.* 1992; Beneduce *et al.* 2003). The second 10 litre sample was prepared by the direct addition of 10^7 *H. pylori* cells already in the VnC stage (as determined by microscopic examination). The total DNA from both samples was extracted as described above. Total DNA was also extracted directly from an actively growing culture of *H. pylori* using a direct method that employs Chelex-100 resin (BioRad Hercules, CA) (Walsh *et al.* 1991).

PCR amplification and detection of *Helicobacter* spp. and *H. pylori*

Extracted DNA template quality was confirmed by PCR amplification using universal bacterial 16S rDNA primers 46f and 519r (Table 3) (Devereux & Willis 1995; Brunk *et al.* 1996). This was necessary in order to determine whether negative PCR results were false due to the presence of PCR inhibitors. DNA extractions that yielded amplification products of the expected size (ca. 490 bp) were used as template for PCR amplification with *Helicobacter* spp. genus-specific and *H. pylori* species-specific primers. To detect the genus *Helicobacter*, we used PCR primers HS1 and HS2 (Table 3) that amplify a 400 bp fragment of the 16S rRNA gene from all known members of this genus (Cantet *et al.* 1999). Species-specific amplification of *H. pylori* was carried out using PCR primers *glm*Mf and *glm*Mr (Table 3) that amplify a 294 bp fragment of the gene for the phosphoglucosamine mutase (*glm*M) enzyme (Bickley *et al.* 1993). A recent comparative study has shown that these primers are currently the most sensitive and specific primers available for the detection of *H. pylori* (Lu *et al.* 1999). The standard PCR reactions were carried out in a 50 μl volume in a GeneAmp 9700 (Perkin Elmer). Optimal PCR conditions were determined for each primer set using a modified Taguchi method (Cobb & Clarkson 1994). The 1XPCR buffer used contained (0.03 M tricine pH 8.4, 0.05 M KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.05% Igepal, 1.2 mg/ml non-acetylated BSA, (Sigma Chemical Company, St. Louis, MO) and 1.25U Taq polymerase (Promega Corp., Madison, WI). Igepal and non-acetylated BSA were added to enhance template access (solubilize DNA), polymerase stability and primer specificity (Innis *et al.* 1990). The primer sequences, primer concentrations, PCR cycle conditions and amplicon sizes of the PCR methods are listed in Table 3.

The sensitivity of each primer set for the detection of *H. pylori* was determined empirically by amplification of serial 10-fold dilutions of purified *H. pylori* genomic DNA (100 ng - 0.001 pg DNA). To demonstrate that these primers were not specifically inhibited by sample DNA preparations and to determine if any reduction in sensitivity occurred in detection from environmental samples, PCR was carried out on a mixture of genomic *H. pylori* DNA and extracted

Table 3 | Conditions for PCR methods

Target (reference)	Primer names and sequences 5′-3′	PCR product size	PCR Primer concentration	PCR conditions
16S RNA gene, *Helicobacter* spp. (Cantet *et al.* 1999)	HS1 5′-AAC GAT GAA GCT TCT AGC TTG CTA G-3′ HS2 5′-GTG CTT ATT CGT TAG ATA CCG TCA T-3′	400 bp	0.25 μM	95°C 5 min (1cycle); 94°C 1 min, 60°C 1.5 min, 72°C 1 min (35 cycles); 72°C 7 min (1 cycle)
glm M gene, H. pylori (Bickley *et al.* 1993)	*glm*M f 5′-AAG CTT TTA GGG GTG TTA GGG GTTT-3′ *glm*M r 5′-AAG CTT ACT TTC TAA CAC TAA CGC-3′	294 bp	0.50 μM	95°C 5 min (1 cycle); 93°C 1 min, 55°C 1 min, 72°C 1 min (35 cycles); 72°C 7 min (1 cycle)
16S rRNA gene, bacteria (Devereux and Wills 1995; Brunk *et al.* 1996)	46f 5′-GCY TAA CAC ATG CAA GTC GA-3′ 519r 5′- GTA TTA CCG CGG CKG CTG-3′	490 bp	0.20 μM	95°C 5 min (1 cycle); 94°C 0.5 min, 56°C 0.5 min, 72°C 1.5 min (30 cycles); 72°C 7 min (1 cycle)

environmental DNA. A 10-fold dilution series of *H. pylori* genomic DNA mixed with 1-10 ng of DNA extracted from an environmental sample (Eagle, 8/9/2001) was used as template. PCR was also used to confirm our ability to detect the DNA from cells of *H. pylori* extracted from the environment (versus genomic *H. pylori* DNA added to the PCR). Total DNA extracted from the *H. pylori* - river water artificial samples (described above) was used as a template for PCR, allowing primer detection limits to be determined from amplification of serial 10-fold dilutions (100 ng - 0.001 pg template).

The presence or absence of *Helicobacter* spp. or *H. pylori* DNA in each extraction was determined by standard PCR using diluted (1:10, 1:100 and 1:1,000) and undiluted environmental samples as a template. These results were compared to quantitative estimates of abundance generated using real-time or "quantitative" PCR (qPCR). The numbers of *Helicobacter* spp. and *H. pylori* cells present in each sample was quantified using an MJ Research Opticon 1 real-time PCR machine (MJ Research, San Francisco, CA.) and the QuantiTect Sybr Green PCR kit (Qiagen Inc., Valencia, CA.). Reaction conditions for qPCR were the same as those used for standard PCR with two modifications. The total number of cycles was increased to 45 and reactions were performed in a total volume of 25 μl according to the manufacturer's instructions. The concentration of *Helicobacter* spp. and *H. pylori* in each environmental sample was quantified by comparison of threshold fluorescence (usually at 20–30 cycles) to a standard amplification curve generated from total DNA extracts containing a known number of *H. pylori* cells (*H. pylori* - river water artificial samples). We scored both PCR methods when reporting our positive results (Table 1).

RESULTS AND DISCUSSION

Water quality

Standard physical and chemical measures of water quality for the rivers sampled are given in Table 1. In particular, we focused our attention on those constituents known to influence or reflect microbial activity (*e.g.*, dissolved oxygen, dissolved organic carbon, ammonia and nitrate) (Brock *et al.* 1991). Ammonium (NH_4) concentrations were consistently low in all the rivers we examined with values ranging from the limits of detection (0.002 mg N/L) at Eagle on the Yukon River to 0.1 mg N/L in the Iroquois River (Table 1). Ammonium levels measured in this study were highest in both rivers from Indiana, probably reflecting fertilizer runoff (Thompson *et al.* 1998). The concentration of dissolved oxygen (DO) measured in all rivers fell within a range of 11.7 mg/l at Eagle (Yukon River) to 5 mg/l at Algonkian Park (Potomac River) (Table 1). The concentration of DO is known to reflect temperature, degree of aeration, respiration and photosynthesis. Nitrate levels in rivers have been used as an indicator of human impact from domestic and industrial wastes, as well as from agricultural practices (Interlandi & Crockett 2003), and is reflected by our data. Nitrate concentrations showed some seasonal variation related to land use practice and changes in flow. Nitrate concentrations were highest in rivers where surrounding land use was agricultural (Sugar Creek and the Iroquois River) and lowest where the surrounding land was predominantly undeveloped (all rivers in Alaska) (Table 1). Concentrations ranged from a high of 11.2 mg N/L in the Iroquois River to the limits of detection (0.01 mg N/L) in several Alaska samples (Table 1).

The levels of suspended sediments in the Yukon River and the Tanana River were extremely high (Table 1). The majority of this sediment is silt and clay associated with glacial meltwater (Brabets *et al.* 2000). In contrast, suspended sediments in the Porcupine River were very low, the result of it draining an area that is underlain continuously by permafrost (Table 1). Levels of suspended solids measured from Sugar Creek, the Iroquois River and the Potomac River were similar and lower than those encountered in the Yukon River Basin sites (Table 1).

Quantification of fecal coliform bacteria

In the Yukon, Tanana and Porcupine Rivers in Alaska, fecal coliform, enterococci and *E. coli* concentrations were below US Environmental Protection Agency (USEPA) and State of Alaska recreational (full body contact) water standards for all but two samples (Table 1) (US

Environmental Protection Agency 2002). Enterococci abundance in single samples from both the Yukon River at Stevens Village 2001 and from the Tanana River at Nenana 2001, exceeded USEPA standards (Table 1). In general, FIB abundance was lower in spring as compared to late summer although more data are required to confirm any seasonal patterns. One possible explanation for this may be related to dilution and rising water volume following the spring melt. The levels of FIB in riverbed sediments from all rivers in Alaska were very low (Table 2).

Fecal indicator bacteria (FIB) in the majority of samples (70%) from both Sugar Creek and the Iroquois River exceeded the State of Indiana and USEPA standards for recreational waters (Table 1). This probably reflects the agricultural land use in this area with the likely presence of multiple sources of FIB contamination; *e.g.* cattle, poultry, wildlife, sewage. In fall, discharge in these rivers is at its lowest due to significant flow reduction occurring over the course of the summer (US Geological Survey 2003a,b). FIB in these rivers were higher (an order of magnitude higher in Sugar Creek) in fall than in spring. A possible explanation for this is that the source of FIB in these rivers is probably from surface runoff and direct delivery. During periods of high water, a significant proportion of the flow in these rivers is shallow groundwater discharge containing lower numbers of FIB. In this system, greater flow acts to dilute FIB abundance.

The highest single level of FIB determined in our study came from the Potomac River, although abundance varied between samplings by three orders of magnitude (250 – 219,000 cells/litre) (Table 1). The sampling site on the Potomac River was within the Middle Potomac watershed. This watershed drains large areas of suburban development, including both residential and light industrial. Microbial contamination observed is probably due to inputs from multiple point and non-point sources related to this development (*i.e.*, septic tanks, sewage) and may vary as a function of changing hydrologic conditions (*e.g.*, precipitation). FIB abundance was the highest in rivers impacted by human activities, either agriculture or suburban-urban development and population density. The presence of low levels of FIB in rivers in Alaska may reflect dilution of the relatively small impacts of human and animal sources to this large environment. Additionally, FIB contamination in these rivers may be highly episodic due to input differences related to the volume of runoff.

PCR detection of *H. pylori* and *Helicobacter* spp.

The two primer sets used in this study were selected based on their reported performance: specificity, sensitivity and robustness. To evaluate their use for detection of *H. pylori* or *H. spp.* in environmental samples and perhaps as an assay for monitoring by public health agencies, their performance was further characterized. Using genomic *H. pylori* DNA alone, the limit of detection by the *Helicobacter* genus-specific primers (HS1, HS2) was 0.1 pg DNA per reaction (ca. 50 cells) in standard PCR reactions and sensitivity was increased to 0.02 pg DNA per reaction (ca. 10 cells) when using qPCR. Detection and quantification of amplification products in the qPCR assay proceeds from fluorescent (SYBR green) labeling of newly synthesized double stranded DNA detected by a photomultiplier tube. Consequently, greater sensitivity of detection from the qPCR assay was expected. The limit of detection of *H. pylori* species-specific primers (*glm*M) using both standard PCR and qPCR protocols was 0.01 pg purified *H. pylori* genomic DNA per reaction. This corresponds to the amount of DNA present in about 5 cells and is in agreement with previously published results from a study using clinical specimens (Lu *et al.* 1999). Amplification reactions combining purified *H. pylori* genomic DNA and mixed environmental template DNA showed reduced sensitivity of detection for both sets of primers. The sensitivity of the *Helicobacter* spp. primers in both standard and qPCR was reduced by a single order of magnitude when environmental template DNA was added to the reaction. However, the *H. pylori* species-specific primers appeared to be more sensitive to environmental template addition and their detection sensitivity was reduced by two orders of magnitude (ca.1 pg per reaction) with standard PCR and one order of magnitude with qPCR.

The environmental DNA template added to control reactions was extracted from the Yukon River near Eagle in August 2001 (Table 1). This DNA yielded consistent PCR amplification products with universal bacterial 16S rDNA primers but no amplification products using either of the *Helicobacter* primer sets. The cause of the reduction in

primer sensitivity in PCR containing environmental template is not well understood. Similar results, however, have been observed previously and a number of studies have shown that template competition in environmental DNA samples can act to reduce the efficiency of primer binding (Kuske *et al.* 1998).

In addition to determining the sensitivity of each primer set based on dilution of genomic DNA and the addition of environmental template, recovery and detection of *H. pylori* DNA from cells in the environment was investigated. In experiments where *H. pylori* cells were added to river water at various concentrations PCR amplification using the *Helicobacter* spp. primers was successful at the limits of detection (ca. 1 pg of *H. pylori* DNA). This is the same sensitivity observed in PCR amplification of purified genomic *H. pylori* DNA and mixed environmental DNA. *H. pylori* sequence, however, was not amplified from these samples although the estimated concentration of *H. pylori* DNA in each reaction was within the limits of detection (10^3 – 10^5 cell equivalents). In addition, the ability to recover and detect *H. pylori* DNA from cells in the VnC stage was confirmed by PCR amplification. Amplification was successful from DNA template extracted from *H. pylori*- artificial river water samples that were held at room temperature for 48 hours. Likewise, amplification was successful from *H. pylori*-artificial river water samples inoculated directly with VnC cells. PCR assay of total DNA extracted simultaneously from uninoculated river water controls was negative using both sets of primers.

The inability of the *glm*M primers to amplify *H. pylori* DNA target sequence from DNA extracts of *H. pylori* - river water artificial samples was unexpected. In contrast, DNA extracted directly from cultured cells using the Chelex 100 method supported amplification of the *H. pylori* target sequence from as few as 10 cells (estimates based on microscopic counts). The differences in the ability to detect the *H. pylori* directly from cultured cells vs. from DNA extracted from those same cells placed in an environmental context may be due to non-specific primer binding or issues related to template competition in complex samples. In addition, these primers may be more sensitive to template purity than the 16S rDNA primers used to confirm template quality. Overall, our results indicate that the amplification of *H. pylori* (using the *glm*M primers) is very sensitive to uncharacterized environmental variables and suggests that these primers may not be sufficiently robust to provide accurate detection or quantification from environmental samples. Although molecular detection using PCR offers distinct advantages over classical culture techniques, this study illustrates the difficulties encountered in the direct application of clinical protocols to environmental samples. Specifically, primers that perform well in assays on well characterized systems can become less sensitive and less specific under complex environmental sample conditions. As a result, negative results from environmental samples must be viewed with caution and primers developed in clinical systems should not be applied to environmental samples without careful optimization of PCR assay conditions.

Environmental results

Based on results from both PCR methods, *Helicobacter* spp. DNA sequence was detected in 58% (19/33) of the river samples and was present at least once in each examined river (Table 1). *H. pylori* DNA was detected as *glm*M sequence in 33% of our water samples (11/33) (Table 1). This bacterium was also present in at least one sample from each river that was tested (Table 1). Quantitative estimates of abundance (qPCR) indicate very low cell numbers in all Alaska river samples as compared to those from Sugar Creek, the Iroquois River or the Potomac River (Table 1).

Detection of *H. pylori* (*glm*M) DNA in water samples from all rivers in Alaska was in agreement using either PCR method. Standard PCR detected *H. pylori* DNA in a single river (the Porcupine River), a result that was confirmed by qPCR. *H. pylori* was detected by qPCR in Yukon River and Tanana River samples, but at a level below the limit of detection for standard PCR. Given the greater sensitivity of the qPCR assay, the observed reduction in detection sensitivity of the *H. pylori* species-specific primers when used with environmental template DNA, and the small number of cells present in these samples, some differences in detection between PCR methods were expected. *H. pylori* was detected in at least one sample from the other three rivers. However, standard PCR detected *H. pylori* in two samples from the Iroquois river that were negative using the qPCR assay (Table 1). Given the greater sensitivity of qPCR

for *H. pylori* detection, this result was unexpected. We note here that *Helicobacter* spp. DNA sequence was detected by both standard and qPCR in these samples. Additionally, total microbial abundance (3×10^9/l, unpublished data) and total suspended solids were higher in the Iroquois than in the other rivers. These factors may have contributed to a reduction in the amplification specificity of the standard PCR assay (as compared to the qPCR assay) due to nonspecific binding of the *H. pylori* primers in complex environmental samples. These conditions could have also resulted in inhibition (substances present that were not removed by the purification steps) of the qPCR reaction and reduced its performance.

Detection of *Helicobacter* spp. (16S rDNA) DNA in water samples using either standard or qPCR was also in agreement although the greater sensitivity of the qPCR method allowed detection of significantly fewer cells in samples (Tables 1, 2). Standard PCR did not detect *Helicobacter* spp. in any river in Alaska although low numbers were detected in several instances using qPCR (Table 1). Similarly, standard PCR detected *Helicobacter* spp. DNA only one of the four samples from Sugar Creek that were positive using qPCR (Table 1). Quantitative estimates of abundance generated by qPCR show that cell numbers in these samples were below the limits of standard PCR detection. The detection of *Helicobacter* spp. DNA cannot be considered *a priori* evidence for the presence of the species, *H. pylori*. This is due to design of the primer set which can detect both *H. pylori* and non-*pylori* species of *Helicobacter*. However, because several non-pylori *Helicobacter* spp. are associated with human disease and because these primers appear to be more robust, the application of *Helicobacter* spp. PCR primers may be more useful and even more appropriate for public health officials (Solnick & Schauer 2001).

The detection of *H. pylori* DNA in 32% of the water samples is low compared to the 60–65% incidence of actively-respiring *H. pylori* cells observed in wells and surface water detected in a previous study employing a combined fluorescent antibody-cyanoditoyl tetrazolium chloride (CTC) staining method (Hegarty *et al.* 1999). That study, however, focused on sampling at sites in Pennsylvania and Ohio where fecal (human or animal) contamination was deemed likely. In contrast, this study examined a wide range of samples from natural environments subject to different surrounding land usage. We did detect both *Helicobacter* spp. and *H. pylori* at a greater frequency than in several other studies that examined rivers (Sasaki *et al.* 1999; Moreno *et al.* 2003). Detection of *H. pylori* in rivers using fluorescence *in situ* hybridization revealed a 20% frequency (2/10 samples) in a single river in Spain (Moreno *et al.* 2003). Similarly, a study by Sasaki *et al.* (1999) used PCR to detect *H. pylori* in about 20% of their samples taken from multiple sources in the natural environment (rivers, ponds, soils, houseflies, cow feces).

An additional result of this study was the detection of *H. pylori* and *Helicobacter* spp. DNA in riverbed sediments (Table 2). Bacteria in the environment are found frequently as members of attached or biofilm communities (Davey & O'Toole 2000). The formation of bacterial films on particles may act to nucleate particulates of increasing size, facilitating greater sedimentation. For these reasons, riverbed sediments may serve to concentrate bacteria and have the potential to act as microbial reservoirs and future sources of inocula (Byappanahalli *et al.* 2003). Riverbed sediment may also serve a protective function and act to extend the persistence of a particular microbe in the environment (Byappanahalli *et al.* 2003). *Helicobacter* DNA was amplified from 57% (8/14) of riverbed sediment samples (Table 2). *Helicobacter* DNA was detected in riverbed sediment from all rivers in Alaska and from Sugar Creek but not in the Iroquois or Potomac Rivers. Quantitative PCR confirmed the standard PCR results for all samples and detected both *Helicobacter* spp. and/or *H. pylori* in four additional samples (Table 2). *Helicobacter* spp DNA was only detected using qPCR in riverbed sediment from Eagle, AK (the Yukon River), Stevens Village upstream and from Sugar Creek. Estimates of abundance indicate that *Helicobacter* spp. DNA in these latter samples was below the level of detection for standard PCR (Table 2). Comparison of *Helicobacter* spp. detection between riverbed sediment and the overlying water reveals detection of *Helicobacter* spp. in water from 7/11 positive sediment samples (Tables 1, 2). Although fewer sediment samples than water samples were analyzed, *Helicobacter* was found in a greater percentage of river sediments then in the overlying water column. Although PCR cannot provide information on the viability of cells present in the environment, these results indicate

that riverbed sediments may serve as potential environmental source of *Helicobacter*.

H. pylori DNA was detected with qPCR in riverbed sediment samples from Sugar Creek site 8 and Stevens Village (3/14, 21%). *H. pylori* DNA was not detected in any sediment sample using standard PCR (Table 2). Quantitative estimates of abundance indicate that *H. pylori* DNA is below the limits of standard PCR detection in the one instance where qPCR yielded a positive result (Table 2). Comparison of *H. pylori* detection from riverbed sediment and the overlying water column, reveals the presence of this bacterium in only 3/7 of the sediment samples with corresponding positive water samples (Tables 1, 2). In general, *H. pylori* DNA was found in fewer riverbed sediments than in water samples, despite the expectation that sediments might harbor higher numbers of bacteria. These results could reflect the difficulties encountered when using the *H. pylori* species-specific primers with sediment samples. Sediment samples are known to be problematic due to various, uncharacterized inhibitors of PCR. Alternatively, *H. pylori* cells may not persist long enough in the environment for sedimentation to occur.

Quantification of *Helicobacter* spp. and *H. pylori* from rivers and riverbed sediments provides insight into the potential for infection in humans. The number of *H. pylori* needed to induce infection in humans is estimated to be about 1×10^4 cells (Graham 2003). Therefore, we estimate that infection by waterborne transmission from the Porcupine River in Alaska (the highest estimated numbers per litre), would require the ingestion of nearly 100 litres. The situation is markedly different in the other rivers we examined. The number of *H. pylori* cells estimated from Sugar Creek, the Iroquois River and the Potomac River, range from about 1×10^5 to 5×10^6 cells per litre (Table 1). This indicates that an individual would need to consume >20 ml (*e.g.*, one good gulp) of water to ingest sufficient *H. pylori* to become infected at either Sugar Creek site #1 or site #9. Based on these estimates, a person drinking from the Potomac River in July 2001 would have had to consume 400 ml of water to reach a minimally infective dose. *H. pylori* was detected in three riverbed sediment samples (Table 2). Numerical estimates of cells per gram from these sites were very low, suggesting that infection from sediments is unlikely.

Environmental co-occurrence of *Helicobacter, H. pylori* and FIB

Total and fecal coliform bacteria have been used extensively for many years as indicators for determining the sanitary quality of surface and recreational water (Griffin *et al.* 2001; Scott *et al.* 2002; Noble *et al.* 2003). In particular, *E. coli* has frequently been used as an indicator of fecal pollution (Griffin *et al.* 2001; Scott *et al.* 2002; Noble *et al.* 2003). There are, however, known problems with the enumeration of coliform bacteria as indicators of sanitary water quality (Griffin *et al.* 2001; Byappanahalli *et al.* 2003). For example, due to differences in survival rates between environments, the numbers of coliform bacteria in the water column may or may not indicate good or poor water quality (Griffin *et al.* 2001). In this study we examined the potential for environmental co-occurrence of pathogenic bacteria (*Helicobacter*) with FIB. A specific aim of this study was to determine if a numerical correlation could be found between counts of FIB in rivers and the presence of *Helicobacter* spp. and/or *H. pylori*.

In all cases where *Helicobacter* spp. or *H. pylori* DNA was detected, some FIB were also found. The converse of this was not true as nearly all samples contained some FIB. A combined total of fifteen samples contained at least one indicator organism or group that exceeded USEPA standards (Table 1). In seven of these (47%), no *Helicobacter* spp or *H. pylori* DNA was detected (Table 1). This does not preclude the presence of other pathogenic organisms that may be associated with the presence of FIB. It does indicate, however, that the predictive value of FIB abundance for *Helicobacter* spp and *H. pylori* is not completely reliable. Additionally, 8/16 samples (50%) that did not exceed USEPA standards for FIB were positive for *Helicobacter* spp. or *H. pylori*. Linear regression analysis revealed no correlation in any sample between the numbers of fecal coliform bacteria, *E. coli* or enterococci and the numbers of *H. pylori* (Table 4). A weak positive correlation was observed only when comparing the inclusive *Helicobacter* spp. with fecal coliform or *E. coli* abundance (Table 4).

In samples from Alaska, a total of 2/16 (13%) samples exceeded USEPA standards for FIB (Table 1). *Helicobacter* spp. and *H. pylori* were detected in one of these samples but not the other. In samples from rural Indiana and

Table 4 | Correlation analysis of the co-occurrence of *Helicobacter* spp., *H. pylori* and fecal indicator bacteria. Linear correlation coefficient (r) is shown[a]

	Helicobacter spp.	*H. pylori*	Fecal coliforms	*E. coli*	Enterococci
Helicobacter spp.	–				
H. pylori	0.466	–			
Fecal coliforms	0.655	0.044	–		
E. coli	0.518	0.177	0.953	–	
Enterococci[b]	0.276	0.371	0.88	0.88	–

[a](r) values were calculated using KaleidaGraph 3.5
[b]Analyses excluded all bacterial numbers from samples where Enterococci were not determined

Illinois, 7/10 (70%) exceeded USEPA standards for FIB. *Helicobacter* spp. was detected in 5/7 (71%) of these samples as well as from a single sample that did not exceed USEPA standards (Table 1). *H. pylori* was detected in 2/7 (29%) of samples where FIB exceeded USEPA standards and was not detected in any sample where FIB did not exceed USEPA standards. FIB abundance in the Potomac River exceeded USEPA standards in 6/7 (85%) samples. *Helicobacter* spp. was detected in 2/6 (33%) of these samples and *H. pylori* was detected in 1/6 (17%). *Helicobacter* spp. or *H. pylori* were not detected in the single Potomac River sample in compliance with USEPA standards. Overall, because of the inconsistencies outlined above, the presence and abundance of FIB cannot be considered a reliable predictor of *Helicobacter* spp. or *H. pylori* and is likely to be of limited use for public health officials.

Helicobacter in the environment

Ultimately, in order to understand the potential for waterborne transmission of *Helicobacter* spp. and *H. pylori* through an environment, greater knowledge of the physiological and metabolic behavior of *Helicobacter* in the environment and the effects of the environment on cells (*e.g.* changes in buoyant densities, sedimentation, etc.) will be necessary. At present, the form that *H. pylori* may adopt in rivers is unknown. This organism responds, however, to unfavorable environmental stimuli by formation of small, metabolically active, "viable-non-culturable" (VnC) coccoid cells that retain all of the antigenic, infective and virulence properties of *H. pylori* vegetative cells (West *et al.* 1992; Velazquez & Feirtag 1999). Previous work on the *H. pylori* VnC stage has shown that temperatures between 4°C and 15°C favor the retention of virulence and that as few as 100 cells per ml are required for establishment of *H. pylori* infection in mice (Velazquez & Feirtag 1999). Comparison of the nutrient and temperature conditions typically found in rivers with those found in the human gut would suggest that the majority of *H. pylori*, if present in environmental samples, would have assumed the VnC cell form. Virulence in this VnC form is maintained at lower cell numbers than in vegetative cells and survival is enhanced at low temperatures such as observed in rivers in Alaska (West *et al.* 1992; Beneduce *et al.* 2003). *H. pylori* infection in humans from the consumption of raw water appears unlikely given the low numbers of cells detected in rivers in Alaska.

Despite the observation that rates of *H. pylori* infection are as high among rural native Alaskans as among peoples of certain developing countries, our study did not identify Alaskan rivers as a potential environmental source for this microorganism. A likely explanation for this is the low density of human population along the large rivers in Alaska. This result can be compared to results from environments where water has been identified as a potential source of *H. pylori* transmission (Sasaki *et al.* 1999; Engstrand 2001; Bunn *et al.* 2002; Nurgalieva *et al.* 2002; Moreno *et al.* 2003). In these studies, the environment being considered was either urban or agricultural, was heavily impacted by human activity and contained population densities that were much higher than those encountered in rural Alaska.

An environmental reservoir of *H. pylori* in surface water is only one of several pathways that have been suggested for the transmission of this pathogen in human populations (Nurgalieva *et al.* 2002). Domestic water sources have also been suggested as a potential mode of *H. pylori* waterborne transmission (Engstrand 2001). Recent epidemiological data that examined the seroepidemiologic pattern of *H. pylori* infection in Kazakhstan showed that both ingestion of river

water and poor household sanitation practices could be important risk factors (Nurgalieva *et al.* 2002). Results of PCR screening of selected domestic water sources (home wells and storage vessels) from Alaskan villages revealed the presence of *Helicobacter* spp. and *H. pylori* DNA in 21% of samples (n = 23) (unpublished data). Waterborne transmission, if it is occurring in these populations, may be proceeding by this mode. Further work characterizing the epidemiological features of these populations should be carried out in order to gain a greater understanding of the potential transmission routes of this pathogen among rural villagers.

CONCLUSION

This study was able to detect and quantify *H. pylori* and *Helicobacter* spp. from five rivers using PCR. Our results indicate that while *Helicobacter* spp. and *H. pylori* are often found in samples containing FIB, the presence and abundance of FIB is not predictive of either *Helicobacter* spp. or *H. pylori*. Amplification of both *H. pylori* and *Helicobacter* spp. DNA sequence from environmental samples varied between PCR methods and primer specificity was reduced from that observed with clinical specimens. Although reported to be sensitive and specific, the primer set designed to specifically detect *H. pylori* (glmM) performed poorly with environmental samples, both water and sediment samples. Further detection and quantification of these organisms from environmental specimens will require specific optimization of each primer set for each environmental sample and/or the use of additional primers that are less sensitive to the complexities of mixed environmental DNA templates. As expected, overall FIB abundance was reflective of different land use patterns. FIB abundance was greater in rivers where a large percentage of the land use of the surrounding watershed was either agricultural or urban. Rivers in Alaska were largely free of FIB, reflecting the relatively undeveloped and pristine nature of these watersheds.

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REFERENCES

Ator, S., Bloomquist, J., Brakebill, J., Denis, J., Ferrari, M., Miller, C. & Zappia, H. 1998 *Water Quality in the Potomac River Basin, Maryland, Pennsylvania, Virginia, West Virginia, and the District of Columbia, 1992-96*. US Geological Survey Circular 1166 online: http://pubs.usgs.gov/circ/circ1166

Beneduce, L., Tarantino, D., Spano, G., Libergoli, M., Labonia, M. & Massa, S. 2003 Survival of *Helicobacter pylori* in well water. *World J. Microbiol. Biotechnol.* **19**, 505–508.

Bickley, J., Owen, R. J., Fraser, A. G. & Pounder, R. E. 1993 Evaluation of the polymerase chain reaction for detecting the urease C gene of *Helicobacter pylori* in gastric biopsy samples and dental plaque. *J. Med. Microbiol.* **39**, 338–344.

Brabets, T. P., Wang, B. & Meade, R. H. 2000 *Environmental and hydrologic overview of the Yukon River Basin, Alaska and Canada*. WRIR-99/4204, United States Geological Survey, 106p.

Brock, T. D., Madigan, M. T., Martinko, J. M. & Parker, J. 1991 *Biology of Microorganisms*, 7th ed. Prentice Hall, Englewood Cliffs, NJ.

Brunk, C., Avaniss-Aghajani, E. & Brunk, C. 1996 A computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. *Appl. Environ. Microbiol.* **62**(3), 872–879.

Bunn, J., MacKay, W. G., Thomas, J. E., Reid, D. C. & Weaver, L. T. 2002 Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. *Lett. Appl. Microbiol.* **34**, 450–454.

Byappanahalli, M., Fowler, M., Shively, D. & Whitman, R. 2003 Ubiquity and persistence of *Escherichia coli* in a midwestern coastal stream. *Appl. Environ. Microbiol.* **69**(8), 4549–4555.

Cantet, F., Magras, C., Marias, A., Federighi, M. & Megraud, F. 1999 *Helicobacter* species colonizing pig stomach: molecular

characterization and determination of prevalence. *Appl. Environ. Microbiol.* **65**(10), 4672–4676.

Chesapeake Bay Program. 2000 online: http://www.chesapeakebay.net.

Cobb, B. D. & Clarkson, J. M. 1994 A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Res.* **22**(18), 3801–3805.

Davey, M. & O'Toole, J. A. 2000 Microbial biofilms: from ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* **64**(4), 847–867.

Devereux, R. & Willis, S. G. 1995 Amplification of ribosomal RNA sequence. In *Molecular Microbiology Ecology Manual* (eds Akkermans A. D. L. & Brujiin F. J. D.). Kluwer, Dordrecht, Netherlands.

Dubois, A. 1995 Spiral bacteria in the human stomach: the gastric Helicobacters. *Emer. Infect. Dis.* **1**, 79–85.

Dunn, B., Cohen, H. & Blaser, M. J. 1997 *Helicobacter pylori*. *Clin. Microbiol. Rev.* **10**, 720–741.

Eddy, N. 2004a Goodbye honeybucket?: wastewater treatment in Alaska Native villages. *Small Flows Q.* **5**(2), 14–17.

Eddy, N. 2004b Life in rural Alaska Native villages. *Small Flows Q.* **5**(2), 18–19.

Engstrand, L. 2001 *Helicobacter* in water and waterborne routes of transmission. *J. Appl. Microbiol.* **30**, 80–84.

Graham, D. Y. 2003 Veterans Administration Medical Center. Houston, TX. Personal communication.

Griffin, D., Lipp, E. K., McLaughlin, M. R. & Rose, J. B. 2001 Marine recreation and public health microbiology: quest for the ideal indicator. *BioSci.* **51**(10), 817–825.

Hegarty, J., Dowd, M. T. & Baker, K. H. 1999 Occurrence of *Helicobacter pylori* in surface water in the United States. *J. Appl. Microbiol.* **87**, 697–701.

Indiana Business Research Center 2000 http: www.stats.indiana.edu.

Innis, M. A., Gelfand, D. H., Sninsky, J. & White, T. J. 1990 *PCR Protocols: A Guide to Methods and Applications*. Academic Press, Inc., San Diego, CA.

Interlandi, S. & Crockett, C. S. 2003 Recent water quality trends in the Schuylkill River, Pennsylvania, USA: a preliminary assessment of the relative influences of climate, river discharge and suburban development. *Water Res.* **37**, 1737–1748.

Krumbiegel, P., Lehmann, I., Alfreider, A., Fritz, G. J., Boeckle, D., Rolle-Kampczyk, U., Richter, M., Jorks, S., Müller, L., Richter, M. W. & Herbarth, O. 2004 *Helicobacter pylori* determination in non-municipal drinking water and epidemiological findings. *Isot. Environ. Health Stud.* **40**(1), 75–80.

Kuske, C., Banton, K. L., Adorada, D. L., Stark, P. C., Hill, K. K. & Jackson, P. J. 1998 Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Appl. Environ.Microbiol.* **64**, 2463–2472.

Lu, J., Perng, C. L., Shyu, R. Y., Chen, C. H., Lou, Q., Chong, S. K. F. & Lee, C. H. 1999 Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. *J. Clin.Microbiol.* **37**(3), 772–774.

Metropolitan Washington Council of Governments (2003). online: http://www.mwcog.org/.

Moreno, Y., Ferrus, M. A., Alonso, J. L., Jimenez, A. & Hernandez, J. 2003 Use of fluorescent *in situ* hybridization to evidence the presence of *Helicobacter pylori* in water. *Water Res.* **37**, 2251–2256.

Noble, R., Moore, D. F., Leecaster, M. K., McGee, C. D. & Weisberg, S. B. 2003 Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. *Water Res.* **37**(7), 1637–1643.

Nurgalieva, Z., Malaty, H. M., Graham, D. Y., Almuchambetova, R., Machmudova, A., Kapsulatanova, D., Osato, M. S., Hollinger, F. B. & Zhangabylov, A. 2002 *Helicobacter pylori* infection in Kazakhstan: effect of water source and household hygiene. *Am. J. Trop. Med.* **67**(2), 201–206.

Parkinson, A. J., Gold, B., Bulkow, L., Wainwright, R., Swaminathan, B., Khanna, B., Petersen, K. & Fitzgerald, M. 2000 High prevalence of *Helicobacter pylori* in the Alaska native population and association with low serum ferritin levels in young adults. *Clin. Diagn. Lab. Immunol.* **7**(6), 885–888.

Parkinson, A. & Butler, J. 2001 Prevention and control of infectious diseases in the Arctic. *Arct. Res. U.S.* **15**, 27–32.

Sambrook, J., Fritsch, E. F. & Maniatis, T. 1989 *Molecular Cloning: A Laboratory Manual*, 2nd. ed. Cold Springs Harbor Laboratory Press, Plainview, NY.

Sasaki, K., Tajiri, Y., Sata, M., Fujii, Y., Matsubara, F., Zhao, M., Shimizu, S., Toyonaga, A. & Tanikawa, K. 1999 *Helicobacter pylori* in the natural environment. *Scand. J. Infect. Dis.* **31**, 275–279.

Scott, T., Rose, J. B., Jenkins, T. M., Farrah, S. R. & Lukasik, J. 2002 Microbial source tracking: current methodology and future directions. *Appl. Environ. Microbiol.* **68**(12), 5796–5803.

Solnick, J. & Schauer, D. B. 2001 Emergence of diverse *Helicobacer* species in the pathogenesis of gastric and enterohepatic diseases. *Clin. Microbiol. Rev.* **14**(1), 59–97.

Standard Methods for the Examination of Water and Wastewater, (1998). American Public Health Association / American Water Works Association / Water Environment Federation, Washington, DC.

Thompson, S. P., Paerl, H. W. & Go, M. C. 1998 *Denitrification dynamics of an estuarine headwater creek receiving agricultural runoff*. WRRI of the University of North Carolina, North Carolina State University, Rayleigh, NC.

US Census Bureau 2000. online: http://factfinder.census.gov.

US Environmental Protection Agency 2000. *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli.* EPA/821/R-97/004, Office of Science and Technology / U.S. Environmental Protection Agency.

US Environmental Protection Agency 2002 online: http://www.epa.gov/waterscience/standard/states/

US Geological Survey National Water Quality Assessment Program. 2002 online: http://water.usgs.gov/nawqa/informing/agriculture.html

US Geological Survey. 2003a. online: http://water.usgs.gov/nasqan/yukon/.

US Geological Survey. 2003b online: http://in.water.usgs.gov/.

Velazquez, M. & Feirtag, J. M. 1999 *Helicobacter pylori*: characteristics, pathogenicity, detection methods and mode of transmission implicating foods and water. *Int. J. Food Microbiol.* **53**, 95–104.

Walsh, P., Metzger, D. A. & Highuchi, R. 1991 Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTech.* **10**, 506–512.

Washington DC Suburban Sanitary Commission. 2003 online: http://www.wssc.dst.md.us.

West, A., Millar, M. R. & Tompkins, D. S. 1992 Effect of physical environment on survival of *Helicobacter pylori*. *J. Clin. Pathol.* **45**, 228–231.

World Health Organization 1996 online: http://www.who.int/whr/en