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Failure To Detect *Helicobacter pylori* DNA in Drinking and Environmental Water in Dhaka, Bangladesh, Using Highly Sensitive Real-Time PCR Assays[▽]

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The main transmission pathway of *Helicobacter pylori* has not been determined, but several reports have described detection of *H. pylori* DNA in drinking and environmental water, suggesting that *H. pylori* may be waterborne. To address this possibility, we developed, tested, and optimized two complementary *H. pylori*-specific real-time PCR assays for quantification of *H. pylori* DNA in water. The minimum detection level of the assays including collection procedures and DNA extraction was shown to be approximately 250 *H. pylori* genomes per water sample. Using our assays, we then analyzed samples of drinking and environmental water ($n = 75$) and natural water biofilms ($n = 21$) from a high-endemicity area in Bangladesh. We could not identify *H. pylori* DNA in any of the samples, even though other pathogenic bacteria have been found previously in the same water samples by using the same methodology. A series of control experiments were performed to ensure that the negative results were not falsely caused by PCR inhibition, nonspecific assays, degradation of template DNA, or low detection sensitivity. Our results suggest that it is unlikely that the predominant transmission route of *H. pylori* in this area is waterborne.

Helicobacter pylori is the most common human bacterial pathogen in the world (15), and it has been estimated that 50% of the world's population is infected. The prevalence of *H. pylori* infection varies greatly worldwide, with infection rates of more than 80% in some developing countries and below 20% in some developed countries (29). *H. pylori* causes peptic ulcers in 10 to 15% and stomach cancer in another 1 to 2% of those infected (29).

H. pylori naturally resides in the human stomach, and except for some primate species, no other host has been identified. Outside its host, *H. pylori* is fastidious and can grow only under microaerophilic conditions at 34 to 40°C in nutrient-rich media (29). Under suboptimal conditions, *H. pylori* transforms into nonculturable spherical or coccoid forms. To date, it is not clear if this process is reversible or if the coccoid form is infectious or even viable, but it has been reported to retain some metabolic activity, its genome, and an intact membrane (1, 6, 12, 28, 38, 47).

Transmission of *H. pylori* has been proposed to occur via gastric-oral, oral-oral, or fecal-oral routes, with studies suggesting transmission through saliva and dental plaque (14, 23), normal and diarrheal stools (18, 23, 41, 43), and vomitus (30, 41). Infected mothers or older siblings, low standards of living, and crowded households have been shown to be major risk factors for contracting *H. pylori* (25, 35, 50). Other studies have shown a relation between infection, water sanitation, and drinking water sources (24, 26, 39), further supported by reports of *H. pylori* DNA in drinking, river, lake, or seawater (3, 7, 16, 19–22, 25, 33, 34, 37, 40, 43, 49).

Since none of the latter group of studies have shown a causative relation between traces of *H. pylori* in water and new infections, our original aim was to perform a 2-year prospective study tracing *H. pylori* in water in a high-endemicity area and relate the findings with new infections in children. For this purpose, we developed highly sensitive and specific quantitative real-time PCR assays for detecting *H. pylori* DNA in water or human samples while allowing analysis of clonal relatedness between samples of different origins by sequencing of recovered DNA. Using these assays, we conducted a study in a slum area in Dhaka, Bangladesh, where we have recently shown a very high rate of *H. pylori* infections, i.e., that 60% of the children were infected by the age of 2 years (4). Drinking, waste, and environmental water samples and natural drinking water biofilm samples were collected and analyzed, with rigorous controls for falsely positive or negative results.

MATERIALS AND METHODS

Culture conditions and strains. To determine the specificity of the real-time PCR assays, 18 *H. pylori* strains and 18 strains of other enterobacterial species were used (Table 1). *Helicobacter* and *Campylobacter* species were grown for 48 h at 37°C under microaerophilic conditions (8) on Columbia agar supplemented with 1% IsoVitaleX. All other species were grown aerobically on horse blood agar for 24 h at 37°C. To obtain a high proportion of spiral *H. pylori*, some strains were further grown in 25 ml brucella broth supplemented with 5% fetal calf serum, 10 μg/ml vancomycin, 5 μg/ml trimethoprim, and 20 U/ml polymyxin B in sterile 250-ml flasks for 20 h at 37°C with shaking at 150 rpm under microaerophilic conditions.

Tap water and seawater microcosms. *H. pylori* strains Hel513 and Hel703 grown in liquid medium were harvested by centrifugation at 10,000 × *g* for 10 min, washed twice in phosphate-buffered saline (PBS), and resuspended in 10 ml

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TABLE 1. Strains used in this study and strain specificities of real-time PCR primers

Group	Detection with primers targeting:		Source[a]
	glmM	*hpaA*	
Aeromonas caviae	−	−	Departmental strain collection
Aeromonas hydrophila	−	−	Departmental strain collection
Aeromonas sobria	−	−	Departmental strain collection
Campylobacter coli	−	−	Clinical isolates
Campylobacter jejuni	−	−	Clinical isolates
E. coli K-12 C600	−	−	Departmental strain collection
Klebsiella spp.	−	−	Clinical isolates
Salmonella enterica serovar Enteritidis	−	−	Departmental strain collection
Salmonella enterica serovar Typhimurium	−	−	Departmental strain collection
Vibrio cholerae			
O1 Classical biotype	−	−	Departmental strain collection
O1 Eltor biotype	−	−	Departmental strain collection
O139	−	−	Departmental strain collection
Vibrio parahaemolyticus	−	−	Departmental strain collection
Vibrio vulnificus	−	−	Departmental strain collection
Yersinia enterocolitica	−	−	Departmental strain collection
Yersinia pseudotuberculosis	−	−	Departmental strain collection
H. acinonychis	+	−	Departmental strain collection
Helicobacter bilis	−	−	CCUG, Culture Collection of the University of Gothenburg
Helicobacter felis	−	−	CCUG
Helicobacter hepaticus	−	−	CCUG
Helicobacter mustelae	−	−	AstraZeneca culture collection
H. pylori			
Ca 14	+	+	Lars Engstrand, Karolinska Institute, Stockholm, Sweden
Ca 57	+	+	Lars Engstrand, Karolinska Institute, Stockholm, Sweden
CAN 13	+	+	Departmental strain collection
CAN 21	+	+	Departmental strain collection
D94	+	+	ICDDR,B, Dhaka, Bangladesh
F18	+	+	ICDDR,B, Dhaka, Bangladesh
Hel226	+	+	Departmental strain collection
Hel230	+	+	Departmental strain collection
Hel264	+	+	Departmental strain collection
Hel312	+	+	Departmental strain collection
Hel340	+	+	Departmental strain collection
Hel436	+	+	Departmental strain collection
Hel513	+	+	Departmental strain collection
Hel515	+	+	Departmental strain collection
Hel703	+	+	Departmental strain collection
Hel709	+	+	Departmental strain collection
J99	+	+	Departmental strain collection
SS1	+	+	Departmental strain collection

[a] "Departmental strain collection" refers to that from the Department of Microbiology and Immunology, University of Gothenburg. "Clinical isolates" refers to those from the Department of Clinical Bacteriology, University Hospital, Gothenburg, Sweden.

TABLE 2. Primers used in this study

Target gene	Primer name	Sequence (5′–3′)
hpaA	HpaA-rt-F1	ACTTTCTCGCTAGCTGGATGGTA
	HpaA-rt-R1	GCGAGCGTGGTGGCTTT
glmM	GlmM-rt-F1	GCTCACTAAAGCGTTTTCTACCATATAG
	GlmM-rt-R1	ATTGCTGCCGGATTGTATTTTAA

sterile filtered tap water or seawater from municipal drinking water facilities in and from the sea outside Gothenburg, Sweden. The bacteria were counted with a Neubauer improved counting chamber (Hausser Scientific, VWR International) at ×40 magnification. The two strains were separately seeded into sterile 1-liter bottles containing 500 ml sterile filtered seawater or tap water to give final densities of 5×10^7 bacteria per ml. The microcosms were sealed and kept dark at an ambient temperature (approximately 21°C) on a rocking platform at 30 rpm and were sampled aseptically after 0, 1, 7, 35, and 100 days. Morphology was studied by phase-contrast microscopy at ×100 magnification. At each sampling occasion, a sample from each seawater or tap water microcosm was diluted 10-fold in seawater or tap water, respectively, from 5×10^7 cells per ml down to 5×10^1 cells per ml. Control samples of seawater and tap water were also included in all analyses. From each dilution, 100 μl was cultured on horse blood agar for 7 days at 37°C under microaerophilic conditions to determine numbers of CFU, 500 μl was centrifuged at 16,000 × *g* for 45 min to collect bacteria, and 500 μl was added to 100 ml sterile PBS and filtered onto 0.22-μm-porosity Millipore filters, also to collect bacteria. Pellets and filters were stored at −70°C. Sampled directly from each microcosm, 100 μl was cultured aerobically on horse blood agar for 48 h at 37°C to check for contamination of other bacterial species and 9 ml was centrifuged for 10 min at 16,000 × *g* to collect bacteria for RNA extraction. Pellets were stored at −70°C.

Hel513 and Hel703 were also incubated for 21 days at approximately 10^7 cells per ml in three drinking water samples and in pond water from Dhaka to detect the presence of PCR inhibitors.

Collection and treatment of water and biofilm samples. The municipal water in Dhaka consists of 83% ground water and 17% treated surface water (44). Drinking and household water is taken from municipal water lines in Mirpur, Dhaka, that are open once or twice a day and stored inside households in jars or open wells for up to 24 h and in water tanks or jars on the rooftops for up to a few days. Forty-five 150-ml drinking water samples from jars and wells and nine 1,000-ml samples from jars and water tanks were collected in Mirpur between October 2005 and April 2006. Fifteen duplicate 150-ml pond and lake water samples and six 50-ml samples from open sewers close to homes and public toilets were collected in Mirpur and in the Mohakhali, Mirpur, Gulshan, Tongi, Jatrabari, and Demra areas in Dhaka between November 2005 and March 2006. Water samples were collected in sterile flasks and transported on wet ice to the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), arriving within 3 h. Drinking water samples were filtered onto 0.22-μm Millipore filters, which were cut with a sterile razor blade into halves or quarters. Lake and pond water samples were filtered first through a Whatman filter to remove large particles and then through 0.22-μm filters. Wastewater samples were centrifuged first at 500 × *g* for 10 min to remove large particles, and the supernatant was then centrifuged at 25,000 × *g* for 10 min to collect bacteria. Twenty-one glass slides were cleaned by 70% ethanol and suspended in water tanks and jars in households for 14 to 30 days before transport on ice to the ICDDR,B, where the biofilm was scraped off using the blunt end of a sterile plastic pipette tip. All filters, pellets, and biofilm samples were stored at −70°C until DNA extraction.

Extraction of nucleic acids. DNA was extracted from bacterial centrifuge pellets, filters, or biofilms by using Qiagen's DNeasy tissue kit (Qiagen GmbH, Helden, Germany) as described previously (32) and eluted in 100 μl elution buffer (buffer AE). To avoid PCR inhibitors, DNA was extracted from wastewater samples with a QIAamp DNA stool kit (Qiagen) and eluted in 200 μl elution buffer. DNA was kept at 4°C for short-term storage or at −20°C for long-term storage. All DNA extractions were performed inside a UV hood. To detect possible template contamination, empty microcentrifuge tubes were included in each DNA extraction. RNA was extracted with Qiagen's RNeasy kit, eluted in 30 μl RNase-free water, and stored at −70°C.

Primer design. The gene sequences of *hpaA* and *glmM* from the completed *H. pylori* genomes of strains J99 (GenBank accession number AE001439) and 26695 (GenBank accession number AE000511) were retrieved from the NCBI nucleotide database, and global alignments were performed with the Web-based EBI alignment tool EMBOSS. Primer pairs targeting conserved regions were then designed using Primer Express (Applied Biosystems, Foster City, CA) software, with default settings (Table 2).

Real-time PCR. Real-time PCRs were run in 96-well plates (Applied Biosystems), with a total volume of 20 μl in each reaction mixture. The optimized PCR mixture contained 10 μl SYBR green real-time PCR master mix (Applied Biosystems), 10 pmol of each primer, 6 μl water (nucleotide, DNase, and RNase free), and 2 μl of a sample. Negative controls and a standard curve were included in each run.

A standard curve was made from RNase-treated genomic DNA extracted from *H. pylori* strain J99. Concentration was determined by measuring the optical density at 260 nm with a spectrophotometer (NanoDrop Technologies, Wilmington, DE), and the molar concentration was calculated using the J99 genome mass. The standard curve was made by 10-fold serial dilutions in elution buffer (Qiagen) from 10^5 down to 10^1 copies per μl. Real-time PCR analysis was

performed as described previously (32), with a model 7500 real-time PCR instrument from Applied Biosystems.

RESULTS

Evaluation of real-time PCR primer pair specificities. In order to accurately quantify *H. pylori* DNA, we established two real-time PCR assays with primers targeting *H. pylori* genes *hpaA* and *glmM* (*ureC*) (Table 2). When evaluated with DNA extracted from a bacterial strain library, neither of the two primer pairs yielded any PCR product with DNA from gastrointestinal pathogens other than *Helicobacter* spp. The *glmM* primers detected *Helicobacter acinonychis* and all *H. pylori* strains but no other *Helicobacter* spp., whereas the *hpaA* primers detected only *H. pylori* strains (Table 1). Furthermore, a BLAST search in the NCBI genome database confirmed that the primer pairs are unique to these species.

Determination of real-time PCR sensitivity. Initial tests were performed with a 10-fold dilution series of purified DNA, with a range of 10^8 down to 10^{-1} genome copies per μl. The minimum detection sensitivity of the real-time PCR using either primer pair was determined to be two or three genomes per reaction with a 20-μl reaction volume and a 2-μl template. The linear, dynamic range of the real-time PCR was at least 8 orders of magnitude, ranging from 2 to 10^8 genomes per μl.

Evaluation of the DNA extraction procedure. Since water samples may contain several bacterial species, the real-time PCR assays were also evaluated with DNA extracted from dilutions of whole *H. pylori* bacteria mixed with *Escherichia coli*. For this purpose, *H. pylori* strain Hel703 was serially diluted in PBS from approximately 10^8 to 10 bacteria, with and without the addition of approximately 10^8 *E. coli* K-12 bacteria to each dilution, followed by collection by centrifugation, DNA extraction, and real-time PCR analysis. The results showed that the lower detection limit of *H. pylori* DNA for this procedure was approximately 100 genomes per sample (corresponding to 2 genomes per PCR). The addition of *E. coli* had no influence on detection or quantification of *H. pylori*.

The recovery of *H. pylori* DNA from dilutions of whole *H. pylori* bacteria collected with 0.22-μm-porosity filters was then evaluated. After collecting bacteria, half of each filter was used directly for DNA extraction and half was washed with 3 ml PBS, followed by centrifugation and DNA extraction from the pellet. The results showed that direct DNA extraction increased recovery approximately 1,000 times. This procedure was then used on all field samples, except for wastewater samples, which were centrifuged to prevent filter clogging.

Determination of filter collection efficiency. To confirm that the filtration and direct DNA extraction accurately collect DNA from *H. pylori* in drinking water and to determine the detection sensitivity of the entire assay and collection procedure, dilutions in municipal tap water from Gothenburg, Sweden, of whole *H. pylori* cells at 5×10^7, 5×10^5, 5×10^3, 5×10^2, 5×10, and 5 cells per ml were prepared. Five hundred microliters of each dilution and a negative control were then collected by parallel centrifugation for 45 min at $16{,}000 \times g$ and filtration through 0.22-μm-porosity filters, followed by DNA extraction and real-time PCR. The results show that collection of bacteria by centrifugation allowed accurate quantification of *H. pylori* in dilutions down to 250 bacteria per sample (500 μl of the dilution with 500 bacteria per ml; this corresponds to five genomes per PCR) and a dynamic range of at least 6 orders of magnitude (Fig. 1). Collection using filters, with half of each filter used for DNA extraction, also allowed quantification for dilutions down to 250 *H. pylori* bacteria per sample or filter (which is 500 μl of the dilution with 500 bacteria per ml, but only half of the filter was used; this corresponds to two or three genomes per PCR) but resulted in an up to 60% lower estimate of the true number of bacteria at high cell densities (Fig. 1). Again, the dynamic range was at least 6 orders of magnitude (Fig. 1). Thus, the final detection sensitivity and quantification range of the method including filtration and DNA extraction were determined to be from 250 bacteria per filter or sample (corresponding to two or three copies per PCR) up to at least 10^7 bacteria per sample. Spiral-shaped (after <24 h of incubation in water) and coccoid (after >2 days of incubation in water) bacteria were recovered and quantified with equal levels of efficiency.

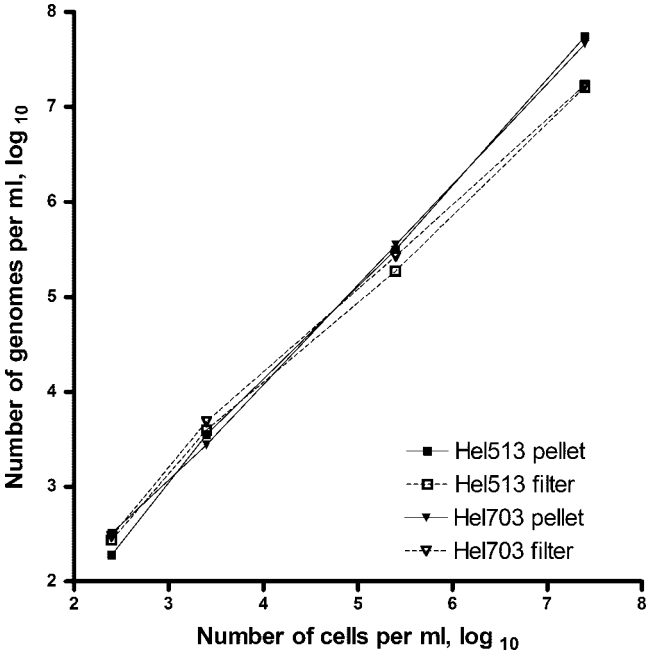

FIG. 1. Efficiency of DNA extraction from filtered or centrifuged suspensions of two *H. pylori* strains. Comparison of the DNA yield between filtration and centrifugation as a method for collecting bacterial cells suspended in water. Data are from tap water microcosms, incubated up to 24 h. The *x* axis shows the numbers of bacterial cells as determined by direct microscopic counting.

Analysis of *H. pylori* DNA in drinking water and natural drinking water biofilms. Using our methodology, a study was performed in Mirpur, a slum area of Dhaka, Bangladesh. Forty-five 150-ml samples were collected from jars or tanks in households with children known to be infected by *H. pylori* or from adjacent wells between September 2005 and April 2006. All 45 samples were negative for *H. pylori* DNA by the two real-time PCR assays. Since *H. pylori* DNA could not be detected in these samples, nine 1,000-ml water samples were collected in March 2006, including two samples from water tanks present on the roofs, where water is contained for longer periods. Again, no *H. pylori* DNA was detected using the real-time PCR

assays. To determine if *H. pylori* was present in natural biofilms, 21 glass slides were submerged in drinking water reservoirs in households for 14 to 30 days and analyzed with the assays. All glass slides had biofilms formed on the surface but were found to be negative for *H. pylori* DNA.

***H. pylori* DNA is not detected in environmental and wastewater samples.** Since *H. pylori* DNA was not detected in the household waters, the presence of *H. pylori* DNA in different environmental water sources in Dhaka was evaluated. A total of 15 water samples from ponds and lakes and 6 wastewater samples were collected in the larger Dhaka area between November 2005 and March 2006. The sample volumes ranged between 150 ml and 1,000 ml. Also, these samples were all negative for the presence of *H. pylori* DNA.

Evaluation of PCR inhibition in samples. To evaluate the possibility that the absence of *H. pylori* DNA in the water samples was caused by substances inhibiting the PCR process, two *H. pylori* strains, Hel513 and Hel703, were incubated at approximately 10^7 bacteria per ml in water samples from three drinking water sources and one pond in Dhaka and in PBS as a control and sampled after 1 and 21 days. The detection rates of less than 100% compared to the level for the control incubated in PBS indicated that PCR inhibitors were present in one of the drinking water sources and in the pond water. However, 10-fold dilution of the purified DNA was shown to remove the effect of the PCR inhibitors.

To further determine the presence of inhibitory factors in the field study water and biofilm samples from Dhaka, the real-time PCR analysis was repeated on all field samples but spiked with 1,000 genomes of *H. pylori* strain J99 in each PCR. Detection rates lower than 30% were found in 41% of the drinking water samples. However, 10-fold dilution of the DNA was shown to remove the inhibitory effect in 90% of samples, but still without positive results. These analyses showed that levels of *H. pylori* DNA were below 250 genome copies in 38% of the samples and below at least 2,500 genome copies in another 52% of the samples. Similar results were found in biofilm and environmental water samples. Interestingly, fewer than 10% of the wastewater samples showed presence of inhibitors, presumably because these were extracted using the Qiagen stool kit, which removes PCR inhibitors but decreases the DNA yield.

***H. pylori* DNA and RNA stability during long-term incubation in tap water and seawater.** Possible degradation of *H. pylori* DNA in water was studied using two different *H. pylori* strains, Hel513 and Hel703. Because of the possible confounding effect of inhibitors in the water in Dhaka, water was taken from Gothenburg, Sweden, for this experiment. Morphology, culturability, *hpaA* and *glmM* gene numbers, and RNA integrity were analyzed at different time points. A majority of the initially spiral-shaped *H. pylori* bacteria converted into the coccoid shape within 24 h, and on day 7, no spiral-shaped bacteria were detected and no viable bacteria were recovered. Real-time PCR assays revealed that the copy numbers of both *H. pylori* genes were constant in tap water for up to 35 days, followed by small decreases at 100 days (Fig. 2), whereas copy numbers in seawater decreased up to 50-fold after 100 days (Fig. 2). However, gel electrophoresis of total RNA showed that RNA was degraded in tap water within 7 days, with no visible 23S or 16S fragments, whereas in seawater, intact 23S and 16S RNAs were visible at 35 days (Fig. 3), showing that metabolic activity may be retained longer in seawater.

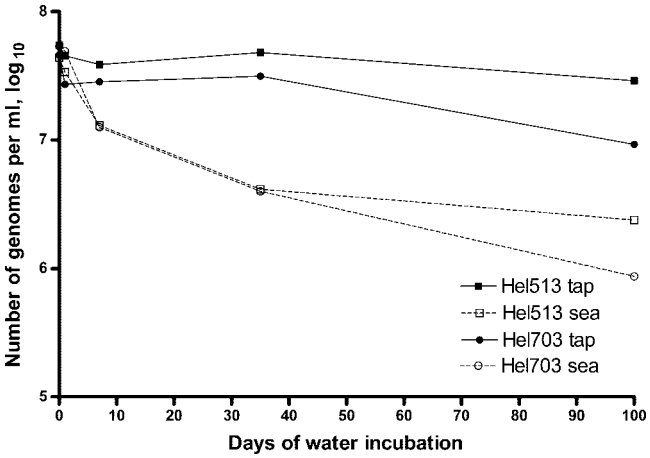

FIG. 2. Recovery of *H. pylori* DNA following long-term incubation in water. Shown are the numbers of genomes per ml as quantified with real-time PCR at different time points in long-term incubations of two *H. pylori* strains in seawater and tap water.

DISCUSSION

Several publications over the past 2 decades have suggested that *H. pylori* may be transmitted through water (2, 3, 7, 16, 19–22, 24, 26, 27, 33, 34, 37, 39, 40, 42, 43, 46, 47, 49), without showing that contaminated water is the source of new *H. pylori* infections. The original purpose of this study was to correlate findings of *H. pylori* in water, using quantitative PCR and phylogenetic analysis, with new *H. pylori* infections in children, using serology, quantitative PCR, and phylogenetic analysis, over a 2-year period in a poor urban area of Dhaka, Bangladesh. With a combination of an extremely dense population, low socioeconomic status, and a high incidence of gastrointestinal infections, including those caused by *H. pylori* and several diarrheal pathogens, this area should constitute a highly suitable field site for showing that new infections with *H. pylori* are caused by contaminated drinking water. However, to our surprise, we were not able to detect any trace of *H. pylori* DNA in any of our water samples, even though the samples were collected between September 2005 and April 2006 in order to include the two peaks of new *H. pylori* infections in the area (4). Several of these water samples were also analyzed by real-time PCR for the presence of toxin genes from enterotoxigenic *Escherichia coli* (32). This analysis showed that 67% of the samples were positive for one or several toxin genes, indicating that enterotoxigenic *E. coli* is present in these samples and that the method can detect bacterial DNA in the water. To confirm that our PCR results were true negatives, a number of control experiments were necessary since there are many possible causes of falsely negative PCR results, including sample collection methods, reaction sensitivity, PCR inhibition, assay specificity, and degradation of template DNA.

Our experiments with dilutions of whole *H. pylori* bacteria showed that the assays were highly sensitive, detecting down to 250 bacteria per sample with the use of standard filters for collecting bacteria in drinking water. There was marked inhi-

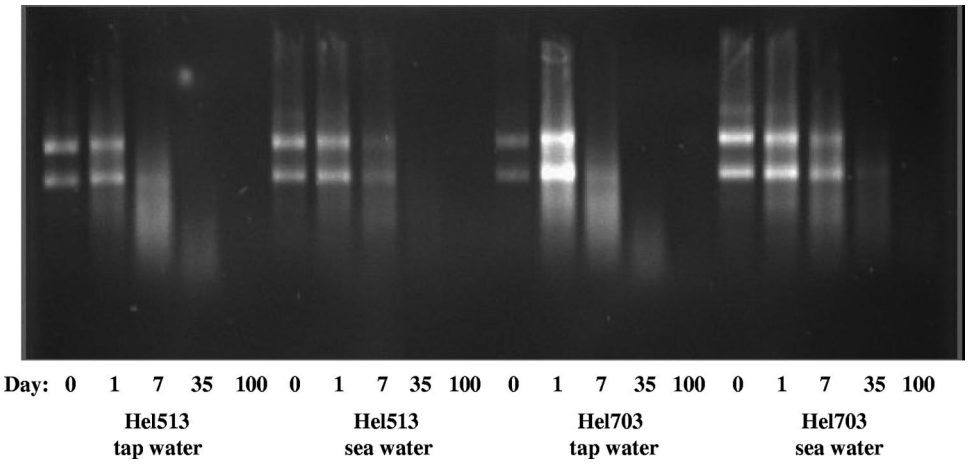

FIG. 3. Recovery of *H. pylori* RNA following long-term incubation in water. Results are shown for gel electrophoresis of RNA extracted at different time points from long-term incubations of *Helicobacter pylori* strains Hel513 and Hel703 in seawater and tap water. Nine milliliters (corresponding to 4.5×10^8 *H. pylori* bacteria) of each microcosm was sampled at each time point.

bition of PCR in approximately 50% of the samples, but this was overcome in an additional 40% of the samples by diluting the extracted DNA 10 times. When tested for specificity, our assays proved to be highly specific for *H. pylori*, with all tested strains being identified by both primer pairs. In addition, no other bacterial species were detected, with the exception of the closely related *H. acinonychis* (13), which was detected by the *glmM* assay. Furthermore, both the *glmM* and the *hpaA* genes have been used in similar previous studies (21, 22, 46), previous phenotypic analyses of >300 clinical *H. pylori* isolates have shown that HpaA was expressed in all strains studied (A.-M. Svennerholm, unpublished results), and HpaA has also been shown to be required for colonization of mice (8). The two genes in combination should thus provide a reliable method for quantification of *H. pylori* genomes in any type of sample.

We further showed that *H. pylori* cells are still detectable at initial numbers after 100 days of incubation in tap water microcosms. However, for practical reasons, Swedish water was used for these studies, and it is possible but unlikely that *H. pylori* cells or DNA is degraded differently in Dhaka tap water. Interestingly, we also showed that the pattern of nucleic acid degradation was different in seawater, where DNA was degraded while rRNA remained intact for at least 1 week rather than 1 day. However, the DNA was still detectable for at least as long as rRNA is intact, again showing that the assays should be capable of detecting cells at least for as long as they stay viable in water.

Some previous studies suggesting waterborne transmission of *H. pylori* have found a positive correlation between *H. pylori* infection and consumption of untreated or low-quality drinking water (24, 26, 31, 35, 39), although these results are sometimes confounded by other factors associated to low socioeconomic status, such as illiteracy and crowded living conditions. In addition, there are a number of studies reporting traces of *H. pylori* in different water sources, mainly using PCR-based methods, although successful isolation of live *H. pylori* from river water (33) or marine zooplankton (10) have been reported. However, some of these studies were performed with river water, lake water, or seawater (16, 33, 43, 49) rather than drinking water, and some used nested PCR (34, 40, 43), which may increase detection sensitivity but is associated with an increased risk for contamination. Unfortunately, none of the studies using PCR-based methods have reported what measures were taken to prevent and, more importantly, detect template contamination of samples. Stringent controls for detecting both falsely negative and falsely positive results are crucial when using PCR-based assays. There are also reports of failure to identify *H. pylori* in drinking water in the United States (36) and in drinking water or reclaimed wastewater in Belgium, Spain, and Italy (5), but these were performed in low-endemicity developed countries. Finally, the viability and putative infectious capability of *H. pylori* under environmental conditions are controversial. Suggestions that *H. pylori* persists in the environment in a viable but nonculturable coccoid form (27, 42, 45) have been challenged (28), and there is only scattered evidence for reversion to the actively dividing form (9, 11, 47). Furthermore, our results show that coccoids, regardless of viability, are readily detected at low numbers by our real-time PCR assays.

Using freshly cultured *H. pylori* administered in small volumes (2 to 10 ml) together with gastric acid-neutralizing agents, the infectious dose of *H. pylori* has been estimated to be at least 10^4 bacteria (17, 48). Considering that *H. pylori* levels were below 2,500 genomes per 150 ml in our field study, we believe that it is unlikely that the analyzed water and biofilm samples contained an infectious dose of *H. pylori*. Although failure to detect *H. pylori* DNA in a limited number of water samples with a relatively small volume does not completely disprove waterborne transmission, our results indicate that the predominant route of *H. pylori* transmission is likely to be other than waterborne in this and other high-endemicity areas.

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