Potential transmission sources of *Helicobacter pylori* infection: detection of *H. pylori* in various environmental samples

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Abstract

Purpose *Helicobacter pylori* is one of the most common human infectious agents which may be transmitted via water. This study was designed to test *H. pylori* presence via molecular methods in various aquatic environments as well as sewage sludge (SS) to understand the role of these environments in the pathogen's transmission.

Methods specific primers for the 16S rRNA and *ureA* genes of *H. pylori* were used in a nested and semi-nested PCR, respectively. Detection sensitivity of *H. pylori* in environmental samples by semi-nested PCR was also compared with real-time PCR. Analysis of fecal coliforms (FC) as pollution indicator bacteria was also performed.

Results *H. pylori* 16S rRNA gene was detected in 36% (14/39) of wastewater samples and 8% (2/25) of water samples, while amplification of *ureA* gene yielded only two positive results. None of the SS samples were positive for *H. pylori* and real-time PCR could not identify *H. pylori* in any of the samples. The results showed no correlation between the presence of *H. pylori* and FC.

Conclusions Our result revealed the widespread presence of *H. pylori* in wastewater samples which indicates wastewater may be a source for dissemination and transmission of *H. pylori* infection. Further research is needed to determine the risk of *H. pylori* in wastewater reuse for irrigation of crops.

Keywords *Helicobacter pylori* · Water · Wastewater · Sludge · PCR

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Introduction

Helicobacter pylori is a common human pathogen which infects up to 50% of the world's population. *H. pylori* colonizes the human stomach and is responsible for peptic ulcers, lymphoma, adenocarcinoma and stomach cancer [1]. In spite of the global distribution of *H. pylori*, transmission route of the bacterium has not yet been precisely identified. However, three pathways which include the oral-oral, fecal-oral and gastric-oral have been suggested as possible transmission routes [1, 2].

Several studies have reported detection of *H. pylori* in various aquatic environments including drinking water, surface water, coastal and marine water and wastewater [3–9]. There are indications of waterborne transmission of this pathogen [3, 10, 11]. Results of one study in Japan showed that *H. pylori* prevalence was strongly associated with the contamination of well water with *H. pylori* [10]. Prevalence of *H. pylori* colonization among a rural area population showed a relationship between the use or drink of *H. pylori* contaminated well water and *H. pylori* infections [11]. *H. pylori* has been considered as

one of the EPA drinking water contaminant candidates [2]. Consumption of wastewater-irrigated vegetables is also a risk factor for acquisition of *H. pylori* infection [12]. Sewage sludge can serve as a reservoir and transmission route for many pathogenic bacteria [2, 12–14]. Widespread land application of biosolids as fertilizer and soil conditioner may entail health risks from the exposure to pathogenic microorganisms through direct contact or consumption of crops grown on biosolids-amended soils [12]. Based on our knowledge, however, there is no study for *H. pylori* detection or its fate in sewage sludge or biosolids. Therefore, it is needed to provide accurate insights into the presence of this bacterium in various environmental samples as potential routes for dissemination and transmission of this pathogen. The investigation is specifically important in arid and semi-arid areas which wastewater is increasingly used as an alternative source for agricultural activities. In other words, investigation about the presence of *H. pylori* in various environments could be used for assessing risks associated with this bacterium especially in the context of wastewater reuse and biosolid application.

However, *H. pylori* is a fastidious, slow-growing bacterium which in exposure to environmental conditions changes from the characteristic vegetative rod-shaped to the viable but non-culturable (VBNC) coccoid form [2, 9]. Bacteria are undetectable by culture method in VBNC form, while can maintain their virulence which is a concern from the public health point of view [15]. Therefore, molecular methods are commonly used to detect *H. pylori* in environmental samples [9]. However, researchers have generally used a specific molecular method to detect *H. pylori* in environmental samples. Since, specificity and sensitivity of selected primers as well as the type of PCR method could influence detection of microorganisms in environmental samples, it is needed to design comparative analysis which providing specific details about the efficiency of PCR method in detection of *H. pylori*.

Based on the potential role of aquatic environments and sewage sludge in transmission of *H. pylori* infections, this study was designed to: 1) determine the presence of *H. pylori* in various aquatic environments and also sewage sludge by PCR based methods 2) compare two sets of *H. pylori* specific primers as well as nested PCR with real-time PCR for detection of *H. pylori* in environmental samples.

Methods

Sampling

A total of 88 samples were collected from various aquatic environments as well as anaerobically digested sewage sludge (SS) in Isfahan, central part of Iran. Water samples (500 ml) were collected from various drinking water sources including wells, springs and aqueducts. Wastewater samples (50–

250 ml) were taken from influent and final effluent of four domestic and hospital wastewater treatment plants (WTPs). SS samples were also taken from two activated sludge treatment plants. Characteristics of WTPs are presented in Table 1. All samples were collected in sterile glasses or bags, transferred to the laboratory and analyzed for the fecal pollution and presence of *H. pylori*.

Fecal pollution analysis

Fecal pollution analysis of samples was performed by multiple-tube fermentation technique for total and fecal coliforms as described in *Standard Methods* and the results were expressed as the most probable number (MPN) per ml or gram [16].

H. pylori detection

Water and wastewater samples were concentrated by filtration-centrifugation and centrifugation, respectively as described previously [17].

For SS, 5 g of each sample was suspended in 45 ml of phosphate buffer solution, shaken for 30 min and then supernatant was concentrated by centrifugation. The resuspended pellets from concentrated samples were subjected to DNA extraction using repeated freeze and thaw process in liquid nitrogen and boiling water, respectively. The DNA was further extracted and purified using Promega DNA Extraction Kit (Promega Wizard_ Genomic DNA Purification Kit, Madison, WI) according to manufacturer's instruction [18].

PCR assay

PCR based method was used for detection of *H. pylori*. We used a nested PCR assay to increase sensitivity detection of *H. pylori* in environmental samples. We also compared the sensitivity detection of nested PCR assay with real-time PCR for detection of *H. pylori* in environmental samples. At first, primer set Eubac 27F and 1492R which amplifies a ~1420 bp fragment of 16S rRNA gene region of bacteria was used [17]. Primer set of HPnesF and HPnesR which targets the 16S rRNA gene of *H. pylori* was used in the nested PCR assay (Table 2). However, after sequencing analysis of PCR positive products of environmental samples; it was defined that this band is not specific for *H. pylori*. Therefore, in the next stage we used species-specific primer sets of Hp2-HP1 and HPnesF-HPnesR which amplify a 520 bp and 139 bp fragments of the 16S rRNA gene in *H. pylori*, respectively in the nested PCR assay (Table 2). We also used species-specific primer pairs of HpyF1-HPU11 and HpyF1-HpyF2 in a semi-nested PCR assay (Table 2). This assay targets the *ureA* gene of *H. pylori*. For comparison

Table 1 Characteristics of wastewater treatment plants (WTPs)

	Municipal wastewater			Hospital wastewater
	WTP A	WTP B	WTP C	WTP D
Types of WTP	Conventional activated sludge	Conventional activated sludge (two step)	Stabilization pond	Extended aeration + high speed sand filtration
Capacity (m ³ /d)	130,000	250,000	11,700	132
Disinfection process	Chlorination	Chlorination	Chlorination	Chlorination+ UV
Final effluent receiving field	River	Land application	Agricultural application	Absorption well

of nested PCR with real-time PCR, primer set of HpyF1- HpyF2 was used in the real-time assay.

The PCR amplification was conducted in a total volume of 25 µl, consisting of 12.5 µl premix (Ampliqon, Denmark), 0.3 µM of each primer, 2 µl and 1 µl of template DNA and amplified product in the first step and second step of nested PCR, respectively and double distilled water. PCR was performed with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, primer annealing at 58 °C for 45 s, and extension at 72 °C for 1 min, and finally one cycle extension at 72 °C for 10 min. All PCR assays included positive and negative controls. A clinical isolate of *H. pylori* which confirmed by 16S rRNA gene sequencing was used as positive control. PCR products were analyzed by electrophoresis using 1.5% (w/v) agarose gel and a 100 bp DNA ladder was used as a molecular weight marker. For confirmation of *H. pylori* detection, the 16S rRNA PCR products from some samples were randomly sequenced.

Real-time PCR was run in 48-well plates (Applied Biosystems), with a total volume of 20 µl in each reaction mixture. The optimized PCR mixture contained 7.5 µl SYBR green real-time PCR master mix, 0.3 µM of each primer and 2 µl of extracted sample. The analysis was performed under following conditions: initial denaturing at 95 °C for 5 min, 40 cycles of 95 °C for 10s and 60 °C for 20s.

Results and discussion

Fecal polluted environmental samples may play a role in transmission of *H. pylori* infection. In this study *H. pylori* was detected with the highest frequency (36%, 14/39) in wastewater samples. PCR detection of *H. pylori* by 16S rRNA gene was positive for 56% (9/16) of influent samples (Table 3). *H. pylori* was detected with the lowest frequency in WTP D which could be related to the presence of disinfectants in hospital wastewater. Furthermore, frequent use of antibiotics in hospitals and subsequently presence of antibiotic residues in hospital wastewater could eradicate *H. pylori*. Among the municipal wastewater treatment plants, the lowest frequency of *H. pylori* detection was related to the WTP A (Table 3) which may in part be related to the socioeconomic status of population served by WTP A. It has been reported that socioeconomic status is an important factor affecting the prevalence of *H. pylori* [1]. The information about the presence of fecal bacterial pathogens such as *H. pylori* in wastewater samples can reflect their prevalence in human population [22]. A study in two sewage treatment plants in the city of Barcelona showed that 66% of raw wastewater samples were positive for *H. pylori* [5]. Our results revealed lower detection (22%, 5/23) of *H. pylori* in effluent samples (Table 3). Similar to our results, Moreno and Ferrus [7] reported more detection of *H. pylori* in raw wastewater than effluent samples. However, Moreno et al. [4] could detect *H. pylori* in only one sample from fifteen wastewater samples of a WTP located in

Table 2 Primers used in the study

Primer	Sequence (5' → 3')	Target gene (expected size (bp))	reference
HpyF1	GGGTATTGAAGCGATGTTTCCT	<i>ureA</i>	[19]
HPUII	CTCCTTAATTGTTTTIAC	<i>ure A</i> (471)	[20]
HpyF2	GCTTTTTTGCCTTCGTTGAT AGT	<i>ureA</i> (135)	[19]
HP2	GCTAAGAGATCAGCCTATGTCC	16S rRNA (520)	[6]
HP1	GGCAATCAGCGTCAGTAATGT		
HPnesF	GAAGATAATGACGGTATCTAAC	16S rRNA (139)	[21]
HPnesR	ATTCACACCTGACTGACTAT		

Valencia, Spain. Although *H. pylori* was detected in effluent samples of municipal WTPs, lower detection of the microorganism in effluent samples (Table 3) shows *H. pylori* removal efficiency in WTPs. Nevertheless, presence of *H. pylori* in effluent samples of the municipal wastewater treatment plants highlights inability of the conventional treatment processes in complete removal of the bacterium. On the other hand, no detection of *H. pylori* in WTP D could be related to the application of UV disinfection system as well as high speed sand filtration systems (Table 1). Furthermore, most positive *H. pylori* samples were identified at lower ambient temperatures of the sampling period from August to March. However, further study is needed to investigate the effect of climate parameters on the presence of *H. pylori* in environmental samples.

Our results showed that only 8% (2/25) of water samples were positive for *H. pylori* which related to samples from well water. No spring and aqueduct samples were positive for *H. pylori* (Table 3). Queralt et al. [5] reported the detection of *H. pylori* in 11% (2/19) of river samples, but in none of the spring water samples. In a study at the Turia river water in Spain, one of ten freshwater samples was positive for

H. pylori by PCR [4]. In analysis of 75 environmental water samples in Bangladesh from an area with a very high rate of *H. pylori* infection, no *H. pylori* was detected. The researchers suggested that waterborne transmission of *H. pylori* is not the predominant route in this area [23]. In contrast, 13 from 22 well water samples were reported positive for *H. pylori* by Baker and Hegarty [3]. The authors have indicated that consumption of untreated well water should be considered a risk factor for *H. pylori* infection [3]. Some studies have reported more frequently detection of *H. pylori* in samples with higher load of fecal pollution [3, 5]. A significant relation between detection of *H. pylori* and the presence of fecal indicators in contaminated drinking water has been reported [3]. Although in the study, higher detection of *H. pylori* was found in wastewater samples, there was no relation between the presence of *H. pylori* and the concentration of fecal indicator bacteria. Fecal indicator bacteria in positive *H. pylori* water samples were not detected (Table 3), whereas some water as well as wastewater samples with the presence or high concentration of fecal coliforms were negative for *H. pylori*. Detection of *H. pylori* in disinfected drinking well waters may be due to its resistance. It was demonstrated that *H. pylori* is more resistant than *E. coli* to chlorine

Table 3 Microbial characteristics of analyzed environmental samples. Data are presented as number of positive samples (percent of positive samples)

Sample type	No. of analyzed samples	No. of positive samples for <i>H. pylori</i> by amplification of			FC concentration (MPN/100 ml) ^a
		16S rRNA gene	<i>ureA</i> gene		
			Nested PCR	Real-time PCR	
Water					
Well	15	2 (13%)	ND ^b	ND	ND
Spring	6	ND	ND	ND	0.2
Aqueduct	4	ND	ND	ND	1.4
Wastewater					
Influent					
WTP A	4	2 (50%)	ND	ND	NA ^C
WTP B	4	3 (75%)	ND	ND	NA
WTP C	3	2 (67%)	ND	ND	NA
WTP D	5	2 (40%)	ND	ND	NA
Effluent					
WTP A	13	3 (23%)	ND	ND	1.2 × 10 ⁶
WTP B	3	1 (33%)	1 (33%)	ND	5.6 × 10 ⁶
WTP C	3	1 (33%)	1 (33%)	ND	NA
WTP D	4	ND	ND	ND	NA
Sewage sludge					
WTP A	12	ND	ND	ND	10 ⁹ ^d
WTP B	12	ND	ND	ND	10 ⁹ ^d

^a Values of mean concentration

^b Not detected

^c Not analyzed

^d MPN per gram dry weight

concentrations used in the disinfection process of drinking water [15]. Our finding is consistent with previous reports which have reported no relation between the occurrence of *H. pylori* and fecal indicator bacteria in estuary and marine waters as well as in coastal fresh water [6, 8]. Therefore, based on the suggestion of Twing et al., conventional microbial analyses may be insufficient in predicting the presence of *H. pylori* in natural waters [6]. Our results also revealed no detection of *H. pylori* in sludge samples with a high concentration of fecal coliforms (Table 3). Although, it has been reported that *H. pylori* is more resistant than coliform bacteria, no detection of this microorganism in sludge may in part be related to the alkaline conditions of sludge digesters; because of *H. pylori* is considered to be a neutrophile [24]. As no *H. pylori* detected in SS, land application of SS may not be a potential risk factor for transmission of *H. pylori* infections. In other words, our finding revealed that SS may not act as a potential vehicle for transmission of *H. pylori*. However, high detection of *H. pylori* in wastewater samples could be a public health concern and may be a threat for consumers of wastewater-irrigated vegetables. Increased risk of *H. pylori* infection with consumption of wastewater-irrigated vegetables was reported [12]. Researches on occupational exposure, however, showed no increased risk from *H. pylori* for wastewater treatment plant workers [25, 26]. Therefore, presence of *H. pylori* in wastewater can be problematic especially in water-scarce regions such as Middle East countries which wastewater irrigation is a particular priority.

The results revealed very higher detection of *H. pylori* by amplification of 16S rRNA gene than *ureA* gene (Table 3). However, detection of *H. pylori* in effluent samples of WTP B and WTP C by amplification of both genes may be related to the higher number of *H. pylori* in these samples. This discrepancy has been also reported by Twing et al. [6]. They detected *H. pylori* in four freshwater and 81% of estuarine samples by 16S rRNA gene, while none of freshwater and 16% of the estuarine samples were positive for *H. pylori* by *ureA* gene amplification. They concluded that the discrepancy could be due to variation in primer specificity and sensitivity. Amplification of 16S rRNA gene for detection of *H. pylori* as a multiple copies gene could be more sensitive, while may not be as specific as *ureA* gene [6]. However, no 100% specificity or sensitivity for primer sets which amplify *ureA* and 16S rRNA genes of *H. pylori* was reported by Sugimoto et al. [27]. Although nested PCR assay increases sensitivity, using the species-specific primer sets for both stages of the semi- or nested PCR assay improved specificity and provided more reliable detection of *H. pylori* in environmental samples as confirmed by sequencing results. BLAST analysis (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) of sequencing data of some amplicons of 16S rRNA gene showed homology of over 98% with *H. pylori*.

Our results showed no detection of *H. pylori ureA* gene by real-time PCR, while two samples were positive for the gene by the semi-nested PCR assay (Table 3). The result suggests that nested or semi-nested PCR may provide more reliable information about the presence of *H. pylori* in environmental samples. However, real-time PCR as a rapid and quantitative method has been frequently used for environmental studies of *H. pylori* [5, 8, 21, 23]. Therefore, more research is needed to determine the reliability of *H. pylori* detection in environmental samples by various types of species-specific primers as well as type of the PCR assay.

Conclusion

We detected *H. pylori* in drinking well water samples without the detection of fecal coliform bacteria which are commonly used as indicator of water quality. Therefore, absence of traditional indicator bacteria may not protect the water consumers from exposure to *H. pylori*. Detection of *H. pylori* in wastewater samples confirms that wastewater may act as a potential source for transmission of *H. pylori* infection. However, no *H. pylori* was detected in SS. Further studies would be necessary to determine the public health risk of *H. pylori* infection from consumption of wastewater-irrigated vegetables.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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