Environmental Health Engineering and Management Journal 2022, 9(3), 305-310
http://ehemj.com
doi 10.34172/EHEM.2022.31

Environmental Health Engineering and Management Journal

Original Article

Environmental Health Engineering and Management

Open Access Publish Free

Detection of *Helicobacter pylori* genes (*CagA* and *VacA*) in municipal drinking water

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Abstract

Background: *Helicobacter pylori* is classified as a carcinogen, and it is also the most common cause of chronic bacterial infection and peptic ulcers. Approximately 45% of people are infected with the bacterium.

Methods: In this study, the *H. pylori* genes, CagA and VacA, were investigated in drinking water, using 100 samples (50 samples from the municipal water supply and 50 samples from the effluent of household water treatment devices). DNA was extracted from colonies with a positive heterotrophic plate count (HPC) for use in molecular testing and microbial identification. The polymerase chain reaction (PCR) was used to identify *H. pylori*.

Results: The study showed that 24% of urban water samples (12% above the World Health Organization [WHO] standards for safe drinking water) and 18% of home water treatment-device samples (4% above the WHO standards) were HPC-positive. The *H. pylori* genes, CagA and VacA, were identified in 2% of the samples from household water treatment devices and 8% of the municipal water supply samples.

Conclusion: The study findings show that *H. pylori* may be transmitted in drinking water. However, there is currently no strong evidence that the bacteria can survive after the disinfection process in the water supply system. Therefore, the health risks of this bacterium in drinking water are still unknown.

Keywords: *Helicobacter pylori*, Drinking water, Polymerase chain reaction, Water supply, Water purification

Citation: Gholami-Borujeni F, Rahimi H, Sajjadi SM. Detection of *Helicobacter pylori* genes (*CagA* and *VacA*) in municipal drinking water. Environmental Health Engineering and Management Journal 2022; 9(3): 305-310. doi: 10.34172/EHEM.2022.31.

Article History:
Received: 3 January 2022
Accepted: 5 March 2022
ePublisged: 10 September 2022

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Introduction

An increase in the incidence and prevalence of water-borne diseases, such as cholera, diarrhea, typhoid, amebiasis, hepatitis (A and E), gastroenteritis, giardiasis, campylobacteriosis, scabies, and parasitic infection, has occurred due to drinking contaminated water. These diseases can lead to death, if correct treatment and contaminant control are not provided. Assuring that drinking water quality is safe has been a crucial challenge for public health. Water contamination with pathogenic microorganisms represents a seriously increased threat to human health (1).

Helicobacter pylori, previously known as *Campylobacter pylori*, is a heterotrophic, gram-negative, spiral bacterium that is associated with human gastritis. It is an oxidase-, catalase-, and urease-positive bacillus that infects more than 50% of the world's population (2). *H. pylori* infection has been identified as a major cause of gastric and duodenal ulcers, and a potential risk factor for gastric adenocarcinoma initiation. The World Health Organization (WHO) considers *H. pylori* as a bacterial carcinogen. In Asia, the bacterium is very common and gastric adenocarcinoma is the most deadly cancer in that region. Studies have shown different patterns in the epidemiology of this pathogen in developed and developing countries (3). In developed countries, the prevalence of infection increases gradually with age, but, in developing countries, most people are infected at young ages (4). Most of the risk factors are related to adverse living conditions, and there is no difference between developed and developing countries. Despite the bacterium's high prevalence, the routes of its transmission to humans have not yet been elucidated (5,6). Much research has been done in this area, and the most common transmission routes are reported as oral-fecal, oral-oral, and sexual (5). *H. pylori* infections arise mainly from the direct human-to-human transmission or environmental contamination, although there have been reports of contamination by

food and water (7).

According to the WHO report, the disease's prevalence is decreasing worldwide due to the improved health conditions, but increasing drug resistance has raised concerns about treatment failure and controlling the major gastrointestinal diseases. The findings suggest that *H. pylori* can survive in water and milk, fresh fruit and vegetables, and fresh meat (poultry, fish, and meat) at temperatures below 30°C (8).

The effect of multiple genes on the severity of bacterial pathogenicity, and the definitive association between the clinical consequences of infection with a particular gene have yet to be determined. It is widely thought that malignant strains are more likely to cause gastrointestinal diseases, especially stomach ulcers (6). Epidemiological studies in Sari, Iran, showed that, on average, 45.5% of people were infected with *H. pylori* (9).

Helicobacter pylori cannot be detected using conventional culture-based methods when isolated from biofilms. Beyond that, the main focus of conventional microbial drinking water analysis methods is coliform group bacteria, shown by numerous studies to be related to the presence of *H. pylori*, using heterotrophic plate count (HPC) methods. The microbial quality index of drinking water is less important in public water distribution networks, but counting and quantification of these bacteria determine how well it performs, and control the water treatment processes and distribution network facilities (10,11).

Helicobacter Pylori is hard to cultivate but biofilm formation in drinking water networks provides conditions that allow it to survive for some time. Numerous studies have used conventional polymerase chain reaction (PCR) and qPCR, to detect VacA, CagA, UreA, and glmM genes, to determine the presence of *H. pylori* in various aqueous environments (surface water, untreated wastewater, seawater, groundwater, and biofilms) (12,13). Previous research shows that the presence of HPC and low residual chlorine in the water distribution network in Sari (Mazandaran, Iran), lead to an increase in the rate of biofilm formation (14).

The purpose of this study was to evaluate heterotrophic microbial contamination and the presence of *H. Pylori* gens (CagA and VacA) in municipal water and household water treatment devices effluent.

Methods

Sampling

One hundred water samples (50 samples from each municipal distribution network and 50 samples from household water treatment devices) were collected from different locations in Sari (Mazandaran Province, Iran) using randomized sampling methods.

Approximately 70% of drinking water in Sari city is groundwater obtained from 27 wells, and the rest is treated surface water obtained from the Shahid Rajaee Dam. Based on the existing maps and mentioned source of the studied water supply network, 100 mentioned samples were collected according to the systematic sampling method. The sampling locations are shown in Figure 1. All samples were transferred to the laboratory using standard cold chain methods in microbial water sampling containers (15). Some physicochemical characteristics of samples, such as pH, free residual chlorine (mg/L), turbidity (NTU), and electrical conductivity (µS/cm), were analyzed according to the standard methods for examining water and wastewater (10).

Heterotrophic plate count (HPC) test

The spread plate method of the HPC (formerly known as the standard plate count) using R2A agar culture medium was used to count the HPC bacteria in samples, based on the following standard methods number (NO = 9215C) (10). In the spread plate method, 0.5 mL of the sample or a diluted portion of the sample is spread on the plate surface. In the present study, after incubation (at 35°C for 72 hours), the colonies that had appeared on the plate surface were counted with a colony counter and the CFU/mL was calculated.

DNA extraction

After incubation on the membrane filter, the colonies that had formed on the R2A agar medium were resuspended with 1 mL of 0.01% Tween solution and pelleted by centrifugation. The DNA was isolated using the SinaPure™ DNA Kit (SinaClon, Iran) according to

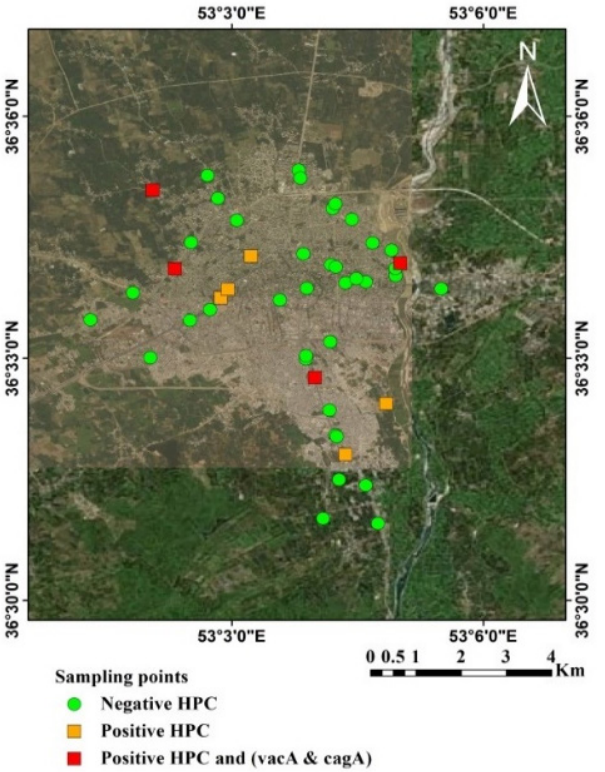

Figure 1. The 50 sampling points in Sari from each household water treatment devices and the municipal water supply network.

the manufacturer's instructions. A collection of 3 to 6 × 106 cells was centrifuged in a 2-ml tube for 5 minutes at 1000 rpm, and the cell pellet was rinsed with PBS. The centrifugation was repeated, and the supernatant was completely discarded by pipetting. Then, 100 µL of pre-lysis buffer and 20 µL of ributinase were added to the tube, and the tube was vortexed and placed at 55°C for 30 minutes or more. The centrifugation was repeated, and the supernatant was discarded by pipetting.

The DNA was eluted in 100 µL of sterile water, and its concentration was measured with a nanodrop spectrophotometer and confirmed by agarose (1%) gel electrophoresis.

Polymerase chain reaction

To amplify the 16S rRNA, CagA, and VacA genes, the primer design and validation were performed using Primer3 and Primer-BLAST, respectively. The PCR reactions were done at a volume of 50 µL with the extracted DNA, 200 M (each) deoxynucleotide triphosphates (dNTPs), 25 pmol of each primer, 1.5 M magnesium chloride, and 1 unit of Taq polymerase (SinaClon, Iran). An Eppendorf thermal cycler was used.

The amplification conditions consisted of initial denaturation at 95°C for 5 minutes followed by 37 cycles of denaturation at 90°C, annealing at 60°C, and extension at 72°C (each of 60 seconds). The final extension phase at 72°C was allowed for 5 minutes to maximize the amplification process according to Table 1.

Primers used to amplify 16S rRNA, CagA, and VacA sequences were according to Table 2.

The negative control used in each PCR assay which lacking DNA. The final result was visualized using 2% agarose gel electrophoresis and staining with RedSafe. A 50 bp ladder was used as the DNA molecular weight standard.

Results

The results of physiochemical analysis such as pH, free residual chlorine (mg/L), turbidity (NTU), electrical conductivity (µS/cm) of the 100 municipal drinking water, and effluent of household water treatment devices samples are summarized in Table 3.

Sampling points, negative HPC samples, positive HPC samples, and positive HPC and (VacA and CagA) in the household water treatment devices and drinking water network of Sari (Mazandaran, Iran) are shown in Figure 1 and Table 4.

The results show that VacA and CagA genes were detected in 2% of the samples collected from household water treatment devices and 8% of the water supply network samples (Figure 2).

Discussion

As shown in the results, some genes of *H. pylori* as an anaerobic bacterium were identified in the colonies of aerobic heterotrophic bacteria. The question is why in the genome of aerobic bacteria, some genes of microaerophilic bacteria such as *H. pylori* have been found. This phenomenon may be as a result of horizontal gene transfer, which is an essential aspect of microbial

Table 1. The PCR amplification reaction program

Step	T (°C)	Time (min)	Number of Cycles
Denaturation 1	95	5	1
Denaturation 2	90	1	
Annealing	60	1	37
Extension	72	1	
Final extension	72	5	1

Table 2. The primer sequences used for the amplification of CagA, VacA, and 16S rRNA genes

Gene	Primer sequences (5'-3')	Amplicon size (bp)	References
CagA	F: 5'AAT ACA CCA ACG CCT CCA-3' R: 5'TTG TTG CCG CTT TTG CTC TC-3'	400	(16)
VacA (s1/s^2)	F: 5'ATG GAA ATA CAA CAA ACA CAC-3' R: 5'CTG CTT GAA TGC GCC AAA C-3'	259/286	(17)
16S rRNA (m1/m^2)	F: 5'CAA TCT GTC CAA TCA AGC GAG-3' R: 5'GCG TCT AAA TAA TTC CAA GG-3'	570/642	(16, 17)

Table 3. Characteristics of municipal drinking water and effluent of the household water treatment devices samples

Sample	Parameter	Mean±SD	Minimum	Maximum
Municipal drinking water	pH	6.63±0.93	5.1	7.99
	Free residual chlorine (mg/L)	0.17±0.29	0	1
	Turbidity (NTU)	0.61±0.37	0.22	2
	Electrical conductivity (µS/cm)	486±23	30	870
Household water treatment devices	pH	6.63±0.93	5.1	7.99
	Free residual chlorine (mg/L)	0.17±0.29	0	1
	Turbidity (NTU)	0.61±0.37	0.22	2
	Electrical conductivity (µS/cm)	486±23	30	870

Table 4. The results of HPC, VacA and CagA in 100 samples

Sample No	X	Y	Municipal water sample	Household water treatment devices samples
1	53.04173	36.557807	ND	ND
2	53.045724	36.560017	ND	ND
3	53.04784	36.562529	ND	HPC
4	53.04925	36.564261	HPC	ND
5	53.056905	36.056918	ND	ND
6	53.063156	36.587199	HPC+VacA & CagA	HPC+VacA & CagA
7	53.094542	36.734391	ND	ND
8	53.070046	36.580877	ND	ND
9	53.069736	36.569284	ND	ND
10	53.070727	36.568797	ND	HPC
11	53.072628	36.565527	HPC	ND
12	53.076642	36.565727	ND	ND
13	53.091662	36.564314	ND	ND
14	53.064729	36.549843	HPC	HPC
15	53.070636	36.534137	ND	ND
16	53.066535	36.546059	HPC+VacA & CagA	ND
17	53.06481	36.550486	ND	ND
18	53.069605	36.553339	ND	ND
19	53.063296	36.58883	ND	ND
20	53.059599	36.562014	HPC	ND
21	53.08259	36.567031	ND	HPC
22	53.082568	36.568478	ND	ND
23	53.081795	36.57222	ND	ND
24	53.077992	36.57377	ND	HPC
25	53.073882	36.578624	ND	
26	53.070638	36.581884	HPC	
27	53.063646	36.587169	ND	
28	53.047241	36.582974	ND	HPC
29	53.045185	36.587677	ND	ND
30	53.034313	36.584731	HPC+VacA & CagA	ND
31	53.041939	36.573889	ND	ND
32	53.038701	36.568499	HPC+VacA & CagA	ND
33	53.030376	36.563461	ND	ND
34	53.021911	36.557931	ND	ND
35	53.033813	36.550073	ND	ND
36	53.069434	36.539254	ND	ND
37	53.072643	36.530125	HPC	ND
38	53.076643	36.523708	ND	ND
39	53.079097	36.515819	ND	ND
40	53.068182	36.516882	ND	ND
41	53.064963	36.56442	ND	HPC
42	53.074794	36.566401	ND	ND
43	53.08352	36.569637	HPC	ND
44	53.051045	36.578428	ND	ND
45	53.053836	36.57111	HPC	ND
46	53.064236	36.571535	ND	ND
47	53.080719	36.540643	ND	ND
48	53.070885	36.533821	ND	HPC
49	53.071351	36.524896	ND	ND
50	53.083412	36.56111	ND	ND

Note. HPC, heterotrophic plate count; ND , not detected.

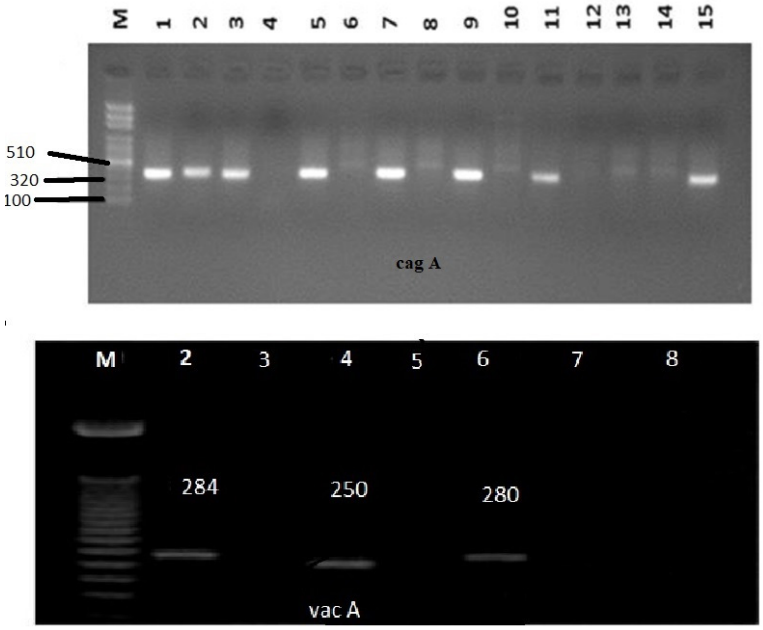

Figure 2. Agarose gel electrophoresis of the PCR products of H. pylori CagA and VacA positive gene from the positive HPC samples agarose gel.

evolution, but its mechanisms, rates, and consequences are poorly understood and difficult to quantify.

The study results show that 24% of the samples from the distribution network had positive HPCs, of which 12% exceeded the WHO drinking water quality standards, and 18% of household water treatment device samples were positive, with 4% exceeding the WHO drinking water quality standards (18).

Due to the nature of *H. pylori*, the possibility of their survival in the drinking water samples requires a microbial culture method. Although cultivated *H. pylori* has never been isolated from drinking water or water distribution systems, molecular techniques like PCR have confirmed its presence, indicating the possibility of its survival in an aquatic environment.

The use of a nucleic acid peptide probe has shown that *H. pylori* within a biofilm was exposed to low chlorine concentrations (0.2 and 1.2 mg/L) for at least 26 days in the lower layers of the biofilm (19). According to the Boehnke et al research, 20.3% of drinking water samples in Lima (Peru) was contaminated with *H. pylori* (20). The results of the study by Vesga et al demonstrate that viable *H. pylori* cells were present in both, influent and effluent water samples obtained from drinking water treatment plants in Bogotá (Colombia) (21). Therefore, it is important to understand its ability to survive in heterotrophic biofilms formed in standard chloride-containing (residual chlorine) waters. If it survives in these conditions, the release of the biofilms into drinking water may pose a health risk.

Conclusion

Water contaminated with *H. pylori* is mainly caused by the contact of water with feces, and preventing the discharge of untreated municipal wastewater into water sources can help prevent microbial contamination of water. Although household treatment devices can contribute to increased water safety and enhance water quality, they can also several undesirable attributes; for example, the commonly used filter membranes and the adjacent interior faucet surface can serve as substrates for the accumulation, growth, and proliferation of pathogenic and non-pathogenic microorganisms that form biofilms and produce extracellular polymeric substances. The findings of the present study reinforce the evidence that *H. pylori* genes (*CagA* and *VacA*) are present in municipal drinking water and the effluent of household water filter devices. There is no current evidence indicating that *H. pylori* can survive the disinfectants used in water distribution networks. The health risks from this source are still unknown. Overall, the results indicate a need for further research regarding the source of water contamination in Sari.

Acknowledgments

The authors would like to thank the Deputy of Research and Technology of Mazandaran University of Medical Sciences for their financial support (IR.MAZUMS.REC.1397.3011).

Ethical issues

The study protocols were approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Ethical code: IR.MAZUMS.REC.1397.3011).

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contributions

All authors equally contributed to the problem suggestion,

experiments design, data collection, and manuscript approval.

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