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Original Article

Potentially Infectious *Helicobacter pylori* in Tap Water in Kermanshah, Western Iran

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Abstract

Background: Although the pathogenesis of *Helicobacter pylori* is well-defined, the origin and transmission of the bacterium have remained largely unknown. The water transmission hypothesis suggested that water acts as a carrier in oral-fecal transmission, especially in high-prevalence areas. We aimed to evaluate the possible contamination of tap water with infective *H. pylori* in Kermanshah, Iran from Sep-Oct 2020.

Methods: Tap water samples were collected from varieties of probable high-alert regions and the viability of *H. pylori* were achieved using culture and real-time PCR techniques (*ureA* gene expression).

Results: Out of 50 tap water samples, 3 were positive for *H. pylori* before enrichment and 6 were positive after enrichment by RT qPCR, while *H. pylori* colonies of two samples were observed on brucella agar plates.

Conclusion: The results of positive samples demonstrated the probable presence of viable *H. pylori* in tap water samples, showing that tap water distribution systems could be a potential route for *H. pylori* transmission.

Keywords: *Helicobacter pylori*; Tap water; Real-time PCR

Introduction

The Joint Monitoring Program (JMP) of the WHO and UNICEF has evaluated tap water, sanitation, and hygiene (WASH). In the report of 2017, a global assessment of tap water service levels has been introduced. JMP has established the comprehensive monitoring for tap water, sanitation, and hygiene as Sustainable Development Goal (SDG) program toward 2030. In a report of 2015, 844 million people were not able to access safe tap water systems (1). In unsafe sanitation conditions, microorganisms can able to enter the tap water system, resulting in waterborne disease outbreaks (2).

The final list of bacterial contaminants transmitted through water based on the US Environmental Protection (EPA) in 2016 included *Helicobacter pylori, Campylobacter jejuni, Legionella pnuemophila, Escherichia coli, Shigella sonnei, Salmonella enterica,* and *Mycobacterium avium* (3). This means *H. pylori* has been proposed as one of the emerging water-

borne bacterial pathogens. For the first time, Warren and Marshall recognized *H. pylori* in 1982 (4). In a short period, water was proven to be the route of transmission of *H. pylori* infection among Peruvian children (5). *H. pylori* is a gram-negative, microaerophilic, and fastidious bacterium colonized in human gastric cells. Infected individuals are the high-risk group for chronic gastritis, gastroduodenal ulcers, gastric carcinoma, and gastric lymphoma associated with type B cells. According to the WHO reports, the mortality of gastric cancer is 650,000, and new cases are estimated to reach more than 900,000 annually in the world (6).

H. pylori infection is one of the most common infections worldwide. More than 50% of the world's population is infected with *H. pylori* and this rate is estimated to reach considerably higher about 80% in developing countries (7). For example, in Colombia, the infected individuals are 70%-80% (8), while in developed countries like the United States, the rate of infection is lower with a prevalence of 10%-25% (9). The transmission pattern of *H. pylori* in developing countries is exposure to public resources instead of person-to-person transmission. Water acts as not only an infection reservoir but also plays a key role in the fecal-oral transmission of *H. pylori* infection (10). Nevertheless, there have been numerous studies on the hypothesis of *H. pylori* transmission through water and the role of water as a possible source of *H. pylori* transmission has been challenged (6, 7).

H. pylori could be cultured from water in vitro. *H. pylori* is capable of adapting to eht extreme environment through conversion from spiral cells to coccoid form in which *H. pylori* enters the viable but nonculturable state (VBNC) (11, 12). In VBNC form, *H. pylori* could remain viable and metabolically active in eht water for several months, during which the organism keeps virulence factors (13). After the entrance of *H. pylori* into the VBNC state in vitro and the culture of *H. pylori*, there was a sharp decrease in the number of bacteria colonies (14). Thus, it is difficult to detect VBNC coccoid form by culture, resulting in the use of molecular techniques to identify *H. pylori* in water.

Several articles have reported the presence of *H. pylori* DNA in water samples such as surface water, treated and untreated wastewater, tap water, and seawater using molecular techniques. *H. pylori* DNA was also isolated from tap water, dental unit water, bottled mineral water, and springs in Iran (15-18).

There have been some treatments for removing microorganisms from water and increasing tap water safety such as chlorination (19). In addition to free chlorine, temperature also affects *H. pylori,* as high temperature decreases the culturability of *H. pylori*. However, the temperature from 4 °C to 15 °C has shown the optimal range for the survival of *H. pylori* in the extended pH range in water (14). *H. pylori* could be able to survive in autoclaved distilled water from 48 h to 20 d (20).

The purpose of this study was to investigate the presence of infectious *H. pylori* in tap water through confirmation of *H. pylori* viability using culture or expressed mRNA using real-time reverse transcription PCR in Kermanshah, Iran during Sep-Oct 2020.

Methods

Helicobacter pylori culture

The number of *H. pylori* reference strain ATCC 43504 were cultured in *Brucella* agar (Merck, Germany) supplemented with 10% egg yolk, 0.4% Isovitalex (Becton Dickinson, USA), antibiotics (5 mg/L trimethoprim, 10 mg/L vancomycin, 5 mg/L amphotericin B) and 10% fetal calf serum (Sigma). Then the plates were incubated at 37°C under microaerophilic conditions (10% carbon dioxide, 5% oxygen, and 85% nitrogen) using the MART system (Anoxamat, Lichtenvoorde, The Netherlands) for 5 days. The plates were examined for the characteristic appearance of *H. pylori* colonies (small size, circular, convex, and translucent). The morphology of the *H. pylori* colony was confirmed using catalase, oxidase, and urease tests. Reference strain was used as a positive control for real-time PCR.

Water sampling

Fifty samples of one L tap water were collected in sterile bottles from 50 different parts of Kermanshah city between Sep and Oct 2020. Water samples were transferred at 4°C to a microbiology laboratory within 2 h. Physical parameters such as sample temperature, pH, and residual free chlorine were determined. Tap water samples were filtered through the membrane filter of 0.45 µm and the membrane was immersed into 10 mL of enrichment Brucella broth with 5% fetal bovine serum. We evaluated the samples using in two ways: before enrichment and after enrichment (21).

Before enrichment evaluation, one mL aliquots were collected in appropriate collection tubes for RNA extraction and Real-time PCR and 100 µL of suspension were spread on Supplemented Brucella Agar (SBA). The plates were incubated under microaerophilic conditions for 5 days at 37°C. After this period, presumptive colonies were identified by morphology, Gram staining, catalase, oxidase, and urease tests.

For after enrichment evaluation, the remaining 10 mL *Brucella* broth was incubated under microaerophilic conditions for 24 h at 37°C. Then, the samples were centrifuged at a speed of 8500 rpm for 10 min. The supernatant was removed, and the pellet was resuspended in 2 mL of phosphate-buffered saline (PBS) and used for culture and RNA extraction. Presumptive Colonies were confirmed by Gram staining, catalase, oxidase, and urease tests.

Primer design

The sequence of *ureA* from the complete genome of the *H. pylori* J99 strain (GenBank accession number AE001439) were retrieved from the NCBI nucleotide database. Primer pair targeted conserved region of type strain sequence with many equivalent sequences. Standard parameters of the primer design for real-time PCR were confirmed using Oligocalculator and Oligoanalyzer online software. The size of the PCR product was 383 bp. The specific primers are listed in Table 1.

Table 1: Primers used in this study

Primer	Sequence
ureA –F	TCGTTGTCTGCTTGCCTATC
ureA –R	CAGGAAACATCGCTTCAATACC

Real-time PCR standard curve

According to the manufacturer's instructions, the DNA of *H. pylori* strain ATCC 43504 was purified using the DNeasy Blood & Tissue kit (Qiagen, USA). DNA quantification was carried out using a NanoDrop (Thermo Scientific, USA). Then, to obtain a standard curve for real-time PCR using primers *ureA*, ten-fold serial dilutions of *H. pylori* reference strain were prepared from 10^6 to 10-copy number/µl. qPCR standard curve of serial dilution based on SYBR green I fluorescence using *ureA* primers was performed to amplify a 383 bp fragment using 72-well rotor gene 6000 (Corbett Life Science, Valencia, CA, USA).

The optimized qPCR mixture in a total volume of 15 µL contained 7.5µL SYBR green real-time PCR master mix (Applied Biosystems, Foster City, CA), 1 µL of each forward and reverse primers (20 mmol/L), 2.5µL DEPC water and 3 µl of a DNA sample. qPCR was run under the following conditions: 95 °C for 10 min, 45 cycles: 95 °C for 15 sec, 60 °C for 10 sec, 72 °C for 15 sec, then 1 extension cycle at 72 °C for 15 sec. All tap water samples, standard curve samples, and controls were run in duplicate. In addition, to confirm the *H. pylori*-specific primers and qPCR standard curve results, qPCR products were also analyzed by gel electrophoresis in a 1% (w/v) agarose gel staining with ethidium bromide.

RNA isolation

Total RNA extraction from water samples (before and after enrichment) was performed using RNeasy mini kit (QIAGEN, Hilden, Germany)

according to the manufacturer's instructions. To eliminate genomic DNA, RNase-free DNase I treatment was used during the isolation procedure and stored at -80 °C. Quantification of RNA after DNase I treatment was examined using NanoDrop (Thermo Scientific, USA).

cDNA synthesis

cDNA synthesis was conducted using 8 µL RNA, 2 µL random hexamer primer, and 10 µL DEPC water added to AccuPower RocketScript RT PreMix tubes (Bioneer, South Korea) in a total volume of 20 µL. Reverse transcription was performed as follows: 30°C for 30 min (Primer annealing), 60 °C for 1 h (cDNA synthesis), and 95°C for 5 min (Heat inactivation). The cDNA was stored at -20 ºC until use. Quantitative amounts of *ureA* gene transcripts were standardized on G3PDH expression.

qPCR Detection

To measure the transcripts level for *H. pylori ureA*, amplification of *ureA* was performed from fifty cDNA samples (both direct and enriched samples) using Real-time PCR as described in the standard curve. Negative and positive controls and 3 to 5 concentrations of standard curve samples were also included in each run. For the determination of the melting curve, the temperature was increased at 1 °C for every 20 sec from 55 °C to 94 °C. To confirm further the qPCR positive results, products of positive samples from both direct and enriched tap water samples were separated by agarose gel electrophoresis (2% agarose gels) then the results were analyzed.

Results

Water characteristics

The amount of residual free chlorine in positive samples was between 0.04 and 0.58. In addition, in the case of pH and temperature, the pH range of all samples was from 7.10 to 7.89. The temperature of tap water samples was within the range of 14.12 to 19.21˚C (Table 2).

H. pylori Culture

No water samples were positive for the culture of *H. pylori* from direct samples. In enriched samples, two samples were observed for real *H. pylori* colonies on *Brucella* agar plates according to the characteristic appearance of *H. pylori* colonies and positive results of biochemical tests (catalase, oxidase, and urease). The results demonstrated enrichment of the membrane filtration was an appropriate strategy for the culture of *H. pylori*.

H. pylori gene expression by real-time RT-PCR analysis

Ten-fold serial dilutions of reference strain *H. pylori* DNA were used as the standard curve. The regression coefficient of the standard curve demonstrated the amplified product. The slope value of the standard curve showed that the efficiency of the reaction was very close to the optimal slope value of -3.32. The R_2 value was 0.98, which indicates a linear PCR system.

The standard curve concentrations were between 1.23×10^{1} and 2.57×10^{6} copy number/µL with cycle threshold values from 33.52 to 14.65 (Fig. 1). Single product-specific melting curves were generated for the *H. pylori* strain. The melting curve analysis demonstrated very little or no primer dimers were produced. Melting temperature (Tm) of the standard curve and positive tap water samples has a similar melting curve with a single peak at a temperature range between 83.24 to 84.91 °C.

Out of 50 tap water samples, 3 samples were positive for *H. pylori* before enrichment and 6 samples were positive after enrichment with *Brucella* broth and 5% fetal bovine serum by RT qPCR (Table 2) (Fig. 2). Ct values of all positive samples were under the reliability threshold (<35 cycles). The percentage of *H. pylori* gene expression in enriched samples was more than in the direct samples.

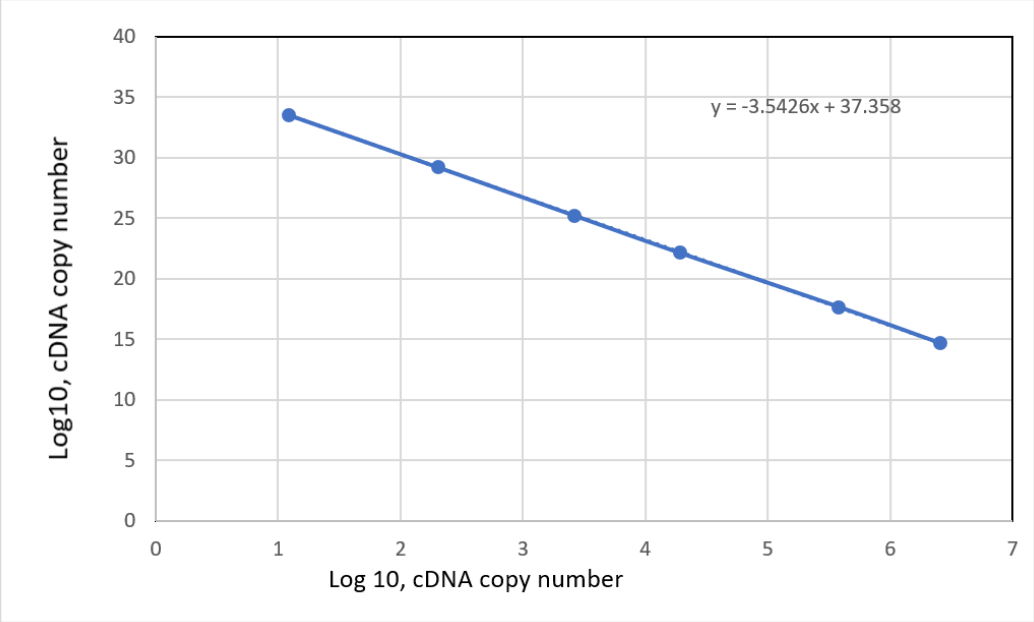

Fig. 1: Linear regression of *H. pylori ureA* gene standard curve DNA from 1.23×10^{1} to 2.57×10^{6}

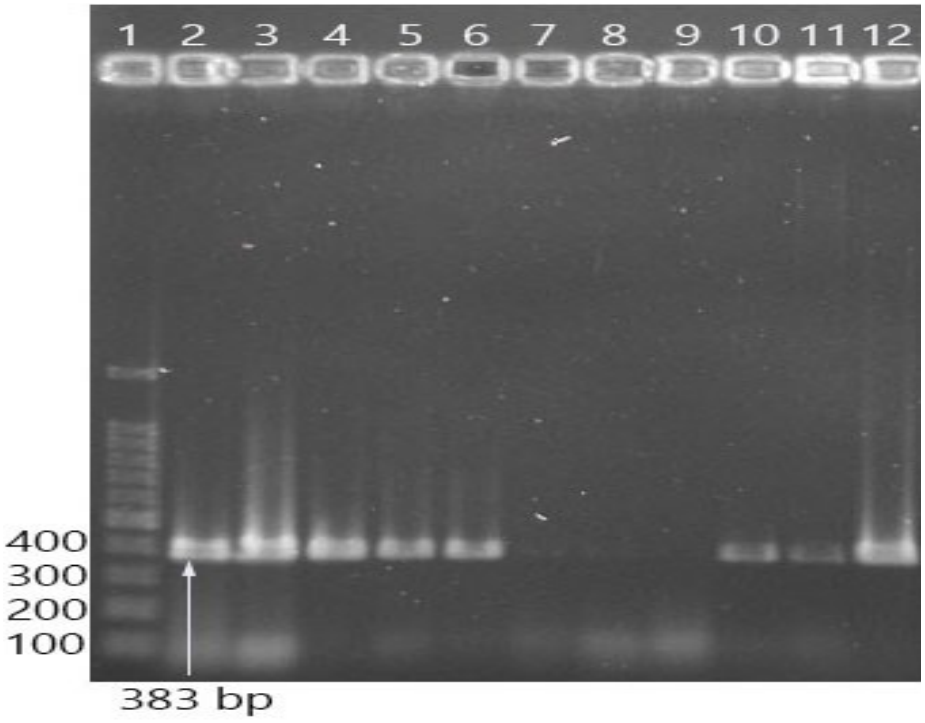

Fig. 2: Agarose gel electrophoresis of the specific *ureA* amplified fragment from positive *H. pylori* tap water samples (after and before enrichment). Lane 1: 100 bp ladder; lane 2: positive control; lanes 3,4,5,6,7,8: enriched water samples (after enrichment); lanes 9: negative control; lanes 10,11,12: direct water samples (before enrichment)

Table 2: Summary of positive *H. pylori* results in tap water samples by culture and RTqPCR strategies

Sample	*RTqPCR*		*Culture*		*Temperature*	*pH*	*FCL*
	Before enrichment	After enrichment	Before enrichment	After enrichment			
1	-	1.95×10^{1}	-	-	17.19	7.33	0.39
2	-	2.35×10^{2}	-	-	15.08	7.58	0.58
3	-	5.53×10^{3}	-	-	17.28	7.98	0.10
4	1.03×10^{3}	7.12×10^{3}	-	+	14.12	7.80	0.21
5	9.7×10^{3}	9.74×10^{4}	-	-	16.65	7.21	0.09
6	1.12×10^{4}	8.51×10^{5}	-	+	14.25	7.15	0.04

Discussion

H. pylori as a microorganism contaminant in tap water systems according to US Environmental Protection Agency (EPA) can able to be a carrier in *H. pylori* transmission (3). The presence of *H. pylori* in tap water biofilms demonstrated water distribution system is a potential reservoir for *H. pylori* (22).Many studies have detected the presence of *H pylori* DNA, but not RNA, in tap water in Iraq (23), Pakistan (24), Spain (25), Sweden (26), Peru (27), Scandinavia (28), Japan (29) using molecular techniques and culture.

In Iran, there have been reports of contamination of tap water with *H. pylori* DNA. The frequency of *H. pylori* DNA in tap water samples in Isfahan was 4% (2/50) (16). In the previous related study in Kermanshah, 25 of 70 tap water samples (35%) were contaminated with *H. pylori* DNA (18).Since molecular techniques based on the detection of DNA can identify both viable and dead *H. pylori* in tap water, we have developed gene expression of *H. pylori* in tap water by RT qPCR based on RNA detection.

Our study demonstrates viable *H. pylori* in three samples before enrichment and 6 samples after enrichment out of 50 tap water samples using RT qPCR. Incubation of enriched samples resulted in increased gene expression of *H. pylori*. For *H. pylori* culture, no *H. pylori* colony was detected in tap water samples before enrichment and the characteristic colonies were observed on two samples enrichment out of 50 tap water samples.

In Santiago's study, the culture result has shown that *H. pylori* in tap water samples can grow on the *H. pylori* selective media after enrichment. Whereas, no culturable *H. pylori* cell was isolated before enrichment. In addition, viable *H. pylori* were detected in 6 out of 24 drinking water samples (25). The presence of viable *H. pylori* in tap water treatment plants was detected in Bogotá, Colombia using culture, qPCR, and FISH techniques (21).

The main challenge in the culture of *H. pylori* is the cultivability of other bacteria in *H. pylori* selective media. Another difficulty of *H. pylori* culture is the entrance of *H. pylori* into the VBNC form in water. Therefore, we need to recover *H. pylori* from tap water to a rich media. In several studies, humans and monkeys received different doses of *H. pylori*. Based on their reports, the infection dose was estimated at 10^4 *H. pylori* cells (30, 31).

In our study, cDNA copy number values of 1 sample before enrichment (1.12×10^4) and 2 samples after enrichment (9.74×10^4 and 8.51×10^5) are close to the infectious dose of *H. pylori* in the stomach (10^4 *H. pylori* cell). This confirmed the probable role of consuming tap water in *H. pylori*-caused infection. *H. pylori* can be viable in low temperatures in water. In our study, there is a relationship between temperature and the presence of *H. pylori*. The temperature range of H. *pylori-positive* samples was between 14 and 17.

Sen et al studied the effects of chlorine exposure on *H. pylori*. They found higher levels of chlorine leading to the degradation of *H. pylori* for 2-3 d (19). According to the WHO, the optimal range of residual free chlorine in tap water is between 0.2 and 1.0 mg/L (1). In our study, residual free

chlorine we found in tap water samples was lower than those recommended by the WHO. In positive samples, residual free chlorine was between 0.04 and 0.58. The Optimal pH in tap water is 6.5-8.5 according to the WHO guidelines. In our study, the pH of tap water samples was within the optimal range of pH above 7.1.
Routine monitoring of tap water has focused on fecal indicator bacteria (FIB) as indicators of contamination in water quality. *H. pylori* has not been assessed in microbiological water quality monitoring. Some studies reported that *H pylori* could be viable in water systems with no FIB contamination. There is no correlation between the presence of FIB indicators and *H. pylori* in water (32).

Conclusion

Our results highlight the role of tap water in the transmission of *H. pylori* in tap water, since the RNA production of our isolates showed the ability of *H. pylori* to survive in water.
These insights suggest a potential menace of contaminated tap water as a source of *H. pylori*, which needs to be considered more precisely in the future.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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