

Detection of *Helicobacter pylori* in Raw and Drinking Water of Ahvaz City, Iran by PCR

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Stomach Cancer is third most prevalent cancer worldwide and most common gastrointestinal cancer in Iran. Several studies showed relationship between *Helicobacter pylori* and Stomach Cancer. Some studies in countries with low socioeconomic status and poor water supply indicated the importance of environmental factors specially water sources. Since prevalence rate of *H. pylori* infection in Khuzestan province was about 57%, we decided to investigate about presence of the bacteria in raw and drinking water of Ahvaz city using Polymerase Chain Reaction (PCR) method. Also physicochemical characteristics of water samples examined to determine role of them on presence or absence of *Helicobacter pylori*. Water samples were collected from River and water taps in consumption points. Obtained water sample examined by PCR method. PCR examination for all 45 water samples was negative, and we could not detect *H. pylori* bacteria in water samples. In River water samples, high turbidity and presence of several organic, inorganic materials, also low concentration of *H. pylori* DNA and transform spiral form of *H. pylori* to coccoid form and in treated water samples, high residual chlorine and pH can destroy *H. pylori* DNA, low concentration of *H. pylori* DNA and low sensitivity of PCR method for detection of *H. pylori* in water samples made detection it difficult by PCR.

Key words: Drinking water, River water, *Helicobacter pylori*, PCR, Ahvaz.

Drinking water is one of most important resources for human life (H. Baker *et al.*, 2002). More than half of globe population is infected by *Helicobacter pylori* (*H. pylori*) (Leclerc, 2003). Although the infection usually is not harmful, but chronic gastritis, stomach ulcer and cancer have increased in infected persons. Prevalence of this infection is high in developing countries. There is

evidence that *H. pylori* infection transmitted from food and water^{6,7} (El Dine *et al.*, 2008, Atherton, 1998, Engstrand, 2001). *H. pylori* is present in 30 to 50 % of globe population, while this present asymptomatic, but acknowledged as risk factor of chronic gastritis, stomach duodenum ulcer. Also there is relationship between *H. pylori* infection and lymphatic tissue lymphoma and cancer. Although natural history of the infection is known but its medical importance and prevalence is less understood. *H. pylori* infection consequences depend on several factors including immunological status, infectious agents prevalence and

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environmental factors (H. Baker *et al.*, 2002, Atherton, 1998, Goodman *et al.*, 1996, Adams *et al.*, 2003, Targosz *et al.*, 2006). *H. pylori* was determined as human carcinogen by International Agency for Research on Cancer (IARC) (IARC, 1997). The bacteria still not isolated from environmental resources including water, but detected by methods like microscopy technics using florescent antibody and molecular technics (PCR) in surface waters. This bacterium at laboratory conditions can survive for several days and weeks in sterile river water, saline solutions and distilled water in wide range of pH and in different temperatures ranged from 4 to 15 Celsius degree. These results proposed water as possible potential source of *H. pylori* transmission. At present human stomach concerned and sole source of *H. pylori* (Targosz *et al.*, 2006, IARC, 1997, Shahamat *et al.*, 1993). *H. pylori* is a microaerophilic organism that can cause of infection in human stomach and despite of immunological and inflammatory reactions and normal responses of stomach epithelium may stay in it for several decades or years. Infection with this bacterium is not only among cause of stomach chronic inflammations and most of stomach ulcers but also has relationship with stomach cancer (Ortiz-Princz *et al.*, 2010). Prevalence rate of the infection is different among populations and depend on living standards and sanitation status. Prevalence risk of this infection is high in people who living in developing countries (Cave, 1997, Olivares and Gisbert, 2006, Perez Perez *et al.*, 2004). Some studies shown drinking water may have possible role in *H. pylori* infection prevalence.

Several studies shown *H. pylori* DNA could identify in stool samples of infected persons or patients with stomach ulcers by polymerase Reaction Chain (PCR). This indicates fecal – oral transmission of the infection. However, because of diverse characteristics of *H. pylori*, which makes it special bacteria in human pathogen world. Long path remain to understand more about transmission epidemiology and environmental events of the pathogen (Leclerc, 2003). *H. pylori* DNA could be identified in water sources, while other organisms are inactivated by residual chlorine in water. Survival capacity of *H. pylori* is related to its uncultivated coccid form that may survive 20 to 30 days in water (Leclerc, 2003). Hypothesis of *H.*

pylori transmission through water needs to prove. Risk of this infection is multifactor's that may due to interaction between polluted environmental resources such as local drinking water, swimming in river and consuming contaminated vegetables. All above mentioned risk factors are reported (Goodman *et al.*, 1996, Xia and Talley, 1997, Zhannat *et al.*, 2002).

Despite of high prevalence of the infection, still reservoir and transmission rout are unknown. Molecular methods identified the bacteria in surface waters and shallow groundwater. There is probability of transmission by water and fecal – oral routs (Adams *et al.*, 2003, Giau *et al.*, 2011).

Some studies reported *H. pylori* more resistant than *Escherichia coli* fecal indicators to disinfection and can tolerate chlorination, thus treated water may be free of coliforms but potentially contain *H. pylori*. *H. pylori* is more resistant to hypochlorite and ozone than *Escherichia coli*, but not to mono chloramines (Castillo-Rojas *et al.*, 2004, McDaniels *et al.*, 2005), thus, some studies indicated fecal coliforms are not good indicators for *H. pylori* in water.

H. pylori is main factor of digestion tract diseases including stomach and duodenum ulcers, stomach cancer and initial gastritis lanfoma. Person to person and fecal – oral routs are proposed as mail transmission routs, while drinking water rout. Water distribution systems biofilms is a possible reservoir of *H. pylori* (McDaniels *et al.*, 2005).

Previous studies questioned survival potential of the bacteria in water distribution system because of its sensitivity to chlorine. However Baker *et al.* argued that *H. pylori* more resistant than *Escherichia coli* fecal indicators to disinfection process. Therefore *H. pylori* could transmit through water. This hypothesis investigated in recent studies which used PCR to identify *H. pylori* in drinking water and biofilms (Degnan *et al.*, 2003, Azevedo *et al.*, 2004).

Trying to cultivate *H. pylori* from environmental samples was unsuccessful and there is question whether the organism in infectious form is exist environment. When water reserved reported this organism morphologically transform from vegetative bacillus form to uncultivated coccid form. Unsuccessful attempts to cultivate *H. pylori*

from environmental water samples lead to use molecular methods for its identification. Some investigations used PCR to identify *H. pylori* from water resources including surface, groundwater, treated and untreated wastewater, recreational waters and distribution system biofilms (McDaniels *et al.*, 2005). United States Environmental Protection Agency recommended more investigation about *H. pylori* in drinking water resources, to obtain more information a rapid and sensitive method like PCR could be helpful (Brown, 2000). *H. pylori* DNA identified in untreated well water, river and stream water, water distribution system, wastewater and drinking water by nested PCR, real time PCR and routine PCR in different countries (Hopkins *et al.*, 1993, Mendall *et al.*, 1992, Fujimura *et al.*, 2004, Gomes and De Martinis, 2004, Percival and Thomas, 2009, Janzon *et al.*, 2009, Watson *et al.*, 2004, Queralt *et al.*, 2005). Klein, *et al.* (1991) identified *H. pylori* in water samples of Lima city, Peru, results showed *H. pylori* can survive in water (Klein *et al.*, 1991). In Japan a study carried out to identify *H. pylori* in different water sources. Results showed none of tap water, sea water and river water samples contain Adhesin UreA antibody and 16SrRNA, Only 2 samples from 6 well samples identified *H. pylori* DNA by PCR (Horiuchi *et al.*, 2001). But investigations in Peru and Sweden identified *H. pylori* by Adhesin in tap water samples (Hulten *et al.*, 1998, Hulten *et al.*, 1996).

Based on above reviewed literature the current investigation carried out to identify *H. pylori* DNA in raw and treated water samples of Ahvaz City, Iran. In addition physico-chemical and biological parameters of water samples analyzed including temperature, turbidity, residual chlorine and pH.

MATERIALS AND METHOD

Sampling and PCR analysis

Sampling carried out in summer season. 30 samples collected from different points of water distribution system and 15 samples collected from Karoon river water (intake points of water treatment plants). Collected water samples volume was 100 liters. Samples passed through 1 micron porous polystyrene filter (Agency, 1995).

Filters transported to laboratory at 4

Celsius degrees. Filter backwashed with 1 liter distilled water (Agency, 1995, Horiuchi *et al.*, 2001).

DNA Extraction

Obtained water samples at first step centrifuged for 2 min at 2000 rpm for removing the mud's. The supernatant was filtered through a 0.2µm-pore-size.

The filters were washed by PBS ((Phosphate buffered saline) containing 8.0 g NaCl, 0.2 g KCl, 1.44 g K₂HPO₄, 0.24 g KH₂PO₄ per liter, and adjusted to pH 7.4.

Genomic DNA from water specimens, was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quantization of DNA from water samples was determined with the Bio Photometer Plus (Eppendorf, Germany). All DNA extracts were stored at -20°C until used. It is necessary to mention that both passed water and washed water analyzed by PCR.

PCR Amplification

Two sets of primers were used for PCR amplification to identify *H. pylori* in water samples. *CagA* (cytotoxin-associated gene A) and Adhesin (*hpa*) genes were selected for PCR (She *et al.*, 2001, Castillo-Rojas *et al.*, 2002). Primers *hpa* 1 (5' GAATTACCATCCAGCTAGCG 3') and *hpa* 2 (5' GTAACCTTGACAAACCGC 3') were used to obtain a 375 bp PCR product of the *H. pylori*. Primers *CagA* 1 (5' ATACACCAACGC CTCCAAG3') and *CagA* 2 (5' TTGTTGCCGC TTTTGCTCTC3') used to amplify a 110 bp region of this gene. The PCR carried out as followed. The PCR mixture contained 3 µM of each primer, 5 mM of 10× PCR Gold buffer, 1.5 mM of MgCl₂, 1 mM of dNTP (CinnaGen), 0.1 U of *tag* DNA polymerase (CinnaGen) and 16.4 µl D.W. The reaction volume was 50 µl containing 20 µl of DNA extracts. PCR amplification was performed according to the following profile: Reactions were preheated in thermal cycler (Bio Rad-USA) for 5 min at 95°C, followed by 35 cycles of 94°C for 1 min, 58°C for 2 min and 72°C for 5 min, with a final extension step at 72°C for 5 min. Then 10 µl of amplified products were electrophoresed in 2% agarose gel. Two control samples were used for each PCR cycle including *H. pylori* (ATCC43504) DNA as positive and distilled water as negative control and photographed under UV illumination with Gel Documentation system (Uvitec, UK).

Physicochemical analyses

Parameters such as temperature, residual chlorine, and pH analyzed by YSI model 3500 portable instrument in sampling points. Water turbidity measured by turbidity meter (Turbi Direc – made in England).

RESULTS

Results of physico-chemical and biological parameters are presented in table's number 1 and 2. Tap water samples pH was between 7.8 and 8.2, temperature ranged from 27 to 33

Table 1. Environmental parameters of Karoon River water Samples

Sample Number	Sample point	Turbidity NTU	PH	Residual Chlorine (mg/l)	Temperature (°C)
1	MelliRah Water treatment Plant	40-110	8	-	26
2	Kian abad Water treatment plant	60-118	7.5-8.2	-	25
3	Aliebn Mahzeiar Water treatment plant	95-145	8	-	26

Table 2. Environmental parameters of water distribution system samples

Sample Number	Sampling points	Turbidity NTU	pH	Residual Chlorine (mg/l)	Temperatur (C°)
1	Mellat	4.9	8	1.5	30
2	Zeitoun	1	8.2	1	30
3	Naft town	6.8	8.2	1.5	30
4	Bahonar	1	8.2	0.5	28
5	Sad dastgah	2.3	8	0.8	27
6	Padadshahr	6	8	1	30
7	Shariati	4.2	8.2	0.5	28
8	Koot abdullah	4.5	8	2.5	30
9	Manbaa e aab	2.5	8.1	0.3	33
10	Sepidar	1	8.2	0.5	30
11	Taleghani	1.92	8.1	0.5	28
12	Akhar esphalt	8	7.8	0.8	30
13	Islam abad	2	8.2	0.2	28
14	Chamran	4.4	8.2	1	30
15	Kian abad	1	8.2	1	30
16	Camp low	3.2	7.9	0.9	30
17	Bani hashem	5.2	8	1	28
18	Aalavi	6	7.8	0.8	30
19	Amanieh	1.4	8	1.3	30
20	Nehzat abad	1	8	1.5	30
21	Golestan	1.4	8.2	1	30
22	Farhang shahr	1	8.2	0.5	32
23	Daneshgah town	1.2	8.2	0.8	30
24	Pardis	4.2	8	1.5	31
25	Baharestan	1	8	0.5	30
26	Choneibeh	6.36	8	1	32
27	Baghaee	6.5	8	0.8	30
28	Mallashieh	2.9	8	0.2	33
29	Ein 2	7	8	0.2	30
30	Sayiahi	4	8	0	29

Celsius degrees, turbidity was from 1 to 7 NTU and residual chlorine was between zero and 2.5 mg/l. River water samples pH was 7.5 – 8.2 , temperature 25 – 26 Celsius degrees, turbidity 40 – 145 NTU.

PCR method could not identify *H. pylori* in all samples of water distribution system and river water. As we mentioned in material & methods section, we tried all possible procedure including concentrating and analyzing passed water and washed water from 1 micron and 0.45 micron filter and centrifuged water samples, but PCR method could not identify *H. pylori* in all samples. To ensure from the results all steps carried out for control bacteria by ATCC43504 , the result was positive.

DISCUSSION

Several investigations for identifying *H. pylori* DNA by PCR in water samples claimed that identifying it by PCR is impossible and they could not isolate it from surface and distribution system water samples (Horiuchi *et al.*, 2001, Fujimura *et al.*, 2004, Zhannat *et al.*, 2002, Castillo-Rojas *et al.*, 2004, Percival and Thomas, 2009, Yamashita *et al.*, 2001).

In a study in England water distribution system investigated to identify *H. pylori*, none of samples were positive for bacteria cultivation, but by using specific PCR, *H. pylori* identified in 15 % of samples only. There was no viable *H. pylori* in water samples. It was not clear whether viable bacteria could pass disinfection process (Watson *et al.*, 2004).

In a study Janzon, *et al* (2009) discussed low DNA concentration of the bacteria in water samples which passed through filters, there was no problem with isolation of bacteria DNA and filtration can not affect DNA isolation performance, but they suggested improving filtration process (Janzon *et al.*, 2009). Also to prevent of passing *H. pylori* through filtration, they used centrifugation. Therefore DNA concentration was not affected by filtration.

In some studies reported *H. pylori* DNA is identifiable by PCR in surface and groundwater samples (Fujimura *et al.*, 2004, Queralt *et al.*, 2005, Cellini *et al.*, 2004, Bahrami *et al.*, 2013), but some studies shown that *H. pylori* DNA identification is

difficult and it should be use alternative methods such as Immune Magnetic Separation (IMP) method for isolation and RT-PCR for identification (Enroth and Engstrand, 1995) or MPN-PCR and RT qPCR methods (Nayak and Rose, 2007). Difficulty in *H. pylori* DNA identification in water samples may relate to short period of its survival in waters (Vale and Vator, 2010).

Some studies shown coccid form of *H. pylori* is responsible for infection transmission in both oral- oral and fecal- oral routs. To identify *H. pylori*, amount of this form needs to be more than bacillus form; therefore coccid form identification is more difficult. There is no explain about inactive or mild form of coccid and remain unknown (Enroth and Engstrand, 1995).

When *H. pylori* expose to water, as normal process, transform from spiral to coccid form. This transformation increases its resistance to osmotic effects. Coccid bacteria in reserved waters may die or inactivate and uncultivable, therefore DNA content will damage (Barer *et al.*, 1993). Results showed identification of *H. pylori* that entered water bodies is difficult (Watson *et al.*, 2004).

Researchers have shown coccid form of *H. pylori* related to spiral form has low concentration. Adams and Shehamat (1993) have shown these bacteria related to other organisms has shortest survival period in water sources. Survival period of *H. pylori* in water sources about 5 to 24 hour in 23 and 4 Celsius degrees respectively. Survivable of bacteria referred to its cultivation, thus *H. pylori* cultivation in water resources will ended in short time (Adams *et al.*, 2003, Shahamat *et al.*, 1993).

H. pylori coccid form out of its reservoir can survive in short time only (Chen, 2004). In some studies attempts have taken to retransform coccid form to spiral form, bur was not successful (Eaton *et al.*, 1995, Sörberg *et al.*, 1996). Kusters (1997) in a study explain that coccid form looks like dead bacteria , in fact coccid form is dead cell residuals (KUSTERS *et al.*, 1997).

In our stuy, the results indicate that residual chlorine and turbidity in most of sampling points of water distribution system and river water samples were more than standards values. Also parameters such as pH, temperature were more than *H. pylori* survival needs. These conditions may destroy *H. pylori* DNA in water samples.

High concentration of residual chlorine and high pH of the collected samples can destroy *H. pylori* DNA. Also several studies shown *H. pylori* can survive about 20 – 25 days in 4 Celsius degrees and 10 -15 days in 15 Celsius degrees and 1 -2 days in 22- 37 Celsius degrees (Watson *et al.*, 2004). Then high temperature can destroy the bacteria because our investigation was in summer. Temperature of this study samples were 27 – 33 Celsius degrees.

H. pylori can survive in acidic environment, optimum pH is about 5.8 – 6.9 (Watson *et al.*, 2004, Xia and Talley, 1997). In this study pH of water samples was 7.8 – 8.2, this pH can affect *H. pylori* survival.

Due to low concentration of nutrients for *H. pylori* in water distribution system, therefore *H. pylori* could not survive (Watson *et al.*, 2004).

H. pylori survival in water sources depends on water source conditions such as pipe type of distribution system and presence of other microorganisms. It is known that recovery percentage of cells from iron pipes is more than glass pipes and natural organism's presence is more, because these organisms are a coat or a cover for *H. pylori* (Queralt *et al.*, 2005). Also *H. pylori* survival in water examined in absence of other organisms, results showed natural organisms have positive role in covering *H. pylori* in water (Queralt and Araujo, 2007).

Water turbidity of natural and environmental samples contains several inhibitors which affect PCR method for identification of *H. pylori*. PCR cannot discriminate between live and dead cells and shows their presence only. It is understood by Taqman real-time PCR that *H. pylori* in water sources can only survive in water sources without growth (Queralt *et al.*, 2005).

This study is the first research in Iran for identification of *H. pylori* in raw and treated water samples by PCR. It is necessary to continue similar researches by using other methods for isolation and identification of *H. pylori* in water resources.

CONCLUSION

Routine and simple Polymerase Chain Reaction (PCR) method is not suitable to identify *H. pylori* bacteria in raw and treated water samples in summer (high temperature), non acidic pH, high

turbidity and high residual chlorine.

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