High frequency of *Helicobacter pylori* DNA in drinking water in Kermanshah, Iran, during June–November 2012

Alvandi Amirhooshang, Abiri Ramin, Aryan Ehsan, Rezaei Mansour and Bagherabadi Shahram

ABSTRACT

To gain a better understanding of transmission and selecting appropriate measures for preventing the spread of *Helicobacter pylori*, the aim of this study was to investigate the prevalence of *H. pylori* in drinking water samples in Kermanshah, Iran. Drinking water samples were collected from around Kermanshah and filtered through 0.45 μm nitrocellulose filters. The bacterial sediment was subjected to DNA extraction and polymerase chain reaction (PCR) for *H. pylori* detection using newly designed primers targeted at the conserved region of the *ureC* gene. The overall detection rates for *H. pylori* DNA in the water samples were 56% (66/118) with a frequency of 36% (25/70) in tap water samples and 85% (41/48) in wells. The detection limit was 50 bacteria per liter of filtered water and a pure *H. pylori* DNA copy number of 6 per reaction. Based on the evidence we may suggest that recontamination occurred and *H. pylori* entered into the water piping system through cracked or broken pipes or was released from established *H. pylori* biofilms on pipes. In conclusion, a high prevalence of *H. pylori* was detected in drinking water samples that strengthens the evidence of *H. pylori* transmission through drinking water.

Key words | drinking water, *Helicobacter pylori*, PCR, *ureC* gene

Alvandi Amirhooshang (corresponding author)
Abiri Ramin
Bagherabadi Shahram
Microbiology Department,
Faculty of Medicine,
Kermanshah University of Medical Sciences,
Shirudi blvd., Parastar blvd,
Postal code: 6714869914, Kermanshah,
Iran
E-mail: *ah_alvandi@kums.ac.ir*

Aryan Ehsan
Antimicrobial Resistance Research Center and Department of Medical Microbiology,
Faculty of Medicine,
Mashhad University of Medical Sciences,
Mashhad,
Iran

Rezaei Mansour
Department of Biostatistics,
Social Development and Health Promotion Research Center Kermanshah University of Medical Sciences, Kermanshah,
Iran

TERMS AND ABBREVIATIONS

PCR	Polymerase chain reaction
Taq	*Thermus aquaticus*
dNTP	Deoxyribonucleotide triphosphates
H. pylori	*Helicobacter pylori*
WHO	World Health Organization

INTRODUCTION

Helicobacter pylori is a motile, microaerophilic and curved Gram-negative bacteria (Percival & Thomas 2009), that was cultured in 1984 for the first time (Marshall & Warren 1984). The bacteria is the etiologic agent of gastritis and peptic ulcer disease, and is considered a risk factor for mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma. Humans are a major reservoir of the bacteria and colonization remains for life unless the person is treated (Percival & Thomas 2009). *H. pylori* is transmitted from person to person by fecal–oral, oral–oral, and/or stomach–oral routes (Bode *et al.* 1993; Madinier *et al.* 1997; Parsonnet *et al.* 1999). In the developing world 70–90% of the population are colonized before the age of ten, but in developed countries the percentage falls to 20–40% (Klein *et al.* 1991; Akcan *et al.* 2000; Engstrand 2001; Benson *et al.* 2004). It has been suggested that exposure to common resources is the leading pattern of *H. pylori* infection in developing countries (Klein *et al.* 1991; Engstrand 2001). Epidemiologic and environmental studies indicate that water can play this role in *H. pylori* transmission (Hegarty *et al.* 1999; Mazari-Hiriart *et al.* 2001; Park *et al.* 2001; Brown *et al.* 2002; Karita *et al.* 2003; Azevedo *et al.* 2004). *H. pylori* can survive more than 96 hours in water (rivers, wells, etc.) during which time its pathogenicity remains (Azevedo *et al.* 2008). Survival time increases at low

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temperature, pH 7–8.5 and in milk (Shahamat *et al.* 1993; Fan *et al.* 1998; Jiang & Doyle 1998). Survival also may depend on the presence of other organisms such as free-living amoeba and plankton, and biofilm formation (Mackay *et al.* 1998; Stark *et al.* 1999; Azevedo *et al.* 2003; Cellini *et al.* 2004). *H. pylori* is known to enter a viable but non-culturable coccoid phase in the environment (Oliver 2000). The coccoid form is metabolically active (Gribbon & Barer 1995; Cellini *et al.* 1998; Mizoguchi *et al.* 1999), and is able to colonize the stomach of mice and induce gastritis (Perez-Perez *et al.* 2004). Therefore, this form can play a major role in the transmission of bacteria from water. However, although the consequences of infection with *H. pylori* have been demonstrated, there are no hygiene measure recommendations to prevent transmission. This is because the mechanism of transmission is not well defined (Parsonnet *et al.* 1999; Brown *et al.* 2002). In order to control *H. pylori* transmission, we need a better understanding of the risk factors and the mechanism of transmission (Engstrand 2001).

Few studies have reported the *in vitro* culture of *H. pylori* from water and sewage samples (Brown *et al.* 2002; Cellini *et al.* 2004; Al-Sulami *et al.* 2010; Moreno & Ferrus 2012; Bahrami *et al.* 2013). Thus, molecular methods for the detection of *H. pylori* have been introduced. Several studies have been carried out to detect *H. pylori* in different water systems around the world (Brown *et al.* 2002; Watson *et al.* 2004; Kusters *et al.* 2006; Minami *et al.* 2006; Janzon *et al.* 2009; Khan *et al.* 2012). In order to gain a better understanding of the environmental epidemiology of *H. pylori*, the aim of this study was to investigate the prevalence of *H. pylori* in drinking water samples in Kermanshah, Iran.

MATERIALS AND METHODS

Sample collection and processing

Municipal and well water samples were collected during June to November 2012. Kermanshah, the capital city of the province, is located in the middle of the western part of Iran. The population of the city is more than one million. The city is mountainous and has a mild climate (Climatology & Geography of Kermanshah 2012). Public water is supplied from 133 wells and Gavshan dam that collects water from 21 reservoirs (Kermanshah Water & Sewage Company 2012). In total, 118 samples including 70 of tap water and 48 of well water were collected from all reservoirs and throughout the city. For each sample, one liter of water (tap and well) was collected in sterile bottles. Physicochemical parameters such as free chlorine, pH, and water temperature were also measured and recorded at the time of sampling. The collected water samples were transported to the laboratory at 4 °C. Water samples were filtered through 0.45 μm nitrocellulose filters (Millipore, Germany). Filters were aseptically removed and transferred to a sterile plate and immersed in 5 mL of sterile deionized water. The suspension was centrifuged and the sediment was used for DNA extraction. DNA was extracted by QIA Amp Mini DNA kit (Qiagen, Germany) according to the manufacturer's instructions.

Primer design and PCR

Several polymerase chain reaction (PCR) technology-based assays have been developed to detect the presence of *H. pylori* DNA in clinical and environmental specimens. Multiple targets have been successfully used for this purpose, including *ureA*, *ureB*, *glmM* (*ureC*), *cagA*, *vacA*, 16S rRNA, and *rpoB* (Bamford *et al.* 1998; Lu *et al.* 1999; Baker & Hegarty 2001; Lim *et al.* 2003; Benson *et al.* 2004; Brooks *et al.* 2004; Liu *et al.* 2008; Bahrami *et al.* 2013). The *glmM* gene appears to be the most promising target and is frequently used for various reasons. This gene is a 'housekeeping' gene and is essential for *H. pylori* growth and survival (Mengin-Lecreulx & van Heijenoort 1996). In addition, *glmM* sequences are relatively well conserved (Stone *et al.* 1997; Burucoa *et al.* 1999; Brooks *et al.* 2004; Raymond *et al.* 2004) and are present in all *H. pylori* isolates (Bamford *et al.* 1998; Brooks *et al.* 2004). Finally, the *glmM* gene has a high degree of sensitivity and specificity (Lu *et al.* 1999; Ho & Windsor 2000; Brooks *et al.* 2004).

To design species-specific primers for detecting *H. pylori*, a multiple alignment of a *ureC* gene sequences database was performed using ClustalW2 software. Specific primers were designed using the conserved region of the *ureC* (*glmM*) gene which was obtained by alignment on the basis of 26695 strain (Accession No. AE000511) as a

template. Sequence and properties of the forward and reverse primers were Hp-ureCF: CAT CGC CAT CAA AAG CAA AG (605–625 positions in 26695 *H. pylori ureC* gene) and Hp-ureCR: CAG AGT TTA AGG ATC GTG TTA G (798–819 positions in 26695 *H. pylori ureC* gene). The primers have 100% identity with most reference strains of *H. pylori* in BLAST searching.

Optimal PCR reaction was done in a final volume of 15 μL containing 20 mM Tris-HCl, 50 mM KCl, 200 μM dNTPmix, 1.5 mM $MgCl_2$, 0.5 μM of each forward and reverse primers, 1 unit *Taq* DNA polymerase, and 5 μL of template DNA. Initial heat denaturation of the target DNA was carried out at 95 °C for 5 min and a 214 base pair target sequence was amplified in the reaction mix through 35 cycles as follows: 92 °C for 30 s, 55 °C for 40 s, and 72 °C for 40 s, followed by 72 °C for 5 min. The PCR products were subjected to electrophoresis through 1.5% agarose gels and stained with ethidium bromide.

Determination of analytical detection limit and sensitivity for PCR assay

In order to determine the sensitivity of the method, the PCR detection limit (detectability) of the newly designed primer was determined using ten-fold serial diluted DNA of *H. pylori* with known concentration. In brief, the concentration of pure extracted DNA of *H. pylori* was measured three times using a NanoDrop device (Thermo Scientific, USA) and the average value was considered as the real concentration. Eventually, a series of ten-fold serial dilutions (100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag) were prepared and PCR was performed on all these dilutions. Ultimately, the last dilution of the *H. pylori* DNA for which the PCR yielded a detectable band on the agarose gel was assigned as the test limit of detection.

For the purpose of calculating the copy number of *H. pylori*, in the last concentration which produced a visible band, the average genome size of this DNA was estimated to be 1.67×10^6 base pairs. In fact, 1.67 fg of DNA is equivalent to a single genome of *H. pylori*. In addition, *H. pylori* cell suspension in phosphate buffered saline (PBS) was prepared from the 3-day-old culture. The bacterial cell number was quantified by Petroff-Hausser Counter. Cell counting was carried out three times and the mean of these was considered as the real number of *H. pylori* in suspension. The *H. pylori* suspension was used to make ten-fold serial dilutions in PBS. The appropriate number of *H. pylori* cells were spiked in 1 liter of filtered and autoclaved tap water in the final concentrations of approximately 100,000, 10,000, 1,000, 100, 50, 10 and 1 cell/L. *H. pylori*-spiked samples were subjected to filtration, DNA extraction and PCR as mentioned above. The last concentration of the *H. pylori* cell with a visible band on the agarose gel was assigned as the analytical sensitivity.

Determination of specificity for PCR assay

The specificity of the primers was first tested by their ability to correctly amplify the gene of interest and then demonstrated by testing the PCR assay on at least 100 ng of genomic DNA isolated from a battery of bacteria other than *H. pylori* which may be present in water, including *Citrobacter*, *Enterobacter*, *Escherichia coli*, *Klebsiella*, *Enterococcus*, *Yersinia*, *Salmonella*, *Shigella*, *Pseudomonas*, and *Campylobacter*. The identification of all strains used in this study were confirmed using biochemical tests. PCR amplification was considered negative if no amplicon was detected. In addition, the primers were subjected to *in silico* analysis against *H. pylori* related bacteria using BLAST.

Statistics analysis

Calculations were performed using the SPSS version 16. Kolmogorov–Smirnov *Z* test was performed to assess normality of data. An independent *t*-test was used to analyze the normal quantitative data, and a Mann–Whitney *U*-test was used in the case of the abnormal quantitative data. A chi-squared test was performed to obtain correlations between kinds of sample and the result of *H. pylori* detection by PCR test. A *p*-value of the significance probability less than or equal to (≤) 0.001 was considered statistically significant.

RESULTS

Sample collection and processing

In total, 118 water samples including 70 tap and 48 well water samples were collected from all the reservoirs and

throughout the city during June to November 2012. Mean values ± SD of physicochemical parameters in both taps and wells are shown in Table 1. The associations between the presence of *H. pylori* and physicochemical parameters were evaluated using a chi square test.

Measurement of free chlorine concentration in the water samples showed that all samples were under optimum concentration of chlorine (Institute of Standards & Industrial Research of Iran 2008; World Health Organization 2011).

Primer design and PCR

PCR with species-specific primers designed on the basis of the conserved region of the *ureC* gene were used for the potential presence of *H. pylori* in water samples. The overall detection rate for *H. pylori* DNA in the water samples was 56% (66/118) with a prevalence of 36% (25/70) in tap and 85% (41/48) in well water samples. Figure 1 shows PCR product gel electrophoresis of some water samples.

PCR analysis with specific designed primers amplified an approximately 214 base pair DNA fragment of *ureC*. Two PCR products, directly amplified from water samples three and four were analyzed by direct DNA sequencing and registered in the GenBank nucleotide database as accession numbers KC833750 and KC865593, respectively.

Determination of analytical detection limit and sensitivity for PCR assay

The limit of detection of the PCR primers for the detection of *H. pylori* was evaluated by PCR amplification of ten-fold serial dilutions of pure extracted *H. pylori* DNA.

Table 1 | Association of *H. pylori* detection by PCR and physicochemical parameters in both taps and wells

Parameter	PCR	No.	Mean ± SD
Temperature (°C)	Positive	66	16.7070 ± 1.85385
	Negative	52	18.8212 ± 2.56227
pH	Positive	66	7.5762 ± 0.27547
	Negative	52	7.7785 ± 0.15574
FCL (mg/L)	Positive	66	0.0120 ± 0.02696
	Negative	52	0.1137 ± 0.31883

FCL, free chlorine; SD, standard deviation.

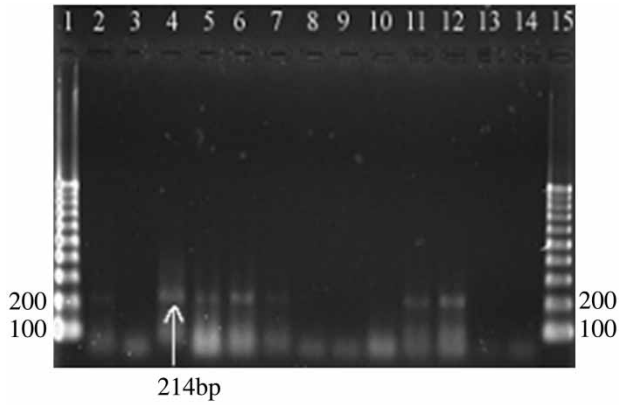

Figure 1 | Agarose gel electrophoresis of PCR products amplified from tap water and wells. Lane 1, 100 bp DNA ladder; lane 2, 50 cell/liter spiked water as positive control; lane 3, negative control, positive control of pure DNA extracted from *H. pylori* colonies; lanes 5–7, tap water sample positive for *H. pylori*, lanes 8–10, tap water negative for *H. pylori*; lanes 11 and 12, wells positive for *H. pylori*; lanes 13 and 14, wells negative for *H. pylori*; lane 15, 100 bp DNA ladder.

The minimum concentration of pure extracted *H. pylori* DNA which yielded a detectable band on the agarose gel after PCR amplification was 10 fg per reaction (Figure 2). Since the average genome size of *H. pylori* DNA is estimated to be 1.67×10^6 base pair, 1.67 fg of DNA is equivalent to a single genome of *H. pylori*. Thus, the limit of detection in the PCR is approximately equivalent to a *H. pylori* copy number of 6 per reaction.

To determine the minimum number of *H. pylori* cells that could be amplified by our PCR primers in water samples, the sensitivity of the assay was evaluated by spiking

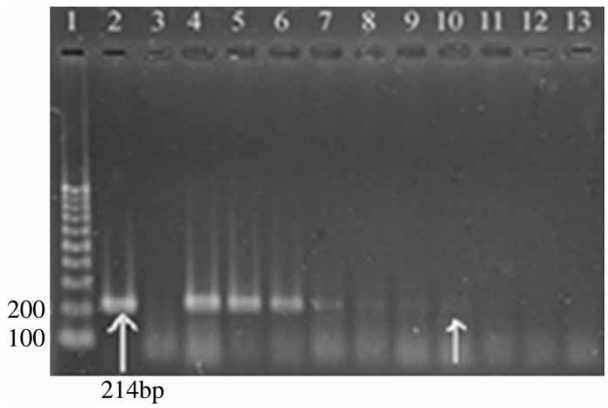

Figure 2 | Agarose gel electrophoresis of PCR products amplified from ten-fold serial dilutions of *H. pylori* pure DNA. Lane 1, 100 bp DNA ladder; lane 2, positive control; lane 3, negative control; lanes 4–13, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, and 100 ag, respectively. Right arrow indicates limit of detection with visible 214 bp PCR product in 10 fg.

one liter of filtered and autoclaved tap water in the final concentrations of approximately 100,000, 10,000, 1,000, 100, 50, 10 and 1 cell/L of *H. pylori* clinical isolate. As shown in Figure 3, the minimum number of *H. pylori* cells that were required to give a visible PCR product on the gel electrophoresis was 50 cells per liter of water.

Determination of specificity for PCR assay

The specificities of the used primers were checked with pure *H. pylori* DNA and a number of bacteria other than *H. pylori* that may be present in environmental and clinical samples. A positive reaction was obtained only with *H. pylori* DNA and there was no detectable band if non-*H. pylori* DNA was subjected to amplification. Furthermore, the primer pairs are unique to the *H. pylori* species and have 100% identity with most reference strains of *H. pylori* in BLAST searching. It should be emphasized that *in silico* analysis showed no significant pairing between the primers and sequences of *H. pylori*-related bacteria.

DISCUSSION

The International Agency for Research on Cancer classified *H. pylori* as a group 1 definite carcinogen. It has been suggested that 50–70% of the world's population is colonized by this bacteria. Several studies have shown the presence of *H. pylori* in the aquatic environment (Adams *et al.* 2003; Cellini *et al.* 2004, 2008; Braganra *et al.* 2007), and it has been suggested that water plays an important role in the spread of the infection in developing countries (Percival & Thomas 2009). Evaluation of chemical and microbial contaminants is considered very important in drinking-water safety but *H. pylori* is not considered in routine microbiological analysis of drinking water. To evaluate the safety of source water, the microbiological analysis of drinking water usually relies on coliform as the bacterial indicator of fecal contamination. It must be noted that *H. pylori* is included in the contaminant candidate list that reflects its potential for transmission by drinking water (Nayak & Rose 2007).

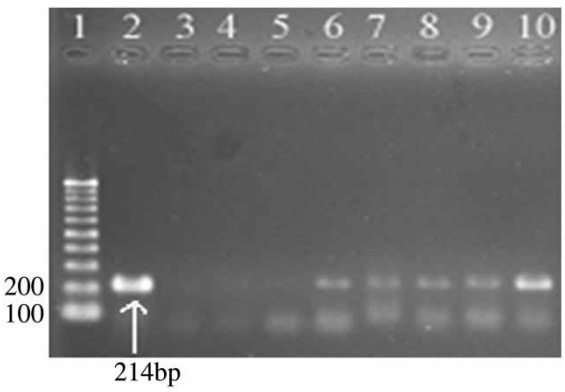

Figure 3 | Agarose gel electrophoresis of PCR products amplified from filtered and autoclaved tap waters spiked by different concentrations of *H. pylori* clinical isolate, approximately 100,000 to one cell/L. Lane 1, 100 bp DNA ladder; lane 2, positive control; lane 3, negative control; lanes 4–10, one cell/L, 10 cell/L, 50 cell/L (minimum concentration with visible 214 bp PCR product), 100, 1,000, 10,000 and 100,000 cell/L. respectively.

Few studies have reported successful culture of *H. pylori* from water and sewage samples (Brown *et al.* 2002; Cellini *et al.* 2004; Al-Sulami *et al.* 2010; Moreno & Ferrus 2012; Bahrami *et al.* 2013). Thus, application of molecular methods for the rapid, sensitive, and specific detection of *H. pylori* in aquatic environments is of the utmost importance. There are several reasons why the presence of *H. pylori* in drinking water should be considered more important than it has been, which include the direct relationship between the consumption of contaminated water and a higher prevalence of *H. pylori* infection. Several studies show consumption from a contaminated water source is one of the risk factors for acquisition of *H. pylori* infection. The prevalence of infection in communities that use domestic wells or lack clean water is higher, while the prevalence of infection is clearly lower in areas where there is access to safe drinking water (Klein *et al.* 1991; Johnson *et al.* 1997; Olmos *et al.* 2000; Brown *et al.* 2002; Nurgalieva *et al.* 2002; Ueda *et al.* 2003; Camargo *et al.* 2004; Krumbiegel *et al.* 2004; Perez-Perez *et al.* 2004; Kusters *et al.* 2006; Samra *et al.* 2011). It should be emphasized that there are some other reasons, including culture of *H. pylori* from feces of infected individuals, viability in water, active respiration of *H. pylori* in surface and groundwater, and ineffectiveness of treatment methods in eliminating this bacteria (Handworker *et al.* 1995; Hulten *et al.* 1996, 1998; Fan *et al.* 1998; Hegarty *et al.* 1999; Mazari-Hiriart *et al.* 2001).

The presence of *H. pylori* DNA in drinking water systems has been reported in the USA, UK, Germany, Japan,

Sweden, Mexico, Pakistan, Iraq, Gambia, and Peru (Klein *et al.* 1991; Hulten *et al.* 1996, 1998; Horiuchi *et al.* 2001; Bunn *et al.* 2002; Benson *et al.* 2004; Krumbiegel *et al.* 2004; Watson *et al.* 2004). In this study, in order to have a better understanding of *H. pylori* epidemiology in Kermanshah, a molecular method based on PCR using primers designed from conserved regions of the urease C gene of *H. pylori* was conducted, and the prevalence of *H. pylori* in tap and well water samples from around the city of Kermanshah was investigated.

The analytical sensitivity of *ureC* based on PCR using the primers was 10 fg of purified *H. pylori* DNA per reaction. Since the weight of the *H. pylori* genome is approximately equivalent to 1.67 fg, the amount of DNA is roughly equivalent to 6 *H. pylori* copy numbers.

The detection limit obtained in this study was much less than in the few previous studies (Enroth & Engstrand 1995; Hegarty *et al.* 1999; Degnan *et al.* 2003; Benson *et al.* 2004; Cellini *et al.* 2004; Queralt *et al.* 2005; Nayak & Rose *et al.* 2007; Janzon *et al.* 2009; Al-Sulami *et al.* 2010; Linke *et al.* 2010; Massoudian *et al.* 2012). Yanez *et al.* (2009) reported the reliable detection and quantification limits of 46.7 and 607 genomic copies, respectively. This means that our method was at least seven times more sensitive than the method by Yanez *et al.* (2009). Nayak *et al.* reported a limit of detection as few as two bacteria per reaction of the plasmid containing the *vacA* gene (Nayak & Rose 2007). However, in their study the reaction was carried out using the *vacA* gene as a target which may be absent in some strains of *H. pylori*. This means that in order to detect *H. pylori* in water samples and other clinical samples this method is more sensitive than the methods used in other studies. Because of the presence of very low numbers of *H. pylori* cells in water, a large amount of water should be filtered or a highly sensitive method should be applied in the detection of *H. pylori* (Al-Sulami *et al.* 2010). With regard to the methods used in other studies which do not have sufficient sensitivity for the detection of low numbers of *H. pylori*, it seems that the prevalence of *H. pylori* in water samples was underestimated. For example, in a study conducted in a high prevalence area using a method with a sensitivity of 250 bacteria per sample, all investigated water samples were negative (Janzon *et al.* 2009). Contamination of domestic water wells in this study was 85.4% (41/48), which was much higher than in other studies. *H. pylori* has been detected in surface and groundwater in areas with a source of fecal contamination all around the world (Handworker *et al.* 1995; Hulten *et al.* 1996, 1998; Hegarty *et al.* 1999; Sasaki 1999; Baker & Hegarty 2001; Horiuchi *et al.* 2001; Mazari-Hiriart *et al.* 2001; Karita *et al.* 2003). The highest prevalence of *H. pylori* in water samples from wells or surface water was less than 65% in all mentioned studies (Hegarty *et al.* 1999). Water contamination with *H. pylori* in Kermanshah may be due to the unsanitary disposal of sewage or because wells are in the vicinity of domestic wastewater disposal wells.

H. pylori contamination of tap water samples was 36% (25/70) which was much higher in comparison with studies on tap water samples from Karachi, Pakistan 4% (Khan *et al.* 2012), Basra, Iraq 2% (Al-Sulami *et al.* 2010), Isfahan, Iran 10% (Bahrami *et al.* 2013), and was lower in comparison with Gambia 80% (Thomas *et al.* 1992), Kazakhstan 71% (Nurgalieva *et al.* 2002; Samra *et al.* 2011). Since the *H. pylori* contamination was not detected in main water sources (data not shown), recontamination occurred and *H. pylori* entered into the water piping system through cracked or broken pipes or was released from established *H. pylori* biofilms on pipes. It must be considered that biofilms may play an important role in the transmission of *H. pylori* (Park *et al.* 2001; Bunn *et al.* 2002). The DNA that has been detected in water samples could be from dead *H. pylori* cells or from coccoid forms. However, a comprehensive study on the prevalence of *H. pylori* has not been done in this area, although Kermanshah is located in an area with a high prevalence of *H. pylori* contamination (Malekzadeh *et al.* 2009). The rate of *H. pylori* in patients referred to the endoscopic ward in Kermanshah is more than 70% using PCR (Abiri *et al.* unpublished data). The authors suggest a high prevalence of *H. pylori* infection in this area may be related to water contamination.

Meanwhile, as mentioned in Table 1, the mean of free chlorine was much lower than the World Health Organization standards (WHO 2011). Based on the findings, there is a statistically significant association between the PCR positive samples and free chlorine concentration, and temperature ($p < 0.001$), *H. pylori* positive water samples showing significantly lower free chlorine concentration and lower temperature.

CONCLUSION

In conclusion, a high prevalence of *H. pylori* was detected in drinking water samples that were collected from Kermanshah. Our study provides evidence to support a possible waterborne route of *H. pylori* transmission through drinking water in Kermanshah. Additional studies are needed to thoroughly detect the viability and number of *H. pylori* cells. It would also be of value to attempt culturing *H. pylori* in these samples.

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