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# Determination of risk factors and transmission pathways of *Helicobacter pylori* in asymptomatic subjects in Western India using polymerase chain reaction

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## ABSTRACT

**Objective:** To determine the prevalence of *Helicobacter pylori* (*H. pylori*) in the salivary samples of asymptomatic subjects, the possible route of transmission and role of hygiene in dissemination of *H. pylori* using polymerase chain reaction. **Methods:** Salivary samples of 1500 asymptomatic subjects were involved to determine the prevalence of *H. pylori*. DNA was extracted from the samples using phenol chloroform, cetyltrimethyl ammonium bromide method and the DNA template was used to amplify *H. pylori* specific genes, 16s rRNA and HSP 60 using *H. pylori* specific primers. Clean water index (CWI), crowding index and information regarding hygiene were recorded using suitable questionnaires in local language. **Results:** The prevalence of infection in male and female subjects was found to be equal to 75.96% and 88.10% respectively. The prevalence in age groups of (20–29), (30–39), (40–49), (50–59) and (60–69) was found to be equal to 80.76%, 81.47%, 74.50%, 86.58 and 80.95% respectively. The prevalence of infection in the subjects using processed and unprocessed water for drinking was found to be equal to 30% and 89.5% respectively. The prevalence of infection in the subjects who belong to low, medium or high CWI status was found to be equal to 26.20%, 65.62% and 86.13% respectively. The prevalence of infection in the subjects who belong to high, medium and low crowding index status was found to be equal to 88.83%, 82.48% and 69.63% respectively. The prevalence of *H. pylori* was significant in the subjects using unprocessed water, having outdoor sanitation practices, belonging to low CWI and high crowding index ( $P < 0.0001$ ). **Conclusions:** *H. pylori* transmission is associated with consumption of unprocessed water, low CWI, outdoor sanitation practices and high crowding index.

## 1. Introduction

*Helicobacter pylori* (*H. pylori*) has been christened as class 1 carcinogen by the World Health Organization and has acquired the shape of an epidemic emerging as principle cause of gastric carcinoma<sup>[1,2]</sup>. *H. pylori* infection is rising at an alarming rate in the developing tropical countries like India<sup>[3]</sup>. In the recent past prevalence of *H. pylori* in the salivary samples of symptomatic and asymptomatic subjects have been investigated by various authors in India<sup>[4–6]</sup>. However, prevalence studies to evaluate the association of risk factors and route of transmission and prevalence of *H.*

*pylori* have not been undertaken among Western Indian population. Hence, the objective of the present investigation was to determine the relation between of processed and unprocessed water, clean water index (CWI), crowding index, sanitation practices and correlate them with presence of *H. pylori* in the salivary samples of asymptomatic subjects in a representative Western Indian population.

## 2. Materials and methods

### 2.1. Chemical preparation

All the chemicals for DNA extraction were procured from S. D. Fine Chemicals, India. The reagents for polymerase chain reaction (PCR), gel preparation, and visualization were purchased from Biolinx India. The forward and reverse

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primer for 16 S rRNA and HSP 60 genes were synthesized at SciFi Biologicals, Pune India. Gel electrophoresis unit (Bangalore Genie, Bangalore) was used to perform gel electrophoresis and gel documentation unit (Alpha Innotech Inc. USA) was used to visualize and capture the gel image.

## 2.2. Sample collection

A total of 1500 asymptomatic healthy subjects devoid of clinical symptoms of acid peptic disease were included in the present investigation. The study was carried out during the months of January to March 2011. All individuals signed an informed consent in order to be included in the study. The study population consisted of men and women of more than 18 years of age. None of the participants had symptoms suggestive of acid peptic diseases. A questionnaire in local language (Marathi) or English was filled by each individual to ascertain the absence of symptoms of acid peptic disease. The medication history of each subject was recorded and it was confirmed that they did not consume proton pump inhibitors, H<sub>2</sub> blockers and antibiotics before one month of saliva sampling. Unstimulated saliva samples were collected by visiting homes, colleges and villages located at various parts in Pune district. Unstimulated saliva in the volume of 1.5 mL was collected in sterile container and stored at -80 °C until processed. Approximately 1.5 mL of non-stimulated salivary flow was collected in a 2-mL microcentrifuge tube. After collection, saliva was homogenized by vigorous shaking with the use of a vortex mixer and clarified by centrifugation (10000 g, 4 °C, 4 min).

## 2.3. Human ethics approval

The study protocol was approved by Institutional Scientific and Institutional human ethics committee Bharati Vidyapeeth Deemed University, Pune.

## 2.4. Collection of data

A questionnaire in local language was prepared for data collection which included gender, water source, information for calculation of CWI and crowding index. CWI and crowding index were calculated using previously reported method<sup>[7,8]</sup>. The details about processing of water in the household of each subject were determined using a suitable questionnaire regarding the practice of purification of potable water used for drinking in their household. Subjects were considered to consume processed water if the tap/well/river water was filtered through a house hold water purifier/automated purification system, or boiled or chlorinated whereas subjects who consumed raw/unfiltered/unboiled water were categorised as consuming unprocessed water.

## 2.5. Study groups

The entire study population was divided into following sub groups: age (20-29), (30-39), (40-49), (50-59), (60-69); gender (male) and (female); nature of drinking water (processed

water), (unprocessed water); sanitation practices (indoor), (outdoor); CWI (low, medium, high); crowding index (low, medium, high). The data regarding these discrete parameters was analyzed to determine the percentage of subjects infected in each sub group.

## 2.6. Preparation of genomic DNA for PCR

DNA isolation from salivary samples was performed according to phenol chloroform CTAB method<sup>[6]</sup>. All the steps were performed in aseptic conditions to minimize contamination. DNA was preserved at -20 °C until amplification was performed.

## 2.7. PCR amplification

### 2.7.1. Sensitivity assay

The detection limits of the PCR assay was determined by preparation of 10-fold serial dilution, from 50 nanogram to 1 femtogram of the isolated genomic DNA from *H. pylori* strain ATCC 26695 in sterile water for injection. An aliquot of each dilution was amplified by PCR, and the amplicons were visualized on 1.5% agarose gel stained with ethidium bromide. Sensitivity of this PCR assay was ascertained based on the maximum dilution of genomic DNA in which the primers were able to amplify their specific gene sequences.

### 2.7.2. Specificity assay

DNA isolated from an entirely sequenced *H. pylori* reference strain DNA (ATCC 26695) was used as a positive control. The specificities of the PCR method was evaluated for three different bacteria obtained from National Centre for Industrial Microbes (NCIM): *Staphylococcus aureus* (*S. aureus*) NCIM 2079, *Escherichia coli* (*E. coli*) NCIM 2345 and *Bacillus subtilis* (*B. subtilis*) NCIM 2063.

### 2.7.3. Amplification of genes of *H. pylori*

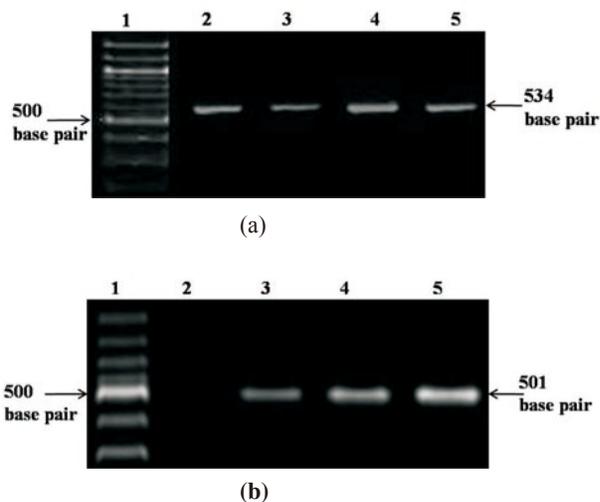
DNA isolated from the salivary sample of each individual was subjected to PCR thermal cycles using specific *H. pylori* primers to amplify 16s rRNA gene to yield an amplicon of 534 bp. The primer sequence for amplification of 16s rRNA gene was as follows: 16s rRNA F-5'-TAAGAGATCAGCCTATGTCC-3' and 16s rRNA R-5'-TCCCACGCTTTAAGCGCAAT-3'<sup>[6]</sup>. Another set of primers was used to amplify HSP 60 genes to yield an amplicon of 501 bp. The primer sequence for amplification of HSP 60 gene was as follows: HSP1-5'-AGGCATGCAATTTGATAGAGGCT-3' and HSP2-5'-CTTTTTTCTCTTTCATTTCCACTT-3', HSPN1-5'-TTGATAGAGGCTACCTCTCC-3' and HSPN2-5'-TGTCAT AATCGC TTGT CGTGC-3'<sup>[4]</sup>. At each amplification step, *H. pylori* DNA isolated from strain ATCC 26695 was used as a positive control, while sterile water for injection instead of *H. pylori* DNA served as a negative control. The PCR products were analyzed by agarose gel electrophoresis unit (Bangalore Genie, India) and the gel image was captured using gel documentation (Alpha Innotech Inc. USA).

## 2.8. Statistical methods

The statistical analysis was carried out to examine the association between the various study variables with saliva PCR positivity for *H. pylori* using Fischer exact test among various study sub groups. Statistical analysis was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA), odds ratio, 95% confidence interval (CI) of odds ratio, relative risk (RR), 95% CI of RR were determined.

## 3. Results

The primer pairs were successful in amplification of the DNA which was equal to 50 nanogram of template DNA on initial amplification step and 10 femtogram following the second. The nested amplification was negative with all the non-*H. pylori* bacteria used in the present study, i.e. *S. aureus*, *E. coli* and *B. subtilis*. 16s rRNA and HSP 60 were amplified in each *H. pylori* positive patient in two discrete polymerase chain reactions using the same DNA template isolated from a particular individual. The gel images captured by gel documentation are shown in Figure 1.



**Figure 1.** Successful amplification of *H. pylori* specific genes. a) 16s rRNA gene (534 base pair); b) HSP 60 gene (501 base pair).

Table 1 indicate the number of *H. pylori* infected individuals from each sub group of subjects. Prevalence of infection in male and female subjects was found to be equal to (75.96%) and (88.10%) respectively ( $P < 0.0001$ ). The prevalence in age groups of (20–29), (30–39), (40–49), (50–59) and (60–69) was found to be equal to 80.76%, 81.47%, 74.5%, 86.58% and 80.95% respectively. The  $P$  value was found to be equal to be equal to 0.0498, 0.0522, 0.0297 and 0.4391 in the age groups (20–29), (30–39), (50–59) and (60–69) when the subjects in the age group (40–49) were considered as referent. The prevalence of infection in the subjects using processed and unprocessed water for drinking was found to be equal to 30% and 89.5% respectively. It was found that *H. pylori* infection had a strong association with consumption

of unprocessed water ( $P < 0.0001$ ). The prevalence in the subjects using outdoor sanitation practices was 81.12% whereas in the subjects using indoor sanitation practices was 42.34% respectively. The prevalence in the subjects using outdoor sanitation practices was significantly ( $P < 0.0001$ ) higher when subjects using indoor sanitation practices were considered as referent. The prevalence of infection in the subjects who belong to low, medium or high CWI status was found to be equal to 26.20%, 65.62% and 86.13% respectively. The prevalence of *H. pylori* was significant in the subjects belonging to lower and middle CWI status ( $P < 0.0001$ ) when the subjects in the high CWI were considered as referent. The prevalence of infection in the subjects who belong to or high medium and low crowding index status was found to be equal to 88.83%, 82.48% and 69.63 % respectively. The prevalence of *H. pylori* was significant in the subjects belonging to lower and middle CWI status ( $P < 0.0001$ ) when subjects in the low crowding index were considered as referent.

**Table 1**

Table showing the *H. pylori* infection status (16s rRNA and HSP 60 gene positive) in various sub groups of asymptomatic subjects.

| Parameters               |                   | Total Subjects | <i>H. pylori</i> + ve | <i>H. pylori</i> - ve |
|--------------------------|-------------------|----------------|-----------------------|-----------------------|
| Age                      | 20–29             | 754            | 609                   | 145                   |
|                          | 30–39             | 394            | 321                   | 73                    |
|                          | 40–49             | 228            | 170                   | 58                    |
|                          | 50–59             | 82             | 71                    | 11                    |
|                          | 60–69             | 42             | 34                    | 8                     |
| Gender                   | Male              | 878            | 667                   | 211                   |
|                          | Female            | 622            | 548                   | 74                    |
| Nature of drinking water | Processed water   | 957            | 287                   | 670                   |
|                          | Unprocessed water | 543            | 486                   | 57                    |
| Sanitation practices     | Indoor            | 822            | 348                   | 474                   |
|                          | Outdoor           | 678            | 550                   | 128                   |
| CWI                      | High              | 248            | 65                    | 183                   |
|                          | Middle            | 480            | 315                   | 165                   |
|                          | Lower             | 772            | 665                   | 107                   |
| Crowding index           | High              | 842            | 748                   | 94                    |
|                          | Middle            | 411            | 339                   | 12                    |
|                          | Low               | 247            | 172                   | 75                    |

The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to gender was (2.343, 1.756 to 3.124) in males when females subjects were considered as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to age was as follows: (1.433, 1.011 to 2.031) for 20–29 years group, (1.500, 1.014 to 2.220) for 30–39 years group, (2.202, 1.092 to 4.442) for 50–59 years group, (1.450, 0.6348 to 3.312) for 60–69 years group when subjects of age group (40–49) years were considered as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to consumption of processed drinking water was as follows: (19.90, 14.64 to 27.07) for unprocessed

water consumers when subjects who consumed processed water were considered as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to low, medium and high CWI status was as follows: (17.50, 12.34 to 24.81) for medium and (5.375, 3.826 to 7.552) for low when subjects belonging to low CWI status were considered as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to sanitation practices was as follows: (5.853, 4.618 to 7.418) for outdoor sanitation practices when subjects who were having indoor sanitation practices were considered as referent. The odds ratio and 95% CI of odds ratio in the population of asymptomatic subjects with respect to high, medium and low crowding index status was as follows: (3.470, 2.455 to 4.904) for high and (12.32, 6.518 to 23.28) for middle when subjects belonging to low CWI status were considered as referent.

The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to gender was (1.160, 1.106 to 1.216) in males when females subjects were considered as referent. The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to age was as follows: (1.083, 0.9965 to 1.178) for 20–29 years group, (1.093, 0.9994 to 1.195) for 30–39 years group, (1.161, 1.036 to 1.302) for 50–59 years group, (1.086, 0.9204 to 1.281) for 60–69 years group when subjects of age group (40–49) years were considered as referent. The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to consumption of processed drinking water was as follows: (2.984, 2.698 to 3.302) for unprocessed water consumers when subjects who consumed processed water were considered as referent. The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to low, medium and high CWI status was as follows: (3.287, 2.662 to 4.058) for medium and (2.504, 2.012 to 3.116) for low when subjects belonging to low CWI status were considered as referent. The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to sanitation practices was as follows: (1.916, 1.755 to 2.092) for outdoor sanitation practices when subjects who were having indoor sanitation practices were considered as referent. The relative risk and 95% CI of relative risk in the population of asymptomatic subjects with respect to high, medium and low crowding index status were as follows: (1.276, 1.171 to 1.390) for high and (1.387, 1.274 to 1.510) for middle when subjects belonging to low CWI status were considered as referent.

#### 4. Discussion

*H. pylori* has intrigued physicians since the dawn of research in gastroenterology and is a menace possessing serious threat to global public health<sup>[9]</sup>. It has been found to have a decisive role in the transformation of ulcers into gastric carcinoma<sup>[10]</sup>. 16s rRNA is the gold standard and has been used to detect the presence of *H. pylori* which serves

as a reproducible and specific biomarker for the detection of *H. pylori*<sup>[6]</sup>. PCR detection technique for the amplification of a *H. pylori* specific gene, HSP 60 has been recently developed<sup>[4]</sup> and has been reproducibly used to determine the prevalence of *H. pylori* using salivary samples<sup>[5]</sup>. Hence, in the present investigation, HSP 60 and 16s rRNA genes were targeted to detect the presence of *H. pylori* in the salivary samples of asymptomatic subjects.

A number of transmission pathways of *H. pylori* have been implicated in population which includes person-to-person, through water, and zoonotic transmission. Out of these transmission through water has been implicated to play a major role in transmission of this bacterium<sup>[7]</sup>.

It is evident from the present investigation that the people consuming unprocessed tap/well/river water were at a greater risk of *H. pylori* infection and a major proportion of people consuming unhygienic water which is not filtered tested positive for *H. pylori*. The findings of the present investigation indicate that *H. pylori* infection status is closely related to water source and nature of water being consumed by the subjects.

CWI has been used by many researchers as a quantitative scale to measure the nature of potable water used by an individual<sup>[7,8]</sup>. It has been associated with *H. pylori* infection in many countries over varied geographical regions and has been useful in assessing the environmental factors which affect the cleanliness of water and hence indirectly affect infection status. The association between water consumption and *H. pylori* infection indicates that *H. pylori* may be transmitted through a waterborne route and previous research has proven that water is the primary source of infection and a major route of transmission<sup>[7]</sup>. *H. pylori* DNA has been detected in the sample of water using various techniques<sup>[11–19]</sup>. Our results clearly demonstrate that the grade of water is closely associated with the infection status in the asymptomatic subjects. The grade of water was determined by CWI of the potable water using a validated procedure<sup>[7,8]</sup>. This study provides a proof to the dictum that impure water is closely associated with transmission and dissemination of *H. pylori*. The literature is teeming with studies carried out to observe oral–oral and fecal–oral transmission and viable *H. pylori* resides in the oral cavity<sup>[20–27]</sup>. Hence, subjects consuming bacteria infected water are at a greater risk of developing a fulminant infection.

*H. pylori* has been reported to reside in water in the biofilms in the viable but nonculturable (VBNC) form which can be detected by PCR<sup>[28,29]</sup>. In this form it is present in a dormant non fulminant non culturable form in biofilms in water. Moreover, it has been shown by various authors that *H. pylori* is capable of surviving in water for a long duration. Biofilm in water is a slimy layer which provides a suitable niche for *H. pylori*. In this form the bacteria is viable but is not detectable by culturing techniques and may be transmitted from an infected to uninfected individual. In the VBNC form *H. pylori* exists in a coccoid form in which the flagella of *H. pylori* is preserved allowing it to be motile and

capable of causing infection<sup>[30,31]</sup>. In this form it is present in drinking water and food articles as well<sup>[32–34]</sup>. Culture or any other technique is incapable to detect such forms of *H. pylori*. PCR has been reproducibly used to detect *H. pylori* in the saliva, water and food articles<sup>[4,6,29,32–34]</sup>. It has been recently, studied that *H. pylori* is present in the surface of water in the form of a VBNC form<sup>[31]</sup>. Researchers have shown that *H. pylori* can survive water microfilms in coccoid form and hence coccoid *H. pylori* in water may be suspected as a transmission mode of the bacteria. Thereafter, it may reside in the oral cavity and again migrate into the gastric milieu<sup>[31]</sup>. Poor hygienic conditions have been reported to be closely associated with the prevalence of *H. pylori*<sup>[7,8]</sup>. This observation was replicated in our findings. It is also experimentally proven by some workers that drinking water contaminated with VBNC form of *H. pylori* is a possible route of transmission of *H. pylori*. Inadequate treatment of potable drinking water may be a major factor relating *H. pylori* infection. Treatment of water with chemicals provides an effective method for the elimination of *H. pylori* from water<sup>[35–39]</sup>.

However recontamination may occur due to unhygienic drinking water contaminated with sewage water<sup>[13]</sup>. In urban and rural India, the municipal water pipes carrying drinking water are in close proximity to sewage water pipes. It is possible that leakage in both the pipelines may provide a path for transmission of *H. pylori* from sewage water to the drinking water pipes. This explains the fecal oral transmission of *H. pylori* via contaminated water. It is worth considering that the *H. pylori* is a gram negative organism requiring micro aerophilic environment to survive. This means that the water is the principle route of transmission of *H. pylori* in this region. In the present investigation, salivary samples were used to assess the *H. pylori* infection and it is evident that subjects consuming raw unprocessed water had a greater prevalence when compared to subjects who consumed processed water. Subjects who implemented a household practice of processing raw water by these methods were found to have a lower prevalence of *H. pylori* indicating that the subjects consuming unprocessed water have a prominent chance of acquiring *H. pylori* infection. Hence, our results are in accordance with a recent study<sup>[12]</sup>. Methodical processing of water by filtration, boiling and chlorination has been reported to have a diminutive effect on the viability, virulence and survival of *H. pylori* which render water pathogen free<sup>[35]</sup>. It could be proposed that proper treatment of drinking water may be the route to prevent transmission of *H. pylori* and also prevent its relapse and reinfection. Crowding index has been documented to be a hallmark of the living conditions of the subjects. It relates to the number of people residing in vicinity to each other. It also serves as a parameter to understand the transmission of *H. pylori* in the members of a family. Several investigations have also revealed transmission amongst people belonging to a particular institution or family indicating the need to decipher whether common household and residence influence the transmission of *H. pylori*, indicating discernable relationship between crowding and infection status of *H. pylori*<sup>[40–42]</sup>. Crowding index was employed in the

present study to determine the correlation between infection status and crowding environment prevalent in the household of the subjects and our findings are in accordance with previous studies carried out in other parts of India<sup>[7]</sup>.

It could be concluded from the present investigation that consumption of unprocessed (unfiltered) water, low CWI, outdoor sanitation practices and high crowding index are major factors that aggravate the infection in urban and rural population in India. These factors pose a serious threat to public health and there is an urgent need to process potable water. Moreover, this study shows that awareness and education programmes are needed to upgrade the sanitation and hygiene practices in India to halt the spread of *H. pylori*.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### References

- [1] Nguyen BV, Nguyen KG, Phung CD, Kremp O, Kalach N, Dupont C, et al. Prevalence of and factors associated with *Helicobacter pylori* infection in children in the north of Vietnam. *Am J Trop Med Hyg* 2006; **74**(4): 536–539.
- [2] Suzuki H, Iwasaki E, Hibi T. *Helicobacter pylori* and gastric cancer. *Gastric Cancer* 2009; **12**(2): 79–87.
- [3] Lehours P, Yilmaz O. Epidemiology of *Helicobacter pylori* infection. *Helicobacter* 2007; **12**(1): 1–3.
- [4] Singh V, Mishra S, Rao GR, Jain AK, Dixit VK, Gulati AK, et al. Evaluation of nested PCR in detection of *Helicobacter pylori* targeting a highly conserved gene: HSP 60. *Helicobacter* 2008; **13**(1): 30–34.
- [5] Mishra S, Singh V, Rao GR, Dixit VK, Gulati AK, Nath G. Prevalence of *Helicobacter pylori* in asymptomatic subjects—a nested PCR based study. *Infect Genet Evol* 2008; **8**(6): 815–819.
- [6] Tiwari SK, Khan AA, Ahmed KS, Ahmed I, Kauser F, Hussain MA, et al. Rapid diagnosis of *Helicobacter pylori* infection in dyspeptic patients using salivary secretion: a non-invasive approach. *Singapore Med J* 2005; **46**(5): 224–228.
- [7] Ahmed KS, Khan AA, Ahmed I, Tiwari SK, Habeeb A, Ahi JD, et al. Impact of household hygiene and water source on the prevalence and transmission of *Helicobacter pylori*: a South Indian perspective. *Singapore Med J* 2007; **48**(6): 543–549.
- [8] Nurgalieva ZZ, Malaty HM, Graham DY, Almuchambetova R, Machmudova A, Kapsultanova D, et al. *Helicobacter pylori*

- infection in Kazakhstan: effect of water source and household hygiene. *Am J Trop Med Hyg* 2002; **67**(2): 201–206.
- [9] Ahmed N, Tenguria S, Nandanwar N. *Helicobacter pylori*—a seasoned pathogen by any other name. *Gut Pathog* 2009; **1**: 24.
- [10] Kamangar F, Qiao YL, Blaser MJ, Sun XD, Katki H, Fan JH, et al. *Helicobacter pylori* and oesophageal and gastric cancers in a prospective study in China. *Br J Cancer* 2007; **96**(1): 172–6.
- [11] Azevedo NF, Almeida C, Fernandes I, Cerqueira L, Dias S, Keevil CW, et al. Survival of gastric and enterohepatic *Helicobacter* spp. in water: implications for transmission. *Appl Environ Microbiol* 2008; **74**(6): 1805–1811.
- [12] Samra ZQ, Javaid U, Ghafoor S, Batool A, Dar N, Athar MA. PCR assay targeting virulence genes of *Helicobacter pylori* isolated from drinking water and clinical samples in Lahore metropolitan. *J Water Health* 2011; **9**(1): 208–216.
- [13] Travis PB, Goodman KJ, O'Rourke KM, Groves FD, Sinha D, Nicholas JS, et al. The association of drinking water quality and sewage disposal with *Helicobacter pylori* incidence in infants: the potential role of water-borne transmission. *J Water Health* 2010; **8**(1): 192–203.
- [14] Moreno Y, Piqueres P, Alonso JL, Jiménez A, González A, Ferrús MA. Survival and viability of *Helicobacter pylori* after inoculation into chlorinated drinking water. *Water Res* 2007; **41**(15): 3490–3496.
- [15] Braganra SM, Azevedo NF, Simões LC, Keevil CW, Vieira MJ. Use of fluorescent in situ hybridisation for the visualisation of *Helicobacter pylori* in real drinking water biofilms. *Water Sci Technol* 2007; **55**(8–9): 387–393.
- [16] Konishi K, Saito N, Shoji E, Takeda H, Kato M, Asaka M, et al. *Helicobacter pylori*: longer survival in deep ground water and sea water than in a nutrient-rich environment. *APMIS* 2007; **115**(11): 1285–1291.
- [17] Gião MS, Azevedo NF, Wilks SA, Vieira MJ, Keevil CW. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. *Appl Environ Microbiol* 2008; **74**(19): 5898–5904.
- [18] Azevedo NF, Pacheco AP, Keevil CW, Vieira MJ. Adhesion of water stressed *Helicobacter pylori* to abiotic surfaces. *J Appl Microbiol* 2006; **101**(3): 718–724.
- [19] Nayak AK, Rose JB. Detection of *Helicobacter pylori* in sewage and water using a new quantitative PCR method with SYBR green. *J Appl Microbiol* 2007; **103**(5): 1931–1941.
- [20] Souto R, Colombo AP. Detection of *Helicobacter pylori* by polymerase chain reaction in the subgingival biofilm and saliva of non-dyspeptic periodontal patients. *J Periodontol* 2008; **79**(1): 97–103.
- [21] Gebara EC, Faria CM, Pannuti C, Chehter L, Mayer MP, Lima LA. Persistence of *Helicobacter pylori* in the oral cavity after systemic eradication therapy. *J Clin Periodontol* 2006; **33**(5): 329–333.
- [22] Zou QH, Li RQ. *Helicobacter pylori* in the oral cavity and gastric mucosa: a meta-analysis. *Oral Pathol Med* 2011; **40**(4): 317–324.
- [23] Silva DG, Tinoco EM, Rocha GA, Rocha AM, Guerra JB, Saraiva IE, et al. *Helicobacter pylori* transiently in the mouth may participate in the transmission of infection. *Mem Inst Oswaldo Cruz* 2010; **105**(5): 657–660.
- [24] Rasmussen LT, Labio RW, Gatti LL, Silva LC, Queiroz VF, Smith Mde A, et al. *Helicobacter pylori* detection in gastric biopsies, saliva and dental plaque of Brazilian dyspeptic patients. *Mem Inst Oswaldo Cruz* 2010; **105**(3): 326–330.
- [25] De Sousa L, Vásquez L, Velasco J, Parlapiano D. Isolation of *Helicobacter pylori* in gastric mucosa, dental plaque and saliva in a population from the Venezuelan Andes. *Invest Clin* 2006; **47**(2): 109–116.
- [26] Salmanian AH, Siavoshi F, Akbari F, Afshari A, Malekzadeh R. Yeast of the oral cavity is the reservoir of *Helicobacter pylori*. *J Oral Pathol Med* 2008; **37**(6): 324–328.
- [27] Silva DG, Stevens RH, Macedo JM, Albano RM, Falabella ME, Veerman EC, et al. Detection of cytotoxin genotypes of *Helicobacter pylori* in stomach, saliva and dental plaque. *Arch Oral Biol* 2009; **54**(7): 684–688.
- [28] Percival SL, Thomas JG. Transmission of *Helicobacter pylori* and the role of water and biofilms. *J Water Health* 2009; **7**: 469–477.
- [29] Linke S, Lenz J, Gemein S, Exner M, Gebel J. Detection of *Helicobacter pylori* in biofilms by real-time PCR. *Int J Hyg Environ Health* 2010; **213**(3): 176–182.
- [30] Azevedo NF, Almeida C, Cerqueira L, Dias S, Keevil CW, Vieira MJ. Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol* 2007; **73**(10): 3423–3427.
- [31] Andersen LP, Rasmussen L. *Helicobacter pylori*—coccoid forms and biofilm formation. *FEMS Immunol Med Microbiol* 2009; **56**(2): 112–115.
- [32] Buck A, Oliver JD. Survival of spinach-associated *Helicobacter pylori* in the viable but nonculturable state. *Food Control* 2010; **21**: 1150–1154.
- [33] Quaglia NC, Dambrosio A, Normanno G, Parisi A, Patrono R, Ranieri G, et al. High occurrence of *Helicobacter pylori* in raw goat, sheep and cow milk inferred by glmM gene: a risk of food-borne infection? *Int J Food Microbiol* 2008; **124**(1): 43–47.
- [34] Vale FF, Vitor JM. Transmission pathway of *Helicobacter pylori*: does food play a role in rural and urban areas? *Int J Food Microbiol* 2010; **138**(1–2): 1–12.
- [35] Gouider M, Bouzid J, Sayadi S, Montiel A. Impact of orthophosphate addition on biofilm development in drinking water distribution systems. *J Hazard Mater* 2009; **167**(1–3): 1198–1202.
- [36] Sasidharan S, Lachumy SJT, Ravichandran M, Latha LY, Gegu SRS. Epidemiology of *Helicobacter pylori* among multiracial community in Northern Peninsular, Malaysia: effect of age across race and gender. *Asian Pac J Trop Med* 2011; **4**(1): 72–75.
- [37] Ishaleku D, Ihiabe HA. Seroprevalence of *Helicobacter pylori* infection among students of a Nigerian University. *Asian Pac J Trop Med* 2011; **3**(7): 584–585.
- [38] Fayed S, S Abd El Dayem, Hussein H, Sherief M, S El-Naghi. Detection of virulent strains of *Helicobacter pylori* and its relation to symptoms and signs in children. *Asian Pac J Trop Med* 2011; **3**(1): 59–62.
- [39] Alsaimary AE, Abdulnbi HM, Laibi A, Jwad AR. The occurrence of *Helicobacter pylori* in hydatid liver disease. *Asian Pac J Trop Biomed* 2012; **2**(3): 233–234.
- [40] Nouraie M, Latifi-Navid S, Rezvan H, Radmard AR, Maghsudlu M, Zaer-Rezaii H, et al. Childhood hygienic practice and family education status determine the prevalence of *Helicobacter pylori* infection in Iran. *Helicobacter* 2009; **14**(1): 40–46.
- [41] Kivi M, Tindberg Y. *Helicobacter pylori* occurrence and transmission: a family affair? *Scand J Infect Dis* 2006; **38**(6–7): 407–417.
- [42] Fujimoto Y, Furusyo N, Toyoda K, Takeoka H, Sawayama Y, Hayashi J. Intrafamilial transmission of *Helicobacter pylori* among the population of endemic areas in Japan. *Helicobacter* 2007; **12**(2): 170–176.