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Full Length Research Paper

Absence of *Helicobacter pylori* in the river waters in the north of Iran

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***Helicobacter pylori* is a helical shaped bacterium that predominantly resides in stomach and duodenal epithelia. It is believed to be responsible for localized inflammations resulting in peptic ulcers as well as other digestive tract complications. Although half of the world's population are carriers of the bacterium, its exact mode of transmission and pathogenic mechanisms are yet to be elucidated. The major purpose of this study was isolation and identification of *H. pylori* from river waters in north of Iran. The preT-KB method was used for isolation and PCR technique for detection of DNA. The presence of the bacterium was not observed in any of the 235 samples of water analyzed. The results of the PCR on the water samples confirmed the absence of *Helicobacter´*s DNA. Considering that the applied technique could identify the number of 10^2 bacteria and 0.1 µg of DNA in each milliliter, we believe that the samples collected from this geographical region were not contaminated with *H. pylori*.**

Key words: *Helicobacter,* river waters, north of Iran.

INTRODUCTION

Helicobacter is the most prevalent human bacterial pathogen in the world (Janzon et al., 2009), and it is estimated that 50% of the world's population is infected with this bacteria. Prevalence of the *Helicobacter* is much diversified all over the world so that the rate of infection is lower than 20% in some developed countries and above 80% in some developing countries. More than one decade ago, the genus Helicobacter has been developed considerably by the diagnosis of 24 species, at least, which can lead to the occurrence of diseases in other organisms, in addition to humans, including dogs, cats, rodents and birds as well (Fox, 2002). Examples of *Helicobacter* species are: *H. pylori*, *H. canadensis, H. cinaedi*, *H. canis*, *H. brantae*, *H. fennelliae. Helicobacter pylori* is the most significant species of the genus and causes the occurrence of stomach ulcer in 10 to 15% of the cases and, also leads to the occurrence of stomach cancer in 1 to 2% of the diseased individuals (Kusters et al., 2006). The over crowded and high-populated houses whose inhabitants are in low levels of living standards have higher probability rates of be affected by diseases caused by *Helicobacter* (Kivi et al., 2005).

Generally, the diseases caused by the *Helicobacter* include the stomach and duodenal ulcer, stomach inflammation, dyspepsia without the ulcer and stomach cancer (Fredrick and Richard, 2002; Olden and Drossman, 2000).

The main rout of transmission of *Helicobacter* has not been diagnosed yet. But, a few reports have described the detection of the *Helicobacter*'s DNA from the drinking and environmental waters and these reports suggest that the *Helicobacter* may be transferred through the water (Janzon et al., 2009). Of course, the waters contaminated by the excreting materials may be a potential source for the transfer of *Helicobacter* (Lu et al., 2002). The *H. pylori* infection is to be acquired in the childhood periods, and it is assumed that this transfer takes place primarily through the oral-excreting cycle from one individual to another (Rowland, 2000). The other ways of transmittance of *Helicobacter* are oral-oral transfers.

Many studies have shown the relationship among the

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Figure 1. Gilan and Mazandaran provinces have been located in the North of Iran.

infection, water quality and the resources of the drinking waters. The problem is supported by the existence of the *Helicobacter pylori*'s DNA in drinking waters, rivers, lakes or the water of seas (Adams et al., 2003). Our main objective is to carry out a prospective 8-month study regarding the presence of the *Helicobacter* genus in the surface waters (river waters) of a region in the north of Iran. In order to achieve this objective, we employed tests with high sensivity to identify the cell and *Helicobacter*'s DNA in the water samples.

MATERIALS AND METHODS

Study areas and sample collection

Gilan and Mazandaran provinces are in northern Iran located on the southern coast of the Caspian Sea (Figure 1). This region possesses the moderate Climate in the various seasons and plentiful rainfalls. In this study, the sampling was conducted from Tonekabon, Ramsar, Rudsar and Lahijan cities. All the river waters have originated from Alborz mountains and reached the Caspian sea after passing from the villages and residential regions. Most rivers have moderate quantities of water.

Between November 2010 and June 2011, 235 samples of the river water from different regions of the north of Iran were collected from the sections which had the state of stagnant waters. The river water samples were collected in 50 ml falcon tubes and transported to the laboratory at ambient temperature and stored at 4°C before they were analyzed within 2 h. All the samples were subjected to detection of *H. pylori* immediately upon arrival in the laboratory (Baserisalehi et al., 2004).

Sample processing and isolation

The preT-KB method was used for isolation of *Helicobacter* spp. (Baserisalehi et al., 2004). In order to carry out this technique, each sample was transferred to the sterile tubes and centrifugation at 4000 rpm for 10 min. After the concentration of the sample, 1 cc of it transferred to the ependorf microtubes assisted by the sterile samplers and located into the micro centrifuge (ependorf-Germany). The samples were centrifuged in 8500 rpm within 10 min, and then the microtubes were taken out of the apparatus gently and placed in the laboratory environment within 10 to 15 min.

After this time, a loopful of supernatant was withdrawn and spread on to the mueller hinton agar (Merk-Germany). In order to compare this technique with the standard methods of the culture, the spread culture was accomplished, from the concentrated water sample without the action of the final centrifuge, on the culture media of skirrow (Qlab-Canada), Brucella agar (Merk-Germany) Campylobacter selective agar (Merk-Germany) enriched by supplement. All plates were incubated at 37°C for one week under microaerophilic conditions.

Phenotyping identification

Helicobacter identification was performed by subjecting all the suspected colonies to microscopic examination of wet mount under dark filed microscopy, Gram staining, glucose fermentation, catalase, oxidase and urease test.

Genotyping identification

DNA extraction

In order to identify the *Helicobacter's* DNA in the collected samples, the concentrated water samples entered into the BHI Broth (Merk-Germany) firstly; then, they were incubated under microaerophil conditions for 5 days. DNA extraction from the bacterium was conducted using phenol-chloroform technique by Sambrook et al. (1989). The purity of the extracted DNA was assessed based on the absorbance of the extracted DNA at 260 and 280 nm wavelengths by biophotometer (Eppendrof-Germany).

Amplification of 16S rRNA gene by PCR

In order to execute the PCR technique, the forward and reverse primers for the 16S rRNA gene of the *Helicobacter* genus were produced by TAG Copenhagen (Denmark). The sequences of forward and reverse primers were 5'-CTATGACGGGTATCCGGC-3' and 5'-ATTCCACCTACCTCTCCCA-3' respectively.

Each reaction was performed in a total volume of 25 μL containing 1 μL of 10 pmol each forward and reverse PCR primers, 2.5 μL of 10 × PCR buffer (Cinagen-Iran), 0.75 μL of 50mm MgCl2 (Cinagen-Iran), 0.5 μL of dNTPs (Cinagen-Iran), 0.5 μL of DNA polymerase (Cinagen-Iran), 4 μL of DNA template and 14.75 μL of molecular biology-grade water (Sigma Aldrich Company Ltd.) Non template control (NTC) tube contained the same PCR reagents as above but had 4 μL of water substituted for template.

Agarose gel electrophoresis

All PCR products were run on a 1.5% (w/v) agarose gels with a 1 kb DNA ladder (Fermentas-Russia). Aliquots of PCR products were electrophoresed at 75 V for 60 min; DNA was visualized using ethidium bromide and photographed after UV transillumination with Uvidoc (England).

Control of the primer's specialization

For this purpose, DNA of a few genus of bacteria which enter into the surface waters through the excretion or soil, including the *Campylobacter*, *Escherichia*, *Pseudomonas* and the *Bacillus* was extracted by the phenol- chloroform method and, after that, the PCR process was implemented using the above mentioned primers.

Minimum quantity of the identifiable bacterium

Then, the serial dilutions from 10^1 to 10^8 were supplied and placed *H. pylori* standard strain (Puno135) was used to study the minimum quantity of the identifiable bacterium in the water samples. At first, Mcfarland's 0.5 dilutions were provided from the desired bacterium. under the action of the DNA extraction and, subsequently, the PCR process with the previous conditions was executed on the samples.

Minimum quantify of the identifiable DNA

In order to study the minimum concentration of the identifiable DNA in the water, the concentration of 100 mg/ml of the DNA was prepared and then the serial dilution from 100 to 0/0001 μg/ml were provided.

Control of the method of the identification in the water

As mentioned earlier, the rate of 1 cc of the water for the inoculation against with the culture media ought to be concentrated for the culture steps and PCR of each sample. Therefore, a quantity of the supernatant was gone out in the different stages. Therefore, in order to control this point that there is not the desired bacterium in the gone-out supernatants, the minimum concentration of the identifiable bacterium was poured in 50 ml of the river water and the isolation operations were conducted upon it. In the next steps, the PCR process was executed upon the gone-out supernatant.

RESULTS

Culture and PCR

With regard to the general attributes of the bacterium, direct examination, gram staining, oxidase, catalase, urease tests, fermentation of the glucose and other biochemical tests from 235 samples cultured on the used media, even one case of the bacterium presence wasn't observed. The results of the PCR on the water samples proved the absence of this *Helicobacter* DNA.

Controlling the primer's specificity

As observed in Figure 2, the result obtained from the control of the primer's specificity which was carried out by a few genus of the bacterium, including *Campylobacter, Escherichia, Pseudomonas* and *Bacillus*, showed that the primers designed for the *Helicobacter* have been specific completely.

Minimum quantity of the identifiable bacterium and DNA

After the preparation of the serial dilutions of 10^1 to 10^8 from the standard strain of *Helicobacter pylori*, the PCR results showed that the minimum quantity of the identifiable bacterium by this method is 10^2 bacterium in each milliliter of water (Figure 3). The minimum rate of

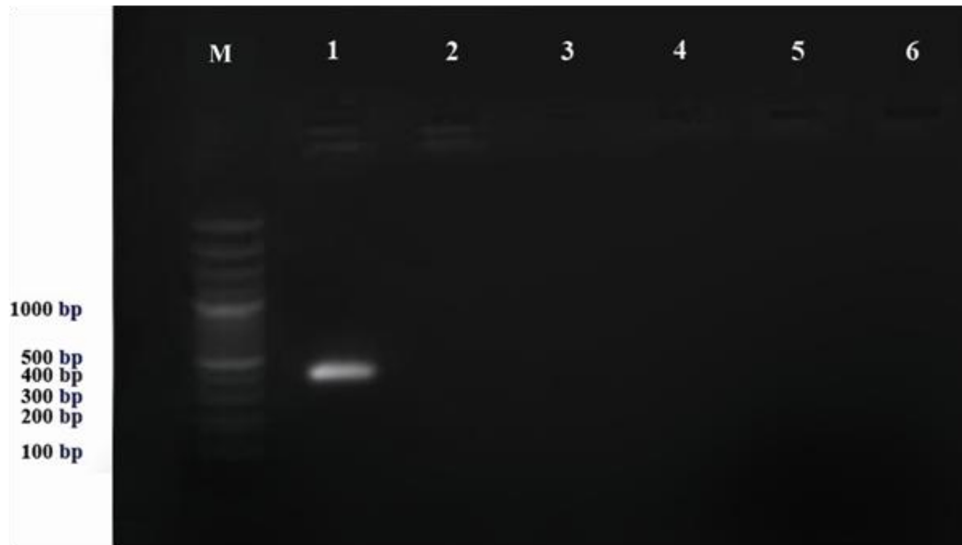

Figure 2. Agarose gel (1.5%) analysis of a PCR test for environmental sample. Lane 1 *Helicobacter pylori* as positive control, Lane 2 *campylobacter,* Lane 3 *Escherichia,* Lane 4 *Pseudomonas,* Lane 5 *Bacillus,* Lane 6 negative control and Lane M size marker 1000 bp.

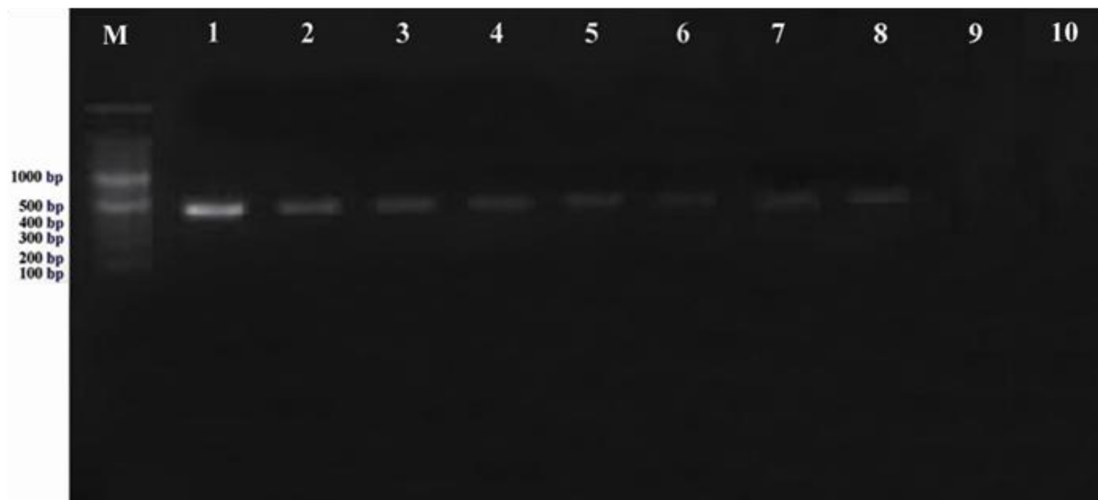

Figure 3. Agarose gel (1.5%) analysis of a PCR test for environmental sample. Lane 1 *Helicobacter pylori as* positive control, Lane 2-9 serial dilution of bacteria from 10^8 to 10^1 , Lane 10 negative control and Lane M size marker 1000 bp.

the DNA which is identifiable by this method of the dilution's preparation as well is reported as 0.1 µg in each milliliter (Figure 4).

Control of method of the bacterium identification in water

As it was imaginable, the results obtained from PCR of the samples were negative, which suggests that the desired bacterium has not been lost in none of the stages of the concentration.

DISCUSSION

Most published articles in two past decades suggested that *H. pylori* might find a way into the surface waters through the feces of the animals and humans. The main purpose of this study has been the identification of the

Figure 4. Agarose gel (1.5%) analysis of a PCR test for environmental sample. Lane 1-7 serial dilution of DNA from 100 to 0/0001 µg/ml, Lane 8 negative control and Lane M size marker 1000 bp.

bacterium and DNA of the *Helicobacter* in river water of the north of Iran using the culture and PCR technique. Ghane et al. (2010) showed that the rivers of these regions of Iran's have been contaminated by dangerous pathogens, including *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Archobacter butzelri*. As a result, it could be presumed that species of *Helicobacter* might also be present in the river waters of this region and that the high levels of infection of this bacterium in the society can be generated by the water. Most surprisingly, in this study *Helicobacter*'s cell and DNA were not identified in the water samples.

In order to confirm the obtained negative results, a number of the controlling test, including the method of the sample's concentration, sensitivity of the primers, specificity of the reaction and the DNA break down was executed in the water.

The test with the sequential dilutions of the *Helicobacter pylori*'s cell and DNA showed that the tests mentioned above are sensitive and c. 100 cells in each sample and c. 0.1 µg of DNA in each sample are identifiable.

In the test of the determination of the primer's sensitivity, it was proved that specificity of the primers is high because none of the bacteria of the used pathogen was paired to DNA and the reaction of PCR for them became negative. Any way, a number of the retrospective studies regarding the transfer of the *Helicobacter* through the contaminated waters were carried out, and it was proved that there exists a relationship between the *H. pylori* infection and the low-quality and not-purified drinking water. Although the successful isolation of the living and culturable cell of the *Helicobacter* has not yet been reported from any place around the world (Fugimura et al., 2004; Watson et al., 2004). There are reports regarding failure in the identification of the *H. pylori* in the drinking water in the United States, Belgium, Spain and Italy (Böckelmann et al., 2009; McDaniels et al., 2005). Nevertheless, a number of the studies carried out with river waters, lake waters and drinking water in Japan and England confirmed the presence of this bacterium's DNA (Queralt et al., 2005; Fugimura et al., 2004; Watson et al., 2004).

At the end, contamination and stability of the *Helicobacter* under the environmental conditions are discussable. The documents show that this bacterium remains Viable but non-culturable (VBNC) coccoids in the water environment. As a result, it's presence can be realized only through the identification of its (bacterium) genome. In addition the studies conducted by Janzon et al. (2009) show that *Helicobacter pylori*'s DNA degrades and becomes undetectable quickly in the environmental waters.

Considering that the applied technique could identify the number of 10^2 bacteria and 0.1 µg of DNA in each milliliter, we believe that the studied samples from this geographical region are not contaminated with *Helicobacter pylori*.

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