Full Length Research Paper

Detection of *Helicobacter pylori* DNA in purified water for drinking

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*H. pylori* is a Gram negative bacterium microaerophilic that is associated to digestive human diseases such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma; this bacterium is considered a risk factor in the development of gastric cancer. Different vias have been proposed by transmission of *H. pylori*. *H. pylori* has been detected in well water, municipal water, treated wastewater and drinking water in developing countries. The present study shows an evidence of the occurrence of *H. pylori* through detection of DNA of this bacterium in purified water for drinking of Puebla city. In this work we apply nested PCR to the detection of *H. pylori* using primers that specifically amplify the gene 16S rRNA.

Keywords: *Helicobacter pylori*, water, 16S rRNA, detection, drinking, PCR, nested.

INTRODUCTION

*H. pylori* is a Gram negative bacterium microaerophilic which is associated to different digestive human diseases such as gastritis, peptic ulcer, gastritis, mucosa-associated lymphoid tissue lymphoma (Brown, 2000; Dunn et al., 1997; Salama et al., 2013; Suzuki et al., 2012; Testerman and Beyond, 2014; Watar et al., 2014). *H. pylori* is considered a risk factor in the development of gastric cancer (Bahrami et al., 2013; Dunn et al., 1997; Hagymási and Tulassay, 2014; Herrera and Parsonnet, 2009; Kim et al., 2011; Polk and Peek, 2010). The epidemiology of *H. pylori* infection appears to be different in developed and developing countries (Cullen et al., 1993; Graham et al., 1991; Khalifa et al., 2010; Mazari-Hiriart et al., 2001; Mitchell et al., 1992; Thomas et al., 1992). The prevalence of *H. pylori* is higher in developing countries than in the developed countries (Graham et al., 1991; Pounder and Ng, 1995). In developing countries, it has been demonstrated that *H. pylori* infection is acquire earlier in life. It is estimated that 70-90% of the population is infected with *H. pylori* in developing countries, while 25-50% of the population of developed countries is infected by this bacterium (Bahrami et al., 2013; Brown, 2000; Lee, 1994). For *H. pylori* different routes of transmission have been proposed, for example: person to person by faecal-oral, oral-tooral and oral-oral routes, gastro-oral route, contaminated foods (*H. pylori* has been detected from foods of animal origin, sea water, drinking...
water) (Allaker et al., 2002; Anand et al., 2014; Dore et al., 2001; Fujimura et al., 2002; Gomes and De Martinis, 2004; Madinier et al., 1997; Parsonnet et al., 1999; Quaglia et al., 2008; Safaei et al., 2011; Thomas et al., 1992; Valdez-González et al., 2014). Also _H. pylori_ has been detected in drinking water, well water, municipal water and treated wastewater in countries as Peru and Sweden (Adams et al., 2003; Benson et al., 2004; Bunn et al., 2002; Casasola-Rodriguez et al., 2013; Cellini et al., 2004; Engstrand, 2001; Glynn et al., 2002; Hegarty et al., 1999; Hulten et al., 1996; Hulten et al., 1998; Klein et al., 1991; Lu et al., 2002). The present study aimed to seek evidence of the occurrence of _H. pylori_ through detection of DNA of this bacterium in purified water for drinking of Puebla city. In this work we apply nested PCR to the detection of _H. pylori_ using primers that specifically amplify the gene 16S rRNA.

**MATERIALS AND METHODS**

**Collection of water samples and microorganism recovery**

Water purified samples were colleted and examined randomly from different comercial establishments, traders dedicated to the purification of drinking water at Puebla city (México), over a period of 6 months from July to December 2014. The number of comercial establishments considered in this study was 20. For each comercial establishment 3 samples were collected in 1,000mL glass steril bottles containing 900 mL of purified water. Samples of purified water collected and transferred to laboratory within 2 hours. Then samples of purified water were filtered through 0.22 micrometer filter membrane (Millipore Co) to collect the microorganisms in them. The membranes containing microorganisms were stored at -20°C until analysis.

**Preparation of genomic DNA**

Bacterial DNA was extracted according to modified methodology described by Ho et al., (1991). Briefly, each membrane was washed with 1 mL of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA) in a 1.5-mL Eppendorf tube and it was added 30 microliters of 10% sodium dodecyl sulfate and 3 microliters (2mg/100 microliters) of proteinase K. Then, tube was incubated at 37°C for 1 h. The mixtures were extracted with an equal volume of phenol-chloroform-isoamyl alcohol and centrifuged at 12,000 x g in a microcentrifuge for 3 min; the aqueous layer was transferred to a fresh Eppendorf tube. Two further extractions were performed with equal volumes of phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. The DNA was precipitated with 0.7 volume of isopropanol at -20°C. The DNA was pelleted by microcentrifugation at 13,000 x g for 5 min, washed with -20°C 70% (V/V) ethanol, desiccated for 30 min, and dissolved in 50 microliters of molecular biology-grade water. The DNA was quantified spectrophotometry. Genomic extracted DNA was stored at -20°C.

**Nested PCR conditions for detection of H. pylori**

Amplification of the DNA template was carried out using primers Hp1, Hp2 and Hp3 previously described by Ho et al., (1991) and Mazari-Hiriart et al., (2001). Assay conditions were following. It was added 0.5 microliters of each oligonucleotide primer (50 pmol/microliter for each primer) in an Eppendorf tube, 1 microliters of extracted DNA, 2.5 microliters of 10X PCR buffer (500 mM KCl, 100 mM Tris-Cl, 15 mM MgCl₂, pH 8.3), 1 microliters of deoxynucleoside triphosphate mixture (final concentration, 1.25 mM each dATP, dCTP, dGTP, and dTTP). 0.3 microliters of Taq DNA polymerase and molecular biology-grade distilled water were added to make a final reaction volume of 25 microliters. For nested PCR, 30 cycles were used for each round of amplification. The temperature profile was as follows: 4 min at 94°C, 45 s at 94°C, 45 s at 60°C, 45 s at 72°C. The last cycle was identical, except that the 72°C extension period was increased to 7 min and the mixture was subsequently refrigerated at 4°C before analysis. Primers were used to amplify _H. pylori_ by nested PCR with the following sequences: Hp1: 5’-CTG GAG AGA CTA AGC CCT CC-3’, Hp2: 5’-ATT ACT GAC GCT GAT TGT GC-3’, and Hp3: 5’-AGG ATG AAG GTT TAA GGA TT-3’.

**Analysis of PCR products**

Aliquots of each PCR product were separated by electrophoresis in a 1% (w/v) agarose gel (Ultra Pure; Invitrogen) with the MBI Fermentas (Amherst NY) 100 bp DNA Ladder Plus used as a size marker, in TAE buffer (90 mM Tris-HCl, 90 mM acetic acid, 2 mM EDTA) and stained in ethidium bromide at 0.06 microgram/mL). Positive and negative controls were included in all assays to monitor specificity and laboratory contamination during the analyses. The specificity of the PCR assays has been previously reported by Ho et al., (1991). Assays on all samples were repeated in duplicate. Samples were interpreted as being positive for the presence of _Helicobacter_ DNA if one or more of the assays produced a fragment comparable in size to that of the positive control DNA (_H. pylori_ 26695).
RESULTS

Between July and December 2014 were collected and examined 20 water purified samples from traders dedicated to the purification of drinking water at Puebla city. 3 samples of purified water (each one 900 mL) were collected and filtered through 0.22 micrometer filter membrane to collect bacteria contained into water. The results shown that filtration method used allowed the total recovery of bacteria suspended into water samples. Colored sediments on filter membrane surfaces were obtained as shown in Figure 1. Figure 1 shows sediment obtained in major amount by filtering samples of purified water. An indirect way of demonstrating the presence of microorganisms in the obtained sediments after filtration of purified water, was carrying out the extraction and quantification of DNA according to Materials and Methods. So each membrane was washed with TE buffer containing sodium dodecyl sulfate and proteinase K. After incubation, DNA was extracted according to common protocol using phenol-chloroform-isoamyl alcohol and ethanol. DNA concentrations determined from sediment samples of filtered water are shown in Table 1. As shown in Table 1, all samples of purified water were positive for the presence of sediment by filtration, which is indicative of the presence of microorganisms. These data are related to the presence of DNA in analyzed sediments. As shown in Table 1, all analyzed water samples containing different concentrations of DNA from 22 to 475 ng/ microliter of DNA. DNA concentration is indicative of the presence of microorganisms in the analyzed water and it should be in proportion to the amount of microorganisms in it. DNA purity of each the samples of analyzed water showed average values of 1.5 (by rate OD260nm / 280nm) (data not shown). Subsequently, it is proceeded to the detection of *H. pylori* using primers Hp1, Hp2 and Hp3 that specifically amplify the 16S rRNA.
Table 1. Concentrations of DNA extracted from filtered water.

<table>
<thead>
<tr>
<th>Purified water Samples</th>
<th>Obtained sediment</th>
<th>DNA concentration (ng/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>197</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>137</td>
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<td>4</td>
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<td>5</td>
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<td>8</td>
<td>+</td>
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<td>12</td>
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<td>19</td>
<td>+</td>
<td>37</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>35</td>
</tr>
</tbody>
</table>

*Average value of DNA concentration from 3 measurements.

gene. The amplification conditions for 16S rRNA gene detected by nested PCR were described in Materials and Methods. PCR analysis amplified an approximately 109 base pair DNA fragment (Figure 2). In this study, 16S rRNA gene of H. pylori was detected in most samples analyzed of purified water: 18 of 20 samples of analyzed water were positive, which resulted novel because the DNA of H. pylori was found in 90% of samples tested. Some H. pylori PCR products isolated from water purified samples are shown in Figure 2. Lanes 2, 3, 4, 5, 7 and 8 are examples of positive PCR; lane 6 represents positive control of H. pylori.

**DISCUSSION**

As mentioned above, the transmission pathways of H. pylori to humans is not clear at all. It has been suggested that the transmission of H. pylori occurs by oral-oral and fecal-oral vias, including contaminated foods and water (Allaker et al., 2002; Anand et al., 2014; Brown, 2000; Dore et al., 2001; Engstrand, 2001; Fujimura et al., 2002; Gomes and De Martinis, 2004; Madinier et al., 1997; Parsonnet et al., 1999; Quaglia et al., 2008; Thomas et al., 1992). Water seems to be an important vehicle to dissemination of bacteria. So H. pylori has been detected from major water reservoirs, for example: sea water, well water, drinking water (Adams et al., 2003; Bahrami et al., 2013; Benson et al., 2004; Bunn et al., 2002; Casasola-Rodríguez et al., 2013; Cellini et al., 2004; Engstrand, 2001; Glynn et al., 2002; Hegarty et al., 1999; Hulten et al., 1996; Hulten et al., 1998; Klein et al., 1991; Lu et al., 2002). In this context, several epidemiological studies have reported that infection with H. pylori is associated particularly to an inadequate water sanitation (Mazari-Hiriart et al., 2001). Some evidences have been provided to sustain the presence of H. pylori in aquatic environments, such as observation of coccoid forms in water samples and survival of H. pylori in water, presence of DNA in water samples, growth of H. pylori from water samples, formation of biofilm by H. pylori in water reservoirs (Azevedo et al., 2007; Bahrami et al., 2013; Bunn et al., 2002; Cellini et al., 2004; Hulten et al., 1996; Hulten et al., 1998; Vale and Vítor, 2010). It has been reported that H. pylori is able to remain viable in water storage systems possibly held in the biofilms and coccoid forms (Azevedo et al., 2007; Bunn et al., 2002; Flores-Encarnación et al., 2015; Percival et al., 2014). It has been reported that H. pylori can survive in aquatic environments (lakes, surface and ground water, wastewater and coastal marine environments, rivers, drinking water (Fernández-Delgado et al., 2008). H. pylori can survive in river water for several months adopting the coccoid morphotype (Azevedo et al., 2007; Chen, 2004; Percival and Suleman, 2014; Vincent, 1995). Thus it was considered that after a time that H. pylori is subject to stressful environmental conditions, it acquires cocccoid form, which has been termed viable non-culturability (Bode...
et al., 1993). In this study the positivity to presence of microorganisms into analyzed samples of purified water were determinated. At first glance it was observed abundant sediment recovered from water filtered samples (Figure 1). Likewise it was possible to carry out the extraction of genomic DNA from collected sediments. These two tests were an evidence of the presence of microorganisms present in the water samples. DNA concentrations were an indicative of the presence of microorganisms in the analyzed water. In the present study, only two water samples (10%) were not found to be contaminated with H. pylori using nested PCR (Table 1). H. pylori was found in 90% of water analyzed samples. There are several papers which demonstrates the presence of H. pylori in drinking water from diverse cities using PCR because the culture from this sources of water results difficult (Benson et al., 2004; Bunn et al., 2002; Gomes et al., 2004; Hulten et al., 1996; Hulten et al., 1998; Klein et al., 1991; Lu et al., 2002; Mazari-Hiriart et al., 2001; Smith et al., 2004; Watson et al., 2004). PCR has been used by many authors as a methodology in detection of H. pylori due to its valuable high sensibility and the variety of primers reported in literature (Ho et al., 1991; Hulten et al., 1996; Lage et al., 1995; Mazari-Hiriart et al., 2001).

The presence of H. pylori in water purified samples indicates the poor microbiologic quality of the water used for human consumption (Mazari-Hiriart et al., 2001). In this study the isolation of bacteria in water analyzed samples was not performed, however previous studies by other authors reported the presence of coliforms and mesophilic aerobic bacteria in the water expended in some places at Puebla city (Cabrera-Maldonado et al., 2009). It indicates the disease potential of the water to be a vehicle for the storage and dissemination of pathogenic bacteria to humans (Bahrami et al., 2013). The use of these purified waters for drinking increase the possibility of acquiring infectious gastrointestinal diseases and in the case of H. pylori, gastritis, peptic ulcer, development of gastric cancer, could be acquired (Brown, 2000; Dunn et al., 1997; Salama et al., 2013; Suzuki et al., 2012; Testerman and Beyond, 2014; Watar et al., 2014). Use of these purified waters represents a potential risk to human health. According to the results of this study, purified water for drinking (distributed for some comertial establishments dedicated to the purification of drinking water at Puebla) are a risk factor to health and a potential vehicle for transmission of H. pylori to the population consuming this water.

CONCLUSION

The presence of H. pylori into water purified samples suggests water contamination because to an inadequate sanitation process to purify water. The presence of amplifiable H. pylori DNA from purified water adds weight to the view that this bacterium may be transmitted through contaminated drinking water. The consumption of contaminated drinking water would be a potential risk of H. pylori infection for the consumer. Further studies will be necessary to determine the presence of H. pylori in purified water and provide further information of the potential risk of human infection with H. pylori via consumption of purified water with poor microbiological quality.

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REFERENCES


