PCR assay targeting virulence genes of *Helicobacter pylori* isolated from drinking water and clinical samples in Lahore metropolitan, Pakistan

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**ABSTRACT**

*Helicobacter pylorus* is considered for chronic gastritis, gastric ulcers and adenocarcinoma and its high infection rate is observed in overcrowded and lower socioeconomic groups in developing countries. This study was designed to identify the role of drinking water in the transmission and prevalence of *H. pylori* (HP). Selective HP medium was developed for enrichment and presumptive identification of *H. pylori* by urease, catalase and species specific 16S rRNA tests. The virulence genes (*vacA* ‘s’ and ‘m’ regions and *cagA*) of *H. pylori* in 90 out of 225 *H. pylori* positive drinking water samples were present (40%). Ten out of 18 biopsies (55.55%) and 15 out of 50 vomiting fluids of gastric disease patients (30%) were also positive for virulence genes. Anti-*H. pylori* antibodies were also detected in 31 out of 50 patients’ sera. The presence of virulence genes was also directly confirmed by hybridization studies using non-radioactive DNA probes of 16S rRNA, *vacA* and *cagA* genes. The presence of *H. pylori* in water is due to poor sanitary conditions, improper waste disposal and lack of public health education. PCR-based analysis and colony hybridization can be used for detection of *H. pylori* in clinical and environmental samples.

**Key words** | 16S rRNA, *cagA*, *H. pylori*, hybridization, PCR, *vacA*

**INTRODUCTION**

*Helicobacter pylori* (*H. pylori*), a Gram-negative microaerophilic bacterium, is recognized as an important gastric pathogen that infects humans worldwide (Parsonnet 1999). It colonizes in the stomach due to acidic pH, which is essential for its propagation and survival. Most infected individuals are asymptomatic but 10–20% of individuals are associated with infection, which may lead to a gastrointestinal disorder or to the development of adenocarcinoma (Westblom et al. 1999; Shiotani et al. 2000; Passaro et al. 2002). Infection is usually acquired in childhood and primarily may spread from person to person via the faecal–oral route (Roosendaal et al. 1993; Brown 2000). The precise route and mode of transmission of *H. pylori* is unknown but studies advocate that the natural reservoir of *H. pylori* outside the human gastrointestinal niche is ground water, surface water and drinking water (Hegarty et al. 1999; Gião et al. 2008; Percival & Thomas 2009). It is also observed that *H. pylori* can also cause natural infection in other primates (Goodman & Correa 1995; Westblom et al. 1999).

*Helicobacter pylori* are present in the stomach or lower bowel sites of many non-human primates as well as in infected humans. It is genetically a panmictic organism associated with different genotypes in different geographic regions (VanDoorn et al. 1999). Understanding the epidemiology of *H. pylori* is an essential step for the development of public health measures due to its association with gastro...
duodenal diseases (Shiotani et al. 2000). The imbalance between the bacterium aggressive factors and the host defence mechanisms may lead to duodenal ulcers, chronic gastritis and stomach cancer (Coghlan et al. 1987; Graham & Yamaoka 1998; Passaro et al. 2002). The risk of H. pylori infection is high in developing countries (Al-Moagel et al. 1990) while the prevalence of H. pylori in developed countries is low due to better industrialization, public health measures/education and living conditions (Bardhan 1997; Percival & Thomas 2009). It is observed that the municipal drinking water supplies are the source for H. pylori infection especially in developing countries, which may be due to contaminated water supplies (Klein et al. 1991; Brown 2000; Baker et al. 2002; Lu et al. 2002; Gião et al. 2008). The detection of H. pylori by PCR in sewage-contaminated drinking water, ponds/river water and shallow ground water indicates the environmental transmission of H. pylori (Hulton et al. 1996; Engstrand 2001; Baker et al. 2002; Lu et al. 2002). It is observed that H. pylori show high tolerance to common disinfectants used in drinking water supplies (Baker et al. 2002).

The cytotoxins secreted by H. pylori induce cytoplasmic vacuolation in eukaryotic cells. The virulence genes of H. pylori can be graded by typing the vacuolating cytotoxin A gene (vacA) and the presence or absence of the cytotoxin-associated gene product (cagA, 128 KDa protein) (Telford et al. 1994; Atherton & Cao 1995; Graham & Yamaoka 1998; Atherton & Cover 1999). The identification of vacA gene in combination with ‘s’ and ‘m’ regions determines the cytotoxic activity of H. pylori (Atherton & Cao 1995). The pathogenicity of H. pylori is also grouped into type I and type II due to the cytotoxin-associated gene (cagA) (Censini et al. 1996; Cesare & Rino 2001). The type I strain has a virulence gene cluster known as cag-PAI (cag Pathogenicity Island), which is involved to induce the interleukin-8 (IL-8) and it may be linked to pro-inflammatory behaviour of the microbe. It is observed that the cag-PAI-treated gastric epithelial cells can induce the activation of proto-oncogenes. The activation of c-fos and c-jun are considered as the progression of H. pylori linked neoplasia (Meyer-Ter-Vehn et al. 2000).

In this study, public drinking water, biopsy samples of chronic gastritis patients suspected of cancer and vomiting fluids of gastric disease patients were screened for the detection of H. pylori. The presence of H. pylori was identified by biochemical tests and confirmed by PCR assays of species specific 16S rRNA, vacA (‘s’ and ‘m’ regions) and cagA genes. In colony hybridization the non-radioactive DNA probes of virulence genes of H. pylori isolated from human biopsy samples were used to screen the H. pylori in drinking water samples, which confirmed the same genus of H. pylori. From this study, it is concluded that the drinking water is the main reservoir for transmission of H. pylori and the hygiene conditions as well as treatment of drinking water need to improve in our local municipal system.

MATERIALS AND METHODS

Materials

Peptone, beef extract, agar and yeast extract were purchased from Oxoid. Helicobacter pylori detecting immunoassay strips were purchased from HUMAN (Germany). Calf serum, vancomycin, trimethoprim, amphotericin B, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate and other chemicals required for buffer preparation were purchased from ICN. Genomic DNA extraction kit was purchased from QIAGEN (Germany). DNA labelling kit and primers were synthesized from Fermentas Inc.

Methods

Sample collection

Drinking water samples (n = 600) were collected from ground-drilled water supplied by water and sanitation agencies in different localities within the Lahore metropolitan area, Pakistan, and were used within six hours for culturing of H. pylori. Eighteen biopsy samples of six chronic gastritis patients (aged 45 to 55 years) suspected of cancer were collected from local hospitals in Lahore. The biopsy samples were taken from three regions of the stomach, namely the antrum, body and fundus parts. The presence of H. pylori in biopsy samples was detected by histological examination of stained sections. Fifty vomiting fluid samples of gastric disease patients (aged 20 to 40 years) with the symptoms of dyspepsia, hyperacidity, bloating and nausea were also
collected from medical clinics. Three to five millilitre blood samples of the same patients were also collected to cross check the immunity against H. pylori. The patients’ samples were collected with the permission of patients or guardians following ethical standards of the Helsinki Declaration. The biopsy sample and 0.5 ml of vomiting fluid were suspended in 1.0 ml normal saline separately and kept in a water bath at 37°C for one hour with constant shaking (100 to 125 rpm). The samples were centrifuged at 5000 × g for 10 minutes at 4°C and the supernatant was separated for culturing H. pylori.

**Preparation of growth medium**

The growth medium for culturing of H. pylori was prepared as described by Degnan et al. (2003) with minor modification. The HP (H. pylori) medium with and without agar was prepared by mixing the following components: (grams per litre) peptone 15, beef extract 5, yeast extract 5, NaCl 5, phenol red 0.1 in water. Agar (15 g/l) was added separately and cooled down to 50°C. Calf serum 7%, antibiotics (mg per litre) i.e. vancomycin 10, trimethoprim 10, amphotericin B 7.5 and urea 600 mg were aseptically added with constant stirring. The pH was adjusted to 6.0 with 1.0 M HCl. The HP-agar medium was poured into the Petri plates and used within three days.

**Growth of H. pylori**

Samples of 0.5 ml each of drinking water, supernatants of tissue biopsy and vomiting fluid (as described above) were applied on HP-agar medium plates separately. The plates were kept in microaerophilic conditions in a humidified incubator at 37°C and 5% CO2 for 3–5 days. The plates were regularly checked during this period to observe the growth of bacteria. The growing colonies were picked up for the preparation of replica plates.

**Characterization of H. pylori**

**Gram staining:** All isolates of H. pylori were processed for Gram staining as described by Benson (1988).

**Catalase and urease tests:** Catalase and urease tests were carried out for the detection of H. pylori. For the catalase test, a small amount of growing culture of H. pylori was placed on a clean microscope slide and a few drops of 2% H2O2 were added (Benson 1988) and the results were noted. For the urease test, HP medium was inoculated with fresh culture of H. pylori and incubated in a humidified incubator at 37°C and 5% CO2 for 72 to 80 hours (Benson 1988).

**Isolation of H. pylori DNA and PCR**

Genomic DNA of H. pylori was extracted by using a genomic DNA purification kit (Gentra) and characterized on 0.8% agarose gel using 0.5 × TBE buffer (Sambrook & Russell 2001). Primers were designed for conserved regions of 16S rRNA (species-specific region), vacA (‘s’ and ‘m’ regions) and cagA of H. pylori (Table 1). The primer sequence for H. pylori 16S rRNA gene was designed as described by Karttunen et al. (1996) while primer sequences of vacA and cagA genes of H. pylori were designed as described by Mukhopadhyay et al. (2000). The restriction sites of Nco1 (CCATGG) and Hind III (AAGCTT) were inserted at the 5’ end of forward and reverse primers respectively of vacA (‘s’ and ‘m’ genes) and cagA genes. PCR of 16S rRNA, vacA (‘s’ and ‘m’ regions) and cagA genes was done separately. In the 50 µl reaction, 0.5 µM of each forward and reverse primer, 0.2 mM of each dNTP, 1 × PCR buffer, 1 U of Taq DNA polymerase, 2.5 mM MgCl2 and 100–200 ng of H. pylori chromosomal DNA of test strain were mixed. The conditions for PCR assay were set as follows: initial denaturation at 95°C for 5 minutes followed by 35 cycles at 95°C for 40 seconds, annealing according to the Tm of the primers for 30 seconds and elongation at 72°C for one minute followed by a final extension step for 15 minutes at 72°C. Amplified PCR products were analysed on 2.5% agarose gel using 0.5 × TBE buffer. The PCR-amplified genes were purified by QIA quick gel extraction kit (Qiagen, Germany). In the control PCR, all the reagents and conditions were the same except the E. coli genomic DNA was used instead of H. pylori genomic DNA.

**Preparation of biotin-labeled DNA probes**

For hybridization studies, PCR amplified and purified DNA of 16s rRNA, vacA gene (‘s’ and ‘m’ regions) and cagA gene
were labelled with Biotin-11-dUTP as described in DNA labelling kit (Fermentas Inc.). The unincorporated biotin labelled dUTP was removed by selective precipitation of labelled DNA with ethanol in the presence of ammonium acetate. The pellet was washed with 70% ethanol and dissolved in TE buffer (Sambrook & Russell 2001).

Colony/slot blot hybridization

The presence of *H. pylori* was also detected in drinking water, biopsy samples of chronic gastritis patients and vomiting fluids of gastric disease patients by hybridization studies. The *H. pylori* colonies were applied onto a nitrocellulose membrane and lysed as described by Sambrook & Russell (2001). The membrane was baked for 4 hours at 80 °C. The fixed DNA on the membrane was treated with non-radioactive biotin-labelled 16s rRNA DNA probes and detected by streptavidin-alakaline phosphatase as described above.

### Immunoassay

Anti-*H. pylori* antibodies were checked in sera of patients by applying it onto the chromatographic immunoassay strips (HUMAN, Germany) and the results were noted.
RESULTS AND DISCUSSION

Characterization of H. pylori

*Helicobacter pylori* was cultured on modified HP medium and detected by biochemical tests. The culture-based method sometimes may lead to false negative results due to contamination of microbial species present in the environment that contain the combination of antibiotic resistant genes. In order to remove this discrepancy the grown bacterial cultures were rechecked on another HP agar plate followed by urease and catalase tests and was also checked under a microscope to minimize the probability of mixed microbial growth.

It was found that 225 out of 600 drinking water samples (37.5%), 10 out of 18 gastric biopsies (55.55%) and 24 out of 50 vomiting fluid samples of gastric disease patients (48%) were positive for *H. pylori* on the HP agar medium. The samples that were positive for *H. pylori* were selected on the basis of the formation of a discrete red colour zone around the bacterial colony during incubation in 5% CO$_2$ at 37°C for 3–5 days. *Helicobacter pylori* colonies were picked up and replica plates were made (Figure 1). The samples were considered negative for *H. pylori* when its growth was not observed during 5 days of incubation under the same conditions. The results of *H. pylori* culture in different samples are shown in Table 2. *Helicobacter pylori* isolated from drinking water, gastric biopsies and vomiting fluids were Gram-negative in nature and this was further confirmed by urease and catalase tests. The sera of six chronic gastritis and 50 gastric disease patients were tested for the presence of antibodies against *H. pylori* and it was observed that five out of six and 31 out of 50 patients were positive for anti-*H. pylori* antibodies, which indicates and confirms the exposure of these patients to *H. pylori* infection.

PCR analysis and hybridization studies

A 139 bp gene segment of 16S rRNA *H. pylori* was amplified from drinking water, gastric biopsies and vomiting fluid samples, which confirmed that the samples were positive for *H. pylori*. (Figure 2). Our results are in agreement with the results of other studies which have reported that species-specific 16S rRNA *H. pylori* can be detected by direct PCR from water, stool and biopsy samples (Mapstone *et al.* 1993; Hulten *et al.* 1996; Li *et al.* 1996). In order to minimize mispriming in direct PCR, *H. pylori* was isolated and characterized by biochemical tests first and then confirmed with PCR assay for 16S rRNA by utilizing the purified *H. pylori* chromosomal DNA. The samples negative for 16S rRNA were analysed again for confirmation of the absence of *H. pylori* in isolated cultures.

PCR analysis showed that 90 out of 225 *H. pylori* positive drinking water samples (40%), 10 out of 18 gastric biopsy

![Figure 1](image1.png)  
*Figure 1* | Culture of *H. pylori*. (a) Growth of *H. pylori* from biopsy sample of chronic gastritis patient on specially formulated HP-agar medium. A red zone is formed around the bacterial colony due to a change in pH of the medium from acidic to alkaline by the hydrolysis of urea by urease enzyme. The plate was incubated for 65–70 hours at 37°C and 5% CO$_2$. (b) Positive colonies of *H. pylori* from drinking water samples were applied on HP-agar medium to make replica plates. The colour of the medium is changed from yellow to red. The plate was incubated for 96 hours at 37°C and 5% CO$_2$. 

![Figure 2](image2.png)  
*Figure 2* | PCR amplification of *H. pylori* from drinking water. (a) 1% agarose gel showing a 139 bp amplicon of 16S rRNA gene segment of *H. pylori*. (b) DGGE analysis showing a single band at 139 bp for *H. pylori*.
samples (55%) and 15 out of 50 vomiting fluid samples (30%) were positive for vacA (‘s’ and ‘m’ regions) and cagA genes (Table 3). The amplified genes of vacA (s-region) and vacA (m-region) are shown in (Figure 3(a, b and c)).

The presence of 16S rRNA, vacA (‘s’ and ‘m’ genes) and cagA genes in the test samples was also confirmed by slot blot and colony hybridization by using the DNA probes prepared from patient samples that confirmed the same type of H. pylori (Figure 5). Other provocative and stimulating results regarding the endoscopic specimens of six symptomatic patients who had undergone gastric biopsy revealed that five out of the six symptomatic patients were positive for H. pylori infection. On the basis of PCR and hybridization analysis it was observed that H. pylori from 90 drinking water samples, 10 gastric biopsies and 15 vomiting fluid samples had the same genotype of

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**Table 2** | Tests of H. pylori in chronic gastritis, vomiting fluids of gastric patients and public drinking water samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Samples</th>
<th>Urease test</th>
<th>Catalase test</th>
<th>PCR Assays</th>
<th>16S rRNA</th>
<th>VacA</th>
<th>CagA genes</th>
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<tr>
<td>Pt1, male, chronic gastritis</td>
<td>Fundus</td>
<td>–</td>
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<td></td>
<td>Antrum</td>
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<tr>
<td>Pt2, female, chronic gastritis</td>
<td>Fundus</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>Antrum</td>
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<td>+</td>
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<tr>
<td>Pt3, female, chronic gastritis</td>
<td>Fundus</td>
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<td>+</td>
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<td></td>
<td>Antrum</td>
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<tr>
<td>Pt4, female, chronic gastritis</td>
<td>Fundus</td>
<td>+</td>
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<td>Antrum</td>
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<tr>
<td>Pt5, male, chronic gastritis</td>
<td>Fundus</td>
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<td>Antrum</td>
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<tr>
<td>Pt6, male, chronic gastritis</td>
<td>Fundus</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>Antrum</td>
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</table>

Vomiting samples positive for H. pylori: $n = 15$

W samples positive for H. pylori: $n = 90$


(+), detected, (-), not detected.

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**Figure 2** | Agarose gel (2.5%) electrophoresis of 16S rRNA of H. pylori. Species-specific 16S rRNA of H. pylori was amplified and 139 bp amplified DNA is shown. Lane M: DNA ladder (bp), Lane 1–3: 16S rRNA amplified from H. pylori of biopsy samples of chronic gastritis patients, Lane 4, 5: 16S rRNA amplified from H. pylori of vomiting fluids, Lane 6, 7: 16S rRNA amplified from H. pylori of drinking water sample. Amplified 139 bp DNA indicates that all bacterial cultures are H. pylori (right arrow).
16S rRNA, vacA (‘s’ and ‘m’ genes) and cagA genes. The remaining 135 drinking water samples and nine from vomiting fluid are under investigation to explore the genotype of virulence genes.

Helicobacter pylori strains from different sources in the Lahore metropolitan area were studied to find the presence of virulence genes and also to correlate with the global genetic structure and evolution of H. pylori. The presence of H. pylori in drinking water samples of Lahore metropolitan city is approximately 40%, which is an alarming situation. The presence of a high percentage of H. pylori contamination in different water sources may be due to low socioeconomic factors such as a lack of public health education, poverty, overcrowding, poor sanitation and unsafe water supplies. The results for the presence of H. pylori in the test samples are in agreement with the results of other studies (Begue & Gonzalez 1998; Stark et al. 1999; Park et al. 2000; Mazari-Hiriart et al. 2001). The epidemiological survey also suggests that water is the potential source for the transmission of H. pylori infection (McKeown et al. 1999; Nurgalieva 2002) and our results advocate that water is one of the vectors required for prevalence and transmission of H. pylori (Sasaki et al. 1999). It is reported that 71% of individuals consuming municipal water acquired H. pylori in comparison with 12% of individuals who consumed boiled or filtered water. It is also explained that subjects who preferred home-cooked food (57%) showed a lower prevalence of H. pylori in saliva and biopsy samples respectively when compared with those (80%) who frequently ate out (Ahmed et al. 2006).

Reports on the survival of H. pylori outside the human niche are limited and need to be expanded. In our study it is concluded that municipal drinking water is the probable source of prevalence and transmission of H. pylori in humans. The PCR or colony hybridization based analysis described here will provide information to clinicians not only to help them to understand the proinflammatory power of the H. pylori strains but also to recommend the correct antibiotic therapy.

Table 3 | Percentage analysis of H. pylori in chronic gastritis, vomiting fluids of gastric patients and public drinking water samples

<table>
<thead>
<tr>
<th>Source</th>
<th>Total sample tested</th>
<th>Positive for 16s RNA</th>
<th>Positive for VacA (s, m), CagA regions</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsies</td>
<td>18</td>
<td>10</td>
<td>10</td>
<td>55.55</td>
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<tr>
<td>Vomiting fluid</td>
<td>50</td>
<td>15</td>
<td>15</td>
<td>30.00</td>
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<tr>
<td>Drinking water</td>
<td>60</td>
<td>225</td>
<td>–</td>
<td>37.50</td>
</tr>
<tr>
<td>Positive for 16s RNA</td>
<td>225</td>
<td>–</td>
<td>90</td>
<td>40.00</td>
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</tbody>
</table>

Figure 3 | Agarose gel (2.5%) electrophoresis of amplified vacA gene (‘s’ and ‘m’ regions) of H. pylori. (a) vacA gene (s-region, 259 bp DNA) was amplified from H. pylori of chronic gastritis patients and from vomiting fluids (right, arrow head). Lane M: DNA ladder (bp), Lane 1–3: H. pylori isolated from chronic gastritis patient 2 (fundus, body and antrum parts respectively), Lane 4, 5: H. pylori isolated from vomiting fluids. (b) vacA gene (s-region, 259 bp DNA) was amplified from H. pylori of drinking water samples (right, arrow head). Lane M: DNA ladder (bp), Lane 1–4: H. pylori from drinking water samples. (c) vacA gene (m-region, 650 bp DNA) was amplified from H. pylori of chronic gastritis patient, vomiting fluids and drinking water samples (right, arrow head). Lane M: DNA ladder (bp), Lane 1, 2: H. pylori isolated from chronic gastritis patient (fundus, and antrum parts respectively), Lane 3: H. pylori isolated from vomiting fluid, Lane 4, 5: H. pylori from drinking water samples.
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REFERENCES


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