

## 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*

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### 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; Gĩao *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 2000; 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 Gentra. PCR product 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 in HP medium for making HP-agar medium. HP and HP-agar media were autoclaved for 20 minutes at 121°C 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% CO<sub>2</sub> 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% H<sub>2</sub>O<sub>2</sub> 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% CO<sub>2</sub> 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 NcoI (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 MgCl<sub>2</sub> 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 T<sub>m</sub> 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

**Table 1** | Oligonucleotide primers for amplification of 16S rRNA, *vacA* and *cagA* genes

	Sequences (5' → 3')	Tm (°C)	Product size (bp)	Reference
For 16S rRNA				
1. RNA-F	GCGACCTGCTGGAACATTAC	56	139	Karttunen <i>et al.</i> 1996
2. RNA-R	GTCGTTAGCTGCATTGGAGA			
For <i>vacA</i> (s-region)				
3. U1-F	CCATGGAAATACAACAAACACAC	55	259	Mukhopadhyay <i>et al.</i> 2000
4. U1-R	CAAGCTTGAATGCGCCAAACTT			Acc. no. AF217727-AF217735
For <i>vacA</i> (m-region)				
5. J2-F	CCATGGCAATCTGTCCAATCAA	55	650	Mukhopadhyay <i>et al.</i> 2000
6. J2-R	GAAGCTTGCCTCAAAATAAATTCC			Acc. no. AF220110-AF220120
For <i>cagA</i>				
7. SG-F	GCCATGGTGGTCCTGGAGCTA	57	325	Mukhopadhyay <i>et al.</i> 2000
8. SG-R	AAGCTTGAAATCTTTAATCTCAGTT			Acc. no. AF202219-AF202225

F = Forward, R = Reverse, *vacA* = vacuolating cytotoxin gene A, *cagA* = cytotoxin associated gene A.

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 2 hours at 80°C under vacuum to fix the DNA on the membrane. The membrane was placed in prehybridization solution (50% formamide, 5 × SSC, 5 × Denhardt's reagent, 20 mM sodium phosphate buffer pH 6.5) and incubated for one hour at 42°C. After one hour, the prehybridization solution was discarded and hybridization solution (45% formamide, 5 × SSC, 1 × Denhardt's reagent, 20 mM sodium phosphate buffer pH 6.5, 20 l of heat-denatured biotin-labelled DNA probes separately) was added and incubated the blot for 20 hours at 42°C. The blot was washed with solutions in the following order: 2 × SSC con-

taining 0.1% SDS, 0.2 × SSC containing 0.1% SDS, 0.16 × SSC containing 0.1% SDS and finally with TBS (100 mM Tris-Cl, pH 7.5, 150 mM NaCl). After washing, the blot was placed in a buffer containing streptavidin-conjugated alkaline phosphatase for one hour with constant shaking. The blot was washed again and incubated with reaction buffer (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 50 mM MgCl<sub>2</sub>) containing nitroblue tetrazolium (5.0 mM) and 5-bromo-4-chloro-3-indolyl phosphate (5.0 mM) for 5 to 10 minutes at 37°C. The reaction was stopped with 20 mM EDTA as described by Thiel *et al.* (2002).

In slot blot hybridization, *H. pylori* chromosomal DNA isolated from gastric biopsy samples was absorbed onto a nitrocellulose membrane through Hybrislot blot apparatus according to the manufacturer's instructions (Bethesda Research Lab., USA). The blot 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-alkaline 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.

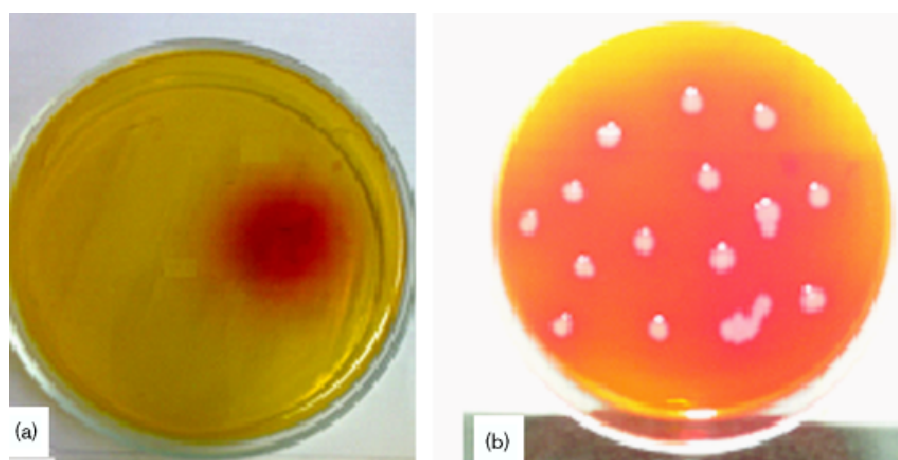
It was found that 225 out of 600 drinking water samples (37.5%), 10 out of 18 gastric biopsies (55.55%) and 24 out of 50 vomiting fluid samples of gastric disease patients (48%) were positive for *H. pylori* on the HP agar medium. The samples that were positive for *H. pylori* were selected on the basis of the formation of a discrete red colour zone around the bacterial colony during incubation in 5% CO<sub>2</sub> 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



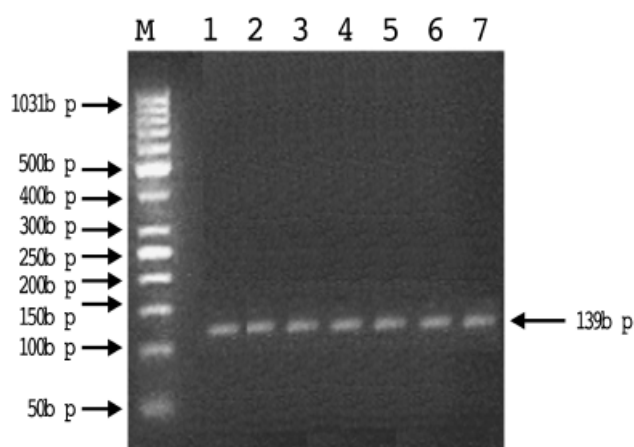
**Figure 1** | Culture of *H. pylori*. (a) Growth of *H. pylori* from biopsy sample of chronic gastritis patient on specially formulated HP-agar medium. A red zone is formed around the bacterial colony due to a change in pH of the medium from acidic to alkaline by the hydrolysis of urea by urease enzyme. The plate was incubated for 65–70 hours at 37°C and 5% CO<sub>2</sub>. (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<sub>2</sub>.



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

No.	Samples	Urease test	Catalase test	PCR Assays		
				16S rRNA	VacA	CagA genes
Pt1, male, chronic gastritis	Fundus	–	–	–	–	–
	Body	–	–	–	–	–
	Antrum	–	–	–	–	–
Pt2, female, chronic gastritis	Fundus	+	+	+	+	+
	Body	+	+	+	+	+
	Antrum	+	+	+	+	+
Pt3, female, chronic gastritis	Fundus	+	+	+	+	+
	Body	+	+	+	+	+
	Antrum	–	–	–	–	–
Pt4, female, chronic gastritis	Fundus	+	+	+	+	+
	Body	+	+	+	+	+
	Antrum	+	+	+	+	+
Pt5, male, chronic gastritis	Fundus	–	–	–	–	–
	Body	+	+	+	+	+
	Antrum	–	–	–	–	–
Pt6, male, chronic gastritis	Fundus	+	+	+	+	+
	Body	–	–	–	–	–
	Antrum	–	–	–	–	–
Vomiting samples positive for <i>H. pylori</i>	<i>n</i> = 15	+	+	+	+	+
W samples positive for <i>H. pylori</i>	<i>n</i> = 90	+	+	+	+	+

Fundus = upper end of the stomach, Body = middle portion of the stomach, Antrum = Lower portion of the stomach, Pt = patient, W = water.  
(+), detected, (–), not detected.



**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).

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 amplified *cagA* gene of *H. pylori* is shown in Figure 4(a and b). 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

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

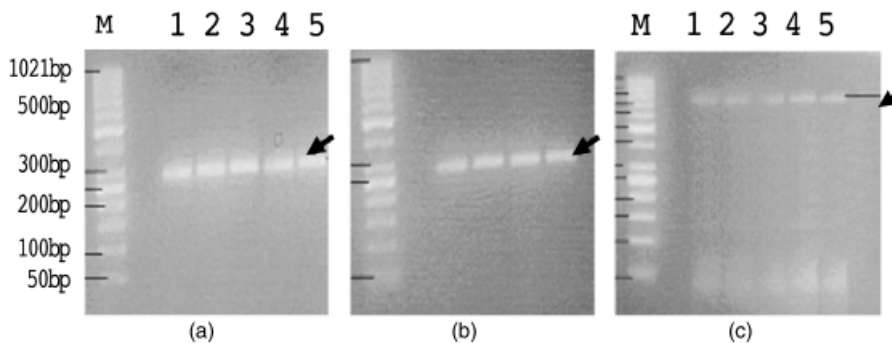
Source	Total sample tested	Positive for 16s RNA	Positive for <i>VacA</i> (s, m), <i>CagA</i> regions	Percentage (%)
Biopsies	18	10	10	55.55
Vomiting fluid	50	15	15	30.00
Drinking water	60	225	–	37.50
Positive for 16s RNA	225	–	90	40.00

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.

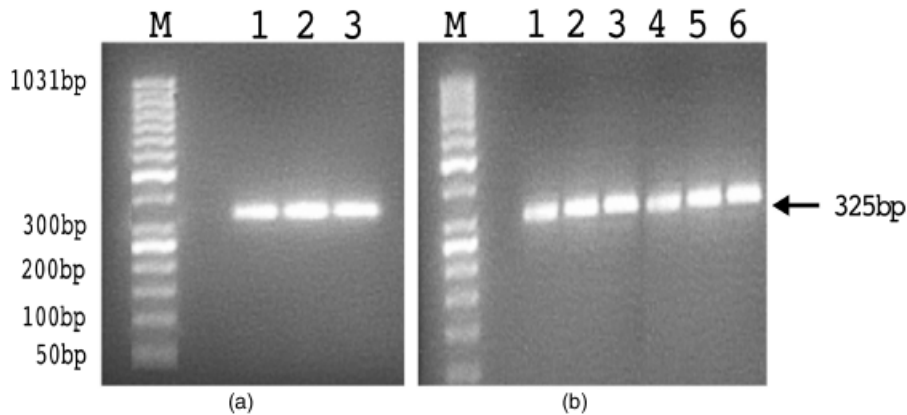
*Helicobacter pylori* strains from different sources in the Lahore metropolitan area were studied to find the presence of virulence genes and also to correlate with the global genetic structure and evolution of *H. pylori*. The presence of *H. pylori* in drinking water samples of Lahore metropolitan 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 & Gonzales 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).

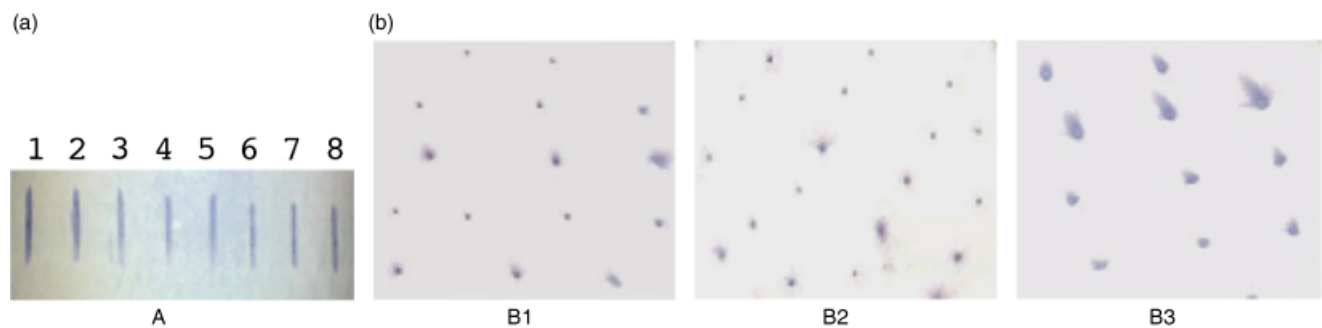
Reports on the survival of *H. pylori* outside the human niche are limited and need to be expanded. In our study it is concluded that municipal drinking water is the probable source of prevalence and transmission of *H. pylori* in humans. The PCR or colony hybridization based analysis described here 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.



**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.



**Figure 4** | Agarose gel (2.5%) electrophoresis of amplified *cagA* gene of *H. pylori*. (a) *cagA* gene (325 bp DNA) was amplified from *H. pylori* of chronic gastritis patient (right, arrow head): Lane M), DNA ladder (bp), Lane 1–3: *H. pylori* isolated from biopsy sample (fundus, body and antrum parts respectively). (b) *cagA* gene (325 bp DNA) was amplified from *H. pylori* of vomiting fluid and drinking water samples (right, arrow). Lane M: DNA ladder (bp), Lane 1–3: *H. pylori* from vomiting fluids, Lane 4–6: *H. pylori* from drinking water samples.



**Figure 5** | Hybridization of 16S rRNA, *vacA* ('s' and 'm' regions) and *cagA* genes. (a) Chromosomal DNA of *H. pylori* was isolated and absorbed on nitrocellulose membrane. The blot was hybridized with biotin-labelled PCR-amplified DNA of 16S rRNA of *H. pylori*. Slot 1–3: chromosomal DNA of *H. pylori* isolated from chronic gastritis patients, Slot 4–6: chromosomal DNA of *H. pylori* isolated from vomiting fluid, Slot 7, 8: chromosomal DNA of *H. pylori* isolated from drinking water samples. (b) Analysis of *H. pylori* by colony hybridization. *H. pylori* was isolated from drinking water samples and applied onto nitrocellulose membrane. The colonies were lysed and hybridized with biotin-labelled DNA probes of *vacA* (s-region), *vacA* (m-region) and *cagA* gene separately. The blue formazan reaction product confirmed the presence of *vacA* (s and m regions) and *cagA* genes. Strip B1: hybridize with *vacA* (s-region) DNA probes, Strip B2: hybridize with *vacA* (m-region) DNA probes, Strip B3: hybridize with *cagA* DNA probes.

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## REFERENCES

- Ahmed, K. S., Khan, A. A. & Ahmed, I. 2006 Prevalence study to elucidate the transmission pathways of *Helicobacter pylori* of a South Indian population. *Singapore Med. J.* **47**, 291–296.
- Al-Moagel, M. A., Evans, D. G. & Abdulghani, M. E. 1990 Prevalence of *Helicobacter pylori* infection in Saudi Arabia. *Am. J. Gastroenterol.* **85**, 944–948.
- Atherton, J. C. & Cao, P. 1995 Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. *J. Biol. Chem.* **270**, 17771–17777.
- Atherton, J. C. & Cover, T. L. 1999 Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. *J. Cell Mol. Med.* **128**, 2979–2982.
- Baker, K. H., John, P., Hegarty, R. B., Reed, N. A. & Diane, S. 2002 Effect of oxidizing disinfectants (chlorine, monochloramine and ozone) on *Helicobacter pylori*. *App. Environ. Microbiol.* **260**, 981–984.
- Bardhan, P. K. 1997 Epidemiological features of *Helicobacter pylori* infection in developing countries. *Clin. Infect. Dis.* **25**(5), 973–978.
- Begue, R. E. & Gonzales, J. L. 1998 Dietary risk factors associated with the transmission of *Helicobacter pylori* in Lima, Peru. *Am. J. Trop. Med. Hyg.* **59**, 637–640.
- Benson, H. J. 1988 *Microbiological Applications: Laboratory Manual in General Microbiology*. 7th edn. McGraw Hill Company Inc., USA.
- Brown, L. M. 2000 *Helicobacter pylori*: epidemiology and routes of transmission. *Epidemiol. Rev.* **22**, 283–297.



- Censini, S., Lange, C., Xiang, Z., Crabtree, J. E., Ghiara, P. & Borodovsky, M. 1996 *Cag*, a pathogenicity of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA* **93**(25), 14648–14653.
- Cesare, M. & Rino, R. 2001 Living dangerously: How *Helicobacter pylori* survives in the human stomach. *Mol. Cell Biol.* **2**, 457–460.
- Coghlan, J. G., Gilligan, D., Humphries, H. & McKenna, D. 1987 *Campylobacter pylori* and recurrence of duodenal ulcer – A 12-month follow-up study. *Lancet* **14**, 1109–1111.
- Degnan, A. J., Sonzogni, W. C. & Standridge, J. H. 2003 Development of a plating medium for selection of *Helicobacter pylori* from water samples. *App. Environ. Microbiol.* **69**(5), 2914–2918.
- Engstrand, L. 2001 *Helicobacter pylori* in water and waterborne routes of transmission. *J. Appl. Microbiol.* **90**, 80S–84S.
- Gião, M. S., Azevedo, N. F., Wilks, S. A., Vieira, M. J. & Keevil, C. W. 2008 Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. *Appl. Environ. Microbiol.* **74**(19), 5898–5904.
- Goodman, K. J. & Correa, P. 1995 The transmission of *Helicobacter pylori*. A critical review of the evidence. *Int. J. Epidemiol.* **24**, 875–887.
- Graham, D. Y. & Yamaoka, Y. 1998 *H. pylori* and *cagA*: relationships with gastric cancer, duodenal ulcer, and reflux esophagitis and its complications. *Helicobacter* **3**, 145–151.
- Hegarty, J. P., Dowd, M. T. & Baker, J. H. 1999 Occurrence of *Helicobacter pylori* in surface water in the United States. *J. Appl. Microbiol.* **87**, 697–701.
- Hulton, K., Han, S. W., Enroth, H., Klein, P. D., Opekun, A. R., Gilman, R. H., Evans, D. G., Engstrand, L. & El-Zaatari, F. A. 1996 *Helicobacter pylori* in the drinking water in Peru. *Gastroenterology* **110**, 1031–1035.
- Karttunen, T. J., Genta, R. M., Yoffe, B., Hachem, C. Y., Graham, D. Y. & El-Zaatari, F. A. 1996 Detection of *Helicobacter pylori* in paraffin-embedded gastric biopsy specimens by in situ hybridization. *Am. J. Clin. Pathol.* **106**, 305–311.
- Klein, P. D., Graham, D. Y., Gaillour, A., Opekun, A. R. & Smith, E. O. 1991 Water source as a risk factor for *Helicobacter pylori* infection in Peruvian children. *Lancet* **337**, 1503–1506.
- Li, C., Ha, T., Ferguson, D. A. Jr, Chi, D. S., Zhao, R., Patel, N. R., Krishnaswamy, G. & Thomas, E. 1996 A newly developed PCR assay of *H. pylori* in gastric biopsy, saliva, and feces. Evidence of high prevalence of *H. pylori* in saliva supports oral transmission. *Dig. Dis. Sci.* **41**, 2142–2149.
- Lu, Y., Redlinger, T. E. & Avitia, R. 2002 Isolation and genotyping of *Helicobacter pylori* from untreated municipal wastewater. *Appl. Environ. Microbiol.* **68**(3), 1436–1439.
- Mapstone, N. P., Lynch, D. A., Lewis, F. A., Axon, A. T., Tompkins, D. S., Dixon, M. F., Quirke, P. 1993 PCR identification of *H. pylori* in faeces from gastritis patients. *Lancet.* **341**, (8842), 447–447.
- Mazari-Hiriart, M., Lopez-Vidal, Y. & Calva, J. J. 2001 *Helicobacter pylori* in water systems for human use in Mexico City. *Water Sci. Tech.* **43**, 93–98.
- McKeown, I. P., MacDonald, A., Kabani, R., Brown, G., Coghlan, M., Dawood, J., Embil, M. & Sargent, G. 1999 *Helicobacter pylori* in the Canadian arctic: seroprevalence and detection in community water samples. *Am. J. Gastroenterol.* **94**, 1823–1829.
- Meyer-Ter-Vehn, T., Covacci, A., Kist, M. & Pahl, H. L. 2000 *Helicobacter pylori* activates MAP kinase cascades and induces expression of the proto-oncogenes *c-fos* and *c-jun*. *J. Biol. Chem.* **275**, 16064–16072.
- Mukhopadhyay, A. K., Kersulyte, D., Jeong, J. Y., Datta, S., Ito, Y., Chowdhury, A., Santra, A., Bhattacharya, S. K., Azuma, T., Nair, G. B. & Berg, D. E. 2000 Distinctiveness of genotypes of *Helicobacter pylori* in Calcutta, India. *J. Bacteriol.* **182**(11), 3219–3227.
- Nurgalieva, Z. 2002 *Helicobacter pylori* infection in Kazakhstan: effect of water source and household hygiene. *Am. J. Trop. Med. Hyg.* **67**, 201–206.
- Park, S. R., Mackay, W. G. & Read, D. C. 2000 *Helicobacter* sp. recovered from drinking water biofilm sampled from a water distribution system. *Water Res.* **35**, 1624–1626.
- Parsonnet, J. 1999 *Helicobacter and Gastric Adenocarcinoma. Microbes and Malignancy: Infection as a Cause of Human Cancers.* Oxford University Press, New York, NY.
- Passaro, D. J., Chosy, E. J. & Parsonnet, J. 2002 *Helicobacter pylori*: consensus and controversy. *Clin. Infect. Dis.* **35**, 298–304.
- Percival, S. L. & Thomas, J. G. 2009 Transmission of *Helicobacter pylori* and the role of water and biofilms. *J. Water Health* **7**(3), 469–477.
- Roosendaal, R., Kuipers, E. J., Brule, V. D., Pena, A. J., Meuwissen, A. J., Walboomers, S. G. & De Graaff, J. 1993 Detection of *Helicobacter pylori* DNA by PCR in gastrointestinal equipment. *Lancet* **341**, 900–910.
- Sambrook, J. & Russell, D. W. 2001 *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, NY, USA.
- Sasaki, K., Tajiri, Y., Sata, M., Fuji, T., Matsubara, F., Zhao, M., Shimizu, A. & Toyonaga, T. 1999 *Helicobacter pylori* in the natural environment. *Scand. J. Infect. Dis.* **31**, 275–279.
- Shiotani, A., Nurgalieva, Z. Z., Yamaoka, Y. & Graham, D. Y. 2000 *Helicobacter pylori*. *Med. Clin. North. Am.* **84**, 1125–1136.
- Stark, R. M., Gerwig, G. J., Pitman, R. S., Potts, L. F., William, N. A., Greenman, J. & Weinzwieg, I. P. 1999 Biofilm formation from *Helicobacter pylori*. *Lett. Appl. Microbiol.* **28**, 121–126.
- Telford, J. L., Covacci, A., Ghiara, P., Montecucco, C. & Rappuoli, R. 1994 Unravelling the pathogenic role of *Helicobacter pylori* in peptic ulcer: potential new therapies and vaccines. *Trends Biotech.* **12**, 420–426.
- Thiel, T., Bissen, S. & Lyons, E. M. 2002 *Biotechnology: DNA to Protein.* 1st edn. McGraw Hill Companies, Inc., NY, USA.
- VanDoorn, L. J., Figueiredo, C., Megraud, F. & Pena, S. 1999 Geographic distribution of *VacA* allelic types *Helicobacter pylori*. *Gastroenterology* **116**, 823–830.
- Westblom, T. U., Czinn, S. J. & Nedrud, J. G. 1999 Gastrointestinal disease and *Helicobacter pylori*: Pathophysiology, diagnosis and treatment. *Curr. Top. Micro. Immunol.* **241**, 311–313.