J. Med. Microbiol. — Vol. 51 (2002), 764–770

ISSN 0022-2615

MICROBIAL PATHOGENICITY

Detection of *Helicobacter pylori* DNA in the oral cavity and gastroduodenal system of a Venezuelan population

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Dental plaque has been suggested as a reservoir for *Helicobacter pylori* but the hypothesis that the oral microflora may be a permanent reservoir of *H. pylori* is still controversial. The aims of this study were to determine the presence of *H. pylori* DNA in the gastric antrum and dental plaque of a Venezuelan population by PCR and to investigate the relationship between this infection and the oral hygiene index. Thirty-two patients from the Hospital Universitario de Caracas, attending for routine gastroscopy, and 20 asymptomatic subjects (control group) were evaluated. The patients' gingiva and plaque were assessed by the gingival and plaque indices of Sillness and Löe. Supragingival plaque was analysed by a PCR for a specific internal urease gene. Gastric antrum biopsies were taken for histological examination and PCR. *H. pylori* was detected in antral samples from 24 (75%) of 32 patients, all of whom had chronic gastritis. *H. pylori* was also detected in dental plaque samples of 12 (37.5%) of the 32 patients. In 7 (58%) of these 12 patients, *H. pylori* was identified in the gastric biopsy. Seven patients with chronic gastritis carried *H. pylori* in dental plaque and antral samples. Of these patients, four also had dysplasia and one had metaplasia. Three subjects in the control group were positive by PCR. In the present study there was no correlation between *H. pylori* infection and dental hygiene, dental caries, periodontal disease or use of dentures. The oral cavity may be a reservoir for *H. pylori* infection and oral secretions may be an important means of transmission of this micro-organism. *H. pylori* in dental plaque may represent a risk factor for gastrointestinal re-infection and ulcer relapse after antibiotic therapy.

Introduction

Helicobacter pylori is one of the most common bacterial infections world-wide. It is associated with chronic gastritis and peptic ulcer disease and constitutes a major risk factor for gastric adenocarcinoma and lymphoma [1–4]. However, these diseases occur only in 15% of infected persons [5]. Of those infected, the development of disease is influenced by the virulence of the infecting *H. pylori* strain, the genetic susceptibility of the host and environmental co-factors [5].

Received 11 June 2001; revised version received 22 Jan. 2002; accepted 12 April 2002.
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Infection prevalence varies widely in different parts of the world, with average rates of 40–50% in western countries rising to >90% in developing countries. The mode of transmission of *H. pylori* is vigorously debated [6], although current evidence suggests that it is transmitted predominantly by direct person-to-person contact. Transmission routes may vary, being largely oral–oral in the industrialised world and faecal–oral in the developing world. More recently, it was suggested that *H. pylori* may exist in the natural environment [7].

Age of acquisition may be critical in determining the clinical outcome of infection [8]. The evidence for and against the oral–oral route of transmission is equivocal at present. Attempts to improve the accuracy of diagnostic tests for *H. pylori* are still being made

[9, 10]. Bacteria have been detected by various methods in dental plaque [11] and saliva [11, 12]. Dental plaque and saliva have been implicated as possible sources of *H. pylori* infection [11–13]. Despite efforts to improve the accuracy of diagnostic tests for *H. pylori* [14, 15], the detection of this bacterium in the oral cavity seems to be complicated.

The recovery rate of *H. pylori* from the oral cavity is controversial; while *H. pylori* could be isolated from the oral cavity in some cases [9, 12, 16], most attempts to culture the organism have failed [17, 18]. Some investigators [18] have suggested that non-cultivable coccoid forms of the organism may survive in the mouth. Many PCR assays have been developed for detecting *H. pylori* in the oral cavity [19], mostly based on the urease gene sequences and 16S ribosomal RNA genes. A high prevalence of *H. pylori* in the mouth has been detected by these PCR assays [11, 20, 21]. In contrast to these observations, no detection or low prevalence of the organism in the mouth has been reported by others [22, 23].

In most cases, patients with positive oral specimens have positive gastric biopsies, but many patients with gastric *H. pylori* do not exhibit oral co-infection [24]. These results are very inconsistent and not in agreement with the world-wide prevalence of *H. pylori* in the stomach [25]. Discrepant results concerning the prevalence of *H. pylori* in the oral cavity may be caused by different methods or different study populations [25]. Technical difficulties, different specificity and sensitivity of the primers used or a variable prevalence of *H. pylori* could explain these differences.

In view of the possibility of re-infection after successful treatment it is of great interest to identify natural reservoirs for this organism other than the stomach. The aim of this study was to evaluate the prevalence of *H. pylori* in dental plaque and gastric antral biopsy samples in a Venezuelan population, and to investigate the relationship between this infection and the oral index.

Materials and methods

Subjects

Thirty-two dyspeptic patients referred for upper gastrointestinal endoscopy and 20 asymptomatic subjects (control group) participated in this study. Informed consent was obtained from all study subjects. The protocol was approved by the ethics committee of the National Scientific and Technology Council (CONICIT). A detailed history and clinical assessment, including information regarding oral care such as teeth cleaning, number of visits to the dentist in the last 12 months and use of dentures, were obtained. The patients' gingiva and plaque were assessed by the gingival and plaque indices of Sillness and Löe [26]. All subjects were interviewed by means of a questionnaire for general demographic details and socioeconomic circumstances, history of peptic ulcer and dyspepsia, smoking, type of diet and alcohol consumption were recorded. The gingival index scores for the mesial, distal, buccal and lingual gingival areas around each tooth were given from 0 (no inflammation) to 3 (severe inflammation, ulceration, spontaneous bleeding). The plaque index measures the thickness of plaque at the gingival margin on the buccal, lingual, mesial and distal aspects of each tooth. The scores used are 0, none; 1, plaque that is not visible to the eye but can be seen on an instrument when scraped along the gingival margin on the tooth surface; 2, plaque that can be seen with the naked eye; and 3, gross accumulation of plaque. The caries index is based on the number of caries lesions for each subject. Exclusion criteria included treatment with antibiotics, bismuth-containing compounds or omeprazole at the time of endoscopy or within the previous 2 weeks.

Sample collection

Plaque samples were collected by scraping tooth surfaces before endoscopy of 32 patients with upper gastrointestinal symptoms and 20 asymptomatic subjects (control group). Two antral biopsy specimens were taken from each dyspeptic patient; one for histological examination and one for PCR assay.

PCR assay

For detection of *H. pylori* DNA by PCR, biopsy specimens and dental plaque samples were dissected and vortex mixed. The suspension was washed with sterile water and centrifuged at 12 000 *g* for 3 min. The pellet was resuspended in 500 μl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 nM EDTA, sodium dodecyl sulphate 0.5%); 10 μl of proteinase K (10 mg/ml) were added and incubated at 50°C for 20 h. The resulting pellet was allowed to dissolve in 100 μl of TE buffer (10 mM), Tris-HCl, pH 7.4, 0.1 mM EDTA, pH 8.0) for 20 h at 37°C. Samples were stored at −20°C before PCR amplification was performed. The following oligonucleotides, which were used as primers, were derived from sequenced urease genes as described by Clayton *et al*. [27]: HPU 1 (5′-GCC-AAT-GGT-AAA-TTA-GTT-3′) and HPU 2 (5′-CTC-CTT-AAT-TGT-TTT-TAC-3′). The expected product after amplification of the target sequence with these primers was 411 bp in length. An internal digoxigenin-labelled probe, HPU II (5′-ATT-GAC-ATT-GGC-GGT-AAC-3′), was used for hybridisation.

PCR amplification was performed in a total reaction volume of 50 μl: 10 μl of sample DNA and 40 μl of reaction mixture (50 mM KCl, 20 mM Tris-HCl, pH 8.3, 3.0 mM $MgCl_2$, gelatin 0.01% w/v, *Taq* polymerase 2.5 U, 0.2 mM dNTPs, 0.5 μM primer HPU 1, 0.5 μM primer HPU 2). After initial denaturation for 4 min at

94°C, 35 cycles of amplification were performed in an automatic thermal cycler. Each cycle consisted of three steps of 1 min each: denaturation at 94°C, annealing at 45°C and extension at 72°C. The PCR-amplified products were analysed by agarose gel electrophoresis; 12 μl of each amplified product was added to 3 μl of loading buffer (20 ml of glycerol 50%, 25 mg of bromophenol blue, 3 drops of 1 N NaOH) and subjected to electrophoresis in an agarose 0.7% gel. The gel was stained with ethidium bromide 0.5 μg/ml and examined under UV light for the presence of the amplified DNA. Samples were scored as positive when a band of 417 bp could be detected in agarose gel (Fig. 1) and were used for the hybridisation procedure.

Hybridisation

The specificity of the amplified DNA was confirmed by hybridisation with a digoxigenin-labelled probe (HPU II; DNA Enzyme Immunoassay). To coat the microtitration plate solid phase with an assay-specific probe, 100 μl of biotinylated probe solution were dispensed into each well. The plate was incubated for 18–22 h at 2–8°C and was washed to remove any excess unbound probe. Before starting the hybridisation procedure, amplified samples were denatured in the thermal cycler for 15 min at 94°C.

At the end of the washing step, 100 μl of ready-to-use hybridisation buffer were dispensed into each well (except the blank well), then 20 μl of amplified and denatured samples were dispensed into their respective wells and incubated for 1 h at 50°C (±1°C). The plate was washed three times and 100 μl of anti-dsDNA working solution were dispensed into each well and incubated for 30 min at room temperature. The plate was washed three times and 100 μl of chromogen substrate solution were dispensed and incubated for 30 min at room temperature in the dark, then 200 μl of blocking reagent were added.

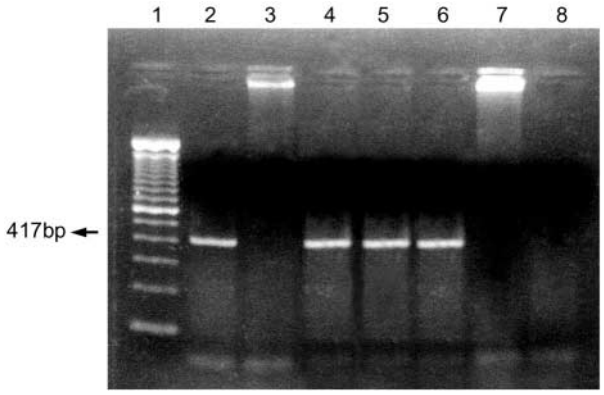

Fig. 1. PCR products generated with the primers HPU 1 and HPU 2 for the *cagA* gene. Lane **1**, mol. wt marker; **2**, DNA of *H. pylori* strain; **3**, total DNA from stomach sample; **4**, total DNA from dental plaque; **5**, total DNA from stomach sample; **6**, total DNA from dental plaque; **7**, total DNA from stomach sample; **8**, negative control (PCR mix without DNA).

The plate was read at 450 nm and 630 nm, the $OD_{630\ nm}$ absorbance value was substracted from the $OD_{450\ nm}$ value. The run was valid when the ratio of the positive control absorbance value to the negative control mean absorbance value was $\geqslant 10$ ($CP/CN \geqslant 10$).

Negative reagent controls consisted of tubes containing distilled water instead of the DNA sample. Positive controls were examined with each batch of amplified product. An *H. pylori* strain isolated from a urease-positive antral biopsy was used as a control for the PCR asssay.

Statistical analysis

The data were analysed by Fisher's test.

Results

Identification of H. pylori from gastric antrum and dental plaque by PCR

H. pylori was detected in antral samples from 24 (75%) of 32 patients, all of whom had chronic gastritis. This organism was also detected in dental plaque samples of 12 (37.5%) of the 32 patients. The prevalence of *H. pylori* in gastric antrum samples was significantly higher than in dental plaque samples from patients ($p > 0.001$). In 7 (58%) of the 12 patients with positive plaque samples, *H. pylori* was detected in the gastric antral biopsy, i.e., seven patients with chronic gastritis had *H. pylori* in dental plaque and antral samples. Of these patients, four had dysplasia and one metaplasia. In all, 5 (41.6%) of the 12 patients with positive *H. pylori* dental plaque assays had negative antral biopsy assays. Dental plaque samples from only three subjects in the control group were positive by PCR.

Association of detection of H. pylori in dental plaque and stomach by PCR with age and sex

Table 1 shows the relationship between *H. pylori* positivity, age of presentation and sex in dyspeptic patients. In the case of dental plaque, 4 (33.3%) of the 12 positive patients were in the age group 20–39 years, 6 (50%) in the age group 40–59 and 2 (16.6%) in the 60–79 age group. As regards the stomach samples, 9 (37.5%) of the 24 positive patients were in the first group (20–39 years old), 12 (50%) in the second group (40–59 years) and 3 (12.5%) in the remaining group (60–79 years). The prevalence of infection was significantly higher in the age group 40–59 years compared with the other groups ($p < 0.001$).

Female patients had a significantly higher prevalence of *H. pylori* infection in the stomach samples ($p < 0.001$). No significant difference was found for dental plaque.

Table 1. Prevalence of *H. pylori* infection in dental plaque and stomach of 32 dyspeptic patients in relation to age, sex, alcohol and smoking habits

Parameter	Number (%) of positive samples	
	dental plaque (n = 12)	stomach (n = 24)
Age (years)		
20–39	4 (33.3)	9 (37.5)
40–59	6 (50)	6 (50)
60–79	2 (16.6)	3 (12.5)
Sex		
Male	6 (50)	9 (37.5)
Female	6 (50)	15 (62.5)
Smoking habits		
Smoker	4 (33.3)	7 (29.6)
Non-smoker	8 (66.6)	17 (79.8)
Alcohol consumption		
Yes	4 (33.3)	4 (16.6)
No	8 (66.6)	20 (83.3)

Prevalence of H. pylori detected by PCR in dental plaque and gastric antrum and association with risk factor

Table 2 shows the prevalence of *H. pylori* detected by PCR in dental plaque and stomach samples with possible risk factors, including eating habits and waterborne spread. The prevalence of *H. pylori* in dental plaque was significantly higher in those who ate at home (11 of the 12 positive patients; 91.6%) compared with those who ate outside (1 of 12; 8.3%) ($p < 0.001$). Similar results were demonstrated for gastric antrum specimens, i.e., 19 (79.1%) of the 24 positive for *H. pylori* ate at home compared with 4 (16.6%) who ate outside ($p < 0.001$). There was no significant correlation with source of water supply.

From Table 1, it is clear that both non-smokers and non-drinkers had a higher PCR-positivity rate for *H. pylori* in both dental plaque and stomach biopsies than smokers and drinkers.

There was no significant correlation between dental hygiene or periodontal disease and the presence of *H. pylori* in dental plaque and stomach (Table 3). Patients with poor oral hygiene and periodontal status had a similar prevalence of *H. pylori* to patients with good-to-moderate dental hygiene. There was no significant correlation between caries index or use of dentures and the presence of *H. pylori* although patients without dental caries had a higher prevalence of *H. pylori* than patients with dental caries.

Table 2. Relationship between eating habits and water supply with presence of *H. pylori* detected by PCR in dental plaque and stomach

Parameter	Number (%) of positive samples	
	dental plaque (n = 12)	stomach (n = 24)
Eating habits		
Home	11 (91.6)	20 (83.3)
Outside	1 (8.3)	4 (16.6)
Water source		
Boiled	1 (8.3)	7 (29)
Municipal water system	5 (41.5)	2 (8.3)
Potable	2 (16.6)	5 (20.8)
Filtered	4 (33.3)	10 (41.6)

Discussion

The transmission routes of *H. pylori* have not been completely elucidated. Among various reports on the transmission of *H. pylori* [13, 28, 29], the faecal–oral and oral–oral routes have been suggested most strongly, as viable *H. pylori* has been isolated from faeces [28], saliva [13], dental plaque [20, 22, 30, 31] and various oral lesions [32, 33].

The present study detected *H. pylori* in 24 stomach specimens (75%) from 32 patients. This organism was also detected in the dental plaque of 12 (37.5%) of the 32 patients. The oral cavity has been proposed as a reservoir of infection on the basis of data from various cultural and PCR-based techniques, with varying results [9, 10, 34]. Some studies have shown frequent detection of *H. pylori* by PCR from oral specimens, particularly dental plaque [20, 31]. Others have reported only occasional detection [11] and many have consistently failed to demonstrate the presence of the organism in the mouth [17, 35]. The conflicting results regarding the incidence of *H. pylori* in dental plaque may be explained by differences in the methods of sample collection and detection techniques, or oral contamination caused by gastro-oesophageal reflux at the time of endoscopy.

The results of the present study demonstrated that the PCR assay was sensitive and specific for the detection of *H. pylori* in clinical specimens. Hammar *et al*. [15]

Table 3. Relationship between gingival and plaque index with presence of *H. pylori* detected by PCR in dental plaque and stomach

Index	Number (%) of positive samples	
	dental plaque (n = 12)	stomach (n = 24)
Gingival index		
0	2 (16.6)	5 (20.8)
1	4 (33.3)	6 (25)
2	2 (16.6)	3 (12.5)
3	4 (33.3)	10 (41.6)
Plaque index		
0	1 (8.3)	5 (20.8)
1	5 (41.6)	7 (29.1)
2	3 (25)	4 (16.6)
3	3 (25)	8 (33.3)

reported that their PCR assay detected the presence of *H. pylori* in four biopsy specimens that were negative by culture. Clayton *et al*. [27] reported that *H. pylori* was detected by PCR in 15 of 23 clinical gastric biopsy samples. Only seven of these samples were positive for *H. pylori* on culture. Mapstone *et al*. [11] found that eight patients with histologically normal gastric specimens had *H. pylori* DNA detectable by PCR. It is difficult to compare these findings because of the use of different primers, PCR protocols and sampling techniques.

In the present study, all patients belonged to families with low socio-economic status. Infection is inversely related to socio-economic class. A low level of education or a low level of socio-economic status, or both, are associated with an increase in the prevalence of *H. pylori* infection [19].

As expected, the prevalence of infection was low in the youngest age group (20–39 years; 37.5% of the positive patients), with a higher incidence of infection in the 40–59 age group (50%). The epidemiological data show that the rate at which a population acquires *H. pylori* infection is higher in developing countries than in developed countries [36].

A higher level of positivity was observed in the stomach samples from female (62.5%) than in those from male patients (37.5%). Similar differences were not observed for dental plaque. Other investigations have reported similar seroprevalences of *H. pylori* in male and female patients [37]. A study in rural Colombian Andes communities found higher infection in young male subjects [38]. Similar results were reported in New Zealand [39].

The results of the present study demonstrated that all the patients positive for *H. pylori* in dental plaque had chronic gastritis. Other diseases including low gastric dysplasia and intestinal metaplasia were observed in these patients. These results confirmed the strong correlation between the presence of *H. pylori* infection and gastritis, duodenitis and other diseases [1], and support the suggestion that dental plaque and saliva may be responsible for the transmission of the bacteria and possibly serve as a source of re-infection after eradication treatment [3]. However, the relationship between gastric symptoms and the presence of *H. pylori* in the oral cavity is unclear. It is possible that the oral cavity is the initial site of infection. *H. pylori* may persist in low numbers in the oral cavity for a long time and not colonise in the stomach [13]. In the present study, five patients whose gastric biopsies were negative for *H. pylori* by PCR were positive for the organism in their oral cavity. Furthermore, *H. pylori* DNA was detected by PCR in the dental plaque of 3 (15%) of 20 asymptomatic subjects. It is unlikely that these specimens were contaminated by extraneous *H. pylori* DNA for the following reasons. First, the samples were collected directly into new sterile containers before the patients underwent endoscopy. Second, sample collection, DNA extraction, pre-PCR preparation and PCR amplification were performed in separate rooms. Finally, PCR assay procedures were performed with a minimum numbers of steps, disposable tips and tubes were used and negative controls were regularly included in each test.

Although faecal–oral and oral–oral routes are probably important in the transmission of *H. pylori*, Klein *et al*. [40] showed that the waterborne spread of *H. pylori* might be an even more important source of infection in developing countries, especially if the water supply is vulnerable to bacterial contamination. Similarly, Hulten *et al*. [41] reported that *H. pylori*-specific DNA was detected in tap water and water in a water tank, but was not detected in well water. Although other investigations in Latin America [42] have confirmed the association of water-related factors with *H. pylori* prevalence, the findings in the present study do not confirm preliminary data on the correlation between *H. pylori* infection and the source of drinking water. In studies in Taiwan, no relationship between infection and water source was found [43].

H. pylori can be transmitted through the consumption of contaminated uncooked vegetables [41]. When eating habits were evaluated as a transmission pathway, a significantly higher prevalence of infection was observed in patients who ate at home. The reason for this finding is unclear, but probably the consumption of uncooked vegetables by these subjects is higher at home than outside and the vegetables may be contaminated by irrigation water. Other reports have shown that *H. pylori* seropositivity increased with consumption of uncooked vegetables in Chile [42], and children in rural Colombia who ate raw vegetables were at increased risk of infection [38]. In the present study there was no correlation of *H. pylori* infection with dental hygiene, dental caries or periodontal disease. Similar results have been reported by Hardo *et al*. [3]. As regards denture use, there was no correlation with *H. pylori* infection. In common with most other restorations, dentures provide hard surfaces that become colonised by oral bacteria; however, it is not known whether their presence increases the risk of infection with *H. pylori* [3].

H. pylori infection, smoking and alcohol consumption are risk factors for acid peptic disorders. However, in the present study, there was no significant association between *H. pylori* infection and smoking and alcohol use. Similar results have been reported by Hardo *et al*. [3], who demonstrated that smoking was not associated with a higher rate of *H. pylori* infection. In contrast, Rajashekhar *et al*. [44] reported that *H. pylori* infection is more common in smokers than in non-smokers. One possible explanation for these results is that the patients with peptic disorders decrease their alcohol consump-

tion and smoking, because these are risk factors for acidity and gastrointestinal pain.

The influence of oral *H. pylori* on the success of eradication therapy against gastric *H. pylori* was studied by Miyabayashi *et al*. [45], who demonstrated that *H. pylori* in the oral cavity affected the outcome of eradication therapy and was associated with a recurrence of gastric infection. If the gastric mucosa is recolonised by *H. pylori* from the oral cavity, which is not accessible to systemic antibiotic therapy, the control of dental plaque and other periodontal procedures should be recommended to these patients with gastritis.

In conclusion, the oral cavity may be a reservoir for *H. pylori* infection and oral secretions may be an important means of transmission of *H. pylori*. The presence of this micro-organism in dental plaque might represent a risk factor for gastrointestinal re-infection and ulcer relapse after antibiotic therapy.

This research was supported by grant no. 10-303792-96 from the Consejo de Desarrollo Científico y Humanístico, Universidad Central de Venezuela and National Council Scientific and Technology Research (CONICIT) S1-96001408 grant. Editorial review by Dr Mario Sánchez Borges is gratefully acknowledged.

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