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Oral cavity is not a reservoir for *Helicobacter pylori* in infected patients with functional dyspepsia

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Introduction: *Helicobacter pylori* infection is very prevalent in Brazil, infecting almost 65% of the population. The aim of this study was to evaluate the presence of this bacterium in the oral cavity of patients with functional dyspepsia (epigastric pain syndrome), establish the main sites of infection in the mouth, and assess the frequency of *cag*A and *vac*A genotypes of oral *H. pylori*.
Methods: All 43 outpatients with epigastric pain syndrome, who entered the study, were submitted to upper gastrointestinal endoscopy to rule out organic diseases. *Helicobacter pylori* infection in the stomach was confirmed by a rapid urease test and urea breath tests. Samples of saliva, the tongue dorsum and supragingival dental plaque were collected from the oral cavity of each subject and subgingival dental plaque samples were collected from the patients with periodontitis; *H. pylori* infection was verified by polymerase chain reaction using primers that amplify the DNA sequence of a species-specific antigen present in all *H. pylori* strains; primers that amplify a region of urease gene, and primers for *cagA* and *vacA* (m1, m2, s1a, s1b, s2) genotyping.
Results: Thirty patients harbored *H. pylori* in the stomach, but it was not possible to detect *H. pylori* in any oral samples using P1/P2 and Urease A/B. The genotype *cagA* was also negative in all samples and *vacA* genotype could not be characterized (s-m-).
Conclusion: The oral cavity may not be a reservoir for *H. pylori* in patients with epigastric pain syndrome, the bacterium being detected exclusively in the stomach.

Key words: dyspepsia; *Helicobacter pylori*; oral cavity

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Helicobacter pylori infection in the stomach of human beings is very common. Approximately 50% of the world's population is infected with *H. pylori* (28, 31, 36). This bacterium is considered the pathogen responsible for gastritis and peptic ulcers, and is a risk factor of gastric cancer (31).

In Brazil, the seroprevalence of *H. pylori* infection has been estimated at 65% (45). Its prevalence is associated with close contact (12), crowded places, poor hygiene (45), and housing conditions that also favor intrafamilial clustering and reinfection after bacterial eradication (14).

Transmission of the bacterium seems to occur from person to person by oral–oral (3), gastric–oral (30), and fecal–oral routes (4, 19) and the oral cavity may play a role in this process, or in the reinfection of the bacterium in the stomach (11). Nonetheless, the transmission process is not established, hindering the successful implementation of preventive measures (12).

It has been suggested that gastric juice may play an important role in the natural route of transmission, such as in gastritis, especially the acute stage, which is often accompanied by increased episodes of

intermittent reflux which may facilitate the passage of viable organisms into the mouth, from where they may be transmitted to other individuals (3). It is possible that oral colonization by *H. pylori* may precede gastric infection (21).

H. pylori has been isolated from saliva (9, 15, 18, 23, 39–41, 44), supragingival dental plaque (3, 19, 35, 40, 44), subgingival dental plaque (9, 16, 41, 44), tongue dorsum (12, 44), and the surface of oral cancer (32); nevertheless, results are controversial and detection of oral *H. pylori* has ranged from 0% (24, 34) to 100% (11, 39).

These discrepant results contribute to the question of whether the oral cavity is a permanent or transient reservoir for *H. pylori*. Methods for *H. pylori* diagnosis in the stomach are well established, such as urease test, histological examination, culture, urea breath test (27), and molecular biology, conversely, to assess *H. pylori* in the oral cavity, polymerase chain reaction (PCR) assays have a much greater sensitivity (39).

To distinguish *H. pylori* strains from each other in the mouth would be of epidemiological importance in the identification of the transmission route and the possible causes of reinfection after eradication therapy (40). The genotypes *cagA* and *vacA* correspond to virulent strains of *H. pylori*. Patients with antibodies against the cagA protein showed higher rates of both peptic ulcers and adenocarcinoma (4). Although studies have been conducted to type *H. pylori* strains in the stomach (14, 25), few data are available concerning the typing of *H. pylori* in the mouth (22, 40).

The aim of the present study was to assess the presence of this bacterium by PCR in the oral cavity of patients with epigastric pain syndrome, and check whether the oral *H. pylori* harbors *cagA* and *vacA* genes.

Materials and methods

Subjects

This study was approved by the local Ethics Committee. Patients gave written informed consent to participate in the study. Forty-three patients (17 men, 26 women; mean age 46.9 years) from the Outpatient Gastroenterology service of the Hospital das Clínicas of the University of São Paulo, School of Medicine were selected for this study. Patients with dyspeptic symptoms were submitted to an upper gastrointestinal endoscopy. Those with a normal examination were considered functional dyspeptic patients, subtype epigastric pain syndrome (42). Subjects with peptic ulcer or diabetes mellitus, those who were human immunodeficiency virus-positive, and patients who had taken antisecretory or antimicrobial agents within the previous 2 months were excluded from the study. Previous gastrointestinal surgery, pregnancy, or lactation and severe comorbidity were other exclusion criteria. Patients with a positive rapid urease test (from antral and corpus biopsies) and ^{14}C-urea breath test were considered *H. pylori*-positive for the stomach.

Oral sample collection

Saliva samples were taken using sterile cotton swabs which were gently rubbed against the oral mucosa (43 samples) (38). Sterile cotton swabs were also used to collect samples of microbiota from the tongue (43 samples) by scraping the posterior third of the dorsum (17). Supragingival plaque (43 samples) was obtained from two different teeth using sterile Grace curettes. Of all the subjects, 15 presented with periodontitis and had subgingival dental plaque collected after removal of supragingival plaque by inserting a sterile paper point into the periodontal pocket (probing depth >5 mm) for 20 s (17). The samples collected from the oral cavity were immediately placed into homemade urease test tubes for the rapid urease test (26). Oral samples positive for the rapid urease test were used for DNA extraction and PCR amplification.

Upper gastrointestinal sampling

All patients, fasted for 8 h then underwent endoscopy after narcosis (7). The channels of the endoscope (Olympus GIF 100–130 V, Tokyo, Japan) were cleaned and disinfected with 2% glutaraldehyde for 30 min after each use. Biopsies of the gastric antrum and corpus were taken for the urease test (14).

DNA extraction

DNA was extracted from the oral samples by a salting-out procedure according to a previously described technique (26).

PCR amplification

The PCR primers are listed in Table 1. Two sets of primers were used to detect *H. pylori* by PCR: P1/P2, which amplifies a 26-kDa antigen gene, present in all strains of *H. pylori* (18) and A/B for urease gene amplification (20): initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 93°C for 1 min, annealing at 57°C for 2 min, extension at 70°C for 2 min, and a final extension at 70°C for 10 min. Amplification conditions for *vacA* and *cagA*2 were: initial denaturation at 94°C for 5 min, followed by 27 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 30 s and a final extension for 7 min at 72°C (6). Two segments of the *cagA* gene were studied, *cagA*1, which is close to the promoter region and present in almost all of the *H. pylori* isolates (25), and *cagA*2 in the middle of the *cagA* gene, described previously by Covacci et al. (8) The amplification conditions for *cagA*1 were performed using 40 cycles with an annealing temperature of 52°C. PCR products were analysed by electrophoresis on 2% agarose gels, stained with ethidium bromide.

Table 1. Polymerase chain reaction primers used to amplify regions of *Helicobacter pylori* genes

Gene	Name	Primer sequence	Size	Location/accession
Ag	P1	5′-TGGCGTGTCTATTGACAGCGAGC-3′	298 bp	
	P2	5′-CCTGCTGGGCATACTTCACCATG-3′		220–242/M55507
Urease	A	5′-AAGCTTTTAGGGGTGTTAGGGGTTT-3′	295 bp	1291–1317/M60398
	B	5′-AAGCTTACTTTCTAACACTAACGC-3′		1563–1586
*m*1	VA3-F	5′-GGTCAAAATGCGGTCATGG-3′	290 bp	2741–2759/U05676
	VA3-R	5′-CCATTGGTACCTGTAGAAAC-3′		3011–3030/U5676
*m*2	VA4-F	5′-GGAGCCCCAGGAAACATTG-3′	352 bp	976–994/U05677
	VA4-R	5′-CATAACTAGCGCCTTGCAC-3′		1309–1327/U05677
*s*1a	SS1-F	5′-GTCAGCATCACACCGCAAC-3′	190 bp	866–884/U05676
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′		1037–1055/U05676
*s*1b	SS3-F	5′-AGCGCCATACCGCAAGAG-3′	187 bp	[1]
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′		1037–1055/U05676
*s*2	SS2-F	5′-GCTAACACGCCAAATGATCC-3′	199 bp	433–452/U29401
	VA1-R	5′-CTGCTTGAATGCGCCAAAC-3′		1037–1055/U05676
*cagA*2	CAG-1	5′-AGACAACTTGAGCGAGAAAG-3′	320 bp	1764–1783/X70039
	CAG-2	5′-TATTGGGATTCTTGGAGGCG-3′		2064–2083
*cagA*1	CagA-F2	5′-GATAACAGGCAAGCTTTTGA-3′	349 bp	157–176/AF001357
	CagA-R2	5′-CTGCAAAAGATTGTTTGGCAGA-3′		505–484

[1]According to Atherton et al. (5) there are no published coordinates for strains of this type.

DNA from gastric biopsies of positive urease tests, which had the genetic profile of *H. pylori* isolates determined in previous publications (25, 26), was used as the positive control for PCRs of each gene. Negative controls consisted of the reagents of PCR without DNA.

Results

H. pylori were found in the stomach of 30 patients (69.7%). A total of 144 samples were collected from the oral cavity, 80 (55.5%) of them were positive for rapid urease test; however, *H. pylori* DNA was not detected in any oral sample by PCR.

The PCRs performed with P1/P2 were not amplified (Fig. 1), even though genomic DNA was extracted from the oral samples (Fig. 2). These negative results were confirmed by a second independent PCR that assesses the urease gene. In spite of this, PCRs with primers for *cagA* and *vacA* genotyping were performed and were all negative.

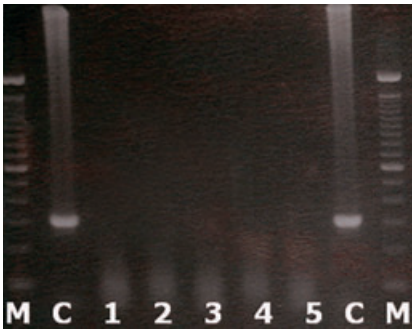

Fig. 1. Agarose gel electrophoresis of the polymerase chain reaction products of 26-kDa species-specific antigen gene for *Helicobacter pylori* (primers P1/P2) from five oral samples and positive controls from gastric biopsies. Lane M, 100-base-pair DNA ladder, Invitrogen (São Paulo, Brazil); lane C, positive controls from gastric biopsies; and lanes 1 to 5, oral samples.

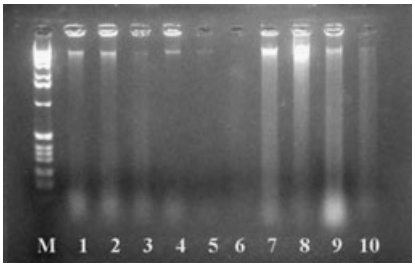

Fig. 2. Agarose gel electrophoresis of the genomic DNA extracted from oral samples. Lane M, 1-kilobase DNA ladder, Invitrogen (São Paulo, Brazil); lanes 1 to 10, genomic DNA extracted from oral samples.

Discussion

H. pylori can survive in the stomach partly because of the large amounts of urease produced by this bacterium compared with the amount produced by other microbes (29). The prevalence of the bacterium detected in the stomach (69.7%) was similar to the range found in a study conducted in São Paulo city, which detected a seroprevalence of 65% of *H. pylori* in blood donors (45). The fact that all patients presented dyspeptic symptoms but not all of them were infected agreed with the literature (13). Many asymptomatic patients have the infection in the stomach; on the other hand, subjects with epigastric pain are not always *H. pylori* hosts (5).

The oral environment is very different from the stomach, the primary residence of *H. pylori* (11). The growth of *H. pylori* in the oral cavity may be influenced by various factors, such as temperature, pH, oxidation–reduction potential, the availability of nutrients, salivary flow, and antimicrobial substances (39).

There are different points of view regarding the contention that the oral cavity is a reservoir of *H. pylori*. Studies that detected low percentages of *H. pylori* in the oral cavity consider that it is not a significant reservoir for this bacterium, which might have only a transient presence in the mouth (17). On the other hand, authors who found high amounts of *H. pylori* in their studied population consider it part of the normal microbiota of the oral cavity (17).

Our findings are in agreement with previous studies that reported an absence of *H. pylori* in the oral cavity (1, 24, 34). Other authors, in comparison, detected this microorganism in several sites in the mouth (3, 9, 19, 22).

Song et al. found from 55% to 82% prevalence of *H. pylori* in dental plaque, using nested PCR with other sets of primers (39). This disagreement with the ranges found in the present study can be attributed to different sample collection procedures and methodology, and the election of different primers. (34, 39).

H. pylori was not isolated on the dorsum of the tongue. This result differs from the prevalence found in the same site by Adler et al.; however, they had only selected patients with tongue pathologies, such as cancer, hyperplasia, and other diseases (1). Therefore, the different patient groups studied might be related to the dissimilar prevalence of *H. pylori* in the oral cavity (34, 39).

Some studies detected *H. pylori* in the oral microbiota of subjects with periodontitis, suggesting that periodontal pocketing and inflammation may favor colonization by this bacterium (16, 17, 41, 44). Fifteen patients presented periodontitis with periodontal pockets with a probing depth of between 4 and 6 mm. The conditions of gingival pockets with this probing depth may favor the growth of *H. pylori*, as reported by Umeda et al. (44), because of the lower oxygen concentration in these sites.

The environment of a gingival pocket seemed to be more appropriate for *H. pylori*, which is microaerophilic, than other sites of the oral cavity, as seen by Gebara et al., who detected higher amounts of *H. pylori* in the dental plaque of periodontal patients (17); nonetheless, *H. pylori* was not detected in the subgingival dental plaque from the 15 patients enrolled in the present study. The work by Asikainen et al. corroborates our findings. They assessed 1000 subgingival dental plaque samples from 336 subjects by PCR and did not detect *H. pylori* in any of them (5).

Okuda et al. found that strains of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* strongly coaggregated with *H. pylori* strains. However, the coaggregation between *H. pylori* and these periodontal disease-associated bacteria can be inhibited by salivary components (33).

Other studies reported that species of oral bacteria could inhibit the growth of *H. pylori* by producing bacteriocin-like inhibitory proteins, which may affect the viability of *H. pylori* in the oral cavity (2, 32). Aiba et al. found that *Lactobacillus salivarius* produced a high amount of lactic acid, which inhibits the growth of *H. pylori* in a mixed culture (2). Okuda et al. obtained similar results in an *in vitro* experiment. The authors found that the growth of *H. pylori* strains isolated from gastric samples and cultivated with supernatants of *Streptococcus mutans* and *Prevotella intermedia* was significantly lower than that of a culture of *H. pylori* without oral bacterium inoculation (32). They also observed that the morphological characteristics of *H. pylori* strains cultivated with this oral bacterium had changed. The viable cell number (colony-forming units) of *H. pylori* was significantly lower than the control, and the coccal cell number was higher (32).

Some investigators proposed that *H. pylori* may exist in a dormant, spore-like state that can be viable but non-culturable. They suggested that under

stress and nutrient deprivation, *H. pylori* undergoes a morphological transformation from actively diving and swimming spiral bacilli to inactive cocci (4). Some authors also suggested that this morphological change could result in failure to isolate *H. pylori* from the oral cavity (32, 37).

The use of PCR to detect *H. pylori* in the stomach and in the oral cavity has been widely accepted and has a greater sensitivity when compared with culture methods, which may underestimate the prevalence of *H. pylori* in clinical samples (39).

Some authors assessed oral *H. pylori* by *in vitro* urease test, inoculating oral samples into tubes containing *Campylobacter*-like organism test. The inoculated solutions that changed from yellow to red in 1 h were reported as being positive for *H. pylori* (35).

The present study showed divergent results: 80 samples were positive after 1 h of inoculation into the urease test tubes, but none of them were positive by the PCRs, demonstrating the possibility of false identification.

The oral cavity harbors a large spectrum of microorganisms, including other urease-producing bacteria, such as *Streptococcus vestibularis* and *Actinomyces viscosus* (35); this is different from the stomach, in which only *H. pylori*, a urease producer can survive (43).

We believe that, with oral samples urease-positive after 1 h, it is unsafe to consider the result as a true positive test. PCR is undoubtably more sensitive and specific (43).

The choice of a set of primers that target the 26-kDa species-specific antigen gene was based on earlier publications that had had positive results in detection of *H. pylori* in gastric biopsy samples (18, 25, 26) and oral samples (18). When used as a probe against numerous *H. pylori* strains and other gram-negative enteric organisms these primers only hybridized with *H. pylori* strains (18), making evident their specificities.

The study design allowed the use of other primers and the amplification of different target sequences along the bacterial genome. The second set of primers used targeted the urease C gene (*ureC*) (20), which was not amplified in any oral sample.

Similar results were obtained using other primers that genotype the cytotoxin gene *vacA* and pathogenicity genes *cagA*2 and *cagA*1. Previous studies detected a frequency of the *cagA*2 genotype in gastric samples from peptic ulcers patients of 75.3%, and of 26.2% in dyspeptic patients. *cagA*1 is the segment close to the promoter, reported by other studies to be present in almost all of the *H. pylori* isolates (25). However, it was not possible to detect these pathogenic genotypes in any oral samples in the present study. These results emphasize the absence of this bacterium in the oral environment of the population studied.

Moreover, results showed no apparent correlation between the prevalence of *H. pylori* in the oral cavity and infection in the stomach, as reported previously (10, 40). Furthermore, the *H. pylori* load in the oral cavity, when swallowed, could be too small to infect the stomach (10).

From the results obtained in the study, it is likely that *H. pylori* is a transient member of the oral microflora (24) of patients with epigastric pain syndrome. Several sites of the human oral cavity, such as the tongue dorsum, saliva, supragingival and subgingival dental plaque, may not be relevant reservoirs of *H. pylori.*

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