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Detection of *Helicobacter pylori* in gastric biopsies, saliva and dental plaques of dyspeptic patients from Marília, São Paulo, Brazil: presence of *vacA* and *cagA* genes

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Abstract: *Helicobacter pylori*, a gram-negative bacterium, possesses two important virulence factors: the vacuolating toxin (*vacA*), and the cytotoxin-associated gene product (*cagA*). The aim of the present study was to evaluate the presence of *H. pylori* in the stomach and oral cavity of humans and compare the *cagA* and *vacA* genotypes of *H. pylori* found in different samples (stomach, saliva and dental plaque) from the same patient. Gastric biopsies, saliva and dental plaques were obtained from 62 dyspeptic adults. DNA was extracted and evaluated for the presence of *H. pylori* and the alleles *cagA* and *vacA*. Persons with gastritis had a higher frequency of *H. pylori*-positive samples in the stomach while positive samples from gastric biopsies were significantly correlated with those from the oral cavity. There was a high *H. pylori* frequency in patients while the *cagA* gene was associated with *vacA* s1 alleles in gastric biopsies. Our results suggest a reservoir of the species in the oral cavity and that, in one patient, more than one *H. pylori* strain may exist in the saliva, dental plaque and stomach. We found a relationship between gastric infection and the bacterium in the oral cavity, with the cytotoxin genotype varying between saliva and dental plaque.

Key words: *Helicobacter pylori*, *cagA*, *vacA*, dental plaque, dyspeptic patient, saliva.

INTRODUCTION

Helicobacter pylori is a spiral-shaped gram-negative flagellate bacterium that has been implicated as a major human gastric pathogen responsible for gastritis and peptic ulcer disease (1). It is estimated that in developing countries like Brazil, the prevalence of *H. pylori* infection approaches 80% in adults versus 30 to 70% in developed countries (2-7).

The high prevalence of the disease has propelled an extensive search for *H. pylori* virulence factors. This research has led to the characterization of a vacuolating cytotoxin A (*vacA*) and an associated cytotoxin A (*cagA*). The *vacA* gene is present in all *H. pylori* strains, and comprises two main regions that show significant sequence variability between strains: the signal region (s1 or s2) and the middle region (m1 or m2). These two parts of *vacA* gene determine cytotoxin production and are associated with pathogenicity of the bacterium. The *vacA* s1/m1 allelic combination exhibits the highest activity, while s2/m2 and the rare s2/m1 combinations may be less toxic (8). The *vacA* protein induces vacuolation and apoptotic processes in epithelial cells, as well as immunosuppressive effects in immunological cells (9).

The *cagA* gene, present in approximately 60 to 70% of *H. pylori* strains, is located within a 40-kilobase DNA fragment known as the *cag* pathogenicity island (*cag* PAI). Several epidemiological studies have revealed that the presence of *cagA*-positive strains is correlated with a higher risk of developing peptic ulceration, gastric atrophy and gastric cancer

(10). In particular, most strains of *H. pylori* carrying the *vacA* alleles s1/m1 and *cagA* have been isolated from patients with severe gastric diseases, including duodenal and gastric ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma (11-13).

In addition to the genetic aspect of *H. pylori,* Nahar *et al.* (14) suggested that low socioeconomic status, crowded living condition, unfavorable dietary habits and poor personal hygiene may be associated with *H. pylori* infection. Although many aspects of the epidemiology of *H. pylori* infection are known, the modes of acquisition and transmission remain unclear; however, fecal-oral, oral-oral, and gastro-oral routes have been most often considered (15).

Some authors have reported the presence of *H. pylori* in the oral cavity, potentially representing a source of *H. pylori* for gastric reinfection after therapy (16). This possibility was supported by Miyabayashi *et al.* (17) who confirmed the relationship between gastritis induced by *H. pylori* infection and oral colonization of the bacterium, besides trying to clarify the resistance mechanisms of oral *H. pylori* to typically triple anti-*H. pylori* therapy employed to eradicate the pathogen from the stomach. They reported that patients with oral *H. pylori* presented a significantly elevated gastric reinfection risk following successful therapy.

Rasmussen *et al.* (18) suggested a relationship between gastric infection and the presence of this bacterium in the oral cavity. Despite this, *H. pylori* was present in the oral cavity with variable distribution between saliva and dental plaques, suggesting the existence of a reservoir for the species and a potential association with gastric reinfection.

Dental plaque and saliva constitute a potential reservoir for infectious microorganisms and have been shown to harbor at least 400 different types of bacteria, including *H. pylori* (19). However, the detection and comparison of the presence of the *cagA* gene and *vacA* alelles of *H. pylori* in dental plaque, saliva and the stomach has not been thoroughly evaluated.

The purpose of this study was evaluate the presence of *H. pylori* in the stomach and oral cavity of patients with gastritis and to compare *cagA* and *vacA* genotypes of *H. pylori* among dental plaque, saliva and the stomach in the same patients, in an attempt to elucidate the infection mechanisms of *H. pylori* and verify its possible transmission route.

MATERIALS AND METHODS

Patients and Collection of Biological Samples

Sixty-two patients (27 males and 35 females with a mean age 52.56 years) presenting with recurrent abdominal pain participated in the study. All subjects were recruited from the Ambulatory Endoscopy Unit of Marília Medical School, São Paulo, Brazil.

All individuals signed an informed consent in order to be included in the study and the local ethics committee approved the study.

Three biopsies of the gastric antrum were collected during gastroscopy. One of the specimens was used for the rapid urease test, the second for histological examination and the other for the molecular analysis. The endoscopic forceps were sterilized in 2% glutaraldehyde solution for a minimum of 20 minutes between each pair of experimental procedures. Saliva and plaque samples were collected prior to the endoscopic examination of each subject. Salivary flow was self-stimulated by each patient and 3 mL of saliva was collected in test tubes. Multiple samples of dental plaque from different sites throughout the oral cavity (incisors, canines, premolars and molars) were taken from each subject with a sterile curette and transferred to a test tube containing 15 mL of PBS (phosphate-buffered saline) pH 7.4.

It is worth mentioning that our group recently published a study suggesting a possible relationship between the presence of *H. pylori* in the oral cavity and the stomach (18). The authors would like to emphasize that the present study is a more detailed analysis than the previous one and aims to elucidate the genetic diversity of *H. pylori* in humans using the same samples.

DNA Isolation and Preparation of DNA Probe

DNA from the gastric biopsies was extracted using the QIAamp® tissue kit (Qiagen, Germany), according to the manufacturer´s instructions**.** DNA extraction from the dental plaques and saliva were performed by the method of Rasmussen *et al.* (18) as previously described (16, 20). The probe was synthesized using the Gene Images AlkPhos® Direct Labelling kit (GE Healthcare, UK), according to the manufacturer´s instructions and the description of Rasmussen *et al.* (18).

Identification of *Helicobacter pylori* by Urease Test, Histological and Molecular Analysis

An antral biopsy from each patient was incubated in premade broth (TUPF; Laborclin, Brazil) for the urease test (RUT) immediately after collection. The test was considered *H. pylori*-positive when the color of the solution changed from yellow to orange, pink, or purple within four hours of incubation at 25°C.

The biopsies for the histological examination were fixed in formalin and stained with HE (hematoxylin and eosin) and Giemsa. The histological parameters were graded using the criteria described in the Sydney system for analysis of chronic inflammation, polymorphonuclear activity and intestinal metaplasia (21).

PCR assays were performed with approximately 100 ng of total DNA using one set of oligonucleotides (Hpx1/Hpx2) ($^{5'}$-CTGGAGARACTAAGYCCTCC-$^{3'}$ and $^{5'}$-GAGGAATACTCATTGCGAAGGCGA-$^{3'}$) that amplifies a 150-bp fragment corresponding to 16S-rRNA from *H. pylori*. The cycling program was initiated with a denaturation step of 94°C for five minutes followed by 40 cycles at 94°C for one minute, 59°C for one minute, 72°C for one minute, followed by a final incubation at 72°C for seven minutes (22). In each experiment, positive (strain 26695) and negative (water) controls were included. After separation with electrophoresis in 2% agarose gels, PCR products were blotted to a Hybond N+ membrane and hybridized with the specific PCR fragments labeled by chemiluminescence (GE Healthcare, UK). The assay was considered positive when the PCR product was present.

Detection of *vacA* and *cagA* Gene

The *vacA* gene, "s" and "m" region genotyping and *cagA* gene detection were performed by PCR and Southern blotting, using one set of oligonucleotides for each gene fragment. For *cagA* detection, the previously described primers Cag1/Cag2 ($^{5'}$-ATGACTAACGAAACTATTGATC-$^{3'}$ and $^{5'}$-CAGGATTTTTGATCGCTTTATT-$^{3'}$) amplified 232 fragments (23). The *vacA* "s" and "m" regions were genotyped with the previously described primer sets SA/SC ($^{5'}$-ATGGAAATACAACAAACACAC-$^{3'}$ and $^{5'}$-CCTGARACCGTTCCTACAGC-$^{3'}$), and MA/MB ($^{5'}$-CACAGCCACTTTYAATAACGA-$^{3'}$ and $^{5'}$-CGTCAAAATAATTCCAAGGG-$^{3'}$), respectively (23-25). The SA/SC primers amplified "s1" fragments of 176 bp and "s2" fragments of 203 bp. The "m1" fragments were 400 bp and "m2" fragments 475 bp (23-25). The same amplification condition was used for every gene. The cycling program was initiated with a denaturation step of five minutes at 94°C, followed by 40 cycles of 94°C for one minute, 53°C for one minute, 72°C for one minute, followed by a final incubation at 72°C for seven minutes.

Statistical Analysis

The chi-square, Fisher's exact and Kappa tests were used to analyze differences in the prevalence and cytotoxin genotypes of *H. pylori* in gastric biopsies, saliva and dental plaque samples. The significance level was set at $p < 0.05$.

Statistical analyses were performed using the statistical package SPSS 11.5.1 (USA).

RESULTS

Detection of *Helicobacter pylori* in Gastric Biopsies, Saliva and Dental Plaque by Urease, Histological and Molecular Analysis

H. pylori was detected in antral biopsies in 50/62 (80.6%) patients via PCR and hybridization by Southern blotting. The histological analysis revealed the presence of *H. pylori* in only 19 subjects (30.6%) whereas the urease test detected *H. pylori* infection in 27 patients (43.5%). All the samples in which the histology and urease test demonstrated the presence of *H. pylori* were also positive by PCR and Southern blot hybridization. The detection of *H. pylori* in gastric antrum samples was significantly higher when the Southern blotting technique was used as compared to the histological or urease tests (chi-square – $p = 0.0001$).

The histology of 45 (72.6%) patients revealed chronic gastritis while 17 (27.4%) had normal gastric mucosa. Of the former, 41 (91%) were found *H. pylori*-positive by PCR and Southern blot hybridization.

H. pylori was found in the saliva of 26 (41.9%) and in the dental plaque of 29 (46.7%) patients (Table 1). The oral cavity was operationally defined as positive when the saliva or dental plaque was positive for *H. pylori*. Sixteen patients presented with *H. pylori* confined to the stomach, while *H. pylori* DNA was concurrently detected in the gastric biopsy, saliva and dental plaques of

Table 1. Southern blotting detection of *H. pylori* DNA and *cagA* and *vacA* genes in *H. pylori* from gastric biopsies, saliva and dental plaque

Samples	n	H. pylori +	cagA +	vacA				
				m1	m2	s1	s2	s1/s2
Gastric biopsies	62	50	*36	*28	22	*38	12	–
Saliva	62	26	13	–	–	14	8	4
Dental plaque	62	29	7	–	–	14	8	7

n: number of individuals; * $p < 0.0001$

ten patients. Twenty-four subjects harbored *H. pylori* in gastric biopsies and the oral cavity. *H. pylori* DNA was not detected in 12 gastric biopsy samples, but six of these 12 cases were found to have *H. pylori* in the oral cavity.

No statistically significant difference was observed between strains in the saliva and dental plaque (chi-square = $p < 0.58$). Despite this, a statistically significant difference was observed between gastric biopsies and the oral cavity (chi-square = $p < 0.0001$).

Detection of *cagA* and *vacA* Alleles of *Helicobacter pylori* Isolates from Gastric Biopsies, Saliva and Dental Plaque

We isolated 25 strains of the common *vacA* genotype s1/m1 and only 12 strains of *vacA* s2/m2. Twenty percent (ten strains) of the *H. pylori* bacterium were found to have *vacA* genotype s1/m2, and three strains showed *vacA* genotype s1/m1/m2, suggesting a coinfection with two different *H. pylori* strains. The *cagA* gene was detected in 36 patients infected with *H. pylori*. Of these 36, 32 were associated with the toxin-producing *vacA* s1 and can be associated with chronic gastritis while only four *cagA*-positive strains were *vacA* s2 ($p < 0.0001$).

The *cagA* gene was detected in 13 saliva samples. Of the 13 patients who were *cagA*-positive, seven were associated with *vacA* s1, two were found to be *vacA* s2 and four were s1/s2. The middle region (m1 or m2) of the *vacA* gene was genotyped only in *H. pylori* gastric isolates. The middle region of *vacA* was not detected in *H. pylori* isolates from these samples, which is likely related to the heterogeneity in the *vacA* gene as previously described (8, 10).

From patients with positive dental plaque, only seven of 29 strains had the *cagA* gene and three were associated with the toxin-producing *vacA* s1. However, 11 of the patients with *cagA*-negative samples demonstrated the *vacA* genotypes s1.

When associated with positivity for *H. pylori*, the presence of *cagA* and *vacA* genes and their alleles, there was approximately 85% disagreement among the three materials, just five patients showed the same genetic profile, according to the genes analyzed, in the oral cavity and biopsies, highlighting a wide variety of strains, a mixed colonization in the same host with an unequal distribution, independent of gastric biopsy, saliva or dental plaque. No statistically significant relationship was found between the presence of *cagA* and *vacA* alelles in saliva or dental plaques. However, there was a statistically significant relationship between the presence of *cagA* and *vacA* genotypes s1/m1 (Fisher = $p < 0.001$). The association between the presence of *cagA* and *vacA* alleles in all strains is described in Table 1.

DISCUSSION

Our methodologies of using PCR in concordance with Southern blotting are supported by Li *et al.* (26) and Song *et al.* (27) who found a significant increase in sensitivity after incorporating the Southern blotting technique. The high sensitivity and specificity of the PCR test with Southern blotting and hybridization in our study did not reveal false positives, false negatives or contamination. This is attributed to the fact that the probe was synthesized from genomic DNA of *H. pylori* culture by PCR, which excludes false results (28, 29). In addition, we used more amplification cycles, optimized buffers and conditions while all *H. pylori*-positive samples were genotyped to confirm the presence of *H. pylori*.

Out of 62 samples, *H. pylori* was detected in 50 of the gastric biopsies in which the most virulent *vacA* genotype s1/m1 was most common. The *cagA* gene was detected in 72% of our patients, a level comparable to two other studies of Brazilian patients where, respectively, 70% and 79% of samples were found to be *cagA*-positive (3, 30). Several studies have described the associations between s2/m2 and *cagA*-negative strains and between s1/m1 and *cagA*-positive strains, both supported by the results of the present study.

Twenty-three and 31 biopsies that were positive as determined by the Southern blotting method were negative when analyzed with the RUT and histological analysis, respectively. This is expected because the sensitivity of the Southern blotting technique is much greater for the detection of *H. pylori* when compared to the RUT and histological analyses. In addition to reduced sensitivity, the histological analysis may be biased by the subjectivity of the assessment, inter-observer variation or human error (21). Researchers and clinicians should be aware of these potentially serious limitations when using histological analysis. The observation of de Francesco *et al.* (31) of *H. pylori* in 20% of histology-negative biopsies by PCR is similar to our findings. This increased the prevalence of the pathogen from 43 to 58% in their patient samples.

In the oral cavity, *H. pylori* had been demonstrated earlier, although the exact time course of the infection is not fully elucidated. It is not clear whether the oral cavity is a permanent or a temporary reservoir for this microorganism. In our study 26 (41.9%) and 29 (46.7%) of the patients had *H. pylori* in saliva and dental plaque, respectively.

Li *et al.* (26) verified 75% positivity among saliva specimens, similar to the findings of Wang *et al.* (32), who found the bacterium in 71% of the saliva samples in their study. Al Asqah *et al.* (33) also found a significant association between the presence of *H. pylori* in the stomach and in dental plaque. In a study of 120 dyspeptic patients, *H. pylori* was detected in all of the samples from gastric biopsies and saliva (34). The results of the present study, along with the previous work, suggest that saliva may serve as a method of infection transmission and may also lead to gastric re-infection after treatment of the disease.

Some studies have suggested that the detection of *H. pylori* in saliva and dental plaque by PCR may reflect the secretion of dead bacteria from the digestive tract. However, the fact that six patients in the current study produced gastric biopsies negative for *H. pylori* by PCR and Southern blotting while testing positive for *H. pylori* in their oral cavity corroborates the hypothesis that the mouth may be a natural reservoir of *H. pylori*.

Berroteran *et al.* (35) also reported that *H. pylori* was detected in the oral cavity of five patients whose gastric biopsies were negative for *H. pylori* by PCR whereas *H. pylori* was also present in 15% of (three of 20) asymptomatic subjects. Li *et al.* (36) suggest that this phenomenon may occur because *H. pylori* may persist in low numbers in the oral cavity for many months or years without colonizing the stomach or because the oral cavity is the initial site of infection.

The *vacA* s1 alelle was found in 53% of the *H. pylori* isolates from saliva and 48% of those from dental plaque, while the *cagA* gene was detected in 13 (50%) saliva samples and only 7 (24.1%) dental plaque samples. Unlike the gastric biopsy samples, no association was found between *vacA* s1 alleles and the presence of *cagA* gene in saliva or dental plaque samples. We detected four saliva and seven dental plaque samples with *vacA* alelles s1 and s2 simultaneously, suggesting the presence of multiple *H. pylori* strains in the oral cavity of the same subject. Wang *et al.* (32) found that the *cagA* gene was present in 23% of the saliva samples whereas the *vacA* s1 allele was present in 77% of the saliva samples. We found the *cagA* gene was highly prevalent in the saliva samples from our subject pool, but the prevalence of the gene was only 23% in the dental plaques. In all, approximately 70% of the oral-cavity samples were positive; however, there was a distinct difference between such positivity in saliva and dental plaques.

We also found significant genotypic diversity among *H. pylori* cytotoxins from the stomach, saliva and dental plaque samples, which corroborates the work of Wang *et al.* (32). The heterogeneity of *H. pylori* may be due to genotypic variation among strains and or variations in *H. pylori* populations within an individual host, as proposed by Blaser (37).

In the present study we verified that the strains found in the stomach are apparently more virulent than those in the oral cavity, an observation not explored in the literature. Thus, we can suggest that

this selection is attributable to two explanations: *H. pylori cagA* + and "s1" have efficient adaptive mechanisms, which allow a rapid and effective colonization of the stomach, thus inhibiting the growth of less adapted organisms and suggesting a possible biological interaction called amensalism among different strains of *H. pylori*. The second explanation may be that the oral cavity can harbor at least 400 different types of bacteria and, given such bacterial heterogeneity, the adaptive mechanisms might not be efficient, thus allowing the growth of other *H. pylori* strains.

The frequency of *H. pylori* in the oral cavity differed in relation to previous studies. The difference in the presence of the pathogen in the oral cavity may be the result of differences in study populations, oral health status, clinical presence of *H. pylori* infection, the type and number of clinical samples, complexity of the oral flora or detection methods (38). Kignel *et al.* (16) reported that the levels of the bacterium in the oral cavity may be too low to be detected by one round of PCR and they emphasized that the region of the mouth from which the samples are collected can influence the prevalence of the microorganism. Song *et al.* (27) determined that the presence of *H. pylori* was 82% in the molar region, 64% in pre-molar region and 59% in the incisor region. Loster *et al.* (39), suggested that this discrepancy may be attributable to the fact that the dental plaque in the posterior region of the oral cavity is less oxygenated, thus providing optimal conditions for the survival of *H. pylori.*

CONCLUSION

In summary, there is a high prevalence of *H. pylori* and the main virulence factor genes in Brazilian *H. pylori* isolates, namely the *cagA* gene, appears to be associated with *vacA* s1 alleles in gastric biopsies. Our results suggest a correlation between gastric infection and the presence of the bacterium in the oral cavity. However, *H. pylori* was present in the oral cavity with variable cytotoxin genotype *cagA* and *vacA* alleles; furthermore, there was a variable distribution between saliva and dental plaque, suggesting the existence of a reservoir of the species, which can lead to gastric re-infection. Additionally, more than one *H. pylori* strain may exist in the saliva, dental plaque and stomach of the same patient.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Ethics Research Committee of Sacred Heart University (process n. 056/2005). Moreover, all study subjects signed an informed consent in order to be included in the study.

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