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BRIEF ARTICLE

Helicobacter pylori in dental plaque and stomach of patients from Northern Brazil

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Abstract

AIM: To establish whether virulence factor genes *vacA* and *cagA* are present in *Helicobacter pylori* (*H. pylori*) retrieved from gastric mucosa and dental plaque in patients with dyspepsia.

METHODS: Cumulative dental plaque specimens and gastric biopsies were submitted to histological examination, rapid urease test and polymerase chain reaction (PCR) assays to detect the presence of *cagA* and *vacA* polymorphisms.

RESULTS: Detection of *H. pylori* from dental plaque and gastric biopsy samples was greater by PCR compared to histological examination and the rapid urease test. DNA from *H. pylori* was detected in 96% of gastric mucosa samples and in 72% of dental plaque samples. Sixty-three (89%) of 71 dental plaque samples that were *H. pylori*-positive also exhibited identical *vacA* and *cagA* genotypes in gastric mucosa. The most common genotype was *vacAs1bm1* and *cagA* positive, either in dental plaque or gastric mucosa. These virulent *H. pylori* isolates were involved in the severity of clinical outcome.

CONCLUSION: These pathogenic strains were found simultaneously in dental plaque and gastric mucosa, which suggests that gastric infection is correlated with the presence of *H. pylori* in the mouth.

Key words: *Helicobacter pylori*; Gastric mucosa; Dental plaque; *cagA*; *vacA*

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INTRODUCTION

Helicobacter pylori (*H. pylori*) gastric infection is considered one of the most common human infections[1]. It occurs in half of the world's population[2,3] and is the most common cause of adenocarcinoma of the distal stomach[4].

The risk in developing gastric cancer is believed to be related to differences among *H. pylori* strains and the inflammatory responses mediated by host genetic factors[3]. Therefore, it is presumed that severe gastric diseases are seen more often in patients who have been chronically infected with *H. pylori* isolates that bear both the *cagA* and *vacA* s1/m1 genes[1,5,6].

H. pylori is known to be acquired early in life[7,8], and is likely to be transmitted from person to person. The transmission is supported by crowded living conditions, accompanied by poor hygiene and intra-familial clustering. Nevertheless, the exact manner of transmission is not completely understood[4,9,10].

The accepted evidence is that the *H. pylori* strains reach the stomach by ingestion through the mouth, and because of its non-invasive nature, the stomach is the definitive site for colonization[10]. Evidence of transmission *via* the oral route includes the high prevalence of *H. pylori* among African children whose mothers premasticate their food before offering it to them[11], and to the higher prevalence of the infection in people who share chopsticks[12].

The presence of *H. pylori* in the oral cavity was first reported in 1989 when the bacterium was cultured from dental plaque of a patient with gastric disease associated with *H. pylori* infection[13].

Dental plaque is a biofilm that is formed by a microbial community of multiple species and represents a strategy to allow survival and evolution of microbial co-aggregation in a dynamic equilibrium, in a favorable environment with selective advantages[14,15]. Hence, bacterial biofilms are considered as sanctuaries, due to being protected from host defense mechanisms and antibiotic therapy. Additionally, antibiotic resistance is increased due to horizontal gene transfer[14,15].

The purpose of this study was to evaluate the prevalence of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, in dental plaque and gastric antral biopsy samples in dyspeptic patients from a North Brazilian population.

MATERIALS AND METHODS

Patients and sampling

We studied the presence of *H. pylori* in the stomach and dental plaque in 99 adult patients (69 women and 30 men; mean age: 37 years, age range: 17-59), who underwent upper gastrointestinal endoscopy due to gastric problems. Most of them were of lower socioeconomic status and lived in the state of Pará, Brazil.

None of the patients had received antimicrobial drugs, H2-receptor antagonists, acid pump inhibitors, non-steroidal anti-inflammatory drugs, or any medication for at least 60 d before sampling, to avoid interference with *H. pylori* detection methods. During endoscopy, three antral gastric biopsy sections were taken from the stomach of each patient. One biopsy was analyzed by molecular methods, and the other sections were also analyzed with histological methods and the rapid urease test. Before endoscopic examination, cumulative dental plaque samples were collected by scraping tooth surfaces with sterile curettes and transferred into tubes that contained physiological saline solution. Dental plaque samples were frozen immediately and stored at -20℃ until required for DNA extraction. This study was approved by the Ethics Committee of Hospital Universitário João de Barros Barreto, Belém, PA, Brazil. All patients gave their informed consent to participate in the study.

Histological evaluation

The biopsy specimens were fixed in 10% buffered formalin solution, embedded in paraffin, cut into sequential 0.4-μm sections, and stained with hematoxylin and eosin (H&E). The presence of *H. pylori* in the sections was determined by using a modified Gram staining protocol and taking into consideration its morphological characteristics: curved and spiral form and intense purple coloring. The histological parameters were graded (0-3) using the criteria described in the update Sydney classification system[16] for analysis of chronic inflammation, polymorphonuclear activity and intestinal metaplasia.

H. pylori detection by the rapid urease test in the stomach and dental plaque

Additional fresh biopsies as well as dental plaque samples were placed in the gel of the CLOtest as recommended by the manufacturer (Uretest; Renylab, Barbacena, MG, Brazil). After 1 h, the CLOtest was inspected for a change in color. Yellow was interpreted as negative, indicating absence of urease. Red or pink was interpreted as positive. For comparison, a similar reading of each biopsy was also performed after 3 and 24 h. Results of the rapid urease test were compared with polymerase chain reaction (PCR) results and histological examination.

DNA isolation

Total DNA was extracted from frozen gastric biopsy and dental plaque specimens using the following procedure[17]: 10 μL proteinase K (Amresco, Cleveland, OH, USA) and 300 μL lysis buffer (200 mmol/L Tris-HCl, 25 mmol/L EDTA, 300 mmol/L NaCl, 1.2% SDS) were added to the biopsy or dental plaque pellets specimens. The mixture was incubated at 55℃ for 12 h. The lysate was extracted with an equal volume of phenol-chloroform, precipitated with isopropanol, and washed with 70% ethanol. The DNA pellet of each sample was dried and resuspended in 200 mL sterile distilled water. DNA extracts were stored at -20℃. Extensive care was taken to avoid contamination during all steps of collecting and preparing the samples.

PCR amplification and detection of amplified DNA products

PCR amplification for detection of *H. pylori* DNA in dental plaque and gastric mucosa was performed as described previously[18]. Briefly, one set of primers (p1-F and p2-R) that amplifies a 26-kDa antigen gene (fragment of 298 bp) present in all strains of *H. pylori* was used to detect bacteria[18]. The previously described F1-F and B1-R primers[19] were used to detect *cagA* gene. The amplification of *vacA* gene was performed by PCR with oligonucleotide primers described by Atherton *et al*[1], *vacA* signal (*vacA s1a, s1b* or *s2,* primers SS1- F, SS3-F, SS2-F/VA1-R, respectively) sequences and middle regions (*vacA m1* or *m2,* primers VA3-F/VA3-R and VA4-F/VA4-R).

All PCR mixtures were prepared in a volume of 25 μL that contained 0.5 nmol/L each primer; 1 × PCR buffer; 1.5 nmol/L $MgCl_2$, sterilized water, 0.2 nmol/L deoxynucleoside, 1.25 μL *Taq* DNA polymerase Platinum (Invitrogen Life Technologies, São Paulo, Brazil), and 2 μL DNA sample. The mixtures were placed in a thermal cycler. PCR amplification was performed under the following conditions: initial denaturation at 95℃ for 2 min, followed by 35 cycles of denaturation at 95℃ for 1 min, annealing and extension for 1 min, and final extension at 72℃ for 10 min. Annealing temperatures were set at 58℃ for primers VA3-F/VA3-R, VA4-F/VA4-R, F1/B1, and at 63℃ for SS1- F, SS3-F, SS2-F/VA1-R. Negative (sterile water) and positive (positive strain) controls were used in all reactions. PCR products were visualized by electrophoresis in 2% agarose gel, stained with ethidium bromide, and examined under UV illumination.

Statistical analysis

Data were analyzed with Biostat 4.0 version software[20]. Mann-Whitney and G tests were used to assess the association between *H. pylori* isolates in dental plaque and stomach and the distribution of *vacA* and *cagA* genotypes in both these specimens. Differences were considered statistically significant for P values less than 0.05.

RESULTS

Identification by PCR of *H. pylori* DNA in gastric biopsies and dental plaque

H. pylori was found in gastric biopsies of 96% (95/99) of the patients and the detected frequency of this organism in dental plaque was 72% (71/99). Both were detected by PCR, but none of the four patients that lacked *H. pylori* in the stomach had this organism in dental plaque. This difference was statistically significant, which indicated that the detection frequency of *H. pylori* in dental plaque was lower than in the stomach ($P < 0.001$).

Relationship of *H. pylori* DNA in dental plaque and stomach with age and sex

In the relative distributions of age and sex of the patients studied, no significant difference was observed between the groups with or without positive DNA for *H. pylori* simultaneously in the gastric mucosa and dental plaque. Nine of 30 (30%) men and 16 of 69 (27.5%) women were *H. pylori*-negative in dental plaque. Among these, 19 of 62 individuals belonged to the 17-39 years age group and eight of 33 to the 40-59 years group. Furthermore, three individuals in the first group and one in the second were *H. pylori*-negative in dental plaque and gastric mucosa.

Relationship of *H. pylori* DNA in dental plaque and stomach with clinical and histopathological parameters

All patients presented an inflammatory process in the gastric mucosa, with a diagnosis of functional dyspepsia. Ulcers or premalignant lesions, such as intestinal metaplasia, which were considered as lesions of higher severity, occurred in 17% (17/99) of the patients. Among these, 82.4% of cases harbored *H. pylori* in the stomach and dental plaque.

The genotypic frequencies of *vacA* and *cagA* of the positive *H. pylori* samples in the gastric biopsies and dental plaque DNA samples in relation to the endoscopic clinical diagnosis and histopathological findings are shown in Table 1. The *vacAs1m1/cagA*-positive genotype was found in 51 of 95 *H. pylori* gastric biopsies, which comprised 44 gastritis specimens and seven peptic ulcer samples, with 46 patients being *H. pylori*-positive in dental plaque DNA samples.

The presence of *H. pylori* mixed strains with *vacA s1*, *s2*, *m1*, *m2* and *cagA*-positive multiple genotypes was detected in 12 of 95 gastric biopsies of patients with gastritis and their corresponding dental plaque DNA samples.

The *vacAs2m2/cagA*-positive genotype was observed in four of 95 *H. pylori* gastric biopsies, which included one patient with peptic ulcer and three with chronic gastritis. The *vacAs2m2/cagA*-negative genotype was found in 28 of 95 *H. pylori* isolates from 27 gastritis specimens and one peptic ulcer sample, with 13 patients being simultaneously *H. pylori* positive in dental plaque DNA samples.

When comparing all these genotypes, there was 66% (63/95) agreement between *H. pylori* biopsy isolates and their corresponding dental plaque DNA samples. In addition, seven of nine patients with peptic ulcer and eight of 86 with chronic gastritis and diagnosed with intestinal metaplasia or gastric atrophy expressed *H. pylori* isolated with *vacAs1m1*- and *cagA*-positive genotypes, which indicated more severe gastritis and peptic ulceration (Table 1). In the same way, in relation to the histopathological data of these patients, a significant association has been observed between the presence of a bacterial virulence genotype and the high levels of inflammation and the neutrophilic activity, as well as an increased risk of developing atrophy and intestinal metaplasia in the gastric mucosa.

Detection of *H. pylori* in dental plaque and gastric biopsies by different diagnostic tests

In addition to the evaluation of *H. pylori* by PCR assay, bacteria in the stomach were also detected by the histological examination and the rapid urease test. However, the analysis of urease test indicated gastric colonization by *H. pylori* in only 49% of the patients. Similarly, evidence of *H. pylori* was detected in 52% of dental plaque speci-

Table 1 Frequency of *vacA* and *cagA* genotypes in patients positive for *H. pylori* DNA in dental plaque and stomach

H. pylori genotypes					Histopathological parameters[a]						Endoscopic diagnosis[b]		Total
Stomach		Dental plaque			DI		NA		IM	*H. pylori*	PU	G (+H)	
vacA	*cagA*	C	D	N	1	2/3	1	2/3	+	+			
s1m1	+	42	4	5	21	30	26	25	8	22	7	34/(10)	51
s1m1, s2m2	+	12	-	-	5	7	6	6	3	5	-	10/(2)	12
s2m2	+	-	-	4	2	2	2	2	1	1	1	2/(1)	4
s2m2	-	9	4	15	25	3	22	6	-	11	1	22/(5)	28
Total		63	8	24	53	42	56	39	12	39	9	72/(14)	95

DI: Degree of inflammation; NA: Neutrophilic activity (histopathological scores: 1, light; 2, moderate; 3, intense); IM: Intestinal metaplasia; *H. pylori*: *Helicobacter pylori*; G: Gastritis; H: Hiatus hernia; PU: Peptic ulcer; N: Polymerase chain reaction (PCR) negative for *H. pylori* in dental plaque; C: Identical genotypes in the stomach and dental plaque; D: Distinct genotypes in the stomach and dental plaque. [a]G tests (Yates) = 16.83, $P < 0.001$ (DI: *s1m1 cagA* + *x s2m2 cagA*-); G tests (Yates) = 4.86, $P = 0.027$ (NA: *s1m1 cagA* + *x s2m2 cagA*-); [b]G tests (Yates) = 4.36, $P = 0.037$ (disease: *s1m1 cagA* + *x s2m2 cagA*-).

Table 2 Results of the different diagnostic tests for *H. pylori* detection in gastric biopsies and dental plaque samples *n* (%)

Techniques used in the specimens	*H. pylori*		Total
	Positive	Negative	
Dental plaque[a]			
PCR	71 (72)	28 (28)	99
Urease	48 (52)	45 (48)	93
Stomach[b]			
PCR	95 (96)	4 (4)	99
Histological[1]	39 (48)	43 (52)	82
Urease	47 (49)	49 (51)	96

[1]31 of 39 were also urease positive; [a]G tests (Yates) = 7.39, $P = 0.006$; [b]G tests (Williams) = 76.82, $P = 0.0001$ (PCR/Histological/Urease); G tests (Yates) = 57.14, $P = 0.0001$ (PCR/Histological); G tests (Yates) = 58.48, $P = 0.001$ (PCR/Urease); G tests (Yates) = 0.001, $P = 0.97$ (Urease/Histological).

Table 3 Comparison of *H. pylori* isolates in the stomach and dental plaque, by PCR assay *n* (%)

Genotypes		Stomach	Dental plaque
vacA	*cagA*		
s1am1	+	16 (17)	16 (22)
s1bm1	+	35 (37)	26 (37)
s2m2	+	4 (4)	-
s1bm1/s2m2 (mixed infection)	+	12 (13)	16 (22)
s2m2	-	28 (29)	9 (13)
s1bm2/s2m2 (mixed infection)	-	-	4 (6)
		95 (100)	71 (100)

Mann-Whitney test: $U = 15.00$, $Z(U) = 0.48$, $P = 0.63$.

mens, using the rapid urease test (Table 2). There were significant differences among *H. pylori* positivity detected by PCR and the other methods used ($P < 0.001$).

Comparison of *H. pylori* vacA and cagA genotypes in dental plaque and gastric mucosa samples

Overall, 63 (89%) of 71 dental plaque samples, which were positive for the presence of the bacteria, revealed identical *H pylori* virulence factors in relation to *vacA* and *cagA* genotypes in the gastric mucosa. *H. pylori* isolates found in 71% (67/95) of gastric mucosa specimens were *cagA*-positive and 82% (58/71) were also *cagA*-positive for dental plaque, with significant correlations among the prevalence of *H. pylori cagA*-positive isolates in the stomach and dental plaque samples (Table 3).

In relation to the *vacA* gene, the *s1m1* genotype had a higher frequency, with 54% (51/95) of gastric biopsies and 59% (42/71) of dental plaque samples However, the *vacAs2m2* subtype combination was found among the patients that harbored *H. pylori* in 34% (32/95) of the stomach and in 13% (9/71) of the dental plaque samples, whereas *vacAs1bm1/s2m2* genotypes were detected in 13% (12/95) of gastric biopsies and 22% (16/71) of dental plaque samples (Table 3). The most common genotype was *vacAs1bm1* and *cagA*-positive, either in dental plaque (37%) or gastric mucosa (37%).

DISCUSSION

H. pylori infections of the stomach are common worldwide. These bacteria are also found in dental plaque, where the presence of this organism may be very low and the numbers of microorganisms appear to vary from one site to another within the mouth[21].

The present study detected DNA from *H. pylori* in 95% of clinical gastric biopsy samples. Likewise, as described in previous studies[6,22,23], the occurrence is very high in this community. In fact, *H. pylori* infection is influenced by precarious sanitation and low socioeconomic level, so that the prevalence of *H. pylori* infected subjects in Brazil can reach over 80%[24,25]. Furthermore, 72% of subjects with stomach infection showed a concomitant presence of *H. pylori* in dental plaque by PCR assay.

The high detection rate for *H. pylori* DNA in the dental plaque is sufficiently significant to support the assumption that *H. pylori* reaches the oral cavity by reflux[21], assisted by the finding that no positive case was found in dental plaque that was negative in gastric mucosa. This result is especially important because, in our sample, only untreated patients were selected, since treatment would likely eradicate the bacteria from the stomach but rarely from dental plaque, due to known resistance to systemic therapy in dental plaque, as has been reported by Gurbuz *et al*[26].

Similar results have been reported by Banatvala *et al*[13], who have verified a 63% correlation between *H. pylori* in the stomach and in dental plaque samples in patients from London. Also, Song *et al*[27] have found *H. pylori* in 97% (41/42) of dental plaque samples obtained in a German population.

Other studies have suggested a positive association between dental plaque and gastric *H. pylori*[28-32], but some others have failed to find *H. pylori* in oral samples[33-35]. In support of the former studies, our results indicate that the frequency of *H. pylori* in dental plaque is related to the existence of gastric infection.

In the patients studied, no significant difference was observed in the rate of *H. pylori* DNA in dental plaque and gastric mucosa of the diverse age groups. These results are similar to the infection patterns in developing countries[7,36]. A similar result was found in relation to sex, which indicated that there was no relation between sex ratio and *H. pylori* infection in the stomach and dental plaque DNA samples. However, Berroteran *et al*[34] have detected a higher level of *H. pylori* in gastric samples from women than from men, but in dental plaque, this difference was not observed.

On the other hand, these data indicate that, in the strains that harbor *vacAs1m1, cagA* genotypes are involved in the severity of gastritis and the final outcome of *H. pylori* infection. In addition, *H. pylori* isolates with *cagA* and *vacAs1m1* genotype are associated with an increased risk of developing distal gastric adenocarcinoma[37].

In the present study, different methods were used for detecting *H. pylori* in dental plaque and gastric mucosa. In this regard, the PCR assay has been demonstrated to be more efficient for the detection of *H. pylori* in clinical specimens, compared to the rapid urease test in dental plaque and gastric biopsies, as well as to the histopathological examination of the gastric mucosa. This result is supported by other studies[27,30,34,38].

Dönmez-Altuntaş and Güven[39] have detected higher specificity and sensitivity of the PCR assay in relation to the urease test, with 93.8% *vs* 65.6% of DNA from *H. pylori* being positive in both tests respectively. Zsikla *et al*[40] have reported that *H. pylori* DNA could be detected by PCR in 20.8% of biopsies with chronic gastritis but without histologically detectable bacteria. These results agree directly with our findings. Despite this, one of the critical points of the studies using PCR is the absence of uniformity of results, due to the establishment of distinct laboratory procedures[27,41-43]. Controlled trials with robust methodology are required to prove if there is any clinical or epidemiological relevance to the colonization of this bacterium in the oral cavity.

The molecular characterization of the *H. pylori* virulence factors, *cagA* and *vacA*, has revealed the existence of indistinguishable genotypes from oral cavity and gastric mucosa samples of the same patient[44]. Our results showed that, in 89% of the 71 cases in which the dental plaque samples were positive for bacteria, the vacA and cagA genotypes were similar to those in the gastric mucosa (Table 3). Thus, these data suggest that similar types of *H. pylori* isolates, in relation to *vacA* and *cagA* genotypes, are present in the mouth and stomach.

We found that the most common genotype was positive for *vacAs1bm1* and *cagA*, either in dental plaque or gastric mucosa (Table 3). In addition, the *H. pylori* isolates that were *vacAs1m1*/*cagA*-positive were detected in dental plaque at a higher percentage than the *vacAs2m2*/*cagA*-negative genotype. It seems that *H. pylori* in dental plaque is related more to severe gastrointestinal diseases, such as gastroesophageal reflux, which could facilitate oral colonization by *H. pylori* with a *vacAs1m1*/*cagA*-positive genotype.

In this context, several investigators have reported that reinfection is caused by the strains in the oral cavity, because they have isolated identical strains of *H. pylori* in the oral cavity and stomach[32,45-49]. Therefore, it seems necessary to apply additional molecular tests in the dental plaque to assist the diagnosis and therapy of gastric infection.

Finally, based on our findings we cannot exclude the possibility that transient colonization of the mouth with *H. pylori* may occur by reflux activities within symptomatic patients, which leads to the spread these organisms by direct transmission from person to person. In this way, it is a risk factor for the occurrence of diseases in the oral cavity and the stomach.

COMMENTS

Background

Infection by *Helicobacter pylori* (*H. pylori*) remains one of the most prevalent chronic bacterial diseases worldwide, with a distribution related to the degree of economic development in each country. The organism is transmitted from person to person, and some researchers have suggested that the oral cavity is an important reservoir of *H. pylori* besides the stomach. Although the oral cavity may be a source of transmission, it is unknown whether the mouth could be a common source for reinfection of the stomach after treatment.

Research frontiers

The variability of clinical manifestations in the population could be related to an interaction between the bacterial virulence factors and genetic susceptibility of the host. *H. pylori* strains that have *cagA*-positive and *vacAs1/m1* genotypes are associated with an intense inflammatory response in the stomach, which leads to peptic ulcer and other severe gastric diseases. For this reason, there is an increasing interest in generating different non-invasive diagnostic tests that allow the epidemiological and clinical study of *H. pylori* infection.

Innovations and breakthroughs

In this study, dental plaque and gastric biopsies were submitted to rapid urease test and polymerase chain reaction (PCR) assays to detect *H. pylori* and the virulence factors, *cagA* and *vacA* genotypes, respectively. In addition, gastric biopsies were evaluated by histological techniques. It was found a high correlation between the most pathogenic strains (*vacAs1m1/cagA*-positive) in dental plaque and gastric mucosa. It seems that the severity of gastrointestinal diseases, such as gastroesophageal reflux, could be facilitated by oral colonization by *H. pylori*.

Applications

The simultaneous presence of the *H. pylori* isolates with similar *cagA* and *vacA* genotypes in dental plaque and gastric mucosa was detected in a high percentage in patients with chronic gastritis and peptic ulcer, which suggests the necessity to apply additional molecular tests to dental plaque to assist with the diagnosis and therapy of gastric infection. This will add to the available knowledge about the pathogenetic relevance of *H. pylori* in the oral cavity.

Terminology

Bacterial virulence factors are expressed by the vacuolating cytotoxin gene (*vacA*) and cytotoxin-associated gene (*cagA*).

Peer review

The authors studied by means of PCR whether *H. pylori*-positive subjects also had the bacterium in the dental plaque. They found that the vast majority of patients with stomach colonization by *H. pylori* also had oral bacterial infection, thus lending further support to the concept that oral to oral transmission of *H. pylori* is highly possible. The manuscript is well written.

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