Helicobacter pylori transiently in the mouth may participate in the transmission of infection

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Helicobacter pylori *infection is associated with peptic ulcer and gastric carcinoma. The oral cavity may be a reservoir for* H. pylori*; however, the results of studies on this subject are controversial. We employed single-step and nested polymerase chain reactions (PCR) to detect the presence of the* vac*A,* ure*A and 16S rDNA genes of* H. pylori *in the stomach, saliva and dental plaque of 30 subjects. The results were confirmed by sequencing. Nested 16S rDNA and* ure*A amplification was achieved in 80% of gastric, 30% of saliva and 20% of dental plaque specimens. Sequencing of 10, seven and four 16S rDNA products from stomach, saliva and dental plaque, respectively, showed > 99% identity with* H. pylori. *Sequencing of the other four oral cavity PCR products showed similarity with* Campylobacter *and* Wolinella *species. Additionally, the* vac*A genotype identified in the samples of different sites was the same within a given subject.* H. pylori *may be found in the oral cavity of patients with gastric infection, thus it could be a source of transmission. However, results obtained with detection methods based only on PCR should be interpreted with caution because other microorganisms that are phylogenetically very close to* H. pylori *are also present in the mouth.*

Key words: *H. pylori* - dental plaque - saliva - oral cavity - transmission - *vac*A

Helicobacter pylori infection, one of the most common chronic bacterial infections worldwide, is considered to play an important role in the pathogenesis of peptic ulcer disease (Mégraud & Lamouliatte 1992), distal gastric carcinoma (Parsonnet et al. 1991) and gastric mucosa-associated lymphoid tissue lymphoma (Wotherspoon et al. 1991). Recently, questions have been raised concerning the possibility that the oral cavity is an *H. pylori* reservoir participating in infection transmission or a nidus for reinfection after eradication therapy or that it plays a role in oral disease. However, the results of the studies on this subject are controversial. Different groups have found discrepant results, ranging from 0-100% positivity for *H. pylori* in the oral cavity by polymerase chain reaction (PCR) methods (Mapstone et al. 1993, Hardo et al. 1995, Parsonnet et al. 1999, Song et al. 2000, Fritscher et al. 2004, Loster et al. 2006, Olivier et al. 2006). In addition, inconsistent correlation between *H. pylori* positivity in the oral mucosa and in the stomach has been found. Many factors likely account for this variability, including the genetic background of the study population, cultural habits, socioeconomic level and differences in the accuracy of the methods used as well as the strain of bacteria. Our goal was to investigate the presence of *H. pylori* in the stomach and oral cavity. We employed highly sensitive PCR assays followed by sequencing to increase the specificity of the PCR results.

PATIENTS, MATERIALS AND METHODS

We included 30 patients (17 males and 13 females; mean age = 53.4 years, standard deviation = 4.3 years) who were consecutively selected among those who underwent upper endoscopy at the Endoscopy Service of the Pedro Ernesto Hospital of Rio de Janeiro State University in Rio de Janeiro, Brazil to clarify the origin of their dyspeptic symptoms. The patients had received neither proton pump inhibitors nor antimicrobials in the two months prior to endoscopy. Those who had less than 20 teeth in their mouth were excluded from the study. The study was approved by the Ethical Committee of the institution and informed consent was obtained from all included patients. Antral biopsy specimens obtained during the endoscopy were kept at -80ºC until DNA was extracted. Saliva and dental plaque samples were collected from each patient immediately before endoscopy using sterile paper points that were placed into 1.5 mL microtubes containing 1.0 mL of sterile water. Approximately 1.0 mL of non-stimulated salivary flow was also collected in a 1.5 mL microtube. For plaque sampling, air drying and sterile cotton rolls were used to avoid saliva contamination. Samples were kept in dry ice, transported to the laboratory and then stored at -80ºC before DNA was extracted.

DNA was extracted with the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations, with minor modifi-

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TABLE I

Oligonucleotide primers used for polymerase chain reaction (PCR) amplification of 16S rRNA and *ure*A genes

Primers and sequence (5'-3')	Amplicon size (bp)	PCR conditions
16S rRNA		
Outer primers		
C70: AGAGTTTGATYMTGGC	1,500	94ºC (45 s), 50ºC (45 s), 72ºC (45 s), 5 s per cycle - 24 cycles
B37: TACGGYTACCTTGTTACGA		
Inner primers		
C97: GCTATGACGGGTATCC	400	94ºC (1 min), 55ºC (2 min), 72ºC (3 min) - 34 cycles
C98: GATTTTACCCCTACACCA		
*Ure*A		
Outer primers		
HPU1: GCCAATGGTAAATTAGTT	411	94ºC (1 min), 45ºC (1 min), 72ºC (1 min) - 35 cycles
HPU2: CTCCTTAATTGTTTTTAC		
Inner primers		
AGTTCCTGGTGAGTTGTTCT	361	96ºC (30 s), 56ºC (15 s), 74ºC (30 s) - 40 cycles
AGCGCCATGAAAACCACGCT		

cations. As a control, we routinely include water in each DNA extraction set. The 16S rDNA gene was amplified by nested PCR using one outer (B37 and C70, a product of about 1500 bp) and one inner (C97 and C98, about 400 bp) primer pair for the *Helicobacter* genus (Table I). Another nested PCR for *H. pylori ure*A (~311 bp) was also used as previously described (Wang et al. 1993) (Table I). An *Escherichia coli* strain (clinical isolate) and an *H. pylori* strain (TX30A) served as negative and positive controls, respectively, and distilled water was used as an internal reaction negative control. PCR targeting the beta-globulin gene was used for testing residual inhibitors as previously described (Verhoef et al. 2003).

The nested 16S rDNA PCR products of 400 bp were purified (Wizard PCR-Prep purification kit, Promega, Madison, WI, USA) and directly sequenced in an Applied Biosystems DNA automated sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA, USA), using and ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and sequencing primers C97 and C98. The sequences were aligned using the cap program at the INFOBIOGEN web server and compared with the GenBank database with the BLAST program on the National Center for Biotechnology Information computer server. The 16S rDNA gene sequences were deposited in GenBank (accession HM246653-HM246661). We also evaluated the presence of the signal sequence of *vac*A, a specific *H. pylori* gene present in all the strains, by one step PCR (Atherton et al. 1995).

RESULTS

The nested PCR for the 16S rRNA gene was positive in 24 of the 30 gastric biopsies (80%). The results were confirmed by the *ure*A nested PCR. In six cases, neither the nested PCRs nor the *vac*A PCR were positive. In the 24 *H. pylori*-positive gastric mucosa samples, *vac*A was detected by one step PCR in 22 samples (91.7%), the s1 genotype was observed in 18 (81.8%), s2 in two (9.1%) and both s1 and s2 in two (9.1%) cases. Although *vac*A was not directly detected by PCR in two samples, the 400 bp product obtained from the 16S rRNA gene nested PCR in these samples was observed to be 99% similar to *H. pylori* by sequencing.

The nested PCR for the 16S rRNA gene was positive in nine (30%) and six (20%) samples of saliva and dental plaque, respectively. The *vac*A gene was observed in two saliva (1 s1 and 1 s2 genotype) and two dental plaque (2 s1 genotypes) samples; the nested 16S rRNA gene was positive in all of them. The *vac*A genotypes were identical to those observed in the corresponding gastric mucosa. The PCR for *vac*A was positive in a significantly higher percentage of gastric mucosa samples than the oral cavity samples ($p < 0.0002$, Chi-square test with Yates correction). The results are summarised in Table II.

TABLE II

Detection of *Helicobacter pylori* genes in the gastric and oral cavity samples of 30 patients by one step and nested polymerase chain reaction (PCR)

Site	*Vac*A PCR n (%)	16S rDNA nested-PCR n (%)	16S rDNA sequencing[a] n (%)
Gastric mucosa	22 (73.3)	24 (80)	10 (100)[b]
Saliva	2 (6.7)	9 (30)	7 (77.8)
Dental plaque	2 (6.7)	6 (20)	4 (66.7)

a: > 99% similarity with *H. pylori*; *b*: 10 PCR products from the gastric mucosa were sequenced.

All 400 bp products obtained from the saliva and dental plaque and 10 obtained from the stomach were sequenced. In seven positive PCR from saliva, four from dental plaque and all from the gastric mucosa, the sequencing showed > 99% identity with *H. pylori*. For all of the positive oral cavity samples, positive results were also observed in the corresponding gastric mucosa sample. In addition, the sequencing of the 400 bp products obtained from two samples of saliva and two samples of dental plaque showed other microorganisms that are closely related to the *Helicobacter* genus phylogenetically: *Campylobacter concisus* ATCC 33237 (96% similarity) and candidatus *Wolinella africans* AS-7 (97.3% similarity) in the saliva samples and *Campylobacter rectus* LMG 7611 (95.8% similarity) and *Campylobacter* sp. oral clone BB.120 (91% similarity) in the dental plaque samples. Therefore, the presence of specific *H. pylori* DNA was truly detected in only 23.3% of the saliva samples and 13.3% of the dental plaque samples. Considering that in two patients the DNA was detected simultaneously in the saliva and dental plaque, the overall detection rate was 30%.

DISCUSSION

In this study, we evaluated the presence of *H. pylori* genes in specimens from the oral cavity (saliva and dental plaque) and gastric mucosa of dyspeptic patients. As shown in Table III, the percentage of saliva or dental plaque *H. pylori* DNA-positive samples we observed was in line with that demonstrated by Parsonnet et al. (1999) and Mapstone et al. (1993), whose study designs, similarly to ours, included PCR amplicon sequencing or control for the presence of the most closely related non-*Helicobacter* spp or other *Helicobacter*. By contrast, the overall detection rate of *H. pylori* in our study was higher than that reported by Hardo et al. (1995) in a low *H. pylori* prevalence area and by Fritscher et al. (2004) who evaluated children. Conversely, in the studies of Song et al. (2000) and Loster et al. (2006) the frequency of *H. pylori* in the oral cavity was higher than that of the present study. However, non-specific methods were used for bacterium identification in these studies. The discordance among the studies may be explained by differences in either the prevalence of gastric *H. pylori* infection or in the accuracy of the methods used. The age of the patients may also be important because the prevalence of infection increases with increasing age. In the present paper, we employed a highly-sensitive nested PCR. This assertion is supported by the results we observed in the gastric mucosa with respect to the prevalence of *H. pylori* infections that were similar to that observed from previous studies in the same geographic region and employing several diagnosis methods such as culture, preformed urease test, stained smears and serology (Queiroz et al. 1999). In addition, saliva and dental plaque samples were truly positive for *H. pylori* by the nested PCR in seven and four specimens, respectively, and by *vac*A one step PCR in only two specimens from each site. This PCR has successfully been used in studies evaluating the presence of *H. pylori* in non-gastric sites by our group (Silva et al. 2003, Oliveira et al. 2004) and others (Fox et al. 1998). Conversely, although the method was highly specific for the diagnosis of *H. py-*

TABLE III

Positivity for *Helicobacter pylori* in the oral cavity by polymerase chain reaction methods

Reference	Sample	Patients n	Gastric *H. pylori* n	Oral cavity *H. pylori* positivity n (%)
Parsonnet et al. (1999)	Saliva	26	16	7 (43.8)
Mapstone et al. (1993)	Dental plaque/saliva	23	13	5 (38.5)
Hardo et al. (1995)	Dental plaque	62	34	1 (2.9)
Song et al. (2000)	Dental plaque	21	10	10 (100)
Fritscher et al. (2004)	Dental plaque/oral mucosa	105	ND	9 (8.6)
Loster et al. (2006)	Oral mucosa	40	40	40 (100)
Olivier et al. (2006)	Dental plaque	79	66	0
Present paper	Dental plaque/saliva	30	24	9 (30)

ND: not determined.

lori infection in the gastric mucosa in this study and in bile or gallbladder and intestinal mucosa in other studies from our group (Silva et al. 2003, Oliveira et al. 2004), the specificity was low in the case of saliva (77.8%) and dental plaque (66.7%). In fact, because the oral cavity is the habitat of many bacterial species very closely related to *H. pylori*, such as several species of *Campylobacter* and *Wolinella* (up to 97% similarity with *H. pylori*), false positive results for *Helicobacter* may occur. We overcame this problem by sequencing the PCR products and demonstrating that a significant proportion of positive PCR for *H. pylori* observed in the oral cavity was, in fact, false-positive results due to the presence of closely related non-*Helicobacter* species.

Different from the data we obtained in the gastric mucosa by single-step PCR, most of the *Helicobacter* DNA was only detected in the oral cavity by nested PCR, indicating that the number of bacteria is smaller at this site than in the gastric mucosa. In addition, there was no positive result with the saliva or plaque specimens from the patients who tested negative in their gastric mucosa specimens and the bacterium *vac*A genotypes observed in the mouth were seen to be the same as those observed in the stomach. These results suggest that *H. pylori* may be transient in the oral cavity, probably due to either pathologic or physiologic gastroesophageal reflux. The lower detection rate of the *vac*A gene in the saliva and dental plaque samples is probably due to a low bacterial load found in the oral cavity as mentioned above.

Our results suggest that *H. pylori* does not establish persistent oral colonisation and, thus, is very unlikely to play a role in oral diseases. Conversely, we may not rule out the possible participation of *H. pylori* present in the oral cavity in the transmission of infection by the oral route. It has to be emphasised that the infection is mainly acquired in childhood and *H. pylori*-positive mothers are an independent risk factor for infection (Rocha et al. 2003). The intimate contact of the mother with her babies and the putative susceptibility of young child to become infected with a small number of the microorganisms may support the hypothesis that *H. pylori* transiently localised to the oral cavity may have a relevant role in the transmission of infection.

In conclusion, *H. pylori* may transiently be found in the oral cavity of patients with gastric infection and it may be a source of the bacterium for the transmission of infection by the oral route. Furthermore, methods based only on PCR for the detection of *H. pylori* at oral sites should be interpreted with caution due to the presence of other microorganisms in the mouth that are phylogenetically related to *H. pylori*.

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