Oral Diseases (2005) 11, 17–21
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Oral Microbiology

Occurrence of *Helicobacter pylori* in dental plaque and saliva of dyspeptic patients

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BACKGROUND: *Helicobacter pylori* infection in the stomach is associated with gastric and duodenal ulcers, gastric cancers and gastric lymphoma. The organism is transmitted by ingestion, but the oral–oral route and the fecal–oral route are also suggested. The prevalence of infection with *H. pylori* in developing countries, including Brazil, is higher than in developed countries.

PURPOSE: This study aimed to evaluate the role of the oral cavity as a reservoir of this species, by evaluating the occurrence of *H. pylori* in supragingival dental plaque and in saliva of Brazilian dyspeptic patients, whether harboring the organism or not in the stomach.

MATERIAL AND METHODS: Forty-nine patients reporting dyspeptic symptoms were subjected to oral clinical examination and collection of saliva and supragingival dental plaque samples prior to the endoscopic examination. The detection of *H. pylori* in oral samples was performed by PCR using 16S rRNA primers. The bacteria were detected in stomach by means of the rapid urease test.

RESULTS: *Helicobacter pylori* was detected in the stomach of 20 of 49 subjects reporting dyspeptic symptoms. The organism was detected in only one supragingival plaque sample, obtained from a patient positive for the urease test in the stomach and in none of the salivary samples.

CONCLUSION: Supragingival dental plaque and saliva may not be relevant reservoirs of *H. pylori*.

Oral Diseases (2005) **11**, 17–21

Keywords: *Helicobacter pylori*; supragingival plaque; saliva

Introduction

The association between spirochetes and peptic ulcers in humans was reported by DOENGUES in 1939. However, it wasn't until 1983 that Warren and Marshall (1983) reported a gram-negative spiral highly motile bacterium found in gastric mucosa and associated with chronic active gastritis, named *Campylobacter pyloridis*. Biochemical and nucleotide sequence differences between this new organism and the species belonging to the genus *Campylobacter* led to the proposal of the genus *Helicobacter* (McNulty and Wise, 1985; Goodwin *et al*, 1989) and the organism being known as *Helicobacter pylori*.

Helicobacter pylori produces large amounts of urease, an enzyme that allows the bacterium to metabolize urea present in the gastric mucosa, establishing a microenvironment favorable to the organism by neutralization of the acid through the generation of ammonia (Dunn and Phadnis, 1998). Beyond the production of urease and the motility, the major determinants of virulence of *H. pylori* are the production of a vacuolating toxin and the presence of the CagA gene, which encodes a protein involved in the induction of an epithelial chemokine response (Moss, 1998; Marais *et al*, 1999).

Once acquired, *H. pylori* establishes chronic, persistent infection. The majority of infected patients may develop a symbiotic relationship with *H. pylori*. In some subjects, the inflammation promoted by *H. pylori* infection predominates in the antrum, leading to pyloric or duodenal ulcers, while for those with diffuse inflammation, involving the antrum and the body of the stomach, a multifocal gastritis will develop promoting hypochloridria. This condition, predisposing to gastric ulcers, gastric cancer or gastric mucosa-associated lymphoid tissue (MALT) lymphoma (McGee and Mobley, 1999; Mukhopadhyay, 1999; Nguyen *et al*, 1999; Veldhuyzen van Zanten and Lee, 1999).

Approximately 50% of the world's population is infected by *H. pylori* (Mitchell, 1999). The prevalence of infection in developed countries ranges from 19 to 57%, whereas the prevalence rates are much higher in developing countries (Souto *et al*, 1998; Mitchell, 1999; Mitchell *et al*, 2003; O'Rourke *et al*, 2003). The organism is transmitted from person to person, and a higher prevalence is found when there is close personal contact and lack of hygiene. The acquisition of *H. pylori* is usually made by ingestion, and an association between prevalence of

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Received 4 February 2004; revised 19 April 2004; accepted 14 May 2004

H. pylori infection and the source of drinking water has been reported (Klein *et al*, 1991). However, the fecal–oral and oral–oral routes are believed to be common means of transmission of *H. pylori* (Mitchell, 1999). Low levels of sanitation and lower socioeconomic status have been associated with an increased prevalence of *H. pylori* infection (Souto *et al*, 1998; O'Rourke *et al*, 2003). In Brazil, the prevalence of the organism in adults can reach over 80% in subjects of low socioeconomical strata (Souto *et al*, 1998; Mitchell *et al*, 2003).

The detection of *H. pylori* infection in stomach is currently made by rapid urease tests, tissue histology or culture, by performing endoscopy. Noninvasive diagnostic tests such as serologic and urea breath tests may also be used to diagnose or monitor eradication of the organism after therapy (Nakamura, 2001). The presence of cross-reactive antigens between the oral organism *Campylobacter rectus* and *H. pylori* may affect the serologic diagnosis of *H. pylori* infections, especially when saliva is used (Ishihara *et al*, 2001). The use of PCR on biopsy specimens does not add much to other techniques employed for the detection of the organism in the stomach (Lage *et al*, 1995; Lin *et al*, 1996). However, the molecular methods are useful when analyzing specimens, such as gastric juice, environmental samples, oral secretions, and stool samples, for which the regular methods lack sensitivity (Westblom and Bhatt, 1999).

Several authors have reported the presence of *H. pylori* in the oral cavity and it was proposed that it would represent a source of *H. pylori* for reinfection of the stomach after therapy. It could also be a route for transmission to other subjects (Li *et al*, 1996; Nguyen *et al*, 1993; Umeda *et al*, 2003). *Helicobacter pylori* could be a part of the dental biofilm (Ishihara *et al*, 1997) making the organism inaccessible to the antibiotics that are used to eradicate the organism from the stomach (Miyabashi *et al*, 2000).

However, there are conflicting data about the prevalence of oral carriers of *H. pylori*, ranging from close to 0 to over 50% among different studies (Asikainen *et al*, 1994; Li *et al*, 1996; Song *et al*, 2000; Okuda *et al*, 2000; Umeda *et al*, 2003). Variations in methods for detection and the prevalence of *H. pylori* infection in the stomach among the sampled populations may account for the conflicting data among studies.

Due to high prevalence of *H. pylori*-infected subjects in Brazil, and the pathogenic potential of this organism, our study aimed to evaluate the role of the oral cavity as a reservoir of this species. We evaluated the occurrence of *H. pylori* in supragingival dental plaque and in saliva of Brazilian patients reporting dyspeptic symptoms, whether harboring the organism or not in the stomach. In order to avoid contamination of the oral cavity by the endoscopic procedure, the oral samples were obtained prior to gastric examination.

Methods

Forty-nine patients were selected for this study among 147 subjects reporting dyspeptic symptoms indicated for endoscopic examination at the Hospital do Servidor Público Estadual, São Paulo, SP, Brazil. The Ethical Committee of the School of Dentistry, University of São Paulo, and of the Hospital do Servidor Público Estadual approved this study. Patients reporting no other medical problems besides dyspeptic symptoms, presenting with at least two premolars and two molar teeth, with no reported antibiotic usage for at least 3 months, who had never been previously subjected to endoscopy and biopsies of the body and antrum were included. Dyspepsia was defined as pain or discomfort in the epigastric region (Drossman *et al*, 2000). After obtaining the signature of an informed consent, and a medical evaluation, the subjects were clinically examined by one of the authors.

Saliva and plaque samples were collected prior to the endoscopic examination from each subject. Salivary flow was stimulated by chewing 1 g of paraffin for one minute, and saliva was collected in test tubes. Supragingival dental plaque on molar and premolars was removed with a sterile curette and transferred to 1.5 ml of phosphate buffered saline (PBS) (pH 7.0). Samples were kept on ice, transported to the laboratory, and then stored in −20°C freezer until analysis.

DNA was extracted from each sample by the method described by Alam *et al* (1999). Aliquots of 1 ml of saliva or 1 ml of the plaque suspensions were centrifuged at 10 000 *g*, for 1 min at 4°C (Eppendorf, Hamburg, Germany) and the supernatant discarded. The pellets were washed again in PBS and resuspended in sterile water. The tubes containing the cell suspensions were immersed in boiling water for 10 min, for cell lyses, and immediately transferred to ice. After centrifugation at 10 000 *g* for 1 min, at 4°C, the supernatants were transferred to another tube and kept at −20°C until use.

DNA extracted from *H. pylori* strain ATCC 43629 was included in every set of reaction as positive control. Negative control reactions with no added template DNA were also used in every set of reactions.

Universal primers UP1 (5′-GATTAGATACCCTGG TAGTCCAC-3′) and UP2 (5- CCCGGGAACGTATT CACCG-3′) which match nearly all bacterial 16S rDNA at the same position, but not the 18S rDNA sequence of eukaryotic cells, served as positive control for the determination of presence of bacterial DNA in the plaque and saliva DNA samples (Tan *et al*, 2002). The reactions consisted of 10 μl of template DNA, and 100 pmol of each primer UP1 and UP2 (Invitrogen, São Paulo, Brazil). Reactions were performed in 50 μl volumes using 1 unit Taq polymerase (Invitrogen), 1.5 mM $MgCl_2$ and were run in a DNA thermal cycler (Gene Amp PCR System 2400, Perkin Elmer Corp., Foster City, CA, USA). DNA was denatured at 95°C for 3 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing at 58°C for 40 s, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min.

Twenty microliters of the amplified products were analyzed on 2.0% agarose gels in Tris-acetate EDTA buffer (TAE) and were detected by staining with 0.5 μg ml^{-1} of ethidium bromide. When DNA from

H. pylori strain ATCC 43629 was used as template, a single product of 608 bp was observed. The reactions using DNA from the oral samples resulted in a wide band around 608 bp, indicating the presence of amplifiable DNA from eubacteria.

PCR was used to detect *H. pylori*, following the protocol described by Riggio and Lennon (1999). Ten microliters of template DNA and 100 pmol of each primer JW 22 (5′-CGT TAG CTG CAT TAC TGG AGA-3′) and JW 23 (5′-GAG CGC GTA GGC GGG ATA GTC-3′) (Invitrogen), homologous to *H. pylori* 16S rRNA gene, were used. PCR reactions were performed in 50 μl volumes using a HotMaster Taq DNA polymerase kit® (Eppendorf). Reagent concentrations were set according to the manufacturer's instructions. The samples were heated at 94°C for 2 min and amplifications were performed at 94°C for 20 s, 58°C for 10 s and 72°C for 30 s for 40 cycles. The amplified products were extended at 72°C for 10 min. In order to avoid contamination, the establishment of the reactions and the product analysis were performed in separate rooms.

Twenty microliters of the amplified products were analyzed on 2.0% agarose gels in TAE and were detected by staining with 0.5 μg ml^{-1} of ethidium bromide. A single PCR product of 295 bp was indicative of the presence of *H. pylori* DNA. Digital images of the ethidium bromide stained gels were obtained with the Photo PC 3100Z (EPSON Hemel, Hempstead, UK).

After oral clinical examination and collection of the oral samples, the subjects were subjected to endoscopic examination. The absence of structural alterations on the mucosa as well as inflammatory signals confirmed the diagnosis of functional dyspepsia. *Helicobacter pylori* detection was performed in stomach by means of the rapid urease test. The rapid urease test was performed on fresh biopsy specimens by the detection of a rise of pH due to ammonia production after hydrolysis of urea. The urease positive test indicates gastric colonization by *H. pylori* (Hazell *et al*, 1987).

Results

Thirty-four female and 15 male subjects, aged 9–72 years old (mean 44 years old) were included in this study. The main reason for exclusion of subjects from the study was absence of at least 4 molars and/or premolars (56 subjects). The evaluation of *H. pylori* in the oral cavity was performed prior to the evaluation of the bacteria in stomach.

These 49 patients were further classified in two subgroups formed by 20 patients harboring *H. pylori* in the stomach, detected by the rapid urease test, and 29 patients tested negative, indicative of absence of *H. pylori* colonization.

Supragingival plaque and saliva samples were taken from all individuals. After DNA extraction, amplification reactions using universal primers for eubacteria revealed that the DNA samples were suitable for PCR, as shown in Figure 1.

Reactions performed with primers homologous to 16S rRNA gene specific for *H. pylori*, revealed that the microorganism was detectable in only one plaque sample, as shown in Figure 2. The positive sample for *H. pylori* was from one of the 20 patients presenting with bacteria in the stomach.

Discussion

Despite the high number of infected subjects in gastric samples, the analysis of oral samples revealed the presence of the organism in only one supragingival plaque sample and in none of the salivary samples.

Several studies reported that the detection frequency of *H. pylori* in the oral cavity by culture methods to be very low (Krajden *et al*, 1989; Bernander *et al*, 1993; Cheng *et al*, 1996; Umeda *et al*, 2003), indicating that, if present, these organisms must be in very low amounts. Thus, the use of a more sensitive technique such as PCR to detect this organism should give more reliable results.

Data based on PCR for detection of *H. pylori* in the oral cavity varies widely (Riggio and Lennon, 1999; Song *et al*, 2000; Umeda *et al*, 2003). These differences may be due to methodological factors, such as sample dilutions, and specificity of the primers. In the present study, DNA from *H. pylori* was amplified from oral samples with a single round of PCR. The levels of *H. pylori* in dental plaque may be too low to be detected by one round PCR (Song *et al*, 2000; Umeda *et al*, 2003), but the importance of such low levels could be questioned (Riggio and Lennon, 1999). Song *et al* (2000) reported that only two of 21 *H. pylori* positive plaque samples presented more than 100 cells of *H. pylori* mg^{-1} dental plaque.

Failure to recover *H. pylori* from oral samples or the low levels of detection indicates that this organism may be found in the oral cavity as part of the transient microflora (Oshowo *et al*, 1998; Okuda *et al*, 2003).

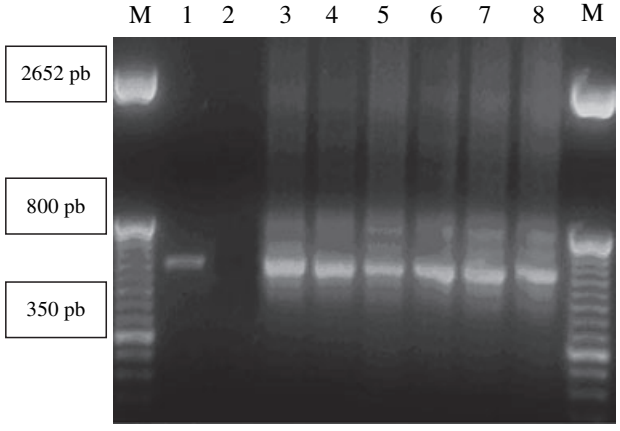

Figure 1 Amplicons after electrophoresis on a 2.0% agarose gel using *16S rRNA universal* primers (608 bp). (Lane 1) Template DNA from strain *H. pylori* ATCC 43629. (Lane 2) Negative control, no template DNA. (Lanes 3–8) DNA extracted from supragingival dental plaque samples was used as template. (Lane M) Molecular weight marker (50 bp DNA Ladder; Gibco/Life Technologies, New York, NY, USA)

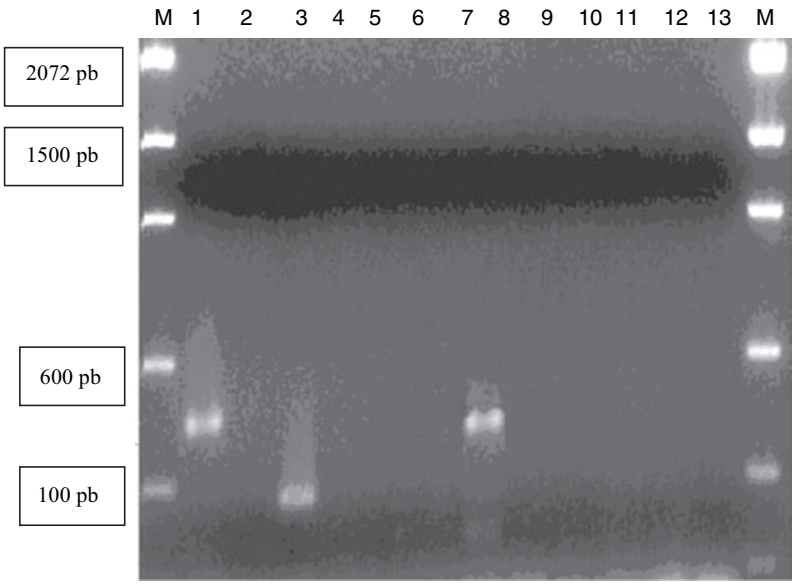

Figure 2 Amplicons after electrophoresis on a 2.0% agarose gel using *16S rRNA* primers specific for *H. pylori* (295 bp). (Lane 1) Template DNA from strain *H. pylori* ATCC 43629. (Lane 2) Negative control, no template DNA. (Lanes 3–13) DNA extracted from supragingival dental plaque samples was used as template. (Lane M) Molecular weight marker (100 bp DNA Ladder; Gibco/Life Technologies)

Patients who had never been subjected to gastric endoscopic examinations formed the study population, and the oral samples were collected prior to endoscopy in order to avoid the carriage of bacteria from the stomach to the oral cavity. This study differs from those evaluated by several authors (Nguyen *et al*, 1993; Umeda *et al*, 2003), whose higher prevalence rates of *H. pylori* could be due to contamination into the oral cavity through the gastric endoscope.

The sites for sample collection in the oral cavity may have also influenced the prevalence of the microorganism. Samples were obtained from supragingival dental plaque and saliva, and other niches could be a better reservoir of *H. pylori*, such as subgingival dental plaque, especially from periodontitis patients (Riggio and Lennon, 1999; Umeda *et al*, 2003).

However, the microaerophilic nature of this organism suggests that the anaerobic environment provided by the subgingival sulcus/pocket would not be suitable for the organism. This hypothesis is reinforced by other findings reporting the absence (Asikainen *et al*, 1994, Shimada *et al*, 1994; Bernander *et al*, 1993) or low prevalence (Oshowo *et al*, 1998) of *H. pylori* in oral samples, including saliva, supra and subgingival dental plaque.

Although 20 subjects harbored *H. pylori* in the stomach, the organism was detected in the oral sample from only one subject (5%). Oshowo *et al* (1998) have reported that *H. pylori* was detected in dental plaque in only 12.9% of patients positive for the bacteria in the stomach. The absence of correlation between infection by *H. pylori* in the stomach and in the oral cavity was also reported in subjects treated with antibiotics (Doré-Davin *et al*, 1999; Okuda *et al*, 2003). Despite the ability to coaggregate with oral organisms such as *Fusobacterium*, *H. pylori* survival in the human oral cavity may be impaired due to inhibition by members of the resident microflora as *S. mutans* and *Prevotella intermedia* (Ishihara *et al*, 1997; Okuda *et al*, 2000, 2003). Furthermore the organism is more frequently found in the oral cavity of subjects professionally exposed to *H. pylori*, which is not reflected by gastric infection, reinforcing the hypothesis that the presence of *H. pylori* in the mouth could be regarded as transitory (Oshowo *et al*, 1998; Doré-Davin *et al*, 1999).

These data should be confirmed by the determination of risk of *H. pylori* infection by dental professionals/personnel due to their close contact with oral fluids, and compared with the increased risk for *Helicobacter pylori* infection by surgical/medical personnel (Upile *et al*, 2002; Melo *et al*, 2003).

As the organisms harvested from saliva are those washed from the oral surfaces, it would be expected that this fluid would reveal the presence of *H. pylori*. However, the organism was not detected in any salivary sample, not even from the patient in whom the organism was found in dental plaque. These results point out that saliva may not serve as a vehicle for these organisms for reinfection after therapy, or even be involved in transmission to other individuals, and that supragingival dental plaque is not a common reservoir of *H. pylori* in patients infected or not by the bacteria in the stomach.

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