J Oral Pathol Med (2004) 33: 129–32

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Association between *Helicobacter pylori* and recurrent aphthous stomatitis in children and adolescents

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BACKGROUND: The recurrent aphthous stomatitis (RAS) is a common disease with unknown etiology. *Helicobacter pylori* has been shown to be the causative factor in peptic ulcers. Considering the similarities of histologic features between gastric and oral ulceration, we studied the possible involvement of *H. pylori* in the development of RAS.

METHODS: A total of 105 children and adolescents were investigated – 53 patients with RAS (case group) and 52 patients without lesions (control group). Specimens obtained by swabbing RAS lesions, intact oral mucosa, and dental plaque were submitted to a polymerase chain reaction (PCR)-based assay.

RESULTS: *Helicobacter pylori* was present in six patients of the case group (11.3%) and in three of the control group (5.8%). When the site of infection was studied, 9.4% of the RAS lesions were PCR positives. In the case group and control group, 5.7 and 1.9% of the specimens from dental plaque, respectively, and 5.7 and 3.8% of the specimens from the intact oral mucosa, respectively, were PCR positives.

CONCLUSION: There was no association between RAS lesions and infection of the oral cavity by *H. pylori* in children and adolescents ($P = 0.254$).

J Oral Pathol Med (2004) **33**: 129–32

Keywords: *Helicobacter pylori*; PCR; recurrent oral aphthous ulcers

Introduction

Recurrent aphthous stomatitis (RAS) is a common multifactorial disease, characterized by the periodic appearance of multiple painful ulcers of the oral mucosa. Etiologic factors, such as stress, trauma, family history, food hypersensitivity, and hormonal and immunologic patterns, have already been associated with the development of these lesions (1, 2), but there is still no evidence of a definite and significant causal factor.

Helicobacter pylori is a microaerophilic, Gram-negative spiral bacillus, which has been pointed out as a causal factor of gastritis and peptic ulcers (3). In developing countries, almost all children are infected up to 10 years of age, and the main routes of transmission appear to be the oral-fecal and oral-oral ones (4).

The aim of the present study was to examine the possible involvement of *H. pylori* in the development of RAS, which was prompted by the fact that the histologic characteristics of gastric lesions are similar to those of oral stomatitis and that *H. pylori* is already associated with the etiology of gastric disease (2, 5, 6).

Patients and methods

The sample comprised 105 children and adolescents originating from the Integrated Child and Adolescent Clinic of the Faculty of Dentistry of PUCRS, having 53 patients with RAS (case group) and 52 patients without these lesions (control group). RAS was identified by clinical examination based on history and characteristics of the lesions according to Scully & Porter (7).

Parents or guardians responsible for the participants signed an informed consent form. Excluded from study were those who had been taking antibiotics 4 weeks preceding the collections of samples.

This study was approved by the research ethics committee of PUCRS.

In the case group, the samples were collected with a *Microbrush*® from three sites: intact oral mucosa, RAS lesion, and dental plaque. In the control group, specimens were collected from the intact oral mucosa and dental plaque.

The specimens were stored in 1.5-ml Eppendorf tubes containing 200 µl of sterile phosphate-buffered saline (PBS) at −20°C until time of processing.

DNA was extracted from the specimens (in 200 µl PBS) utilizing the QIAGEN® QIAamp Tissue Kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions.

The DNA amplification reaction was performed by means of nested PCR, according to the method recommended by Li

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Accepted for publication September 11, 2003

et al. (8) and Song et al. (9, 10). Utilized for the primary PCR reaction were the pair of primers EHC-U (5′-CCCTCACG-CCATCAGTCCCAAAAA-3′) and EHC-L (5′-AAGAAGT-CAAAAACGCCCCAAAAC-3′) (Gibco BRL, Rockville, MD, USA), which amplifies a DNA fragment of 417 bp homologous to a DNA fragment of *H. pylori* of 860 bp (8–10) using the following amplification protocol: initial denaturation (94°C for 5 min); 40 cycles of denaturation (94°C for 45 s), annealing (59°C for 45 s) and extension (72°C for 30 s); and final extension (72°C for 10 min).

In the second PCR (nested PCR), an additional pair of primers was utilized, ET-5U (5′-GCCAAATCATAAGT-CCGCAGAA-3′) and ET-5L (5′-TGAGACTTTCCTAGAA-GCGGTGTT-3′) (Gibco BRL), which amplifies an internal fragment from the 417-bp fragment generated by amplification with the EHC-U and EHC-L primers, producing a 230-bp fragment. Many authors have proven the high sensitivity and specificity of this method (8–10). The following amplification protocol was used in this reaction: initial denaturation (95°C for 5 min); 30 denaturation cycles (94°C for 45 s), annealing (59°C for 45 s) and extension (72°C for 45 s); and final extension (72°C for 10 min).

Each PCR reaction set had a positive control (*H. pylori* ATCC 43504D) and a negative control (without *H. pylori*).

The results obtained were analyzed by means of descriptive statistics and Fischer's exact test, at a significance level of 5%.

Results

In the group of patients with RAS, there were six cases (11.3%) that were PCR positive for *H. pylori*, and in the control group, three cases (5.8%). No statistically significant relationship was found between the presence of *H. pylori* and the occurrence of RAS (Table 1).

Of the samples originating from RAS lesions, five (9.4%) showed *H. pylori* positivity, while two positive cases (3.8%) were found among the samples from the intact oral mucosa of the patients in the control group. The difference in the frequency of *H. pylori* observed between RAS and intact mucosa of the control group was not statistically significant (Table 2).

In patients with RAS, *H. pylori* positivity was shown in three cases (5.7%) in relation to oral mucosa without lesions. Yet, in the oral mucosa of the control group, two positive cases (3.8%) were also found. Statistical analysis showed that there was no significant difference between these two groups in the incidence of *H. pylori* in the intact mucosa (Table 3).

The analysis of specimens of dental plaque showed positive findings of *H. pylori* in three cases (5.7%) in the group of patients with RAS, while in the control group, there was one positive case (1.9%). Statistical analysis did not show a significant difference between the two groups studied in relation to the incidence of *H. pylori* in dental plaque (Table 4).

PCR-positive results for *H. pylori* were found in eight males (15.7%) and one female (1.9%), which was shown to be a statistically significant difference (Table 5).

The representative results following agarose gel electrophoresis of PCR products obtained from patients' samples are showed in Fig. 1.

Table 1 Prevalence of *H. pylori* infection of oral cavity in patients with and without RAS lesions

H. pylori	*Case group*	*Control group*	*Total*
PCR+	6 (11.3%)	3 (5.8%)	9 (8.6%)
PCR−	47 (88.7%)	49 (94.2%)	96 (91.4%)
Total	53	52	105

Fisher's exact test, $P = 0.254$.

Table 2 Prevalence of *H. pylori* in RAS lesions and intact oral mucosa

H. pylori	*Case group (RAS)*	*Control group (intact mucosa)*	*Total*
PCR+	5 (9.4%)	2 (3.8%)	7
PCR−	48 (90.6%)	50 (96.2%)	98
Total	53	52	105

Fisher's exact test, $P = 0.226$.

Table 3 Prevalence of *H. pylori* in oral mucosa of patients with and without RAS lesions

	Intact oral mucosa		
H. pylori	*Case group*	*Control group*	*Total*
PCR+	3 (5.7%)	2 (3.8%)	5
PCR−	50 (94.3%)	50 (96.2%)	100
Total	53	52	105

Fischer's exact test, $P = 0.509$.

Table 4 Prevalence of *H. pylori* infection of oral cavity in patients with and without RAS lesions

	Dental plaque		
H. pylori	*Case group*	*Control group*	*Total*
PCR+	3 (5.7%)	1 (1.9%)	4
PCR−	50 (94.3%)	51 (98.1%)	101
Total	53	52	105

Fischer's exact test, $P = 0.316$.

Table 5 Prevalence of *H. pylori* infection of oral cavity bases on sex of the patients studied

	H. pylori		
Sex	*PCR+*	*PCR−*	*Total*
Male	8 (15.7%)*	43 (84.3%)	51
Female	1 (1.9%)*	53 (98.1%)	54

Fischer's exact test, $P = 0.012$.
*Statistically significant difference.

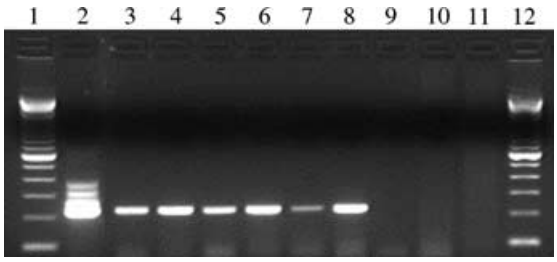

Figure 1 PCR product analysis. The agarose gel (2%) illustrates the pattern obtained after nested PCR on different samples. Lanes 1 and 12: 100-bp DNA ladder (Invitrogen); lane 2: positive control (*H. pylori* ATCC 43504D); lanes 3–8: illustrate positive samples for *H. pylori*; lanes 9 and 10: illustrate negative samples; lane 11: negative control.

Discussion

In the group of patients with RAS, the prevalence of *H. pylori*-positive specimens was 11.3%, and in patients without RAS, it was 5.8%. Such results demonstrate that *H. pylori* can be present in the oral cavity, but its presence in the mouth, independently of the site examined, is not associated with the occurrence of RAS. In addition, there was no significant difference in the incidence of *H. pylori* positivity between specimens collected from the lesions and those collected from intact oral mucosa of the control group. Similar results were obtained by Riggio et al. (11) who identified *H. pylori* in 11% of RAS biopsies by PCR. This incidence was not statistically significantly higher than in oral lichen planus or normal tissue samples. Mravak-Stipetic′ et al. (12) also demonstrated the presence of *H. pylori* in 3.04% of oral lesions, showing no association between *H. pylori* and ulcerated and non-ulcerated lesions.

The relation between RAS lesions and *H. pylori* was demonstrated by Birek et al. (13), who obtained 71.8% *H. pylori* positivity using PCR in RAS lesions of 39 patients. These authors utilized primers based on the sequence of urease genes, which could produce false positives (14). Moreover, the group of patients studied comprised 16 HIV-infected patients, which could place the RAS diagnosis in doubt (15).

There is an important controversy on the significance of the presence of *H. pylori* in the oral cavity. In the gastric mucosa, the only bacterium identified has been *H. pylori*, but more than 350 different species can grow in dental plaque, including microorganisms similar to *H. pylori*, capable of inducing false-positive results, depending on the method of identification applied (16). According to Desai et al. (17), the acid conditions and warm temperature near 37°C in dental plaque offers an ideal medium for the growth of *H. pylori*. Cammarota et al. (18) observed a prevalence of 3.2% for *H. pylori* in dental plaque, and Kamat et al. (19) found a prevalence of 4.4%, suggesting that dental plaque is not reservoir for these bacteria.

The possibility of dental plaque being a source of *H. pylori* in gastric infection was pointed out in a study by Riggio & Lennon (20), who detected the presence of the bacteria in periodontal pockets in 33% of cases, and in a study by Song et al. (21), who showed using nested PCR a high prevalence of *H. pylori* in oral specimens. The authors identified *H. pylori* DNA in 97% of samples of dental plaque and in 55% of saliva samples, while finding this bacterium in the stomach of 26% of the patients. However, according to these authors, these results were evidence that *H. pylori* belonged to normal oral flora and that this oral infection occurs independently of the bacterial status in the gastric mucosa.

Song et al. (22) analyzed quantitatively samples of dental plaque positive for *H. pylori* using cPCR, and determined that 79% of the samples of dental plaque contained less than 50 *H. pylori* per mg. Patients with gastric infection had between 1 and 213 *H. pylori* per mg of dental plaque, while healthy children who participated in the study had 4–94 *H. pylori* per mg of dental plaque, suggesting that this microorganism belongs to the normal flora of the oral cavity in humans. Using PCR in the study of 53 healthy children, Santamaría et al. (23) also could not identify *H. pylori* in the dental plaque of any of the children.

H. pylori infection occurs mainly in children between 1 and 2 years of age (24). On the other hand, in children between 2 and 6 years of age, this bacterium is not identified in the oral cavity, despite that this age group is especially susceptible to infection. This could be explained by the occurrence of small numbers of *H. pylori* in healthy children (22) and by the instability of the oral flora at this age.

Majmudar et al. (25), using the campylobacter like organism (CLO) test, found 100% *H. pylori* positivity in samples of dental plaque collected from healthy volunteers. Similarly, Desai et al. (17) reported 98% positive cases of *H. pylori* in samples of dental plaque using the same diagnostic test. According to the authors of these two studies, dental plaque is a more important reservoir for *H. pylori* than is the gastric mucosa. In the study by Namavar et al. (26), microorganisms similar to *H. pylori* were detected based on positive results for urease, catalase, and oxidase, but were shown to be negative in a PCR assay for this bacterium. Such findings demonstrate the possibility of false identification, as the oral cavity contains other bacteria that produce urease, which could lead to false positives when the method of detection for oral samples is the CLO test. Shimoyama et al. (27) tested lesions from patients with RAS and lichen planus for *H. pylori* by culturing and did not find any positive cases. The authors argued that *H. pylori* from the oral cavity could be in an uncultivable state and that commensal microorganisms present in this cavity could inhibit their growth in culture medium. Ishihara et al. (28), in comparing culture assays and RT-PCR for gastric samples, saliva, and dental plaque, demonstrated that the majority of oral bacteria, primarily *Streptococcus mutans* and *S. sobrinus*, inhibit the growth of *H. pylori.*

The identification of bacteria by DNA analysis is the method most indicated for samples from the oral cavity (29, 30). Performing a second PCR step utilizing nested primers augments, the sensitivity of the test similar to that obtained with pure culture, and qualifies nested PCR as the test indicated for the detection of *H. pylori* in highly contaminated clinical material (31), whereby the primers EHC-U/EHC-L are the most sensitive and specific for this purpose (9).

The results of this study suggest that RAS lesions in children and adolescents are not associated with the presence of *H. pylori* in the oral cavity, and thus the involvement of *H. pylori* in the etiopathogenesis of RAS appears unlikely.

Conclusions

There is no association between *H. pylori* infection in the oral cavity and the occurrence of RAS in children and adolescents ($P = 0.254$); the prevalence of *H. pylori* in RAS lesions are not statistically different from those found in the integral oral mucosa of patients with this disease ($P = 0.226$); there is no statistically significant difference in the prevalence of *H. pylori* in neither the integral oral mucosa ($P = 0.509$) nor the dental plaque ($P = 0.316$) of children and adolescents with RAS, when compared to patients without this disease.

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Acknowledgement

Hereby I thank Dr Diogenes Santiago Santos for his substantive contribution to this study.