Detection of *Helicobacter pylori* in recurrent aphthous ulceration by nested PCR

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Abstract: Recurrent aphthous ulceration (RAU) is the most common lesion of the oral mucosa. Although many factors have been postulated as etiological factors for RAU, the role of *Helicobacter pylori* as a causative agent of RAU remains controversial. We therefore investigated the association of *H. pylori* and RAU by a highly sensitive technique, nested polymerase chain reaction (PCR), in 22 patients with RAU with ages ranging from 12-36 years. Samples were brushed from the lesions and the dorsum of the tongue of each patient. In addition, samples from the dorsum of the tongue of 15 normal individuals with ages ranging from 13-40 years were used as controls. The results showed that only one sample from a lesion (4.5%) and one sample from the tongue (4.5%) of two different patients with RAU were positive for *H. pylori*. In the control group, 3 samples (20%) were positive for *H. pylori*. These findings suggest that *H. pylori* does not play a role in the pathogenesis of RAU and the dorsum of the tongue may be a reservoir of *H. pylori* in some individuals. (J. Oral Sci. 45, 107-110, 2003)

Key words: recurrent aphthous ulceration; *Helicobacter pylori*; nested polymerase chain reaction.

Introduction

Recurrent aphthous ulceration (RAU) is the most common lesion affecting the oral mucosa (1). The prevalence of RAU varies from 2% to 50% in the general population. RAU is clinically characterized by painful recurrent solitary or multiple ulcers usually appearing on non-keratinized oral mucosa. RAU is classified according to clinical features into three types: minor, major and herpetiform RAU (2).

Although the basic cause of RAU remains unknown, several factors including local, microbial, systemic, nutritional, immunological and genetic factors have been suggested as potential etiologic factors (1). Several studies of the role of microorganisms including viruses and bacteria in the pathogenesis of RAU have been undertaken (3-7). Recently, a bacterium, *Helicobacter pylori*, has attracted the interest of many investigators as a possible cause of RAU. *H. pylori* is a microaerophilic, gram-negative spiral bacterium commonly found in gastric mucosa (8). *H. pylori* is known to be a causative factor in chronic active gastritis and has been found to be associated with peptic ulcer disease and gastric cancer (8,9). *H. pylori* has been detected in dental plaque, saliva, the subgingival region and in oral mucosal ulcers (10-13). These data have suggested that the oral cavity may be an alternative reservoir for the organism and a possible route of transmission to other sites (14,15). Since the histological features of RAU are similar to those of gastric ulcers, a relationship between *H. pylori* and RAU has been suggested. However, previous studies of this relationship using various techniques have shown conflicting results (13,16-19). We therefore undertook an additional series of investigations to measure...
the prevalence of *H. pylori* in RAU by means of a highly sensitive and specific polymerase chain reaction (PCR) method.

**Materials and Methods**

**Subjects and sample collection**

Twenty-two patients with RAU (age range 12 to 36 years) and 15 volunteers with normal mucosa (age range 13 to 40 years) who attended the Oral Diagnostic Clinic, Faculty of Dentistry, Chiang Mai University, were included in the present study. None of the patients had prior treatment for their current episode of RAU or had any symptoms of chronic gastritis or peptic ulcer disease. Samples were collected from patients with the classic history and appearance of RAU. Samples were collected when the lesions exhibited well circumscribed margins surrounded by a red halo. The size of the lesions varied from 1 mm to 5 mm. Multiple ulcers were also observed in some patients. Written informed consent was obtained from each patient prior to enrolment in the study, according to the guidelines of the Human Subject Protection Committee, Faculty of Dentistry, Chiang Mai University, Thailand.

The oral samples were collected by brushing the ulcer surfaces and/or the dorsum of the tongue with sterile blunt toothpicks, placed in microcentrifuge tubes, and then stored at -20°C until processing for PCR.

**DNA extraction**

Microcentrifuge tubes containing the samples (blunt toothpicks) were filled with 1 ml of distilled water, held at room temperature for 30 min, and centrifuged at 14,000 r.p.m. for 2 min. The supernatant was removed, and 100 μl of Chelex-100 chelating resin (20% w/v) and 2 μl of proteinase K (100 mg/ml) were added to the pellet. The tubes were vortexed for 10 min, centrifuged at 14,000 r.p.m. for 10 min, and boiled for 8 min. The resulting supernatant was transferred to fresh microcentrifuge tubes in preparation for PCR.

**Standard PCR**

The oligonucleotide primers used in the present study were specific for a 375 base-pair fragment of HpaA gene encoding an adhesin subunit of *H. pylori*, as described previously (20). The primers were custom synthesized at the Institute for Biotechnology, Thailand: forward primer with 20 bases (5'-GA ATT ACC ATC CAG CTA GCC-3') and reverse primer with 20 bases (5'-GT AAC CTT GAC AAA ACC GGC-3'). The PCR product was confirmed by digestion with restriction enzyme Hinf I.

For each aliquot of 10 μl containing an oral sample (5 μl of DNA extraction), a reaction mixture containing 20 mM Tris (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.01% BSA, 0.05% Tween 20, 10% glycerol, 0.2 mM each deoxynucleotide triphosphate, 0.25 μM each oligonucleotide primer, and 0.25 units of *Taq* DNA polymerase (QIAGEN, GmbH, Germany) was added. The reaction mixture was overlaid with 20 μl of mineral oil to prevent evaporation during thermal cycling. The initial denaturation was done at 95°C for 2 min 30 s. The amplification cycles comprised DNA denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min, and extension at 72°C for 1 min. The final cycle included extension at 72°C for 7 min. All the samples were amplified through 35 consecutive cycles.

**Nested PCR**

Nested PCR was performed by transferring 2 μl of the standard PCR product to the second round reaction mixture and reamplifying it with the inner pairs of primers, consisting of a forward primer with 30 bases (5'-GCC AGC TTT CCA ATA CAG CGA TAA CAT TGC-3') and a reverse primer with 26 bases (5'-CCA AAC CAG TGG AGA ATA ATG ACC CG-3'). The annealing temperature was 62°C. The remaining procedure was identical to that of standard PCR.

**Agarose gel electrophoresis**

Each reaction product (5 μl) was added to 2 μl of gel loading buffer [0.25% (w/v) xylene cyanol, 30% (v/v) glycerol, 100 mM EDTA, pH 8.0], electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml), visualized under ultraviolet (UV) illumination, and photographed. A 100-bp DNA ladder (New England Biolabs, USA) was used as a size marker.

With each set of PCR amplification of the oral samples, a corresponding amplification was performed with positive and negative controls. *H. pylori* genomic DNA from a Thai patient was used as the positive control. The negative control was a reaction mixture in which the DNA template was replaced by distilled water.

**Results**

**PCR test results**

Amplification of *H. pylori* genomic DNA from a Thai patient gave an expected 375-bp fragment. Digestion of PCR product with restriction enzyme Hinf I generated specific fragments of 259 bp and 116 bp.

By employing standard PCR assays, none of the samples showed positive results. We were able to obtain positive results when using a more sensitive method, nested PCR. Positive results were obtained from one sample (4.5%) from an ulcer and one sample (4.5%) from the dorsum of the
tongue of 2 different patients with RAU, and three samples (20%) from the dorsum of the tongue of the control group. A representative photograph of the electrophoretic patterns of nested PCR is shown in Fig. 1. An amplification product of the expected size (269 bp) is visible as a well-defined electrophoretic band in the lane corresponding to a sample from the patient with RAU (lane 16). The negative and positive controls, run in parallel with samples from the ulcer and the tongue, showed appropriate results, therefore excluding the possibility of contamination and validating the PCR results obtained.

Discussion

Our study showed that *H. pylori* was not significantly associated with RAU since *H. pylori* DNA was detected in only 4.5% of ulcers by nested PCR assay. These findings were consistent with most recent studies of the association between *H. pylori* and RAU by various techniques. Porter et al. (21) conducted a study of the frequency of serum anti-*H. pylori* IgG antibodies in patients with RAU and found that the frequency of anti-*H. pylori* seropositivity was not significantly greater in patients with RAU compared with other ulcerated oral mucosal lesions and the controls. In a study with a small sample size, Chapman et al. found that all four RAU biopsies were negative for urease activity (22). By using a culture technique recognized as the gold standard for diagnosis of *H. pylori* infection (23), Shimoyama et al. reported that all twelve samples from patients with RAU were found to be culture-negative (19). By employing a more sensitive PCR technique, other researchers found that only 3 of 28 (11%) biopsies (18) and 4 of 32 cytobrushed specimens (12.5%) from RAU lesions were positive (17) for *H. pylori* DNA. On the other hand, a study by Birek et al. (13), the only study to have shown a significant relationship between *H. pylori* and RAU, revealed that the majority of swabbed samples from RAU lesions (71.9%) were positive for *H. pylori* DNA. These discrepancies in the results among these studies remain unexplained. Many factors may account for these discrepancies; for example, variations in techniques used in the studies, variations in collection of the specimens, bacterial density of the samples, differences in the patient populations studied, and variations in primers and target DNA used in the PCR assay (13). However, it appears that the majority of the data including the present study do not support the idea that *H. pylori* plays a role in the pathogenesis of RAU.

Recently, an attempt to look for an association between *H. pylori* and RAU in patients with chronic gastritis was performed and revealed that 14 out of 27 RAU samples from patients with chronic gastritis were positive for *H. pylori* and all 29 RAU samples from patients with the absence of chronic gastritis were negative for *H. pylori* (24). These findings suggested a connection between *H. pylori* in RAU and chronic gastritis. Hence, the low frequency of detection of *H. pylori* in the present study may be partially explained by the fact that our patients did not have chronic gastritis.

Previous studies have suggested that the oral cavity might be a reservoir for *H. pylori* and the source of infection/reinfection after eradication of gastric infection and transmission. Having looked for the presence of *H. pylori* in dental plaque, saliva and the tongue, these studies again showed extreme variations in findings. For example, the detection rate of *H. pylori* by PCR assay from dental plaque and saliva samples varied from 0% (13) to 97% (10) and from tongue samples varied from 0% (25) to 25% (13). In addition, *H. pylori* was detected in cheek samples of 3 out of 20 dyspeptic patients (15%) (26). In the present study we were able to detect *H. pylori* in 4 out of 37 samples from the dorsum of the tongue (10.8%) using PCR techniques. Collectively, these data suggest that the oral cavity may be a reservoir for *H. pylori* in some individuals and transmission of the disease may be via an oral-to-oral route. Nonetheless, the issue of whether *H. pylori* is a permanent or transient member of the oral microflora will need to be further investigated.

![Fig. 1 Nested PCR amplification of *H. pylori* HpaA-specific sequences from RAU samples. Lane 1 represents a 100-bp DNA ladder; lane 2, negative control; lane 3, *H. pylori* genomic DNA from a Thai patient; lanes 4, 6, 8, 10, 12, 14, samples from the dorsum of the tongue; lanes 5, 7, 9, 11, 13, 15, 16, samples from RAU.](image)

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