Prevalence of *Helicobacter pylori* DNA in Recurrent Aphthous Ulcerations in Mucosa-Associated Lymphoid Tissues of the Pharynx

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**Objective:** To determine the presence of *Helicobacter pylori* and, if detected, its potential prevalence in causing recurrent aphthous ulcers confined to mucosa-associated lymphoid tissues of the pharynx.

**Design:** Prospective, controlled clinical trial.

**Setting:** Otolaryngology Department of Tanta University Hospitals, Tanta, Egypt.

**Patients:** A total of 146 patients with recurrent multiple aphthous ulcers of the oral cavity and pharynx and 20 normal control subjects.

**Interventions:** Patients were assigned to group 1 (n=58), in which the ulcers were strictly limited to the lymphoid tissues, or group 2 (n=88), in which the ulcers were randomly distributed in the oral cavity and pharynx. *Helicobacter pylori* DNA was extracted from 3-mm-diameter tissue samples, and polymerase chain reaction amplifications were performed for the 16S ribosomal RNA gene.

**Main Outcome Measure:** Positivity for *H pylori*.

**Results:** In group 1, 39 patients (67%) were positive for *H pylori* DNA, while in group 2, 9 patients (10%) were positive (χ² test, P<.001). It was not detected in any of the 20 control samples.

**Conclusion:** Our results support a possible causative role for *H pylori* in recurrent aphthous ulcerations with a characteristic distribution and affinity to mucosa-associated lymphoid tissues of the pharynx.

Many studies have been published that support and contradict this theory. The histologic similarities between gastric and oral ulcerations have suggested a possible role of *H pylori* in the pathogenesis of RAS, which has been sought and identified in a small proportion (11%) of the tested samples.4

A variety of diagnostic techniques for the detection and identification of bacteria in clinical samples, based on characteristic DNA sequences, have been documented. Nucleic acid–based detection systems can allow the identification of bacteria without the need for isolation in pure culture or the propagation of living organisms.5 This technique is especially useful for the detection of organisms that cannot easily be grown in vitro,6 as is the case for *H pylori*.7 Polymerase chain reaction (PCR), a technique for the amplification of DNA sequences in vitro, has been widely used to assist in the diagnosis of infectious diseases.8,9 The speed and sensitivity of the technique make it ideal for “high-throughput” automated screening of blood and tissue samples. Furthermore, PCR can detect a single copy of a target DNA sequence and therefore requires only small samples for analysis.

Because *H pylori* has an affinity to colonize MALTs and it has been observed clinically that some RAS lesions are sharply confined to MALTs, we aimed to study the presence of *H pylori* and, if detected, its potential viability in the etiology of recurrent aphthous ulcers, confined to MALTs of the pharynx, by using molecular methods.

### METHODS

This prospective study included 146 patients who presented with idiopathic recurrent multiple aphthous ulcers of the oral cavity and pharynx. They were recruited from the Otolaryngology Department of Tanta University Hospitals, Tanta, Egypt, from October 1, 2001, to July 31, 2004. By clinical examination, patients were assigned to group 1 (n=58) (age range, 16-43 years; mean age, 23±6 years; 27 male and 31 female), in whom the ulcers were strictly limited to the lymphoid tissues, or group 2 (n=88) (age range, 15-46 years; mean age, 24±5 years; 41 male and 47 female), in whom the ulcers were randomly distributed in the oral cavity and pharynx. Control samples (n=20) were obtained from surgical specimens of uvulopalatopharyngoplasty and tonsillectomy of sex- and age-matched patients, who demonstrated no symptoms of gastritis, peptic ulcer disease, or RAS, and were treated for unrelated medical entities.

Patients were subjected to an assessment protocol that included a careful history review; full ear, nose, and throat examination; and a general assessment of health, including the immune system. All patients were questioned about the classic symptoms of gastroesophageal reflux (heartburn, acid taste, and regurgitation). In addition, they were asked whether they had been treated previously for gastroesophageal reflux disease or *H pylori* infection in their stomachs. Follow-up was between 6 and 27 months (mean follow-up, 15±6 months). Study protocol and consent forms were approved by the research review committee of Tanta University.

Patients who had used antibiotics during the previous month and who had used bismuth-containing drugs or proton pump inhibitors during the previous 3 months were excluded from the study.

### COLLECTION OF BIOPSY SPECIMENS AND TISSUE DNA EXTRACTION

Within 48 hours of ulceration, multiple biopsy specimens were obtained (each containing about one 3-mm-diameter sample). All samples were analyzed for detection of *H pylori* organisms. If one of the samples from a patient was confirmed to be positive, that patient was recorded as positive for *H pylori*.

Biopsy specimens were immediately frozen and stored at –20°C until used. For DNA extraction, tissue samples were manually homogenized in 0.5 mL of Tris–EDTA–sodium chloride lysis buffer (10-mmol/L Tris hydrochloride [pH 8.0], 1-mmol/L EDTA, 100-mmol/L sodium chloride [pH 8.0]) per 100 mg of tissue. Proteinase K was added at a final concentration of 100 µg/mL. The mixture was incubated at 56°C for 3 hours before the enzyme was inactivated by heating the sample for 10 minutes at 95°C. The mixture was centrifuged (13 000g, 1 minute) and the supernatant retained, and genomic DNA was purified by the phenol-chloroform method.10 The DNA was then precipitated with ethanol, pelleted (13 000g, 5 minutes), washed in 70% ethanol, and dried. The dried pellet was resuspended in 20 µL of sterile water and stored at 4°C.

### H pylori PCR PRIMERS AND PCR AMPLIFICATION

The primers selected for PCR targeted the 16S ribosomal RNA (rRNA) gene of *H pylori*. These primers were previously described and tested in other studies, in which their specificity for *H pylori* was confirmed.11,12 The primers used were *H pylori* forward primer (5’-GTAGCCGTACAGCTACGGAGA-3’) and *H pylori* reverse primer (5’-GACGCGTGAGGCGGGAATGC-3’) (Genosys, Cambridge, England). The expected size of the amplified product was 293 base pairs (bp). Before PCR amplification and to avoid DNA contamination as well as cross-contamination, all pipettes, tubes, and racks were exposed to UV light for 20 minutes before setup in a cabinet using filtered air. The reaction components were assembled on ice in sterile 0.5-mL thin-walled Eppendorf tubes and mixed by vortexing. The PCR amplification was carried out in a total volume of 50 µL comprising 1.0 µL of Taq polymerase in reaction buffer (Boehringer Mannheim Biochemica, Mannheim, Germany), 1.5-mmol/L magnesium chloride, 30 pmol of each primer, 0.2-mmol/L of each deoxyribonucleotide triphosphate, and 5 µL of extracted tissue DNA. The reaction mixtures were overlaid with mineral oil (Sigma–Aldrich Corp, St Louis, Mo), incubated at 96°C for 2 minutes, then subjected to 40 cycles of 96°C for 60 seconds, 60°C for 60 seconds, and 72°C for 90 seconds, followed by a 10-minute extension at 72°C. In addition, each of the reagents incorporated a positive control, DNA extracted from an *H pylori* isolate, and a negative control with the DNA template replaced with double-distilled water. Amplification products were analyzed, after electrophoresis at 80 V, in 1.5% (wt/vol) agarose gels stained with ethidium bromide, along with a 100-bp DNA ladder (Promega, Madison, Wis) as a size marker.

### STATISTICAL ANALYSIS

The results of the study and control groups were statistically analyzed by χ² test; statistical significance was indicated by a level of P<.05.
assigned to either group 1 (n = 58, 40%), in which the ulcers were strictly limited to the lymphoid tissues, or group 2 (n = 88, 60%), in which the ulcers were randomly distributed in the oral cavity and pharynx. In addition, 20 healthy subjects were used as control subjects. Demographic data and clinical manifestations of the study population are presented in the Table.

On examination of tissue samples from the study patients, H pylori DNA was detected in 39 patients (67%) in group 1; 38 of them had the clinical presentation of minor aphthous ulcers and only 1 clinically had herpetiform ulcers. In group 2, 9 patients (10%) were shown to be PCR positive for H pylori DNA (all with clinically minor aphthous ulcers), while it was not detected in any of the control samples. The detection rate of H pylori DNA was significantly higher in group 1 than in group 2 or the control group (χ² test, P < .001). The presence of the amplified 16S rRNA gene products migrating at approximately 300 bp was visualized and photographed under UV light (Figure).

The cause of RAS is not entirely clear, and aphthae are therefore termed idiopathic. The RAS may be the manifestation of a group of disorders of quite different etiology, rather than a single entity. Immune mechanisms appear at play in persons with a genetic predisposition to oral ulceration. Possible predisposing factors seen in a minority include trauma, hematnic deficiency, emotional stress, hormonal state, food allergies, and human immunodeficiency virus infection.2 3

The lesions are usually noted in childhood or adolescence and recur with decreasing frequency and severity with age. The prevalence of RAS varies from 5% to 50% in the general population. Women are affected more commonly than men.2 Lesions are classified into 3 groups: minor, major, and herpetiform ulcers. Minor aphthous ulcers are most common, less than 1.0 cm, and resolve without scarring in 1 to 2 weeks. Major aphthous ulcers are less common, usually greater than 1.0 cm, and deeper, and they heal slowly in 10 to 30 days with scarring. Herpetiform ulcers are the least common variant, with numerouse 1- to 2-mm grouped ulcers that coalesce and heal in 7 to 30 days.1 3

Although H pylori is probably the most common chronic bacterial infection of humans and is present in almost half of the world’s population, the exact mode of transmission and natural reservoirs are unknown. There are 3 proposed routes of transmission: oral-oral, gastric, and fecal-oral. Gastritis, especially in the acute stage, is often accompanied by increased episodes of intermittent gastroesophageal reflux or vomitus, and the tonsils might be colonized with H pylori and thus act as a reservoir.6 Helicobacter pylori may also exist in the nasal and maxillary sinus tissue specimens of some patients with chronic sinusitis and gastric H pylori infection.1 7

It is also possible that the reverse is true. The oral cavity may be a reservoir for H pylori infection and oral secretions may be an important means of transmission of this microorganism. Helicobacter pylori in dental plaque may represent a risk factor for gastrointestinal reinfection and ulcer relapse. In addition, the therapeutic results suggest that recrudescence of infection after cessation of therapy may occur owing to recolonization of the stomach from the H pylori present in dental plaque, because the latter would be unaffected by such treatment. Thus, a small number of organisms probably survive a treatment course only to multiply and recolonize when the treatment regimen is finished, rendering the patient susceptible to ulcer relapse. These studies taken together strongly suggest that the oral cavity in general is a reservoir site of H pylori.1 8 19

Many invasive and noninvasive methods are used to diagnose H pylori infections. Bacteriologic culture and histologic staining of biopsy specimens are the conventional ways used to detect H pylori. Although H pylori culture can be carried out in most laboratories, it has some constraints, including the long delay (>4 days) in obtaining results, the low sensitivity of the culture isolation method, and the need for strict transport conditions because of the fastidious nature of the bacterium.2 0

Table. Demographic and Clinical Data of the Study Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 58)</th>
<th>Group 2 (n = 88)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>23 ± 6</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Sex, No. M/F</td>
<td>27/31</td>
<td>41/47</td>
</tr>
<tr>
<td>Ulcer distribution</td>
<td>MALT</td>
<td>Random</td>
</tr>
<tr>
<td>Ulcer size, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minor</td>
<td>47 (81)</td>
<td>70 (80)</td>
</tr>
<tr>
<td>Major</td>
<td>6 (10)</td>
<td>13 (15)</td>
</tr>
<tr>
<td>Herpetiform</td>
<td>5 (9)</td>
<td>5 (6)</td>
</tr>
<tr>
<td>GER-related complaints, No. (%)</td>
<td>42 (72)</td>
<td>22 (25)</td>
</tr>
<tr>
<td>H pylori positive, No. (%)</td>
<td>39 (67)*</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

Abbreviations: GER, gastroesophageal reflux; MALT, mucosa-associated lymphoid tissues.

*Significant (P < .001).

Figure. Electrophoresis on a 1.5% agarose gel of polymerase chain reaction products obtained from tissue samples with Helicobacter pylori 16S ribosomal RNA-specific primers. Lanes 1 and 7 show a 100–base pair (bp) DNA ladder; lane 2, negative control; lanes 3 to 5, recurrent aphthous ulcer samples; and lane 6, positive control. Polymerase chain reaction positivity is indicated by the presence of 295-bp product. The bottom arrow indicates the 100-bp mark; top arrow, the 600-bp mark.
On the other hand, histologic analysis is time consuming and requires an expert pathologist. The special stains, such as modified Giemsa and silver, have good specificity and sensitivity, but false-positive readings can occur if abundant mucus and contaminating organisms resembling H pylori are present.20,21

Serologic tests inexpensively detect circulating IgG or IgA antibodies. However, despite the cost attractiveness, the diagnostic significance of the enzyme-linked immunosorbent assay test is limited because it cannot discriminate between current and old infections.21 Likewise, in the urea breath test, a patient drinks an oral preparation containing urea labeled with carbon 13 or 14. In the stomach, H pylori bacteria metabolize the urea to produce carbon, which is absorbed into the bloodstream. The carbon travels through the bloodstream into the lungs. When the lungs exhale the carbon, measurement of carbon 13 or 14 determines the presence or absence of H pylori infection, but false-positive results due to the presence of other enteric bacteria remain its main disadvantage.11,22

The PCR is one of the most widely used molecular techniques for detecting specific pathogens. Compared with histologic and cultural methods, PCR offers unprecedented sensitivity in detection of H pylori. It was used for the detection of H pylori in gastric tissue samples, where it provides a rapid, sensitive, and specific test result and is particularly useful for a gastroenterologist who does not have access to local routine laboratory facilities. Furthermore, because the H pylori organisms need not be alive when tested, there are no special requirements in the handling, transport, and storage of the biopsy specimens. The PCR technique also has made it possible to detect DNA in samples that are too small or too degraded to permit other types of analysis.23 For these reasons, it has been suggested that PCR should be considered as a reference test for H pylori after antibiotic therapy. However, care has to be taken in interpreting the PCR result if the follow-up period is too brief, as PCR may falsely amplify DNA from H pylori that have been killed by the antibiotic therapy but remained in the tissues.24

Several genes have been used to detect and identify H pylori, such as UreA and UreB, which encode urease; UreC, which encodes phosphoglucomutase enzyme; and CagA, a cytotoxin-associated gene; as well as 23S rRNA25 and 16S rRNA26 genes. In the present study, 16S rRNA gene primers were used for the detection of H pylori. These primers were demonstrated to be highly sensitive and specific in previous studies.4,10 Amplification of these gene segments has a theoretical advantage, as the high copy number of rRNA per bacterial cell increases the target DNA copies (templates) by several thousandfold. Therefore, this amplification was suggested to give more reliable results.27

Various microorganisms have been examined for a causal association with recurrent aphthous ulcers; however, studies investigating possible bacterial involvement in RAS have been limited. Early investigations suggested that coagulase-negative staphylococci, ß-hemolytic streptococci, and Neisseria species are the predominant bacteria in RAS tissues.28

In the present study, H pylori DNA was detected in 67% of patients in whom the ulcers were strictly limited to the lymphoid tissues, but in only 10% of patients in whom the ulcers were randomly distributed in the oral cavity and pharynx. These results support a possible etiologic role for H pylori in recurrent aphthous ulcers with special affinity for MALTs.

Riggio et al detected H pylori DNA in 11% of RAS samples but not in any of the normal samples. Although their results did not support a definitive etiologic role for H pylori in RAS, the possibility that H pylori may be involved in a small proportion of RAS cases could not be excluded. Likewise, the possible pathogenic significance of H pylori in oral ulcerations had also been reported by Birek et al.29 In their study, 71.8% of patients with oral aphthous ulcers were found to be positive for H pylori DNA, while the saliva and plaque samples of the same patients were consistently negative.

On the other hand, some studies did not support the assumption that H pylori could be involved in RAS development. Unfortunately, those studies showed overall controversial results; the incidence of H pylori DNA in oral aphthous ulcers varied from 4.5% to 38.9%, and in none of them was this incidence rate significant compared with their controls.30-32 All of these studies were carried out at dental research centers, where samples were obtained from the oral cavity, while oropharyngeal mucosa was not tested. In addition, specimens were obtained by swabbing or brushing of RAS lesions.

Using molecular techniques, Cirak et al, in their study of patients who had undergone adenoidectomy and/or tonsillectomy, demonstrated that the colonization rate of H pylori in tonsil and adenoid tissues was 30%. The authors postulated that the tonsil and adenoid tissue may be an ecological niche of the mouth regardless of transient or permanent colonization, and oral-oral transmission may be a possible mode of spread of H pylori. They concluded that the risk of peptic ulcer disease, gastric cancer, and MALT lymphomas of the stomach can be decreased with therapies for eradicating the bacteria. Moreover, they speculated that tonsillectomy and adenoidectomy may protect the host against H pylori infestation of the stomach.

The present study could not establish causality because this would require rigorously controlled epidemiologic studies to clarify the potential underlying pathogenetic mechanisms. In addition, long-term follow-up is required to show that eradication of H pylori alters the course of the disease.

Although many aspects of the epidemiology of H pylori infection are known, the mode(s) of transmission remains unclear. Recently, the oral environment has been suggested to be one of the many potential pathways for transmission. Our results support a possible etiologic role for H pylori in recurrent aphthous ulcerations with a characteristic distribution and affinity to MALTs of the pharynx.

Submitted for Publication: March 13, 2005; final revision received April 3, 2005; accepted April 8, 2005.
REFERENCES