



RAPID COMMUNICATION

***Helicobacter pylori* infection in the pharynx of patients with chronic pharyngitis detected with TDI-FP and modified Giemsa stain**

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pharynx, which was remarkably higher ($P=0.042$) than that in the patients without stomach ailment history (1 case, which was 2.9%).

CONCLUSION: *H. pylori* may not be detected in the pharynx of healthy people. Chronic pharyngitis may be related to *H. pylori* infection. The infection rate with *H. pylori* in the pharynx is higher in patients with stomach ailment histories than in patients without stomach ailment histories, suggesting that chronic pharyngitis may be related to stomach ailment history.

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Key words: Chronic pharyngitis; *H. pylori*; Modified Giemsa stain

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Abstract

AIM: To detect whether there is *Helicobacter pylori* (*H. pylori*) colonization in the pharynx mucous membrane of healthy people and whether chronic pharyngitis is related to *H. pylori* infection.

METHODS: Fifty cases of chronic pharyngitis refractory over three months were prospectively studied from March 2004 to August 2004 in the otolaryngology outpatient department of the Second Hospital of Xi'an Jiaotong University. Template-directed dye-terminator incorporated with fluorescence polarization detection (TDI-FP) and modified Giemsa stain were used to examine pharynx mucous membrane tissue for *H. pylori* colonization in the patients with chronic pharyngitis and the healthy people as a control group.

RESULTS: In the control group, no people were detected to have *H. pylori* in the pharynx. In contrast, in 50 cases with chronic pharyngitis, 19 (38.0%) cases were *H. pylori* positive with a TDI-FP assay and 4 (8%) cases were TDI-FP positive with Giemsa staining in the pharynx. Sixteen of the 50 pharyngitis cases had stomach ailment history, 11 cases (68.8%) of these 16 patients were determined to be *H. pylori* positive in the pharynx with the TDI-FP assay. χ^2 test showed that this infection rate was remarkably higher ($P=0.0007$) than that in the cases without stomach ailment history. Giemsa staining showed that 3 cases (18.8%) of the patients with stomach ailment history were infected with *H. pylori* in the

INTRODUCTION

Chronic pharyngitis is a common disease in the otolaryngology clinic. Pharyngitis, bronchitis, and pneumonia represent the most common respiratory tract infections. Upper respiratory tract infections are common and important. These include sinusitis, otitis media, and pharyngitis/tonsillitis. Many patients visiting an office-based physician report "sore throat, foreign-body sensation in the throat" as their primary reason for the visit^[1]. Acute pharyngitis may be caused by a wide variety of microbial agents, but some of the most common and potentially dangerous microorganisms are group A beta-hemolytic streptococci (GABHS). For example, streptococcal pharyngitis is responsible for about 5% to 17% of sore throats in adults. However, the causative microorganisms in many cases remain unclear^[2]. An important risk factor for chronic pharyngitis is gastroesophageal reflux disease (GERD). GERD is a common disorder in the Western population and is also common in Xi'an's adult population. The etiology and pathogenesis of GERD are probably associated with other conditions that are also risk factors

for chronic pharyngitis, including functional dyspepsia (FD), irritable bowel syndrome (IBS), and some respiratory and laryngopharyngeal diseases^[3].

The Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) is associated with severe gastric pathologies, including peptic ulcers, chronic active gastritis and gastric cancer. This microorganism is able to invade and colonize in the human stomach, gastric juice, saliva, and faeces of patients^[4-6]. Moreover, as relates to the current study, investigations using the CLO (Campylobacter-like organism) test and PCR showed that there was a high rate of *H. pylori* colonization in tonsil and adenoid tissues^[7,8], however, another investigation indicated the opposite result^[9]. Therefore, we sought to detect whether there was *H. pylori* colonization in the pharynx in healthy people, as compared to patients suffering from chronic pharyngitis. We used two methods to detect *H. pylori* infection in the pharynx: template-directed dye-terminator incorporated with fluorescence polarization detection (TDI-FP) and modified Giemsa stain. From this study, it can be determined more definitively whether there is a relationship between *H. pylori* infection of the pharynx and chronic pharyngitis; moreover, it can be determined if a history of certain gastric diseases may contribute to this infection.

MATERIALS AND METHODS

Patients

Two groups were studied, a group with chronic pharyngitis and a control group without chronic pharyngitis. The group with chronic pharyngitis consisted of 50 cases of chronic pharyngitis refractory over three months that were studied from March, 2004 to August, 2004 in the otolaryngology outpatient department. Among them, 32 cases were females and 18 cases were males, with ages ranging from 23 to 52 years old. The shortest disease history was 3 mo and the longest was 8 years. Major symptoms included foreign body sensation, dry sensation, angina in the pharynx, nausea, cough and belching. Clinical examination showed congestion in the pharynx mucosa, lymph follicle hyperplasia, and slightly swollen tonsils. Of these 50 cases, there were 34 cases with no specific stomach ailment history and 16 cases with gastric ulcers or chronic active gastritis. There were 20 cases in the control group. Of these, 12 cases were females and 8 cases were males. Ages ranged from 20-51 years old. The people in the control group had no specific pharyngitis or stomach ailment history, such as gastric ulcer or chronic active gastritis. In addition, all the people in the above two groups (1) had no other system diseases; (2) had no antibiotics treatment 10 d before examination; and (3) had no clear history of metronidazole, amoxicillin or tetracycline treatment. To collect tissue from the pharynx of each patient, the surface of the pharynx mucous membrane was sprayed with 20 g/L amethocaine for anaesthesia. Epithelial tissue in the pharynx was then collected with a sterilized curette. The protocol was approved by the Institutional Human Subject Committee at Xi'an Jiaotong University.

Methods

The biopsied epithelial tissue from the pharynx of each patient was routinely fixed in 4 g/L formaldehyde and embedded in paraffin wax. Histological sections were stained with routine modified Giemsa.

Fluorescence Polarization-Capable Instrument-Victor, AmpliTaq DNA Polymerase, shrimp alkaline phosphatase, *E. coli* exonuclease I, mixtures of TAMRA-ddTTP and RP110-ddGTP were purchased from PerkinElmer. The pGEM-T-Easy Vector System TA Cloning Kit was purchased from Promega. The ABI 377 and BigDye Terminator Cycle Sequencing Kit were purchased from Applied Biosystem(s). All reactions were run and read in 96-well black-skirted plates purchased from MJ Research. The specific probes and terminators of *H. pylori* were designed by DNA Star based on a specific target sequence. All probes were synthesized by Sbsbio (Beijing, China).

Scrapes or biopsies were collected and washed into 5 mL PBS (pH 7.2). In each case, the cell suspension was centrifuged for 5 min at 10 000 r/min. The cell pellet was re-suspended and mixed in 1.5 mL TE buffer (200 mg/L proteinase K, 10 mmol/L Tris, 0.1 mmol/L EDTA, 1 g/L SDS, 3 g/L Triton X-100) at 37°C for 12 h. The suspension was incubated at 95°C for 10 min to inactivate proteinase K, and then it was centrifuged for 5 min at 10 000 r/min. The supernatant DNA was used as a template for PCR.

Amplification of *H. pylori* DNA by using a conservative primer pair

For extensive detection of *H. pylori* DNA, each sample DNA was first amplified in a 25 µL reaction mixture containing 1 µL of DNA extract, 2 µL of 6.25 pmol/L common conservative primer pair (P1, 5' tgccccgttc-cactaacccca 3'; P2, 5' gtcagccactttgccacttctacag 3', *H. pylori* urease B (*ureB*) gene (gene bank accession number AY295085), 10×PCR reaction buffer (10 mmol/L Tris-HCl, 50 mmol/L KCl), 2.5 µL of 2.5 mmol/L dNTP, 1.5 µL of 25 mmol/L MgCl₂, and 2.5 µL of 0.25 U AmpliTaq DNA Polymerase. The mixture was denatured initially for 5 min at 94°C, followed by 30 cycles of amplification in a PCR processor. Each cycle included a denaturing step of 94°C for 1 min, an annealing step of 45°C for 1 min and a chain elongation step of 72°C for 1.5 min. The final elongation step was then prolonged for another 5 min. The size of all *H. pylori* genotyping PCR products was predicted to be 413bp according to gene bank accession number AY295085. Sample containing other plasmid DNA was the negative control.

All PCR products were analyzed using a template directed dye-terminator incorporation assay (TDI) with fluorescence polarization (FP). In order to degrade excess dNTP and common conservative primers in the PCR product, 1µL of PCR product, 16.67 nkat of shrimp alkaline phosphatase, 1U of *E. coli* exonuclease I in 1 µL of shrimp alkaline phosphatase buffer (0.5 mol/L Tris-HCl, pH8.5, 50 mmol/L MgCl₂), and 8 µL of distilled water were mixed and incubated at 37°C for 60 min before enzymes were heat-inactivated at 80°C for 15 min. Thirteen microliters of TDI-FP mixture containing 10× reaction

Table 1 *H pylori* infection in the pharynx of patients with chronic pharyngitis

Group	Case	TDI-FP(%)	Giemsa(%)
Chronic pharyngitis	50	19 (38.0) ^a	4 (8.0)
With stomach ailment history	16	11 (68.8) ^b	3 (18.8) ^b
Without stomach ailment history	34	8 (23.5)	1 (2.9)
Normal (Control)	20	0	0

^a $P \leq 0.05$ vs Control; ^b $P \leq 0.01$ vs group without stomach ailment history.

buffer, TAMRA-ddTTP, RP110-ddGTP, *H pylori* probes and 7 μ L of the enzymatically treated PCR product were mixed and denatured at 95°C for 2 min, followed by 25 cycles of 95°C for 15 s and 50°C for 30 s. At the end of the cycles, the mixture was held at room temperature. *H pylori* genes were acquired by reading FP (mp) at wavelengths of 535 nm and 595 nm, and analyzed by Fluorescence Polarization-Capable Instrument-Victor.

Statistical analysis

All the data were analyzed by the χ^2 test.

RESULTS

H pylori infections in the pharynx of the people in the control group and the patients suffering from chronic pharyngitis were examined with TDI-FP and modified Giemsa stain. In the control group, analysis with TDI-FP showed that none of the 20 cases was infected with *H pylori* in the pharynx. The examination using modified Giemsa stain revealed that no cases were infected with *H pylori* in the pharynx.

Regarding the 50 cases of the patients who were suffering from chronic pharyngitis, 19 cases (38%) were determined using TDI-FP to be infected with *H pylori* in the pharynx. When the modified Giemsa stain procedure was used, 4 cases (8.0%) of patients were determined to be infected with *H pylori* in the pharynx. In the 16 pharyngitis cases with stomach ailment history, 11 cases (68.8%) were determined using TDI-FP to be infected with *H pylori* in the pharynx, which was remarkably higher ($P \leq 0.01$) than that in the cases without stomach ailment history (8 cases, 23.5%) as determined by the Statistical χ^2 test. From using the modified Giemsa stain method, 3 cases (18.8%) of patients with a stomach ailment history were determined to be infected with *H pylori* in the pharynx, which was remarkably higher ($P \leq 0.01$) than that in patients without a stomach ailment history (1 case, 2.9%), as determined by the statistical χ^2 test (Table 1). This showed the following: (1) that *H pylori* is not detected in the pharynx of healthy people; (2) that chronic pharyngitis is often related to *H pylori* infection; and (3) that a stomach ailment history is associated with a higher rate of *H pylori* infection of the pharynx.

DISCUSSION

We used two methods to detect *H pylori* infection,

template-directed dye-terminator incorporation with fluorescence polarization (TDI-FP), and modified Giemsa stain. TDI-FP is a single base extension technique in which an oligonucleotide probe anneals to a polymerase chain reaction (PCR)-amplified product adjacent to a single nucleotide polymorphism (SNP) of interest^[3,10]. In the presence of DNA polymerase and fluorescently labeled dideoxynucleoside triphosphates (ddNTPs), the probe is extended by a single base dictated by the polymorphic site in the target sequence. Fluorescence polarization, the property that fluorescent molecules emit polarized fluorescent light when excited by plane polarized light, is used to identify incorporated ddNTP, and to assign a genotype^[10]. With this design, the PCR primers will not interfere with the primer extension step of the assay. This method is simple, rapid, sensitive, specific and could also be used for detecting pathogen DNA in the clinic^[11,12]. The modified Giemsa stain is very straightforward, inexpensive, and takes about five minutes to perform, excluding the time in solution, and rarely requires repeat stains^[13].

In the current study, we examined for *H pylori* infection in the pharynx with TDI-FP and the modified Giemsa stain methods. These two methods are rapid, sensitive, specific, easily reproducible and easy to perform technically. The secretion in the pharynx in our study was washed out by water from the epithelial tissue in the checked pharynx before examination so that the results were not affected by gastric juice and saliva.

Sore throat is one of the most common reasons for visits to family physicians. While most patients with sore throat have an infectious cause (such as pharyngitis), fewer than 20 percent have a clear indication for antibiotic therapy (i.e., group A beta-hemolytic streptococcal infection). Useful, well-validated clinical decision rules are available to help family physicians care for patients who present with pharyngitis^[14]. Because of recent improvements in rapid streptococcal antigen tests, throat culture can be reserved for patients whose symptoms do not improve over time or who do not respond to antibiotics. Pharyngitis and the common cold are among the most frequent diseases. Two thirds of the patients consulting for respiratory infection received antibiotic treatment, and antibiotics confer relative benefits in the treatment of sore throat^[16]. Pharyngitis may be caused by a wide variety of microbial agents. *Burkholderia cepacia* is a Gram-negative bacillus that is widely distributed in nature; pharyngitis due to *Burkholderia cepacia* was reported for person-to-person transmission^[17]. The main aetiological agents reportedly causing acute pharyngitis were adenovirus, respiratory syncytial virus (RSV), *Mycoplasma pneumoniae*, *Streptococcus pyogenes* and *Chlamydia pneumoniae*. *M. pneumoniae* was the agent found most frequently as a single pathogen. A history of recurrent pharyngitis, having older siblings and a negative outcome were significantly more common among patients with acute *M. pneumoniae* infection than among those with infections due to other pathogens, or healthy controls. That study demonstrates that: (1) adenovirus and RSV have a prominent role in acute pharyngitis; (2) *S. pyogenes* is found frequently, but it is not possible to distinguish simple carriers from patients with a true infection; and (3) *M. pneumoniae* appears to be

able to cause acute pharyngitis^[18]. In our daily work, we use antibiotics to treat pharyngitis and sore throat and find antibiotics are effective, although we are not clear about the mechanism of the treatment.

H pylori is one of the world's most widespread microorganisms. The abrupt increase of *H pylori* during high school may result from a marked increase of interpersonal social activities^[19]. Its acquisition in humans remains poorly understood, however, epidemiological studies have identified drinking water as a reservoir for the bacterium^[20]. *H pylori* has been associated with the development of gastritis, peptic ulcers and gastric cancer. Although *H pylori* infects up to more than half of the world's population, to date the precise modes of transmission have not been fully understood^[13]. *H pylori* has been investigated in several other organ systems and localizations besides the gastrointestinal cavity, such as the oral cavity. For example, in one study, it was found that the majority of patients with oral diseases have possible *H pylori* colonization in dental plaque; while about two-thirds have *H pylori* associated chronic active gastritis. The oral cavity may be the first place for colonization by *H pylori*, and then the infection involves the gastric mucosa^[21]. Oral hygiene (the frequency of dental visits and teeth cleaning) did not have a significant influence on the presence of *H pylori* in dental plaque. Other investigators supported the hypothesis that *H pylori* infection begins in the oral cavity by concluding that dental plaque is the reservoir of *H pylori* with no relationship to gastric infection^[22]. In our study, the pharynx may be a place for *H pylori* colonization, but the number of *H pylori* was so few that the modified Giemsa stain was not very useful. Therefore, we made an amplification of *H pylori* DNA by using a conservative primer pair, then detected the *H pylori* colonization using TDI-FP and acquired a good result.

Other investigators have studied the localization of *H pylori* as well. Akbayir *et al*^[23] demonstrated that *H pylori* was not present in laryngeal squamous cell carcinoma tissue or in benign lesions. They could not find any evidence indicating that *H pylori* played a role at the tissue level in the pathogenesis of laryngeal carcinoma. Their results may indicate that *H pylori* does not colonize in either adenoid or tonsils and that these tissues do not constitute a reservoir for *H pylori* infection. On the contrary, Cirak *et al*^[7] detected *H pylori* and its *CagA* gene in tonsil and adenoid tissues by PCR. These authors found: 7 of 23 (30%) patients to be positive for *H pylori* DNA, 5 (71%) of whom also possessed the *CagA* gene. In another study, specimens from 208 dyspeptic patients were collected from saliva, supra- and subgingival dental plaque, tongue scrapings, and oropharyngeal swabs. *H pylori* was detected from multiple sites (dental plaque, gastric juice, gastric biopsy, duodenal aspirate, and the oropharynx^[26]). As in our study, the authors used more than one method for detecting *H pylori*. When PCR was used, 15 of 208 patients (7%) tested positively for *H pylori* by PCR in dental plaque. In contrast, only 2 samples were positive by culture. This demonstrates the importance of using more than one method for detecting *H pylori* infection. We also found this to be true in our own study, where we used two methods, TDI-FP and modified Giemsa stain,

to detect *H pylori* infection. As shown in Table 1, TDI-FP consistently detected *H pylori* infections that were missed by the modified Giemsa stain method. These authors also found that four of the dental plaque strains had restriction patterns similar to those of the stomach and duodenal sites, providing evidence that these sites were infected with the same strain of *H pylori*. The detection in dental plaque could indicate that the oral cavity may act as a reservoir or sanctuary for the organism. Likewise, this finding also relates to our data. We found that *H pylori* infection in the pharynx was significantly more likely in patients with a history of stomach ailments than in patients without a history of stomach ailments. That is, just as there is a relationship between the occurrence of *H pylori* in dental plaque and its occurrence in the stomach and duodenum, there may be a relationship between infection of the pharynx with *H pylori* and a history of stomach ailments. However, whether *H pylori* is a resident or transient oral microorganism is still unclear, although it is more likely to be transient in nature^[27].

In our study, the goal was to determine if there was a relationship between *H pylori* infection in the pharynx and chronic pharyngitis, and, in addition, to determine if such infections correlated with a history of stomach ailments. We determined both of these relationships to be true. In addition, we utilized two methods for detecting *H pylori*, TDI-FP and modified Giemsa stain, and found the TDI-FP method to be the most sensitive. These findings can be applied to the clinical setting: doctors will be able to use the appropriate method to test if a patient with chronic pharyngitis is infected in the pharynx with *H pylori*, aiding in prescribing the appropriate antibiotic. These findings also should cause doctors to examine a patient with chronic pharyngitis for ailments in the digestive system. But on the other hand, the TDI-FP test can yield false positive results.

To determine whether *H pylori* infection is related to other upper respiratory tract infections, such as sinusitis, or with otitis media, further investigations will need to be performed.

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