Helicobacter pylori in Dental Plaque and Saliva

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Background : *About half of the world population is infected with H. pylori, but the transmission and the source of this infection are still unclear. Recently, dental plaque (DP) and saliva have been implicated as possible sources of H. pylori infection. This study was done to investigate the detection rates of H. pylori in the DP and saliva by use of PCR depending on H. pylori infection state of gastric mucosa.*

Methods : *In 46 subjects, gastric H. pylori colonization was evaluated with CLO test, microscopy of Gram stained mucosal smear, culture and histology after modified Giemsa staining in the antrum and body, respectively. A patient was regarded as H. pylori positive if one or more of the four aforementioned test methods demonstrated H. pylori colonization of the gastric mucosa. For detection of H. pylori in the DP and saliva, PCR assay was done with ET4-U and ET4-L primers. To estimate the sensitivity and specificity of this PCR, H. pylori positivity was evaluated in the antrum and body, separately.*

Results : *The sensitivity of mucosal PCR was 50.0% (27/54) and the specificity 86.8% (33/38). When a subject was regarded as H. pyloi positive, if either antrum or body mucosal H. pylori was is positive, the positive rate of mucosal PCR was 62.1% (18 subjects) in the 29 H. pylori-positive and 17.6% (3 subjects) in the 17 H. pylori-negative subjects. DP PCR was positive in 2 of 29 H. pylori-positive subjects (6.9%) and none in the 17 H. pylori-negative (0%). Saliva PCR was positive in 4 of 14 H. pylori-positive subjects (28.6%) and none of 6 H. pylori-negative (0%).*

Conclusion : *The detection rates of H. pylori in DP and saliva by PCR were rather low, 6.9% and 28.6%, respectively, and these rates might have been underestimated by low sensitivity of the PCR method used in this study. However, the results that H. pylori was found in the DP and saliva suggest that the oral cavity can perform a role as a reservoir of H. pylori in Korea.*

Key Words : *Helicobacter pylori; Dental plaque; Saliva; Polymerase chain reaction*

INTRODUCTION

Helicobacter pylori (*H. pylori*), first isolated from a human gastric biopsy specimen in 1983, is now considered as a common worldwide gastric pathogen. It causes chronic type-B gastritis and is considered

as a major cause of peptic ulcer disease and, presumably gastric malignancy[1]. About half of the world population is infected with *H. pylori*[2] and oral-oral and fecal-oral modes of transmission have been postulated. However, the transmission and the source of this infection are still unclear. Recently, dental plaque (DP) and saliva have been implicated as possible sources of *H. pylori* infection[3,4]. In addition, the failure of triple therapy to clear *H. pylori* infection from DP, despite its clearance from gastric mucosa[5], raised the possibility that DP is a potential source of reinfection of gastric mucosa. In our country, the reinfection rate of *H. pylori* was 12.8% per year[6], higher than those of developed countries, 0.36-1.2% per year[7-9], raising the necessity of investigating whether or not oral cavity such as DP or saliva performs does a role as a reservoir of *H. pylori*.

While *H. pylori* could have been isolated from the oral cavity in a few cases[10-14], most attempts to culture it have failed[15-18]. Polymerase chain reaction (PCR)-based assays may be convenient tools for detecting *H. pylori* in saliva and DP because of their high sensitivity and specificity[3,19]. However, the detection rates of *H. pylori* by PCR ranged from 0% to 100%[3,10,12,16,17,20-25], suggesting that these variations may reflect variable prevalence of *H. pylori* in the oral cavity but also that it can be originated from different specificity and sensitivity of the primers used. This study was done to investigate the detection rates of *H. pylori* in the DP and saliva by use of PCR depending on infection state of gastric mucosa. For this aim, we used a new nested PCR assay probe which has been proved to be very sensitive and specific for *H. pylori*[26].

MATERIALS AND METHODS

A total of 46 subjects who visited the endoscopy room of Kangnam General Hospital from September 1997 to October 1998 participated in the study. They were selected in three kinds of ways: 32 subjects were by high suspicion of peptic ulcer disease by past ulcer history or upper GI series, and they were diagnosed as active duodenal ulcer (DU) (10 patients), benign gastric ulcer (BGU) (13 patients), DU scar (5 patients), active pyloric channel ulcer (3 patients), and both of DU and BGU (1 patient) after endoscopy; 11

were peptic ulcer patients undertaking follow-up gastroendoscopy at least 4 weeks after triple therapy (7 patients of BGU and 4 patients of DU); three subjects were spouses of peptic ulcer patients. Their mean age was 45.8± 13.2 years old, and 36 (78.3%) were male. All subjects were informed of this study and consent was received.

DP and saliva specimens were always taken prior to the endoscopic procedure to exclude the possibility of contamination of the tooth surface and saliva with *H. pylori* during the withdrawal of the endoscope. After the frequency of dental visits during the previous 1 year was assessed, the subjects' gingiva and plaque were assessed by using the gingival and plaque indices of Silness and Loe[27].

The gingival index was as follows: 0, normal gingiva; 1, mild inflammation, slight change in color, slight edema, and no bleeding on probing; 2, moderate inflammation, redness, edema, and bleeding on probing; and 3, severe inflammation, marked redness and edema, ulceration, and tendency to spontaneous hemorrhage.

The plaque index was as follows: 0, no plaque; 1, film of plaque, visible only on removal on probe or by disclosing with color indicator system; 2, moderate accumulation of deposits within the pockets or on the margins which can be seen with the naked eye; and 3, heavy accumulation of material filling the niche between the gingival margin and the tooth surface, and the interdental region is filled with debris. DP was obtained from the incisor teeth with universal curette. The currette was immersed in 2% glutaraldehyde when not in use and was thoroughly rinsed first with glutaraldehyde and then with distilled water, before use in each subject. Supragingival plaques, collected by an upward scrape against the tooth surface, were immediately placed in sterile tube containing 0.1 mL of saline for PCR, and part of plaque was innoculated into CLO test gel. In 39 of 46 participating patients, about 0.1 mL of saliva was innoculated into CLO test gel, and in 20 subjects about 1 mL of saliva was collected in sterile tube for PCR. Both of DP and saliva for PCR were stored at -70°C until they were processed. After getting DP and saliva, the subjects undertook endoscopy. Six biopsy specimens were taken within 3 cm of the pyloric ring and in the middle body, respectively. These biopsy specimens were analyzed with CLO test (one specimen), microscopy of Gram stained mucosal smear (one

specimen), culture (one specimen), and histology after modified Giemsa staining (2 specimens). Remaining one specimen from antrum and body, respectively, was immediately placed in sterile tube containing 0.3 mL of saline for PCR, and stored at -70°C until they were processed. A patient was regarded as *H. pylori* positive if one or more of the four aforementioned test methods demonstrated *H. pylori* colonization of the gastric mucosa[6].

Extraction of DP was done as follows[28]: after thawing of frozen plaque samples, DP was suspended in 100 μL of TE containing Mutanolysin (final concentration, 0.1 μg/μL) and lysozyme (final concentration, 5 μg/μL) (both enzymes were purchased from Sigma Chemical Co., St. Louis, MO. USA). After 1 hour at 37°C, 900 μL of a lysis buffer (which contained following per 100 mL of 0.1 M Tris [pH 6.4]: 120 g of guanidine isothiocyanate, 22 mL of a 0.2 M EDTA solution adjusted to pH 8.0 with sodium hydroxide, and 2.6 mL of Triton X-100) was added together with 40 μL of diatomaceous earth (Celite; Sigma Chemical Co.). The samples were mixed and then incubated at room temperature for 10 min. The samples were again mixed and centrifuged at 12,000 x *g* for 20 sec. The DNA containing pellet was washed twice with 1 mL of guanidine isothiocyanate in Tris, twice with 1 mL of 70% ethanol, and once with 1 mL of 100% acetone. The pellets were dried by incubation at 56°C for 10 min, and then the DNA pellet was eluted in 300 μL of TE by incubation at 56°C for 10 min. The supernatant was extracted with 300 μL of phenol:isoamylalcohol:chloroform (25:1:24) two times. The upper phase was mixed with two volumes of cold 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 4.8), and stored at -70°C for 20 min. The pellet was washed with 1 mL of 70% ethanol and dried at 56°C, and resuspended with 55 μL of TE. To prepare the genomic DNA for PCR from saliva and gastric biopsy, gastric biopsy samples were transferred to 0.5 mL of digestion buffer [20 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS] containing protease K (final concentration, 100 μg/mL), and for saliva 0.5 mL of this digestion buffer was added. After incubation at 52°C for 3 hours, DNA was extracted with 0.5 mL of phenol, 0.5 mL of chloroform, and 0.5 mL of phenol:isoamylalcohol: chloroform (25:1:24), sequentially. The upper phase (about 0.4 mL) was mixed with 2 volumes of cold 100% ethanol and 1/10 volume of 3 M sodium

acetate (pH 5.2). After centrifugation, the DNA pellet was washed with 70% ethanol, and finally resuspended with 100 μL of TE. The primers (ET4), developed and sequenced by Dr. Eapen Thomas (East Tennessee State University)[26] were synthesized at GenoTech (Daejeon, Korea). The sequences of the primers were: ET4-U (24 bp) 5' AAA ATC AGG CCT ATC GCT TTG TAT 3'; and ET4-L (21 bp) 5' GCC CCC ATA AAC ACC AAG AGT 3'. This pair of primers had a span of 203 bp. The buffer of the PCR contained 1.5 mM MgCl, PCR buffer, 0.4 mM dNTP, 0.25 μM ET4-U and ET4-L primers each, and 0.25 units of *Taq* polymerase. The total volume of the PCR was 20 μL. One microliter of 200 ng DNA specimens of gastric mucosa and saliva, and as much as possible up to 113 ng of DP specimen were used as templates. For each pair of primers, the following conditions were the same: initial denaturation at 95°C for 10 min: denaturation, annealing, and extension by 40 cycles, with each cycle consisting of 95°C/45 sec, 60°C/30 sec, and 72°C/45 sec. There was another longer extension of 6 min at 72°C. Positive and negative controls were performed for each batch of amplifications. The DNA extracted from *H. pylori* ATCC 43629 served as a positive control, and water as a negative control. The amplified products were analyzed by 1% agarose gel electrophoresis with ethidium bromide, and observed under ultraviolet light.

RESULTS

Among 46 participating subjects, gastric *H. pylori* was positive in 25 (54.3%) in the antrum, and 29 subjects (63.0%) in the body by CLO test, touch print of Gram stain, Giemsa stain and/or culture. In 4 subjects, *H. pylori* was found only in the body. When mucosal PCR was done in these 92 mucosal specimens (antrum and body in each subject), 203 bp fragment was found in the 32 specimens (Figure 1). To estimate the sensitivity and specificity of the PCR, *H. pylori* positivity was evaluated in the antrum and body, separately. In the antrum, the PCR was positive in 12 of 25 *H. pyloi*-positive subjects (sensitivity: 48.0%), and negative in 18 of 21 *H. pyloi*-negative subjects (specificity: 85.7%). In the body, the PCR was positive in 15 of 29 *H. pyloi*-positive subjects
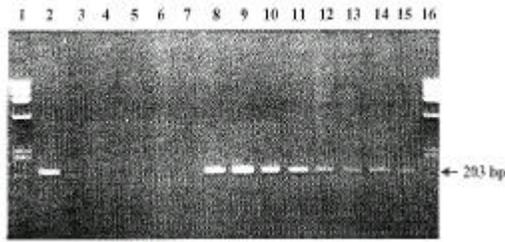
Figure 1. Representative PCR products from the gastric mucosal DNA preparations. The amplified DNA fragments were 203 bp in the electrophoresis on 1% agarose gel. Lane 1 and 16: Ladder DNA markers (Phix174 DNA Hae Three Marker, Promega Co.).

Lane 2: positive control.

Lane 3: negative control.

Lane 4-7: negative PCR.

Lane 8-15: positive PCR.

(sensitivity: 51.7%), and negative in 15 of 17 *H. pyloi*-negative subjects (specificity: 88.2%). Taken together, the sensitivity of mucosal PCR was 50.0% (27/54) and the specificity 86.8% (33/38) (Table 1).

When a subject was regarded as *H. pyloi* positive if either antrum or body mucosal *H. pylori* was positive, the positivity rate of mucosal PCR was 62.1% (18 subjects) in the 29 *H. pylori*-positive subjects, and 17.6% (3 subjects) in the 17 *H. pyloi*-negative subjects (Table 2). Among these 18 mucosal PCR positive subjects, 9 subjects showed PCR positive in both antrum and body, 4 in the antrum only and 5 in the body only. All three mucosal PCR positive subjects in the 17 *H. pyloi*-negative were followed up cases of two DU and one BGU patients after triple therapy, and one showed PCR positive in both antrum and body, one in the antrum and another in the body only. The dental visits in the previous one year was 0.13± 0.30, and the mean indexes of gingiva and plaque were 1.54± 0.70, and 2.02± 0.70, respectively. When

Table 1. The sensitivity and specificity of gastric mucosal PCR

| PCR	True positive	True negative
Positive	27	5
Negative	27	33
Total	54	38

sensitivity : 50.0% (27/54), specificity : 86.8% (33/38)

Table 2. Comparison of *H. pylori* tests in the gastric mucosa and dental plaque

	Gastric Mucosal Positive	*H. Pylori* Negative
No.	29	17
Mucosal PCR positive	18 (62..1%)	3 (17.6%)
Dental plaque CLO test positive	29 (100%)	16 (94.1%)
Dental Plaque PCR positive	2 (6.9%)	0 (0%)

gastric mucosa and DP were compared, DP CLO test was positive in 29 gastric *H. pylori*-positive subjects and in 16 of 17 *H. pylori*-negative subjects (94.1%)(Table 2). However, DP PCR was positive in only two of 29 gastric *H. pylori*-positive subjects (6.9%) and none in the 17 *H. pylori*-negative subjects (Table 2, Figure 2A). Two with DP PCR positive were active BGU and active pyloric channel ulcer patient, respectively. These two patients showed gastric mucosal *H. pylori* test positive in both the antrum and body, but mucosal PCR was all negative.

When gastric mucosa and saliva were compared, saliva CLO test was positive in 21 of 25 *H. pylori*-positive subjects (84.0%) and positive in 11 of 15 *H. pylori*-negative subjects (73.3%) (Table 3). However, saliva PCR was positive in 4 of 14 *H. pylori*-positive subjects (28.6%) and none of 6 *H. pylori*-negative subjects (Table 3, Figure 2B). Four saliva PCR positive subjects were all positive in saliva CLO test. They were diagnosed as DU in two, BGU in one and pyloric channel ulcer in one, and all of them showed active ulcers. Three of them were mucosal PCR positive in both antrum and body, but
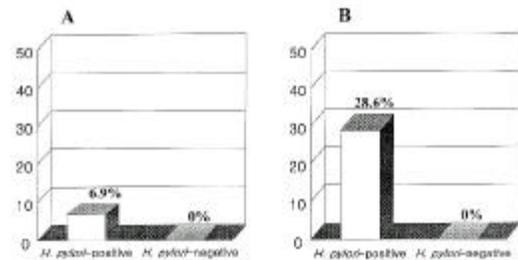
Figure 2. The positive rates of H. pylori PCR in the dental plaque (A) and saliva (B) depending on H. pylori positivity of gastric mucosa. PCR of dental plaque was positive in 6.9%, and that of saliva 28.6% in gastric H. pylori-positive subjects. In contrast, there was none in gastric H. pylori-negative subjects.

was remaining one was negative in both areas. The patient with negative mucosal PCR showed DP PCR positive, and, in the remaining three, all DP PCR were negative.

DISCUSSION

It has been hypothesized that *H. pylori* infection is acquired by either oral-oral or fecal-oral transmission, or by common source exposure. Although *H. pylori* has been isolated from feces by culture[29], detected in fecal samples[30] and in drinking water by PCR assay[31,32] and also isolated from cats[33,34], to date no environmental source of *H. pylori* has been recognized with certainty. However, many studies have provided evidence that there is a significant correlation of *H. pylori* in the stomach and the mouth, and it is thought to be possible that *H. pylori* in the mouth plays an important role in transmission and recurrence after eradication therapy. In our country, the reinfection rate of *H. pylori* was rather high, 12.8% per year, suggesting that oral cavity might have some role as a reservoir of *H. pylori*.

H. pylori infection in the stomach is easily detected by the rapid urease test, histology, urea breath test and serology[2], but detection of this bug in the oral cavity seems to be complicated. Several reports showed high positivity rate of urease test in the DP, saliva and gingival pockets, such as 84%-100%[4,5,12]. Similarly, our study showed the positivity rates of CLO test in the DP and saliva as 100% and 84.0%, respectively, from subjects with gastric *H. pylori* infection, but they were also high, 94.1% and 73.3% from subjects without gastric *H. pylori* infection (Table 2, 3). However, the PCR positivity rates in the DP and saliva were so low, 6.9% and 28,6%, respectively, even in gastric *H. pylori*-positive subjects, that CLO test in the DP and saliva looked like not reflecting the real *H. pylori* in each area. It is known that a positive urease test on a specimen obtained from the oral cavity should be interpreted with caution.

One study[22] showed that a patient had *H. pylori*-like organisms in samples collected from tongue and palate. Both strains were urease, catalse and oxidase positive and grew microaerophilically, but they were negative on *H. pylori*-specific PCR analysis, demonstrating the possibility of false identification[22]. There

Table 3. Comparison of *H. pylori* tests in the gastric mucosa and saliva

	Gastric Mucosal Positive	*H. Pylori* Negative
No.	25	15
Saliva CLO test positive	21(84.0%)	11(73.3%)
Saliva PCR positive	4/14(28.6%)	0/6(0%)

are other urease-producing bacteria in the oral cavity, such as *Actinomyces Viscosus* and *Strecptococcus Vestibularis*, which may cause false-positive results[35]. Culture of *H. pylori* is recognized as the "gold standard" for the diagnosis of the infection. However, we could not succeed in *H. pylori* culture from DP and saliva specimens in any of these 46 participating subjects (data not shown) mainly due to overgrowth of other bacteria. However, recently there have been reports of successful culture from samples of oral cavity, 11% from saliva[11], 19% from DP[36], and 13% from oral cavity[22]. It is speculated that nonculturable coccoid forms of the organisms may survive in the mouth, and more specific and sensitive culture methods are required for the detection of *H. pylori* in the oral cavity[37].

To date, many PCR assays have been developed for detecting *H. pylori* in the oral cavity. Most of them have been based on the sequence of urease genes and 16S ribosomal RNA genes. However, reports of high prevalence of *H. pylori* in the oral cavity as detected by urease gene-based PCR assay[10,21,22] have been questioned because urease-positive organisms are commonly present in cultures from the oral mucosa[38]. It is suggested that to confirm the presence of *H. pylori* DNA in DP with PCR assays, a sequence that is not part of the urease gene should be used. In addition, results from the oral cavity have shown a great variation of PCR-positive rates: from 0 to 92.9%[3,21,23-25]. It is possible that sets of primers designed from different sequences may have different sensitivities and specificities and cause conflicting data. In our study, we used a new nested PCR assay which was developed from *H. pylori* genomic DNA with no homologous sequences in GenBank[26,37]. With this nested PCR assay with ET4-U and ET4-L primers, a 203 bp

DNA fragment was amplified in 33 of 45 saliva samples collected from patients with gastric *H. pylori* infection. However, in our study, the sensitivity of the PCR in the gastric mucosa was rather low, 50.0%, and the specificity was relatively high 86.8%, based on *H. pylori* tests such as CLO test, touch print of Gram stain, Giemsa stain and culture. There is the possibility that this low sensitivity of the PCR might have caused an unerestimation of the detection rates of *H. pylori* in DP and saliva like 6.9% and 28.6%, respectively (Table 2, 3, Figure 2). In one study using three pairs of primers, the detection rates of *H. pylori* DNA in DP samples were 26.5%(9/34) for HPD1/HPD2, 78.9%(30/38) for HP1/HP2, and 100%(40/40) for EHC-U/EHC-L, showing that PCR primers are very important[39]. Because the primers, ET4-L and ET4-U, were very sensitive and specific for Western *H. pylori*[26], there is the possibility that *H. pylori* harbored in Koreans may be different from that in Westerners, especially in the amplifed 203 bp DNA sequence by these primers. In addition, several other factors are known to cause decrease of detection rates of *H. pylori* in DP and saliva. First, proper sampling is important: that is, saliva samples collected in the morning before teeth were brushed gave a higher rate of detection of *H. pylori*[37]. Second, collecting saliva samples directly into digestion buffer was important[37]. Third, the number of PCR cycles, the amount of template DNA and optimal buffers might have affected the detection rates of PCR. For example, we used 200 ng of DNA as templates for gastric mucosa and saliva, but below 50 ng for most a cases of DP, because collected DP usually showed very scanty amount of DNA. Therefore, there is the possibility that the lower detection rate of *H. pylori* in DP, 6.9%(2/29) than in saliva, 28.6%(4/14) might be caused by less amount of DNA in DP than in saliva, especially with low sensitivity rate of our PCR method. In addition, it is speculated that *H. pylori* in saliva is derived from DP because the positive rate of DP *H. pylori* was higher (100%) than that of saliva (59-70%)[3,20].

In conclusion, the detection rates of *H. pylori* in DP and saliva by PCR were rather low, 6.9% and 28.6%, respectively, and these rates might have been underestimated by low sensitivity of the PCR method used in this study. However, the results that *H. pylori*

was found in the DP and saliva suggest that oral cavity can preform a role as a reservoir of *H. pylori* in Korea.

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