J. Korean Soc. Microbiol., Vol. 35, No. 4, 2000

A Novel PCR Primers HPU185 and HPL826 Based on 16S rRNA Gene for Detection of *Helicobacter pylori*

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The PCR primer set JW21-JW22 of Weiss et al. (19), which was reported to amplify a 139-bp fragment of the 16S rRNA gene of *Helicobacter pylori*, has been recently used for the detection of *H. pylori* in clinical specimens. However, when we applied JW21-JW22 PCR to other members of the genus *Helicobacter* and unrelated microorganisms, all of these bacteria produced a 139-bp PCR product. Therefore, we designed a novel primer set, HPU185-HPL826, which produced a 642-bp amplicon of the 16S rRNA gene of *H. pylori*. Then we further examined the specificity of the novel PCR assay using Southern blot hybridization with an internal probe, HPP225. The PCR assay described in this study was shown to be highly sensitive and specific only to the *H. pylori* 16S rRNA gene sequences.

Key Words: *Helicobacter pylori*, PCR, 16S rRNA

INTRODUCTION

Helicobacter pylori has been established as an etiological agent of chronic active gastritis, gastroduodenal ulcers, and carcinoma of the stomach (2,5,12). *H. pylori* infection has been found in all areas of the world, but the rate of *H. pylori* infection is known to vary in different populations. The prevalence of *H. pylori* infection can be close to 100% in certain developing countries (17). The definitive diagnostic tool of *H. pylori* infection is therefore of great significance in the treatment and control of these diseases. Several diagnostic tests are usually performed in association with endoscopy of the upper gastrointestinal tract. Recently, many PCR-based methods which use different gene targets of *H. pylori* to detect the organism directly in clinical specimens have been described (1,4,8,9, 10,15,18).

Because some segments of rRNA are functionally and evolutionally conserved while others are variable to different degrees, phylogenetic comparison of rRNA sequences has become a powerful method for the systematic classification and detection of microbial organisms (7). Furthermore, the 16S rRNA gene of *H. pylori* could be a highly specific target for PCR amplification because of the high copy number of rRNA per bacterial cell. As such, it has been previously used to help reclassify and detect the organism.

Ho et al. (9) designed a set of PCR primers, Hp1-Hp2 which has been widely used to detect *H. pylori* in various studies (11,13,14,16). But Chong et al. (3) demonstrated that the PCR method with the primer set, Hp1-Hp2, was not specific and should

Received for Publication : October 11, 2000, Accepted for Publication : November 24, 2000
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not be used to detect *H. pylori* in clinical specimens. Weiss et al. (19) also designed a set of PCR primers, JW21-JW22 (JW21, 5'-GCG ACC TGC TGG AAC ATT AC -3'; JW22, 5'-CGT TAG CTG CAT TAC TGG AGA -3'), which amplify a 139-bp fragment of the *H. pylori* 16S rRNA gene. The PCR primer set, JW21-JW22 has been recently used for the detection of *H. pylori* in clinical specimens. Gramley et al. (6) have used the same primer-based PCR of gastric tissue specimens and fecal samples to detect *H. pylori* DNA, and reported that this assay had a sensitivity of 100%. However, when we applied JW21-JW22 PCR to other members of the genus *Helicobacter* and unrelated microorganisms, we observed an unexpected and controversial result of the PCR, in which all of these bacteria produced a 139-bp PCR product.

In this study, we carried out experiments to determine the specificity of JW21-JW22 primers for the detection of *H. pylori* and developed a novel and specific PCR assay for *H. pylori* based on the 16S rRNA sequence alignments.

MATERIALS AND METHODS

Organisms. Three strains of *Helicobacter pylori* were obtained from following sources; *H. pylori* ATCC 43504 from American Type Culture Collection (ATCC), *H. pylori* strain 51 from Dr. Kwang-Ho Rhee (Dept. of Microbiology, College of Medicine, Gyeongsang National University, Chinju, R. O. K.) and *H. pylori* WCH1 which were isolated from human gastric biopsy specimens in Korea. Other bacterial strains of *Helicobacter felis* ATCC 49479, *Helicobater canis* ATCC 51401, *Helicobacter pullorum* ATCC 51864, *Helicobacter bilis* ATCC 51631, and *Helicobacter hepaticus* ATCC 51448, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 13565, *Salmonella typhi* ATCC 19430, and *Campylobacter jejuni* ATCC 33560 were obtained from ATCC. Three strains of *H. pylori* and 5 *Helicobacter* species were inoculated on trypticase soy agar supplemented with 5% bovine serum, 10 μg/ml vancomycin, 25 μg/ml nalidixic acid, 1 μg/ml amphotericin B, and 8 IU/ml polymyxin B, then incubated at 37℃ under microaerophilic conditions for 5 to 7 days. Other bacterial strains were maintained in appropriate media.

Design of novel PCR primers. On the basis of 16S rRNA sequence alignments, the PCR primers HPU185 and HPL826 were designed by comparing the nucleotide sequences of the 16S rRNA gene of *H. pylori* with those of other members of the genus *Helicobacter, Escherichia coli, Salmonella typhi, Campylobacter jejuni*, and *Staphylococcus aureus*. *H. pylori*-specific 16S rRNA sequences were selected on the basis of alignments performed with Oligo software (National Biosciences, Polymouth, Minn.) and BLASTN 2.1.1 program (20). All of the sequence data were obtained from GenBank. The GenBank nucleotide sequence accession numbers for the 16S rRNA sequences for *H. pylori* are U01-328 to U01332, and U00679. And the accession numbers of other members of the genus *Helicobacter* are U51873 (*H. hepaticus*), and AF047850 (*H. pullorum*). The novel primers, designed in this study, consisted of two oligonucleotides, HPU185-HPL826 (HPU185, 5'-CCT ACG GGG GAA AGA TTT AT-3'; HPL826, 5'-AGC TGC ATT ACT GGA GAG ACT-3'), which amplify a 642-bp 16S rRNA gene product of *H. pylori*.

PCR and Southern blot analysis. For the PCR, bacterial genomic DNA was extracted using the boiling lysis method. Gastric mucosa tissue was ground with Stomacher Lab-Blander 80 (Seward Medical, London), and DNA was extracted with a QIAamp DNA Mini Kit (QIAGEN Inc., U.S.A) according to the manufacturer's instructions. The DNA pellet was dissolved in autoclaved distilled water and frozen at -20℃ until use. The 20 μl of PCR mixture contained 2 μl of template DNA, PCR buffer (50 mM KCl, 10 mM Tris-HCl), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 40 pmol of each PCR primer, and 0.5 U of *Taq* polymerase. The reaction mixture was subjected to 35 cycles

Figure 1. Agarose gel electrophoresis of products generated by JW21-JW22 PCR primers with *H. pylori* strains and other microorganisms. Lanes: 1, DNA molecular size standards (100-bp ladder [GenoTech Corp., Taejon, R.O.K.]); 2, *H. pylori* ATCC 43504; 3, *H. pylori* strain 51; 4, DNA extracted from gastric biopsy specimen; 5, *H. felis* ATCC 49479; 6, *H. canis* ATCC 51410; 7, *H. pullorum* ATCC 51864; 8, *H. bilis* ATCC 51631; 9, *H. hepaticus* ATCC 51448; 10, *E. coli* ATCC 25922; 11, *S. aureus* ATCC 13565; 12, *S. typhi* ATCC 19430; 13, *C. jejuni* ATCC 33560; 14, negative control.

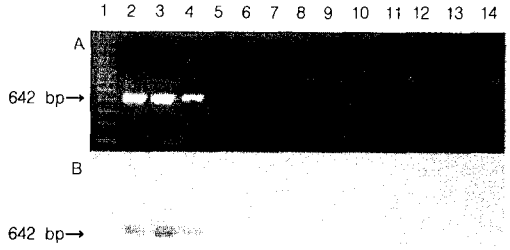

Figure 2. Agarose gel electrophoresis of HPU185-HPL826 PCR amplification products **(A)** and corresponding Southern blot hybridization with a probe, HPP225 **(B)**. Lanes are the same as described in Fig. 1.

of 94℃ for 1 min, 52℃ for 1 min, and 72℃ for 1 min 30 s, and an additional extension step for 15 min at 72℃ was performed at the end of the amplification. For confirmation of the PCR-amplified DNA by Southern blot hybridization, 10 μl of the PCR product was electrophoresed through a 1.5% agarose gel and transferred onto a nylon membrane using the capillary transfer method. The membrane was baked at 80℃ for 3 hours, hybridized with a 3'-end flourescein-11-dUTP-labeled probe, and exposed in the presence of luminol onto Hyperfilm-ECL (Amersham, RPN 2130; Buckinghamshire, England) according to the manufacturer's instructions. The probe, HPP225, was a 22-bp oligonucleotide (5`-CTT GTT GGT AAG GTA ATG GCT T-3'), which corresponds to an internal region of the 642-bp fragment in the 16S rRNA gene of *H. pylori*.

To determine whether the primer JW21-JW22 can be used to specifically detect *H. pylori*, PCR was performed as described previously (6).

The sensitivity and specificity of novel PCR primers. To investigate the sensitivity and specificity of this HPU185-HPL826 PCR assay, specimens of gastric mucosa were obtained by endoscopy from the 61 patients with complaints of abdominal pain. The sensitivity and specificity of PCR assay developed in this study was further investigated by comparing the biopsy specimen-based diagnostic assays of the urease test in relation to the results of cultivation and/or the direct demonstration of *H. pylori* by Warthin-Starry silver stain as a gold standard. Five antral gastric biopsy specimens were obtained: two for culture, one for urease test (*Campylobacter*-like organism test; Delta West, Perth, Australia), one for histology, and one for PCR. Gastric mucosal biopsy specimens were directly inoculated onto trypticase soy agar supplemented with 5% bovine serum, 10 μg/ml vancomycin, 25 μg/ml nalidixic acid, 1 μg/ml amphotericin B, and 8 IU/ml polymyxin B, then incubated at 37℃ under microaerophilic conditions for 5 days.

RESULTS

JW21-JW22 primer-based PCR. The result of PCR amplification with JW21-JW22 primer set produced a 139-bp fragment for *H. pylori* strains along with other species of the genus *Helicobacter*, *E. coli*, *S. aureus*, *S. typhi*, and *C. jejuni* as shown in Fig. 1.

PCR analysis with novel primer set HPU185-HPL826. Under *H. pylori* specific HPU185-HPL826 primer-based PCR, a product of 642-bp was observed with DNAs extracted from the three *H. pylori* strains (Fig. 2A). Non-specific band was not obtained with other species of the genus *Helicobacter*, the five closely related species on the basis of 16S rRNA sequences. Moreover, no PCR products were observed with DNAs isolated from *E. coli*, *S. aureus*, *S. typhi*, and *C. jejuni*. Specificity

Table 1. Summary of the PCR results for the detection of *Helicobacter pylori* with reference to urease test

Assay	Results of *H. pylori* detection							
	H. pylori present[a]				*H. pylori* absent[b]			
	Pos.	Neg.	Sensitivity	PPV[c]	Pos.	Neg.	Sensitivity	NPV[d]
Urease test	48	5	91%	97%	1	7	88%	58%
JW21-JW22 PCR	47	6	89%	89%	6	2	25%	25%
HPU185-HPL826 PCR	49	4	93%	100%	0	8	100%	67%

[a] Positive by culture or Warthin-Starry silver stain, [b] Negative by culture or Warthin-Starry silver stain.
[c] PPV, positive predictive value, [d] NPV, negative predictive value

of this primer set was further examined by Southern blot hybridization with a probe, HPP225, directed against an internal region of the 642-bp fragment in the 16S rRNA gene of *H. pylori* (Fig. 2B). The corresponding product of Southern blot hybridization indicates that these bands were specific to *H. pylori* strains.

The sensitivity and specificity of novel PCR primers. Among the total of 61 patients, *H. pylori* was identified in 44 (83%) by histology, and in 36 (68%) by culture of 53 positive subjects, which were determined with gold standard of culture and/or histology. The PCR results for the detection of *H. pylori* are shown in Table 1 with reference to the urease result. The sensitivity of HPU185-HPL826 PCR assay was 93%, and the specificity of this PCR was 100%, while those of JW21-JW22 PCR assay were 89% and 25%, respectively. The 6 false positive cases of 8 *H. pylori*-negative subjects were observed in JW21-JW22 PCR assay system.

DISCUSSION

In this study, we have developed a PCR assay system for *H. pylori* from gastric mucosa, and have evaluated the clinical accuracy of a novel primer set with previously reported JW21-JW22 primers (19).

HPU185-HPL826 primer-based PCR, developed in this study, produced an amplicon of 642-bp only in 3 reference *H. pylori* strains as shown in Fig. 2A. Non-specific band was not obtained with other species of the genus *Helicobacter,* the five closely related species on the basis of 16S rRNA sequences, and with the bacterial strains of *E. coli, S. aureus, S. typhi,* and *C. jejuni*. Furthermore, this results were confirmed by Southern blot hybridization with a probe, HPP225, directed against an internal region of the 642-bp fragment in the 16S rRNA gene of *H. pylori* as shown in Fig. 2B. The corresponding product of Southern blot hybridization indicates that these bands were specific only to *H. pylori* strains.

In contrast to this result, the JW21-JW22 primer-based PCR amplification produced a 139-bp fragment for *H. pylori* strains along with other species of the genus *Helicobacter, E. coli, S. aureus, S. typhi,* and *C. jejuni* as shown in Fig. 1. Weiss et al. (19) reported JW21-JW22 primers did not cross-react with other common microorganisms, such as *E. coli, Campylobacter* species, and other members of the genus *Helicobacter*. However, the observation in the present study that the JW21-JW22 PCR amplified a 139-bp fragment from other members of the genus *Helicobacter* and unrelated microorganisms indicates that our unexpected positive results might be due to nonspecific amplification of 16S rDNAs by JW21-JW22 PCR. This would suggest that JW21-JW22 PCR assay should not be applied to detect *H. pylori* in clinical specimens.

The plausible reasons for this nonspecific reaction in JW21-JW22 PCR might be attributed to two reasons. One might be the carry-over pheno-

mena during PCR due to the contamination and the other might be the presence of identical nucleotide sequences in the genomic sequences of related bacteria other than *H. pylori*. However, the first possibility of carry-over phenomena during PCR amplification could be ruled out because reagent mixing, sample addition and thermocycling were done in separated laboratory to avoid the false positivity in PCR due to laboratory contamination, and the precautions, such as using micropipette tips with aerosol barrier, were also taken throughout this study. And unexpected nonspecific PCR amplification had not been observed in other PCR reactions undertaken in our laboratory until now.

Then the second possibility of the presence of identical sequence was examined using BLASTN 2.1.1 program (20). The JW21 primer sequence is completely aligned to the sequences of 16S rDNA of *H. cinaedi, H. bilis, H. canis, H. hepaticus* and *H. pullorum* as well as that of *H. pylori*. The completely identical sequence of JW22 primer was found in the sequences of 16S rDNA of *H. hepaticus*, and almost identical sequences with one to four base pair mismatch were found in the sequence database of 16S rDNA of *H. canis, H. felis, H. pullorum* and *C. jejuni*. These results of nucleotide sequence alignments of 16S rDNA database indicate that the primer sequences of JW21 and JW22 primer were shown to be completely or almost identical to the sequences of 16S rDNA in related bacteria, which was easily confirmed with alignments of primer sequences in the database of GenBank. Therefore, the reasons for these nonspecific reactions in JW21-JW22 PCR might be attributed to the presence of identical sequences in the sequence of 16S rDNA of related bacteria.

Among the total of 61 patients with complaints of abdominal pain, 53 (86.9%) of the patients were considered to harbor infections with *H. pylori*, while 8 (13.1%) were uninfected using the gold standard of culture and/or histological findings. Diagnosis of *H. pylori* infection was made if culture, histology, or both were positive. Of the 53 biopsy specimens harboring the infections with *H. pylori*, 49 specimens gave rise to amplification with HPU185-HPL826 PCR primers described in this paper, while only 36 specimens were positive in the isolation of *H. pylori*. With these results, the HPU185-HPL826 PCR was shown to be specific (specificity of 100%) only to *H. pylori* and to be sensitive (sensitivity of 93%) for detection of *H. pylori* in the clinical gastric biopsy specimens. This observation was re-confirmed by the Southern blot hybridization.

In summary, compared with other primer based-PCR assays, the enhanced specificity of the HPU185-HPL826 PCR assay allows rapid and accurate assessment of *H. pylori* detection in clinical specimens.

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