Detection of *Helicobacter pylori* in Gastric Mucosa of Patients with Gastroduodenal Diseases by PCR-Restriction Analysis Using the RNA Polymerase Gene (*rpoB*)


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A novel PCR restriction analysis method using the RNA polymerase β-subunit-coding gene (*rpoB*) was employed to both detect and identify *Helicobacter pylori* in biopsy specimens and culture isolates. The *rpoB* DNAs (458 bp) were specifically amplified by PCR with the *Helicobacter*-specific primers (HF and HR). Based on the determined *rpoB* sequences of the culture isolates, an *H. pylori*-specific restriction site, *Tru*I, was found. *H. pylori* can be identified by observing two discernible DNA fragments (288 and 138 bp) after *Tru*I digestion and agarose gel electrophoresis. The *rpoB* PCR and subsequent restriction analysis (PRA) enabled the specific detection and identification of *H. pylori* in biopsy specimens from patients with gastroduodenal diseases. The *rpoB* PRA conferred a compatible or a slightly higher positive rate (53.7%) than did the *Campylobacter*-like organism (CLO) test (50.4%) and *glmM* PCR (48.8%), suggesting that it is useful for diagnosing an *H. pylori* infection without culture in the clinical laboratory.

*Helicobacter pylori* is one of the most prevalent microorganisms and is a major cause of gastrointestinal disease in humans. *H. pylori* is found in the stomach of patients with gastroduodenal diseases, such as duodenal and gastric ulcers (9). In addition, *H. pylori* is thought to be one of the major causes of stomach cancer (10, 11). It can be cultivated from biopsy specimens in 2 to 8% O₂ and 10% CO₂ after 3 to 5 days and is identified through means such as Gram staining, a catalase test, and a urease test. Although the culture method is a "gold standard" for diagnosing many infectious diseases, it is not easy to cultivate *H. pylori* in 2 to 8% O₂ and 10% CO₂ after 3 to 5 days and is identified through means such as Gram staining, a catalase test, and a urease test. In addition, *H. pylori* is thought to be one of the major causes of stomach cancer (10, 11). It can be cultivated from biopsy specimens in 2 to 8% O₂ and 10% CO₂ after 3 to 5 days and is identified through means such as Gram staining, a catalase test, and a urease test. Although the culture method is a "gold standard" for diagnosing many infectious diseases, it is not easy to cultivate *H. pylori* in biopsy specimens. The sensitivity of the *CLO* test and/or histological examination are not always a guarantee of a positive culture (19). Usually under normal atmospheric conditions, it vanishes easily (13); nor is it easy to cultivate *H. pylori* by traditional methods. Therefore, in addition to the noninvasive methods, many different kinds of methods, such as CLO testing (14), special staining (19), and PCR (2, 24), have been used to detect *H. pylori* in biopsy specimens.

PCR is one of the most widely used molecular methods for detecting specific pathogens. Several genes have been used to detect and identify *H. pylori* (2, 17, 24). However, *H. pylori* is a microorganism with marked genetic diversity. Although it is described as a quasispecies (25) because isolates show genetic diversity and because genetically different isolates have been cultured in the same patients, it appears that genetic identification by using housekeeping *H. pylori* genes is needed to accurately identify *H. pylori* and evaluate the causes of gastroduodenal diseases resulting from an *H. pylori* infection. For the genetic identification of *H. pylori*, several PCR methods that employ the 16S rRNA gene (ribosomal DNA [rDNA]), *rpoD*, *ureA*, *ureB*, and *ureC* have been used (2, 24). Most are protein-encoding genes except for 16S rDNA. Among them, *ureC* (*glmM*) PCR is known to be specific to *H. pylori* and is frequently used (17).

In this study, the RNA polymerase β-subunit-coding gene (*rpoB*) (1) was used for the detection and identification of *H. pylori* by specific PCR restriction analysis (PRA). *rpoB* is an important transcription apparatus in all microorganisms. It is a kind of housekeeping gene and is as stable as 16S rDNA. Recently, partial *rpoB* DNA sequences containing the Rif region, which is related to rifampin resistance of *Escherichia coli* and *Mycobacterium tuberculosis*, were used to either differentiate or identify closely related species in *Enterobacteriaceae* (19), *Mycobacterium* (12), and *Borrelia* (15). Because this region is highly conserved, a novel PCR amplifying 458-bp DNA and a subsequent restriction analysis method were developed. This was applied to culture isolates as well as gastric biopsy specimens from patients with gastroduodenal diseases and was compared to those of *glmM* PCR and CLO testing.
agar containing 10% bovine serum, 10 biopsy specimens were smeared directly on Mueller-Hinton from patients with gastric disorders or from normal adults. The All strains were isolated from the gastric biopsy specimens Hospital, and S.-Y. Kim at Chungbuk University, all in Korea. Gyeong-Sang University, M.-W. Chang at Kosin University ing to codons 432, 441, and 487.

The gastric biopsy specimens were obtained from 123 patients who were diagnosed with gastroduodenal diseases by gastroscopy at the Department of Internal Medicine, Dankook University Hospital. Six pieces were obtained from the biopsy specimens from the antrum of stomach. Two pieces were used for the rapid urease test, two pieces for the histological examination, and two pieces for the rpoB PCR and glmM PCR. The patients were diagnosed by using histological examinations.

H. pylori, isolates (157 strains) were provided by M.-J. Cho at Gyeong-Sang University, M.-W. Chang at Kosin University Hospital, and S.-Y. Kim at Chungbuk University, all in Korea. All strains were isolated from the gastric biopsy specimens from patients with gastric disorders or from normal adults. The biopsy specimens were smeared directly on Mueller-Hinton agar containing 10% bovine serum, 10 µg of vancomycin ml⁻¹, 25 µg of nalidixic acid ml⁻¹, and 1 µg of amphotericin B ml⁻¹. The inoculated contents of plates were incubated at 37°C for 4 or 5 days in 10% CO₂. All isolates were identified as being H. pylori based on the culture characteristics, such as small translucent colonies under a dissecting microscope, gram-negative spiral organisms, and urease production. D. E. Berg at Washington University Medical School kindly provided the five H. pylori strains. The Helicobacter hepaticus (ATCC 51448) strains were provided by Y.-G. Choi at the Korea Research Institute of Bioscience and Biotechnology, and the Helicobacter felis (ATCC 49179) strains were provided by J.-S. Yum at Mogam Biotechnology Research Institute. The Helicobacter cinaedi (ATCC 35683) and Helicobacter mustelae (ATCC 43772) strains were purchased from the American Type Culture Collection.

The H. pylori DNAs were prepared by using the previously described bead beater-phenol extraction method (12). A loopful of each isolate was suspended in 200 µl of Tris-EDTA-NaCl buffer (10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl [pH 8.0]). A bacterial suspension was placed in a 2.0-ml screw-cap microcentrifuge tube filled with 100 µl (packed volume) of glass beads (diameter, 0.1 mm; Biospec Products, Bartlesville, Okla.) and 100 µl of phenol-chloroform-isomyl alcohol (25:24:1) (P-2069; Sigma Chemical Co.). The tube was oscillated on a Mini-Bead Beater (Biospec Products) for 1 min and was centrifuged (12,000 × g, 5 min) to separate the phases. The aqueous phase was subsequently transferred into another clean tube; 10 µl of 3 M sodium acetate and 250 µl of ice-cold absolute ethanol were added. To precipitate the DNA, the mixture was kept at −20°C for 10 min. The harvested DNA pellets were dissolved in 60 µl of Tris-EDTA buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]) and were used as a template DNA for PCR. A set of primers (HF, 5’-AAGCTTTTTAGGTCAGGTTTGTTT-3’; and reverse primer, 5’-AAGC TTACTTTCTACCTAAACATG-3’) was used to amplify the glmM DNA (294 bp) (17). The PCR mixture was subjected to 30 amplification cycles (1 min at 93°C, 1 min at 55°C, and 1 min at 72°C).

The nucleotide sequences (363 bp) of the purified rpoB PCR products (458 bp) were determined directly by using an Applied Biosystems model 373A automatic sequencer and a Big-Dye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). For the sequencing reaction, 60 ng of PCR-amplified DNA, 3.2 pmol of either the forward or the reverse primer, and 4 µl of BigDye Termina tor RR mix (part no. 4303153; Perkin-Elmer Applied Biosystems) were mixed. The final volume was adjusted to 10 µl by the addition of distilled water. The reaction was run with 5% (vol/vol) dimethyl sulfoxide for 30 cycles of 15 s at 95°C, 10 s at 50°C, and 4 min at 60°C.

The sequences were aligned by using the multiple-alignment algorithm in the MegAlign package (Windows version 3.12e; DNASTAR, Madison, Wis.). The enzyme restriction sites for the DNA fragment on each rpoB sequence of H. pylori and five Helicobacter species were generated by using the Map-Draw program (MapDraw version 3.14; DNASTAR).

Ten microliters of the rpoB PCR products was transferred to a fresh microcentrifuge tube and was digested with restriction enzyme according to the supplier’s instruction. One microliter of Tru9I (no. R7011, 10 U/µl; Promega), 1 µl of the enzyme buffer, and 4 µl of distilled water were added to the PCR products and were placed in a 65°C water bath for 1 h. The mixture was electrophoresed (100 V for 25 min) on a 1.2% (wt/vol) agarose gel, and the DNA bands were visualized by using ethidium bromide staining and were then photographed.
with gastritis (41 patients), a duodenal ulcer (42 patients), a benign gastric ulcer (21 patients), and a gastric cancer (19 patients). The DNAs were extracted from the biopsy specimens as described above and were used for rpoB PCR and glmM PCR.

The rapid urease test kit (CLO test; Delta West, Bentley, Australia) was used. It was stored in the refrigerator (2 to 8°C) and was used after warming. The gastric biopsy specimens were immediately embedded into the gel in the kit. The kit was placed at room temperature for 24 h and was observed for any color change. If a color change from yellow to red was observed, it was interpreted as a positive reaction.

Two pieces of the biopsy specimens were fixed in 10% (vol/vol) buffered formalin, embedded in paraffin, and then sectioned. Hematoxylin-and-eosin staining and modified Giemsa staining were used for routine histology and to detect H. pylori, respectively.

rpoB PCR for the 5 Helicobacter species and 162 H. pylori isolates including Spanish strains was performed. The PCR products (458 bp) were observed only from the Helicobacter species. There was no amplification from other bacteria, suggesting that the primers (HF and HR) are Helicobacter specific (Fig. 2). The nucleotide sequences of the amplified DNAs were determined, and portions of them (363 bp) were aligned.

The percentage similarities among the rpoB sequences of 162 H. pylori strains, H. pylori 26695 (AE000625), and H. pylori 399 (AE001540) were more than 91.5%. The nucleotide sequences of the rpoB DNA among the H. pylori strains and H. cinaedi showed a 68.3% similarity. There was no significant clustering observed among the culture isolates, which had been isolated from three different areas in Korea. Through the computer-aided analysis of the rpoB DNA sequences from 164 H. pylori strains, including H. pylori 26695 and H. pylori 399, Tru91 was found to distinguish H. pylori from the other Helicobacter species (Fig. 3). Digestion with Tru91 yielded four bands (288, 138, 28, and 4 bp) from H. pylori rpoB DNA. Because of the fairly different sizes (247, 207, and 4 bp) of the DNA fragments from other species, such as H. mustelae, H. pylori can be easily identified by using the two major DNA fragments (288 and 138 bp) (Fig. 3A). Tru91 PRA for the gastric biopsy samples showed identical results, suggesting that the amplified DNAs were amplified from H. pylori (Fig. 3B). The results of rpoB PRA and glmM PCR results were compared with that of the CLO test, which was performed on the same gastric biopsy specimens. The concordance rate of rpoB PRA with the CLO test was 83.0%, while that of glmM PCR was 81.3% (Table 1). Considering the CLO test as the gold standard, the sensitivity and specificity of rpoB PRA were 85.7 and 80.0%, while those of glmM PCR were 79.3 and 83.0%, respectively. In general, the detection rate of rpoB PRA was slightly higher than those of the CLO test and glmM PCR, especially in the gastric cancer (Table 2). However, it was not statistically significant (P > 0.05). Nevertheless, this may be due to the small sample numbers. Among the 123 gastric biopsy specimens, 53 samples were histologically examined. The concordance rate between rpoB PCR and histological examination was 69.8%, while those of the CLO test and glmM PCR were 71.7 and 69.8%, respectively (Table 3).

There are many invasive and noninvasive methods used to diagnose an H. pylori infection. Although they may be convenient to use and safe for patients, noninvasive methods such as the urea breath test, which detects the metabolic end product, are considered to be nonspecific, as the results may be affected by other enteric bacteria (14, 19). However, gastrofiberscopy is commonly used to directly observe a pathological lesion and

![FIG. 2. Amplification of rpoB DNAs (458 bp) from Helicobacter species (A) and other bacteria (B). PCR products were electrophoresed on a 1.2% agarose gel. Lane M in both panels, φ X174 RF DNA/HaeIII digest. (A) Lanes: 1 to 6, H. pylori isolates; 7, H. felis; 8, H. cinaedi; 9, H. mustelae; and 10, H. hepaticus. (B) Lanes: H, H. pylori isolate; 2, E. coli; 3, C. diphtheriae; 4, S. aureus; 5, B. subtilis; 6, M. fortuitum; 7, B. catarrhalis; 8, H. influenzae; 9, N. sicca; and 10, E. faecalis.](http://jcm.asm.org/)

![FIG. 3. Identification of H. pylori culture isolates (A) and H. pylori in the gastric biopsy specimens (B) by PRA using Tru91. PCR products were digested and electrophoresed on a 1.2% agarose gel. All strains showed two major bands (288 and 138 bp), which are specific for H. pylori. Lane M in both panels, DNA marker φ X174 RF DNA/HaeIII digest. (A) Lanes 1 to 5, H. pylori culture isolates; lane 6, H. mustelae. (B) Lanes 1 to 9, gastric biopsy specimens.](http://jcm.asm.org/)
TABLE 2. Comparison of methods of diagnosing \textit{H. pylori} according to the patterns of gastroduodenal diseases (\textit{n} = 123)

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of patients with illness/no. of patients (%)</th>
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<tbody>
<tr>
<td></td>
<td>Gastritis</td>
</tr>
<tr>
<td>CLO</td>
<td>16/41 (39.0)</td>
</tr>
<tr>
<td>\textit{rpoB}</td>
<td>18/41 (43.9)</td>
</tr>
<tr>
<td>\textit{glmM}</td>
<td>16/41 (39.0)</td>
</tr>
</tbody>
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\textsuperscript{a}DU, duodenal ulcer. 
\textsuperscript{b}BGU, benign gastric ulcer.

Although \textit{H. pylori} is a homogeneous population, the intraspecies variation of the \textit{rpoB} DNA sequence would be much higher than those observed in \textit{Mycobacterium} (12) and \textit{Borrelia} (15). It is not surprising that \textit{H. pylori} \textit{rpoB} shows such high variation, because its genetic diversity is well known. However, the \textit{rpoB} sequences of five \textit{Helicobacter} species differed markedly from each other. The similarity of the nucleotide sequences of \textit{H. pylori} and of \textit{H. cinaedi} was only 68.3\%, which is the lowest. Therefore, specific primers for \textit{rpoB} PCR and the restriction site for \textit{H. pylori} could be selected.

TABLE 3. Concordance of results from \textit{rpoB} PCR, \textit{glmM} PCR, and CLO test with pathology (\textit{n} = 53)

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
<th>No. of specimens with pathology result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>11</td>
</tr>
<tr>
<td>\textit{rpoB} PCR</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>10</td>
</tr>
<tr>
<td>\textit{glmM} PCR</td>
<td>+</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>14</td>
</tr>
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</table>

Note: In this study, small numbers (\textit{n} = 53) of gastric biopsy tissues were included. The concordance rate between the histological examination and the CLO test was similar to the concordance rate between the \textit{rpoB} PRA and the \textit{glmM} PCR. The concordance rate between the CLO test and the \textit{rpoB} PRA was 83.0\%. It is interesting that the positive rate of the \textit{rpoB} PRA was higher in the gastritis and gastric cancer samples than in the duodenal and benign gastric ulcer samples. The \textit{rpoB} PRA had an \textit{H. pylori} detection gene PCR with the primer set Hp1 and Hp2 was reported to be nonspecific and cannot be used to detect \textit{H. pylori} in clinical specimens (2). However, in general, the sensitivity and specificity of presently available PCR methods ranged from 92 to 100\% and 69 to 100\%, respectively (8, 17, 18, 26, 27). Among them, PCR amplifying \textit{glmM}, which was formerly called \textit{ureC} and encodes phosphoglucomutase (3), was reported to be the most sensitive method (17, 21).

Therefore, it is clear that PCR methods targeting a stable gene such as 16S rRNA (6) or \textit{rpoB} (12) would give more reliable results. The \textit{rpoB}, a kind of housekeeping gene, was newly used to detect \textit{H. pylori} in this study. \textit{rpoB} encodes the RNA polymerase \textbeta{} subunit and is related to rifampin resistance. The targeted region of \textit{rpoB} is highly conserved in gram-positive and -negative bacteria (20). According to the sequence analysis of 162 Korean strains, the sequence dissimilarity among the \textit{H. pylori} isolates was less than 8.5\%. If \textit{H. pylori} is a homogeneous population, the intraspecies variation of the \textit{rpoB} DNA sequence would be much higher than those observed in \textit{Mycobacterium} (12) and \textit{Borrelia} (15). It is not surprising that \textit{H. pylori} \textit{rpoB} shows such high variation, because its genetic diversity is well known. However, the \textit{rpoB} sequences of five \textit{Helicobacter} species differed markedly from each other. The similarity of the nucleotide sequences of \textit{H. pylori} and of \textit{H. cinaedi} was only 68.3\%, which is the lowest. Therefore, specific primers for \textit{rpoB} PCR and the restriction site for \textit{H. pylori} could be selected.

The results of PRA for the 162 \textit{H. pylori} strains and 123 biopsy specimens suggest that it can be an \textit{H. pylori}-specific method. PRA has frequently been used for molecular typing of \textit{H. pylori} strains in several studies (4, 5, 7, 16, 22, 23). It could specifically amplify the DNA fragments of the urease-encoding genes. However, although restriction fragment length polymorphism analysis was efficiently used to type the \textit{H. pylori} strains, it could not be applied to identify \textit{H. pylori}, due to the marked complexity. The complexity, which may have originated from the size and sequence diversity of the target DNA, may be helpful for distinguishing the genotype of the \textit{H. pylori} strains identified but not for the actual identification. That is why \textit{rpoB} PRA is useful. Compared to other methods, \textit{rpoB} PRA uses small DNA fragments, which are highly conserved. There are two or three \textit{Trn91} restriction sites on the amplified \textit{rpoB} DNA of \textit{Helicobacter} species. According to these results, all of the \textit{H. pylori} isolates tested in this study had three identical restriction sites. Therefore, they had a simple and unique restriction pattern, which has the advantage of being able to be used for the specific identification of \textit{H. pylori}. However, though only one type strain was analyzed, \textit{H. felis}, which is a cat-associated species and rarely infects humans, showed the same restriction pattern.

A histological examination is known to have high sensitivity and specificity for detecting \textit{H. pylori} (19). In this study, small numbers (\textit{n} = 53) of gastric biopsy tissues were included. The concordance rate between the histological examination and the CLO test was similar to the concordance rate between the \textit{rpoB} PRA and the \textit{glmM} PCR. The concordance rate between the CLO test and the \textit{rpoB} PRA was 83.0\%. It is interesting that the positive rate of the \textit{rpoB} PRA was higher in the gastritis and gastric cancer samples than in the duodenal and benign gastric ulcer samples. The \textit{rpoB} PRA had an \textit{H. pylori} detection.
rate similar to those of the other tests. Therefore, rpoB PRA may be useful in detecting and identifying \textit{H. pylori} from gastric biopsy specimens without a culture in a clinical laboratory.

PRA may be considered an expensive, laborious, and thus impractical procedure for many samples in clinical laboratory settings. Thus, the rpoB PCR can be used alone without subsequent restriction analysis, because it is \textit{Helicobacter} specific and because \textit{Helicobacter} species other than \textit{H. pylori} can be hardly detected in human samples. In addition, the cost, which is higher than those of other methods, including culture, will be reduced.


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