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Evaluation of 16S rRNA Gene PCR with Primers Hp1 and Hp2 for Detection of *Helicobacter pylori*

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The PCR primer set Hp1-Hp2, which amplifies a 109-bp fragment of the 16S rRNA gene of *Helicobacter pylori*, has been widely used for the detection of *H. pylori* in clinical specimens. We have examined 34 stool samples and 50 human tissue samples from *H. pylori*-infected and uninfected patients, five human leukocyte samples, and one human cell line by this PCR method. All of these specimens produced a 109-bp PCR product. When *Escherichia coli* DNA was used as the template, several nonspecific bands, but not the 109-bp band, were observed. No PCR products were generated when DNA samples from five different fungi were used as templates. These results indicate that this 109-bp PCR product was amplified from the human genome. The 109-bp PCR product generated from various clinical specimens also hybridized with the probe pHp, corresponding to a region internal to the PCR product of Hp1-Hp2. We conclude that the 16S rRNA gene PCR with the primer set Hp1-Hp2 is not specific and cannot be used to detect *H. pylori* in clinical specimens.

The entire nucleotide sequence of the 16S rRNA gene of *Helicobacter pylori* has been determined. On the basis of that sequence, Ho et al. (4) designed a set of PCR primers, Hp1-Hp2 (Hp1, 5′-CTG GAG AGA CTA AGC CCT CC-3′; Hp2, 5′-ATT ACT GAC GCT GAT TGT GC-3′), which amplifies a 109-bp fragment of the *H. pylori* 16S rRNA gene. This PCR method has been widely used to detect *H. pylori* in various clinical specimens including gastric biopsy (9, 12), gastric juice (9), dental plaque (9), saliva (9), and stool (10, 14, 17) specimens. Using this method, several investigators (9, 10, 13) have obtained positive PCR results with gastric biopsy specimens in which no *H. pylori* organisms were detected by culture or histology. In the present study, we performed experiments to determine the specificities of these primers for the detection of *H. pylori* in various clinical specimens.

MATERIALS AND METHODS

Stool specimens. Thirty-four stool specimens were collected from 4 *H. pylori*-seropositive patients, 4 *H. pylori*-seronegative patients, 4 *H. pylori*-seropositive adult patients who had completed the triple therapy (1, 2) with Pepto-Bismol, tetracycline, and metronidazole 1 week prior to stool collection, 5 newborn babies (3 to 7 days old), and 17 randomly selected volunteers. The dosages of the drugs were as follows: bismuth subsalicylate (Pepto-Bismol), two 262-mg tablets four times a day; tetracycline, 500 mg four times a day; and metronidazole, 500 mg twice a day. Stool samples (3) were stored at −20°C until use and were processed for PCR by the procedures described previously (7).

Approximately 100 mg of a fecal sample was suspended in 1 ml of sterile water. After a low-speed centrifugation (2,800 × *g*) for 2 min, 500 μl of the supernatant was boiled in a water bath for 5 min to lyse the bacteria and was further clarified by centrifugation at 12,000 × *g* for 5 min. Fifty microliters of the clarified supernatant was then purified by spun-column chromatography with Sepharose CL-6B.

Tissue specimens. Most of the tissue specimens used for the study were remnants of tissues left after other diagnostic tests had been completed. The biopsy specimens from juveniles were obtained with the consent of the patients' parents. The gastric biopsy specimens used for the study were the same pieces of tissues that were used for CLO tests (11). Twenty CLO test-positive and 20 CLO test-negative gastric biopsy specimens were recovered from the CLO tests after the results had been finalized. Three liver biopsy specimens were taken from three infants with biliary atresia, and three colon biopsy specimens were obtained from three children with colitis. Four fetal liver specimens were obtained from the Indiana University Fetal Tissue Bank. All 50 human tissue samples were processed for PCR by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation as described previously (6).

Other specimens. DNAs from two strains of *Escherichia coli* (strains DH5α and INVαF1) and five different fungi including *Cryptococcus neoformans*, *Candida albicans*, *Saccharomyces cerevisiae*, *Blastomyces dermatitidis*, and *Histoplasma capsulatum* were examined. Bacterial DNA was extracted as described by Ho et al. (4), and fungal DNA was isolated by the procedures described previously (8). Five human leukocyte samples and one human cell line (HeLa) were also used; DNAs were isolated from these samples with the Puregene DNA Isolation Kit (PC Gentra, Research Triangle Park, N.C.) according to the manufacturer's instructions.

PCR conditions. PCRs with both the Hp1-Hp2 and U1-U3 primer sets were performed as described by Ho et al. (4). The 50-μl PCR mixture contained 100 ng of template DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin), 0.2 μM (each) PCR primers, 0.2 mM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase. The reaction mixture was overlaid with 50 μl of mineral oil and was then subjected to 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR products were electrophoresed on 6% polyacrylamide gels. The DNA bands were visualized by UV transillumination after the gels were stained with ethidium bromide.

Southern hybridization. The PCR products were electrophoresed on an agarose gel (2%) and were then transferred onto a Nytran membrane by the procedures of Southern (15). The membrane was baked, prehybridized, and hybridized with ^{32}P-labeled probes as described previously (5). The oligonucleotide probe pHp was labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase.

RESULTS

To determine whether the primer set Hp1-Hp2 can be used to detect *H. pylori*, PCR was performed with four stool specimens from patients who were seropositive for *H. pylori*. As controls, four stool specimens each from patients who were *H. pylori* seronegative and from patients who were *H. pylori* seropositive but who had completed the entire course of triple therapy were also examined by PCR with Hp1-Hp2 primers. All 12 specimens generated a positive PCR result. Examples of the PCR product bands electrophoresed on a polyacrylamide gel are shown in Fig. 1 (lanes 3 and 4). Two prominent bands of 260 and 109 bp were produced from all stool specimens. Four bands (approximately 1,400, 1,100, 600, and 500 bp) with much weaker intensities were also generated from most of the stool specimens. These results were quite surprising since stool specimens from *H. pylori*-seronegative patients were not expected to produce the 109-bp PCR product. To explore the

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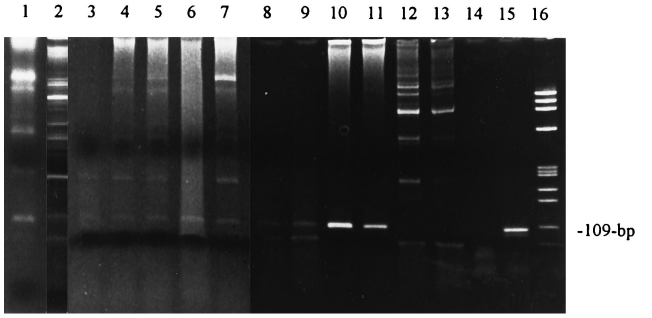

FIG. 1. Hp1-Hp2 PCR results for various clinical specimens. PCR products from the following specimens were electrophoresed on 6% polyacrylamide gels in different lanes. Lane 1, human leukocyte; lane 2, human HeLa cell line; lanes 3 and 4, stool specimens; lane 5, colon biopsy specimen; lane 6, liver biopsy specimen; lane 7, fetal liver specimen; lanes 8 and 9, CLO test-negative gastric biopsy specimens; lanes 10 and 11, CLO test-positive gastric biopsy specimens; lane 12, *E. coli* DH5α; lane 13, *E. coli* INVαF1; lane 14, distilled water (negative control); lane 15, *H. pylori* DNA (positive control); lane 16, DNA size marker.

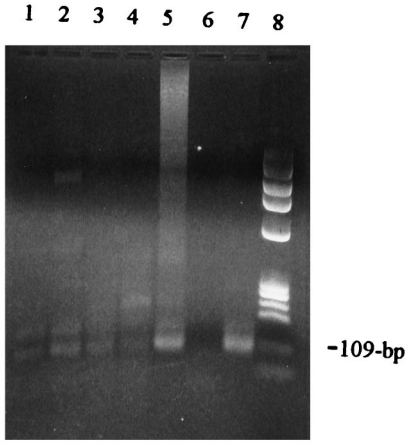

FIG. 2. Hp1-Hp2 PCR products from the indicated specimens were electrophoresed on a 2% agarose gel in different lanes. Lane 1, stool specimen from an *H. pylori*-seropositive patient; lane 2, stool specimen from an *H. pylori*-seronegative patient; lanes 3 and 4, CLO test-negative gastric biopsy specimens; lane 5, CLO test-positive gastric biopsy specimen; lane 6, distilled water (negative control); lane 7, *H. pylori* DNA (positive control); lane 8, DNA size marker.

possibility that the results were false positives, stool specimens from 5 newborn babies and 17 healthy volunteers who had no signs of *H. pylori* infection were examined by the same PCR method. The 109-bp PCR product was again produced from all of these stool specimens, suggesting that the Hp1-Hp2 PCR is not specific for *H. pylori*.

To determine whether the Hp1-Hp2 PCR nonspecifically amplifies the 109-bp fragment from *E. coli* strains that are normal flora of the colon, DNAs isolated from *E. coli* DH5α and INVαF1 were used as templates for the Hp1-Hp2 PCR. Another PCR with universal primers U1 (5′-CGGTTACCTT GTTACGACTT-3′) and U3 (5′-CAGCAGCCGCGGTAATA C-3′) (4), which amplify a 992-bp DNA fragment of the 16S rRNA gene of a wide range of eubacteria, was also performed with the *E. coli* DNA to ensure that the DNA was amplifiable. The 992-bp DNA fragment produced by the U1-U3 PCR was amplified from the DNAs of both strains of *E. coli* (data not shown). The Hp1-Hp2 PCR did not produce the 109-bp PCR product from either strain of *E. coli* (Fig. 1, lanes 12 and 13). It did produce several other bands; the most noticeable ones were approximately 1,400, 1,100, 800, 500, 260, and 70 bp. These results indicate that the 109-bp product of the Hp1-Hp2 PCR was not amplified from the *E. coli* genome. To determine whether the 109-bp DNA fragment was amplified from fungi, DNAs isolated from five different fungi including *C. neoformans*, *C. albicans*, *S. cerevisiae*, *B. dermatitidis*, and *H. capsulatum* were amplified by the Hp1-Hp2 PCR. None of the fungal DNAs tested produced any band by this PCR, indicating that the 109-bp product of the Hp1-Hp2 PCR was not derived from fungi.

To determine whether the 109-bp DNA fragment was amplified by the Hp1-Hp2 PCR of DNA from the human genome, DNA samples isolated from human tissues were examined. The tissues included 20 CLO test-positive and 20 CLO test-negative gastric biopsy specimens, 3 colon biopsy specimens, 3 liver biopsy specimens, and 4 fetal liver specimens. DNA samples from all of these tissues produced the 109-bp Hp1-Hp2 PCR product. The intensities of the 109-bp bands from CLO test-positive samples were much stronger than those of the bands from CLO test-negative samples. A band of approximately 90 bp was also seen with CLO test-negative samples (Fig. 1, lanes 8 and 9). The banding patterns of PCR products from liver and colon biopsy specimens were similar to those from stool specimens (Fig. 1, lanes 5 to 7). To further confirm that the 109-bp DNA fragment was amplified by the Hp1-Hp2 PCR of DNA from the human genome, DNAs from five human leukocyte samples and the HeLa cell line were examined by this PCR. All five human leukocyte samples produced the 109-bp band as well as bands of approximately 2,000, 1,000, and 500 bp. The DNA from the HeLa cell line also produced the 109-bp band and bands of about 2,000, 1,000, 600, 500, 400, 230, and 90 bp. These results further indicate that the 109-bp fragment was amplified by the Hp1-Hp2 PCR of DNA from the human genome. A Southern hybridization was performed to determine whether the 109-bp PCR products generated from various types of tissues would react with the probe pHp, which was designed by Ho et al. (4) to identify the product of the Hp1-Hp2 PCR. The sequence of the pHp probe is 5′-CATCCATCGTTTAGGGCGTG-3′, which corresponds to an internal region of the 109-bp fragment in the *H. pylori* genome. PCR products from two stool specimens, two CLO test-positive tissue specimens, two CLO test-negative tissue specimens, and the *H. pylori* genome (positive control) were examined. The 109-bp PCR bands from all of these different types of tissues seen on the gel (Fig. 2) were found to react with the probe (Fig. 3). None of the bands of other sizes reacted with the probe.

DISCUSSION

The PCR primers Hp1 and Hp2 were designed by comparing nucleotide sequences of the 16S rRNA genes of *E. coli*, *Wolinella succinogenes*, and many species of *Helicobacter* and *Campylobacter* (4). The primer sequences that were used are located in areas that have the least homology among all the sequences compared (4). The Hp1-Hp2 PCR appeared to be specific for *H. pylori* when it was run with various bacteria (4, 9, 16). The observation that the two *E. coli* strains and the five fungal strains tested in the study did not produce the 109-bp DNA band supports that conclusion.

When the Hp1-Hp2 PCR was applied to stool specimens from *H. pylori*-infected patients and from healthy individuals, a 109-bp PCR product was generated from all 34 stool specimens tested, suggesting that the Hp1-Hp2 PCR produces false-positive results. These results are contradictory to those of van Zwet et al. (17); they found that none of the stool specimens from 24 *H. pylori*-infected patients produced a positive result

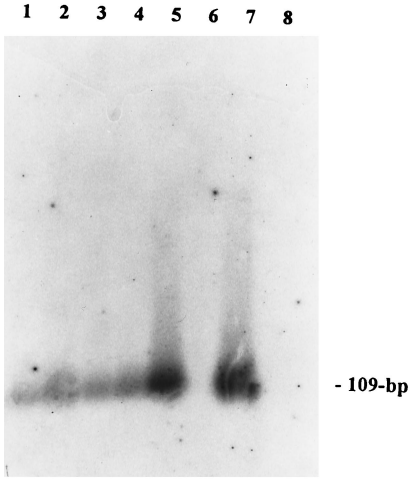

FIG. 3. Southern blot hybridization of the PCR products of the specimens electrophoresed for Fig. 2, displaying hybridization of the pHp probe with the 109-bp PCR products.

by the Hp1-Hp2 PCR (17). On the basis of that observation, they concluded that *H. pylori* organisms are not shed into the feces of infected patients. Although this conclusion may be true, the lack of a positive Hp1-Hp2 PCR result is very likely due to the presence of PCR inhibitors in stool specimens, as described previously (7).

Nonspecific amplification was also observed when the Hp1-Hp2 PCR was applied to human tissue specimens. Although similar results were also noted by Mapstone et al. (9) and Roosendaal et al. (13), the specificity of the Hp1-Hp2 PCR has not been questioned. Mapstone et al. (9) speculated that these unexpected positive PCR results were due to the presence of trace amounts of *H. pylori* organisms in the stomach or to contamination at the time of biopsy. Roosendaal et al. (13) attributed the unexpected positive Hp1-Hp2 PCR results to contamination that occurred during the PCR or the endoscopic procedures (13). The observation in the present study that the Hp1-Hp2 PCR amplified a 109-bp fragment from liver tissues that are not known to harbor *H. pylori* organisms indicates that these unexpected positive results are due to nonspecific amplification of human genomic DNA by Hp1-Hp2 PCR. The finding that the pHp probe, which was also designed by Ho et al. (4) to identify the 109-bp Hp1-Hp2 PCR products, reacted with the 109-bp PCR products generated from human tissues indicates that the pHp probe is also not specific to *H. pylori*.

To conclude that the Hp1-Hp2 PCR is not specific, contamination of specimens by *H. pylori* or previous PCR products must be ruled out. In the present study, the negative PCR control, which contained all components of the PCR except the template DNA, was always negative. This finding rules out the possibility that the *Taq* polymerase, Hp1 and Hp2 primers, deoxynucleoside triphosphates, and PCR buffer were contaminated. The DNA samples isolated from two strains of *E. coli* and five different fungi were also negative by the Hp1-Hp2 PCR, indicating that the reagents used to isolate DNA were not contaminated. The possibility that all 40 gastric biopsy specimens used in the study were contaminated by *H. pylori* during endoscopy cannot be ruled out directly, but this possibility is not supported by the fact that three liver biopsy specimens, four fetal liver specimens, five leukocyte samples, and one human cell line, which were not obtained by endoscopy, also produced positive Hp1-Hp2 PCR results.

In the present study, the intensities of the 109-bp PCR bands from the CLO test-positive biopsy specimens were stronger than those from CLO test-negative biopsy specimens (Fig. 1). It is possible that the positive PCR products in the CLO test-positive biopsy specimens were derived from both *H. pylori* DNA and human DNA, whereas the positive PCR results in the CLO test-negative biopsy specimens were derived from human DNA only. According to the results obtained in the present study, we conclude that the PCR method with the primer set Hp1-Hp2 is not specific and cannot be used to detect *H. pylori* in clinical specimens.

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