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Use of PCR with Feces for Detection of *Helicobacter pylori* Infections in Patients

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PCR was performed for the detection of *Helicobacter pylori* in feces from 24 patients with proven infections. Several precautions were taken to overcome possible inhibition of PCR with feces. In the first 12 patients, feces were examined shortly after endoscopy. In another group of 12 patients, who were treated during 2 weeks with omeprazole (40 mg each day) to increase gastric pH, feces were examined as well. *H. pylori* target DNA could not be detected in the stools of any of the 24 infected patients. It was concluded that there was no substantial shedding of *H. pylori* in feces from either group of patients.

The exact mode of transmission of *Helicobacter pylori* is unknown. The concept that *H. pylori* is transmitted by the fecal-oral route is adhered to widely (9, 17). Some, however, consider the oral-oral route as the most likely way that *H. pylori* is transmitted (10, 14). Arguments against the hypothesis of the fecal-oral route of transmission are presented by studies in animals with related *Helicobacter* spp. Germ-free mice and rats infected with *Helicobacter felis* did not transmit their infection to uninoculated mice after prolonged contact in the same cage, even though these animals are coprophagous (10). Until recently, no one was able to culture *H. pylori* from the feces of infected patients. These negative culture results were also regarded as an argument against the fecal-oral route of transmission. Recently, however, the microorganism was successfully cultured by Thomas et al. (19) from the feces of a group of African children. This encouraging finding has not yet been confirmed by other laboratories. In fact, culture of *H. pylori* from feces is tremendously difficult, because overgrowth of other bacteria can occur. Fox et al. (3) showed that suppression of acid production by omeprazole, in order to induce a hypochlorhydric state, can increase the recovery of *Helicobacter mustelae* in the feces of ferrets. It is possible that hypochlorhydria also occurred in the group of very young children who Thomas et al. (19) studied. However, the problems of culturing *H. pylori* from feces can be avoided by the use of PCR for *H. pylori* detection. PCR enables the amplification of minimum quantities of target DNA and provides a very sensitive detection method. We looked for *H. pylori* DNA using PCR with feces from patients with a proven infection to test the hypothesis of the fecal-oral route of transmission. Since hypochlorhydria could allow the transition of the microorganism from the stomach to the feces (3), some patients were first treated with omeprazole before their feces were examined.

Two groups of patients, all with culture-proven *H. pylori* infections, were examined. All patients gave informed consent to participate in the study. Each member of the first group of 12 patients (6 with duodenal ulcer [DU], 2 with gastric ulcer [GU], 1 with both a DU and a GU, and 3 with nonulcer dyspepsia) was asked to bring their feces to the laboratory shortly after endoscopy. The second group of 12 patients (DU, 7; GU, 2; nonulcer dyspepsia, 3) was first treated with 40 mg of omeprazole daily to induce hypochlorhydria. They were requested to bring their feces to the laboratory on day 14 of this treatment course. All patients were urged to deliver their feces as soon as possible after defecation. The mean time delay between defecation and delivery of the sample at the laboratory was 1 h and 40 min for the first group and 55 min for the second group. The samples were stored at −20°C in the laboratory until the specimens were processed.

Endoscopy was performed after patients had fasted overnight. The endoscopes (Olympus GIF IT 20, GIF Q20, and GIF K10) and biopsy equipment were cleaned with a detergent and were disinfected between all procedures by using 2% glutaraldehyde in the ETD Olympus washing machine. Antral biopsy samples for culture, taken about 2 cm from the pylorus, were placed in 1 ml of thioglycolate broth and were immediately sent to the bacteriology laboratory, which is located near the endoscopy department. Detection of *H. pylori* in antral biopsy samples by culture was carried out as described elsewere (20).

PCR of fecal samples can easily give false-negative results because a variety of chemicals in the stool inhibit the reaction (13, 21). In the assay described here, several precautions were taken to avoid the problem of inhibition. First, hexadecyltrimethyl ammonium bromide was added. This detergent has been described to effectively remove PCR-inhibiting factors from stools (8) and has been used by others for the detection of *H. pylori* in fecal samples (7, 12). Furthermore, our method for purification of nucleic acids was based on the nucleic acid-binding properties of silica particles in the presence of guanidinium thiocyanate (GuSCN), an agent with strong nuclease-inactivating properties (1). Inhibiting chemicals are washed away after the binding of the nucleic acids to these silica particles.

To verify whether inhibition of the PCR by feces was effectively controlled, after we took the precautions described above, for all fecal samples that scored negative, PCR was repeated after the addition of 10^3 bacteria from an *H. pylori* culture. These *H. pylori* bacteria were seeded directly onto the untreated fecal suspensions before processing was carried out.

In the PCR, primers for the specific detection of *H. pylori* were taken from the gene encoding 16S rRNA as described by Ho et al. (7): Hp1 (5′-CTG-GAG-AGA-CTA-AGC-CCT-CC-3′) and Hp2 (5′-ATT-ACT-GAC-GCT-GAT-TGT-GC-3′).

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Hybridization was performed by using a digoxigenin-labelled probe (pHp, 5′-CAT-CCA-TCG-TTT-AGG-GCG-TG-3′) in the region of the product of amplification, which was 109 nucleotide pairs in length.

Fecal samples were thawed at room temperature. A total of 100 mg of feces was taken and a suspension was made in 1,000 μl of buffer (0.1 M Tris-HCl [pH 7.5], 12.5 mM EDTA, 0.15 M NaCl). This suspension was vortexed and centrifuged at 1,000 × *g* for 90 s. A total of 750 μl of supernatant from the centrifuged fecal suspension was taken. A total of 100 μl of 10% sodium dodecyl sulfate and 100 μl of proteinase K (10 mg/ml) were added. After incubation at 56°C for 1 h, 150 μl of 10% cetyltrimethylammonium bromide (hexadecyltrimethyl ammonium bromide; Sigma) was added. Then, 100 μl of 5 N NaCl was added and the mixture was incubated at 65°C for 10 min. A total of 50 μl of silica particles was added to 900 μl of a lysis buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl [pH 6.4] with 22 ml of a 0.2 M EDTA solution [pH 8.0] and 2.6 g of Triton X-100 [Packard Instrument Co., Inc.]). A total of 100 μl of the prepared mixture was added to this buffer along with silica particles. Afterward, the vessel was vortexed (approximately 5 s). After 10 min at room temperature, the vessel was vortexed again and centrifuged (12,000 × *g* for 15 s), after which the supernatant was disposed of by suction. The pellet was subsequently washed twice with washing buffer (120 g of GuSCN in 100 ml of 0.1 M Tris-HCl [pH 6.4]), twice with 70% ethanol, and once with acetone. Pellets were dried in a heat block for 10 min at 56°C. A total of 100 μl of TE elution buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) was added, and the vessel was closed, vortexed, and incubated for 10 min at 56°C. The vessel was briefly vortexed again and centrifuged at 12,000 × *g* for 2 min. A total of 1 μl of the supernatant was used in the PCR assay.

Amplification was performed in a reaction volume of 50 μl in a thermal cycler (Hybaid TR1). The reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 U of *Taq* polymerase (Boehringer Mannheim), 0.2 mM (each) deoxynucleotide, 1 μM primer Hp1, and 1 μM primer Hp2. PCR amplification consisted of an initial denaturation step at 94°C for 2 min and then an annealing step at 60°C for 30 s and an extension step for 1 min at 72°C. Afterward, another 39 cycles were performed at the temperatures indicated above, as follows: a denaturation step for 30 s, an annealing step for 1 min, and an extension step for 1 min, with the exception of the last cycle, in which the extension period was increased to 5 min. The PCR-amplified products were analyzed by agarose gel electrophoresis as described elsewhere (20). Samples were scored positive when a band of 109 nucleotide pairs could be detected on the gel. In all positive samples, the specificity of the amplified product was confirmed by Southern blot hybridization with a digoxigenin-labelled probe (pHp).

Positive and negative controls were examined with each batch of amplified product. Positive controls consisted of an *H. pylori* suspension in distilled water as well as spiked fecal samples from *H. pylori*-negative patients. Negative controls consisted of tubes containing distilled water and tubes containing fecal samples from *H. pylori*-negative patients.

The sensitivity of the PCR assay was tested in different samples. Tenfold serial dilutions of an *H. pylori* suspension from a culture in distilled water as well as vessels containing serial dilutions of spiked fecal samples from different noninfected patients were amplified. These fecal suspensions from noninfected patients were spiked prior to the processing of the samples.

The sensitivity of the assay was high, since 10 bacteria could be detected in distilled water and approximately 10 to 100 bacteria per reaction vessel could be detected in fecal samples. This means that the PCR assay could detect at least as few as 1,660 bacteria per mg of feces since a reaction vessel contained about 0.06 mg of feces. In the first group of 12 patients with proven *H. pylori* infection, PCR of fecal samples appeared to be negative for all 12 patients. For the second group of patients treated with omeprazole (40 mg each day during 2 weeks), PCR signals were negative as well. The specificity of the amplified DNA, which was detectable in samples from all positive controls in both groups, was confirmed by Southern blot hybridization.

After the addition of 10^3 bacteria to the fecal suspensions of each of the 24 *H. pylori*-infected patients whose fecal samples scored negative, PCR signals became positive for all samples. The specificities of the amplified DNAs from these reaction vessels were also confirmed by Southern blot hybridization.

Since the PCR result became positive for all fecal samples after the addition of 10^3 *H. pylori* bacteria, the problem of inhibition seemed to be controlled, making false-negative results unlikely. In the PCR, a reaction vessel contained about 0.06 mg of feces. Since the PCR assay was able to detect the DNAs from as few as 10 to 100 *H. pylori* bacteria in each vessel, we concluded that there was no substantial fecal shedding of *H. pylori* at the time of examination of the 24 infected patients. Therefore, we believe that the use of fecal samples for the detection of *H. pylori* in patients is precarious. Our findings seem to argue against the hypothesis of the fecal-oral route of spread of the organism. The successful culture of *H. pylori* from a group of African children (19) and the detection by PCR of *H. pylori* DNA in feces from patients suffering from gastritis (12) seem to contradict our findings. We do not have a reasonable explanation for the differences between our negative PCR results and the positive results of PCR with feces presented by other investigators (12). In fact, the same primers used in the present study were used previously (7), and in the method described here inhibiting factors were eliminated as much as possible, resulting in a sensitivity of the PCR of 10 to 100 bacteria per reaction vessel. Concerning the positive culture results for feces from African children, it must be emphasized that the fecal samples for culture were mainly obtained from infants (mean age, 13.8 months) (19). It can be argued that the microorganism can be detected only in the feces of *H. pylori*-infected people with hypochlorhydria (3). Hypochlorhydria caused by *H. pylori* mainly occurs during the acute stage of the infection (4, 16). In developing countries, the acquisition of infection occurs at an early age (15). It is conceivable that in the group of children in whom *H. pylori* could be cultured from their feces, infection still was in the acute hypochlorohydric stage, a period that can last for several months (2, 18). Fox et al. (3) could promote the fecal transmission of gastric *H. mustulae* in some ferrets treated orally with 0.7 mg of omeprazole per kg of body weight once a day (3). In a similar experiment in our second patient group, we were not able to detect fecal shedding of *H. pylori* in these infected patients. However, since we did not monitor the gastric pH during the experiment, we were not informed about whether the gastric pH was indeed effectively elevated in all patients.

We suggest that in contrast to the situation in developing countries, in developed countries, the fecal-oral route of transmission of *H. pylori* probably does not occur frequently. Recently, support for this hypothesis came from a small study of *H. pylori*-infected patients in whose feces the microorganism could not be detected (11). Moreover, the evidence suggests

that in the United States today, *H. pylori* is not a waterborne infection (5, 6), in contrast to the situation in Peru (9).

In conclusion, if the fecal-oral route of organism spread is an important means of transmission, it seems to be rare among adults in developed countries.

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