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Immunomagnetic Separation and PCR for Detection of *Helicobacter pylori* in Water and Stool Specimens

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The detection of *Helicobacter pylori* in clinical and environmental samples by PCR sometimes requires removal of polymerase inhibitors. We have used a magnetic immunoseparation technique as pre-PCR treatment to facilitate direct detection of *H. pylori* in stool and water specimens. Rabbit hyperimmune antiserum was produced and magnetic beads were coated with purified immunoglobulin G, which reacted with and bound to both coccoid and rod-shaped forms of *H. pylori*. When PCR was applied for the detection of *H. pylori* from cultured samples, the number of organisms that was required for positive scores varied significantly. For a 3-day culture of *H. pylori*, samples containing 10^2 bacteria per ml are needed for a positive score; for a 6-day culture, samples containing 10^4 bacteria per ml are needed; and for a 10-day culture, samples containing 10^6 bacteria per ml are needed. These results indicate that the coccoid forms of *H. pylori* may have a different antigenicity and DNA content and are therefore more difficult to detect by immunomagnetic separation and PCR than the rod-shaped forms. Spiked samples with the addition of feces, spiked water samples, and a patient stool specimen were all scored positive with this technique.

Immunomagnetic separation (IMS) has been shown to be efficient for recovering certain eucaryotic cells from fluids, as well as for separation of procaryotic pathogens from heterogeneous samples such as blood, food, and fecal samples (21). Integrated systems for IMS and PCR have recently been presented to facilitate DNA diagnosis of some bacteria, viruses, and parasites (4, 18, 23, 27). Paramagnetic beads coated with antibodies to surface antigens of bacteria are used to separate and concentrate organisms from the sample. Bacteria bound to beads form aggregates which are drawn to the side of the test tube by a magnet, and inhibitory factors in the test sample can be removed by changing the medium. The bacteria, still attached to the beads, are lysed, and DNA is released into the supernatant. The analytical procedure then continues by PCR with the supernatant, and positive amplified samples are easily detected as specific bands on an agarose gel.

The transmission of *Helicobacter pylori* from one host to another has been extensively studied but is yet not fully understood (11–14, 16, 24). Two different hypotheses are possible: oral-oral and fecal-oral transmission between individuals. The IMS method discussed here is a suitable tool for studies of the transmission of *H. pylori* in nature. Because the rod-shaped *H. pylori* cells change with time into coccoid forms, we performed the experiments with old cultures of bacteria kept in water for several weeks to imitate the natural state in feces and freshwater. We have shown that *H. pylori* can be detected in heterogeneous samples from the natural environment by using this simple and rapid IMS technique as a pre-PCR step.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* (laboratory strains 88-23 and NCTC 11637) and a clinical isolate (SVA 40) were cultured on Columbia agar II BBL plates containing 7% horse blood and 8% horse serum. The plates were incubated in a moist microaerophilic atmosphere containing 7% CO_2, 6% O_2, and 87% N_2 at 37°C. Bacteria were harvested and suspended in phosphate-buffered saline (PBS [pH 7.2]) or water.

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Cultures of stool specimens and water from the Fyris River in Uppsala, Sweden, were performed on selective medium containing 0.4% Skirrow's antibiotic supplement, Oxoid blood agar base 2, and 7% horse blood.

Gram staining and immunofluorescence. Gram staining was performed to check the purity and verify the morphology of *H. pylori* cultures of different ages. For immunofluorescence studies, three different cultures of *H. pylori* (2- and 6-day cultures and a 10-day culture which had been harvested and then suspended in water for 1 month) were applied to three different microscope slides. Ten microliters of each bacterial suspension was applied to eight wells and dried. A twofold serial dilution of purified polyclonal rabbit anti-*H. pylori* immunoglobulin G (IgG) was prepared in PBS. Ten microliters of each dilution was added to the bacteria dried on slides and incubated at 37°C in a moist chamber for 30 min. Slides were washed three times for 5 min in PBS. Ten microliters of fluorescein-conjugated swine anti-rabbit Ig (Dakopatts, Glostrup, Denmark) was added to each well at a dilution of 1:40, and then the mixture was incubated and washed under the conditions described above. An additional wash in water was performed before cells were mounted with PBS-buffered glycerine. Slides were then examined with a UV microscope at a magnification of ×1,000.

Electron micrographs. A 7-day culture of *H. pylori* NCTC 11637 cells, containing both rods and cocci confirmed by Gram staining, was used for the electron micrographs (Fig. 1). IMS was performed. The supernatant was changed from PBS to electron microscopy fixative containing 3% glutaraldehyde in lactate buffer.

IMS-PCR techniques: rabbit immunization and IgG purification. A clinical isolate of *H. pylori*, SVA 40, isolated from a patient with a long history of peptic ulcer disease was used for immunization of the rabbit (8). Bacteria were grown for 5 to 6 days, and morphological characteristics were determined by Gram staining. The bacteria were harvested for immunization when approximately 50% coccoid forms were found. Rabbit hyperimmune antiserum was produced by repeated subcutaneous and intravenous immunization in two rabbits as described previously (7) and stored in aliquots at −70°C until used. For the separation of polyclonal antibodies and purification of IgG, an affinity chromatography gel, protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology), was used according to the manufacturer's instructions.

Coating of the immunomagnetic beads. Three microliters of purified polyclonal rabbit anti-*H. pylori* IgG (5.53 mg of protein per ml) was incubated with 500 μl of magnetic beads [(6 to 7) × 10^8 beads per ml] precoated with sheep anti-rabbit IgG (Dynabeads M-280; Dynal) for 24 h at 4°C with gentle agitation. With the aid of a magnet, beads were drawn to one side of the vial and rinsed three times in 1 ml of PBS containing 0.1% bovine serum albumin (BSA) for 30 min at a time with gentle agitation at 4°C. After the third washing, beads were resuspended in 500 μl of PBS containing 0.1% BSA and 0.02% sodium azide and stored at 4°C.

IMS and lysis of bacteria. For the standard IMS operation, 20 μl of coated beads was used per ml of test sample. The samples were gently agitated for 1 h at 4°C, and then a magnet was used to separate the beads with bound bacteria from the sample solution. The beads could then be washed if needed. The bead-bacterium aggregates were then resuspended in 50 μl of sputum lysis buffer (sputum sample preparation kit; Roche Diagnostic Systems) and heated for 45

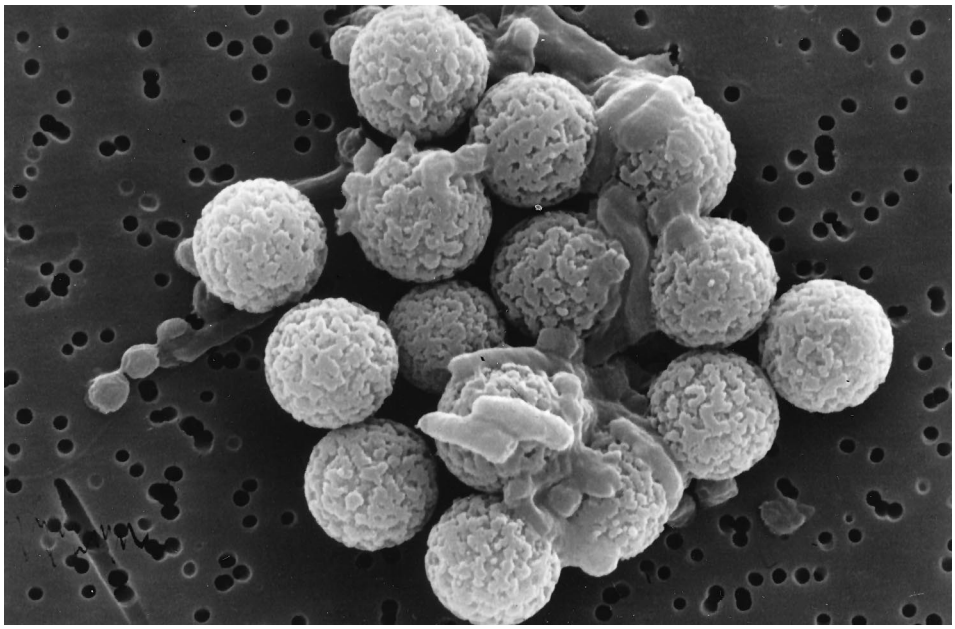

FIG. 1. Electron micrograph of *H. pylori* NCTC 11637 cells in a 7-day culture. The micrograph shows that both rods and coccoid forms of *H. pylori* bind to the paramagnetic beads. Magnification, ×5,500.

min at 60°C. After lysis, 50 μl of sputum neutralization buffer was added to each sample, and then the sample was stored at 4°C until the PCR was performed.

PCR. Primers for the detection of *H. pylori* DNA span a 375-bp segment of the adhesin-encoding gene (10). The following primers (20 bases each) were used: Hp A (5′-GAA-TTA-CCA-TCC-AGC-TAG-CG-3′) and Hp B (5′-GTA-ACC-TTG-ACA-AAA-CCG-GC-3′) (Scandinavian Gene Synthesis, Köping, Sweden). Ten microliters of supernatant containing bacterial DNA and 3 μl of each primer were used for amplification in each 50-μl reaction mixture under standard conditions (22). The amplification was performed in a thermal cycler (Perkin-Elmer). The temperature profile for the PCR was as follows: an initial step of 5 min at 95°C, followed by a denaturation step for 1 min at 94°C, an annealing step for 2 min at 55°C, and a primer extension step for 2 min at 72°C. After the 30th cycle, the extension step was prolonged for 10 min and then the samples were kept at 4°C until analysis. The amplified DNA was analyzed by electrophoresis on a 1.5% (wt/vol) agarose gel containing ethidium bromide and visualized with a UV transilluminator. Test samples were scored as positive when a 375-bp band was detected on the gel. *H. pylori* DNA from a clinical isolate and water were used as positive and negative controls, respectively, for the PCRs.

Applications of the IMS-PCR method: *H. pylori* dilution series. Three different cultures of *H. pylori* 3, 6, and 10 days old were suspended in 5 ml of PBS. The oldest culture was stored for 4 weeks in water to ensure that it contained only the coccoid forms. The concentration of bacteria per milliliter was calculated with a Bürker chamber.

Tenfold serial dilutions of bacteria were prepared: 10^8 to 10^2 bacteria per ml for the 3-day culture, 10^7 to 10^1 bacteria per ml for the 6-day culture, and 10^8 to 1 bacterium per ml for the 10-day culture. The IMS method was used as a prelude to PCR. The samples from the 3- and 6-day cultures were divided into two 500-μl aliquots before lysis of the bacteria to make it possible to compare the specificity of the PCR for washed and unwashed beads: 500 μl of PBS with BSA was used for washing once prior to lysis. Only unwashed beads were used with the 10-day culture.

Investigation of stool specimens. A 10-fold serial dilution (10^8 to 10^4 bacteria per ml) from a 10-day culture of *H. pylori* kept in water for an additional 6 weeks was prepared in PBS. A series of samples, 500 μl each, were removed from each dilution. These samples were not washed. A 10-μl amount of feces was added to the remainder of each dilution and mixed thoroughly and then was left to stand. After 5 min, when the solution was clear, a 1-ml sample was removed. IMS was performed on two different series, one series with only bacteria and another with bacteria and feces. The samples containing feces were divided further into two tubes after incubation, one of which was washed before lysis to investigate the inhibitory effect of feces on the PCR. Two extra controls were used for PCR: a sample containing only bacteria and feces not separated with the magnetic beads, in which the pellet was lysed directly after 10 min of centrifugation at 9,980 × *g*, and a sample containing only feces in PBS to ascertain that the feces did not contain *H. pylori* when IMS was performed.

A total number of 25 randomly collected stool specimens from patients born before 1930, without verified *H. pylori* infection, were also analyzed by the IMS and PCR method described above. Fecal samples were cultured on selective medium.

Investigation of water specimens. To check that the IMS method was sensitive for *H. pylori* in water samples, two 50-ml water samples were taken from the Fyris River, near the outflow from the sewage treatment plant. One sample was spiked with a 7-day culture of *H. pylori*. The other water sample was kept as a negative control. The samples were centrifuged at 9,980 × *g* for 30 min, the supernatant was removed, and the pellets were resolved in 1 ml of PBS. Twenty microliters of coated beads was added to the sample, IMS was performed, and after incubation with the beads, the samples were divided into two aliquots of 500 μl each. One of the negative and one of the positive aliquots were washed once in PBS-BSA. The supernatant was removed, and all of the samples were lysed in 50 μl of lysis buffer. After preparation, the four samples were investigated by PCR as previously described.

RESULTS

Rabbit immunization. The rabbit antiserum produced was tested against 45 clinical isolates of *H. pylori* by indirect immunofluorescence and gave strong, clear-cut reactions with serum titers in the range of 1:320 to 1:1,250 in all cases. Both coccoid and rod-shaped forms of *H. pylori* were detected.

Electron micrographs. The magnetic beads used bind to both the coccoid and rod-shaped forms of *H. pylori* (Fig. 1).

PCR of serial dilutions containing *H. pylori*. The three serial dilutions of *H. pylori* from bacteria of different ages clearly show these differences in sensitivity of PCR between rods and cocci. For the 3-day culture, samples containing 100 bacteria per ml could be detected by PCR (Fig. 2), and for the 6-day culture, samples containing 10,000 bacteria per ml were necessary to give a positive result (Fig. 3). For the 10-day culture kept in water for 4 weeks, a positive result was obtained with 10^5 to 10^6 bacteria per ml (Fig. 4). With Gram staining, it was confirmed that the 3-day culture consisted of rod-shaped bacteria, the 6-day culture was a mixture of both rods and cocci, and the 10-day culture kept in water contained bacteria exclusively in the coccoid form.

Stool specimens. Investigation of the stool specimens seeded with a 10-fold serial dilution of *H. pylori* showed that positive results were obtained only when the beads were washed before

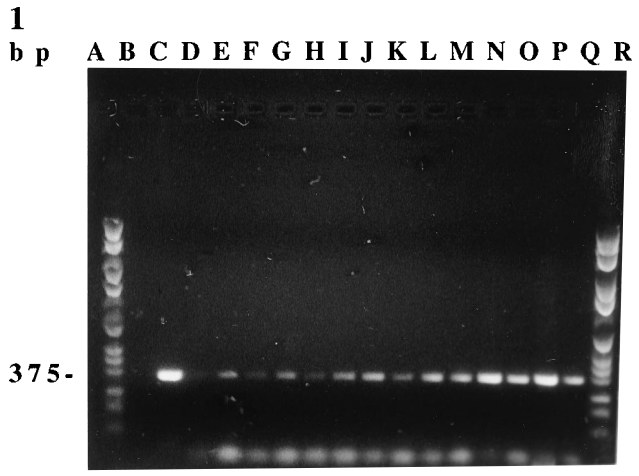

FIG. 2. Serial dilution 1. Ethidium bromide-stained agarose gel with amplified DNA from a serial dilution of a 3-day-old culture of *H. pylori*. Lanes: A and R, DNA molecular weight marker VI (Boehringer Mannheim); B, negative control; C, positive control; D to Q, serial dilution starting with 10^2 bacteria per ml in lane D to 10^8 bacteria per ml in lane Q, with double samples of each dilution (washed and unwashed beads, respectively).

PCR analysis (Fig. 5). Inhibitory factors in the feces were removed, and *H. pylori* could be detected at a level of 10^7 bacteria per ml. Samples without feces were positive with PCR at a concentration of 10^5 to 10^6 bacteria per ml. In the control sample with feces and the sample with 10^8 bacteria per ml not separated by IMS, no bacteria could be detected. One of the patients' fecal samples was scored positive by PCR, but no *H. pylori* was detected on culture (data not shown).

Water specimens. Seeded water samples from the Fyris river, prepared by lysis of bacteria from both washed and unwashed beads, were scored positive by PCR, showing that detection of *H. pylori* is possible in environmental water samples.

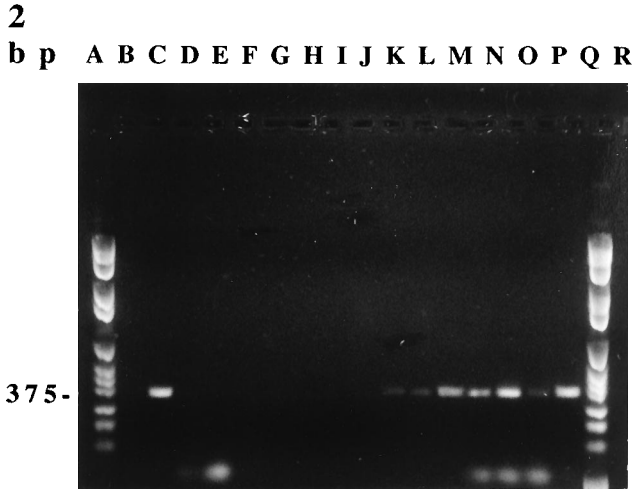

FIG. 3. Serial dilution 2. Ethidium bromide-stained agarose gel with amplified DNA from a serial dilution of a 6-day-old culture of *H. pylori*. Lanes: A and R, DNA molecular weight marker VI (Boehringer Mannheim); B, negative control; C, positive control; D to Q, serial dilution starting with 10^1 bacteria per ml in lane D to 10^7 bacteria per ml in lane Q, with double samples of each dilution (washed and unwashed beads, respectively).

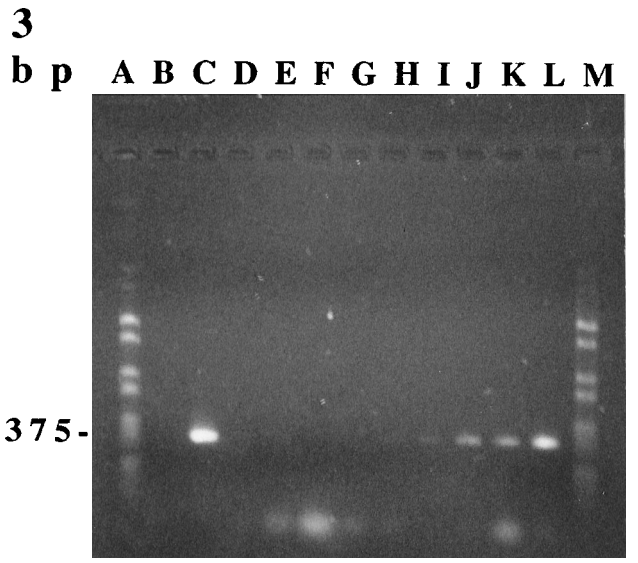

FIG. 4. Serial dilution 3. Ethidium bromide-stained agarose gel with amplified DNA from a serial dilution of a 10-day-old culture of *H. pylori*. Lanes: A and M, DNA molecular weight marker VI (Boehringer Mannheim); B, negative control; C, positive control; D to L, serial dilution starting with 1 bacterium per ml in lane D to 10^8 bacteria per ml in lane L, with single samples of each dilution (unwashed beads only).

DISCUSSION

The IMS method as a pre-PCR step. Our results show that the immunomagnetic separation method followed by PCR is an easy way to detect *H. pylori* in water and stool specimens. No additional DNA extraction is needed, and inhibitory factors in the stool specimens are readily removed by washing, allowing PCR detection in environmental samples. The magnetic beads used bind to both the coccoid and rod-shaped forms of *H. pylori*. This was confirmed by both electron micrographs and immunofluorescence. *H. pylori* in the coccoid form was more difficult to detect by PCR, probably because of different antigenicity and/or DNA content compared with the rods. The numbers of bacteria in the 6- and 10-day cultures used for the dilution series are approximations based on bacteria seen in

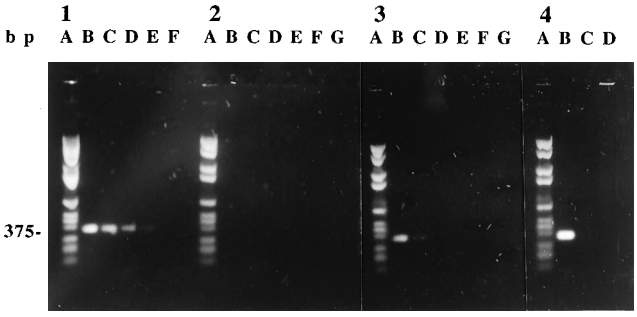

FIG. 5. Ethidium bromide-stained agarose gel with amplified DNA from a serial dilution of a 10-day-old culture of *H. pylori* with or without the addition of feces. Series 1 contained only the serial dilution of *H. pylori*, series 2 contained the serial dilution with addition of feces (unwashed samples), series 3 contained the serial dilution with addition of feces (washed samples), and series 4 contained controls. Lanes: A in all four series, DNA molecular weight marker VI (Boehringer Mannheim); B to F in series 1 to 3, serial dilutions starting with 10^8 bacteria per ml in lanes B to 10^4 bacteria per ml in lanes F; G in series 2 and 3, feces and beads without bacteria added; B in series 4, positive control; C in series 4, negative control; D in series 4, 10^8 bacteria per ml, with the addition of feces but without beads.

the Bürker chamber, because coccoid forms of *H. pylori* form large bacterial aggregates which are difficult to count.

The morphologic conversion of *H. pylori* from the bacillary to the coccoid form has been studied extensively (6, 17, 20, 26), and DNA has been extracted from the coccoid forms of the bacteria (15). Ultrastructural analysis of coccoid forms showed that the majority of bacteria, although not all, have an intact cell wall, cell membrane, and cytoplasm (5).

This IMS method followed by PCR analysis could be useful to detect *H. pylori* in water and stool specimens when most of the bacteria are possibly in the coccoid form. Our results show that the coccoid form of *H. pylori*, which is thought to be the form responsible for transmission in both fecal-oral and oral-oral routes, must be present in larger numbers than rods to be detected and scored positive by PCR. We have shown that both rods and cocci bind to the beads; therefore the IMS was not specific for either rods or cocci, yet the cocci are still more difficult to detect. We have, to this date, no clear explanation for this phenomenon, because it is not known whether coccoid forms of *H. pylori* are dormant or degraded. The commercial lysis buffer used is optimized for lysis of *Mycobacterium* sp.; i.e., this solution fulfills our requirements for lysis of rod-shaped *H. pylori* cells. However, we currently do not know enough about the coccoid cell wall, which may be thicker and harder to break than the cell wall of rod-shaped *H. pylori*. It may be that some of the coccoid bacteria are dead after storage in water, not dormant or viable but nonculturable, and therefore do not contain intact DNA (2). If feasible, samples as fresh as possible should be analyzed to avoid the conversion of rod-shaped *H. pylori* to the coccoid form.

PCR is a method that is very useful for the detection of a wide range of microorganisms present in the environment (1), including *H. pylori* in various types of specimens. This has been shown by a number of primers and PCR methods available for detection of *H. pylori* (3, 9, 15). We have shown that IMS followed by PCR is sensitive in stool and water specimens when the samples are spiked with cultures of *H. pylori*. For investigation of dental plaque, gingiva samples, saliva, biopsy samples, and stool specimens from patients with which ordinary diagnostic techniques have been used (3, 11, 19), the IMS method could be an alternative or a complement for the detection of *H. pylori*. Detection of *H. pylori* from human feces has been performed by isolation on selective media (24), direct PCR of a feces-water mixture (16), and PCR after treatment with detergent and silica particles to remove contaminants (25). Detection of *Helicobacter mustelae* from feces of ferrets has been performed by isolation on selective medium (12). IMS is an alternative to the often-used DNA extraction techniques; providing that the beads are coated with antibodies specific to the target bacteria, IMS may be a useful tool for future transmission and epidemiological studies of different microorganisms, including *H. pylori*. Direct lysis of *H. pylori* by using sputum lysis buffer or boiling water without the use of IMS as a pre-PCR step gave lower detection rates by PCR (data not shown). Our IMS method is easy to perform, because no DNA extraction is needed, and it is a rapid method: all steps in the method can be performed within 6 h.

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