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Detection of *Helicobacter pylori* in Fecal Samples of Gnotobiotic Mice Infected with *H. pylori* by an Immunomagnetic-Bead Separation Technique

TAKAKO OSAKI,[1]* HARUHIKO TAGUCHI,[2] HIROYUKI YAMAGUCHI,[2] AND SHIGERU KAMIYA[2]

Division of Flow Cytometry[1] and Department of Microbiology,[2] Kyorin University School of Medicine, Mitaka, Japan

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By an immunomagnetic-bead (IMB) separation technique, isolation of *Helicobacter pylori* from gastrointestinal and fecal samples of gnotobiotic mice infected with the microorganism was tried. The isolation rate of *H. pylori* from stomach samples after IMB separation was not higher than that of direct culture of the samples. After IMB separation of feces, *H. pylori* was detectable by PCR, although *H. pylori* was not culturable.

Helicobacter pylori, the etiologic agent of gastritis and peptic ulceration, may infect the gastric mucosae of over half the world's population (7, 21). While persistent infection with *H. pylori* in gastric mucosae has been reported worldwide, the mode of transmission of *H. pylori* is not clearly understood and the environmental reservoir is not known. Two hypotheses, oral-oral and fecal-oral transmission, have been reported (5, 6, 8–11, 14, 18). The culture of *H. pylori* from feces is difficult, and the detection of *H. pylori* in feces by PCR is rare due to the presence of inhibitory substances (15, 19). Few investigators have reported the successful culture of *H. pylori* from feces (13, 20).

The immunomagnetic-bead (IMB) separation technique was reported to be useful for detecting pathogens from water, food, feces, and clinical materials (3, 4). Magnetic beads coated with antibodies against bacteria are used to separate and concentrate microorganisms in a sample. We have used the IMB separation technique and tried to isolate *H. pylori* from intestinal samples and feces of gnotobiotic mice infected with this organism.

H. pylori TK1029 and TK1402 were used as described before (22). Both strains were isolated from gastric biopsy specimens of gastric ulcer patients. *H. pylori* strains were cultured under microaerophilic conditions at 37°C on brain heart infusion agar containing 7% horse blood (BHI-blood agar) for 3 or 4 days (16). The coccoidal form of *H. pylori* TK1029 was produced by 6-month culture in sterile distilled water at 4°C after microaerophilic cultivation. *H. pylori* TK1402 (2.5×10^8 to 10.0×10^8 CFU) was daily inoculated into 10 germfree mice (IQI/Jic, 8 weeks old, female) per os for 3 days. Gnotobiotic mice were sacrificed 1 and 4 weeks after inoculation. Gastric mucus layers (100 mg), upper bowels (100 mg), cecal contents (200 mg), and feces (100 mg) were suspended in 400 μl of Hanks' balanced salt solution (HBSS). Each sample was mixed and diluted with HBSS and cultured on BHI-blood agar at 37°C under microaerophilic conditions. The remainder (350 μl) was resuspended in HBSS to a volume of 2 ml for IMB separation.

H. pylori-containing feces were also prepared for IMB separation by using feces of uninoculated GF mice. Feces (200 mg) from GF mice were suspended in 1.6 ml of HBSS and mixed with either the helical or the coccoid form of *H. pylori* suspended in 200 μl of HBSS (approximately 10^5 to 10^7 CFU/g of feces).

IMB separation was carried out by the method of Enroth and Engstrand (4) with some modification. Rabbit hyperimmune antiserum was produced by immunization with formalized *H. pylori* NCTC11638 and purified by using a packed column with GammaBind G-Agarose (Genex Co., Gaithersburg, Md.). Magnetic Dynabeads (1.4×10^8 beads [M-280 precoated with sheep anti-rabbit immunoglobulin G; Dynal A.S., Oslo, Norway] per ml of HBSS with 1% gelatin [HGS]) were preincubated and agitated with 200 μg of purified polyclonal rabbit anti-*H. pylori* NCTC11638 immunoglobulin G at 4°C overnight. After being washed with HGS, the beads were resuspended in 1 ml of HGS. Each sample was prepared by the method described above. After mixing, a sample was put through a nylon mesh (pore size, 40 μm; Becton Dickinson Labware, Franklin Lakes, N.J.) (approximately 1.5 ml of sample was recovered) and incubated with the beads (approximately 1.4×10^7 beads in 100 μl of HGS) for 20 min at 37°C with gentle shaking. After incubation, the beads were collected with a magnet and washed twice with HBSS. The collected beads were resuspended in HBSS. Half of the suspension was cultured on BHI-blood agar under microaerophilic conditions at 37°C, and the other half was stored at −20°C until it was used for PCR.

Collected beads were incubated with a solution containing lysozyme (3 mg/ml), 1% sodium dodecyl sulfate, and RNase A (50 μg/ml). DNA was extracted once with phenol-chloroform and precipitated with isopropanol. DNA extract was resuspended in 10 μl of 10 mM Tris-HCl (pH 7.4)–1 mM EDTA. PCR was performed with 1 μl of sample DNA and 1 μl of primers that were based on the sequence of the UreA-encoding gene (2R, 5′-ATGGAAGTGTGAGCCGATTTG-3′; 2F2, 5′-ATATTATGGAAGAAGCGAGAGC-3′; or 2F3, 5′-CATGAAGTGGGTATTGAAGC-3′) (12) in 50 μl of reaction mixture containing Premix ex*Taq* (Takara Shuzo Co. Ltd., Otsu, Japan). Thirty-five cycles of amplification were performed with a DNA thermal cycler 480 (Perkin-Elmer, Norwalk, Conn.). Each cycle included a 1-min denaturation step at 96°C, a 1-min annealing step at 57°C, and a 1.5-min extension step at 72°C. A sample which was negative by the first PCR was amplified again with the products of the first PCR by the method described above by using primers 2F3 and 2R (second PCR). PCR products (314 bp [first PCR] and 204 bp [second PCR]) were analyzed by agarose gel electrophoresis with molecular weight markers.

* Corresponding author. Mailing address: Department of Microbiology, Kyorin University School of Medicine, 6-20-2, Shinkawa, Mitaka 181, Japan. Phone: 81-422-47-5511, ext. 3464. Fax: 81-422-44-7325. E-mail: osaki@kyorin-u.ac.jp.

TABLE 1. Detection of *H. pylori* from *H. pylori*-containing feces of germfree mice by the IMB separation technique followed by culture and PCR

No. (CFU) of *H. pylori* in feces	Test result for:					
	Helical form			Coccoidal form[a]		
	No. (CFU) of *H. pylori* cultured	First PCR	Second PCR	Culture of *H. pylori*	First PCR	Second PCR
2.0×10^6	TNTC[b]	+	ND[c]	−	+	ND
2.0×10^5	110	−	+	ND	ND	ND
2.0×10^4	20	−	+	−	−	+

[a] The coccoidal form of *H. pylori* TK1029 was produced by 6-month culture in sterile distilled water at 4°C after microaerophilic cultivation.
[b] TNTC, too numerous to count.
[c] ND, not determined.

Recovery of *H. pylori* from *H. pylori*-containing feces of germfree mice after IMB separation was performed by microaerophilic cultivation with and without PCR amplification. After IMB separation of a helical-*H. pylori* (2×10^4 CFU)-containing fecal sample, 20 CFU of *H. pylori* was recovered by cultivation. The microorganisms were detectable by the second PCR but not by the first PCR (Table 1). To detect the coccoid form of *H. pylori*, IMB separation was followed by PCR. *H. pylori* organisms were detectable in feces containing 2×10^4 CFU of unculturable *H. pylori* by the second PCR (Table 1). We were able to detect *H. pylori* from the feces of germfree mice containing 5.0×10^1 CFU of *H. pylori* by a combination of the IMB separation technique and PCR but not from feces containing 5 CFU of *H. pylori.*

Detection of *H. pylori* from the gastrointestinal tracts and feces of gnotobiotic mice monoassociated with *H. pylori* was tried by IMB separation (Table 2). Although *H. pylori* was isolated from a stomach sample by the IMB separation technique at 1 week after inoculation, its cultivation was unsuccessful at 4 weeks after the inoculation (Table 2). Colonization of *H. pylori* in gastric mucus was observed 5 weeks after infection, and more than 10^4 CFU of *H. pylori* per g of mucus was detected in all gnotobiotic mice by direct culture of their gastric mucosal layers (data not shown). All the data from the mice at 1 week and 4 weeks after inoculation were evaluated by statistical analysis. The sensitivities of IMB separation combined with culture, the first PCR, and the second PCR were 60% (6 of 10 samples), 20% (2 of 10 samples), and 90% (9 of 10 samples), respectively. Positive predictive values were all 100%. Specificity and negative predictive values could not be calculated, as all the stomach samples were positive by direct culture. Furthermore, *H. pylori* was recovered by a combination of IMB separation with culture and PCR (Table 2) 1 week or 4 weeks after inoculation from the stomachs and upper bowels of gnotobiotic mice. From fecal samples, *H. pylori* was detectable by the combination of IMB separation and PCR, although *H. pylori* was not culturable.

TABLE 2. Isolation of *H. pylori* by IMB separation followed by culture and PCR from the gastrointestinal tracts and feces of gnotobiotic mice monoassociated with *H. pylori*

Wk after inoculation	Sample type examined	No. of positive samples/total no. of samples tested by:		
		Culture of *H. pylori*	First PCR	First and second PCRs
1	Stomach	4/5	2/5	5/5
	Upper bowel	0/5	1/5	2/5
	Feces	0/7	2/7	4/7
4	Stomach	2/5	0/5	4/5
	Upper bowel	0/5	0/5	3/5
	Cecal contents	0/5	1/5	2/5
	Feces	0/5	0/5	4/5

This study has indicated that the IMB separation procedure is useful for the detection of *H. pylori* from intestinal and fecal samples. However, the isolation rate of *H. pylori* from stomach samples after IMB separation (4 of 5 and 2 of 5 samples) was not as high as that by direct culture. In preliminary experiments, the isolation rate by direct culture was 100% at 1 week and 4 weeks after inoculation (data not shown). After IMB separation of the samples containing the viable helical form of *H. pylori*, the microorganisms were culturable. In addition, after a combination of PCR and IMB separation of nonculturable *H. pylori*-containing feces, *H. pylori* was detectable.

Direct cultivation of *H. pylori* from the stomachs of gnotobiotic mice is possible. However, the culture of *H. pylori* in samples from conventional animals was difficult due to the presence of other bacteria. From this aspect, IMB separation has an advantage in that other concomitant bacteria could be removed from the specimens to be examined. It is possible that the IMB separation technique is useful for monitoring *H. pylori* not only in germfree animals but also in conventional animals. In this study, a lower rate of detection was obtained by a combination of culture and IMB separation than by direct culture. Our results suggested that IMB separation does not recover *H. pylori* perfectly or that during IMB separation, the viability of *H. pylori* might be decreased.

A combination of PCR and IMB separation increased the rate of detection of *H. pylori* from *H. pylori*-containing HBSS. However, with feces containing 2.0×10^4 CFU of helical *H. pylori*, both cultivation and the second PCR were positive but the first PCR was negative in the recovery of the microorganism. It was implied that the IMB separation technique is inhibited artificially by substances in feces. In addition, DNA loss during extraction was also implied.

Recently, it was shown that complex polysaccharides present in feces act as PCR inhibitors (15). In our preliminary study, we could not detect *H. pylori*-homologous DNA by PCR amplification from the feces of gnotobiotic mice infected with the organism (data not shown). However, in the present study, it is likely that such PCR inhibitors in fecal samples were removed or decreased by the IMB separation technique, since the first and second PCRs of fecal samples of gnotobiotic mice monoassociated with *H. pylori* were positive.

It is well-known that an altered environment leads to the morphological change of *H. pylori*, from a helical to a coccoid form which is viable but nonculturable (1, 2, 17). Although the biological role of the viable but nonculturable coccoid form of *H. pylori* is unclear, it is thought to be a survival form in the human intestine and various environments (2, 17). Although our data indicate that nonculturable *H. pylori* organisms exist in the intestinal tract, their viability was not shown. In order to clarify the transmission route of *H. pylori*, further investigation of the viable but nonculturable form is needed.

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