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Absence of Mucosa-Associated Colonic *Helicobacters* in an Australian Urban Population

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Application of nested PCR for *Helicobacter* species to 416 samples obtained at colonoscopy from 15 patients with Crohn's disease, 12 with ulcerative colitis, and 43 controls revealed *H. pylori* DNA in only 6 individuals with no disease association. No other *Helicobacter* species were detected in ileal or colonic samples.

Whether *Helicobacter* species are present in the human colon or hepatobiliary system and participate in disease pathogenesis is controversial (1–3, 5–7, 15, 16, 23; R. K. Linskens, J. G. Kusters, X. W. Huijsdens, L. A. Dieleman, J. Stoof, M. M. Gerrits, C. M. Vandenbroucke-Grauls, S. G. Meuwissen, and E. J. Kuipers, abstr. 1771, Gastroenterology, p. A325, 2000). The hypothesis that mucus-adapted helicobacters might perpetuate colitis in humans with a genetic predisposition to inflammatory bowel disease (IBD) is attractive, given the colitis-inducing potential of several murine *Helicobacter* species in immunodeficient mice (4, 8–11, 20). *Helicobacter* 16S ribosomal DNA (rDNA) has been detected in biopsy specimens from normal and diseased human ileum and colon by PCR (2, 23; Linskens et al., abstract, 2000). In this study we sought to confirm the presence of *Helicobacter* colonization of the ileum and colon of IBD patients and controls by using a nested PCR.

Seventy patients undergoing colonoscopy ($n = 64$) or sigmoidoscopy ($n = 6$) at the Inner West Endoscopy Centre, Sydney, Australia, participated in a study approved by the Human Research Ethics Committee. Fifteen subjects met standard diagnostic criteria for Crohn's disease (CD) (7 males and 8 females; mean age, 40 years), 12 met standard criteria for ulcerative colitis (UC) (6 males and 6 females; mean age, 49 years), and 43 subjects who did not have IBD formed the control group (23 males and 20 females; mean age, 61 years). The ethnicity of the subjects was 25 Australian, 30 Southern European, 4 Southeast Asian and 11 "other." The indications for colonoscopy included previous colonic polyps (27%), change in bowel habit (24%), rectal bleeding (19%), and a family history of colonic carcinoma (11%). Review of clinical records revealed that four subjects were known to have gastric *Helicobacter pylori* infection at the time of examination, 40 were negative, and 26 were of unknown infection status. At colonoscopy, biopsy specimens from the ileum, cecum (in duplicate), transverse colon, descending colon, and rectum were collected into separate sterile tubes. At sigmoidoscopy, biopsy specimens were collected from the sigmoid (in duplicate) and

rectum. An aspirate of luminal fluid was collected when present.

To determine if 16S rDNA from the genus *Helicobacter* was present in any of the samples, a nested PCR was performed. Two positive controls were set up for each subject to account for fecal inhibition of PCR (12, 13). One of the duplicate biopsy specimens and a 100- μ l aliquot of luminal fluid were spiked with approximately 10^2 and 10^3 CFU of *H. muridarum* (ATCC 49282), respectively. DNA was extracted from all samples by using the Puregene kit (Gentra Systems, Minneapolis, Minn.) and amplified in the first-round hot-start PCR with the bacterial primers F27 (25) and R1494 (14) on a Sprint thermal cycler (Hybaid, Ashford, Middlesex, United Kingdom). Cycling consisted of 94°C for 4 min, 30 cycles of 94°C for 15 s, 50°C for 20 s, and 72°C for 2 min, and finally 72°C for 7 min. A 1:25 dilution of the first-round product (including the negative controls) was amplified in the second round with the *Helicobacter* genus-specific primers H276f and H676r (19). Cycling consisted of 94°C for 4 min, 35 cycles of 94°C for 5 s, 57°C for 5 s, and 72°C for 30 s, and finally 72°C for 2 min. The presence of the PCR product was assessed by agarose gel electrophoresis, and all positive samples were sequenced.

A total of 360 biopsy specimens (mean, 5.1 per subject; range, 0 to 6) and 56 luminal fluid aspirates were collected from the 70 subjects. *Helicobacter* DNA was detected in nine samples obtained from 6 of the 70 subjects: 2 controls, 2 with UC, and 2 with CD (Table 1). For three of the patients with positive samples, two samples were positive (two biopsy specimens for two patients and a biopsy specimen and a luminal fluid aspirate for the other), and for the remaining three, only one sample was positive (a biopsy specimen for two patients and a luminal fluid aspirate for the other). Three of the subjects with positive samples had known gastric *H. pylori* infection, two were of unknown status, and one was negative. This negative individual had undergone a previous Bilroth II partial gastrectomy and had documented *H. pylori* infection in 1995, with subsequent eradication. The other 338 samples were negative. In 68 of 70 subjects, the biopsy specimen and luminal fluid aliquot spiked with *H. muridarum* were positive. In the other two subjects, only the spiked luminal fluid sample was positive.

Sequencing of the PCR product from the nine positive samples revealed an identical 356-bp DNA sequence that com-

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TABLE 1. Results of nested PCR

Subject category	No. of subjects	Mean age (yr)	No. of subjects with <i>H. pylori</i> -positive samples			No. of subjects with all samples negative				
			Total	Gastric <i>H. pylori</i> infection status		Total	Gastric <i>H. pylori</i> infection status			
				Positive	Unknown		Negative	Positive	Unknown	Negative
Controls	43	61	2	1	0	1	41	1	10	30
UC patients	12	49	2	1	1	0	10	0	6	4
CD patients	15	40	2	1	1	0	13	0	7	6

pletely matched the 16S rDNA of *H. pylori*. This sequence differed from the corresponding sequences of *H. cinaedi* (AF348745), *H. fennelliae* (AF348746), *H. heilmannii* (AF058768), *H. bilis* (AF047843), and *H. hepaticus* (AF302103) by 2.5, 2.5, 3.6, 3.9, and 4.2%, respectively. There was no significant difference in the proportion of subjects with IBD who had detectable *Helicobacter* DNA in ileal or colonic samples compared with controls (χ^2 test, $P = 0.32$).

Samples from 8.6% of the subjects in this study contained *Helicobacter* 16S rDNA compared with those from 33 to 60% of subjects in previously reported studies (2, 23; Linskens et al., abstract, 2000). In all of the positive samples, the resulting sequence was identical to *H. pylori* 16S rDNA. Although these sequence data are insufficient to state with certainty that wash-down of gastric *H. pylori* was the source of this DNA, this conclusion would appear highly likely (21, 24). The use of spiked control samples for each individual means that the presence of *Helicobacter* species, as defined by current primer sequences, in numbers greater than 10^2 per biopsy specimen and 10^4 per ml of luminal aspirate can be excluded. Previous studies have shown that a substantial layer of colonic mucus remains after bowel preparation, so that loss of mucus is unlikely to explain the negative results (18, 22). Therefore, there does not appear to be a significant population of *Helicobacter* species colonizing the colonic mucus of urban Australian IBD patients or controls.

This observation is consistent with that of Bohr et al. (2) but contrasts with the results of Linskens et al. (abstract, 2000) and Tiveljung et al. (23), who detected *Helicobacter* DNA in ileal or colonic tissue from a large proportion of IBD patients and controls. The specificity of the assay performed by Tiveljung et al. must be questioned because other pathogenic bacteria were also detected in a large proportion of CD patients and controls. The study by Linskens et al. is reported as an abstract only.

In PCR studies, problems with reaction specificity and contamination of nested assays can mislead clinicians and stimulate further research of limited value (17). To make these studies more robust, we recommend that PCR studies reporting negative results include spiked controls from each patient and that the results of positive studies be confirmed by culture or fluorescent in situ hybridization.

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