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Detection of Species-Specific *Helicobacter* Ribosomal DNA in Intestinal Biopsy Samples from a Population-Based Cohort of Patients with Ulcerative Colitis

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The inflammatory bowel diseases are considered an abnormal host immune response to an environmental stimulus. Evidence suggests a role for intestinal bacteria in initiating and/or providing an ongoing stimulus for inflammation in inflammatory bowel disease. *Helicobacter pylori* is the major cause of active chronic gastritis and peptic ulcers in humans and has been linked to gastric carcinoma and lymphoma. Studies in various animal models, particularly mice, have identified enterohepatic *Helicobacter* species that are capable of causing hepatitis and enterocolitis. We hypothesize that *Helicobacter* species may have a role in maintaining inflammation in humans with inflammatory bowel disease. In order to investigate this, biopsy specimens were obtained from patients with and without inflammatory bowel disease. DNA was extracted from the tissues and subjected to PCR with primers designed to detect the ribosomal DNA of members of the *Helicobacter* species. DNA from six biopsy samples from 60 inflammatory bowel disease patients tested positive. This included 5 of 33 ulcerative colitis patients that were positive compared to 0 of 29 age-matched controls ($P < 0.04$). Sequencing of the bands produced by PCR amplification revealed ≥99% homology with *H. pylori*. These results indicate that a member of the *Helicobacter* species may be involved in some cases of ulcerative colitis.

Inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are chronic diseases characterized by exacerbations and remissions of mucosal inflammation and ulceration. These diseases cause significant morbidity and are associated with an increased risk of colon cancer (4, 17). The cause(s) of inflammatory bowel disease is not known. The current hypothesis is that genetically susceptible individuals develop an abnormal immune response to an environmental stimulus or agent (5, 19, 25). The factor(s) that initiates the inflammation or contributes to exacerbations is not known. A number of clinical observations suggest that bacteria or gut flora play a role in these diseases; however, the data linking specific infectious agents remains inconclusive (8, 11, 15, 20, 21, 25). On the other hand, studies in rodent models of inflammatory bowel disease have suggested that elements of the normal gut flora may be involved in the pathogenesis of the intestinal inflammation (10, 26).

Gastric infection by *Helicobacter pylori* is a well-recognized cause of chronic active gastritis and gastric and duodenal ulcers and is linked to the development of gastric malignancies (14). Studies in mice have suggested that persistent intestinal infection by related organisms such as *Helicobacter hepaticus* and *Helicobacter bilis* is associated with the development of chronic inflammation and enterocolitis (6, 7, 12, 13, 18). These findings raise the possibility that intestinal infection by *Helicobacter* species may be involved in the pathogenesis of the intestinal inflammation in patients with inflammatory bowel disease. To date, no evidence is available that confirms a relationship between *Helicobacter* species and the development of human inflammatory bowel disease. In this report, primers which amplify *Helicobacter* species-specific sequences of ribosomal DNA were used for PCR analysis to search for the presence of *Helicobacter* DNA in endoscopically obtained biopsy tissue.

MATERIALS AND METHODS

Colonic biopsy samples were obtained from patients enrolled in a population-based case-control study of inflammatory bowel disease. In developing an administrative definition of inflammatory bowel disease, subjects identified through the administrative database of Manitoba Health, the single health insurer for the province, were mailed questionnaires regarding their histories of inflammatory bowel disease and willingness to participate in future studies. Sixty percent of the subjects responded and mailed back the questionnaires (3). This group was used to develop the population-based University of Manitoba Inflammatory Bowel Disease Epidemiology Database (3). Those who returned questionnaires and thereby consented to be known to the researchers were logged in the University of Manitoba Inflammatory Bowel Disease Research Registry. At the beginning of the present study, there were 2,890 subjects in the research registry. Persons in this research registry who agreed to participate in future studies furnished a mailing address and a telephone number to facilitate contact. We accessed the registry in search of subjects under the age of 50 years and mailed them information sheets and questionnaires for a case-control study examining possible etiologic risk factors for inflammatory bowel disease.

A population-based set of controls was developed from the Manitoba Health (MH) population registry. The Manitoba Health population registry contains demographic information on all persons registered with the Manitoba Health public health insurance system. The registry is regularly updated with vital registrations and information from medical and hospital transactions and closely matches population estimates derived from the Canadian census (Statistics Can-

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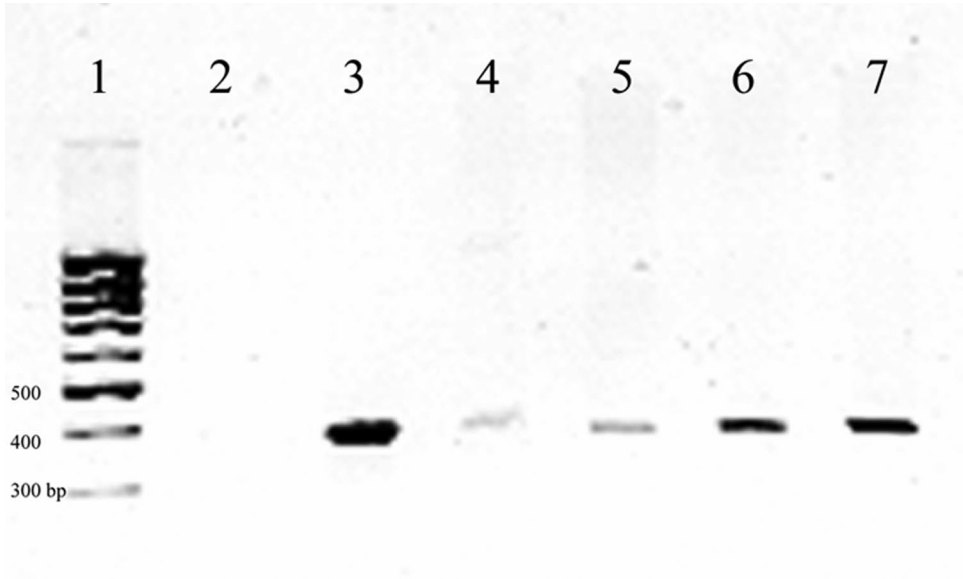

FIG. 1. Assessment of tissue extraction efficiency for the detection of *Helicobacter* rRNA gene sequences. DNA extracts from tissues treated with serially diluted *Helicobacter pylori* bacteria were amplified with pan-*Helicobacter* species PCR primers, followed by electrophoresis of the PCR product on an ethidium bromide-containing agarose gel. Lane 1, 1-kb DNA ladder; lane 2, negative control; lane 3, positive control; lane 4, DNA from tissue with 10 ± 10 bacteria present; lane 5, DNA from tissue with 100 ± 10 bacteria present; lane 6, DNA from tissue with 1,000 ± 100 bacteria present; lane 7, DNA from tissue with 10,000 ± 1,000 bacteria present.

ada) (22). A random sample of registered persons was selected with stratification for age (5-year intervals) and gender to achieve balance with the case series for those two variables. With the specified stratification, Manitoba Health's Information Services generated a mailing list of eligible controls and sent an information package prepared by the investigators explaining the study and requesting participation. The investigators did not know the identity of the controls unless they received a mailed response. For a second control group we asked patients with inflammatory bowel disease to refer us to one or more siblings.

All cases and controls completed a questionnaire and consented to provide a blood sample. Controls were invited to participate in colonoscopy plus biopsies, and those agreeing were paid an honorarium. Approximately 10% of controls who were enrolled in our study collecting questionnaire data and blood agreed to participate in the colonoscopy plus biopsy study. Cases were asked to contact the study personnel when they were to undergo their next colonoscopy for clinical reasons. All patients who were to undergo colonoscopies consented to providing extra biopsy tissue for study purposes.

Each of the cases and controls provided eight biopsy samples from the cecum and eight biopsy samples from the rectum at colonoscopy. In subjects with a previous cecal resection, biopsy samples were obtained from the right colon distal to the ileocolonic anastomosis. All biopsy samples were snap frozen in liquid nitrogen and stored at −70°C. These studies were approved by the research ethics board of the University of Manitoba

Nucleic acid purification and amplification. Nucleic acids were extracted from tissue samples essentially as previously described (16), except that tissues were homogenized in only 1 ml of lysis buffer in 10-ml polypropylene snap cap tubes. The tissue lysis buffer that was used in this study is a modification (16) of the commonly used Chomczynski lysis buffer (9). Specifically, 0.2 M sodium acetate was used at pH 7.0 instead of at pH 4.0. DNA was quantified by spectrophotometry at 260 nm.

The *Helicobacter* genus-specific primers 5′TATGACGGGTATCC GGC-3′ and 5′-ATTCCACCTACCTCCTCCCA-3′ were designed to amplify a 375-bp sequence within an area of the 16S rRNA gene conserved among members of the *Helicobacter* genus (2). An NCBI BLAST analysis confirmed that these primers identify the target sequence within the genome of known *Helicobacter* species including *H. pylori*, *H. felis*, *H. muridarum*, *H. bilis*, and *H. hepaticus* strains. PCR primers were constructed at the Institute for Molecular Biology and Medicine (Mobix Laboratory, McMaster University, Hamilton).

PCRs were prepared with 2.8 μM each primer, 3 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 5 μl of Amplitaq Gold Buffer (Applied Biosystems), 1.25 μg of template DNA, and 1.6 U of *Taq* polymerase (Applied Biosystems) plus distilled water to a total volume of 50 μl. PCR mixtures were heated to 94°C for 10 min, followed by 45 cycles of denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s, and extension at 72°C for 45 s, followed by 10 min at 72°C in a PTC 200 DNA Engine (MJ Research, Waltham, Mass.). PCR products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide, and the size of the product was confirmed by using DNA molecular size standards.

The specificity of the PCR primers designed for these studies was evaluated. The primer set amplified DNA extracted from cultured *H. pylori*, *H. hepaticus*, and *H. felis* (data not shown). No bands were observed with these *Helicobacter* PCR primers with DNA extracts from *Escherichia coli* or *Campylobacter jejuni.* PCR analysis detected bands in mouse gastric tissue known to be infected with *H. pylori* (data not shown). To confirm that the method of extracting DNA allowed identification of bacteria adherent to the mucosa, human gastric tissue with histologically identifiable *H. pylori* infection was obtained; DNA extracted from this tissue had a strong positive band for *Helicobacter* DNA (data not shown).

To assess the sensitivity of the PCR for the detection of *H. pylori* DNA, serially diluted samples of *H. pylori* DNA were subjected to the PCR. Strong bands were detected in samples diluted down to 0.000125 μg of *H. pylori* DNA (data not shown)

To ensure that the DNA extraction method would allow extraction of bacterial DNA from tissue, tissue known to be negative for *Helicobacter* was combined with cultured *H. pylori* bacteria in decreasing amounts from 10^8 to 10^0 bacteria ($\pm 10^1$ bacteria in each sample). After DNA extraction, PCR analysis of the DNA from the bacteria-spiked tissues detected *H. pylori* DNA with bacterial concentrations of between 10 and 100 bacteria per sample (Fig. 1).

Controls were included in each set of PCR amplifications of patient sample DNA. A negative control lacking DNA template was used as well as a positive control containing DNA extracted from cultured *H. pylori* Sydney strain. In addition, each specimen was run with and without a DNA spike consisting of the addition of 1 μg of *H. pylori* DNA to the PCR mixture to exclude the presence of PCR inhibitors. *H. pylori* DNA was used for this in preference to human gene internal controls as some inhibitors may be specific for *Helicobacter* DNA. PCR products from tissue specimens that produced a positive band were sequenced by a fluorescence-based DNA sequencing method (Mobix Laboratory, McMaster University, Hamilton). The sequences were compared to that of known members of the *Helicobacter* genus with the Blast program at the National Center for Biotechnology Information.

Fisher's exact test was used to compare proportions of positive results between cases and controls. Student's *t* test was used to compare clinical information between groups.

Nucleotide sequence accession numbers. Sequences M28, M36, M37, and M38

TABLE 1. Clinical information for subjects undergoing biopsy

Group (*n*)	Mean age (yr) ± SD	Sex (M:F)[a]	Mean duration of disease (yr) ± SD
Crohn's disease (25)	38.8 ± 10.9	14:11	10.2 ± 5.9
Ulcerative colitis (33)	43.3 ± 13.6	18:13	14.4 ± 9.0
Indeterminate colitis (2)	31.5 ± 14.0	0:2	7.0 ± 7.1
Controls (29)	42.0 ± 8.1	14:15	

[a] M, male; F, female.

were submitted to GenBank and assigned accession numbers AY426557, AY426558, AY426559, and AY426560, respectively.

RESULTS

Biopsy specimens were obtained from 29 age-matched control patients and 60 patients with inflammatory bowel disease, of which 33 had ulcerative colitis, 25 had Crohn's disease, and 2 had indeterminate colitis. The mean age of the ulcerative colitis patients was 43 years, that of the Crohn's disease patients was 38.8 years, and that of the indeterminate colitis patients was 31.5 years, compared to a mean age of 42.0 years for the controls (Table 1). Information on all previous antibiotic treatment was not available for these patients, although no subjects had used antibiotics within 1 week of tissue collection.

None of the cases or control specimens contained inhibitors of the PCR, as evaluated by the presence of a band on the spiked sample. The 29 control subjects were all negative for *Helicobacter* DNA. Within the inflammatory bowel disease group, 60 patients were tested. On PCR amplification, the DNA extracted from six patients produced bands of the size appropriate for extracted species (Table 2, Fig. 2). Five of these positive results were from subjects with ulcerative colitis and one was from a patient with active Crohn's disease. The PCR amplification product from each of these patients was sequenced. Four (M28, M36, M37, and M38) of the six sequences showed >95% identity compared to the corresponding 16S rRNA gene sequence of the *H. pylori* type strain ATCC 43504 and the sequenced strains, J99 and 26695. The partial 16S rRNA gene fragments M28 (345 bases), M36 (342 bases), M37 (339 bases), and M38 (342 bases) all showed one base change at nucleotide 296 (A to G) compared to that of strains ATCC 43504, J99, and 26695.

When compared to the 16S rRNA gene sequence of *H. hepaticus* ATCC 51449 (sequenced strain) and *H. bilis* (accession number U51873), these four sequences all showed ≤95% identity. The remaining two PCR sequences also showed the highest match (85% identity) to *H. pylori* 16S rRNA gene sequences, but with the limited DNA obtained from the biopsy samples, we were unable to obtain a good clean sequence for submission to GenBank.

The proportion of ulcerative colitis patients showing *Helicobacter* sp. DNA was greater than the proportion of controls ($P < 0.04$ by Fisher's exact test). The patients who tested positive were significantly older than the patients who were negative for *Helicobacter* DNA (mean age, 52 years, versus 39.8 years for the negative ulcerative colitis patients; $P = 0.024$) and had a significantly longer duration of disease (mean, 19.7 years, versus a mean of 11.8 for *Helicobacter*-negative patients; $P = 0.025$). Patients with ulcerative colitis who were positive for *Helicobacter* DNA also tended to have inactive disease (four of six positive patients), although this was not statistically significant.

[^{13}C]urea breath testing and serology for *Helicobacter* were not performed at the time the biopsy samples were taken: after these results were obtained, the patients who tested positive for *Helicobacter* DNA were contacted and requested to undergo further testing. Five of the six patients (all of the ulcerative colitis patients) agreed to urea breath testing, which was performed approximately 1 year postendoscopy. Only one of these patients tested positive, suggesting active gastric *H. pylori* infection.

DISCUSSION

H. pylori is well recognized as the major cause of chronic active gastritis, gastric and duodenal ulcers, and gastric malignancies. Other members of the *Helicobacter* family have been identified in various species, where they reside throughout the gastrointestinal tract. Some of these have been termed the enterohepatic helicobacters for their abilities to persistently colonize and cause inflammation within the liver, small intestine, and colon. In particular, *H. hepaticus* and *H. bilis* have been linked to chronic colitis in a variety of mouse models (6, 7, 24, 28). These observations suggest a possible link to human inflammatory bowel disease.

One previous study searched for microbial agents such as *Helicobacter* in biopsy samples taken from normal or inflamed mucosa of 11 patients with Crohn's disease. Of the five biopsy samples from inflamed mucosa, three contained numerous bacteria, including *Helicobacter*, as assessed by broad-range and genus-specific hybridization analysis of the PCR products obtained by amplification of fragments of the 16S rRNA gene (27). However, species-specific primers were unsuccessful at identifying which members of the extracted genus were present. The authors suggested that, considering the mixture of bacterial species present in inflamed tissue, it was unlikely that *Helicobacter* as a single agent would be responsible for Crohn's disease in these patients. However, their failure to confirm the presence of *Helicobacter* extracted by species-specific primers raises questions about the specificity of the original genus-

TABLE 2. Clinical information for patients with and without PCR evidence of *Helicobacter* DNA

Patient group	No. positive for *Helicobacter*	Diagnosis[a] (*n*)	Mean duration of disease (yr) ± SD	Mean age (yr) ± SD	Sex (M:F)[b]	Disease activity (no. noted)
PCR positive	6	UC (5), CD (1)	19.7 ± 9.8	52 ± 9.6	2:4	Active (2)
PCR negative	54	UC (28), CD (24), I (2)	11.8 ± 7.7	39.8 ± 12.5	30:24	Active (12)

[a] UC, ulcerative colitis; CD, Crohn's disease; I, indeterminate colitis.
[b] M, male; F, female.

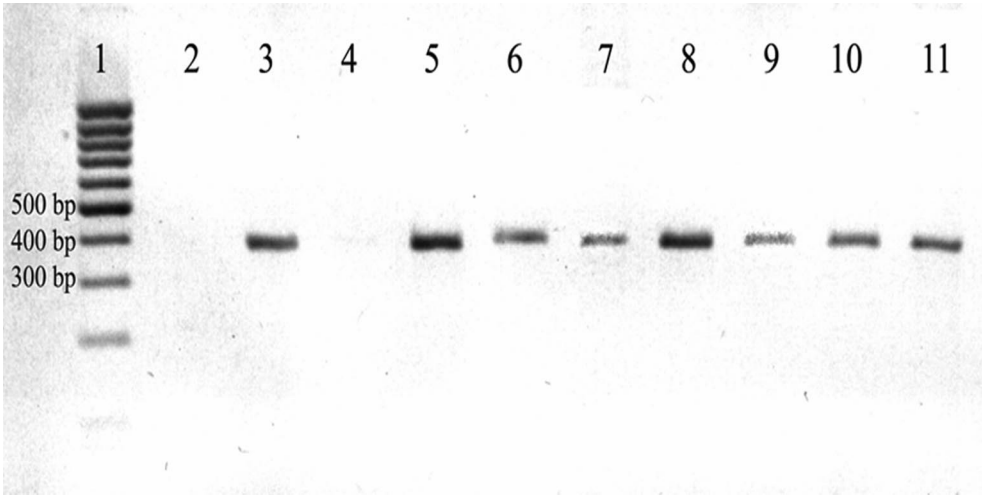

FIG. 2. Assessment of DNA extracted from endoscopic biopsy samples taken during evaluation of the intestines of patients with and without inflammatory bowel disease: PCR products obtained from amplification of DNA with pan-*Helicobacter* species PCR primers. Lanes: 1, 1-kb DNA ladder; 2, negative control; 3, positive control; 4, ulcerative colitis subject negative for extracted DNA; 5, ulcerative colitis subject from lane 4 with *H. pylori* DNA spike; 6, positive subject with ulcerative colitis; 7, positive subject with ulcerative colitis; 8, positive subject with active ulcerative colitis; 9, positive subject with active Crohn's disease; 10, positive subject with ulcerative colitis; 11, positive subject with ulcerative colitis.

specific primer set. It is possible that the primers were detecting a closely related bacterium such as *Campylobacter*. It should be noted that none of the patients included in this study had ulcerative colitis.

We examined biopsy samples from a more extensive population-based cohort of patients with both active and inactive inflammatory bowel disease. For this study, we used a PCR primer set designed to amplify an area of the 16S rRNA gene common to *Helicobacter* species identified on Blast search. We defined precisely the specificity and sensitivity of this primer set to ensure that the PCR amplification method would detect only *Helicobacter* species and that DNA from as few as 10 helicobacters in 100 μg of tissue was detectible. We also tested each sample for inhibitors that might interfere with PCR amplification, to ensure that each negative was truly devoid of *Helicobacter* DNA.

The lack of *Helicobacter* sp. DNA in the control patient groups is of interest. Considering the age of the control population, a proportion (approximately 20%) of these patients might be expected to have gastric infection with *H. pylori* (23), which might have been detectable by PCR testing of feces. However, patients undergoing colonoscopy have a bowel cleansing preparation prior to endoscopy, and this would minimize fecal material in the biopsy samples taken for DNA isolation. It may be that this would prevent detection of any *Helicobacter* species not adherent to the mucosa.

Our studies identified six patients who had *Helicobacter* DNA present in their colonic biopsy samples. Five of these had ulcerative colitis and one had Crohn's disease. Statistically, there was a significant difference between the ulcerative colitis group and controls ($P < 0.04$). Though the sequencing of the amplified fragments from the inflammatory bowel disease patients showed homology with *H. pylori*, this does not rule out the possibility of the presence of a member of the *Helicobacter* family with significant sequence homology to *H. pylori*. Gastric *H. pylori* status for all subjects at the time of colonoscopy was not known. However, only one of the ulcerative colitis patients whose tissue contained *Helicobacter* DNA had a positive urea breath test approximately 1 year postendoscopy, suggesting that the presence of gastric *Helicobacter pylori* was not a significant factor in these patients.

In conclusion, *H. pylori*-like DNA sequences were identified in biopsy samples from 5 of 33 patients with ulcerative colitis and in 1 of 25 with Crohn's disease, while all 29 age-matched controls were negative. These patients with positive findings had a longer history of disease and tended to have inactive disease. Though sequencing of the PCR product indicated the DNA detected showed homology to *H. pylori*, it remains entirely possible that this organism is distinct from gastric *H. pylori*. While the DNA in these tissues may be due to bacteria or bacterial DNA in transit from the stomach, these results raise the possibility that a proportion of inflammatory bowel disease patients, particularly those with ulcerative colitis, have transient infection or colonization of the lower gastrointestinal tract with a *Helicobacter* sp. which may contribute to the persistence of colonic inflammation.

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