JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1995, p. 28–32
0095-1137/95/$04.00+0
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Vol. 33, No. 1

Detection of *Helicobacter pylori* Gene Expression in Human Gastric Mucosa

RICHARD M. PEEK, JR.,[1]* GERALDINE G. MILLER,[2] KYI T. THAM,[3] GUILLERMO I. PÉREZ-PÉREZ,[2] TIMOTHY L. COVER,[2,3] JOHN C. ATHERTON,[2] G. DEWEY DUNN,[1,3] AND MARTIN J. BLASER[2,3]

Division of Gastroenterology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2279[1]; Division of Infectious Diseases, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2605[2]; and U.S. Department of Veterans Affairs Medical Center, Nashville, Tennessee 37212[3]

Received 8 August 1994/Accepted 7 October 1994

Mucosal and systemic immunologic recognition of *cagA* by *Helicobacter pylori*-infected individuals is associated with peptic ulcer disease; however, in the laboratory, expression of *cagA* is subject to artificial conditions which may not accurately reflect the conditions in host tissues. Gastric antral and body biopsy specimens and serum for anti-*H. pylori* immunoglobulin G serology were obtained from 42 patients. Biopsy specimens were studied by histology, culture, and reverse transcription PCR (RT-PCR). Oligonucleotide primers specific for *H. pylori* (16S rRNA, *ureA*, and *cagA*) were used to detect bacterial mRNA in gastric biopsy specimens. PCR was performed on DNA from corresponding *H. pylori* isolates to detect genomic 16S rRNA, *ureA*, and *cagA*. Of the 42 patients from whom clinical specimens were obtained, 25 were infected with *H. pylori* on the basis of both serology and histology or culture (i.e., tissue positive); 13 were negative by serology, histology, and culture; and 4 were positive by serology only. RT-PCR with 16S rRNA primers detected 24 of 25 tissue-positive and 0 of 17 tissue-negative patients ($P < 0.001$). RT-PCR with *ureA* primers detected 16 of 25 tissue-positive and 0 of 17 tissue-negative patients ($P < 0.001$). *CagA* mRNA was detected by RT-PCR in 14 of 25 gastric biopsy specimens in the tissue-positive group and in 0 of 17 gastric biopsy specimens in the tissue-negative group. PCR of genomic DNA for the presence of the *cagA* gene in the corresponding bacterial isolates correlated absolutely with *cagA* gene expression in gastric tissue. These results indicate that RT-PCR is a sensitive and specific method for the detection of the presence of *H. pylori* and the expression of *H. pylori* genes in human gastric tissue. Detection of *H. pylori* gene expression in vivo by this approach may contribute to improving the diagnosis and understanding the pathogenesis of *H. pylori* infections.

Helicobacter pylori is now recognized as the major etiologic agent of chronic gastritis, and infection with *H. pylori* plays an important role in the pathogenesis of peptic ulceration and is a risk factor for the development of adenocarcinoma of the distal stomach (8, 10). Although essentially all infected persons develop gastritis, the clinical sequelae of *H. pylori* infection are recognized in only a minority of persons (14).

H. pylori isolates demonstrate a high level of genotypic diversity, but nearly all phenotypic characteristics of the organism are well-conserved (1, 16). Currently, the phenotypic characteristics known to differ among strains include the production of a vacuolating cytotoxin and the presence of a high-molecular-weight protein encoded by *cagA* (9, 12, 18, 21). About 60% of *H. pylori* strains possess *cagA*, and this is the first *H. pylori* gene found to be nonconserved among strains (2, 21). The presence of antibodies to the *cagA* protein is found in the sera of 100% of *H. pylori*-infected patients who have peptic ulcer disease but only 60 to 62% of patients who have gastritis only (9, 12, 13). Thus, the presence of *cagA* in a strain is associated with the tendency for *H. pylori* infection to cause peptic ulceration and is statistically and clinically significant, and methods to determine the *cagA* status of *H. pylori* isolates in pure culture or in biopsy specimens are potentially important.

The identification of most bacterial virulence factors in the past has been dependent on the ability to reproduce host environmental conditions in the laboratory. However, expression of these virulence factors in vitro may not accurately reflect the expression occurring in host tissues (19). In vivo detection of *H. pylori cagA* expression may improve understanding of the mechanisms by which *H. pylori* causes disease. For these reasons, we sought to develop a sensitive method for detecting *H. pylori* in gastric mucosa and to distinguish among strains on the basis of the presence of *cagA*. Reverse transcription PCR (RT-PCR) on gastric biopsy specimens with primers specific for *H. pylori* 16S rRNA has previously been used successfully for the detection of *H. pylori* in gastric biopsy specimens (15). We now report on the relative utility of RT-PCR with primers specific for 16S rRNA or urease (*ureA*) for detecting *H. pylori* infection. In addition, we describe the in vivo detection of *H. pylori cagA* expression by RT-PCR on gastric biopsy specimens and demonstrate that the in vivo expression of *cagA* correlates with the presence of *cagA* in genomic DNA from the corresponding *H. pylori* isolate. The sensitivity and specificity of RT-PCR should make it useful for the detection of *H. pylori* and aid in delineating bacterial factors that influence the clinical outcome of infection.

MATERIALS AND METHODS

Preparation of genomic DNA from reference strain and clinical isolates. *H. pylori* reference strain 60190 was grown in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 0.5% charcoal under microaerobic conditions at 37°C for 72 h as described previously (11). To determine the lower limit of detection of *H. pylori* by PCR, serial 10-fold dilutions of 1-ml aliquots of the culture were made in phosphate-buffered saline (PBS; pH 7.6). One-milliliter aliquots of the dilutions were centrifuged at 10,000 × *g* for 5 min,

* Corresponding author. Mailing address: Division of Infectious Diseases, Vanderbilt University, 1161 21st Avenue South, A-3310 Medical Center North, Nashville, TN 37232-2605. Phone: (615) 322-2035. Fax: (615) 343-6160.

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TABLE 1. Primers used for PCR

Gene (reference)	Nucleotide positions	Primer[a]	Expected product size (bp)
16S rRNA (15)	219–240	5′-GCTAAGAGATCAGCCTATGTCC-3′	522
	719–740	5′-TGGCAATCAGCGTCAGGTAATG-3′	
ureA (7)	304–321	5′-GCCAATGGTAAATTAGTT-3′	491
	697–714	5′-CTCCTTAATTGTTTTTAC-3′	
cagA (21)	1228–1249	5′ GATAACAGGCAAGCTTTTGAGG 3′	349
	1555–1576	5′ CTGCAAAAGATTGTTTGCGAGA 3′	

[a] For each pair, the 5′ sense primer is listed first.

the supernatant was discarded, and the bacterial pellets were stored at −70°C for subsequent DNA and RNA extraction. A parallel set of dilutions was cultured on 5% sheep blood agar plates, and the plates were incubated for 3 to 5 days under microaerobic conditions (Campy Pak envelope [BBL], in a GasPak jar [BBL] with catalyst) before *H. pylori* colonies were counted. Genomic DNA was prepared by boiling the frozen cell pellets in 0.5 ml of sterile distilled water at 95°C for 1 min. Samples then were centrifuged at 14,000 × *g* for 5 min, and the supernatants were stored in sterile vials (Nunc, Roskilde, Denmark) at −70°C until they were used as PCR templates.

Gastric biopsy specimens were placed immediately in normal saline at 4°C and were coarsely homogenized in 250 μl of normal saline with a tissue grinder (Micro Kontes, Vineland, N.J.). Fifty microliters was plated onto 5% sheep blood agar and was allowed to grow for 96 h under microaerobic conditions as described above. Genomic DNA from clinical isolates was prepared by vortex mixing a 10-μl loopful of colonies in 1 ml of PBS, centrifuging at 14,000 × *g* for 2 min, and boiling the pellet in 1 ml of distilled water for 1 min. The samples were then centrifuged at 12,000 × *g* for 4 min at 4°C, and the supernatants were stored in sterile vials (Nunc) at −70°C until they were used as PCR templates.

Preparation of cDNA from reference strains. RNA extraction from frozen pellets of *H. pylori* 60190 was performed with guanidinium thiocyanate-phenol chloroform in 500 μl of homogenization buffer (6). Total RNA was dissolved in 10 μl of diethyl pyrocarbonate-treated distilled water. cDNA was synthesized with 1 μg of random hexamers (Pharmacia LKB Biotech, Piscataway, N.J.) added to the total RNA, and the mixture was incubated at 70°C for 10 min and placed on ice for 2 min. The mixture was incubated with 5 μl of 5× RT reaction buffer (Bethesda Research Laboratories, Gaithersburg, Md.)–2.5 μl of 100 mM dithiothreitol (Bethesda Research Laboratories)–1.25 μl of 10 mM deoxynucleotides (Promega, Madison, Wis.)–10 U of RNase inhibitor (Promega) at 42°C for 2 min and was then incubated at 42°C for 60 min with 200 U of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories); this was followed by heating at 70°C for 10 min (22).

Clinical specimens. Patients scheduled for upper endoscopy were enrolled prospectively from the Nashville Department of Veterans Affairs Medical Center Gastroenterology Clinic between February 1993 and April 1994. Patients were excluded if they had a history of gastric surgery, were receiving steroids or other immunomodulating drugs, were abusing alcohol or illicit drugs, had an active infection or malignancy, had taken antimicrobial agents within the prior 2 weeks, were hepatitis B virus surface antigen or human immunodeficiency virus positive, or had active gastrointestinal bleeding. After obtaining written consent, blood was obtained to measure the serum immunoglobulin G (IgG) response to *H. pylori* infection by enzyme-linked immunosorbent assay (ELISA) (20). Each serum sample was run in duplicate in parallel with known positive controls, and the cutoff for positivity was established as an optical density ratio of >1.0 as described previously (20). Upper endoscopy was performed, and endoscopic findings were recorded. Seven biopsy specimens were obtained from each patient: one biopsy specimen each from the duodenal bulb, gastric antrum, and gastric corpus was submitted for histologic evaluation by a single pathologist who was unaware of the PCR results; one biopsy specimen each from the gastric antrum and gastric corpus was cultured under microaerobic conditions at 37°C for isolation of *H. pylori* as described previously (21); and one biopsy specimen each from the gastric antrum and gastric corpus was frozen at −70°C for RNA extraction and RT-PCR to detect the presence of mRNA for *H. pylori* genes. Pathologic specimens were stained with a modified Giemsa stain to detect *H. pylori* (17).

RNA extraction and RT for gastric biopsy specimens. Individual gastric biopsy specimens were placed in sterile vials (Nunc) and were stored at −70°C until they were used for RNA preparation for RT-PCR. Total RNA was isolated by the guanidinium thiocyanate-phenol chloroform method in 500 μl of homogenization buffer (6). The RNA was dissolved in 12 μl of diethyl pyrocarbonate-treated distilled water, and 2 μl of RNA was used for first-strand cDNA synthesis with random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) as described above (22).

PCR. PCR primers were designed on the basis of published sequences of *H. pylori* 16S rRNA, *ureA*, and *cagA* (7, 15, 21) (Table 1). Amplification of *H. pylori* genomic DNA sequences was carried out in a total volume of 50 μl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleotides, 2 U of AmpliTaq polymerase (Perkin-Elmer Cetus), and 1 μl of boiled *H. pylori* supernatant as DNA template. The 16S rRNA, *ureA*, and *cagA* primers were each used at a final concentration of 0.5 μM. Each reaction mixture was overlaid with 25 μl of light mineral oil and was amplified for 39 cycles as follows: 1 min at 94°C, 1 min of annealing at 55°C (for 16S and *cagA* PCRs) or 45°C (for *ureA* PCR), and 2 min at 72°C. After the 39th cycle, extension was continued for another 7 min. PCR of cDNA from gastric biopsy specimens was performed exactly as described above. Agarose gel electrophoresis with ethidium bromide staining was performed on an aliquot from each PCR mixture. To avoid PCR contamination, PCR mixtures were prepared in a dedicated area used only for PCR, and PCR products were opened in a laminar flow hood separated from the PCR preparation area. Negative and positive controls were performed in every experiment.

Statistical analysis. Two-tailed Fisher's exact test was used to make statistical comparisons between patient groups.

RESULTS

Sensitivity of the PCR assay. The sensitivity of the PCR assay for the detection of *H. pylori* 16S rRNA sequences was investigated by performing PCR on serial dilutions of both genomic DNA and cDNA from the *H. pylori* control strain 60190. The lower limit of detection by PCR with cDNA was 2 CFU/ml (Fig. 1). The sensitivity of PCR with *H. pylori*-specific *cagA* primers also was tested in parallel experiments. The sensitivity of PCR for *cagA* detection was identical to that for 16S rRNA detection (Fig. 1). The lower limit of detection with genomic DNA for 16S rRNA and *cagA* was 5 CFU/ml. These data indicated that PCR could detect small numbers of *H. pylori* cells by using both genomic DNA and cDNA as templates. An alternate explanation could be that repeated freezing and thawing could reduce the viable counts but allow for the detection of nucleic acid, and thus, the high degree of sensitivity noted could be due to the detection of released nucleic acid by lysed, nonviable organisms. However, we attempted to minimize freezing-thawing, and in the majority of cases extracted DNA or RNA was used immediately as tem-

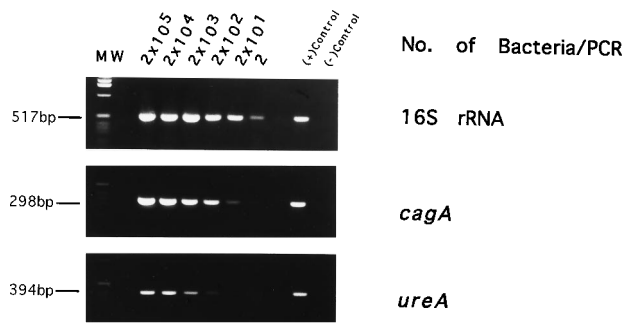

FIG. 1. Sensitivity of PCR assay for detection of *H. pylori*. Dilutions of cDNA from the *H. pylori* control strain 60190 were used as templates for PCR with *H. pylori*-specific 16S rRNA, *cagA*, and *ureA* oligonucleotide primers. The results shown are for dilutions corresponding to cDNA from 2×10^5 to 2 CFU. The first lane contains molecular weight (MW) markers. The limit of detection for 16S rRNA and *cagA* by this assay was 2 CFU; the limit of detection for *ureA* was 200 CFU.

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TABLE 2. RT-PCR for *H. pylori* detection in gastric biopsy specimens from 42 dyspeptic patients

Diagnosis of *H. pylori* infection		No. of patients	No. positive by RT-PCR		
Tissue[a]	Serum[b]		16S rRNA	*ureA*	cagA
+	+	25	24	16	14
−	−	13	0	0	0
−	+	4	0	0	0

[a] Tissue positive defined as histologically and/or culture positive.
[b] Serum positive as measured by IgG ELISA, defined as an optical density ratio of >1.0.

plate for PCR or RT-PCR prior to freezing. PCR with *ureA* primers on serial dilutions of both genomic DNA and cDNA from *H. pylori* control strain 60190 was less sensitive; the lower limits of detection with cDNA and genomic DNA were 200 and 2×10^5 CFU/ml, respectively. To assess specificity, PCR was performed with *H. pylori*-specific 16S rRNA, *ureA*, and *cagA* primers on genomic DNAs from control strains of *Escherichia coli*, *Campylobacter fetus*, and *Campylobacter jejuni*, and no amplification products were obtained.

Detection of *H. pylori* in gastric biopsy specimens by PCR. Gastric biopsy specimens obtained prospectively were tested by PCR by using primers for *H. pylori* 16S rRNA and *ureA*. Forty-two patients were studied; they ranged in age from 29 to 81 years (median age, 61 years), 98% were men, and 14 had duodenitis or duodenal ulcer, 1 had gastric ulcer, 21 had nonulcer dyspepsia, and 6 had esophagitis. Of 42 patients, 25 were positive for *H. pylori* infection by both serology and histology and/or culture, and these patients were designated "tissue positive." All of the 25 tissue-positive patients also had positive anti-*H. pylori* IgG serology (Table 2). The serologic IgA response to *H. pylori* was not examined in the present study because of the lower sensitivity and specificity compared with those of the IgG response to the organism (70% sensitivity and 88% specificity for the IgA response versus 93% sensitivity and 93% specificity for the IgG response). There were no situations in our patient cohort in which the IgA response was positive in an infected person and the IgG response was negative. Thirteen patients were negative for *H. pylori* infection by serology, histology, and culture, and four patients were positive by serology only. RT-PCR with primers specific for *H. pylori* 16S rRNA was positive for 24 of 25 tissue-positive and 0 of 17 tissue-negative patients, respectively (Table 2) ($P < 0.001$). Stratification by biopsy location revealed that detection was slightly better in the gastric antrum compared with that in the gastric corpus; 22 (96%) of 23 antral biopsy specimens from tissue-positive patients were positive by PCR, compared with 21 (84%) of 25 gastric body biopsy specimens from which *H. pylori* could be grown or visualized. For the sole subject whose *H. pylori* infection was not detected by RT-PCR for 16S rRNA a gastric antral biopsy specimen was not available for analysis. RT-PCR with primers specific for *H. pylori ureA* mRNA also was highly specific but less sensitive. Samples from only 16 (64%) of 25 tissue-positive patients were positive by RT-PCR with *ureA* primers (Table 2), and samples from 0 of 17 tissue-negative patients were positive ($P < 0.001$).

Detection of *H. pylori cagA* gene expression in gastric biopsy specimens and correlation with genomic *cagA*. With *cagA* primers, RT-PCR was positive for 14 (56%) of 25 gastric biopsy specimens obtained from patients in the tissue-positive group but for 0 of the 17 specimens from patients in the tissue-negative group. To confirm that PCR detection of *cagA* expression in vivo reflected the genotype of the infecting strain, genomic DNA was prepared from the *H. pylori* isolates from 19 patients in the tissue-positive group and was used as the template for PCR. These represented the total number of isolates available for analysis. cDNA was prepared from the corresponding gastric biopsy specimens from the same patient whose specimen was used as the template for PCR. PCR on genomic DNA with primers for 16S RNA and *ureA* primers yielded the expected products in all 19 *H. pylori* isolates. PCR with *cagA* primers was positive on genomic DNAs of 10 of the 19 *H. pylori* isolates. Each of the 10 corresponding gastric biopsy specimens also was positive by RT-PCR with *cagA* primers, and for each of the 9 *cagA*-negative *H. pylori* isolates, all of the corresponding gastric biopsy specimens also were negative. These results demonstrate that detection of *cagA* by RT-PCR of gastric tissue and PCR of bacterial genomic DNA were 100% correlated.

Correlation of *cagA* status with clinical outcome. *CagA* status was determined by RT-PCR with specific *cagA* oligonucleotide primers. Tissue-positive patients were stratified into two groups on the basis of endoscopic findings: those with peptic ulcer disease (PUD) or duodenitis and those without PUD or duodenitis. PUD was defined as a circumscribed break in the duodenal or gastric mucosa which measured at least 10 mm in diameter, had apparent depth, and was covered by an exudate. Duodenitis was defined as erythema of the duodenal mucosa associated with superficial erosion. Eleven tissue-positive individuals had PUD or duodenitis at the time of endoscopy, and 9 of these 11 individuals harbored $cagA^+$ strains ($P = 0.005$). Of the 14 tissue-positive individuals without PUD or duodenitis, 5 harbored $cagA^+$ strains and 9 possessed *cagA*-negative strains.

DISCUSSION

The clinical importance of *H. pylori* in PUD is clear, and infection with this organism has been established as a risk factor for gastric adenocarcinoma (8). Because of the fastidious growth characteristics of *H. pylori* and the prolonged incubation period required, several alternative approaches have been developed for the accurate and rapid detection of *H. pylori* in gastric mucosa. Engstrand et al. (15) first demonstrated that RT-PCR on gastric biopsy specimens with 16S rRNA primers is a sensitive method for the detection of *H. pylori*. We have confirmed this observation and have demonstrated that as few as 2 CFU/ml can be detected from in vitro cultures. The likely explanation for this high degree of sensitivity is the large number of 16S rRNA template molecules present in each bacterial cell (5). In our study, the sensitivity of RT-PCR on antral biopsy specimens with primers specific for *H. pylori* 16S rRNA was 96% in patients who were serologically positive for *H. pylori* and positive by either histology or culture. The specificity of this assay with antral biopsy specimens was 100%, in that only patients who were positive by histology and/or culture were positive by RT-PCR. Detection of *H. pylori* in gastric corpus specimens by RT-PCR was slightly less sensitive compared with detection in gastric antral specimens, but it was still completely specific. The accuracy of detection by this method and the rapidity of the assay substantiate the fact that RT-PCR is a useful technique for the diagnosis of *H. pylori* infection from gastric biopsy specimens.

RT-PCR with *H. pylori ureA* primers was less sensitive than RT-PCR with 16S rRNA primers in detecting *H. pylori*, possibly because of the lower quantities of *ureA* RNA within each bacterial cell. These findings are consistent with the hypothesis that the amount of *H. pylori* urease production in

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vivo may be low (4). However, the sensitivity of RT-PCR with *ureA* primers remained low when genomic DNA from *H. pylori* 60190 was used as the template, suggesting that *ureA* primer efficiency may be the issue. The sensitivity of detection of *H. pylori ureA* mRNA may possibly be improved if alternate *ureA* primers are designed.

In addition to demonstrating the presence of *H. pylori* in gastric specimens, the ability to identify *H. pylori* genes by a single technique would be of substantial benefit. Engstrand et al. (15) used the 3′ *H. pylori*-specific 16S primer HP1 to prime RT and followed this with PCR amplification to detect *H. pylori* in gastric biopsy specimens (15). In contrast, use of random hexamers to prime cDNA synthesis is advantageous because it allows for the study of the expression of multiple *H. pylori* genes, both conserved and nonconserved. This may permit a correlation between the in vivo expression of putative virulence factors and the clinical and pathologic sequelae of infection.

Four patients in the present study who were seropositive for *H. pylori* infection were negative by histology, culture, and RT-PCR. For two of these patients antral erythema was noted at endoscopy and chronic gastritis was noted histologically. The third patient had normal mucosa endoscopically and chronic gastritis by histology. The fourth patient had endoscopic evidence of mild duodenal bulb erythema with chronic inflammation of the duodenal mucosa on histologic evaluation. Three hypotheses may explain the dichotomy between the tissue-based methods for *H. pylori* detection and the serologic results. First, the serology results may have been falsely positive. However, in three of the patients, the IgG ELISA optical density ratio was consistently >2.5, which far exceeds the cutoff; in previous studies, values this high were rarely falsely positive (20). Second, there may be patients who harbor numbers of organisms below the limit of detection, which may represent a paucibacillary form of infection. Third, *H. pylori* infection may have a patchy distribution in some patients (3), and thus, the areas sampled by biopsy may not have contained organisms. The clinical significance of this pattern of *H. pylori* infection has not been established.

One perplexing feature of *H. pylori* infection is that although essentially all infected individuals develop gastritis, clinical consequences are recognized in only a minority of individuals, suggesting that strain differences may result in differences in virulence (14). *H. pylori* strains may differ by the presence or absence of the *cagA* gene and its high-molecular-weight protein product. About 60% of *H. pylori* strains have this gene (2, 21); however, antibodies to its product are present in the sera and mucosae of essentially all patients with duodenal ulceration (9, 12, 13). Mucosal IgA recognition of this protein was associated with the activity of gastritis and with the extent of surface degeneration (13). In the present study, we have demonstrated that RT-PCR with primers specific for *H. pylori cagA* is a sensitive and accurate technique for identifying infection with *cagA*-positive strains. We have also shown a significant association between PUD or duodenitis and infection with $cagA^+$ strains. The prognostic implication of harboring an *H. pylori* strain associated with particular clinical sequelae such as peptic ulceration could have an impact on therapeutic decisions. One noted problem with the treatment of *H. pylori* is the cumbersome regimens necessary for successful eradication, which involve multiple dosing schedules, a moderately high incidence of side effects, and poor patient compliance. The identification of particular subsets of infected individuals who are at increased risk for developing clinical complications such as peptic ulceration would enable physicians to focus eradication therapy and therefore reduce the number of patients receiving unnecessary therapy. In addition, knowledge of potential bacterial virulence factors may contribute to the delineation of pathogenic mechanisms involving *H. pylori* infection.

In conclusion, we have developed a highly sensitive and rapid technique for diagnosing the presence of *H. pylori* in gastric biopsy specimens and for detecting the in vivo expression of *cagA*. The cDNA prepared by this method also can be used to examine the expression of other genes and may be useful for correlating bacterial virulence factors with host response to infection.

ACKNOWLEDGMENTS

The study described here was supported in part by grant R01CA58834 from the National Cancer Institute, the Medical Research Service of the U.S. Department of Veterans Affairs, and the Andrew Woodfin-Miller Foundation.

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