

PCR-Based Restriction Pattern Typing of the *vacA* Gene Provides Evidence for a Homogeneous Group among *Helicobacter pylori* Strains Associated with Peptic Ulcer Disease

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The results of PCR-based molecular typing of *Helicobacter pylori* strains by restriction fragment length polymorphism analysis of a 1,161-bp nucleotide sequence of the midregion of the *vacA* gene are reported. A total of 48 *H. pylori* strains isolated from gastric biopsy specimens obtained from 18 patients with peptic ulcer dyspepsia, 15 patients with nonulcer dyspepsia, and 15 asymptomatic *H. pylori*-infected subjects were studied. Highly heterogeneous restriction patterns were obtained by digestion of PCR products with *SauII*, *BglII*, and *HhaI*, whereas *HaeIII* digestion resulted in a strictly homogeneous profile for *H. pylori* strains isolated from 14 of 18 (77.7%) patients with peptic ulcer dyspepsia, but a strictly homogeneous profile was found for strains from only 8 of 15 (53.3%) patients with nonulcer dyspepsia ($P = 0.163$) and 5 of 15 (33.3%) asymptomatic *H. pylori*-infected subjects ($P = 0.014$). A potentially important aspect of the results obtained is the clinical relevance, since a single restriction pattern seems to be able to identify the majority of *H. pylori* strains associated with peptic ulcer disease.

Helicobacter pylori is linked to gastritis, peptic ulcer, and gastric cancer (4, 6, 14). Peptic ulcer disease, as distinct from chronic asymptomatic infection, is strongly associated with the expression of bacterial virulence markers (5, 30), including cytotoxin-associated gene A (*CagA*) (12, 28) and the vacuolating cytotoxin (*VacA*) that induces the formation of intracellular vacuoles in eukaryotic cells in vitro (9, 20).

Most people infected with *H. pylori* are asymptomatic, with only a few patients developing peptic ulcer or gastric cancer. A possible explanation is that patients with serious gastroduodenal lesions are infected with virulent *H. pylori* strains, whereas those patients who are asymptomatic and who present with simple chronic gastritis and no ulcer are infected with organisms with low pathogenic potentials.

Although *H. pylori* isolates show high levels of genotypic diversity (16), almost all phenotypic characters of the microorganism are conserved with the exception of the production of the vacuolating cytotoxin encoded by *vacA* (10) and the presence of the 128-kDa cytotoxin-associated protein encoded by *cagA* (7, 10). These two factors are therefore potentially important virulence determinants that affect the clinical outcome of *H. pylori* infection. In particular, the *vacA* gene is present in almost all strains tested (11, 22), and about 50% of clinical isolates produce inactive or less active toxins due to the presence of alleles characterized by differences in the signal peptide and/or middle region of the gene of *H. pylori* isolates obtained from U.S. subjects (8). By PCR typing and DNA sequencing, Atherton et al. (2) demonstrated that s1 *vacA* genotypes are associated with a higher level of in vitro cytotoxin activity than the levels of activity with which other genotypes are associated and that type s1 strains are more frequently observed among patients with past or present peptic

ulceration than patients without peptic ulcer (2, 3). Recently, the existence of different allelic variants has also been described in *H. pylori* strains obtained from European (23, 24, 29) and Japanese (18) subjects.

We report here on a simple PCR-based method of typing *H. pylori* that uses restriction fragment length polymorphism (RFLP) analysis of a 1,161-bp fragment of the midregion of *vacA*. Using this analysis, we found a simple fingerprinting pattern that identifies most *H. pylori* strains isolated from patients with peptic ulcer disease.

MATERIALS AND METHODS

Patients and clinical specimens. Forty-eight subjects (23 men and 25 females; mean age, 46 years; age range, 22 to 75 years) were admitted to the study. Thirty-three patients had undergone gastroduodenoscopy for dyspepsia, and 15 asymptomatic subjects were the partners of *H. pylori*-infected patients. Gastric specimens were cultured for *H. pylori*, as described previously (27). Briefly, biopsy samples were homogenized and cultured on Columbia agar base (Oxoid, Milan, Italy) supplemented with 7% horse blood and Dent's selective supplement (Oxoid). The cultures were incubated in a microaerophilic atmosphere at 37°C in GasPak jars and CampyPak II envelopes (BBL Microbiology System, Cockeysville, Md.), and the isolates were identified as *H. pylori* by their morphology upon Gram staining and by positive urease, oxidase, and catalase tests. Histological sections of formalin-fixed biopsy specimens were stained with hematoxylin-eosin to evaluate the morphology and whether *Helicobacter*-like organisms were present. A serum sample was obtained by routine venipuncture for serological studies.

Assay for cytotoxicity. Supernatants from broth cultures of *H. pylori* isolates were concentrated by using Centrprep-100 ultrafiltration units (Amicon, Beverly, Mass.) and were incubated with HeLa cells at twofold dilutions ranging from 1:5 to 1:160 as described previously (13). Cell vacuolization was assessed by light microscopy after 48 h of incubation. Wells in which 50% or more of the cells were vacuolated were defined as showing a cytotoxic effect.

Neutralization of *H. pylori* cytotoxin activity. Human sera were heated at 56°C and diluted with Eagle's minimal essential medium. Sera diluted twofold (from 1:10 to 1:160) were incubated for 1 h at 37°C with an equal volume of the concentrated type strain *H. pylori* CCUG 17874 (Culture Collection of the University of Göteborg, Göteborg, Sweden) culture supernatant. Adherent HeLa cells were incubated for 18 h at 37°C in 96-well plates with 50- μ l mixtures of serum and *H. pylori* plus 50 μ l of minimal essential medium. The neutralization titer was defined as the highest dilution of a serum sample that completely neutralized vacuolization, as assessed by light microscopy.

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TABLE 1. Genetic, phenotypic, and serological properties of *H. pylori* isolates from 33 patients with gastritis and endoscopically defined gastroduodenal pathology and 15 asymptomatic *H. pylori*-infected subjects

Clinical diagnosis	No. of patients	No. (%) of <i>H. pylori</i> isolates with the following genetic and phenotypic properties ^a :				No. (%) of serum samples with the following:		
		<i>vacA</i> , ho	<i>vacA</i> , he	<i>cagA</i> positive (%)	Tox+	Neutralizing activity	Anti-VacA IgG ^b	Anti-CagA IgG
Ulcer dyspepsia	18	14 (77.8)	4 (22.2)	17 (94.4)	9 (50.0)	13 (72.2)	13 (72.2)	17 (94.4)
Nonulcer dyspepsia	15	8 (53.3)	7 (46.7)	13 (86.7)	7 (46.7)	10 (66.7)	10 (66.7)	12 (80.0)
Asymptomatic, infected	15	5 (33.3)	10 (66.7)	12 (80.0)	7 (46.7)	8 (53.3)	8 (53.3)	10 (66.7)

^a Strains with homogeneous (ho) and heterogeneous (he) restriction pattern profiles for PCR-based amplification of the *vacA* gene and digestion with *Hae*III are indicated (see Fig. 1a). Tox+, in vitro cytotoxin production.

^b IgG, immunoglobulin G.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) analysis for antibodies against VacA and CagA antigens. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (19) with a 7.0% acrylamide gel, as described previously (13). Supernatants from the type strain *H. pylori* CCUG 17874 broth culture was concentrated by using Centrprep-100 ultrafiltration units (Amicon) and were used as antigen, as described previously (13).

The Western blot procedure of Towbin et al. (26) was performed as described previously (13). Briefly, after electrophoretic transfer, the blots were incubated for 12 h at room temperature with human sera diluted 1:1,000 in phosphate-buffered saline containing 0.05% (vol/vol) Tween 20. Antigen-antibody complexes were detected with anti-human peroxidase-labeled immunoglobulin G (DAKO, Copenhagen, Denmark) diluted 1:500 in phosphate-buffered saline and with 4-chloro-1-naphthol (Bio-Rad, Hercules, Calif.) as the enzyme substrate. The VacA and CagA antigens were recognized as clear bands of 87 to 94 and 128 kDa, respectively (13).

PCR. The oligonucleotides used as PCR primers in this study have been used previously by Xiang et al. (30). The primers for the *vacA* gene are derived from the sequence of the *vacA* gene. Briefly, the amplification product of *H. pylori vacA* primers 5'-GCTTCTCTTACCACCAATGC and 5'-TGTCAGGGTTGTT CACCATG was 1,161 nucleotides in length and was derived from the middle region of *vacA*, from nucleotides 1468 to 2629 (25). *H. pylori cagA* primers 5'-AGTAAGGAGAAACAATGA and 5'-AATAAGCCTTAGAGTCTTTTG GAAATC amplify a 1,350-bp DNA fragment (30). The PCR mixtures (50 μ l) contained 50 mM KCl, 10 mM Tris, 200 μ M (each) deoxynucleoside triphosphate, 30 pmol of each primer, 2.5 U of Amplitaq (Perkin-Elmer, Norwalk, Conn.), and 10 ng of DNA obtained from each bacterial strain by phenol-chloroform extraction and ethanol precipitation. Amplifications were performed on a PCR 9600 thermocycler (Perkin-Elmer) as follows: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Five microliters of the PCR product was electrophoresed on a 2% agarose gel (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 1 \times Tris-borate-EDTA buffer containing 1 μ g of ethidium bromide per ml. The gels were examined by transillumination and were photographed. A *vacA*- and *cagA*-positive *H. pylori* strain (strain CCUG 17874) and a *cagA*-negative and *vacA*-positive *H. pylori* strain (strain HPG21) were used as controls in the PCR experiments.

RFLP analysis. A 10- μ l sample of the PCR product was digested with 10 U of the restriction enzymes *Hae*III, *Sau*III, *Bgl*II, and *Hha*I (Boehringer Mannheim) for 4 h at 37°C in the buffer, as recommended by the supplier. The digest was analyzed by electrophoresis in a 2% agarose gel with 1 \times Tris-borate-EDTA buffer containing 1 μ g of ethidium bromide per ml. DNA molecular size marker VI (Boehringer) was used.

Statistical analysis. Prevalence rates were compared by the χ^2 test and Fisher's exact test. Probability levels (*P*) of <0.1 were considered statistically significant.

RESULTS

The endoscopic diagnoses for 33 symptomatic patients with dyspepsia were peptic ulcer dyspepsia (*n* = 18) and nonulcer dyspepsia (*n* = 15). The gastric histology of the latter 15 patients was active (*n* = 5) or chronic active (*n* = 10) gastritis. The 15 asymptomatic partners of *H. pylori*-infected patients were either histologically negative (*n* = 7) or the partners presented with simple chronic histological gastritis (*n* = 8). All 48 patients studied were positive for *H. pylori* by culture. Supernatants from 23 of 48 *H. pylori* isolates produced vacuolization in HeLa cells, whereas supernatants from the remaining 25 did not. The supernatant dilution that produced vacuolization ranged from 1:5 to \geq 1:160.

The specific sequences of the *vacA* and *cagA* genes of the

H. pylori isolates were looked for by PCR: all 48 strains were *vacA* positive, whereas 42 strains possessed the *cagA* gene (data not shown). The relationship between the genetic, phenotypic, and serological properties of the *H. pylori* strains isolated from the 48 subjects is reported in Table 1.

From the restriction enzyme digestion of the 1,161-bp products of amplification of the *vacA* gene from the 48 *H. pylori* strains studied, we found highly heterogeneous restriction patterns with *Sau*III, *Bgl*II, and *Hha*I (data not reported), whereas *Hae*III digestion of the 1,161-bp *vacA* fragment resulted in a strictly homogeneous profile (Fig. 1) for strains from 77.8% of the patients (14 of 18) with peptic ulcer dyspepsia, whereas a strictly homogeneous profile was found for strains from 53.3%

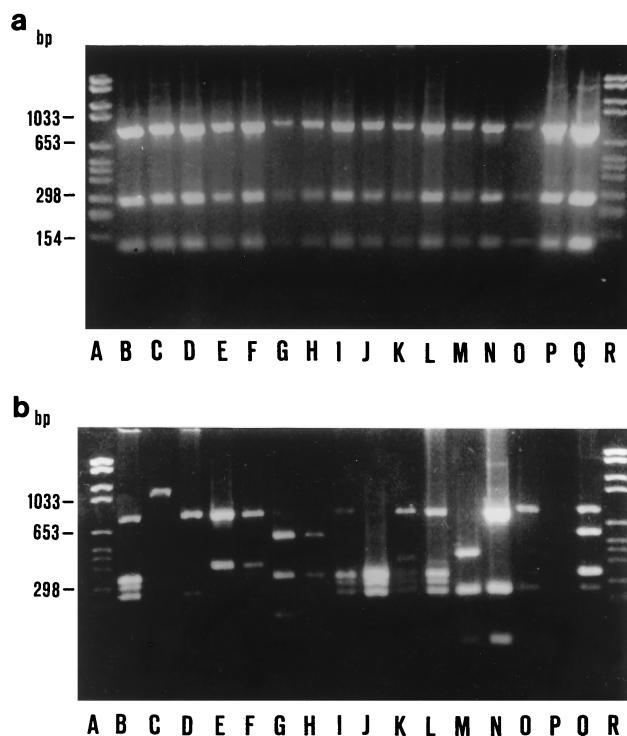


FIG. 1. RFLP analysis of *H. pylori vacA* gene. A 1,161-bp region was amplified by PCR and digested with *Hae*III, and the fragments were separated in a 2% agarose gel. (a) Homogeneous pattern characterized by three bands. Lanes A and R, molecular size marker VI; lanes B to O, isolates from 14 patients with ulcer dyspepsia, respectively; lanes P and Q, isolates from patients with nonulcer dyspepsia. (b) Heterogeneous profiles characterized by several different patterns. Lanes A and R, molecular size marker VI; lanes B to I, isolates from asymptomatic *H. pylori*-infected patients; lanes J to O, isolates from patients with nonulcer dyspepsia; lane P, running buffer alone; lane Q, isolate from a patient with ulcer dyspepsia.

of the patients (8 of 15) with nonulcer dyspepsia and chronic active gastritis ($P = 0.163$) and 33.3% of the asymptomatic subjects infected with *H. pylori* (5 of 15), who had either a negative histology result or simple chronic gastritis ($P = 0.014$) (Table 1). All 27 *H. pylori* strains with the homogeneous profile were *cagA* positive, and 17 (63.0%) produced cytotoxin in vitro. Of 21 strains with the heterogeneous profile, 15 (71.4%) were *cagA* positive and 6 (28.6%) produced cytotoxin in vitro.

DISCUSSION

Although molecular biological typing methods with genomic DNA or gene probes usually require laborious sample preparation and processing steps, the use of PCR-based RFLP analysis affords a simple and rapid typing technique. The molecular typing approach for *H. pylori* that uses the PCR-based RFLP analysis has been used previously. The genes encoding urease and its accessory proteins have been preferential targets for PCR (1, 17, 21) since these genes are conserved in *H. pylori*. The adhesin gene *hpaA* (15) has also been used as the target for comparison of the effects of genetic changes of *H. pylori* isolates.

In our study, the *vacA* gene was the target for the PCR, and we decided to use a fragment of the midregion of the gene large enough to permit the detection of diversity but small enough to allow regular amplification. Previous studies by Cover et al. (11) and Atherton et al. (2) have shown that several cytotoxin-producing strains and cytotoxin-nonproducing strains of *H. pylori* have substantially different sequences within the middle region of the *vacA* gene. Our results by RFLP analysis of the PCR products with several restriction enzymes confirmed the high degree of diversity of the genomic structure of the *vacA* gene among *H. pylori* strains isolated from gastric biopsy specimens. However, the digestion of PCR products with the *Hae*III enzyme allowed us to identify a genetic correlation for 27 of 48 *H. pylori* strains examined, thus resulting in a homogeneous group of strains with identical *vacA* gene restriction patterns characterized by the presence of three distinct bands. In addition, these strains were strongly associated with the presence of the *cagA* gene and cytotoxin activity and occurred more frequently in patients with peptic ulcer dyspepsia. In fact, the genetically related strains were isolated from 77.8% of patients with peptic ulcer dyspepsia but significantly ($P = 0.014$) less frequently (33.3%) from asymptomatic *H. pylori*-infected subjects. Although the homogeneous *H. pylori* strains were more likely to be isolated from patients with peptic ulcer dyspepsia (77.8%) than from patients with nonulcer dyspepsia and chronic active gastritis (53.3%), the differences were not significant ($P = 0.163$). The detection of strains with this characteristic restriction pattern not only in patients with ulcer dyspepsia but also in patients with a less serious disease such as nonulcer dyspepsia and chronic active gastritis or in *H. pylori*-infected but as yet asymptomatic subjects showed that the specificity (57.7%) of the pattern for ulcer disease was lower than its sensitivity (77.8%). This observation, however, does not seem to variance with the presumed ulcerogenic potential of these strains. It is well known that chronic infection often occurs without symptoms and that with time some individuals develop severe features of upper gastrointestinal diseases. Untreated chronic infection with these *H. pylori* strains may progress from simple chronic gastritis in asymptomatic subjects to chronic active gastritis in patients with nonulcer dyspepsia and, finally, to peptic ulcer disease in ulcer patients.

In conclusion, the results of this study by PCR-based restriction pattern analysis suggest that the specific RFLP profiles reported here may be indicators of the pathogenic potential of *H. pylori* strains. A potentially important aspect of this simple

method may be its clinical relevance, since a single restriction pattern for the *vacA* gene seems to be able to identify *H. pylori* strains strongly associated with peptic ulcer disease.

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