Use of Polymerase Chain Reaction-Amplified *Helicobacter pylori* Urease Structural Genes for Differentiation of Isolates

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Helicobacter pylori has been demonstrated as an etiologic agent of human gastritis and peptic ulcer formation. However, there is no straightforward basis to distinguish different isolates. We used the polymerase chain reaction (PCR) to amplify the urease structural subunit genes, *ureA* and *ureB*, which, when digested with appropriate restriction endonucleases, allow the differentiation of patterns on agarose gels. PCR amplification was possible with DNA rapidly extracted from *H. pylori* by alkaline lysis and phenol-chloroform. The 2.4-kb PCR products amplified from 22 clinical isolates and subjected to *Hae*III restriction endonuclease digestion produced 10 distinct patterns on agarose gels, with two patterns being shared between five and six strains. PCR amplification of the urease genes may enable the differentiation of closely related *H. pylori* strains by restriction digest analysis of PCR-amplified *ureA* and *ureB* genes.

Helicobacter pylori has been demonstrated as an etiologic agent of gastritis and peptic ulcer disease in humans (10, 15). A number of putative virulence factors, including hemagglutinins, flagella, cytotoxins, and urease, are thought to enable the survival of the organism in the gastric mucosa (2). *H. pylori* produces a highly active urease that both catalyzes the hydrolysis of urea, yielding ammonia and carbon dioxide, and elicits an immune response in patients (5, 16). Urease is produced by many bacterial genera, including *Proteus*, *Klebsiella*, and *Morganella*, and by the jack bean plant, *Canavalia ensiformis* (17). The urease enzymes in these species have highly conserved amino acid sequences; however, the genes encoding the urease enzyme in *H. pylori* provide an opportunity to differentiate isolates of this species.

Molecular epidemiological studies of *H. pylori* isolates have previously been done with restriction endonuclease analysis of chromosomal DNA to distinguish strains, as no biochemical typing scheme has been devised (12, 14, 18).

With the advent of the polymerase chain reaction (PCR), rapid amplification of specific DNA fragments is possible from single colonies or even biopsy material, foregoing the necessity of primary culturing or subculturing. This technique has been used in the identification of fastidious bacteria requiring prolonged incubation (1, 22), including *H. pylori* (3, 24). We report on the application of PCR amplification to the analysis of the restriction digest patterns of the urease structural genes, *ureA* and *ureB*, amplified from clinical isolates of *H. pylori*. This technique offers a sensitive epidemiological tool for differentiating or confirming the identities of clinical isolates. Preliminary accounts of this work have appeared elsewhere (7, 8).

Clinical isolates of *H. pylori* were obtained by biopsy from 21 patients with suspected gastritis at the time of endoscopic examination at the University of Maryland Hospital (UMAB) or the Johns Hopkins Hospital (JH), both in Baltimore, Md. Biopsy tissue was used to inoculate brucella agar plates (Difco, Detroit, Mich.) containing 5% sheep blood and Skirrow's supplement (21), and the plates were cultured for 4 days at 37°C under microaerobic conditions

(CampyPak; BBL Microbiology Systems, Cockeysville, Md.). Pure cultures were stored at -70°C.

Purified chromosomal DNA was isolated from *H. pylori* by standard procedures (20), except for the incubation of the resuspended bacterial cells at 37°C for 20 h in the following lysis buffer: 10 mM Tris-HCl (pH 8.5)-100 mM EDTA-3 mg of lysozyme per ml-1% sodium dodecyl sulfate-0.05 mg of RNase A per ml-0.8 mg of pronase E per ml-0.5 mg of proteinase K per ml.

Rapid isolation of chromosomal DNA from *H. pylori* was carried out with a 10- μ l loopful of confluent growth resuspended in 400 μ l of 25 mM glucose-1 mM EDTA-2 mM Tris-HCl (pH 8.0). Lysozyme (100 μ g/ml) was added, and the mixture was incubated for 30 min at 37°C; 300 μ l of 50 mM sodium chloride, 1% sodium dodecyl sulfate, and proteinase K (2.5 mg/ml) were added (9). Incubation was continued for 60 min. The sample was extracted with phenol-chloroform and, after centrifugation, the aqueous phase was ethanol precipitated and the pellet was resuspended in 50 μ l of sterile distilled water. Typically, 5- μ l volumes were used in PCR amplifications.

Oligonucleotides were synthesized on an Applied Biosystems automated DNA synthesizer (model 380B) and purified on a Pharmacia fast protein liquid chromatography system with a Mono Q column; elution was done with a 500 to 900 mM sodium chloride gradient. After ethanol precipitation, oligonucleotides were stored in aliquots at -70°C.

The PCR was carried out in accordance with instructions supplied with the reagents (Perkin Elmer Cetus, Norwalk, Conn.) in a Twinblock temperature cycler (EriComp, San Diego, Calif.) with chromosomal DNA concentrations of 2 ng/ μ l. To amplify the 2.4-kb fragment containing the *ureA* and *ureB* genes, we used two oligonucleotides with recognition sequences of 5'-AGGAGAATGAGATGA-3' (bp 308 to 322) (11) and 5'-ACTTTATTGGCTGGT-3' (bp 2718 to 2703) (11). A 30-cycle amplification was used with these primers and consisted of annealing at 50°C for 2 min, extension at 72°C for 4 min, and denaturation at 94°C for 1 min.

PCR products (0.2 μ g) were run on 0.7% agarose gels. Prior to restriction digest analysis, amplified DNA was ethanol precipitated and DNA pellets were washed twice with 70% ethanol. Digests were incubated with 5 to 10 U of

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FIG. 1. *Hae*III restriction digest patterns of the amplified urease genes on a 2,409-bp PCR product from representative *H. pylori* clinical isolates. Amplified DNA was digested with *Hae*III and electrophoresed on a 3.5% Nusieve GTG agarose gel. Lanes: S, 1-kb ladder, sizes in kilobases; A to J, *H. pylori* UMAB 529 (A), JH 18 (B), UMAB 24a (C), UMAB 218 (D), UMAB 41 (E), UMAB 85 (F), UMAB 92 (G), UMAB 97 (H), JH 13 (I), and JH 31 (J).

enzyme in the appropriate buffer recommended by the enzyme manufacturer at 37°C for 3 h. Restriction patterns were compared after the PCR products were run on 3.5% Nusieve GTG agarose gels (FMC Bioproducts, Rockland, Maine).

All 22 *H. pylori* isolates produced a 2.4-kb PCR product that hybridized to an oligonucleotide internal to the *ureA-ureB* sequence (bp 2010 to 1989) (11) (data not shown). Of these, 12 were amplified from purified chromosomal DNA preparations and 10 were amplified from rapidly isolated chromosomal DNA preparations.

Restriction digest analysis was carried out on the 2.4-kb PCR product amplified from the clinical isolates. Ten different *Hae*III restriction patterns were seen for the 22 clinical isolates (Fig. 1). Two of these patterns were shared between five and six strains. Pattern A (Table 1) was observed in *H. pylori* UMAB 12, UMAB 96, UMAB 519, UMAB 529, UMAB 534, and JH 9. Pattern B was produced by *H. pylori* UMAB 84, JH 14, JH 18, JH 27, and JH 35. Pattern C was seen with *H. pylori* UMAB 24a and JH 11, and pattern D was seen with *H. pylori* UMAB 218 and UMAB 535. Patterns E to J were only seen with the single isolates reported in Fig. 1.

Other restriction digest fragment patterns were identical for all strains. *Mbo*I digestion of the 2.4-kb PCR product yielded six fragments, of 630, 580, 430, 370, 250, and 180 bp, while *Bam*HI digestion produced two fragments, of 1,900 and 500 bp.

The PCR-amplified *ureA* and *ureB* genes from the 22 strains unexpectedly showed sequence divergence, as manifest by the different restriction patterns with *Hae*III. This divergence was not due to base substitutions during PCR amplification, as evidenced by the identical restriction fragment patterns obtained for isolates tested after 30 serial

TABLE 1. *Hae*III restriction digest fragment lengths of *ureA-ureB* 2.4-kb PCR products from clinical isolates of *H. pylori*

Pattern (reference)	No. of strains	<i>Hae</i> III restriction fragment length (bp)
A	6	870, 800, 530, 235
B	5	1100, 530, 460, 320
C	2	870, 800, 539, 310
D	2	870, 540, 460, 330, 310
E	1	800, 530; 530, 330, 225
F	1	940, 530, 380, 330, 310
G	1	1550, 850
H	1	610, 540, 480, 330, 310, 240
I	1	810, 640, 530, 440
J	1	810, 530, 480, 320
<i>ureA-ureB</i> (4) ^a		515, 457, 440, 315, 232, 218, 165, 63
<i>ureA-ureB</i> (11) ^a		1516, 517, 315, 63

^a *Hae*III restriction digest fragment lengths were predicted from the published nucleotide sequences.

passages or by the identical patterns obtained for a clinical isolate and its corresponding cloned urease genes maintained on a plasmid in *Escherichia coli* (data not shown). When the predicted restriction fragment patterns of the published *ureA* and *ureB* gene sequences were compared with those obtained in this study, the only correlation was between the *Bam*HI pattern, involving one restriction site, and the sequence reported by Labigne et al. (11). No restriction site was present in the sequence reported by Clayton et al. (4). As such, the published DNA sequences of the *ureA* and *ureB* genes may not be applicable to all strains.

The clinical isolates used in this study were obtained over a 12-month period in the case of the JH isolates and over a 19-month period in the case of the UMAB isolates. The grouping of isolates in patterns A and B could be explained by reinfection of patients with a single strain at the time of endoscopy or by contact spread to cohorts from a single patient. These possibilities can be discounted, as no patient cohorts were enrolled in either study and only two pairs of isolates were sampled on the same day, all others having been sampled on separate days. Cross-contamination upon endoscopy can further be dismissed, since both pairs of isolates sampled on the same day, *H. pylori* UMAB 218 and UMAB 519 and *H. pylori* UMAB 84 and UMAB 85, produced different *Hae*III restriction fragment patterns.

It was thought that the nucleotide sequence of such an important virulence factor as urease would be conserved among strains. It can be postulated that the nucleotide sequence variations detected in the *ureA* and *ureB* genes may be base substitutions that nevertheless conserve encoded amino acids or allow amino acid substitutions that do not affect enzyme activity. These data suggest that variations are present in genes thought essential for the virulence of *H. pylori*. Analysis of the nucleotide sequence of an accessory gene involved in the expression of *H. pylori* urease, *ureC*, has also revealed variations among strains (6).

These ideas are consistent with observations of considerable genomic variations between *H. pylori* strains detected by whole-cell DNA restriction digest analysis. Previous studies have used gross differences in the restriction digest patterns of *H. pylori* chromosomal DNAs to differentiate strains (12). However, interpretation of these results is difficult because of complicated restriction digest patterns, even with densitometric analysis of one signature area of a pattern. The use of the *ureA-ureB* PCR product for restric-

tion digest analysis produces readily identifiable differences in restriction digest patterns. It also does not require high concentrations of chromosomal DNA, and the PCR product produced upon amplification is sufficient material for a number of restriction digests. *Hae*III digestion of the PCR-amplified *ureA* and *ureB* genes produced 10 distinct restriction digest patterns for 22 isolates (Fig. 1). Two sequential clinical isolates from the same patient were included in this study. *H. pylori* JH 18 and JH 31 were isolated from biopsy materials taken 9 weeks apart from the same patient. Both isolates exhibited *Hae*III restriction digest pattern B. This result suggests that the patterns are stable, unlike chromosomal digest patterns, which have been reported to be highly diverse for each isolate and to vary between sequential isolations of the same strain (14, 18).

We propose that *Hae*III digestion of PCR-amplified urease genes can be used as the basis for the typing of *H. pylori* isolates. Similar alternative typing schemes based on restriction fragment length polymorphisms of PCR products have been proposed to type clinical isolates of avian infectious bronchitis virus (13) and *Chlamydia trachomatis* (19) as well as to identify variant toxin genes in *E. coli* (23). We do not suggest that these PCR-amplified urease genes be used for the primary identification of *H. pylori*, since urease genes are well conserved among bacterial species. Previous work by Valentine et al. (24) has provided primers that serve the purpose of primary identification with high sensitivity and specificity. However, once an isolate has been identified as *H. pylori*, the scheme suggested in this report can be used to compare multiple isolates cultured from a single biopsy, single isolates obtained from several patients, or isolates obtained from one patient over a period of time.

These unsuspected restriction fragment length polymorphisms confirm DNA sequence variations within the highly conserved urease genes. The restriction digest patterns of the amplified urease genes may serve as a sensitive epidemiological tool for the typing of *H. pylori* clinical isolates.

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REFERENCES

- Bernet, C., M. Garret, B. de Barbeyrac, C. Bebear, and J. Bonnet. 1989. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. *J. Clin. Microbiol.* **27**:2492-2496.
- Buck, G. E. 1990. *Campylobacter pylori* and gastroduodenal disease. *Clin. Microbiol. Rev.* **3**:1-12.
- Clayton, C., K. Kleanthous, and S. Tabaqchali. 1991. Detection and identification of *Helicobacter pylori* by the polymerase chain reaction. *J. Clin. Pathol.* **44**:515-516.
- Clayton, C. L., M. J. Pallen, H. Kleanthous, B. W. Wren, and S. Tabaqchali. 1990. Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. *Nucleic Acids Res.* **18**:362.
- Clayton, C. L., B. W. Wren, P. Mullaney, A. Topping, and S. Tabaqchali. 1989. Molecular cloning and expression of *Campylobacter pylori* species-specific antigens in *Escherichia coli* K-12. *Infect. Immun.* **57**:623-629.
- Courcoux, P., C. Freuland, Y. Piemont, J. L. Fauchere, and A. Labigne. 1990. Polymerase chain reaction and direct DNA sequencing as a method for distinguishing between different strains of *Helicobacter pylori*, abstr. P49. *Rev. Esp. Enferm. Dig.* **78**(Suppl. 1):29-30.
- Foxall, P. A., L.-T. Hu, and H. L. T. Mobley. 1990. Amplification of the complete urease structural genes from *Helicobacter pylori* clinical isolates and cosmid gene bank clones, abstr. P282. *Rev. Esp. Enferm. Dig.* **78**(Suppl. 1):123-129.
- Foxall, P. A., L.-T. Hu, and H. L. T. Mobley. 1991. Detection of *Helicobacter pylori* urease structural genes by PCR amplification, abstr. B78, p. 38. *Abstr. 91st Gen. Meet. Am. Soc. Microbiol.* 1991.
- Frankel, G., L. Riley, J. A. Giron, J. Valmanoi, A. Friedmann, N. Strockbine, S. Falkow, and G. K. Schoolnik. 1990. Detection of *Shigella* in faeces using DNA amplification. *J. Infect. Dis.* **161**:1252-1256.
- Jiang, S. J., W. Z. Lui, D. Z. Zhang, Y. Shi, S. D. Xiao, Z. W. Zhang, and D. Y. Lu. 1987. *Campylobacter*-like organisms in chronic gastritis, peptic ulcer, and gastric carcinoma. *Scand. J. Gastroenterol.* **22**:553-558.
- Labigne, A., V. Cussac, and P. Courcoux. 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J. Bacteriol.* **173**:1920-1931.
- Langenberg, W., E. A. J. Rauws, A. Widjokokusumo, G. N. J. Tytgat, and H. C. Zanen. 1986. Identification of *Campylobacter pyloridis* isolates by restriction endonuclease DNA analysis. *J. Clin. Microbiol.* **24**:414-417.
- Lin, Z., A. Kato, Y. Kudou, and S. Ueda. 1991. A new typing method for the avian infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. *Arch. Virol.* **116**:19-31.
- Majewski, S. I. H., and C. S. Goodwin. 1988. Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. *J. Infect. Dis.* **157**:465-471.
- Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**:1311-1315.
- Mobley, H. L. T., M. J. Cortesia, L. E. Rosenthal, and B. D. Jones. 1988. Characterization of urease from *Campylobacter pylori*. *J. Clin. Microbiol.* **26**:831-836.
- Mobley, H. L. T., and R. P. Hausinger. 1989. Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* **53**:85-108.
- Oudbier, J. H., W. Langenberg, E. A. J. Rauws, and C. Bruin-Mosch. 1990. Genotypical variation of *Campylobacter pylori* from gastric mucosa. *J. Clin. Microbiol.* **28**:559-565.
- Rodriguez, P., A. Vekris, B. de Barbeyrac, B. Dittl, J. Bonnet, and C. Bebear. 1991. Typing of *Chlamydia trachomatis* by restriction endonuclease analysis of the amplified major outer membrane protein gene. *J. Clin. Microbiol.* **29**:1132-1136.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Skirrow, M. B. 1977. *Campylobacter* enteritis: a new disease. *Br. Med. J.* **2**:9-11.
- Starnbach, M. N., S. Falkow, and L. S. Tompkins. 1989. Species-specific detection of *Legionella pneumophila* in water by DNA amplification and hybridization. *J. Clin. Microbiol.* **27**:1257-1261.
- Tyler, S. D., W. M. Johnson, H. Lior, G. Wang, and K. R. Rozee. 1991. Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **29**:1339-1343.
- Valentine, J. L., R. R. Arthur, H. L. T. Mobley, and J. D. Dick. 1991. Detection of *Helicobacter pylori* by using the polymerase chain reaction. *J. Clin. Microbiol.* **29**:689-695.