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PCR-Based Restriction Fragment Length Polymorphism Typing of *Helicobacter pylori*

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We applied a molecular typing approach for *Helicobacter pylori* that uses restriction fragment length polymorphism (RFLP) analyses of an 820-bp PCR-amplified portion of the *ureC* gene in *H. pylori*. The PCR products were digested with restriction enzyme *Hha*I, *Mbo*I, or *Mse*I, and the fragments generated were analyzed by agarose electrophoresis. Among 25 independent clinical isolates, each showed a different pattern when a combination of the three RFLP patterns was used. Using this method, we studied isolates from the antrum or the body of the stomach of 14 patients before and after antibiotic therapy. Before treatment, successful isolation of *H. pylori* from the two sites of the stomach was possible for 12 of the 14 patients. For 10 of these 12 patients, each pair of isolates had identical RFLP profiles. For the other two patients (16.7%), however, isolates from the antrum and the body of the stomach had different RFLP profiles. Treatment was successful for 6 of the 14 patients; of the 8 patients with treatment failures, 5 had identical isolate pairs. In each case, the isolates found posttreatment were the same as the pretreatment isolates. For one of the patients who was colonized with two different isolates pretreatment, one of the isolates was identified at both sites after unsuccessful treatment. We also studied six long-term follow-up patients who had sequential biopsies at intervals of up to 5 months. Each follow-up isolate from each patient had the same RFLP profile as the initial isolate. This typing method provides a reliable and reproducible typing scheme for the study of *H. pylori* infections and indicates that infection with more than one *H. pylori* isolate is not rare.

There is mounting evidence that *Helicobacter pylori* plays a significant role in the etiology of chronic superficial gastritis (14), duodenal ulcers (3, 13), and gastric cancer (6). However, little is known about the transmission of *H. pylori* infections, in part because of the absence of a sensitive, reproducible, and widely used typing scheme for *H. pylori*. A rapid and simple method for subclassifying *H. pylori* strains is urgently required.

A variety of conventional typing schemes, based on hemagglutination (9), enzymatic activity (15), cytotoxin activity (7), plasmid profiles (19, 22), sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins (15), and immunoblot studies (4), have been used for *H. pylori* isolates; however, each of these methods has important limitations.

Molecular techniques also have been applied to the identification of *H. pylori* isolates. Restriction endonuclease analysis of genomic DNA has been found to be highly discriminatory in that *H. pylori* isolates from different patients all exhibit unique profiles (11, 12). This method has illustrated the considerable genomic diversity that exists within *H. pylori*, although the patterns produced can be complex and difficult to interpret. Simpler fingerprint patterns can be obtained for *H. pylori* by the use of rRNA gene probes and Southern hybridization (ribotyping) (18) to identify restriction fragment length polymorphisms (RFLPs), but the method is time-consuming and technically more demanding.

Since the PCR can selectively amplify the copy number of a target sequence more than 10^6-fold within hours, PCR-based typing systems for *H. pylori* have been developed. The genes encoding urease and its accessory proteins have been targets for PCR because these genes are conserved in *H. pylori*. A variety of different fragments (*ureA-ureB* [2.4 kb] and *ureC-ureD* [1.7 kb] [1], *ureA-ureB* [2.4 kb] [8], *ureC* [1.1 kb] [16], and *ureB* [933 bp] [5]) have been used to differentiate isolates.

We report here a PCR-based typing scheme for *H. pylori* that uses RFLP analyses of an 820-bp region of *ureC* (10). Using this typing method, we examined multiple isolates from patients with gastritis to determine whether DNA heterogeneity is present in organisms found at different anatomical sites and whether relapse after therapy represents reinfection by a different isolate or recrudescence. We found that this method represents a simple and sensitive tool for differentiating or confirming the identities of clinical isolates.

MATERIALS AND METHODS

Bacterial strains. The 80 clinical isolates of *H. pylori* that were used in this study had been obtained by biopsies from 25 patients with gastritis at the time of endoscopic examination. In brief, 39 isolates were from 14 patients who had biopsies as part of an investigation of the treatment of *H. pylori* infections with a single antimicrobial agent (clarithromycin) (20). For these 14 patients, who were enrolled at the University of Virginia Hospital, biopsy samples were taken from both the antrum and the body of the stomach before and 4 weeks after the end of a 14-day course of the treatment. Another 39 isolates were obtained by sequential biopsies (40 to 150 days between the initial biopsy and each follow-up biopsy) from 10 patients with gastritis at Vanderbilt University Hospital and for whom therapy with bismuth

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subsalicylate and metronidazole had been used. The remaining two isolates were obtained from a human volunteer 11 and 463 days after *H. pylori* injection; these isolates had previously been shown to have identical protein, lipopolysaccharide, and restriction endonuclease profiles (17). The isolates, which all were confirmed as *H. pylori* (20), were stored at −70°C until examined.

Boiled supernatant of bacterial cells. *H. pylori* isolates were grown to stationary phase on Trypticase soy agar with 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) at 37°C under microaerobic conditions with the Campypak system (BBL). Three 1-μl loopfuls of bacteria were mixed well with 0.5 ml of sterile double-distilled water in a 1.7-ml microcentrifuge tube, and the mixture was heated for 10 to 15 min on a heat block at 95°C. The bacteria were sedimented by centrifugation, and the supernatant was removed for testing.

PCR. The oligonucleotides used as PCR primers were derived from the known sequence of *ureC*, which encodes a required accessory protein for urease expression (10). The amplification product of the forward (5'TGGGACTGATG GCGTGAGGG) and reverse (5'AAGGGCGTTTTTAGATT TTT) primers was 820 nucleotides in length and represented an internal portion of *ureC* beginning 12 nucleotides downstream from the ATG start codon. PCR amplification was performed with a 50-μl reaction volume by use of a model N801-0150 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). A mixture of 30 μl of the boiled supernatant as a DNA template and 40 pmol of each primer was first incubated for 5 min at 95°C and then kept at 80°C for the addition of the reagent mixture (1.25 U of GeneAmp *Taq* polymerase, 10× PCR buffer I [Cetus], and 10 pmol of each deoxynucleotide). The reaction included an initial denaturation of target DNA at 94°C for 1 min and then 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The final cycle used 10 min at 72°C to ensure full extension of the product. To examine for the homogeneity and yield of the *ureC* amplicon, 5 μl of the PCR product was electrophoresed on a 1.5% agarose gel (electrophoresis grade; Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 1× Tris-acetate-EDTA (TAE) buffer containing 1 μg of ethidium bromide per ml.

RFLP analysis. A 10-μl sample of the PCR product was digested with 10 U of restriction enzyme *Hha*I, *Mbo*I, or *Mse*I (New England Biolabs, Beverly, Mass.) for 3 h at 37°C in the buffer recommended by the supplier. The digest was analyzed by electrophoresis with a microgel system (model C2; Owl Scientific, Cambridge, Mass.) that uses a 5% low-melting-temperature gel (SeaPlaque; FMC Bioproducts, Rockland, Maine) containing 1 μg of ethidium bromide per ml. A 100-bp DNA ladder and a 10-bp DNA ladder (GIBCO BRL, Gaithersburg, Md.) were used as standards for molecular size determinations. The gel was run at 70 V with TAE buffer for 1 to 2 h until the dye front reached a point 3 cm (*Mse*I) or 4 cm (*Hha*I and *Mbo*I) from the wells. The gel was then examined by transillumination and photographed.

RESULTS

The PCR protocol successfully amplified 820-bp products from boiled supernatants obtained from all 25 *H. pylori* isolates initially examined (data not shown). From the restriction enzyme digestion of these PCR products, we found 10, 10, and 11 different patterns with *Hha*I, *Mbo*I, and *Mse*I, respectively (Fig. 1). These *H. pylori* isolates from 25 unrelated patients were classified on the basis of combinations of these three independent RFLP patterns to test the usefulness of the typing method. Combinations of the restriction enzyme digestion patterns obtained for the *ureC* gene segment with *Hha*I, *Mbo*I, and *Mse*I divided these 25 isolates into 25 distinct RFLP patterns (Table 1).

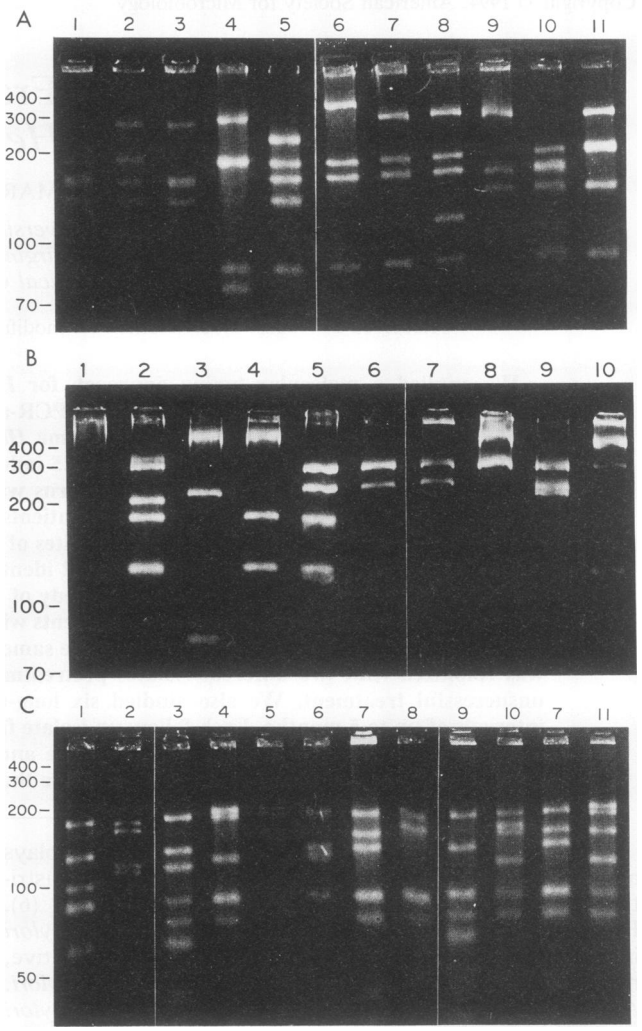

FIG. 1. Representative restriction endonuclease digests of the PCR-amplified, 820-bp *ureC* region of *H. pylori*. Molecular size markers (base pairs) are shown at left. (A) *Hha*I digests (11 patterns). (B) *Mbo*I digests (10 patterns). (C) *Mse*I digests (11 patterns; pattern 7 is shown twice).

Next, using this method, we examined all of the isolates from the 14 patients in the treatment study, including those from the gastric antrum or body, before and after antibiotic therapy (Table 2). Before treatment, *H. pylori* was successfully isolated from both sites of the stomach in 13 of the 14 patients, and 25 of the 26 isolates were recovered for our tests. For 10 of the 12 patients who were evaluable, each pair of isolates had identical RFLP profiles. In contrast, for the other two patients (214 and 216), isolates from the antrum and from the body had completely different RFLP profiles.

Treatment was successful for 6 of the 14 patients. Of 15 isolates obtained from patients failing therapy, 7 were resistant to clarithromycin. For five of the eight patients with unsuccessful treatment, isolates from both biopsy sites were available for analysis; for each patient, both isolates had

TABLE 1. Restriction enzyme digestion profiles of 25 *H. pylori* isolates from 25 different patients with gastritis

Strain	Restriction pattern number		
	*Hha*I	*Mbo*I	*Mse*I
93-36	1	1	9
93-39	5	3	3
93-41	10	4	6
93-42	5	2	8
93-43	1	5	4
93-45	5	10	6
93-48	1	2	3
93-57	10	6	9
93-60	3	6	9
93-157	1	6	5
93-159	4	5	5
93-161	4	4	9
93-171	3	10	9
93-175	5	4	1
93-190	1	7	2
93-191	2	8	9
93-192	6	7	10
93-193	7	7	10
93-194	8	8	7
93-195	8	10	7
93-196	1	9	11
93-197	1	2	7
93-198	9	8	7
93-199	1	7	5
93-200	10	8	9

identical RFLP profiles. For each patient, the isolates found posttreatment had the same profiles as the pretreatment isolates. Of note is that patient 216 was colonized with two different isolates before treatment. After unsuccessful treatment, only one isolate, the isolate originally from the body of the stomach, was identified in both biopsy sites.

We also examined isolates obtained by sequential biopsies from six patients in the long-term follow-up study, who had biopsies between 40 and 150 days apart (Table 3). Two of these patients had *H. pylori* isolated on two occasions, one patient had the organism isolated on three occasions, and three patients had the bacterium recovered from four consecutive biopsies over a period of 7 months. For all six patients, the RFLP profiles of the *H. pylori* isolates from both sites of the stomach for both the initial and the follow-up biopsies were identical (Table 3). The two isolates obtained 452 days apart from the human volunteer showed identical (10-8-9) profiles.

DISCUSSION

Although molecular biological typing methods using genomic DNA or gene probes usually require laborious sample preparation and processing, the use of the *ureC* PCR product for restriction digestion analysis does not require high concentrations of chromosomal DNA, and the amount of the PCR product is sufficient for a number of restriction digests. That supernatants from boiled cells rather than purified chromosomal DNA can be used as templates allows this to be a relatively simple and rapid technique. For PCR, it is often difficult to reproducibly amplify products of >1 kb; thus, we sought to use a fragment sufficiently large to permit the detection of diversity but small enough that amplification could be regularly achieved. In this study, RFLP analysis of PCR products derived from boiled cell supernatants showed that *H. pylori* is highly diverse in genomic structure (12), even when the 820-bp fragment of the highly conserved *ureC* gene is used. The results presented here demonstrate that our typing scheme for *H. pylori* is a simple and reliable method because it is only necessary to amplify a single product before endonuclease digestion.

Using this method, we showed that most patients appear to harbor a single isolate of *H. pylori* in their stomach, but 2 (10%) of the 20 patients were concurrently infected with at least two isolates. This datum is consistent with the observation of Prewett et al. of two isolates of *H. pylori* colonizing the gastroduodenal mucosa of 2 (13%) of 15 patients (21). Fox et al. (7a) reported that of their 11 patients, three distinct strains were found in isolates from 1 patient and two strains were found from each of 4 patients. Beji et al. also described one patient who had three different DNA patterns for *H. pylori* organisms cultured from three biopsy specimens obtained during a single endoscopy (2). These data indicate that some patients with gastritis are infected by multiple isolates of *H. pylori*. Examination of larger numbers of isolates from individual patients will permit a better quantitation of the extent of multiple infections. That the paired pre- and posttreatment *H. pylori* isolates had identical RFLP profiles further confirmed that short-term infection recurrence represents relapse. After unsuccessful treatment, the patient who was colonized with two different isolates before treatment had only one isolate, which was originally from the body of the stomach, in both biopsy sites. While this phenomenon may be caused by sampling error, persistence in the body might be a mechanism for therapeutic failure.

The long-term follow-up study showed that all isolates from the same patient yielded identical RFLP profiles, regardless of the time interval between biopsies. These data

TABLE 2. Restriction enzyme digestion profiles of *H. pylori* clinical isolates from 14 patients with gastritis

Phase of treatment and biopsy site	Restriction profile[a] of isolates from patient													
	201	202	203	206	207	208	209	210	212	213	214	215	216	217
Pretherapy														
Antrum	1-1-9	1-2-3	3-6-9	5-3-3	10-4-6	5-2-8	1-5-4	1-6-5	5-8-6	10-6-9	5-10-6	4-5-5	4-4-9	5-4-1
Body	1-1-9	1-2-3	3-6-9	5-3-3	10-4-6	5-2-8	1-5-4	NG[b]	5-8-6	10-6-9	3-8-9	ND[c]	11-4-9	5-4-1
Posttherapy														
Antrum	1-1-9	1-2-3	3-6-9	NG	NG	NG	NG	1-6-5	5-8-6	NG	NG	4-5-5	11-4-9	5-4-1
Body	ND	NG	3-6-9	NG	NG	NG	NG	1-6-5	5-8-6	NG	NG	ND	11-4-9	5-4-1

[a] Profiles are derived from the restriction patterns of the PCR-amplified 820-bp *ureC* region as shown in Fig. 1. The first number in the profile represents the *Hha*I pattern, the second number represents the *Mbo*I pattern, and the third number represents the *Mse*I pattern.
[b] NG, no growth from the biopsy specimen.
[c] ND, the isolate originally grew from the specimen but could not be recovered for further testing.

TABLE 3. Restriction enzyme digestion profiles of *H. pylori* clinical isolates from six gastritis patients in a long-term follow-up study

Patient	Interval between biopsies (days)	Restriction profile[a] of isolates from:	
		Antrum	Body
1	0	1-7-2	1-7-2
	70	1-7-2	1-7-2
	143	1-7-2	1-7-2
	233	1-7-2	1-7-2
2	0	6-7-10	6-7-10
	64	6-7-10	6-7-10
	140	6-7-10	6-7-10
	239	6-7-10	ND[b]
3	0	8-10-7	8-10-7
	52	8-10-7	8-10-7
	119	8-10-7	8-10-7
	216	8-10-7	8-10-7
4	0	NG[c]	8-8-7
	40	8-8-7	8-8-7
	190	8-8-7	8-8-7
5	0	9-8-7	9-8-7
	74	9-8-7	9-8-7
6	0	1-7-11	NG
	85	1-7-11	1-7-11

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *c*.
[c] See Table 2, footnote *b*.

indicate that an *H. pylori* infection with a single isolate was stable and persisted for at least 7 months in this population; the study with the human volunteer extended this observation.

We conclude that the method reported is useful for epidemiological studies of *H. pylori* infections and for clinical applications. In particular, these would include monitoring treatment regimens to differentiate between a new infection or recrudescence, to examine the persistence of individual isolates during long-term carriage and antibiotic therapy, to identify the frequency of coinfection, and to characterize anatomical distribution of isolates.

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