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NOTES

Molecular Typing of *Helicobacter pylori* Isolates from a Multicenter U.S. Clinical Trial by *ureC* Restriction Fragment Length Polymorphism

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The molecular typing of 81 pretreatment *Helicobacter pylori* isolates and the comparison of 18 pretreatment-posttreatment pairs is described by restriction fragment length polymorphism (RFLP) of the *ureC* gene. The results of our study show the extreme genomic diversity of *H. pylori* and indicate that infection by *H. pylori* in the United States does not appear to be limited to a small number of RFLP types.

Helicobacter pylori has been recognized as an etiologic agent for gastritis and duodenal ulcers in humans. A multicenter double-blinded clinical trial was conducted in the United States from 1993 to 1994 to examine the efficacy of clarithromycin, omeprazole, and clarithromycin with omeprazole for the treatment of duodenal ulcers. Patients with confirmed duodenal ulcer disease were randomly assigned to one of three treatment groups: omeprazole only (omeprazole 40 mg once a day [q.d.] for 14 days [days 1 to 14]), clarithromycin only (clarithromycin 500 mg three times a day [t.i.d.] for 14 days [days 1 to 14]), or clarithromycin and omeprazole (clarithromycin 500 mg t.i.d. and omeprazole 40 mg q.d. for 14 days [days 1 to 14] plus omeprazole 20 mg q.d. for an additional 14 days [days 15 to 28]). Gastric biopsies taken before and after treatment were cultured for *H. pylori*. The large number of isolates presented an opportunity to investigate the distribution of strain types associated with active ulcer disease in the United States. In this study we describe the molecular typing of 81 pretreatment isolates and the comparison of pretreatment-posttreatment pairs of isolates from 18 patients by a previously described method, *ureC* PCR-restriction fragment length polymorphism (RFLP) typing (1, 6). The 81 patients were unrelated and lived in different regions throughout the United States.

Clinical *Helicobacter* strains were isolated as described by Versalovic et al. (12). Susceptibility testing was performed by the agar dilution method as previously described (7).

The primers and conditions for *ureC* amplification were those used by Fujimoto et al. (6). Presence of *vacA* was determined by amplification with primers 1 and 2 as described by Cover et al. (4). Presence of *cagA* was determined by amplification with the following primers: 5′-AACGCTGTCGCTTC ATACG (forward) and 5′-TCTGCTTTTTCTTTGTCAT (reverse), amplifying 374 bp from 1173 to 1528 (11). Primers were chosen with Oligo 4.0 (NBI, Plymouth, Minn.) by using sequence deposited in GenBank.

Boiled bacterial lysates were prepared as described by Fujimoto et al. (6). One microliter of lysate was used in the amplification reaction with PCR Supermix (Gibco-BRL, Gaithersburg, Md.) according to the manufacturer's protocol on a Perkin-Elmer 9600 thermocycler (Perkin-Elmer, Foster City, Calif.).

Following completion of the amplification, 5 μl of product was examined by electrophoresis on a 2% agarose (Gibco BRL) gel with 1× Tris–acetate–EDTA (1× TAE) (9). The *ureC* product was 820 bp, the *vacA* product was 631 bp and the *cagA* product was 374 bp.

For RFLP typing, the *ureC* product was digested with either *Sau*3A or *Hha*I restriction enzyme in the supplied buffer according to the manufacturer's protocol (Gibco-BRL). Restriction products were separated on a 3% Metaphor agarose (FMC Bioproducts, Rockland, Me.) gel in 1× TAE (70 V for 2 h) or on a 12% nondenaturing polyacrylamide gel (9). The gel was stained with ethidium bromide and examined on a UV light box. Fragment sizes were determined by comparison with a 100- or 123-bp ladder (Gibco-BRL).

All strains tested gave the expected 820-bp *ureC* product (data not shown). *ureC* is an open reading frame which is not required for urease activity or expression and which is located upstream of the *ure* operon (5). Digestion of the product with *Sau*3A resulted in 1 to 5 fragments ranging in size from 820 to 75 bp in length. *Hha*I digestion resulted in smaller fragment sizes ranging from 380 to 75 bp in length. Representative restriction patterns are shown in Fig. 1. The 81 pretreatment strains gave a total of 17 *Sau*3A patterns (A to Q) and 11 *Hha*I patterns (A to K). Small variations (≤5 bp) in the size of a restriction fragment were not considered a different pattern. A unique combination of a *Sau*3A and a *Hha*I pattern was called an RFLP type. The 81 strains were divided into 44 RFLP types. As shown in Fig. 2, the majority of types had only 1 to 2 strains and three types had a small cluster of strains: type 2 with eight strains and types 5 and 7 with five strains.

In two cases the total size of the restriction fragments added up to twice the size of the undigested PCR product. This would be expected to occur if a patient were infected with two strains

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TABLE 1. Typing comparison of pretreatment and posttreatment isolates

Patient no.	Treatment status[a]	Treatment[b]	*Sau*3A pattern[c]	*Hha*I pattern	PCR-RFLP type	Clarithromycin MIC (μg/ml)
3	Pre	Clari	L	A	9	0.06
3	Post	Clari	L	A	9	32
3	1 mo	Clari	L	A	9	32
3	3 mo	Clari	L	A	9	0.03
3	6 mo	Clari	L	A	9	0.06
43	Pre	Clari	C	A	4	0.008
43	1 mo	Clari	C	A	4	0.008
55	Pre	Clari	A	A	7	0.008
55	Post	Clari	A	A	7	0.008
58	Pre	Clari	A	A	7	0.03
58	1 mo	Clari	A	A	7	0.03
92	Pre	Clari	B	A	5	0.03
92	Post	Clari	B	A	5	2
92	1 mo	Clari	B	A	5	4
92	3 mo	Clari	B	A	5	4
119	Pre	Clari	A	Q	11	0.03
119	1 mo	Clari	A	Q	11	64
125	Pre	Clari	B	A	5	0.06
125	Post	Clari	B	A	5	8
167	Pre	Clari	M	A	38	0.008
167	Post	Clari	M	A	38	16
288	Pre	Clari	D	C	16	0.03
288	Post	Clari	D	C	16	16
288	1 mo	Clari	D	C	16	16
288	3 mo	Clari	D	C	16	8
288	6 mo	Clari	D	C	16	16
399	Pre	Clari	Mixed	A	19	0.06
399	Post	Clari	Mixed	A	19	0.06
116	Pre	Clari + omep	C	A	4	0.03
116	Post	Clari + omep	C	A	4	32
132	Pre	Clari + omep	B	A	5	0.03
132	Post	Clari + omep	B	A	5	128
138	Pre	Clari + omep	M	J	40	0.015
138	1 mo	Clari + omep	M	J	40	16
80	Pre	Omep	P	E	41	0.12
80	Post	Omep	P	E	41	0.06
117	Pre	Omep	D	A	2	16
117	Post	Omep	D	A	2	16
134	Pre	Omep	M	Q	70	0.03
134	1 mo	Omep	M	Q	70	0.06
170	Pre	Omep	M	A	38	0.06
170	Post	Omep	B	E	3	0.06
194	Pre	Omep	D	A	2	0.06
194	Post	Omep	D	A	2	0.06

[a] Pre, pretreatment; post, posttreatment. Numbers of months are months posttreatment.
[b] Clari, clarithromycin only; omep, omeprazole only; clari + omep, clarithromycin and omeprazole. See text for dosing regimen.
[c] Restriction fragment pattern. Mixed, more than one strain present (see text for discussion).

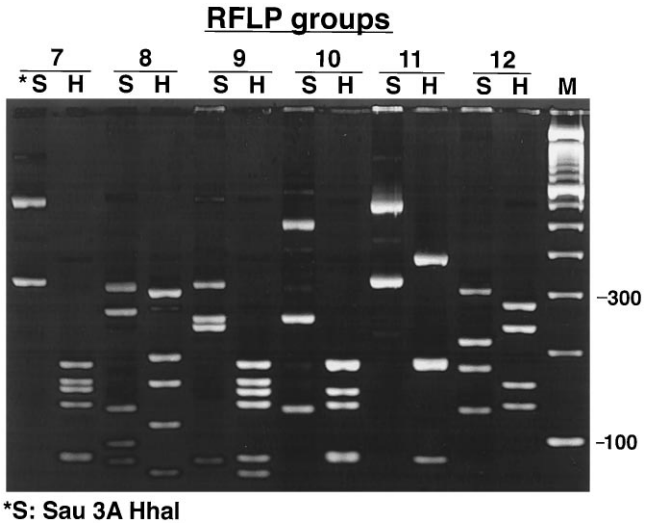

FIG. 1. Six clinical isolates showing RFLP types 7 to 12. The restriction patterns shown for *Sau*3A (S) and *Hha*I (H), respectively, are: type 7, AA; type 8, DB; type 9, LA; type 10, JD; type 11, AQ; and type 12, BG. Lane M, 100-bp ladder (Gibco BRL).

that had different RFLP patterns (8). Only 2 of 81 patients had apparent mixed infections, suggesting that in the United States patients are typically colonized with a single strain although mixed infections can occur.

The pretreatment RFLP type was compared with the posttreatment RFLP type in 18 cases of treatment failure from the three treatment groups (Table 1). In all but one patient, the same RFLP type was present following treatment. For three patients, from whom four or five sequential isolates over a 3- to 6-month period were examined, the *ureC* pattern remained unchanged throughout the study period. The strain from patient 3 developed resistance to clarithromycin posttreatment and then reverted to being susceptible 3 months after treatment even though the RFLP type remained constant. This patient was described by Versalovic et al. (12). The two resistant posttreatment isolates were shown to have a rRNA point mutation associated with macrolide resistance which was not present in the susceptible pre- and posttreatment isolates. Versalovic proposed that either the mutation was unstable or a resistant subpopulation was unable to compete for growth with the wild-type population after treatment had been concluded (12).

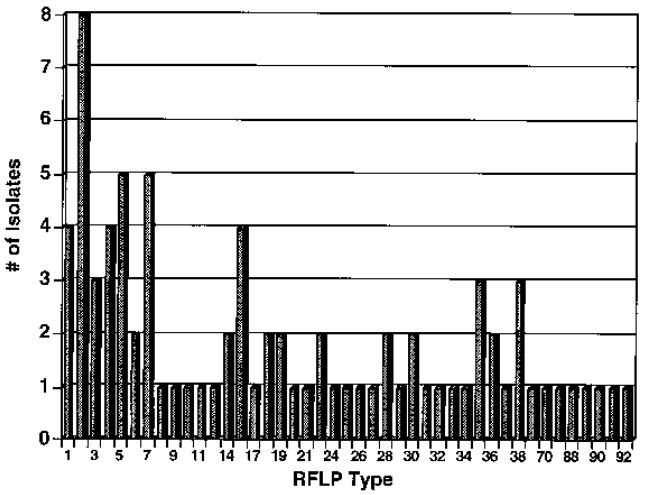

FIG. 2. Distribution of *ureC* RFLP types. Eighty-one pretreatment *H. pylori* isolates were divided into 44 RFLP types by using combinations of *Sau*3A and *Hha*I restriction digestion patterns of an 820-bp *ureC* product. Not all 92 types shown in the graph are represented in this study; some types were assigned to isolates from other clinical trials (10).

The *cagA* gene, which encodes the CagA antigen, has been associated with a more virulent phenotype in *H. pylori* (2, 3). As this group of isolates came from patients with active duodenal ulcers, the presence of *cagA* was determined. In 81% of the isolate lysates, a product of the expected size was amplified with *cag*-specific primers. The presence of *vacA*, the vacuolating cytotoxin gene, was also investigated, using primers in a conserved region of the gene (4). A total of 94% of the isolates had an amplified product of the expected size for that region of the *vacA* gene. Production of cytotoxin was not examined.

Our data suggest that ulcers in the United States are not caused by a particular *ureC* RFLP type. Infection by multiple strains also seems to be uncommon in this country, although it is impossible to say that mixed cultures were not overlooked in the extensive incubation and subculturing steps prior to typing. We also observe that treatment failure is not generally due to reinfection with a different strain but rather to the persistence of a single strain, whether through development of antibiotic resistance, lack of patient compliance, or other unknown reasons for treatment failure.

Our results are consistent with previously published work suggesting that $cagA^+$ strains are likely to be associated with active disease (3).

In summary, *ureC* PCR RFLP is a rapid and useful method for comparison of *H. pylori* strains isolated before and after clarithromycin treatment from a single patient as well for comparison of isolate relatedness in large-scale clinical trials.

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