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PCR-RFLP typing of *ureC* from *Helicobacter pylori* isolated in Argentina from gastric biopsies before and after treatment with clarithromycin

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SUMMARY

A clinical trial was conducted in Argentina to determine the efficacy of clarithromycin plus lansoprazole for the treatment of *Helicobacter pylori* in duodenal ulcers and non-ulcer dyspepsia. PCR–RFLP was conducted on an 820-bp amplified product of the *ureC* gene of *H. pylori* to determine the genetic heterogeneity of 83 pretreatment and 21 post-treatment isolates. Twelve different restriction patterns were observed when digested with *Sau* 3A or *Hha* I, resulting in 40 different RFLP types. Comparison of isolates before treatment to after treatment showed that 20 of 20 patients had the same RFLP type. In addition, the presence of the cytotoxin-associated gene (*cagA*) and the vacuolating gene (*vacA*) were determined. All pretreatment isolates were positive for *vacA* whereas 75% of the pretreatment isolates were positive for *cagA*. The results of this study indicate that a high degree of heterogeneity exists among *H. pylori* and that infection is not limited to a small number of RFLP types.

INTRODUCTION

The association between *Helicobacter pylori* and chronic gastritis and peptic ulcers [1, 2] is well established. Studies have also shown that eradication of an *H. pylori* infection cures duodenal ulcers [3] and reduces the risk of adenocarcinoma [4]. However, recurrence of ulcers after the apparent eradication of *H. pylori* has been reported in many cases [5, 6].

Conventional typing methods based on cytotoxin [7], plasmids [8], and haemagglutination [9] have all been used for epidemiologic studies of *H. pylori*. A number of different molecular typing techniques also have been shown to distinguish strains of *H. pylori*. Pulsed-field gel electrophoresis [10] and ribotyping using RFLP of rRNA genes as targets for hybridization [11] have been used with success. However, a high degree of genomic diversity exists in *H. pylori* [10] making it difficult to type by molecular methods.

PCR-based techniques offer a simple and rapid method for the analysis of genetic relatedness [5, 12–14]. In this study, we utilized a simple PCR–RFLP typing method of an 820-bp region of the *ureC* gene from *H. pylori*. Isolates of *H. pylori* from patients suffering from duodenal ulcers or dyspepsia were analysed before and after treatment with clarithromycin and lansoprazole for DNA heterogeneity to determine the diversity of strains causing disease and if reinfection occurs by a different strain or recrudescence occurs after treatment. In addition to PCR-RFLP typing, we analysed the *H. pylori* isolates for the presence of two genes reported to be associated with pathogenesis, *cagA* and *vacA* [7, 15].

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MATERIALS AND METHODS

Patient population and treatment

Patients with confirmed active duodenal ulcers and non-ulcer dyspepsia from different localities in Argentina were entered into a single-blind, randomized, multicentre study sponsored by Abbott Laboratories. Biopsies were performed prior to treatment and 4–6 weeks after completion of treatment for examination. Patients were randomly assigned to receive either clarithromycin (500 mg t.i.d.) with lansoprazole (60 mg q.d.) for 14 days or clarithromcyin (500 mg t.i.d.) with lansoprazole (20 mg b.i.d.) for 14 days.

Isolation of *H. pylori*

Antrum mucosal biopsies were frozen at −70 °C in 1 ml of Brucella broth (Difco Laboratories, Detroit, MI) containing 20% glycerol and shipped overnight to the laboratory for culture. Each specimen was minced and a suspension was made in 0·1 ml Brucella broth (Difco Laboratories). The suspension (50 μl) was streaked onto Skirrow agar plates (BBL Microbiology Systems, Cockeysville, MD) and brain heart infusion agar (Difco Laboratories) containing 7% defibrinated horse blood. Incubation was under microaerophilic conditions at 37 °C and 12% CO_2 for 3–10 days. Isolates were confirmed as *H. pylori* based on colony morphology, Gram-stain, and production of catalase, oxidase, and urease. Cultures were frozen at −70 °C in 10% skim milk containing 20% glycerol.

Preparation of DNA

DNA was extracted from *H. pylori* as previously described [14]. Briefly, *H. pylori* was cultured on sheep blood agar plates at 37 °C containing 12% CO_2. A loopful of bacteria was suspended in 50 μl of sterile double-distilled water in a 0·2 ml thin-walled thermocycle tube. The suspension was heated for 15 min at 95 °C in a thermocycler. Cellular debris was sedimented by centrifugation and the supernatant was removed for testing.

DNA amplification

Oligonucleotides used for amplification of the *ureC* gene in this study were previously described [14]. The primers were derived from the published sequence of the *ureC* gene, which encodes an accessory protein for urease expression [14] and results in an 820 bp amplified product. Primers for amplification of a 631-bp product of the *vacA* gene were previously described [16]. Primers for amplification of a 506-bp product of the *cagA* gene (5′-GATCTCGGTGGGTCTTTCC, cognate to residues 3990–4009 and 5′-TCTTTTA-CGGCATTGTTCA cognate to residues 4495–4477) were derived from the published sequence [15]. The amplification mixture (50 μl) consisted of 20 mM Tris-HCl (pH 8·4), 50 mM KCl, 1·5 mM $MgCl_2$, 100 μM each of the four deoxynucleoside triphosphates, 1·0 μM each primer, and 2·5 U of *Taq* DNA polymerase (Gibco-BRL, Gaithersburg, MD). Amplification was conducted on a DNA thermocycler (model 9600; Perkin-Elmer, Foster City, CA), which included an initial denaturation of DNA at 94 °C for 2 min and then 40 cycles of 15 s at 94 °C, 30 s at 45 °C, and 30 s at 72 °C, followed by a 10 min extension period at 72 °C. Amplified products (10 μl) were visualized electrophoretically in ethidium bromide-stained, 2% agarose gels.

RFLP analysis

A 40-μl aliquot of the PCR product was removed and ethanol precipitated. The DNA pellet was resuspended in 20 μl of water. A 10-μl aliquot was then removed and digested with 10 U of either *Sau* 3A (Gibco-BRL) or *Hha* I (Gibco-BRL) restriction enzymes as recommended by the manufacturer. Digests (30 μl) were analysed in 3% metaphor agarose (FMC Bioproducts). A 100 bp DNA ladder and a 1 kb DNA ladder were used as molecular weight standards. Gels were run at 70 V in TAE buffer for 4–5 h.

Statistical analysis

Statistical analysis was done by use of chi-square analysis to determine the association of *cagA*+ strains with treatment failure and the association of *cagA*+ strains with ulceration [17].

RESULTS

Recovery of *H. pylori*

A total of 83 isolates of *H. pylori* were recovered from gastric biopsies of 88 patients prior to treatment with clarithromycin and lansoprazole for a 94% recovery rate. Of the 83 isolates of *H. pylori*, 11 were recovered from patients with confirmed duodenal ulcers and 72 were recovered from patients with non-ulcer dys-

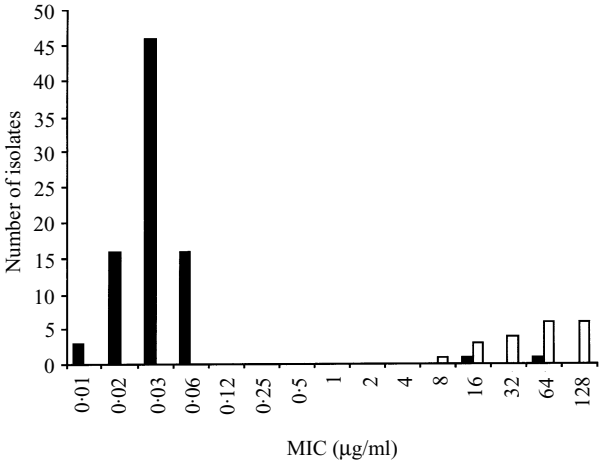

Fig. 1. MIC distribution of clarithromcyin to *H. pylori* before (■) and after (□) treatment.

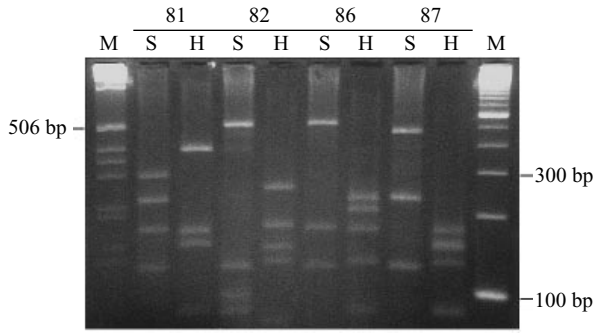

Fig. 2. Representative restriction endonuclease digests of the 820-bp amplified product of the *ureC* gene from *H. pylori*. PCR–RFLP types are indicated by numerals. (M) Molecular size standards. (S) *Sau* 3A digests. (H) *Hha* I digests.

pepsia. A total of 81 (98%) of the pretreatment isolates were susceptible to clarithromycin (Fig. 1). *H. pylori* was unrecoverable from 5 of the gastric biopsies prior to treatment. Of the 5 culture negative pretreatment biopsies, 1 isolate was recovered from the corresponding post-treatment biopsy but unrecoverable from the other 4 post-treatment biopsies. A total of 21 isolates were recovered from biopsies of 78 patients after treatment for a 27% recovery rate. Of the 21 isolates, 3 were recovered from patients with duodenal ulcers and 18 were recovered from patients with dyspepsia. All the isolates recovered from post-treatment biopsies were shown to be resistant to clarithromycin (Fig. 1).

Analysis of *ureC* amplified product

An 820-bp amplified product from the *ureC* gene was successfully obtained from all the *H. pylori* isolates examined. The amplified product was digested with

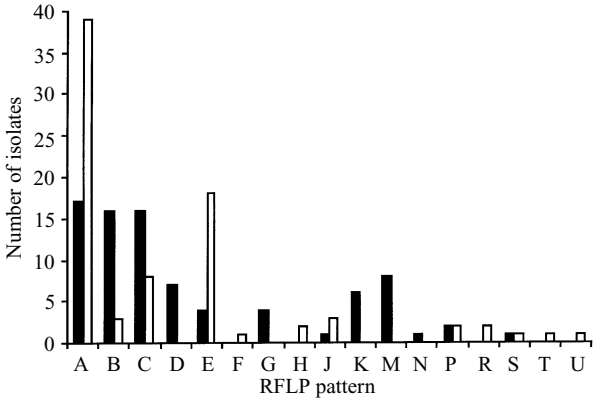

Fig. 3. Distribution of restriction endonuclease patterns of the 820-bp amplified product of the *ureC* gene from *H. pylori* isolated prior to treatment. Patterns from each restriction enzyme were assigned alphabetically. Patterns were not consecutively lettered because some patterns were not observed in this study but were observed in previous studies (unpublished observations). ■, *Sau* 3A; □, *Hha* I.

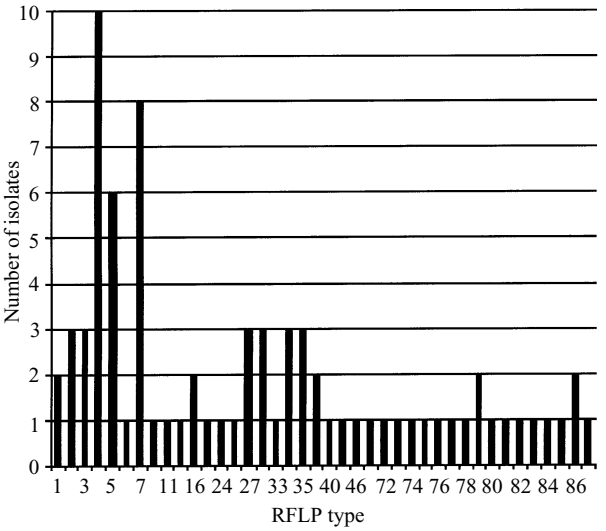

Fig. 4. Distribution of PCR–RFLP types of 820-bp amplified product of the *ureC* gene from *H. pylori*. Each type was a combination of restriction patterns and assigned numerically. Types were not numbered consecutively because some types were not observed in this study but were observed in previous studies (unpublished observations).

Sau 3A and *Hha* I to determine the genetic heterogeneity between the pretreatment and post-treatment isolates (Fig. 2). A total of 12 different restriction patterns were observed when the product was digested with *Sau* 3A and 12 different patterns were also observed with *Hha* I (Fig. 3). In combining the restriction patterns, 40 different RFLP types were observed for pretreatment isolates and 17 different RFLP types were observed for the post-treatment isolates (Fig. 4). For 20 of 20 patients from where *H.*

Table 1. *Analysis of pre and post-treatment pairs using PCR–RFLP or the* ureC *gene of* H. pylori

Patient	Diagnosis	Treatment status	*Sau* 3A	*Hha* I	PCR–RFLP type	Clarithromycin MIC (μg/ml)
1	Dyspepsia	pre*	K	C	73	0·03
		2 mo†	K	C	73	128
2	Dyspepsia	pre	D	A	2	0·015
		1 mo	D	A	2	16
4	ulcer	pre	D	E	6	0·03
		1 mo	D	E	6	128
7	Dyspepsia	pre	C	A	4	0·03
		2 mo	C	A	4	8
9	Dyspepsia	pre	B	E	3	0·06
		1 mo	B	E	3	> 128
13	Dyspepsia	pre	A	E	27	64
		2 mo	A	E	27	64
14	Ulcer	pre	C	T	75	0·03
		1 mo	C	T	75	64
18	Dyspepsia	pre	A	A	7	0·06
		2 mo	A	A	7	64
29	Dyspepsia	pre	C	A	4	0·03
		2 mo	C	A	4	> 128
37	Dyspepsia	pre	D	C	16	0·03
		2 mo	D	C	16	16
39	Dyspepsia	pre	D	A	2	0·03
		2 mo	D	A	2	64
41	Dyspepsia	pre	N	A	85	0·015
		2 mo	N	A	85	64
42	Dyspepsia	pre	C	A	4	0·03
		2 mo	C	A	4	128
44	Dyspepsia	pre	B	A	5	16
		2 mo	B	A	5	64
48	Dyspepsia	pre	B	R	81	0·03
		2 mo	B	R	81	32
49	Dyspepsia	pre	A	C	1	0·06
		2 mo	A	C	1	128
53	Dyspepsia	pre	M	E	35	0·03
		2 mo	M	E	35	128
64	Ulcer	pre	M	J	40	0·03
		2 mo	M	J	40	16
73	Dyspepsia	pre	A	A	7	0·03
		2 mo	A	A	7	32
94	Dyspepsia	pre	G	J	82	0·015
		2 mo	G	J	82	32

* Strain isolated prior to treatment.
† Strain isolated 1–2 months after completion of treatment.

pylori was recovered from biopsies before and after treatment, each pair had identical RFLP types (Table 1).

Analysis of *cagA* and *vacA*

In addition to PCR–RFLP of the *ureC* gene, the presence of the cytotoxin-associated gene (*cagA*) and the vacuolating gene (*vacA*) was analysed by PCR. A total of 62 of 83 (75%) pretreatment isolates and 14 of 21 (67%) post-treatment isolates were positive for the presence of the appropriate size amplified product of *cagA*. Therefore, no significant difference was observed ($P > 0{\cdot}05$) for the presence of *cagA* between pretreatment and post-treatment isolates of *H. pylori*. Of the 11 isolates from patients with active duodenal ulcers, 10 (91%) were positive for the presence of *cagA* and 61 of 71 (86%) isolates from patients with dyspepsia were positive for the presence of *cagA*. Thus, no significant difference was observed ($P >$

0·05) for the presence of *cagA* between dyspepsia and ulceration. All of the isolates (100%) were positive for *vacA*.

DISCUSSION

In this study, *H. pylori* isolated from patients before treatment and after treatment with clarithromcyin were analysed for: (i) reinfection *vs.* recrudescence, (ii) prevalence of strains in relation to ulceration or dyspepsia, and (iii) presence of genes (*cagA* and *vacA*) related to pathogenesis. An 820-bp amplified product of the *ureC* gene was analysed by restriction digestion as a method to epidemiologically type *H. pylori* isolated from patients before and after treatment.

Analysis of the digested 820-bp amplified product of the *ureC* gene showed the heterogeneity of *H. pylori*. When the digestion patterns were combined, we obtained 40 different types. This is consistent with other reports demonstrating the genetic diversity of *H. pylori*. Fujimoto and colleagues [14] found 25 different isolates were divided into 25 different distinct patterns when the same 820 bp product was digested with 3 different enzymes, *Hha* I , *Mbo* I, and *Mse* I. In a related study, 15 of 21 clinical isolates of *H. pylori* showed different patterns when a 1·1 kbp amplified product of the *ureC* gene was digested with *Alu* I and *Pvu* II [18].

The identification of strains by PCR–RFLP of the *ureC* gene can discriminate between reinfection with a new strain or recrudescence of the original strain that has developed resistance. The present study showed that *H. pylori* isolated from the same patient before and after treatment with clarithromycin and lansoprazole had identical RFLP types. This indicates that recrudescence occurred with the original strain rather than reinfection with a different strain. Other studies have shown that strains of *H. pylori* isolated before and after treatment had identical DNA profiles. Fujimoto and colleagues [14] showed that 10 of 12 patients had identical *ureC* PCR–RFLP types before and after treatment with clarithromcyin. Other methods using random amplified polymorphic DNA [5] or ribotyping [19] have shown similar results.

All 21 of the post-treatment isolates were shown to be resistant to clarithromcyin. Additional antimicrobial data on the isolates before treatment showed that only two of the pretreatment isolates were resistant to clarithromycin, indicating that resistance to clarithromycin developed during treatment. Similar studies have reported that resistance to clarithromcyin developed during treatment. Fujimoto and colleagues [14] recovered 15 isolates of *H. pylori* from 14 patients after treatment with clarithromycin and 7 were shown to be resistant.

There was no relationship found between *ureC* PCR–RFLP type and resistance to clarithromycin. There were 17 different PCR–RFLP types among the 23 resistant strains recovered in this study (2 pretreatment and 21 postreatment). We also found no relationship between *ureC* PCR–RFLP type and ulcer disease or recurrence of ulcers. From the 11 patients with confirmed ulcers, 10 different types were isolated. From the 10 different types, 4 were also found in non-ulcer dyspepsia patients. This would indicate that ulcer disease is not limited to a single or a few *ureC* PCR–RFLP types.

There is evidence to suggest that the presence of cytotoxin-producing strains of *H. pylori* is associated with the ability to cause peptic ulceration [20]. In mice, cytotoxic strains of *H. pylori* have been shown to cause gastric lesions similar to those seen in humans [21, 22]. Nearly all strains have been shown to possess the *vacA* gene [16] but only 50% of *H. pylori* produce a vacuolating cytotoxin [23]. Although in this study we did not analyse for the existence of the vacuolating toxin, we did analyse by PCR for the presence of the *vacA* gene. Our findings were consistent with others in that 100% of the strains possessed *vacA* [16].

An additional product that is associated with the production of the vacuolating toxin is the product of the *cagA* gene. CagA is an immunodominant antigen with a molecular mass of 96–138 kDa [24]. It has been shown that 60–80% of *H. pylori* isolates possess CagA. We found that 75% of the pretreatment isolates possessed *cagA* and that 67% of the post-treatment isolates possessed *cagA*. No correlation was observed for the presence of *cagA* in *H. pylori* from patients who failed treatment with clarithromycin and lansoprazole. The function of CagA is not known; however, it has been shown that a strong association with production of CagA by *H. pylori* and peptic ulcer exists. Covacci and colleagues [24] demonstrated that 100% of the *H. pylori*-infected patients with peptic ulcer disease have antibodies to CagA, but only about 60% of the patients suffering from gastritis have antibodies to CagA. We did not observe a significant correlation between the presence of *cagA* and ulceration. Our findings show that 91% of the isolates from patients with confirmed duodenal ulcers were *cagA*+ and 86% of the patients with dyspepsia were *cagA*+. This is slightly higher than the findings of Peek and colleagues [25], where 82% of the patients with peptic ulcers harboured *cagA*+ strains of *H.*

pylori and 36% of patients without peptic ulcers had *cagA*+ strains of *H. pylori*. Further research is needed to elucidate the role CagA has in peptic ulcer disease.

The PCR–RFLP typing of *ureC* is a rapid method that can be used for epidemiological studies to differentiate between *H. pylori* isolates. The PCR–RFLP method used in this study supports other studies that a high degree of heterogeneity exists among *H. pylori*. This study appears to indicate that recrudescence occurred in patients due to development of resistance to clarithromycin. However, the potential for reinfection due to acquisition from a non-gastric site can not be excluded. This study also demonstrated that infections and ulcerations are not limited to a single or few types.

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