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PCR-Based RFLP Analysis of *ureC* Gene for Typing of Indian *Helicobacter pylori* Strains from Gastric Biopsy Specimens and Culture

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Since culture of *Helicobacter pylori* is relatively insensitive and cumbersome, molecular detection and typing of *H. pylori* isolates are gaining importance for strain differentiation. In the present study genomic DNA of 42 gastric biopsies and *H. pylori* isolates from corresponding patients were analyzed and compared by PCR-based RFLP assay. The 1,132-bp product representing an internal portion of *ureC* gene of *H. pylori* was amplified by PCR and digested with restriction enzymes *Hind*III, *Alu*I and *Pvu*I. The *Hind*III, *Alu*I and *Pvu*I digestion produced 4, 7, and 2 distinguishable RFLP patterns respectively from 42-*H. pylori* isolates. By combining all three restriction enzyme digestions, 15 RFLP patterns were observed. However, when PCR products from 42 gastric biopsy specimens were digested by restriction enzymes *Hind*III, *Alu*I and *Pvu*I, we observed 5, 8 and 2 RFLP patterns, respectively. Patterns from 34 of 42 gastric biopsy specimens matched those of corresponding *H. pylori* isolates from respective patients. Patterns from the remaining eight biopsy specimens differed and appeared to represent infection with two *H. pylori* strains. The patterns of one strain from each of these biopsies was identical to that of the isolate from corresponding patients and the second pattern presumably represented the co-infecting strain. From the study, it appears that PCR-based RFLP analysis is a useful primary tool to detect and is distinguish *H. pylori* strains from gastric biopsy specimens and is superior to culture techniques in the diagnosis of infection with multiple strains of *H. pylori*.

Key words: *Helicobacter pylori*, PCR-RFLP, *ureC*

Helicobacter pylori is a gram-negative, microaerophilic organism that colonises human gastric mucosa. It has been proved that *H. pylori* is the causative agent of chronic gastritis and peptic ulcer diseases and is also a risk factor for gastric cancer, Nomura *et al.*, 1991; Parsonnet *et al.*, 1991; The World Health Organization has classified *H. pylori* as a class I carcinogen (IARC Working Group. 1994). Epidemiological studies have shown that *H. pylori* infection occurs worldwide at a high prevalence rate. Recurrence of infection after apparent eradication has also been reported and is associated with the recurrence of ulcers. However, it is unclear whether the recurrence of ulcers following *H. pylori* eradication therapy is due to recrudescence of the previous infection or to exogenous re-infection by another strain. An accurate method for the detection and differentiation of *H. pylori* strains in patients both before and after therapy is therefore of great importance for diagnosis, monitoring of treatment, and reduction of the long-term consequences of continued but undetected disease.

Attempts to differentiate *H. pylori* strains have been made using a variety of conventional typing schemes, like haemagglutination (Huang *et al.*, 1998), biotyping (Megraud *et al.*, 1985), cytotoxin activity (Figura *et al.* 1989), plasmid profile (Penfold *et al.*, 1988), sodium dodecyl sulphate- polyacrylamide gel electrophoresis of proteins (Megraud *et al.*, 1985) and immunoblotting (Burnie *et al.*, 1988). However, each of these methods has been of little use for precise differentiation of *H. pylori* strains. Although several nucleic acid techniques, such as restriction endonuclease analysis of genomic DNA (Majewski and Goodwin, 1988; Clayton *et al.*, 1991) and Southern blot hybridization with rRNA gene probes (Owen *et al.*, 1991) have been applied to type *H. pylori* clinical isolates from different patients, the patterns obtained by these methods are complex and difficult to interpret, particularly for large scale analysis of clinical isolates. Recently, PCR-based restriction fragment length polymorphism (RFLP) analysis and randomly amplified polymorphic DNA (RAPD) methods have been developed for typing of *H. pylori* clinical isolates (Akopyanz *et al.*, 1992; Foxall *et al.*, 1992; Clayton *et al.*, 1993; Moore *et al.*, 1993; Desai

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et al., 1994). PCR based RFLP schemes have been used to analyze *H. pylori* genes, which encode urease and its accessory proteins, including the 2.4-kb *ureA-ureB*, 1.7-kb *ureC-ureD* (Forman *et al.,* 1991;Akopyanz *et al.,* 1992), 933-bp *ureB* (Clayton *et al.,* 1993) and 820-bp *ureC* (Fujimoto *et al.,* 1994) genes. The published data have shown PCR-based RFLP typing is a rapid, sensitive method and capable of discriminating among clinical isolates, but the studies were limited to *H. pylori* isolates cultured from gastric biopsy specimens.

In this study, we report a PCR-based RFLP analysis to differentiate *H. pylori* strains directly from gastric biopsy specimens and the results were compared with *H. pylori* isolates from corresponding patients.

Materials and Methods

H. pylori strains and patients

Forty-two *H. pylori* strains were isolated from the gastric biopsy specimens of 42 patients (23 men and 19 females; mean age 46 years; age range 22 to 75 years) attending the endoscopy units in the gastroenterology department, for upper gastrointestinal complaints. Patients taking antibiotics, with bleeding ulcers or an acute hemorrhage from other sites in the upper gastrointestinal tract and a patient who had recently had surgery of the stomach were excluded. All patients gave informed consent to be biopsied. The known strain, CCUG 17874 (provided by Dr. B. Kaijser, University of Goteborg, Sweden) was used as a *ureC* positive control in this study. Three gastric biopsy specimen from each patient were collected from a similar location in the antrum of the stomach from each patient. One piece each of the specimens from each patient was cultured for *H. pylori*, examined histologically by Giemsa and subjected to PCR-based RFLP analysis.

Culture of H. pylori from gastric biopsy specimens

For bacterial culture, the gastric biopsy sample was homogenized and cultured on Brucella chocolate agar containing antibiotic supplement (vancomycin 10 mg/L, polymyxin-B 2500 IU/L, and amphotericin-B 5 mg/L) with 7% sheep blood (Shanjana *et al.,* 1996). Plates were incubated at 37°C in microaerophilic atmosphere with CO_2 10%, O_2 5% and N_2 85%. Plates were opened after 72 h and every 48 h afterwards if no growth was obtained. Plates were discarded only after 7 days of incubation. Organisms were identified as *H. pylori* based on colony morphology, modified Gram staining, and positive oxidase, catalase and rapid urease tests. Histological sections of formalin-fixed biopsy specimens were stained with haematoxylin-eosin to evaluate the presence of *H. pylori*.

Processing of the sample for PCR assay

Genomic DNA from *H. pylori* isolates was extracted and purified from freshly harvested bacterial cells by the alkali lysis method and was dissolved in distilled water (Sambrook *et al.,* 1989). DNA of biopsy specimens from patients positive for *H. pylori* by culture and histology was prepared as follows: the frozen biopsy specimen was thawed, crushed and approximately 10 volumes of extraction buffer [10 mM Tris-Cl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% SDS, 20 μg/ml pancreatic RNAse and proteinase K 200 μg/ml] and 200 μl of 0.5% N-acetyl-L-cysteine (Sigma, USA) solution were added and stirred for 1 h in 2-ml cryo tubes. After centrifugation, the supernatant was collected and proteinase K was inactivated by heating at 98°C for 10 min. DNA was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1) followed by precipitation with a double volume of absolute ethanol. Samples were incubated for 1 h at 70°C and then were centrifuged at 133×g for 15 min at 4°C. The DNA pellet was washed in 70% ethanol, dried and resuspended in 40 μl water. The quality and quantity of DNA were determined spectrophometrically at 260 nm and 280 nm.

Primers

The oligonucleotide primers forward and reverse from the known sequence of *ureC* that encodes the urease structural gene (Labigne *et al.,* 1991; Richard *et al.,* 1993) were used for PCR based RFLP and synthesized from Genset, Lithuania.

Length	Sequences	Annealing Position
26	5′-TTTGGGACTGATGGCGTGAGGGGTAA-3′	519-544
28	5′- GGACATTCAATTCACCAGGTTTTGAGG-3′	1650-1623

PCR amplification

Amplification was performed in a 50 μl reaction mixture volume containing a 20 μM concentration of each oligonucleotide primer, 200 μM each dATP, dCTP, dGTP and dTTP (MBI Fermantas, Singapore), 10X reaction buffer (MBI Fermantas, Singapore), 10 μl of template DNA sample (100 ng DNA) and 2.5 units of Taq polymerase (MBI Fermantas, Singapore). Twenty-five cycles of amplification were performed in a DNA thermal cycler (Perkin Elmer Cetus, USA). The following cycle conditions were used: 25 cycles at 94°C for 1.5 min, 55°C for 1 min, and 72°C for 2 min, and a final extension step at 72°C for 5 min. DNA extracted from the type strain and plain distilled water were used as the positive and negative controls respectively in each batch of PCR assays.

For identification of the amplified products, 10 μl of the PCR mixture was analyzed by electrophoresis on 1.5% agarose gels (Sigma, USA), stained with ethidium bromide 0.5 μg/ml and visualized under UV illumination. Electrophoresis was performed in TBE buffer.

Restriction digestion of amplified DNA

The amplified DNA product was extracted from low melting point (LMP) agarose gel electrophoresis as the method of Sambrook *et al.* (1989). A 10 µl purified amplified DNA was digested with 10 U of the restriction enzyme *Hind*III, *Alu*I, and *Pvu*I for 6 hrs at 37°C in the appropriate buffer solution as recommended by the manufacturer (Bangalore Gene, India). The digests were analyzed by electrophoresis in 2% agarose gels (metaphor agarose; Sigma, USA). The gels were examined under transillumination and photographed. The sizes of digested DNA fragments were estimated from migration distances of a 100-bp DNA ladder molecular weight standard (MBI Fermentas, Lithuania).

Results

A total of 42 strains were isolated from biopsy specimens of 14, 22 and 6 patients with gastritis, peptic ulcers and portal hypertension (PHT), respectively.

ureC PCR assay for H. pylori

The PCR assay successfully amplified a fragment of the expected 1,132 bp from the DNA preparation of the *H. pylori* type strain, CCUG 17874, with the *ureC* primers. Genomic DNA of the 42 *H. pylori* isolates and the gastric biopsy specimens from corresponding patients also yielded the amplified product of the same molecular size as that of *H. pylori* type strains CCUG 17874 (Fig. 1).

Human genomic DNA and DNA from closely related non-*H. pylori* species (*Campylobacter jejnui*), and 4 urease-positive bacterial species not related to *H. pylori* (*Klebsiella pneumoniae, Serratia marcescens, Proteus mirabilis, Providentia rettgeri*) were used to test the specificity of the PCR assay. The Desired product could not be amplified from the DNA of all the non-*H. pylori* species when tested by agarose gel electrophoresis.

RFLP analysis

In order to determine whether the PCR-based RFLP analysis method could differentiate *H. pylori* strains directly from gastric biopsy specimens without culturing, the PCR assay was performed on genomic DNA of 42 *H. pylori* isolates and gastric biopsy specimens from their corresponding patients. The 1,132-bp fragments obtained by PCR were further digested with three restriction enzymes, *Hind*III, *Alu*I, and *Pvu*I, and analyzed by agarose gel electrophoresis. The PCR products from 42 *H. pylori* isolates subjected to RFLP analysis showed 4, 7, and 2 distinguishable digestion patterns by *Hind*III, *Alu*I, and *Pvu*I respectively (Table 1). By combining all three restriction enzyme digestion patterns, we observed 15 distinct RFLP patterns from 42 *H. pylori* isolates (Table 2).

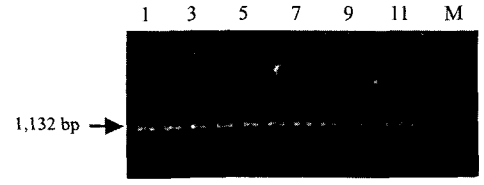

Fig. 1. PCR amplification product of H. pylori ureC gene. Lanes: 1,3,5,7 and 9-from biopsies and 2,4,6,8 and 10 - from their corresponding isolates respectively, 11 - positive control H. pylori CCUG 17874, 12 - negative control and M- molecular weight marker (ϕx174 DNA/ *Hae*III digest, Bangalore Genei Pvt. Ltd. India).

Table 1. Restriction digest profiles of 42 clinical *H. pylori* isolates

Enzyme	RFLP patterns (frequencies)*
*Hind*III	H1 (4), H2 (16), H3 (16), H4 (6)
*Alu*I	A1 (4), A2 (10), A3 (12), A4 (2), A5 (2), A6 (4), A7 (8)
*Pvu*I	P1 (32), P2 (10)

*The H1 in the restriction pattern profile refers to the *Hind*III pattern, the A1 refers to the *Alu*I pattern, and the P1 refers to the *Pvu*I pattern. Figures in parentheses indicates number of *H. pylori* strains.

However, when the PCR products from 42 gastric biopsy specimens were digested by the restriction enzymes *Hind*III, *Alu*I, and *Pvu*I, we observed 5, 8, and 2 RFLP patterns respectively. The patterns from 34 of 42 gastric biopsy specimens matched those from the corresponding *H. pylori* isolates from respective patients. The three enzyme digest combination patterns of the remaining eight biopsy specimens from patients 4, 7, 8, 11, 17, 26, 35, and 37 appeared to represent infections with two *H. pylori* strains; one pattern was identical to that of the isolate from the corresponding patient (Table 2). The gastric biopsy specimen from patient 28, *Hind*III and *Pvu*I generated combination RFLP patterns different from that of the isolate, while *Alu*I generated a pattern identical to that of the corresponding isolate. Representative results of restriction enzyme digestion of PCR-amplified products from *H. pylori* isolates and their corresponding gastric biopsy specimens from each patient are shown in Fig. 2.

Relationship between H. pylori RFLP types and gastrointestinal disease

The large number of isolates presented an opportunity to investigate the distribution of strain types associated with peptic ulcers, gastritis, and portal hypertension in north India. Of 22 strains of peptic ulcer, four strains were of RFLP type 6, three each were of type 10 and type 15 and two strains of each type 4, 7, 11, or type 14 (Table 3). In contrast, three, two, and four strains from a patient with gastritis were of RFLP type 8, type 9 or type 12. In PHT patients out of six strains, most of the strains were RFLP type 12 (two strains). RFLP type 4, 6, 7, 10 and 11 were found only in ulcer patients. In contrast, RFLP type 9 was found only isolates from gastritis patients (Table 3). Thus the results suggest that duodenal ulcers, gastritis and PHT in India are not associated with the same *ureC* RFLP type.

Table 2. PCR-based RFLP analysis of 42-*H. pylori* clinical isolates and their corresponding biopsy specimens by *HindIII*, *AluI* and *PvuI*

Patients	RFLP Pattern (s) with-					
	*Hind*III		*Alu*I		*Pvu*I	
	Isolate	Biopsy specimen	Isolate	Biopsy specimen	Isolate	Biopsy specimen
1	H1	H1	A7	A7	P1	P1
2	H1	H1	A7	A7	P1	P1
3	H1	H1	A7	A7	P2	P2
4*	H1	H1, H5	A7	A7, A3	P2	P2, P1
5	H2	H2	A1	A1	P1	P1
6	H2	H2	A1	A1	P1	P1
7*	H2	H2, H1	A2	A2, A7	P1	P1, P2
8*	H2	H2, H4	A2	A2, A7	P1	P1, P2
9	H2	H2	A2	A2	P2	P2
10	H2	H2	A2	A2	P2	P2
11*	H2	H2, H5	A3	A3, A7	P1	P1, P2
12	H2	H2	A3	A3	P1	P1
13	H2	H2	A3	A3	P1	P1
14	H2	H2	A3	A3	P1,P2	P1
15	H2	H2	A3	A3	P2	P2
16	H2	H2	A3	A3	P2	P2
17*	H2	H2, H3	A6	A6, A8	P1	P1, P2
18	H2	H2	A6	A6	P1	P1
19	H2	H2	A6	A6	P1	P1
20	H2	H2	A6	A6	P1	P1
21	H3	H3	A1	A1	P2	P2
22	H3	H3	A1	A1	P2	P2
23	H3	H3	A2	A2	P1	P1
24	H3	H3	A2	A2	P1	P1
25	H3	H3	A2	A2	P1	P1
26*	H3	H3, H2	A2	A2, A3	P1	P1, P2
27	H3	H3	A2	A2	P2	P2
28	H3	H3	A2	A2	P2	P2
29	H3	H3	A3	A3	P1	P1
30	H3	H3	A3	A3	P1	P1
31	H3	H3	A3	A3	P1	P1
32	H3	H3	A3	A3	P1	P1
33	H3	H3	A3	A3	P1	P1
34	H3	H3	A3	A3	P1	P1
35*	H3	H3, H2	A4	A4, A8	P1	P1, P2
36	H3	H3	A4	A4	P1	P1
37*	H4	H4, H3	A5	A5, A7	P1	P1, P2
38	H4	H4	A5	A5	P1	P1
39	H4	H4	A7	A7	P1	P1
40	H4	H4	A7	A7	P1	P1
41	H4	H4	A7	A7	P1	P1
42	H4	H4	A7	A7	P1	P1

*Gastric biopsies infected with two strains when compared with the corresponding culture isolate.

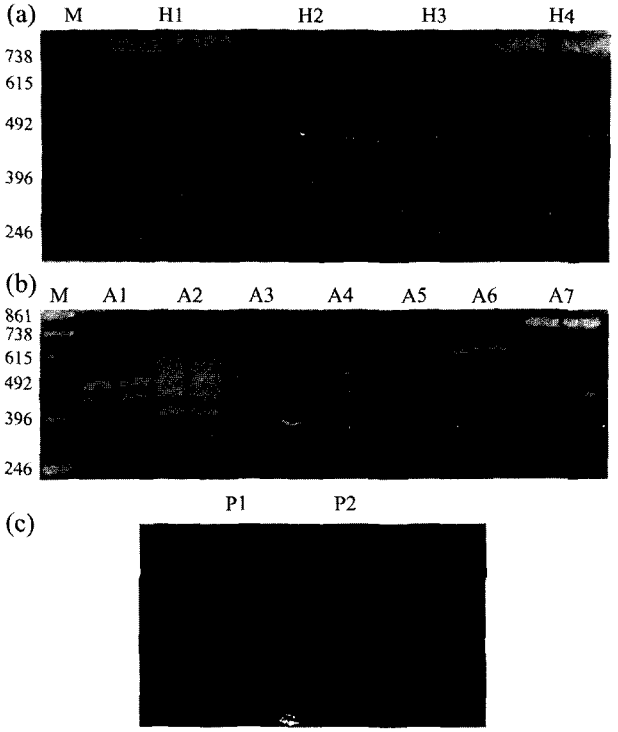

Fig. 2. PCR-based RFLP patterns from the 42-*H. pylori* isolates and their corresponding gastric biopsy specimens with three different restriction enzymes. **A.** *Hind*III digests (H1 to H4), **B.** *Alu*I digests (A1 to A7), and **C.** *Pvu*I digests (P1 and P2). The left lane of each pair is an *H. pylori* isolate and the right lane is the corresponding gastric biopsy specimen. Lane M is a 100-bp DNA ladder.

Table 3. PCR-RFLP types from different gastroduodenal diseases

RFLP type (No. of strains)	No. of isolates from different disease groups		
	Gastritis	Ulcer	PHT
1 (2)	1	0	1
2 (2)	1	1	0
3 (2)	0	1	1
4 (2)	0	2	0
5 (2)	1	1	0
6 (4)	0	4	0
7 (2)	0	2	0
8 (4)	3	0	1
9 (2)	2	0	0
10 (4)	1	3	0
11 (2)	0	2	0
12 (6)	4	0	2
13 (2)	0	1	1
14 (2)	0	2	0
15 (4)	1	3	0
Total 42	14	22	6

Discussion

In recent years, many investigators applying molecular techniques have revealed that *H. pylori* possesses a remarkable degree of genetic diversity, which is closely related with its epidemiological and pathological characteristics and dynamics of transmission. The PCR-based RFLP analysis has been widely accepted for typing and differentiation of *H. pylori* strains from clinical specimens. This method has been used to analyze conserved *H.*

pylori genes, especially those encoding urease structural and accessories proteins (Foxall *et al.,* 1992; Fujimoto *et al.,* 1994; Shortridge *et al.,* 1997 and Owen *et al.*, 1998).

In our study, a PCR assay employing a primer pair derived from the *ureC* gene sequence successfully amplified a 1,132-bp fragment. from 42 clinical isolates and their corresponding gastric biopsy specimens but not from DNA of closely related non-*Helicobacter* species (*C. jejuni*), and four other urease-positive bacterial species not related to *H. pylori*. Richard *et al.,* 1993 had also tested *ureC* gene primers on bacterial strains of closely related genera and various urease-positive bacterial species and found them *H. pylori* specific. The present study corroborates the earlier data that *ureC* gene is an excellent target for PCR assay of *H. pylori* and the results have demonstrated a great diversity in urease genes among *H. pylori* clinical isolates.

In the present study, *H. pylori* isolates were grouped on the basis of restriction enzyme analysis of the 1,132 bp PCR product of *ureC* gene. *Hind*III restriction digest patterns differentiated *H. pylori* isolates into four groups (Table 1). Further, the isolates were differentiated on the basis of *Alu*I or *Pvu*I restriction digest patterns. *Alu*I and *Pvu*I differentiate each isolate into seven and two groups respectively (Table 1). When RFLP patterns obtained by three different enzymes, *Hind*III, *Alu*I and *Pvu*I were combined, we observed 15 distinct RFLP patterns among the 42 *H. pylori* clinical isolates (Table 2). In a recent comparable study it was suggested that a portion of the *ureC* gene could be a reliable scheme for differentiating *H. pylori* isolates (Richard *et al.*, 1993).

By using this method, 5, 8, and 2 distinct RFLP patterns were generated by three restriction enzymes *Hind*III, *Alu*I, and *Pvu*I, respectively from 42 gastric biopsy specimens. The RFLP patterns from 34 (81%) of 42 gastric biopsy specimens were found to be identical to those of *H. pylori* isolated from the corresponding patients. The combination of RFLP patterns from the remaining eight gastric biopsy specimens, suggested the colonization of stomach by two *H. pylori* strains in these eight patients, the with one of the two patterns from biopsy specimen always matching that from its corresponding culture isolate. As shown in Table 2, gastric biopsy specimens from patients 4, 7, 8, 11, 17, 26, 35, and 37 were probably colonized by two strains of *H. pylori*. Only 8 (19%) of 42 patients had mixed infections, suggesting that a majority of our patients was typically colonized with a single strain.

It has been previously reported that two strains of *H. pylori* have been found in the stomachs of some patients (Fujimoto *et al.,* 1994; Owen *et al.,* 1994; Prewett *et al.*, 1992). These data are consistent with the observation of Fox *et al.* 1993; three distinct strains were found in isolates from the same patients and two strains were found from each of 4 patients. Beji *et al.* (1989) also described one patient who had three different DNA patterns from three biopsy specimens obtained during a single endoscopy. These data indicate that some patients are infected by multiple isolates of *H. pylori*. Examination of large numbers of isolates from individual patients will permit a better quantitation of the extent of multiple infections. However, infection by multiple strains seems to be uncommon in India, and mixed cultures are likely to be missed in the extensive incubation and sub-culturing steps prior to typing.

Although several PCR-based RFLP protocols have been developed for the differentiation of *H. pylori* strains, most of the studies have been carried out on *H. pylori* isolates from gastric biopsy specimens. Culture of *H. pylori* is time consuming and not very sensitive. Our PCR-based RFLP protocol provides a means for direct detection and differentiation of *H. pylori* strains in gastric biopsy specimens without culture. Clayton *et al.* 1993 also applied the PCR-restriction endonuclease analysis method directly to biopsy samples and suggested its use for epidemiological investigation on the transmission of this pathogen. This shows that PCR-based RFLP assay may be useful as a primary approach for the identification of specific *H. pylori* strains in gastric biopsy specimens without culturing.

The present study also showed that there is a relation between the RFLP type of the *ureC* gene and the clinical outcome, a finding in agreement with previous reports (Moore *et al.,* 1993;Gzyl *et al.,* 1999). Of 22 strains of peptic ulcer, most of the strains were RFLP type 6, 10 or 15. In contrast, of 14 strains isolated from patients with gastritis, most were RFLP types 12, 8 or 9; most of the strains in PHT patients were RFLP types 12. RFLP types 4, 6, 7, 10 and 11 were found only in ulcer group. In the contrast, RFLP type 9 was found in patients with gastritis (Table 3). Thus our results suggest that ulcer, gastritis and portal hypertension in India are not associated with the same *ureC* RFLP type and that the RFLP type of the *ureC* gene is unrelated to the disease status such as ulcers, gastritis and PHT.

Several studies have confirmed that PCR-based RFLP analysis of the *ureC* gene can differentiate *H. pylori* clinical isolates. Fujimoto *et al.* 1994 have shown that digestion of an 820-bp PCR amplified portion of *H. pylori ureC* gene by the restriction enzymes *Hha*I, *Mbo*I, and *Mse*I resulted in 10, 10, and 11 different patterns, respectively. The 25 *H. pylori* clinical isolates, however, could be grouped into 25 distinct RFLP patterns when the three digestion patterns were combined. Chuanfu *et al.* (1997) have also shown that digestion of an 1,179-bp PCR amplified portion of *ureC* gene of *H. pylori* by the restriction enzymes *Hha*I, *Mbo*I, and *Alu*I, generated 11, 10, and 6 digestion patterns respectively. The 19 *H. pylori* clinical isolates, however, could be grouped into 19 distinct RFLP patterns when the three digestion patterns were combined. De Sai *et al.* (1994) found that twenty-four combined *ure-*

ACD profiles were generated with *Hind*III, subdividing the 64 strains into 11 types and 13 single profiles.

The present findings suggest that the PCR-based RFLP technique may be useful for epidemiological studies of *H. pylori* infection directly from gastric biopsies. It appears to be more discriminatory and superior to culture-based studies in detecting mixed infection. Infection by multiple strains seems to be uncommon in this country and treatment failure is not generally due to reinfection with a different strain but rather due to the persistence of the same infecting strain.

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