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PCR and RFLP Analysis for Identification and Typing of *Helicobacter pylori* Strains Isolated from Gastric Biopsy Specimens

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Şimşek, İ.Ş., Menevşe, S. and Şahin, F.İ. *PCR and RFLP Analysis for Identification and Typing of* ***Helicobacter pylori*** *Strains Isolated from Gastric Biopsy Specimens.* Tohoku J. Exp. Med., 2000, **190** (3), 213-222 —— *Helicobacter pylori* (*H. pylori*) infection is the most common gastrointestinal tract infection which plays an important role in the ethiopathogenesis of peptic ulcer and gastritis. In recent years, molecular biological methods have been presented for detection of *H. pylori* in addition to histopathological and microbiological methods. Among these methods, polymerase chain reaction (PCR) and following restriction fragment length polymorphism analyses (RFLP) are highly sensitive methods for diagnosis and follow up of patients. In this present study our aim was to amplify *H. pylori* urease A and B genes by PCR and perform RFLP analysis. Gastric biopsy specimens from 17 female and 18 male patients were included in the study. Amplified PCR products were subjected to RFLP analysis and typing of the bacteria in pre and posttreatment specimens were performed. *H. pylori* urease A and B gene amplification was observed in 32 pretreatment samples and in 8 of 21 posttreatment specimens. As a result, PCR is a sensitive method to determine the *H. pylori* infection. RFLP, which is another effective method in order to demonstrate the reinfection of *H. pylori*. ———— *Helicobacter pylori*; PCR; RFLP; duodenal ulcer © 2000 Tohoku University Medical Press

Helicobacter pylori is important in pathogenesis of peptic ulcer and gastritis (Blum 1996) and is the most common bacterial gastrointestinal disease in the world with an incidence of 70-80% (Türet 1993; Axon 1997).

Various diagnostic methods used in diagnosis of *H. pylori* have been studied and molecular biological tools have been found to be more sensitive (Coates et al. 1991). Restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products is a protocol which is used in both diagnosis of the bacteria and detection of reinfections (Foxall et al. 1992; Dzierzanowska et al. 1996).

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In this study we aimed to amplify urease genes by PCR and genotype the pathogen by RFLP analysis. We also targeted to use these methods as routine diagnostic and typing tools.

Materials and Methods

Patients

Thirty five patients with gastroduodenal symptoms were included in the study. Gastric biopsy specimens from 17 female and 18 male patients were taken into sterile culture tubes containing phosphate buffered saline solution (PBS) and sent to our laboratory for DNA isolation. At the same time, samples for pathologic examination and microbiological methods were taken. The organisms were identified as *H. pylori* on the basis of morphology in negative Gram stains and by positive oxidase, catalase and rapid urease tests. All samples were inoculated onto special agar plates and cultured for 7–10 days as described before (Clayton et al. 1993). Identification of cultured bacteria were performed as stated above. Tissue specimens were kept at −20°C in PBS until DNA isolation. Omeprazole and claritromycin treatment was given to the patients and posttreatment specimens were obtained 6 months after the end of therapy.

DNA isolation

Uncultured samples were minced under sterile conditions in a petri dish and taken into 1.5 ml ependorf tubes and 100 μl distilled water was added to the samples. After boiling for 15 minutes, centrifugation at 14 000 g for 5 minutes was performed and supernatant was taken into another tube to be kept at −20°C prior to PCR amplification. For cultured bacteria a similar protocol was followed.

PCR amplification

Two urease genes, A and B were amplified by two sets of primers. HPU 1 and HPU 2 (IDT, Coralville, IA, USA) were used to amplify a 411 bp product of urease A gene and HPU 25 and HPU 50 (IDT) were used to amplify a 933 bp product of urease B gene (Clayton et al. 1993).

A PCR mixture of 10 mM Tris-HCl (pH: 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine (MBI Fermentas, Vilnius, Lithuania) containing 0.2 mM of each nucleotide (MBI Fermentas), 0.5 μM of each oligonucleotide primer and 2.5 U Taq polymerase (MBI Fermentas) also contained 1 μl of DNA from cultured samples or 10 μl of DNA from direct tissue specimens. A total of 100 μl reaction volume was obtained. Hp 11 637 strain was used as the positive control.

Each PCR cycle consisted of denaturation at 96°C for 30 seconds, annealing at 56°C for 15 seconds and elongation at 74°C for 30 seconds and the whole reaction was 35 cycles totally.

PCR products were visualised in 2% agarose gel electrophoresis.

RFLP analysis

In order to detect RFLP patterns of *H. pylori* DNA amplified by urease A and B primers, various restriction enzyme digestions were applied. Alu I (MBI Fermentas), Mlu I (MBI Fermentas) and Hinf I (MBI Fermentas) enzymes were used for urease A gene products whereas Sau 3A (MBI Fermentas), Hae III (MBI Fermentas) and Alu I (MBI Fermentas) enzymes were used for urease B gene products. Enzyme digests were again visualized in 2% agarose gel electrophoresis.

Results

A total of 35 patients' antral biopsy specimens consisting of 17 female and 18 male cases were included in the study. The clinical and laboratory findings of the patients are shown in Table 1. Histopathological examination was positive in 22 patients and bacterial cultures were positive in 15 patients whereas PCR was positive in 32 patients (91%).

PCR results

H. pylori urease A and urease B gene primers were used and *H. pylori* was detected in 32 (91%) patients. Urease A primers amplified a 411 bp PCR product and urease B primers amplified a 933 bp PCR product. PCR amplification was observed in 21 samples, and the relapse ratio for 8 of them was found to be 38%

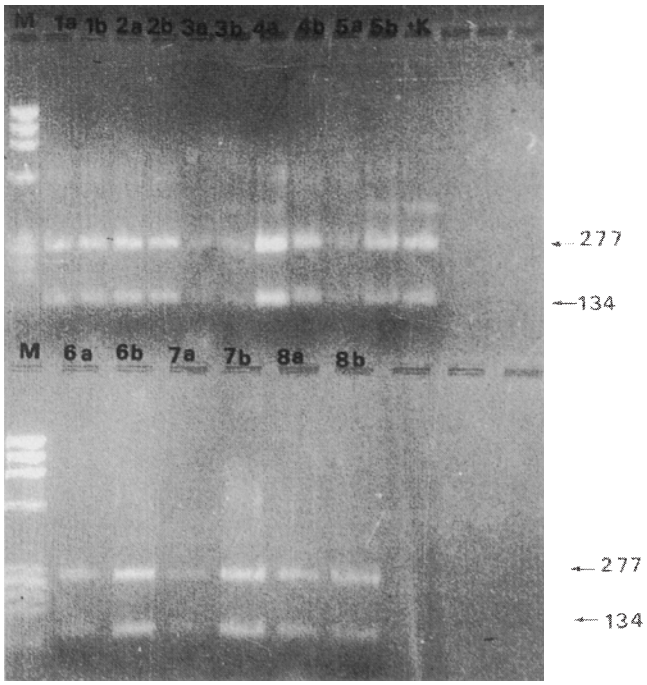

Fig. 1. Hinf I digested PCR products of patients 1–8 amplified with urease A primers. M, Φx 174 DNA marker digested with Hae III; 1a-8a, pretreatment and 1b-8b, posttreatment specimens; +K, Hp 11 637 strain PCR product digested with Hinf I.

TABLE 1. *Clinical and laboratory finidngs of the patients included in the study*

Patient No.	Sex	Age	Endoscopic diagnosis	Histopathology before treatment	Histopathology after treatment	Culture before treatment	Culture after treatment	PCR before treatment	PCR after treatment
1	M	24	Active gastritis	+	+	+	−	+	+
2	F	32	Peptic ulcer	+	−	+	+	+	+
3	F	33	Chronic gastritis	+	+	−	−	+	+
4	M	22	Antral gastritis	+	+	+	+	+	+
5	F	21	Antral gastritis	−	−	−	−	+	+
6	M	53	Peptic ulcer	+	−	+	+	+	+
7	F	40	Peptic ulcer	+	+	−	−	+	+
8	F	21	Antral gastritis	Unavailable	−	−	−	+	+
9	M	47	Acute gastritis	+	−	+	−	+	−
10	M	44	Gastroduodenitis	+	−	−	Unavailable	+	−
11	M	32	Peptic ulcer	+	−	+	−	+	−
12	M	73	Chronic gastritis	+	−	−	−	+	−
13	M	36	Chronic duodenitis	−	−	+	Unavailable	+	−
14	F	24	Prepyloric ulcer	Unavailable	−	−	−	+	−
15	F	53	Peptic ulcer	+	−	+	−	+	−
16	M	22	Antral gastritis	Unavailable	−	Unavailable	−	+	−
17	M	22	Acute gastritis	−	−	−	−	+	−
18	F	21	Gastroduodenitis	−	Unavailable	−	−	+	−
19	F	32	Peptic ulcer	+	−	−	−	+	−
20	M	25	Peptic ulcer	+	−	−	−	+	−

previously reported that 5 mg/kg CDDP caused maximal, significant increases in BUN and lipid peroxidation in rat kidney 5 day after a single injection (Satoh et al. 1998). A side effect of CDDP, gastrointestinal damage, is caused by doses greater than 5 mg/kg. Ward and Fauvie (1976) also reported that many rats receiving an LD_{50} of CDDP lost weight after 24 hours and died after 2 to 7 days. In our present study, it was shown that animals treated with 6 mg/kg CDDP alone and CDDP plus 2.75 mg/ kg ebselen resulted in marked elevation of BUN and Cr, significant loss of body weight, and diarrhea after 4 to 5 days, which suggests dysfunction of the renal tissue and the gastrointestinal system. However, all rats survived during the experimental period. In contrast, when either 5.5 mg or 11.0 mg/kg ebselen was administered to rats before the CDDP injection, the CDDP-induced increase in BUN and Cr levels were well suppressed, but these values were still higher than those of control rats (Table 1). Ebselen doses of 5.5 and 11.0 mg/ kg also decreased loss of body weight and reduced diarrhea caused by CDDP. These results indicate that ebselen provides effective protection against CDDP-induced nephrotoxicity and gastrointestinal toxicity when administrated daily oral dose one or two molar, which equivalents greater than CDDP.

In our present study, morphologic changes in the kidney after injection of 6 mg/kg CDDP was characterized by selective proximal tubular damage and regeneration of the effected tubular epithelia on day 5. These observations are in agreement with numerous earlier reports (Babu et al. 1995; Matsushima et al. 1998; Satoh et al. 1998). However, we did not note histopathological changes in the kidney of rats treated with CDDP and 11.0 mg/kg ebselen, suggesting that ebselen may be useful for prevention of CDDP-induced nephrotoxicity.

Lipid peroxidation is known to increase in rat kidney following injection in vivo (Hannerman and Baumann 1988; Husain et al. 1998; Satoh et al. 1998). Our results also showed that renal lipid oxidation, as determined by renal MDA content, was significantly increased 5 days after CDDP injection. However, MDA levels were decreased in kidneys of rats pretreated with doses of 5.5 and 11.0 mg/ kg ebselen but not with 2.75 mg/kg ebselen. Ebselen showed a dose-dependent effect in decreasing the CDDP-induced lipid peroxidation, similar to its ability to decrease BUN and Cr levels. These data indicate that ebselen dose-dependently and markedly reduces oxidative renal damage caused by free radical production.

The selenium-containing enzyme GSH-Px protects cells against oxygen free radicals. In this study, the activity of GSH-Px in the kidney was reduced significantly in CDDP-injected rats. This result indicates that the increase in lipid peroxidation in the kidney of rats treated with CDDP may be related to the decrease in the activity of GSH-Px, which scavenges hydroperoxides and lipid peroxides. Prior to CDDP injection, however, daily administration of ebselen, which exhibits GSH peroxidase-like activity, prevented the decrease of GSH-Px activity in the kidney. Husain et al. (1998) and Satoh et al. (1998) showed that although the reduction of GSH activity in the renal tissue of rats treated with

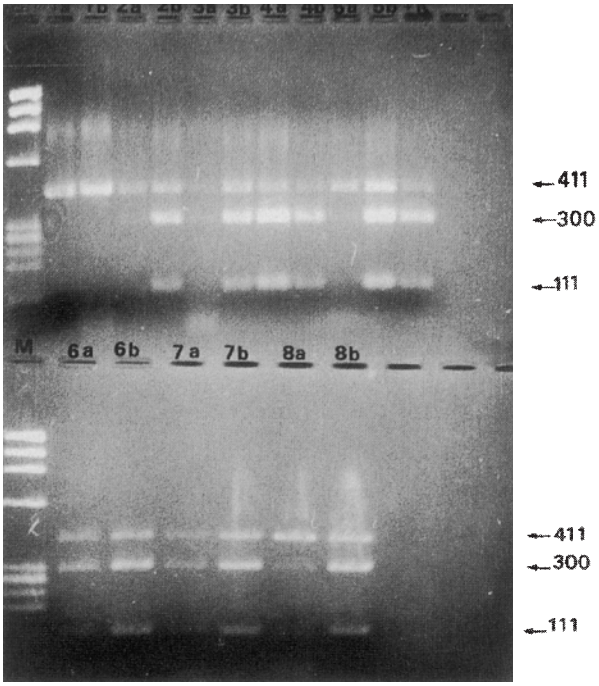

Fig. 2. Mlu I digested PCR products of patients 1-8 amplified with urease A primers. M, Φx 174 DNA marker digested with Hae III. 1a-8a, pretreatment and 1b-8b, posttreatment specimens. +K, Hp 11 637 strain PCR product digested with Mlu I.

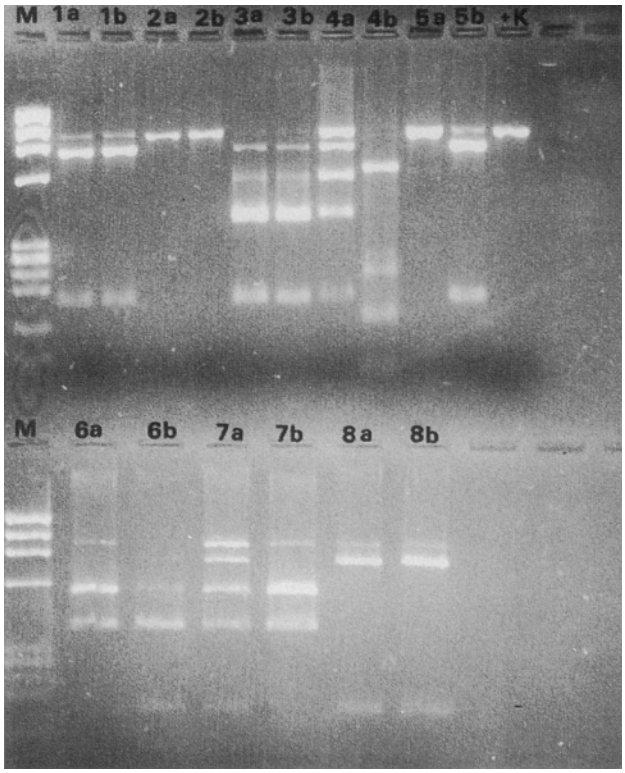

Fig. 3. Hae III digested PCR products of patients 1-8 amplified with urease B primers. M, Φx 174 DNA marker digested with Hae III. 1a-8a, pretreatment and 1b-8b, posttreatment specimens. +K, Hp 11 637 strain PCR product digested with Hae III.

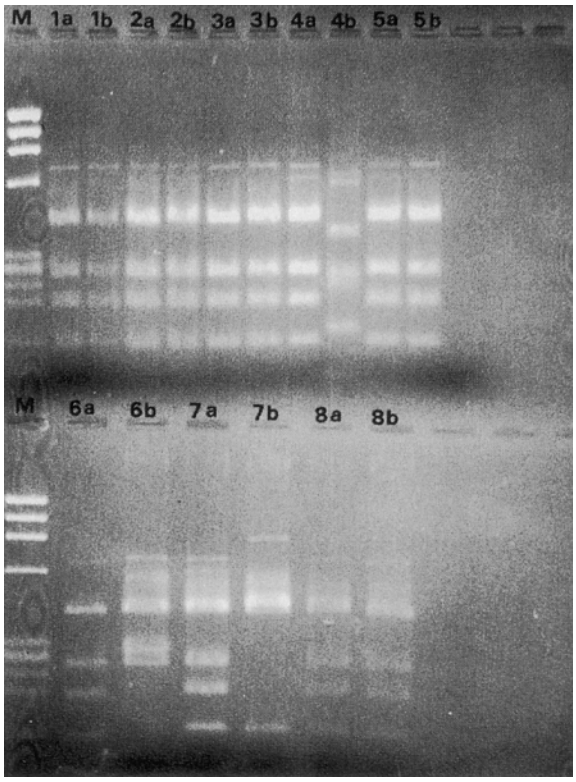

Fig. 4. Sau 3A digested PCR products of patients 1–8 amplified with urease B primers. M, Φx 174 DNA marker digested with Hae III. 1a-8a, pretreatment and 1b-8b, posttreatment specimens. +K, Hp 11 637 strain PCR product digested with Sau 3A.

after therapy. The remaining 13 posttreatment samples did not show any amplified PCR products by urease A and B primers.

RFLP analysis results

Urease A amplified and Hinf I digested samples and the Hp 11 637 strain revealed two bands of 277 and 134 bp (Fig. 1). Alu I digested PCR products also revealed two bands of 347 and 64 bp. The Mlu I site within the 411 bp product gave rise to two bands of 300 and 111 bp. In patient 1 no digestion products were observed after Mlu I digestion (Fig. 2).

Hae III site within the urease B fragment revealed identical bands in pre and posttreatment samples of patients 1, 2, 3, 6 and 8. In patients 4, 5 and 7 different sized bands were observed in pre and posttreatment PCR products. Hp 11 637 strain remained as a 933 bp fragment after digestion (Fig. 3).

Digestion of urease B gene PCR products with Alu I gave rise to identical bands in pre and post treatment samples of patients 1, 2, 3, 5 and 7. In patients 4 and 6 different bands were observed in two samples before and after therapy.

Sau 3A digestions revealed identical bands in patients 1, 2, 3, 5 and 8 but different bands in patients 4, 6 and 7 (Fig. 4).

Discussion

H. pylori infection is a major public health problem as it is widespread and frequent. Different methods used in diagnosis have advantages and disadvantages when compared (Ho et al. 1991). In our study, PCR based RFLP analyses seemed to be more sensitive and specific than histopathological and microbiological methods. Our findings showed that PCR method is more successful in diagnosis of *H. pylori*, which supported other investigators' reports (Valentine et al. 1991; Wang et al. 1993).

Another point is that, after therapy the number of bacteria in specimens decrease and microbiological and histopathological examinations may give false negative results (De Boer 1997). PCR is a sensitive method, which enables to amplify small amounts of bacterial DNA in the samples (Hammar et al. 1992).

The major problem in *H. pylori* infections is the recurrence of disease in a short time. In developed countries recurrence rates have been reported to be 11% or lower however in developing countries it has been reported to be over 30% (Sung et al. 1994). In Turkey this frequency is 35 to 56% (Türet 1993). In our study, 6 months after therapy the recurrence rate was 38%.

In this study, we aimed to determine whether reinfections occur with the same or different strains. RFLP analysis results with Hinf I and Alu I revealed the same bands in pre and posttherapy specimens, whereas, Mlu I site revealed more than one digestion pattern which made us to conclude a single base mutation in the same strain, rather than an infection with a new strain. Single base mutations, however are frequent in *H. pylori* and give rise to restriction site differences, correlating with these urease RFLPs detected in our study. The DNA base changes observed within the urease genes of the bacterium generally cause aminoacid substitutions and these differences do not affect enzyme function (Labigne et al. 1991; Hammar et al. 1992; Clayton et al. 1993). In patient 1 no digested bands were seen in the 411 bp region which showed reinfection with the same strain.

As Hinf I and Alu I digests revealed identical bands within the urease A region, in order to determine different strains, another gene urease B primers were used and restriction analysis were performed. When all the patients in the study were regarded, we determined many strains with different digestion patterns. Our findings support other investigators' reports about genomic variability of *H. pylori* (Clayton et al. 1991). Genomic variations in the bacterium have been reported to originate from adjustment of bacterial DNA against natural selection. This adjustment results in genomic recombination of the bacterial DNA (Akashi et al. 1996). As a result every patient reveals a different digestion pattern. Genomic variability enables *H. pylori* infections to be widespread all over the world (Majewski and Goodwin 1988).

Restriction enzyme digestion patterns of urea B gene in patients 4, 6 and 7

revealed different bands in pre and posttreatment samples. These findings supported other investigators' reports and we concluded that three possible conclusions could be drawn; such as infection with a new strain, infection with more than one strain or in vivo mutation (Clayton et al. 1991; Taylor et al. 1995; Shortridge et al. 1997).

Genomic changes in *H. pylori* cause development of drug resistance, which result in decrease in therapy effectiveness (Sorensen et al. 1990). This is because genotyping is necessary in diagnosis and planning therapeutic regimens of *H. pylori* infections. RFLP analysis has been reported as the best method for genotyping *H. pylori* (Akashi et al. 1996).

As a result we could suggest that in *H. pylori* typing studies at least two gene regions should be studied. PCR-RFLP is a promising method for diagnosis and typing of *H. pylori*, especially in epidemiological studies.

Acknowledgments

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