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Original Article

Differentiation between Reinfection and Recrudescence of *Helicobacter pylori* Strains using PCR-based Restriction Fragment Length Polymorphism Analysis

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The aim of this study was to evaluate whether PCR-based restriction fragment length polymorphism (RFLP) analysis was effective in differentiating between reinfection and recrudescence of *H. pylori* strains. Following a 1-2 week regimen of omeprazole 20 mg, amoxicillin 1.0 g, and clarithromycin 500 mg twice daily, twenty patients with duodenal ulcer were enrolled in the study. Ten patients (group 1, control) were not successfully treated, and another 10 patients (group 2) exhibited recurrence of infection 6-24 months following the therapy. Follow-up diagnosis was performed by Giemsa stain and CLO test. RFLP profiles of antral and midbody biopsy specimens were compared before and after therapy. PCR products using the *ureC* gene were digested with restriction enzymes *Hha* I, *Mbo* I, and *Hind* III, and the fragments generated were analyzed by agarose gel electrophoresis. *Hha* I, *Mbo* I, and *Hind* III digestion produced 13, 7, and 2 distinguishable digestion patterns, respectively. There was no difference in RFLP profiles seen before and after the therapy in 17 duodenal ulcer patients, while different RFLP profiles were discovered in 3 patients. Following treatment, one (group 2) patient differed in *Mbo* I, and two (one each from both groups) patients differed in *Hha* I and *Mbo* I RFLP patterns. Eight of group 2 patients showed recrudescence of previous infection and two patients had reinfection by another strain. This study supports the hypothesis that PCR-based RFLP analysis can be effective for differentiating reinfection and recrudescence of *H. pylori* strains following triple therapy.

Key Words: *Helicobacter pylori*, polymorphism, restriction fragment length, duodenal ulcer, drug therapy

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INTRODUCTION

Helicobacter pylori is linked to chronic active gastritis, peptic ulcer and gastric cancer.[1] Its worldwide distribution at a high prevalence rate and the importance of associated pathologies make the eradication of *H. pylori* a very useful approach for treating and controlling these gastroduodenal diseases.[2] The recurrence of infection after apparent eradication has also been reported and is associated with the recurrence of ulcers.[3,4] However, it is unclear whether the recurrence of ulcers following *H. pylori* eradication therapy is due to the recrudescence of the previous infection or due to exogenous reinfection by another strain.[5] Therefore, an accurate method for the detection and differentiation of *H. pylori* strains, both before and after therapy, is of great importance for diagnosis and monitoring of treatment.[6] Recently, many genotypic methods have been suggested for the purpose of allowing high levels of discrimination between bacterial strains.[7] As a result of improved PCR technology, a new DNA fingerprinting technique such as the analysis of restriction fragment length polymorphism (RFLP) has considerable potential for fingerprinting isolates of *H. pylori*.[8-10] Therefore, the purpose of this study was to determine the effectiveness of PCR-based RFLP analysis for differentiating reinfection and recrudescence of *H. pylori* strains following triple therapy in patients with duodenal ulcer.

MATERIALS AND METHODS

Patient characteristics

Twenty duodenal ulcer patients (male : female= 19 : 1, mean age 38.25 years old) treated with a 1-2 week regimen of omeprazole 20 mg, amoxicillin 1.0 g, and clarithromycin 500 mg (OAC) twice daily were enrolled over a three-year period from March 1996 and divided into two groups. Ten patients (group 1, all male) were not successfully treated, and another 10 patients (group 2, M : F = 9 : 1) experienced recurrence of infection 6-24 months following triple therapy. The mean follow-up period of both groups were 2.1 months and 13.9 months, respectively. Two of the group 2 patients (patient 1, 7) showed recurrence of ulcer after 16 and 13 months following the therapy. They were referred to the outpatient clinic at regular intervals; 1 month, 6 months, and 1 year after the triple therapy. However, patients did not come regularly because they were not experiencing gastrointestinal symptoms until they revisited our hospital. Follow-up diagnoses were performed by CLO test (Delta West, Bentley, Australia) and Giemsa staining. The endoscopic apparatus, GIF-Q230 gastroscopes (Olympus, Tokyo, Japan) including all channels, and biopsy forceps (Olympus FB 25K-1, Tokyo, Japan) were carefully disinfected by immersion in a 3% peroxygen/organic acid surfactant blend (Virkon, Galderma Ltd., Seoul, S. Korea) solution for 10 min, rinsed in water, and dried after use with each patient and at the end of each endoscopic session. A biopsy specimen of each patient was taken with a different set of sterilized biopsy forceps. Eight biopsies were taken from the antrum and midbody greater and lesser curvature at follow-up endoscopies. Four of the biopsy specimens from each patient were used for Giemsa stain, and two biopsy specimens were used for CLO test. Two specimens from antral and midbody biopsy were stored in -70℃ freezer until being used for RFLP analysis before and after therapy.

Preparation of genomic DNA for PCR

Two biopsy samples from antrum and midbody were homogenized in 500 μℓ of STE buffer, and digested with 100 μg of proteinase K (Sigma Chemical Co., St. Louis, MO, USA) per mL in 1.0% sodium dodecyl sulfate (SDS) for overnight at 56℃. The solution was purified by phenol-chloroform extraction, ethanol precipitation, and resuspension in 60 μℓ of distilled water, and used as a PCR template.

PCR

The primers for the *ureC* gene were derived from the sequence of the *ureC* gene. Briefly, the amplification product of *H. pylori ureC* primers 5′-TGGGACTGATGGCGTGAGGG and 5′-AAGG GCGTTTTTAGATTTTT was 820-bp in length (Fig. 1). PCR amplification was performed in 100 μℓ, using genomic DNA 10 μℓ, 1 X reaction buffer (100 mM Tris-HCl pH 8.3, 400 mM KCl), 1.5 mM $MgCl_2$, 200 uM each dATP, dCTP, dTTP, and dGTP, 100 pM of each primer, and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA). The reaction was carried out in a thermal cycler (Gene Amp 9600 system, Perkin-Elmer Cetus, Norwalk, CT, USA) programmed for an initial denaturation of the target DNA at 95℃ for 5 minutes, and then 35 cycles at 95℃ for 1 min, 45℃ for 1 min, and 72℃ for 1 min 30 sec. The final cycle used 10 min at 72℃. The PCR products were electrophoresed on a 1.5% agarose gel (Gibco-BRL, Gaithersburg, MD, USA) for 30 minutes at 100 volts. The gels were examined by transillumination and photographed.

RFLP analyses

Two μg of the amplified DNA were digested with 20 U of the restriction enzymes *Hha* I, *Hind* III, and *Mbo* I (Boehringer Mannheim Gmbh, Germany) for 1 hr (*Hha* I, *Hind* III) and 3 hrs (*Mbo* I) at 37℃ in the buffer. The digested product was analyzed by electrophoresis in a 3% agarose gel (Metaphor agarose, FMC Bioproducts, Rockland, ME, USA) for 2 hrs at 50 volts. The gels were examined using transillumination and photographed.

RESULTS

PCR amplified an 820 bp DNA fragment of the

ureC gene from gastric biopsy specimens (Fig. 1) digested with the restriction enzymes *Hha* I (H), *Mbo* I (M), and *Hind* III (Hn). Each DNA fragment underwent electrophoresis and was compared with a standard DNA size marker to infer the strain of *H. pylori*. After analyzing several DNA fragments, even the same number of DNA fragments can be produced in different sizes, and the number and size of DNA fragments may all be different. Therefore, the strain of *H. pylori* can be distinguished by the combination of band patterns obtained from specific restriction enzymes. The figure showed somewhat indistinguishable band patterns because of the relatively small amount of DNA samples; however, the figure did not effect the result of grossly confirmed band patterns. Restriction enzyme digestion profiles from 20 different patients with duodenal ulcer are reported in detail in table 1. *Hha* I, *Mbo* I, and *Hind* III digestion produced 13, 7, and 2 distinguishable RFLP patterns, respectively. Overall, 23 distinct RFLP profiles were obtained from the 20 patients (Table 1). The combined RFLP profiles of paired specimens before and after treatment from 17 patients showed an infection with a single strain of *H. pylori*. This included nine of the group 1 and eight of the group 2 patients. These eight, including two patients with recurrent ulcer that showed re-

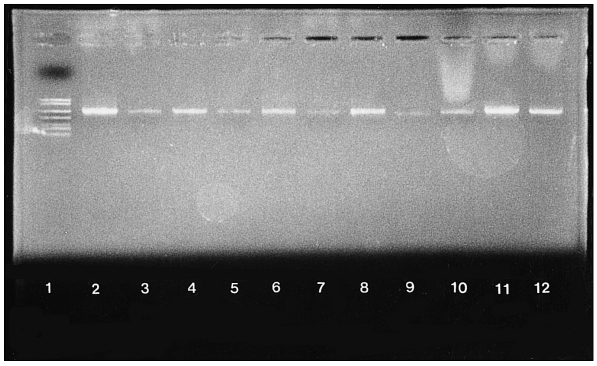

Fig. 1. Representative results for the PCR-amplified 820 bp *ureC* region of *H. pylori*. Lane 1 shows molecular size markers, lanes 2 to 12 show gastric biopsy specimens.

Table 1. Restriction Enzyme Digestion Profiles from 20 Different Patients with Duodenal Ulcer

Enzyme Pt code	*Hha* I Pre	*Hha* I Post	*Mbo* I Pre	*Mbo* I Post	*Hind* III Pre	*Hind* III Post	Subtype	Follow-up biopsy (mo)	Group
1	H4	H4	M1	M1	Hn1	Hn1	1	16	2
2	H7	H7	M3	M3	Hn2	Hn2	2	1.5	1
3	H8	H8	M3	M3	Hn1	Hn1	3	1.5	1
4	H5	H5	M1	M1	Hn2	Hn2	4	3.5	1
5	H5	H5	M5	M5	Hn2	Hn2	5	3	1
6	H1	H1	M3	M3	Hn2	Hn2	6	1.5	1
7	H9	H9	M2	M2	Hn1	Hn1	7	13	2
8	H2	H2	M3	M3	Hn1	Hn1	8	2	1
9	H5	H5	M2	M2	Hn2	Hn2	9	9	2
10	H11	H11	***M6***	***M7***	Hn2	Hn2	10,21	18	2
11	H1	H1	M4	M4	Hn1	Hn1	11	20	2
12	H3	H3	M2	M2	Hn2	Hn2	12	8	2
13	H10	H10	M1	M1	Hn2	Hn2	13	12	2
14	***H9***	***H12***	***M1***	***M7***	Hn1	Hn1	14,22	13	2
15	H4	H4	M1	M1	Hn2	Hn2	15	6	2
16	H6	H6	M3	M3	Hn1	Hn1	16	3	1
17	H9	H9	M6	M6	Hn2	Hn2	17	2	1
18	H6	H6	M3	M3	Hn2	Hn2	18	1.5	1
19	H9	H9	M1	M1	Hn1	Hn1	19	24	2
20	***H4***	***H13***	***M7***	***M6***	Hn2	Hn2	20,23	1.5	1

mo, months.

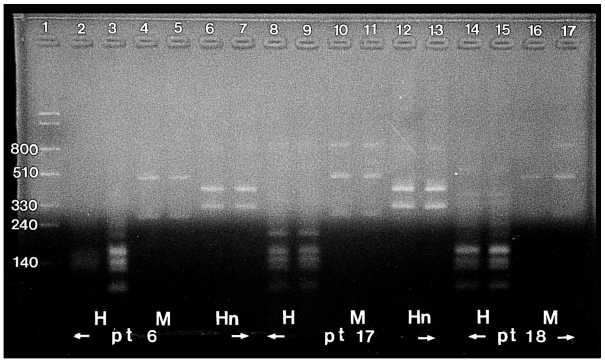

Fig. 2. PCR amplified 820 bp DNA fragment from gastric biopsy specimens digested with the restriction enzymes *Hha* I (H), *Mbo* I (M), and *Hind* III (Hn). Lane 1 represents molecular size markers. Lanes 2 to 7 represent patient 6, lanes 8 to 13 represent patient 17, lane 14 to 17 represent patient 18. The left lane of each pair represents pre-treatment results and the right lane represents post-treatment results. The combination patterns from the pre-treatment gastric biopsy specimens (patient 6, 17, 18) matched those of post-treatment.

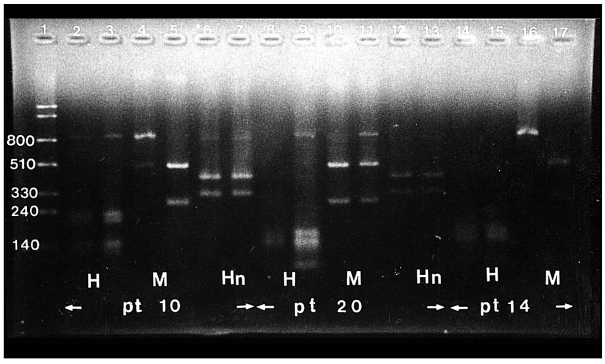

Fig. 3. Lanes 2 to 7 represent patient 10, lanes 8 to 13 represent patient 20, lane 14 to 17 represent patient 14. The left lane of each pair represents pre-treatment results and the right lane represents post-treatment results. Patient 10 (group 2) showed a combination pattern of H11, M6, Hn2 before the therapy and differed in *Mbo* I pattern after the therapy. Patient 20 (group 1) showed a combination pattern of H4, M7, Hn2 before the therapy and differed in *Hha* I and *Mbo* I patterns after the therapy. Patient 14 (group 2) showed a combination pattern of H9, M1, Hn1 before the therapy and differed in *Hha* I and *Mbo* I patterns after the therapy.

crudescence of previous infection. Before and after the triple therapy, patient 6 showed the combination pattern of H1, M3, Hn2, patient 17 showed that of H9, M6, Hn2, and patient 18 showed that of H6, M3, Hn2 (Fig. 2). Different RFLP profiles before and after the therapy were revealed in 3 patients. In these 3 patients (patient 10, 14, 20), the RFLP profiles after treatment were different for *Mbo* I (patient 10), or *Hha* I and *Mbo* I (patient 14, 20) RFLP patterns (Fig. 3). Two group 2 patients (patient 10, 14) showed reinfection of another strain.

DISCUSSION

This study shows that PCR-based RFLP analysis using gastric biopsy specimens can be effective for differentiating *H. pylori* strains and whether the recurrence of infection following *H. pylori* eradication therapy is due to the recrudescence of the previous infection or due to reinfection by another strain. The identification of strains using PCR-based RFLP of the *ureC* gene has been shown to be an effective method for differentiating between reinfection with a new strain and recrudescence of the original strain that failed to respond to the treatment.[5,11] In the present study, *Hha* I, *Mbo* I, and *Hind* III digestion produced 13, 7, and 2 distinguishable RFLP patterns, respectively. Compared with other studies, *Hha* I, *Mbo* I, and *Alu* I digestion produced 11, 10, and 6 distinguishable RFLP patterns from the 19 gastric biopsy specimens, respectively.[5] Additionally, by using *Hind* III, all *H. pylori* strains of study subjects[12] and two distinct types[13] were fingerprinted in agreement with this study. Our study reconfirms the regional variance and genetic diversity of *H. pylori* strains. Two of eight group 2 patients showing recrudescence of the previous infection revealed the recurrence of ulcer with epigastric pain. The other two patients showed reinfection of a different strain without recurrence of ulcer. One group 1 patient showed different RFLP profiles after therapy. This result might be explained by the possible initial concurrent *H. pylori* infection of both areas or by a treatment factor.

Treatment may be a factor in promoting minor changes in *H. pylori*.[14] Omeprazole treatment specifically induces genomic changes in *H. pylori* which result in modified stable genotypes.[14] In this study, the PCR-based RFLP patterns were compared before and after OAC triple therapy. Identical PCR-based RFLP profiles were reported following treatment with clarithromycin,[9] or with clarithromycin and/or omeprazole.[11] Owen RJ et

al.[15] showed that only two patients had multiple subtypes of *H. pylori* before treatment; however four patients displayed new or different subtypes subsequent to treatment. In their study, molecular typing methods showed that the failed therapy almost certainly resulted from recrudescence of infection due to incomplete eradication of *H. pylori*, which in most of the patients was originally a mixed population of types or subtypes.[15] It is reported that these minor genomic variations may have arisen from point mutations or inversions occurring over time.[13]

Although the PCR-based RFLP method provides for an easy typing scheme of isolates, it does not reveal the true extent of genetic diversity. This method is limited when it comes to differentiating isolates because it detects only a single base change at a specific restriction site of the enzymes, even if many other regions differed throughout the entire genome.[6]

In conclusion, the results of this study support the effectiveness of PCR-based RFLP analysis for differentiating reinfection and recrudescence of *H. pylori* strains following triple therapy.

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