Genotypic Variation of *Helicobacter pylori* Isolated from Gastric Antrum and Body in Korean Patients

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Although most persons infected with *Helicobacter pylori* harbor a single strain of the organism, multiple strain colonization in the same patient is also occasionally reported in developed countries. The aims of this study were to determine the prevalence of multiple strain colonization in Korean patients and to detect the *cagA*, *iceA*1, and *babA* status of *H. pylori* isolated from the antrum and body of the stomach. *H. pylori* was obtained from 35 patients from the antrum and body of the stomach. The genomic diversity of *H. pylori* was determined by random amplified polymorphic DNA analysis. The status of *cagA*, *iceA*1, and *babA* genes of *H. pylori* was assessed by polymerase chain reaction with appropriate primers. Clearly different diversity patterns were identified among the isolates from 35 individual patients. Eighteen (51.4%) patients had a single strain of *H. pylori*. Eight (22.9%) and nine (25.7%) patients had subtypically (one or two bands difference) and typically (clearly different pattern) different strains of *H. pylori* in the antrum and body, respectively. Among the 70 isolates of *H. pylori* from 35 patients, the positive rates of 349-bp and 208-bp *cagA* gene fragments and the *iceA*1 gene were 68/70 (97.1%), 68/70 (97.1%), and 58/70 (82.9%), respectively. However, the *babA* gene was found in 22/66 cases (31.4%). In five out of 18 patients with a single strain, the genetic status of *cagA*, *iceA*1, and *babA* varied between the isolates from the antrum and the body. In 8/17 patients with subtypically or typically different strains, the gene status differed between antrum and body isolates.

The prevalence of co-colonization with typically or subtypically different strains is high in Korea, and sub-clones with different pathogenic gene status exist within strains of identical RAPD patterns.

Key Words: *H. pylori*, Genomic diversity, *cagA*, *iceA*1, and *babA*

INTRODUCTION

Various DNA-based methods have been used to identify and type *H. pylori*, and it is recognized that great genetic diversity exists between isolates from different persons (13,17,26). Although most infected persons harbor a single *H. pylori* strain, mixed infections have been reported in some cases (13,15,17,22,25,26). The reported prevalence of multiple strain colonization varies and is usually below 20% (13,15,25,

26). However, most studies have been carried out in countries where the prevalence of *H. pylori* infection is relatively low. In Korea, *H. pylori* infection and the diseases related to *H. pylori* infection are highly prevalent (30). We hypothesized that the rate of multiple-strain infection should be high among carriers of *H. pylori* in Korea, who have frequent opportunities for exposure to this organism after childhood.

It is important to recognize that co-colonization with a mixture of pathogenic and non-pathogenic strains may occur in clinical settings, because treatment may only be necessary for the pathogenic ones. Several genes have been identified that may play a role in the pathogenicity of *H. pylori*. The cytotoxin-associated gene (*cagA*) is considered a marker for genomic pathogenicity island (9). Several genes of this *cag* island encode proteins that enhance the virulence of the strain, by inducing cytokine production by the host (4,9,28). A novel gene has been recently discovered (23), designated *iceA* (induced by contact with epithelium). There are two main allelic variants of the gene: *iceA*1 and *iceA*2. The function of *iceA* is not yet clear, but the expression of *iceA*1 is up-regulated on contact between *H. pylori* and human epithelial cells and may be associated with peptic ulcer disease. Doom et al. (12) reported that *cagA* and *iceA*1 are markers of *H. pylori* strains that are more likely to lead to ulcer disease. Ilver et al. (16) identified the *H. pylori* blood group antigen-binding adhesin gene, *babA*, related to the binding activity between bacterial adhesin and a host receptor.

The aims of this study were to determine the prevalence of mixed infections, using random amplified polymorphic DNA (RAPD) analysis, and to detect the *cagA*, *iceA*1, and *babA* status of *H. pylori* samples isolated from the antrum and body of the stomach within individual patients.

MATERIALS AND METHODS

***H. pylori* Isolates.** *H. pylori* was isolated from biopsy specimens at the antrum and body of stomach from the patients during endoscopic examination in Chungbuk National University Hospital, Korea. The 35 patients (24 males; 11 females; median age 52 years, ranging from 13~84 years) were enrolled in this study. None had taken bismuth, antibiotics, or omeprazole within the previous six weeks. The histological diagnosis were duodenal ulcer in 15 (42.8%), gastric ulcer in 8 (22.9%), gastric cancer in 3 (8.6%), chronic atrophic gastritis in 2 (5.7%), and chronic active gastritis in 7 (20.0%) patients. *H. pylori* was cultured and subcultured on Mueller-Hinton agar (Difco, Sparks, Maryland, U.S.A.) plates containing 10% sheep blood, vancomycin (10 μg/ml), nalidixic acid (25 μg/ml), and amphotericin B (1 μg/ml) for 3 to 5 days at 37°C under 100% humidity and 10% CO_2. *H. pylori* was identified with gram negativity and positive activities of urease (within 1~2 min) and catalase. One-half of colonies was used for DNA extraction and the other was stored in liquid nitrogen.

DNA Isolation. *H. pylori* DNA was isolated using GeneReleaser (GR; BioVentures, Inc., Murfreesboro, Tenn., U. S. A.) One loop of each isolated bacterial strain was added to 20 μl of GR into the thermocycle tube. Samples with GR were denatured (65°C for 30 s, 8°C for 30 s, 65°C for 90 s, 97°C for 180 s, 8°C for 60 s, 65°C for 180 s, 97°C for 60 s, 65°C for 60 s) in the thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, Conn., U.S.A.).

Polymerase Chain Reaction. All primers used in this study are presented in Table 1. For the genomic fingerprinting by RAPD, PCR was performed in the 50 μl reaction mixture containing 2 μl of genomic DNA released by GR, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 100 pmol primers (primer 1254 and

**Table 1.** Nucleotide sequences of oligonucleotide primers for RAPD analysis and PCR

Primer designation	Primer sequence (5'-3')	Size (bp)	Genotype
1281	AACGCGCAAC		RAPD
1254	CCGCAGCCAA		RAPD
Cf1	GATAACAGGCAAGCTTTTGAGG	348	*cagA*
Cr1	CTGCAAAAGATTGTTTGCGAGA		
Cf2	GAATCAGTATTTTTCAGAC	208	*cagA*
Cr2	GGGTTGTATGATATTTTCC		
LBf	AATCCAATTTAATCCAAA	190	*babA*
LBr	ATAGTTGTCTGAAAGATC		
ice1f	GTTGGGTAAGCGTTACAGAATTT	557	*iceA*1
ice1r	CATTGTATATCCTATCATTACAAG		

primer 1281), 200 μM of each dNTP, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo Co., Ltd., Kyoto, Japan). The PCR program comprised 5 min of predenaturation at 94℃, followed by 30 cycles of 30 s at 94℃, 30 s at 36℃, and 1 min at 72℃ and a final incubation at 72℃ for 5 min. PCR products were inspected by electrophoresis on 2% agarose gels. For the detection of *cagA, iceA*1 and *babA* PCRs were performed in a volume of 50 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, 200 μM of each dNTP, 2 μl of genomic DNA released by GR, 2.5 U of *Taq* DNA polymerase, and 25 pmol of speicific primer sets (Table 1). The PCR program for *cagA* comprised 5 min of predenaturation at 94℃, followed by 32 cycles of 1 min at 94℃, 1 min 30 s at 55℃, and 2 min at 72℃ (349-bp *cagA* fragment) or 1 min at 94℃, 1 min 30 s at 48℃, and 2 min at 72℃ (208-bp *cagA* fragment) and a final incubation at 72℃ for 5 min. The PCR program for *babA* comprised 5 min of predenaturation at 94℃, followed by 30 cycles of 1 min at 94℃, 1 min at 38℃, and 1 min at 72℃ and a final incubation at 72℃ for 5 min. The PCR program for *iceA*1 comprised 5 min of predenaturation at 94℃, followed by 40 cycles of 30 s at 94℃, 45 s at 50℃, and 45 s at 72℃

and a final incubation at 72℃ for 5 min. After amplification 10 μl of PCR product was electerophoresed on 1.7% agarose gel and examined under UV illumination. DNA types were defined on the basis of clearly distinct DNA fingerprints with multiple band differences, and subtypes were defined when only one or two bands differed.

RESULTS

Clearly different DNA fingerprints were identified among the isolates from 35 individual patients. In 18 patients (51.4%), RAPD patterns of *H. pylori* isolates from stomach antrum were identical to those of *H. pylori* isolates from stomach body (Fig. 1). In eight patients (22%), however, *H. pylori* isolates from antrum showed different RAPD patterns from those of body isolates (Fig. 2), and in nine patients (25.7%) clearly different RAPD patterns were observed between *H. pylori* isolates from antrum and body (Fig. 3). Representative examples of the genetic status of *cagA, iceA*1, and *babA* among the *H. pylori* isolates are shown in Fig. 4. Among the 70 isolates of *H. pylori* from 35 patients, the 349-bp and 208-bp fragments of the *cagA* gene, and the *iceA*1 gene were found

1254 primer

1281 primer

A

B
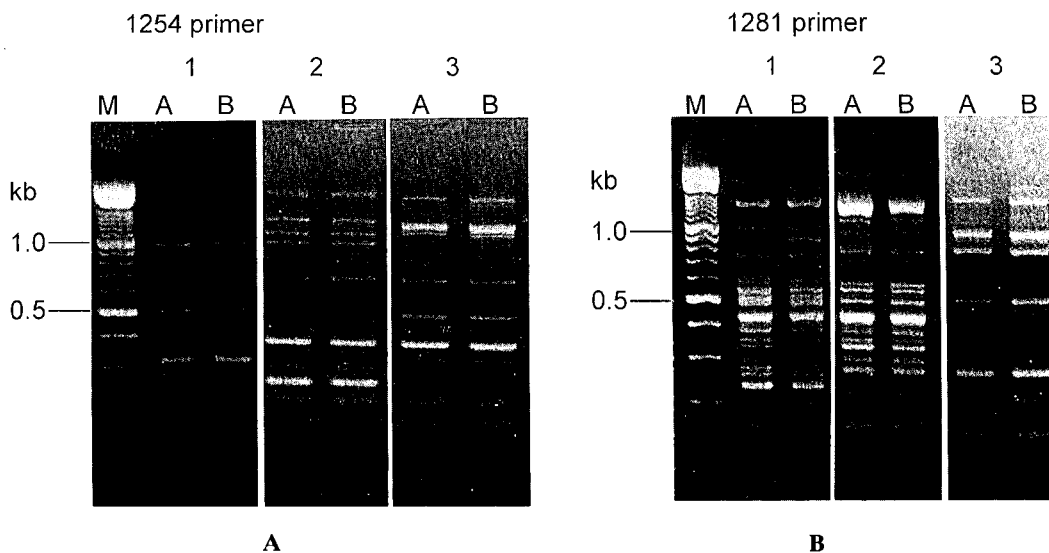
Figure 1. Representative RAPD patterns of identical *H. pylori* isolates from 3 individual patients using 1254 and 1281 primers. RAPD fingerprinting shows identical patterns between antrum and body isolates. M, 100 bp DNA step ladder marker; 1 to 3, *H. pylori* isolates (**A**, antrum; **B**, body).

1254 primer

1281 primer

A

B
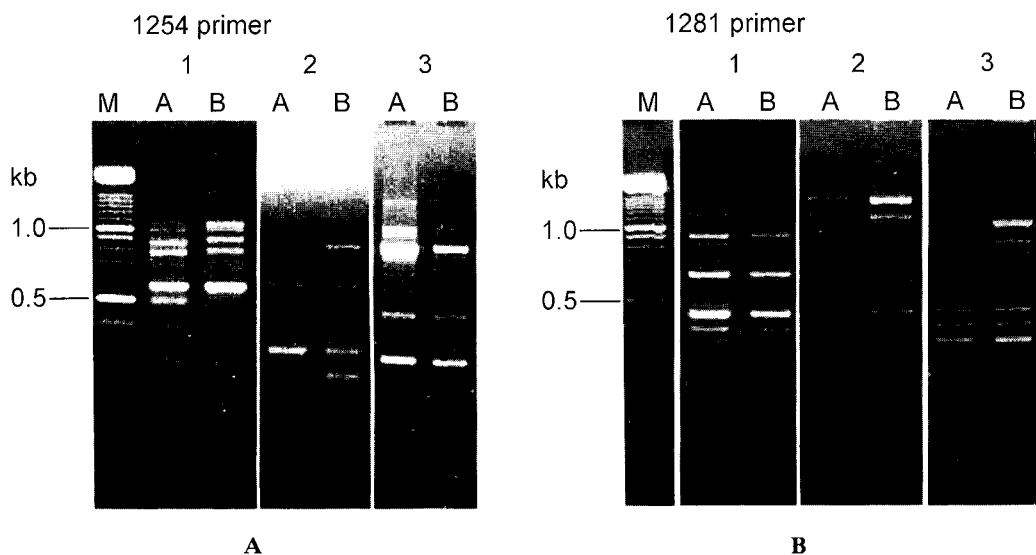
Figure 2. Representative RAPD patterns of subtypically different *H. pylori* isolates from 3 individual patients using 1254 and 1281 primers. RAPD fingerprinting shows only one or two band difference (subtypical variation) between antrum and body isolates. M, 100 bp DNA step ladder marker; 1 to 3, *H. pylori* isolates (**A**, antrum; **B**, body).

in 68 (97.1%), 68 (97.1%), and 58 (82.9%) isolates, respectively (Table 2). However, the *babA* gene was found in only 22 of 66 isolates (31.4%) (4 isolates were lost before *babA*

gene analysis). In five out of 18 patients whose stomach antrum and body were infected with identical *H. pylori* strains in RAPD fingerprinting, the genetic status of *cagA, iceA*1, and

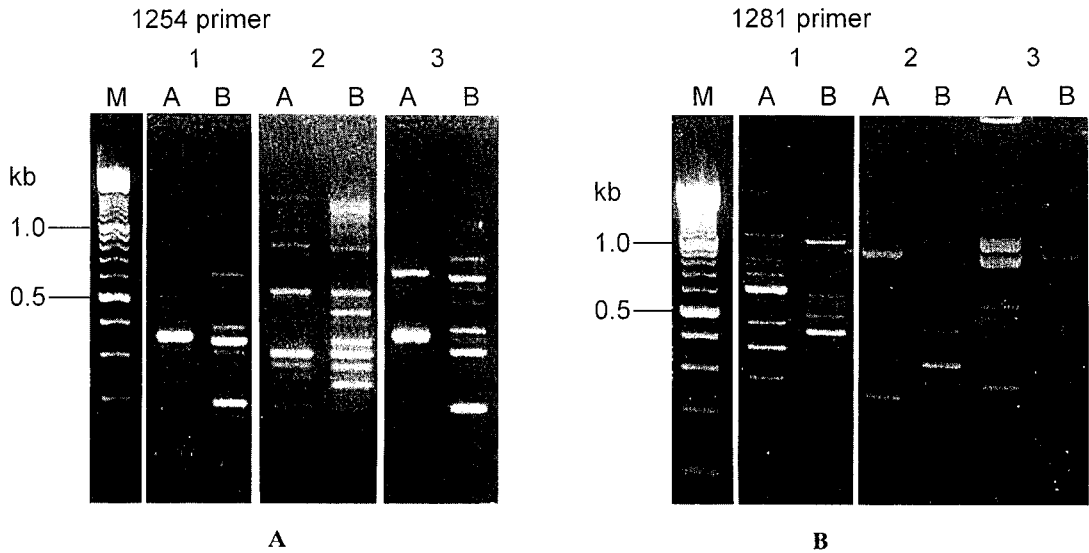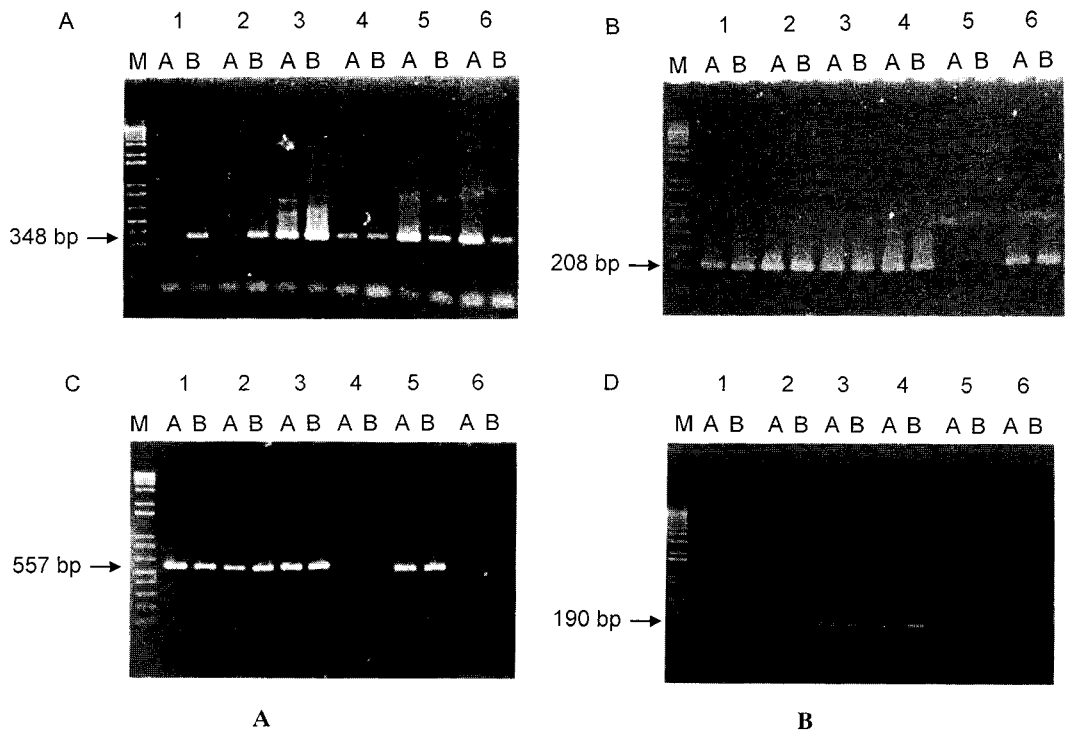**Figure 3.** Representative RAPD patterns of typically different *H. pylori* isolates from 3 individual patients using 1254 and 1281 primers. RAPD fingerprinting shows clearly distinct patterns (typical variation) between antrum and body isolates. M, 100 bp DNA step ladder marker; 1 to 3, *H. pylori* isolates (**A**, antrum; **B**, body).
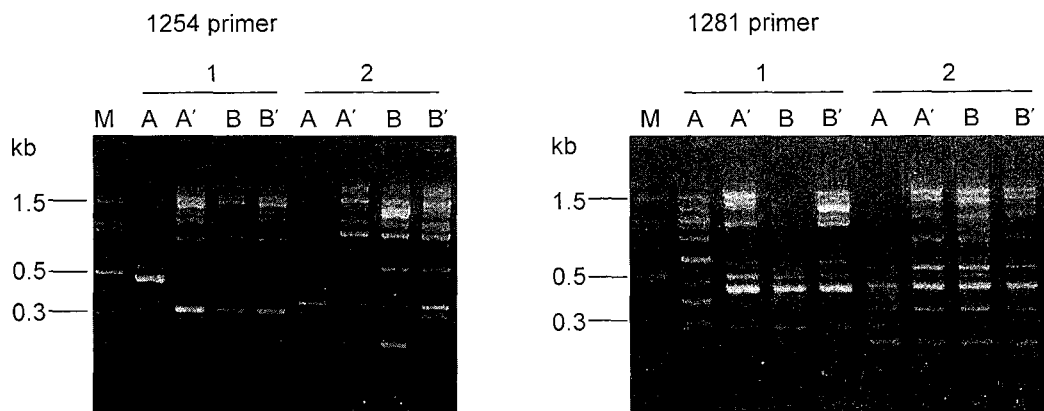

Figure 4. Representative PCR patterns of pathogenicity related genes. **A**, 348-bp *cagA* gene; **B**, 208-bp *cagA* gene; **C**, *iceA*1 gene; **D**, *babA* gene; M, 1 kb ladder marker; 1 to 6, *H. pylori* isolates. (A, antrum; B, body).

Table 2. Characteristics of patients and genotype variation of *H. pylori* isolates by RAPD and genetic status of *cagA*, *iceA*1, and *babA* genes

Genotype patient No.	Sex/Age	Diagnosis	cagA-349 bp antrum/ body	cagA-208 bp antrum/ body	iceA1 antrum/ body	babA antrum/ body
Patients whose antrum and body were infected with identical strains						
1	F/28	GC	+/+	+/+	+/+	+/+
2	M/84	GC	+/+	+/+	+/+	−/+
3	F/15	CAG	+/+	−/−	−/−	−/+
4	F/66	DU	+/+	+/+	−/−	−/−
5	M/48	GU	+/+	+/+	+/+	−/+
6	M/56	GU	+/+	+/+	+/+	−/−
7	M/71	DU	+/+	+/+	+/+	−/−
8	F/13	CAG	+/+	+/+	−/+	−/−
9	M/55	GC	+/−	+/+	+/+	−/−
10	M/62	DU	+/+	+/+	+/+	−/−
11	M/47	DU	+/+	+/+	+/+	+/+
12	M/30	DU	+/+	+/+	+/+	−/−
13	M/51	GU	+/+	+/+	+/+	+/+
14	F/51	CAG	+/+	+/+	+/+	−/−
15	M/43	GU	+/+	+/+	+/+	−/−
16	M/52	DU	+/+	+/+	+/+	+/+
17	M/49	DU	+/+	+/+	+/+	−/+
18	M/39	DU	+/+	+/+	+/+	
Patients whose antrum and body were infected with subtypically different strains						
1	M/69	GU	+/+	+/+	+/+	−/−
2	F/64	AG	+/+	+/+	−/−	−/+
3	M/47	GU	+/+	+/+	+/+	−/+
4	M/57	DU	+/+	+/+	+/+	−/+
5	F/18	CAG	+/+	+/+	+/+	−/−
6	F/53	CAG	+/+	+/+	+/+	−/−
7	M/77	GU	+/+	+/+	+/+	+/+
8	F/53	AG	+/+	+/+	−/+	−/−
Patients whose antrum and body were infected with typically different strains						
1	M/62	DU	+/+	+/+	+/+	−/−
2	F/59	DU	+/+	+/+	+/+	−/−
3	F/42	CAG	+/+	+/+	−/−	−/+
4	M/29	CAG	+/+	+/+	+/+	−/−
5	M/52	DU	+/+	+/+	+/+	−/+
6	M/44	GU	+/+	+/+	+/+	+/−
7	M/65	DU	+/+	+/+	−/−	+/+
8	M/44	DU	−/+	+/+	+/+	−/−
9	M/64	DU	+/+	+/+	+/+	

Abbreviations: DU, duodenal ulcer; GU, stomach ulcer; GC, stomach cancer; CAG, chronic active gastritis; AG, atrophic gastritis

Figure 5. RAPD patterns of *H. pylori* isolates obtained from initial and 6 months follow-up of two patients. M, 100 bp DNA ladder marker; 1 to 2 patient number; A or B, initial isolates from antrum or body; A' or B' follow-up isolates from antrum or body after 6 months.

babA varied between the isolates from the stomach regions. In eight of 17 patients with subtypically or typically different strains in their stomach antrum and body, the gene status of the isolates differed between the regions sampled. We evaluated the time-dependent changes in RAPD patterns in two patients harboring different strains. After six months, subtypical or almost identical strains were isolated from the antrum and body, but RAPD analysis showed that they differed from the first isolates of both patients (Fig. 5).

DISCUSSION

This study demonstrates that mixed infection or colocalization with more than one strain of *H. pylori* is higher in Korea than in Western countries, probably because of more frequent re-exposure to infection. Differing types of *H. pylori* between the antrum and body were discovered in 25.7% of patients, and subtypical variation was seen in 22.9%. In five out of 18 patients whose stomach antrum and body were infected with a single strain, their isolates showed different patterns of pathogenic genes between the regions sampled. This indicates the possible existence of multiple strains in patients

apparently having only a single strain, according to RAPD analysis. Although RAPD has high discriminatory power, it only samples a minority of genomic sequences and thus only rarely detects any particular mutation (5,7). If more than two random primers (1254 and 1281) had been used for RAPD analysis, then more differences could probably have been demonstrated in these patients (3).

The prevalence of multiple strain colonization in the same individual differs between studies. Hirschl et al. reported a noticeable discrepancy between the pronounced inter-patient heterogeneity and the rare intra-patient variations of *H. pylori* (15). In the developed countries, multiple strain colonization is only rarely found. Prevalences of 2.5% have been reported by Shortridge et al. (25), 13.3% by Prewett et al. (24), 20% by Tayor et al. (26), and 16.7% by Fujimoto et al. (13). In contrast, Owen et al. (22) reported that only two of 13 patients had identical *H. pylori* in the stomach antrum, corpus and fundus. Jorgensen et al. (17) reported that most patients were colonized with more than two strains, with up to five variants. These differences may be due either to the different incidence of *H. pylori* infection in the populations studied, or to methodological factors.

Lee et al. (20) reported multiple strain infection in five of 24 patients (20.8%) in Korea using pulsed-field gel electrophoresis (PFGE), which was similar to our results here. As *H. pylori* was not isolated from individual colonies and only one biopsy sample was obtained from each site in our study, the prevalence of multiple strains may be even higher. Because treatment with bismuth and antibiotics may induce different DNA patterns (26), we only enrolled patients who had not been treated with these drugs.

In the present study, the high positive rate of identification of *cagA* in Korean patients suggests that this gene does not play a role in the differentiation of pathogenic and non-pathogenic strains, nor in genetic diversity. The *iceA*1 gene, related to the onset of peptic ulcers, was also frequently detected in the present study (58 of 70 strains). The frequency (82.9%) of *iceA*1 gene was higher than that (25% and 69%) of the westerns countries (23). Positive identification of the *babA* gene (22 of 70 strains) was not as high as for the other two genes. However, *babA* showed higher substrain genetic diversity (four of 18 strains) in strains with the same RAPD patterns. It is not clear if the *babA* gene is less stable than the other two genes or if it exhibits more frequent mutation or genetic drift (19).

Several typing systems are used to discriminate between isolates of *H. pylori* for epidemiological and clinical purposes. Methods include restriction enzyme analysis (21), ribotyping (11), PFGE (15), restriction fragment length polymorphism analysis of polymerase chain reaction product (PCR-RFLP analysis) (2), and PCR-based RAPD analysis (3). Among these techniques, PCR-RFLP analysis and RAPD analysis give optimal typing capacity and yield excellent discriminatory powers (8). However, RAPD is more convenient than PCR-RFLP. In the present study, we characterized different RAPD patterns between clinical isolates as be-

ing subtypical (one or two bands differed) and typical (clearly different pattern) strains. It is difficult to determine whether bacteria with subtypical differences are variants of one strain or should be considered wholly different strains. Because a difference of more than one band in RAPD patterns suggests that there is sufficient polymorphic DNA diversity between isolates (29), those strains with subtypical differences may be considered different strains. Experimently induced mixed infections with two different *H. pylori* strains in gnotobiotic piglets showed non-random distributions of the input strains (1), suggesting a microcolonial mode for *H. pylori* growth with relatively little migration between different sites. It is assumed that some individuals may become infected by strains specific to various sites, or it is quite possible that recent ingestion of a strain that is better suited to the patient than the first strain may occur in highly prevalent *H. pylori* infection areas, such as Korea. However, there is also the possibility that subtypical differences could represent quasi-species variation (19). *H. pylori* has high genomic diversity (14) and a number of explanations for this have been advanced. It may reflect a long period of evolution for *H. pylori* and its ancestors (6). Diversity could also reflect a process of persistent accumulation of mutations within individual strains, either from spontaneous mutations or resulting from horizontal uptake and incorporation of homologous (*H. pylori*) or heterologous DNA (19). *H. pylori* strains are naturally competent (27) and possess a conjugation-like mechanism for DNA uptake (18). These properties allow for the efficient incorporation of foreign DNA. In one animal study, bacteria recovered from mice given a mixture of strains 12 months previously developed minor, but consistent, differences in their RAPD patterns (10).

In our study, the sequential differences in RAPD patterns at a six-month interval in two patients showed the emergence of new strains

in both. It is not clear if the new strains already existed but were not isolated initially. This finding may represent the adaptation and selection of *H. pylori* to changing gastric environments. Persistent accumulation of mutations could provide a pool of *H. pylori* variants that can be selected for optimal colonization of particular gastric niches that develop because of host changes during the long duration of colonization.

In the present investigation, only 13 (37.1%) patients showed identical RAPD patterns and pathogenic gene status regardless of the regions sampled, suggesting a true single strain in their stomachs. However, it has also been reported that even strains with the same RAPD pattern can have mixed populations of metronidazol-susceptible and -resistant variants (5). Therefore, the high prevalence of multiple strain colonization should be considered as part of planning therapeutic approaches as well as in studying the pathogenesis of *H. pylori* infection.

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