High Prevalence of Multiple Strain Colonization of *Helicobacter pylori* in Korean Patients: DNA Diversity Among Clinical Isolates from the Gastric Corpus, Antrum and Duodenum

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Background : The aims of our study were to determine the correlation of the strain variation and degree of homogeneity of infecting *Helicobacter pylori* (*H. pylori*) with their disease outcomes, and the relevance of duodenal *H. pylori* expression of *cagA* and/or *vacA* gene to the development of duodenal ulcer in Korean patients.

Methods : One hundred and twenty bacterial colonies isolated from different anatomical sites of the stomach and duodenum were used. The study population was consisted of 40 Korean patients, 21 with duodenal ulcer, 7 with gastric ulcer, 3 with combined gastric and duodenal ulcer, and 9 with chronic gastritis. Genomic characteristics of each strain were analyzed by random amplified polymorphic DNA (RAPD) fingerprinting. The *cagA* and *vacA* genes were detected by polymerase chain reaction (PCR).

Results : PCR-based RAPD was proved to be a reliable method for the discrimination of individual bacterial genomic characteristics. Genomic fingerprinting showed a varying degree of inter- and intra-patient variation. Thirteen patients (32.5%) were colonized by a single strain throughout the corpus, antrum and duodenum, whereas the other 27 (67.5%) harbored multiple *H. pylori* strains. Thirty-six isolates (90.0%) each from the corpus and antrum, and 34 (85.0%) from the duodenum, expressed the *cagA* gene. The prevalence of duodenal *H. pylori* expression of the *cagA* gene was not different between patients with chronic gastritis and those with duodenal ulcer. All isolates were positive for both genes *vacA s1* and *vacA s1a*.

Conclusion : These results suggested that many of the *H. pylori*-infected Korean patients were actually colonized with mixed populations of different *H. pylori* strains and that the prevalence of duodenal *H. pylori* expression of the *cagA* and/or *vacA* gene was not correlated with the development of duodenal ulcer in Korean patients.

Key Words : *H. pylori*, DNA diversity, Genomic fingerprinting, *cagA*, *vacA*

INTRODUCTION

Long-term colonization with genotypically single, homogeneous or multiple heterogeneous strains of *Helicobacter pylori* (*H. pylori*), causes chronic inflammation of the stomach and duodenum, although the magnitude of inflammation varies from strain to strain and from host to host. In 20 to 30% of infected patients, clinical end results range from chronic active gastritis to peptic ulceration, gastric adenocarcinoma and gastric MALToma[1-3].

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The data on the genomic characteristics of infecting *H. pylori* have shown that an individual person can be colonized by a strain with the same genomic DNA throughout the stomach and duodenum[4-8], or by different strains with DNA heterogeneity in each organism found at different anatomical sites[1, 9-14]. Multiple strain infection of *H. pylori* occurs in both developed and developing countries. In addition, Campbell et al. suggested that separate bacterial lineages might have evolved in parallel with race-specific specialization[15]. Since the virulence differs between *H. pylori* strains, discrimination between the genomic characteristics of each isolate found in an individual with multi-strain infection may be important for diagnosis and treatment of infected patients[16]. Of the two representative techniques to discriminate between isolates of *H. pylori*, genotyping studies may be more useful since phenotypic expression of virulent determinants may be affected by storage and culture condition[17]. Among the most currently used techniques of typing systems based on polymerase chain reaction (PCR), random amplified polymorphic DNA (RAPD) analysis and repetitive extragenic palindromic DNA sequence-based PCR (REP-PCR) demonstrated optimal typeability (100%) and excellent discriminatory powers (0.99 to 1 and 0.99, respectively), while PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis demonstrated a lower discriminatory power (0.70 to 0.97). Chromosome restriction-based typing methods, such as ribotyping and pulsed-field gel electrophoresis (PFGE), are limited by a low typeability (12.5 to 75%) that strongly decreases their discriminatory power (0.92 and 0.24 to 0.88, respectively)[18].

Although there have been increasing reports opposed to the disease-specific significance of *cagA* and *vacA*[19-23], several investigators still consider that these two genes are important risk factors in certain ethnic populations for developing more serious gastroduodenal diseases[24-29].

The present study aimed to determine the strain variation of *H. pylori* by studying clinical isolates from four different patient groups and to determine the degree of homogeneity in organisms found at different anatomical sites within an individual patient. We also evaluated the significance of *cagA, vacAs1* and *vacAs1a* as virulent determinants for developing peptic ulcer diseases.

MATERIALS AND METHODS

1. Population Studied

Among *H. pylori*-infected Korean patients with verified diagnoses, 40 patients were selected who simultaneously showed positive results for the culture of mucosal biopsies from all three of the corpus, antrum, and duodenal bulb.

The 40 patients consisted of 21 with endoscopically confirmed duodenal ulcer (DU, mean age 43.2 years; age range 13-69 years; 14 men and 7 women), 7 with benign gastric ulcer (GU, mean age 51.6 years; age range 30-66 years; 6 men and 1 woman), 3 with combined gastric and duodenal ulcer (GDU, mean age 53.7 years; age range 48-58 years; all men), and 9 with chronic gastritis (CG, mean age 49.7 years; age range, 30-73 years; 3 men and 6 women). Patients were excluded if they had a history of gastric surgery, were receiving steroids or NSAIDs, had taken H_2-receptor antagonists, proton pump inhibitor, or antimicrobial agents within 30 days prior to study, or had active gastrointestinal bleeding. Patients with any other chronic illness were also excluded.

2. Clinical Isolation of *H. pylori*

A mucosal biopsy specimen was taken from each of the antrum, corpus and duodenal bulb in each patient. Antral and corpus biopsy specimens were taken at the greater curvature of the prepyloric region and the proximal corpus, respectively. Duodenal biopsy was taken at least 1 cm away from any ulcer margin. Each biopsy specimen was taken with a different pair of biopsy forceps. Between patients, the endoscope and biopsy forceps were washed automatically with a cycle including soaking in 2% glutaraldehyde for at least 4 minutes. In addition, the duodenal bulb was flushed several times with sterile water before sampling.

3. *H. pylori* Culture

One hundred and twenty clinical isolates were grown at 37℃ in microaerophilic condition for 3 to 7 days. *H. pylori* were confirmed by typical Gram stain morphology and biochemical tests positive for urease, oxidase, and catalase.

4. Extraction of Genomic DNA from Clinical Isolates

The best-growing colony from each biopsy was incubated with STE buffer, 20% sodium dodecyl sulphate and proteinase K solution. An equal volume of phenol: chloroform solution and 1/10 of final volume of a 3M SDS solution, pH 5.3, was added. Double stranded DNA was precipitated with ethanol, the reaction mixture was allowed to stand at -80℃ for 1 hour, and after centrifugation for 30 minutes at 12,000 g the resultant pellet was dissolved in 50 to 100 L of deionized water.

5. Detection of *vacA* and *cagA* Genes by PCR

PCR primers were designed on the basis of the published sequence of *H. pylori vacA s1* (VA1-F/VA1-R ATGGAAATACAACAAACACAC and CTGCTTGAATGCGCCAAAC, nucleotide position 797-1055, size of product 259 bp), *vacA s1a* (SS1-F/VA1-R GTCAGCATCACACCGCAAC and CTGCTTGAATGCGCCAAAC, nucleotide position 866-1055, size of

product 190 bp)[26], and two different sets of *cagA* gene (set 1: primer *cagA1*, GATATAGCCACTACCACCACCG, nucleotide position 1249-1270; and primer *cagA2,* GGAAATCTTTAATCT-CAGTTCGG, nucleotide position 1797-1819; size of product 570bp; set 2: *cagA7*, AGGAATCTCGCAATTAAGGG, nucleotide position 710-731; and *cagA8* TTCTATGCCATTATGAC-TCCCC, nucleotide position 1446-1467; size of product 757bp)[28]. The *cagA* genes were amplified with 5 minutes at 94C; 40 cycles of 1 minute at 94℃, 1 minute at 55℃, and 1 minute at 68℃ or 72℃; followed by 5 minutes at 72℃ for extension. The reaction conditions for *vacA s1* and *s1a* were as follows: 5 minutes at 94℃; 40 cycles of 1 minute at 52℃, 1 minute at 52℃, and 1 minute at 68C or 72℃; followed by 5 minutes at 72℃ for extension.

6. *H. pylori* Genome Typing by RAPD-PCR

PCR-based RAPD fingerprinting was performed using the method described by Akopyanz et al[1]. The reaction was carried out in 50 L containing 20 ng *of H. pylori* genomic DNA, 3 mM $MgCl_2$, 20 pM of primer, 2U of AmpliTaq DNA polymerase, 250 μM of each of dCTP, dGTP, dADP and dTTP in 1mM Tris-HCl, pH8.3, 50 mM KCl. For each primer, primer 1247 (AAGAGCCCGT), 1254 (CCGCAGCCAA), 1281 (AACGCGCAAC), or 1283 (GCGATCCCA), the following cycling program was used: 4 cycles of 94C for 5 minutes, 36℃ for 5 minutes, and 72℃ for 5 minutes; 30 cycles of 94℃ for 1 minute, 36℃ for 1 minute, and 72℃ for 2 minutes; and then 72℃ for 10 minutes. After PCR, 20 L aliquots of products were electrophoresed in 2% agarose gel containing 0.5 g/mL ethidium-bromide in the gel, and photographed under UV light.

7. Statistical Analysis

Chi square test and Fisher's exact test were used to determine the significance of differences between the four groups. Unless stated otherwise, a *p* value less than 0.05 was taken to be significant. All statistical analyses were performed using SPSS for windows ver 9.0 (Chicago, Ill., U.S.A).

RESULTS

Genomic Fingerprinting of *H. pylori* Isolates

RAPD-PCR amplification resulted in 2 to 10 resolved DNA bands; 4 to 8 bands with primer 1281, 2 to 9 bands with primer 1283, 2 to 8 bands with primer 1254, and 2 to 10 bands with primer 1247. In this study, primers 1281 and 1283 yielded clearly distinct DNA fingerprints with multiple band differences but primers 1247 and 1254 did not (Figure 1A, 1B). Discrimination of individual isolates and their DNA typing was based on the characteristics of DNA fingerprints, and band differences and subtypes were defined when only one or two bands differed (Figure 2).

RAPD-PCR fingerprintings of 120 isolates from 40 patients showed variable divergence of both inter-patient and intra-patient bacterial DNA characteristics. Many patients showed slight variations in the banding pattern, i.e., bands missing or bands appearing with different intensity or at slightly different molecular weights. On the basis of the fingerprint results, DNA patterns of the all isolates could be categorized into 5 different groups (Table 1).

Table 1. Summary of RAPD-PCR fingerprints results according to disease status

Disease Status \ Group	I	II	III	IV	V	Total
Chronic Gastritis	4	1	2	2	0	9
Duodenal Ulcer	4	10	3	1	3	21
Gastric Ulcer	3	3	0	1	0	7
Gastroduodenal Ulcer	2	1	0	0	0	3
Total	13	15	5	4	3	40

Group Ⅰ, *H. pylori* from the corpus, antrum and duodenum are all identical strains.
Group Ⅱ, *H. pylori* from the corpus, antrum or duodenum are totally different strains from each other.
Group Ⅲ, *H. pylori* only from the corpus and antrum are identical strains.
Group Ⅳ, *H. pylori* only from the antrum and duodenum are identical strains.
Group Ⅴ, *H. pylori* only from the corpus and duodenum are identical strains.

Group I patients (13; 4 DU, 3 GU, 2 GDU and 4 CG) were colonized by *H. pylori* with the same genomic DNA throughout the corpus, antrum and duodenum (Figure 3). Group II patients (15; 10 DU, 3 GU and 1 GDU and 1 CG) showed multi-strain infection, i.e., DNA patterns of *H. pylori* from the corpus, antrum and duodenum were totally different from each other within an individual patient (Figure 4). In five patients (Group III, 3 DU and 2 CG), only *H. pylori* from the corpus and antrum were an identical strain (Figure 5). Group IV patients (4; 1 DU, 1 GU and 2 CG) showed only infecting *H. pylori* from the antrum and duodenum were identical (Figure 6). Three DU patients (Group V) showed only *H. pylori* from the corpus and duodenum were identical (Figure 7).

Detection of *cagA and vacA* Genes

Thirty-six isolates (90.0%) each from the corpus and antrum, and 34 (85.0%) from the duodenum, expressed *cagA* gene. *H. pylori* strains positive for *cagA* from the corpus were found in all of the patients with CG (100%), in 18 of 21 (85.7%) with DU, and in 6 (85.7%) of 7 with GU. Antral strains

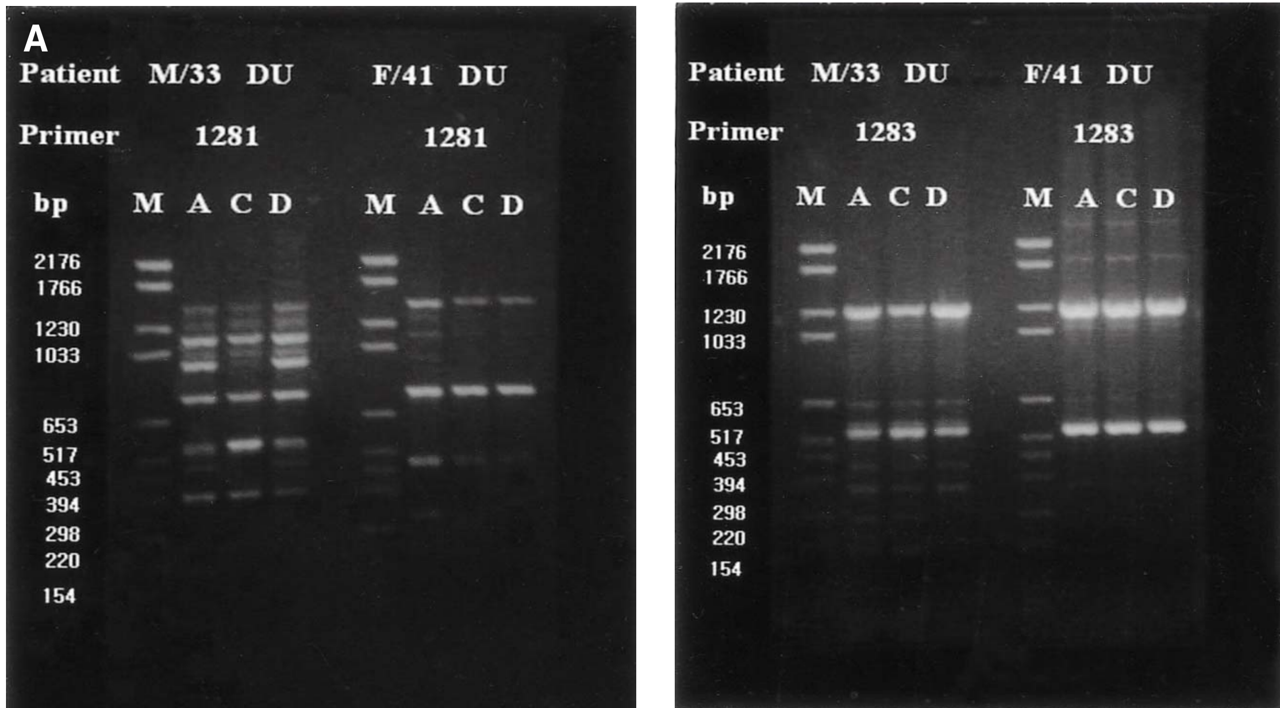

Figure 1. (A) Representative chromosomal DNA profiles of *H. pylori* isolated form 2 unrelated patients by RAPD-PCR DNA fingerprints obtained with primer 1281 and 1282. Column A, C, and D represent fingerprints of antral, corpus, and duodenal isolaeds of each patient, respectively. Primer 1281 and 1283 yielded clearly distinct DNA fingerprints with band differences.

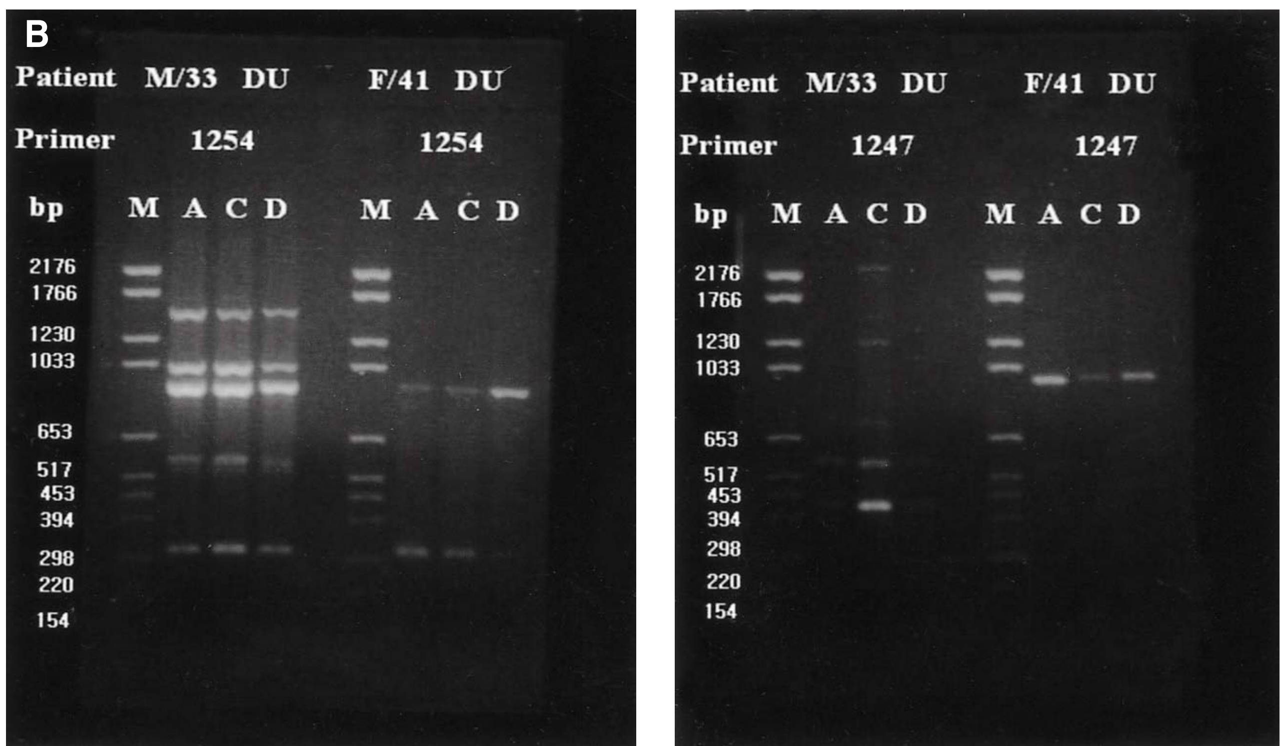

Figure 1. (B) Representative chromosomal DNA profiles of *H. pylori* isolated form 2 unrelated patients by RAPD-PCR DNA fingerprints obtained with primer 1254 and 1247. Column A, C, and D represent fingerprints of antral, corpus, and duodenal isolaeds of each patient, respectively. DNA fingerprints obtained with primer 1254 and 1247 wrer less distinct than those of 1281 and 1283.

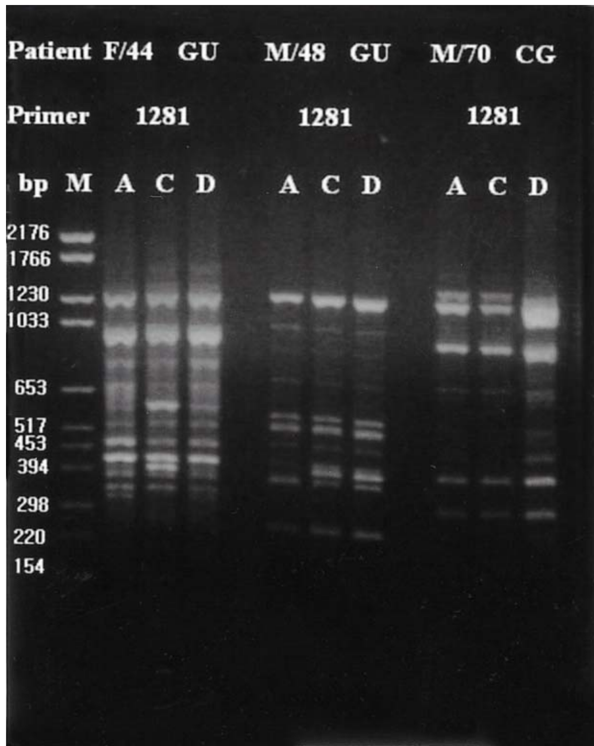

Figure 2. Representative chomosomal DNA subtypes of *H. pylori* isolated from 2 unrelated patients by RAPD-PCR DNA fingerprints obtained with primer 1281. Column A, C, and D represent fingerprints of antral, coupus, and duodenal isolates of each patient, respectively. DNA fingerprints showed slight variation in the banding pattern, i.e., bands missing or bands appearing with different intensity or at slightly different molecular weights.

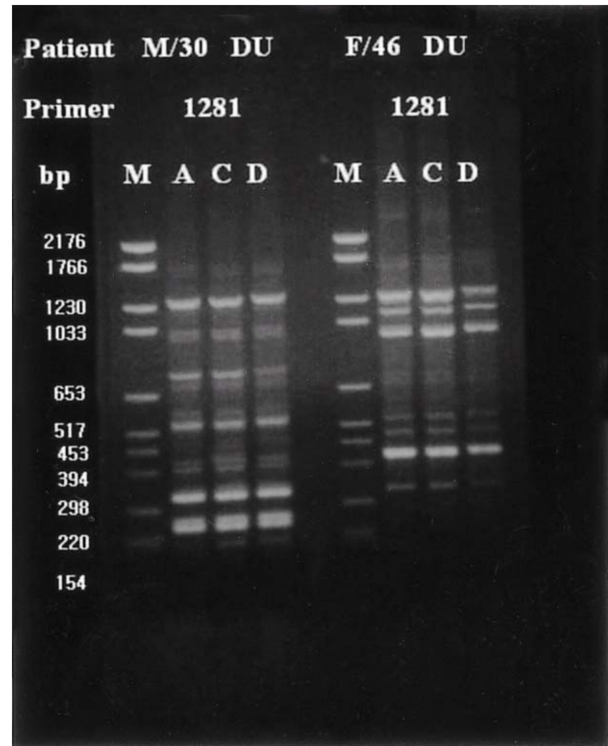

Figure 3. Representative chromosomal DNA profiles of antral (column A), corpus (column C), and duodenal (column D) *H. pylori* isolated from 2 unrelated patients. RAPD-PCR DNA fingerprints from different andtomical sites of each patient showed homegeneous genomic patterns.

positive for *cagA* were found in 8 (88.9%) patients with CG, 18 (85.7%) with DU and 7 (100%) with GU. *cagA*$^{+}$ duodenal strain were found in 8 CG (88.9%), 18 DU (85.7%) and 5 GU patients (71.4%) (Table 2). Out of thirty-six patients who were infected by *cagA*$^{+}$ *H. pylori*, 11 (30.6%) were below the age of 29.

All of the 120 strains isolated from the gastric corpus, antrum and duodenum were positive for both the *vacA s1* and *vacA s1a* genes.

Table 2. Relevance of cagA expression to clinical outcomes

Disease	No.	*cagA* Expression					
		Corpus		Antrum		Duodenum	
Status	Patients	No. Positive	No. Negative	No. Positive	No. Negative	No. Positive	No. Negative
Chronic Gastritis	9	9 (100%)	0	8 (88.9%)	1 (11.1%)	8 (88.9%)	1 (11.1%)
Duodenal Ulcer	21	18 (85.7%)	3 (14.3%)	18 (85.7%)	3 (14.3%)	18 (85.7%)	3 (14.3%)
Gastric Ulcer	7	6 (85.7%)	1 (14.3%)	7 (100%)	0	5 (71.4%)	2 (28.6%)
Gastroduodenal Ulcer	3	3 (100%)	0	3 (100%)	0	3 (100%)	0
Total	40	36 (90.0%)	4 (10.0%)	36 (90.0%)	4 (10.0%)	34 (85.0%)	6 (15.0%)

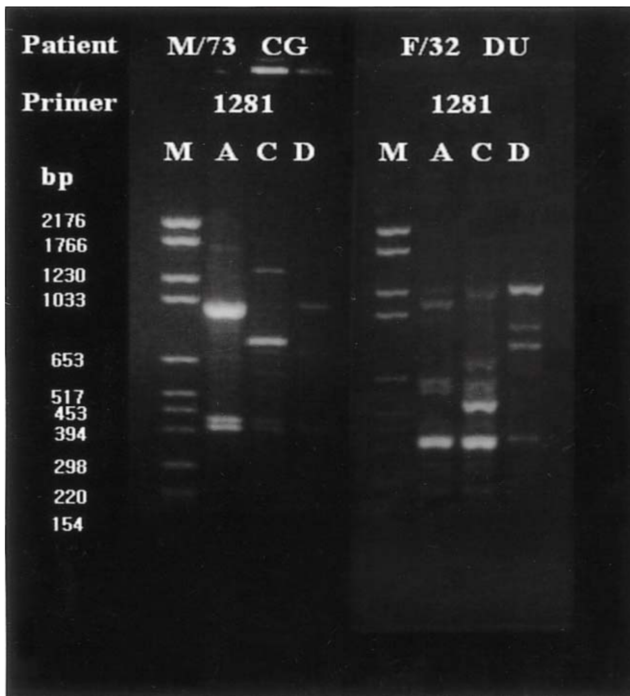

Figure 4. Representative chromosomal DNA profiles of antral (column A), corpus (column C), and duodenal (column D) *H. pylori* isolated from 2 unrelated patients. RAPD-PCR DNA fingerprints from different anatomical sites of each patient showed totally different genomic patterns.

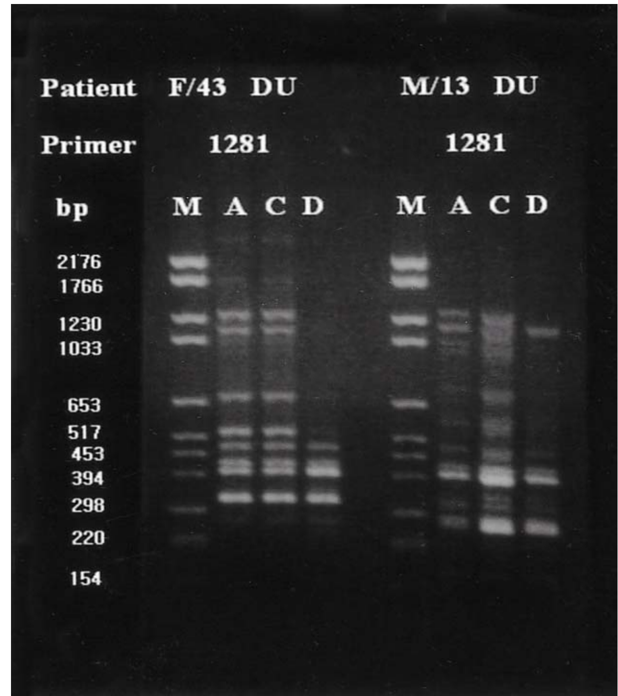

Figure 5. Representative chromosomal DNA profiles of antral (column A), corpus (column C), and duodenal (column D) *H. pylori* isolated from 2 unrelated patients. Isolates only from and corpus showed identical RAPD-PCR DNA fingerprints.

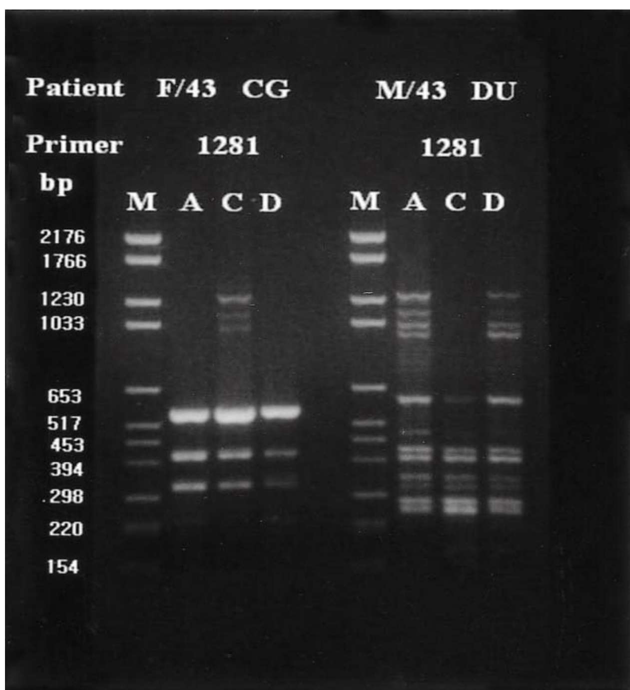

Figure 6. Representative chromosomal DNA profiles of antral (column A), corpus (column C), and duodenal (column D) *H. pylori* isolated from 2 unrelated patients. Isolates only from antrum and duodenum showed identical RAPD-PCR DNA fingerprints.

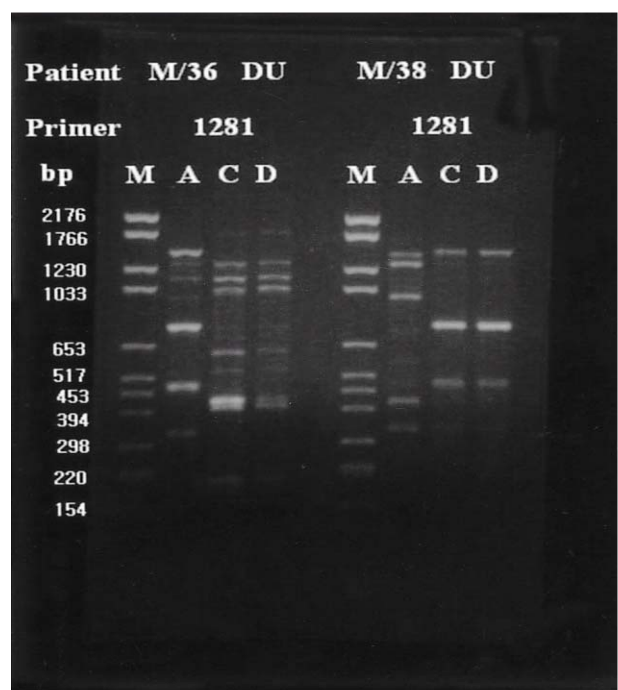

Figure 7. Representative chromosomal DNA profiles of antral (column A), corpus (column C), and duodenal (column D) *H. pylori* isolated from 2 unrelated patients. Isolates only from antrum and duodenum showed identical RAPD-PCR DNA fingerprints.

DISCUSSION

There have been two opposite opinions on the genomic characteristics of *H. pylori* strains colonizing the gastroduodenal mucosa. An individual patient can be colonized by either a single strain or a genetically predominant strain with the same genomic DNA, or by different strains which show the genomic heterogeneity of their DNA sequences found at different anatomical sites within an individual patient. Furthermore, some researchers have reported that infecting *H. pylori* in the duodenal mucosa in a particular gastroduodenal disease (i.e., DU) are genomically different strains from those of simple gastritis patients and that specific PCR products are associated with isolates from a particular disease and may contain genes encoding potential virulence factors[9, 15, 28].

The principal objectives of our study were to determine the prevalence of inter- and intra-patient variation of genomic characteristics of *H. pylori* colonizing at different anatomical sites of the stomach and duodenum and their relevance to clinical outcomes. Along with this aim, we also evaluated the significance of *cagA* and *vacA* genes as virulent determinants for developing peptic ulcer diseases in Korean patients. We analyzed DNA patterns of 120 *H. pylori* strains cultured from different anatomical sites of the stomach and duodenum of patients with DU, GU, GDU, and CG by PCR-based RAPD fingerprints.

The results confirmed that the genotypic comparison by RAPD-PCR fingerprinting between isolates collected from different patients or different anatomic sites within the same patient is a reliable method for discrimination of their genomic characteristics since the PCR results obtained with the primers 1281 and 1283 yielded clearly distinct DNA patterns with multiple band differences. We speculated that *H. pylori* subtypes showing minor genomic variations might have resulted from the existence of a subclone that probably arose from the same original strain.

Our study revealed that there were variable degrees of genomic DNA diversity between clinical isolates. Thirteen (4 CG, 4 DU, 3 GU & 2 GDU) out of 40 patients (32.5%) harbored a single strain of *H. pylori* throughout their stomach and duodenum, while 27 (67.5%) showed heterogeneous DNA fingerprints, indicating multi-strain infection at different anatomical sites within an individual patient. Fifteen of the 40 (37.5%) harbored totally different strains of *H. pylori*, i.e., the genomic DNA fingerprints of the organisms isolated from the corpus, antrum and duodenum were all different from each other. In the remaining 12 patients (30.0%), one or two isolates from different biopsy sites showed genetically different fingerprints. Although the patient sample was small, our study showed an interesting result that duodenal *H. pylori* isolated from 13 out of 21 DU patients (61.9%) were genotypically different from those isolated either from the corpus or antrum. However, the prevalence of multi-strain infection in the duodenum and stomach of either CG (3 of 9, 33.3%) or GU patients (3 of 7, 42.9%) was not different from that of DU patients (p>0.1). These findings suggested that the strain variation of infecting *H. pylori* and the degree of homogeneity in organisms within an individual Korean patient were not correlated with their disease outcomes. Thoreson et al. also reported similar results of a mixed population of different *H. pylori* strains with marked variation, both genotypically and phenotypically, colonizing the same DU patient[14].

The genetic diversity in the *H. pylori* population could be due to, i) the diversity of the species worldwide, ii) the large number of co-existing variants, or iii) the reports of natural transformation and genetic rearrangements or alterations in *H. pylori* within an individual host during colonization or adaptation to the host[30]. Furthermore, the case of maintaining the same DNA fingerprints may be a result of, i) the continuous evolution which occurs within the stomach of the infected person, because of nucleotide mutations, ii) excision of the *cagA* pathogenicity island (PAI), iii) transposition of insertion elements of *cag* PAI, iv) recombination with DNA from incoming strains that do not establish a chronic infection, and v) horizontal transfer of new genes[3, 31-34].

The present study showed that the majority of patients irrespective of their disease states were infected with *cagA*$^+$ *H. pylori*, and that the frequency of infection with *cagA*$^-$ strains was very low. Hamlet et al. suggested that a high density of *cagA*$^+$ strains in the duodenum with severe duodenitis was an important determinant of DU disease, because their DU patients had a much higher prevalence of *cagA*$^+$ strains in the duodenum than the asymptomatic subjects did (81% vs. 30%), despite a similar prevalence of *cagA*$^+$ strains in the antrum (86% vs. 75%) [28]. In the present study, however, no association between the presence of *cagA*$^+$ strain in the duodenum and the development of DU in Korean patients was evident since the prevalence of *cagA*$^+$ duodenal *H. pylori* of DU patients was not significantly higher than that of CG patients (88.9% vs. 85.7%, p>0.05).

The reason for the low frequency of infection with *cagA*$^-$ strains in Korean patients is difficult to explain. We proposed two possibilities: the predominant strains naturally found in Korea might be *cagA*$^+$ *H. pylori*, or, as seen in Covacci's proposal, the decreased frequency of excision of *cag* PAI by the *cagA*$^+$ strains with reduced generation of isogenic *cagA*$^-$ strains or to certain host factors that restricts the growth of *cagA*$^-$ strains[35]. Following from our previously reported results[23], this study also showed that infection with *H. pylori* strains positive for *vacA* gene was not correlated with clinical outcomes since

both *vacA s1* and *vacA s1a* genes were also expressed in all of the 120 isolates.

On the basis of these observations, we concluded that the majority of *H. pylori*-infected Korean patients were actually colonized with mixed populations, but often with a single strain, and that the prevalence of duodenal *H. pylori* expression of *cagA* and/or *vacA* was not correlated with the development of DU disease in Korean patients.

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