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Genotyping *CagA*, *VacA* Subtype, *IceA1*, and *BabA* of *Helicobacter pylori* Isolates From Korean Patients, and Their Association with Gastroduodenal Diseases

The genetic status of *cagA, vacA* subtype, *iceA1*, and *babA*, and the relationship to gastroduodenal diseases were assessed in *Helicobacter pylori* isolates in Korea. Seventy-six strains of *H. pylori* were isolated from the antrum and the corpus of 41 adult patients (22 with peptic ulcer and 19 with gastritis). The *cagA, iceA1,* and *babA* genes were assessed by polymerase chain reaction and the *vacA* subtypes were determined by reverse hybridization-line probe assay. The positive rates of 349-bp *cagA*, 208-bp *cagA*, *iceA1*, and *babA* genes were 97.4%, 96.1%, 84.2%, and 36.1%, respectively. The *vacA* s1a, s1b, s1c, and s2 variants were detected in 11.8%, 3.9%, 80.4%, and 1.3%, respectively. m1 (78.9%) is more prevalent than m2 (5.3%). The most common *vacA* genotype was s1c/m1 (61.9%), and 14 isolates (18.4%) contained mixed *vacA* genotypes from a single biopsy specimen. Twenty-one (60%) of 35 patients were infected with more than two strains of different *cagA, iceA1, babA*, and *vacA* genotypes. None of *cagA*, *iceA1, babA*, and *vacA* s1/m1 were associated with peptic ulcer. In conclusion, most *H. pylori* isolates in Korea carry *cagA, iceA1*, and *vacA* s1c/m1 genes, and reside with multiple strains. These genes do not correlate with the peptic ulcer in the Korean patients.

Key Words : *Helicobacter pylori; CagA; VacA; IceA1; BabA; Peptic Ulcer*

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INTRODUCTION

Although more than 50% of the world's population is infected with *Helicobacter pylori*, only a minority of carriers develop serious gastroduodenal diseases (1). There is increasing evidence that the genetic variability of *H. pylori* may have a clinical importance. Several genes have been identified that may play a role in the pathogenesis of *H. pylori*, such as *cagA*, *vacA* s1/m1, *iceA1*, and *babA*.

The cytotoxin-associated gene (*cagA*) is considered to be a marker for a genomic pathogenicity island (2). Several genes of this *cag* island encode proteins that enhance the inflammatory responses such as interleukin (IL)-8 production in gastric epithelial cells (3). The vacuolating cytotoxin gene (*vacA*) is present in all *H. pylori* strains (4). *vacA* genotype is composed of a hypervariable signal sequence and a mid-region allele. The *vacA* subtypes are determined by the combination of s1a, s1b, s1c, and s2, and m1, m2a, and m2b (5). Cytotoxin production and virulence are higher in the s1/m1 subtypes than in the s1/m2 subtype, and lower still in the s2/m2 subtype (6). Previous studies have shown that the *vacA* subtypes show regional and racial differences, and that the *vacA* s1a/m1 is associated with peptic ulcer disease (7). Because of the clinical importance of these virulence factors, *H. pylori* strains are classified as type 1 (VacA-positive and CagA-positive) and type 2 (VacA-negative and CagA-negative) (8), but this classification alone does not allow a proper clinical distinction between pathogenic and non-pathogenic strains, because of the high prevalence of type 1 strains in Korean populations (9).

A novel gene has recently been discovered, designated *iceA* (*i*nduced by *c*ontact with *e*pithelium) (10). There are two main allelic variants of the gene: *iceA1* and *iceA2*. The expression of *iceA1* is up-regulated on contact between *H. pylori* and human epithelial cells, and may be associated with peptic ulcer disease (11). Ilver et al. (12) identified the *H. pylori* blood group antigen-binding adhesin gene, *babA*, involved in the binding activity between bacterial adhesin and human Lewis^b blood group antigens on gastric epithelial cells. Several lines of evidence suggest that the presence of *babA* is

related to the occurrence of peptic ulcer (13, 14).

H. pylori strains may differ in various geographical regions (7), and studies of different populations may clarify the importance and universality of putative virulence factors. In the present study, the prevalence of *cagA*, *iceA1*, and *babA* genes, and the subtyping of the *vacA* gene were investigated in 76 *H. pylori* isolates recovered from patients with peptic ulcer and gastritis in Korea. The correlation between the genetic status of the isolates and the occurrence of peptic ulcer was assessed.

MATERIALS AND METHODS

H. pylori was isolated from biopsy specimens sampled from the antrum and the corpus of 41 patients (26 men, 15 women; median age, 52 yr; age range, 20 to 77 yr), in Chungbuk National University Hospital, Cheongju, Korea. Informed consent was obtained from all patients, and research protocols were approved by the Ethics Committee of Chungbuk National University Hospital. No patient had taken bismuth, antibiotics, or omeprazole within the six weeks before sampling. The final diagnoses were duodenal ulcer in 15 (36.6%) patients, gastric ulcer in 7 (17.1%) patients, and gastritis in 19 (46.3%) patients. In six gastritis patients, only one isolate from the antrum could be taken. Therefore, 76 isolates of *H. pylori* were assessed. Isolates were 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℃, under 100% humidity and 10% CO_2. *H. pylori* was identified by Gram staining, urease test (within 1-2 min), and catalase tests.

Table 1. PCR primers for amplication of *cagA*, *iceA1*, *babA*, and s and m sequences of *vacA*

Gene and segment	Primer designation	Primer Sequence (5′→3′)
cagA-349 bp	Cf1	GATAACAGGCAAGCTTTTGAGG
	Cr1	CTGCAAAAGATTGTTTGCGAGA
cagA-208 bp	Cf2	GAATCAGTATTTTTCAGAC
	Cr2	GGGTTGTATGATATTTTCC
iceA1-557 bp	ice1f	GTTGGGTAAGCGTTACAGAATTT
	ice1r	CATTGTATATCCTATCATTACAAG
babA-190 bp	LBf	AATCCAATTTAATCCAAA
	LBr	ATAGTTGTCTGAAAGATC
vacA S	VA1F	Bio*-ATGGAAATACAACAAACACAC
vacA S	VA1XR	Bio-CCTGAR*ACCGTTCCTACAGC
vacA M	MF1.1	Bio-GTGGATGCCCATACGGCTAA
vacA M	MF1.2	Bio-GTGGATGCTCATACAGCTW*A
vacA M	MF1.3	Bio-GTGGATGCCCATACGATCAA
vacA M	MF1.4	Bio-GCGAGCGCTCATACGGTCAA
vacA M	MR1	Bio-R*TGAGCTTGTTGATATTGAC

R*=G or A, W*=A or C, Bio*=biotin

DNA Isolation

H. pylori DNA was isolated using GeneReleaser (GR; Bio Ventures, Inc., Murfreesboro, Tenn., U.S.A.) (15). One loop of each isolated bacterial strain was added to 20 μL of GR in the thermocycle tube. Samples with GR were denatured (65℃ for 30 sec, 8℃ for 30 sec, 65℃ for 90 sec, 97℃ for 180 sec, 8℃ for 60 sec, 65℃ for 180 sec, 97℃ for 60 sec, and 65℃ for 60 sec) in the thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer, Norwalk, Conn., U.S.A.).

Polymerase Chain Reaction for *cagA, vacA , iceA1,* and *babA* genes

For the detection of *cagA*, *vacA*, *iceA1*, and *babA*, polymerase chain reactions (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 specific primer sets (Table 1). The PCR primers for *cagA*, *vacA*, *iceA1* were synthesized as described (5, 10, 16). The *babA* primers were designed on the basis of the recently published signal sequence of the *babA* gene (12). The PCR program for *cagA* comprised 32 cycles of 1 min at 94℃, 1 min 30 sec at 55℃, and 2 min at 72℃ (349-bp *cagA* fragment) or 1 min at 94℃, 1 min 30 sec at 48℃, and 2 min at 72℃ (208-bp *cagA* fragment). The PCR program for *vacA* was 40 cycles of 30 sec at 94℃, 30 sec at 50℃, and 30 sec at 72℃ and that for *iceA1* comprised 40 cycles of 30 sec at 94 ℃, 45 sec at 50℃, and 45 sec at 72℃ The PCR program for *babA* comprised 30 cycles of 1 min at 94℃, 1 min at 38 ℃, and 1 min at 72℃. After amplification 10 μL of PCR product was electrophoresed on 1.7% agarose gel and examined under UV illumination.

Table 2. Allele-specific probes for *vacA* s and m regions used for reverse hybridization

Gene segment and probe designation	Probe sequence (5′→3′)	Specificity
vacA-s		
P1S1	GGAGCRTTRGTCAGCATCAC	sla
P22S1a	GCTTTAGTAGGAGCR*TTRGTC	sla
P1S1b	GGAGCGTTGATTAGY*K*CCAT	slb
P2S1b	GTTTTAGCAGGAGCGTTGA	slb
P3s1	GGGYTATTGGTYAGCATCAC	slc
P4s1	GCTTTAGTR*GGYTATTGGT	slc
P1S2	GCTAAYACGCCAAAY*CATCC	s2
P2S2	GATCCCATACACAGCGAGAG	s2
vacA-m		
P1M1	TTGATACGGGTAATGGTGG	m1
P2M1	GGGTAATGGTGGTTTCAACA	m1
P1M2a	ACGAATTTAAGAGTGAATGGC	m2a
P2M2a	AGAGCGATAACGGGCTAAACA	m2a
P2M2b	AGGGTAGAAATGGTATCGACA	m2b

R*=G or A, Y*=C or T, K*=G or T

Reverse hybridization-line probe assay (LiPA) for *vacA* subtypes

PCR products from the *vacA* s and m regions were analyzed by reverse hybridization on a line probe assay. A nitrocellulose strip that contains a number of oligonucleotide probe for type-specific detection of *H. pylori* genotypes was prepared as described earlier (5) (Table 2). Ten μL of each of the PCR products was placed in a plastic trough, and 30 μL of 400 mmol/L NaOH and 10 mmol/L ethylene diamine tetraacetic acid was added to denaturate the DNA. After 5 min, 1 mL of preheated hybridization buffer (2X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM Tris-HCl [pH 7.5], 0.1% sodium dodecyl sulfate [SDS]) was added, and an LiPA strip was submerged into the solution, followed by incubation in a shaking water bath at 50 ±0.5℃ for 1 hr. The strips were washed with 1 ml of 2X SSC-0.1% SDS for 30 min at 50℃. Subsequently, the strips were rinsed three times in phosphate buffer, and streptavidin-alkaline phophatase was added. After 30 min of incubation at room temperature, the strips were rinsed again and 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate substrate was added. Positive hybridization was visible as purple probe lines. Interpretation of the results was performed visually.

Statistical analysis

Prevalences were compared using two tailed Fisher's exact test (SPSS 9.0, Chicago, U.S.A.). A p value <0.05 was considered statistically significant.

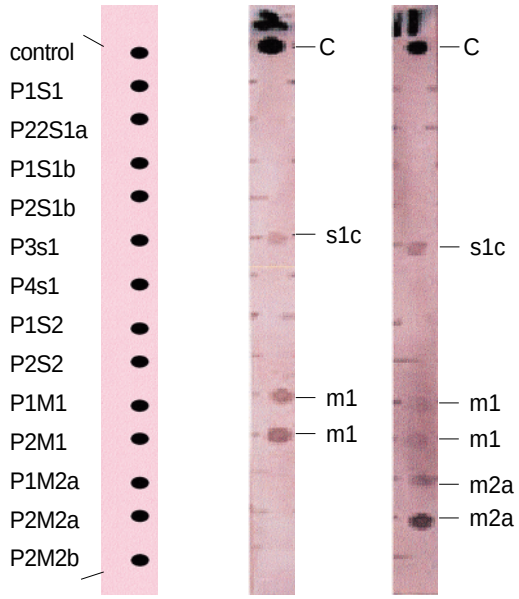

Fig. 1. The positions of the specific probes and examples of the LiPA for the *vacA* genotypes. *vacA* s1c/m1 and multiple strains carrying s1c/m1 and s1c/m2a are shown.

RESULTS

The genotypes of *cagA, iceA1, babA,* and *vacA* subtypes

Of the 76 isolates, 97.4% (74/76) were positive for the 349-bp *cagA* gene fragment, and 96.1% (73/76) for the 208-bp *cagA* gene fragment (Table 3). Either the 349-bp or the 208-bp *cagA* gene fragment was amplified from all strains. The *iceA1* gene was present in 64 of the 76 strains (84.2%). However, the *babA* gene was found in only 36.1% (26/72) of samples. Examples of *vacA* genotypes among the *H. pylori* isolates are shown in Fig. 1. The *vacA* genotypes were identified in all the *H. pylori* isolates. Of the *vacA* s subtypes, all but one were s1. Only one strain presented as s2. The proportion of s1a, s1b, and s1c subtypes was 11.8% (9/76), 3.9% (3/76), and 80.4% (61/76), respectively. Two (2.6%) isolates contained mixed s subtypes: one, s1c and s1a, and the other, s1c and s1b. Within the m subtypes, m1 and m2a occurred in 78.9% (60/76) and 5.3% (4/76) of isolates, respectively. Twelve (15.8%) strains displayed mixed m subtypes: m1 and m2a. No strain belonged to the m2b subtype. The most common *vacA* genotype was s1c/m1 (61.9%). The *vacA* genotypes s1a/m1, s1b/m1, s1c/m1, s1c/m2, and s2/m1 were found in 11.8% (9/76), 3.9% (3/76), 61.9% (47/76), 2.6% (2/76), and 1.3% (1/76), respectively. More than one *vacA* subtype were identified in 14 (18.4%) of the 76 isolates, so it represents the mixed strains with different *vacA* subtypes from single biopsy specimens (Table 4). Seventy *H. pylori* strains were isolated from both the antrum and the corpus of 35 patients. When the genetic differences within the 35 strain pairs (isolates from the antrum and the corpus of the same patients) were compared, the 208-bp *cagA*, 349-bp *cagA*, *iceA1*, and *babA* genotypes var-

Table 3. Genotypes of *cagA*, *iceA1*, *babA*, and *vacA* genes of 76 *H. pylori* strains isolated from 41 patients

Genotype		Peptic ulcer (n*=44)	Gastritis (n=32)	Total
cagA	(+)	43	31	74* (97.4%)
349 bp	(–)	1	1	2 (2.6%)
cagA	(+)	44	29	73 (96.1%)
208 bp	(–)	0	3	3 (3.9%)
iceA1	(+)	40	24	64 (84.2%)
	(–)	4	8	12 (15.8%)
babA	(+)	12	14	26 (36.1%)
	(–)	28	18	46 (61.8%)
	not determined	4	0	4 (2.1%)
vacA	s1a/m1	5	4	9 (11.8%)
	s1b/m1	1	2	3 (3.9%)
	s1c/m1	28	19	47 (61.9%)
	s1c/m2a	1	1	2 (2.6%)
	s2a/m1	0	1	1 (1.3%)
	multiple	9	5	14 (18.4%)

n*, number of strain (%)

Table 4. Fourteen isolates with multiple *vacA* subtypes from single biopsy specimen

Number	Diagnosis	*vacA* (s)	*vacA* (m)
1	DU*	slc	m1+m2a
2	DU	slc	m1+m2a
3	DU	sla+slc	m2a
4	DU	slc	m1+m2a
5	DU	slc	m1+m2a
6	DU	slc	m1+m2a
7	DU	slc	m1+m2a
8	GU†	slc	m1+m2a
9	GU	slc	m1+m2a
10	Gastritis	slc	m1+m2a
11	Gastritis	slc	m1+m2a
12	Gastritis	slb+slc	m2a
13	Gastritis	slc	m1+m2a
14	Gastritis	slc	m1+m2a

DU*, duodenal ulcer; GU†, gastric ulcer

ied in 0, 2, 2, and 9 patients, respectively. Overall, in 21 (60%) of the 35 strain pairs, the genetic status of *cagA*, *iceA1*, *babA*, and the *vacA* subtype varied between the isolates taken from the different stomach locations. The four pathogenicity-related genes, *cagA*, *vacA* s1c/m1, *iceA1*, and *babA*, did not correlate with other genes ($p>0.05$).

Relationship between the genetic status (*cagA*, *vacA* subtype, *iceA1*, and *babA*) and peptic ulcer

The incidence of *cagA*, *vacA* s1/m1, *iceA1*, and *babA* genes relative to clinical outcome is described in Table 5. We compared the genetic status of *H. pylori* isolates taken from the antrum, in a comparison of 22 peptic ulcer patients and 19 gastritis patients. In the cases of mixed *vacA* subtypes, the presence or absence of s1/m1 in the multiple strains was identified. All isolates from the antrum possessed more than one fragment of the *cagA* gene. There was no significant difference in the presence of the *iceA1* and *babA* genes in *H. pylori* isolates from peptic ulcer and gastritis patients (90.9% vs 73.7%, $p=0.148$, and 27.3% vs 26.3%, $p=0.578$, respectively). There was no statistical difference in the presence of *vacA* s1/m1 between peptic ulcer and gastritis groups (95.5% vs 94.7%, $p=0.718$).

Table 5. Prevalences of *cagA, iceA1, babA* and *vacA* s1/m1 of *H. pylori* isolated at the gastric antrum from 22 peptic ulcer and 19 gastritis patients

Genotype		Peptic ulcer (n*=22)	Gastritis (n=19)	*p*-value
cagA	(+)	21 (95.5%)	19 (100%)	0.539
349 bp	(–)	1 (4.5%)	0 (0%)	
cagA	(+)	22 (100%)	17 (89.5%)	0.209
208 bp	(–)	0 (0%)	2 (10.5%)	
iceA1	(+)	20 (90.9%)	14 (73.7%)	0.148
	(–)	2 (9.1%)	5 (26.3%)	
babA	(+)	6 (27.3%)	5 (26.3%)	0.578
	(–)	15 (68.2%)	14 (73.7%)	
	not determined	1 (4.5%)	0 (0%)	
vacA (s1/m1)	(+)	21 (95.5%)	18 (94.7%)	0.718
	(–)	1 (4.5%)	1 (5.3%)	

n*, number of patients

DISCUSSION

The present study demonstrates that the genetic status of *H. pylori* isolates in Korean is characterized by a high incidence of *cagA*, *vacA* s1c/m1, and *iceA1*, but a relatively low incidence of the *babA* gene, and that these virulence-associated determinant genes are not related to clinical outcome.

Our result that all strains contained more than one fragment of the *cagA*, would seem to resemble the other Asian countries, where a higher percentage of *cagA*-positive isolates are found (17, 18). This finding suggests that *cagA* is not a useful single marker for the discrimination between pathogenic and non-pathogenic strains of *H. pylori* in the Korean population.

All strains can be classified into *vacA* subtypes, and seven *vacA* subtypes (s1a/m1, s1b/m1, s1c/m1, s2/m1, s1a/m2a, s1b/m2a, and s1c/m2a) were identified in the present study. van Doorn et al. (5) expanded the allelic diversity of *H. pylori vacA* by further discriminating the m1, m2a, and m2b subtypes. They found the m2b variant in eight of 70 Asian, s1c strains (7). However, we did not find m2b subtype in the 76 isolates from Korean patients in the present study. The distribution of *vacA* subtypes differs between races, or over geographic locations (7, 18). van Doorn et al. (7) reported that s1c was predominant in Asian, and m1 and m2a were equally represented. In our study, the s1c and m1 variants were predominant among the *vacA* subtypes, and the presence of s1/m1 was not associated with peptic ulcer. The relation between s1/m1 and peptic ulcer has been controversial, because studies reported in western countries have shown that the *vacA* s1 or m1 genotypes correlate with clinical outcomes (7, 19). However, data from Asian including our study suggest various genotypes in *H. pylori* isolates are not associated with peptic ulcer (20, 21).

The *iceA1* gene, which may be related to the onset of peptic ulcers (10, 11), was also frequently detected in the present study (64 of 76 strains). Consistent with this, recent reports from Japan (18, 22) and Singapore (21) have shown that the incidence of *iceA1* did not correlate with clinical outcomes.

BabA-mediated adherence of *H. pylori* to the gastric epithelium plays a critical role in the efficient delivery of bacterial virulence factors that damage host tissue (12). Gerhard et al. (13) reported that the presence of *babA2* could be regarded as a good indicator of the ability of strains to express the Lewis^b antigen-binding adhesin, and that *babA2* is signifi-

cantly associated with duodenal ulcer in *H. pylori* isolated from a German population. The incidence of the *babA2* genotype was about 72% in their study (duodenal ulcer 100%, gastric cancer 77.8%, and gastritis 51.4%). However, in our study, the incidence of *babA* was low, and was not related to peptic ulcer disease. This may result from the possibility that Korean strains have sequence variations in the *babA* PCR primer regions and this affect the low incidence of *babA*. But, according to the reports (12, 23, 24) about variations within *bab* genes, the 5′ and 3′ regions are well conserved and it also suggests that there may be rarely any variations in our *babA* PCR primer regions located in the 5′ region of *babA* gene.

The pathogenicity-related genes tend to be coexpressed with other genes. Ilver et al. (12) found expression of both *cagA* and *babA* in 70% of subjects. van Doorn et al. (11) showed that both *cagA* and *vacA* s1 were strongly associated. Gerhard et al. (13) showed that the *vacA* s1 genotype was also significantly associated with the presence of *babA2*, and that Lewis^b-antigen-binding activity strongly correlated with the presence of the *cag* pathogenicity-associated islands. In contrast to these results, we did not find associations among virulence-related markers.

As *H. pylori* carries only a single copy of *vacA* (19), detection of multiple genotypes implies the presence of multiple strains in a clinical sample. The frequency of multiple genotypes of *vacA* in a single biopsy specimen in our study was 18.4%. Comparing the genetic differences in 70 isolates of *H. pylori* from the antrum and the corpus of 35 patients, 21 patients were infected with at least two strains of different *cagA*, *iceA1*, *babA*, and *vacA* genotypes. *H. pylori* infection with multiple genotypes is more common in Korea than in western countries (5, 25, 26). This results may be related to the high prevalence of *H. pylori* and higher rates of childhood infection in Korea (27). In Korea, approximately 71.5-91% of adults (27, 28) and 50% of children (29) are infected, so coinfection or superinfection with different strains may be common (30, 31). And *H. pylori* may also have the opportunities to evolve genetic variations during the long-term colonization from childhood (32). The prevalence of multiple-strain colonization should be considered when planning therapeutic strategies, as well as in any study of the pathogenesis of *H. pylori* infection.

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