

CagA and VacA Polymorphisms Are Associated with Distinct Pathological Features in *Helicobacter pylori*-Infected Adults with Peptic Ulcer and Non-Peptic Ulcer Disease[∇]

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Polymorphic variability in *Helicobacter pylori* factors CagA and VacA contributes to bacterial virulence. The presence of one CagA EPIYA-C site is an independent risk factor for gastroduodenal ulceration (odds ratio [OR], 4.647; 95% confidence interval [CI], 2.037 to 10.602), while the presence of the *vacA* i1 allele is a risk factor for increased activity (OR, 5.310; 95% CI, 2.295 to 12.287) and severity of gastritis (OR, 3.862; 95% CI, 1.728 to 8.632).

Helicobacter pylori, colonizing the gastric mucosa of 35 to 70% of people worldwide, is the etiologic factor for peptic ulcer development and increases the risk for gastric cancer. *H. pylori* pathogenesis is exerted via distinct virulent factors such as the secreted cytotoxin VacA (vacuolating cytotoxin A), the *cag* pathogenicity island (*cagPAI*) encoding the type IV secretion system (T4SS), and the cytotoxin-associated gene A (CagA) protein (6). We analyzed *H. pylori* clinical isolates from the antrum of 144 Greek adults (mean age \pm standard deviation [SD], 52.6 \pm 13.7 years; 78 male) diagnosed with peptic ulcer (gastric, $n = 21$; duodenal, $n = 44$) and non-peptic ulcer disease (nonulcer dyspepsia, $n = 61$; esophagitis, $n = 18$) on the basis of functional CagA EPIYA motifs as well as *vacA* alleles for signal, intermediate, and middle regions, as described previously (13, 15), and assessed putative associations with disease parameters and gastric inflammatory response.

Approximately 27% of the strains were found to be *cagA* negative with complete absence of the *cagPAI*. Among the 96 *cagA*-positive isolates, 15 (10.4%) lacked a functional T4SS as they induced minimal interleukin-8 (IL-8) levels (Fig. 1A), and no phosphorylated CagA was detected (Fig. 1B) following infection of gastric epithelial AGS cells (15). Infection with strains possessing a functional T4SS led to significantly higher IL-8 secretion, irrespective of the number of EPIYA-C sites, and to CagA phosphorylation (Fig. 1A and B). Hence, for univariate and multivariate logistic regression analysis, *cagA*-positive isolates with a nonfunctional T4SS were grouped together with *cagA*-negative cases, comprising the “None” category. In single *H. pylori* strain infections, the majority of isolates ($n = 59$, 41.0%) were of the ABC EPIYA type (15), with a second EPIYA-C repeat observed in 19 (13.2%) strains,

while ABCCC strains were also identified ($n = 2$, 1.4%). In 11 cases (7.7%), the presence of mixed infection by isogenic strains differing solely with regard to the number of EPIYA-C repeats was identified as shown before (13).

The dominant *vacA* polymorphisms for the signal, intermediate, and middle regions were s1, i1, and m2, respectively, as reported for Western-type *H. pylori* strains (5, 17). More specifically, 102 (70.8%) isolates were identified as *vacA* s1, with 57 (39.6%) carrying the *vacA* m1 allele simultaneously. No strain with *vacA* s2/m1 was recorded. Of the 91 *vacA* i1 strains, 54 (59.3%) were also typed as *vacA* s1/m1, whereas 38/53 (71.7%) *vacA* i2 strains were s2/m2 ($P < 0.001$). Depending on the *vacA* genotype, strains were further classified into three categories (7, 14), namely, nonvacuolation (s2/i2/m2, s1/i2/m1, s1/i2/m2, and s2/i1/m2), low vacuolation (s1/i1/m2), and high vacuolation (s1/i1/m1). Vacuolating *vacA* s1/i1/m1 or s1/i1/m2 types were present in strains harboring functional CagA variants with more EPIYA-C phosphorylation repeats, with frequencies reaching approximately 90% in cases of multiple infections, whereas *cagPAI*-defective strains were almost exclusively related to a nonvacuolating *vacA* genotype ($P < 0.001$).

vacA s1 and i1 polymorphisms were found to be associated with marked chronic inflammatory infiltration and activity of chronic gastritis in the antrum (Table 1). No association was observed with the density of *H. pylori* colonization or the presence of gastric atrophy and intestinal metaplasia (IM), even though in 41/57 (71.9%) of recorded IM the strain carried the *vacA* i1 allele ($P = 0.111$). However, the risk for IM development increased 2-fold upon infection with *vacA* m1 strains (odds ratio [OR], 2.182; 95% confidence interval [CI], 1.098 to 4.338; $P = 0.026$). Heavy *H. pylori* colonization (OR of 6.866 and 95% CI of 3.072 to 15.344 [$P < 0.001$] and OR of 8.476 and 95% CI of 3.633 to 19.777 [$P < 0.001$], respectively) and infection with *vacA* i1 strains (OR of 3.862 and 95% CI of 1.728 to 8.632 [$P = 0.001$] and OR of 5.310 and 95% CI of 2.295 to

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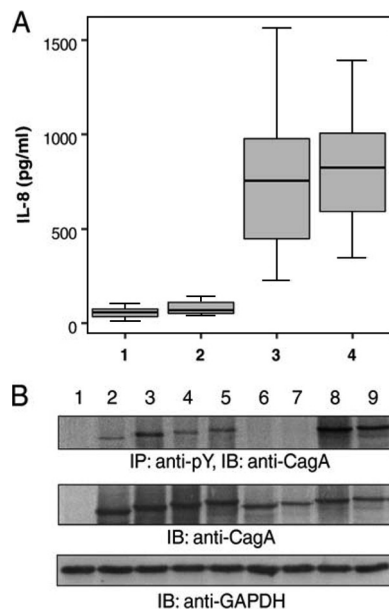


FIG. 1. (A) Levels of secreted IL-8 following infection of gastric epithelial AGS cells with *H. pylori* clinical strains (1, *cagA* negative; 2, *cagPAI* defective; 3, 1 EPIYA-C repeat; 4, ≥ 2 EPIYA-C repeats). No difference was observed between *cagA*-negative and *cagPAI*-defective strains ($U = 32.500$ and $P = 0.201$ by the Mann-Whitney U test). *cagA*- and *cagPAI*-positive strains induced higher levels of IL-8 than *cagPAI*-defective strains ($U = 0.000$ and $P < 0.0001$ by the Mann-Whitney U test), irrespective of the number of EPIYA-C sites ($U = 224.500$ and $P = 0.627$ by the Mann-Whitney U test). (B) Tyrosine phosphorylation and expression patterns of CagA protein following infection of AGS cells with representative *H. pylori* clinical strains. CagA tyrosine phosphorylation was detected by immunoblotting (IB) following immunoprecipitation (IP) with PY20 antiphosphotyrosine antibody. The expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was utilized as a total protein loading control. Lanes: 1, CagA-negative clinical isolate; 2 to 5, CagA-positive isolates with functional *cagPAI* harboring 2 (AB), 3 (ABC), 4 (ABCC), and 5 (ABCCC) motifs in CagA, respectively; 6 and 7, CagA-positive *H. pylori* strains carrying 3 (ABC) and 4 (ABCC) EPIYA motifs with defective *cagPAI*, respectively, as depicted by the absence of phosphorylated CagA; 8 and 9, CagA-positive strains with 5 (ABCCC) EPIYA motifs.

12,287 [$P = 0.001$], respectively) were recognized as independent risk factors for the development of severe chronic inflammatory infiltration and marked activity of chronic gastritis in the antrum. This is the first report associating *vacA* intermediate region polymorphisms with increased activity of antral gastritis. *vacA* s1 strains of Western origin have previously been associated with more-severe gastric inflammation (5, 11, 16). The only reports relating specific *cagA* polymorphisms with histological lesions involve strains of East Asian origin carrying the ABD EPIYA sites, which present distinct biological properties compared to Western-type ABC EPIYA motifs (4).

The development of gastric ulcers (GU) or duodenal ulcers (DU) was associated with the occurrence of *H. pylori* strains harboring functional EPIYA-C repeats in CagA ($P = 0.001$) as well as with the *vacA* s1 allele ($P = 0.004$) and marked activity of chronic gastritis ($P = 0.014$). Despite that, CagA EPIYA polymorphisms were found to be the only independent risk factor for ulcer disease (Table 2), with over 50% of strains with 1 (57.6%) or 2 or more (52.4%) EPIYA-C repeats found to be isolated from ulcer cases (8 GU/26 DU and 4 GU/7 DU, respectively) and the majority (8/11, 72.8%) of multiple infections with isogenic strains (4 GU and 4 DU) (Table 2). To date, infection with *cagA*-positive *H. pylori* has been well associated with gastroduodenal ulcers (2, 6, 12), whereas variability in the EPIYA phosphorylation sites and in particular CagA variants with an increased number of EPIYA-C repeats or of East Asian origin have been reported to augment the risk for gastric adenocarcinoma (1, 4, 8–10, 20). In our study, we observed that the presence of single or multiple infecting strains rather than the number of EPIYA-C sites in CagA *per se* is probably crucial in determining the type of gastric disease, since the majority of mixed infections with isogenic strains expressing CagA with various numbers of EPIYA-C repeats were isolated from peptic ulcer patients. Previous reports relate ulcer lesions with the presence of *vacA* s1 and i1 types (3, 5, 14), although in our sample, the association of VacA determinants with peptic

TABLE 1. Univariate logistic regression analysis showing association of *vacA* and *cagA* polymorphisms with severity and activity of chronic gastritis in the antrum of 144 Greek adults

Risk factor	Chronic inflammatory infiltration				Activity of chronic gastritis			
	No. (%) of isolates with mild/moderate severity	No. (%) of isolates with marked severity	OR (95% CI) ^a	<i>P</i>	No. (%) of isolates with mild/moderate activity	No. (%) of isolates with marked activity	OR (95% CI) ^a	<i>P</i>
<i>vacA</i> alleles								
s2	25 (17.4)	17 (11.8)	Reference		27 (18.8)	15 (10.4)	Reference	
s1	34 (23.6)	68 (47.2)	2.941 (1.402–6.171)	0.004	38 (26.4)	64 (44.4)	3.032 (1.435–6.405)	0.004
i2	30 (20.8)	23 (16.0)	Reference		34 (23.6)	19 (13.2)	Reference	
i1	29 (20.1)	62 (43.1)	2.789 (1.385–5.614)	0.004	31 (21.5)	60 (41.7)	3.463 (1.704–7.040)	0.001
<i>cagA</i> EPIYA status								
None	32 (22.2)	21 (14.6)	Reference		33 (22.9)	20 (13.9)	Reference	
1 EPIYA-C repeat	18 (12.5)	41 (28.5)	3.471 (1.589–7.58)	0.002	23 (16.0)	36 (25.0)	2.583 (1.204–5.539)	0.015
≥ 2 EPIYA-C repeat	6 (4.2)	15 (10.4)	3.810 (1.274–11.389)	0.017	6 (4.2)	15 (10.4)	4.125 (1.376–12.363)	0.011
Mixed infections	3 (2.1)	8 (5.6)	4.063 (0.966–17.091)	0.056	3 (2.1)	8 (5.6)	4.4 (1.044–18.542)	0.044
Vacuolation potential								
None	32 (22.2)	24 (16.7)	Reference		35 (24.3)	21 (14.6)	Reference	
Low	12 (8.3)	22 (15.3)	2.444 (1.014–5.895)	0.047	11 (7.6)	23 (16.0)	3.485 (1.418–8.566)	0.007
High	15 (10.4)	39 (27.1)	3.467 (1.563–7.690)	0.002	19 (13.2)	35 (24.3)	3.070 (1.411–6.681)	0.005

^a Reference, used as the reference category for the calculation of risk in each case.

TABLE 2. Multivariate logistic regression model depicting parameters relating to the development of peptic ulcers

Risk factor	No. (%) of cases with non-peptic ulcers	No. (%) of cases with peptic ulcers	OR (95% CI) ^a	P
<i>cagA</i> EPIYA status				
Defective <i>cagPAI</i>	41 (28.5)	12 (8.3)	Reference	<0.001
1 EPIYA-C repeat	25 (17.4)	34 (23.6)	4.647 (2.037–10.602)	0.015
≥2 EPIYA-C repeat	10 (6.9)	11 (7.6)	3.758 (1.288–10.969)	0.003
Mixed infections	3 (2.1)	8 (5.6)	9.111 (2.085–39.810)	<0.001
<i>vacA</i> alleles				
s2	30 (20.8)	11 (7.6)	Reference	
s1	49 (34.0)	54 (37.5)		0.24
i2	34 (23.6)	19 (13.2)	Reference	
i1	45 (31.3)	46 (31.9)		0.76
Activity of chronic gastritis				
Mild	11 (7.6)	1 (0.7)	Reference	
Moderate	32 (22.2)	21 (14.6)		0.383
Marked	36 (25.0)	43 (29.9)		0.129

^a Reference, used as the reference category for the calculation of risk in each case.

ulcer disease was not sustained through multivariate analysis, possibly reflecting geographical differences in the prevalence of the various genotypes (17–19).

Collectively, our data indicate a distinct yet coordinated activity of virulence factors associated with *H. pylori* pathogenesis, with CagA contributing to the development of particular disease phenotypes, such as peptic ulcer, and VacA differentially affecting the inflammatory process. Our findings emphasize the necessity to meticulously assess the functionality of virulence factors in *H. pylori* clinical strains so as to discern the true biological significance that lies beneath the plasticity of the *H. pylori* genome.

Nucleotide sequence accession numbers. Partial *cagA* nucleotide sequences were submitted to the GenBank/EMBL/DDBJ databases under accession numbers FN561978 to FN562025.

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