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Source / Izvornik: **Croatian Medical Journal, 2009, 50, 124 - 132**

Journal article, Published version
Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

https://doi.org/10.3325/cmj.2009.50.124

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:396500

doi: 10.3325/cmj.2009.50.124

Serum Antibodies Positivity to 12 *Helicobacter pylori* Virulence Antigens in Patients with Benign or Malignant Gastroduodenal Diseases – Cross-sectional Study

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Aim To investigate the association of gastric histological and endoscopic findings in patients with *Helicobacter pylori (H. pylori)*, according to presence of seropositivity to 12 bacterial virulence antigens.

Methods This is a cross-sectional single-center study of 360 consecutive outpatients referred in the period of one year to upper gastrointestinal endoscopy because of dyspeptic complaints. Patients sera were tested by Western blot method to determine the presence of serum antibodies to bacterial virulence antigens – p120 (CagA – cytotoxin-associated antigen), p95 (VacA – vacuolating cytotoxin), p67 (FSH – flagellar sheath protein), p66 (UreB – urease enzyme heavy subunit), p57 (HSP homologue – heath shock protein homologue), p54 (flagellin), p33, p30 (OMP – outer membrane protein), p29 (UreA – urease enzyme light subunit), p26, p19, and p17. Upper gastrointestinal endoscopy was performed, endoscopic diagnosis recorded, and 4 mucosal biopsy samples were obtained and assessed according to Updated Sydney protocol.

Results The sera of 207 patients were analyzed. Thirty patients had gastric adenocarcinoma, 126 peptic ulcers, and 51 normal finding. p120 (CagA) seropositivity was significantly more often present in patients with higher activity grade in the antrum ($P=0.025$), p30 in patients with greater inflammation in the antrum ($P=0.025$) and the corpus ($P=0.010$), p33 in patients with greater inflammation in the corpus ($P=0.050$), and p19 (OMP) in patients with lower intestinal metaplasia grades in the corpus ($P=0.025$). Seroreactivity to all other bacterial proteins showed no association with the histological status of the stomach mucosa. Except for the seropositivity to protein p95 (VacA), which was more often present in patients with duodenal ulcer ($P=0.006$), there was no difference in seroreactivity to other bacterial proteins and upper gastrointestinal endoscopic findings.

Conclusions p120 (CagA), p33, p 30 (OMP), and p19 (OMP) seropositivity was more often present in patients with higher grades of the histological parameters of gastritis and seropositivity to protein p95 (VacA) with endoscopic presence of duodenal ulcer. Histological parameters of gastritis are more associated with bacterial virulence than endoscopic findings.

Received: October 27, 2008

Accepted: February 27, 2009

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Helicobacter pylori (H. pylori) is recognized as a major gastric pathogen with worldwide distribution (1). Estimated prevalence of *H. pylori* infection in Croatia is 60.4% (2). The persistent colonization induces gastritis and in some patients peptic ulcer disease, atrophic gastritis, or gastric carcinoma (3). However, most of patients infected with *H. pylori* never develop cancer or ulcer disease and there is a need to identify additional factors that may influence the risk for development of such diseases. The outcome of *H. pylori* infection is associated with specific (virulence associated) bacterial genotypes, environmental factors, and host's immune status (4). A number of putative virulence factors for *H. pylori* infection have been identified, including CagA (cytotoxin-associated antigen), VacA (vacuolating cytotoxin), IceA (induced by epithelium antigen), BabA (blood-group antigen-binding adhesin), etc. It is likely that every other factor that results in increased inflammation will also consequently increase the risk of a disease occurrence.

Presently, the only reliable way to identify the disease associated with *H. pylori* infection remains endoscopic examination combined with histological assessment of the gastric mucosa (5). The bacterial virulence antigens elicit specific antibodies during infection. However, the value of these antibodies as predictive factors for the severity of the disease remains controversial (6-15). So far, several investigations on the subject have been done, such as detecting the level, specificity, or presence of isotypes of serum *H. pylori* antibodies (16-22). Because disease outcome depends on both the strain characteristics and the host's response, the serum antibody response to virulence antigens could provide clues in predicting the presence and severity of associated diseases (23,24). On the other hand, since subjects without manifest disease also have strains bearing this or other virulence antigens, it seems that the disease could not be attributed to one virulence antigen alone. Thus, other virulence antigens may also be important. The exact role of other bacterial virulence antigens – p67 (FSH – flagellar sheath protein), p66 (UreB – urease enzyme heavy subunit), p57 (HSP homologue – heath shock protein homologue), p54 (flagellin), p33, p30 (OMP – outer membrane protein), p29 (UreA – urease enzyme light subunit), p26, p19 (OMP), and p17 in the pathogenesis of gastrointestinal diseases is still unclear.

In this study, we aimed to investigate the association of gastric histological and endoscopic findings in *H. pylori*-positive patients according to presence of seropositivity to 12 bacterial virulence antigens. Since both bacterial virulence antigens and pattern of *H. pylori* gastritis may contribute to development of clinically relevant gastroduodenal disease, we wanted to determine the antibodies which are most associated with higher grades of histology findings of gastritis, atrophy, or intestinal metaplasia and different clinical diseases (peptic ulcer, gastric cancer, and non-ulcer dyspepsia).

METHODS

Patients

In 2000, 360 consecutive outpatients referred to upper gastrointestinal endoscopy because of dyspeptic complaints were screened for *H. pylori* infection. Before entering the study, all patients provided written informed consent. The research protocol was approved by the Clinical Research Ethical Committee of the University Hospital Merkur in Zagreb. All procedures were in accordance with the Declaration of Helsinki, revision 2008 (25).

Two hundred and seven patients were eligible for the study. Inclusion criteria for the study were age over 18, being positive to *H. pylori* (proven by histology and serology), and no data about previous eradication treatment for *H. pylori* infection. The exclusion criteria were previous eradication treatment; any antimicrobial treatment 2 months preceding the study; concomitant medication with bismuth preparations, proton pump inhibitors, H_2-receptor antagonists, or non-steroid anti-inflammatory drugs; other serious illnesses; and history of gastric surgery. *H. pylori* positive status was proven by histology and serology (Western blot).

At examination, upper gastrointestinal endoscopy was performed and 4 gastric mucosa biopsy samples were obtained (2 from the antrum and 2 from the corpus). Additionally, 1 vial of venous blood was obtained for serological examination. According to endoscopic findings, patients were divided into 5 groups as follows: normal mucosa (N), duodenal ulcer (D), gastric ulcer (V), duodenal and gastric ulcer (VD), and gastric adenocarcinoma (C).

Histology

For histological examination, 4 biopsy samples were obtained: 2 from the antrum and 2 from the corpus of gastric mucosa. The biopsy samples were embedded in paraffin wax and stained with hematoxylin and eosin, modified 2% Giemsa stain, and periodic acid Schiff (PAS). All samples were analyzed for *H. pylori* density, activity (in-

tensity of polymorphonuclear cells infiltrates), inflammation (intensity of mononuclear cells infiltrates), atrophy, and intestinal metaplasia, as stipulated by Updated Sydney system classification (26). All parameters were graded as 0 – none, 1 – mild, 2 – moderate, or 3 – marked. Intestinal metaplasia was recognized morphologically with the presence of goblet cells, absorptive cells, and cells resembling colonocytes. The results from two antrum or corpus biopsy samples were combined and whenever differences were observed, the highest score was considered for statistical analysis.

Serology

Sera analysis was performed with commercially available Anti-*H. pylori* -Western blot test kit (Western blot, Euroimmun Medizinische Labordiagnostika AG, Lubeck, Germany). The procedure was performed according to manufacturer's instructions. Test is based on detection of anti *H. pylori* antigens IgG antibodies to 12 *H. pylori* specific and unspecific (cross-reacting or undefined) antigens. Antigen source for this commercially available anti-*H. pylori*-Western blot test is provided by the *H. pylori* type strain (27).

Serum samples were assayed for 12 antibodies to *H. pylori* antigens: p120 (CagA), p95 (VacA), p67 (FSH), p66 (UreB), p57 (HSP homologue), p54 (flagellin), p33, p30 (OMP), p29 (UreA), p26, p19 (OMP), and p17 by Western blot method. Proteins p 120 (CagA), p 95 (VacA), p33, p30 (OMP), p29 (UreA), p26, p19 (OMP), p17 are *H. pylori* strain specific antigens and p67 (FSH), p66 (UreB), p57 (HSP homologue), p54 (flagellin) are cross-reacting or undefined antigens.

Statistical analysis

The associations between *H. pylori* individual virulence antigens and presence of mucosal histological changes or clinical disease were analyzed using χ^2 test. Odds ratios (OR) with confidence intervals were assessed by bivariate logistic regression. The ***t*** test was used to analyze the age distribution between the different patients groups. STATISTICA for Windows, release 5.5 H (StatSoft, Tulsa, OK, USA) and SPSS for Windows, release 17.01 (SPSS Inc., Chicago, IL, USA) were used in statistical analysis.

RESULTS

Study population

All 207 patients included in the study were *H. pylori* positive, confirmed by histology and serology. Among them, 113 were men, with mean age ± standard deviation of 58.0 ± 14.1 years and 94 were women, 57.9 ± 14.7 years old ($P = 0.960$). In Table 1, demographic data, endoscopic diagnosis, and serological and pathohistological findings of all 207 patients are presented.

Inflammation of the corpus mucosa was present in 91.1% patients, activity (intensity of polymorphonuclear cells in-

TABLE 1. Demographic, endoscopic, serological, and patohistological findings of all 207 Croatian patients with benign and malignant gastroduodenal diseases

Demographic or endoscopic parameter	No. (%) patients	Virulence antigens	Finding No. patients, %		Pathohistological finding *	Finding (No. patients, %)	
			positive	negative		positive	negative
Age (mean ± standard deviation)	54.89 ± 14.3	P 120 (CagA)	189 (91.3)	18 (8.7)	Corpus:		
Men	113 (54.6)	P 95 (VacA)	157 (75.8)	50 (24.2)	inflammation	201 (97.1)	6 (2.9)
women	94 (45.4)	P 67 (flagellin)	83 (40.1)	124 (59.9)	activity	104 (50.2)	103 (49.8)
Endoscopic finding:		P 66 (ureB)	130 (62.8)	77 (37.2)	atrophy	15 (7.2)	192 (92.8)
gastric ulcer (V)	46 (22.2)	P 57 (heath shock	149 (71.9)	58 (28.1)	intestinal metaplasia	23 (11.1)	184 (88.9)
duodenal ulcer (D)	68 (32.8)	protein homologue)			Antrum:		
gastric carcinoma (C)	30 (14.5)	P 54 (flagellin)	50 (24.1)	157 (75.9)	inflammation	203 (98.1)	4 (1.9)
normal mucosa (N)	51 (24.6)	P 33	52 (25.1)	155 (74.9)	activity	153 (73.9)	54 (26.1)
gastric and duodenal ulcer (VD)	12 (5.8)	P 30 (outer membrane protein)	57 (27.5)	150 (72.5)	atrophy	22 (10.6)	185 (89.4)
		P 29 (ureA)	93 (44.9)	114 (55.1)	intestinal metaplasia	48 (23.2)	159 (76.8)
		P 26	87 (42.1)	120 (57.9)			
		P 19 (outer membrane protein)	111 (53.6)	96 (46.4)			
		P 17	76 (36.7)	131 (63.3)			

*According to Updated Sydney system classification (26).

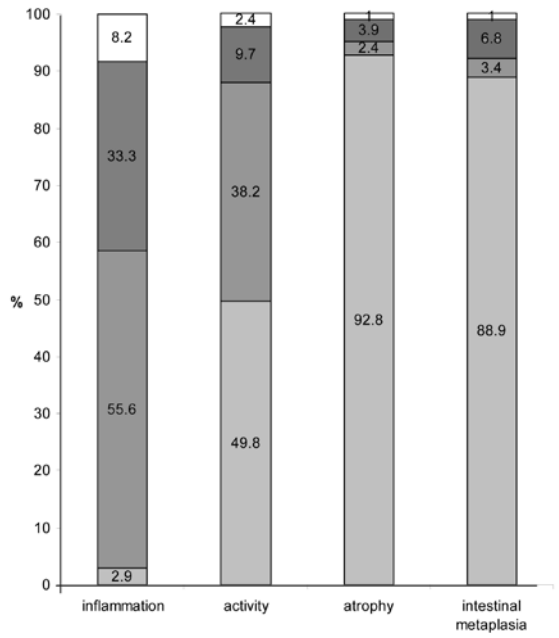
filtrates) was found in 50.1%, atrophy in 7.2%, and intestinal metaplasia in 11.1%. Figure 1 represents distribution of different grades of pathohistological parameters of gastritis in the corpus of patients with benign and malignant gastroduodenal diseases.

Pathohistological analysis of the antrum mucosa revealed inflammation in 51.2% patients, activity (intensity of polymorphonuclear cells infiltrates) was found in 74.9%, atrophy in 10.6%, and intestinal metaplasia in 23.2%. Figure 2 shows the distribution of different grades of pathohistological parameters of gastritis in the antrum mucosa of patients with benign and malignant gastroduodenal diseases. Out of 30 patients with gastric adenocarcinoma, 23 had intestinal and 7 diffuse cancer form. Of all patients, 18 had tumor in the antrum and 12 in the corpus part of the stomach.

Correlation of H. pylori virulence antigens and gastric histology

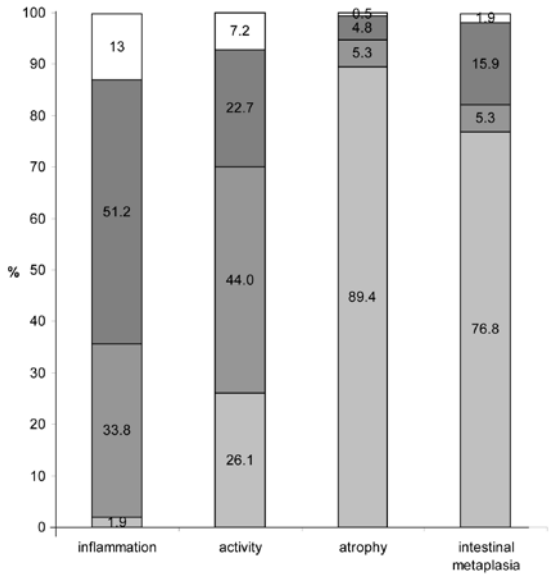
The correlation of histological features and seropositivity status was significant for proteins p120 (CagA), p30 (OMP),

Figure 1.



Distribution of different grades (light grey – no change, medium grey – mild, dark grey – moderate, white – marked) of histological parameters (inflammation, activity, atrophy, and intestinal metaplasia) of gastritis in corpus mucosa of 207 Croatian patients with benign and malignant gastroduodenal diseases.

Figure 2.



Distribution of different grades (light grey – no change, medium grey – mild, dark grey – moderate, white – marked) of histological parameters (inflammation, activity, atrophy, and intestinal metaplasia) of gastritis in the antrum mucosa of 207 Croatian patients with benign and malignant gastroduodenal diseases.

p33, and p19 as follows: p120 (CagA) seropositivity was associated with higher activity grades ($P=0.025$) in the antrum, p30 (OMP) seropositivity with higher inflammation grades in both the corpus ($P=0.010$) and the antrum ($P=0.025$), and antigen p33 with higher inflammation grades in the corpus ($P=0.050$). Contrary to this, seropositivity to p19 (OMP) antigen was associated with lower grades of intestinal metaplasia in the corpus ($P=0.025$) of gastric mucosa (Table 2). There was no significant difference in the distribution of histological parameters of gastritis with respect to the serological status of other bacterial virulence antigens.

Correlation of H. pylori virulence antigens and endoscopic diagnosis

Seropositivity to antigen p95 (VacA) was significantly more often observed in patients with duodenal ulcer and less often in those with normal mucosa and gastric carcinoma ($P=0.006$). Sensitivity and specificity of positive serology finding to p95 (VacA) to detect patients with duodenal ulcer were 91.2% and 31.7%, respectively. Also, sensitivity and specificity of positive serology finding to detect patients with gas-

TABLE 2. Presence of different grades of histological parameters of gastritis in patients serologicaly positive and negative to *H. pylori* virulence proteins p120 (CagA), p19, p30 (OMP), and p33 (n = 207)

		Antibodies				χ^2 test	Bivariate logistic regression	
Histology*	grade	negative	positive	sensitivity	specificity	*P*	*P*	odds ratio (95% CI)
		p 19						
Intestinal metaplasia, corpus	0	79	105					
	1	4	3	2.70	95.83			
	2	11	3	2.70	88.54	0.025	0.005	0.441 (0.248-0.785)
	3	2	0	0.00	97.92			
	all	96	111					
		p 30 (outer membrane protein)						
Inflammation, corpus	0	6	0					
	1	88	27	47.37	41.33			
	2	49	20	35.09	67.55	0.010	0.003	1.956 (1.252-3.055)
	3	7	10	17.54	95.33			
	all	150	57					
		p 30 (outer membrane protein)						
Inflammation, antrum	0	1	3					
	1	54	16	28.07	64.00			
	2	80	26	45.61	46.67	0.025	0.367	1.224 (0.789-1.900)
	3	15	12	21.05	90.00			
	all	150	57					
		p 33						
Inflammation, corpus	0	4	2					
	1	95	20	38.46	38.71			
	2	45	24	46.15	70.97	0.050	0.026	1.666 (1.061-2.616)
	3	11	6	11.54	92.90			
	all	155	52					
		p 120 (CagA)						
Activity, antrum	0	4	50					
	1	14	77	40.74	22.22			
	2	0	47	24.87	100.00	0.025	0.095	2.295 (0.911-3.175)
	3	0	15	7.94	100.00			
	all	18	189					

*According to Updated Sydney system classification (26).

tric carcinoma were 60.0% and 21.5%, to detect patients with gastric ulcer were 71.7% and 23.0%, to detect patients with simultaneous gastric and duodenal ulcer were 66.7% and 23.6%, and to detect patients with normal mucosa finding were 70.6% and 22.4%, respectively. There was no association between seropositivity to any other of 11 *H. pylori* virulence proteins and endoscopic finding. The distributions of different endoscopy findings and antibodies to *H. pylori* virulence antigens are summarized in Table 3.

DISCUSSION

The results of our study demonstrated the association of seropositivity to p120 (CagA), p33, and p30 (OMP) with higher grades of the histological parameters of gastritis, and seropositivity to p19 (OMP) with lower grades of intestinal metaplasia. Only seropositivity to p95 (VacA) was strongly associated with endoscopic presence of duodenal ulcer.

TABLE 3. Serological findings for 12 *H. pylori* virulence antigens in various benign and malignant gastroduodenal diseases (n = 207)

		Endoscopic finding*					χ^2 test	Bivariate logistic regression	
Antigen		D	C	N	V	VD	*P*	odds ratio (95% confidence interval)	*P*
p120	positive	65	28	43	42	11	0.301	0.794 (0.511-1.233)	0.304
	negative	3	2	8	4	1			
p95	positive	62	18	36	33	8	0.006	1.147 (0.862-1.524)	0.347
	negative	6	12	15	13	4			
p67	positive	24	11	27	15	6	0.209	1.264 (0.981-1.628)	0.070
	negative	44	19	24	31	6			
p66	positive	42	23	32	27	6	0.455	0.854 (0.663-1.101)	0.233
	negative	24	7	19	19	6			
p57	positive	48	22	40	30	9	0.691	1.128 (0.860-1.479)	0.384
	negative	20	8	11	16	3			
p54	positive	14	10	13	11	2	0.680	0.872 (0.656-1.159)	0.347
	negative	54	20	37	35	10			
p33	positive	14	5	19	12	2	0.174	1.199 (0.902-1.593)	0.211
	negative	54	25	32	34	10			
p30	positive	20	10	17	7	3	0.291	1.092 (0.831-1.434)	0.529
	negative	48	20	34	39	8			
p29	positive	28	16	24	19	6	0.790	0.983 (0.770-1.256)	0.894
	negative	40	14	27	27	6			
p26	positive	28	13	28	12	6	0.391	1.272 (0.989-1.635)	0.061
	negative	40	17	23	34	6			
p19	positive	41	11	31	20	8	0.076	1.409 (1.094-1.814)	0.008
	negative	27	19	20	26	4			
p17	positive	28	12	19	13	4	0.699	1.023 (0.795-1.316)	0.859
	negative	40	18	32	33	8			
all		68	30	51	46	12			

*Abbreviations: C – carcinoma, D – duodenal ulcer, N – normal mucosa, V – gastric ulcer, VD – gastric and duodenal ulcer.

So far, many attempts have been made to find serological markers of *H. pylori* virulence, which could be correlated with the severity of the *H. pylori*-associated diseases. These studies indicate that seropositivity to some *H. pylori* antigens could be used as serological marker for bacterial virulence (17-20,28,29).

When we compared serology findings to 12 *H. pylori* virulence antigens with gastric mucosa histopathological findings, we noticed that p120 (CagA) seropositivity was associated with higher activity grades in the antrum, p30 (OMP) seropositivity with higher inflammation grades in both the antrum and corpus, p33 seropositivity with higher inflammation grades in the corpus, and p19 (OMP) seropositivity with lower grades of intestinal metaplasia in the corpus. Protein CagA is the most investigated *H. pylori* virulence factor, but its connection with the presence of various mucosal histological changes is still controversial and there are many studies with contradictory results (30-33). Our results are consistent with previous observations, in which CagA elicited high neutrophil accumulation, measured as inflammation activity in gastric mucosa (29). According to some studies, this can be related to strong stimulation of IL-8 production by CagA positive strains (34,35). There are no data on the association of seropositivity to proteins p30 (OMP), p33, and p19 (OMP) and different patterns of gastritis. The first step in all *H. pylori*-related diseases is emergence of gastritis. Possible pathogenetic mechanisms involved in the association of these proteins with higher grades of gastritis should be additionally investigated regarding implications in the occurrence of gastroduodenal diseases.

Proteins p95 (VacA), p67 (FSH), p66 (UreB), p57 (HSP homologue), p54 (flagellin), p29 (UreA), p26, and p17 seropositivity were not associated with the histological patterns of gastritis. Several studies consistently showed that different *vacA* genotypes were associated with disease appearance in patients from various countries. On the other hand, there are different VacA isoforms with

distinct antigenic properties and, consequently, multiple forms of VacA could elicit different antibody responses in *H. pylori*-positive humans (36). Based on our findings, we can conclude that present serology testing to antigen VacA was not sensitive enough to predict toxic and histolopathological effects of vacuolization toxin. Obviously. distinct serological response to VacA toxin cannot differentiate between more and less toxic isoforms of VacA related with different grades of histopathological findings. Better results in this field of research could be provided with investigation of different *vacA* genotypes in relation with presence of various histopathological findings.

Also, none of the tested serologic markers have been associated with appearance of atrophic gastritis. It may be necessary to look for other antigens, or the atrophy could be unrelated to serologic status to *H. pylori* virulence antigens.

In the present study, we found that only antibodies to the protein p95 (VacA) were more frequently than other endoscopic findings present in patients suffering from duodenal ulcers. Thus, the anti-VacA antibody is a more powerful marker of ulcers than anti-CagA. This is not surprising, because the vacuolizating toxin has been suspected to be involved in the development of mucosal ulcerous lesions (37-39). It has been reported that the toxin causes epithelial damage, but appears to have little effect on inflammatory cell infiltration. These results are in agreement with previous studies, which showed that anti-CagA, anti-VacA, and anti-p35 appear to be the best markers of ulcers (40). However, the clinical relevance of this association remains controversial (15). Contrary to this, we found no association of anti-CagA antibodies to endoscopic finding. Explanation of the discrepancy could be that in some areas there is high prevalence of CagA- and VacA-positive individuals. In our study, 91.3% of patients were CagA and 75.8% VacA positive. Another explanation could be the fact that peptic ulcers may be intermittently present and certain patients may be non-ulcerous at the time of endoscopic examination but may later evolve to an ulcerous state. Other authors have attempted to establish correlations between certain antibody patterns and gastric cancer (20). We also found anti-p95 (VacA) to be less frequently detected in patients with endoscopic finding of gastric carcinoma or normal mucosa than peptic ulcer. Possible clinical implications of the observed phenomena could be used in future in order to distinguish patients infected with more virulent strains of bacteria, especially those infected with therapy resistant strains. Also, proteins which elicit strong serological response in greater number of patients could be possibly used as a part of anti *H. pylori* vaccine. However, in order to confirm its real prognostic significance and clinical usefulness of these observations, a larger study population is needed.

A possible limitation of the study is that sample size calculation or power analysis was not performed. Marginal results of statistical significance were observed in difference of distributions of various grades of corpus inflammation and intestinal metaplasia according to anti-p120 (CagA) finding (data not shown). Additional investigation with greater number of patients could answer the question on true association of protein CagA and histopathological patterns of gastritis. Moreover, the presence of the bacterial virulence antigens was evaluated indirectly by host serological response only. Many of the tested bacterial virulence antigens are highly immunogenic and can induce antibody response in almost all patients (41). But, it is generally known that serological response depends on antigen type and immunological reactivity of the host. Some patients are infected with more than one type of bacteria possessing a variety of different (more or less virulent) proteins encoded by different genotypes of virulence genes. All of this explains why negative serological response does not always implicate *H. pylori* negativity to tested virulence antigen. Seropositivity to specific antigen does not absolutely indicate the relationship with pathogenesis of specific disease. Furthermore, these observations indicate that there is a need for future research into the pathogenesis of gastroduodenal diseases.

In conclusion, histological parameters of gastritis show better association with bacterial virulence than an endoscopic finding. However, no single entity or array of antigens has yet been identified that can serve as a serological marker(s) for predicting the development of specific gastric disease. This could be attributed to the role of other factors that govern disease expression, eg, other undetected virulence antigens, changes in gastric physiology, or host immune-inflammatory response (42).

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