Original Article

Helicobacter pylori cagA Status, vacA Subtypes and Histopathologic Findings in Iranian Patients with Chronic Gastritis

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ABSTRACT

***Background and Objective*: *Helicobacter pylori* has several strains with different degrees of virulence. The aim of this study was to detect two major important virulence factors, cagA/vacA genotypes, and to determine correlations among different cagA/vacA genotypes and histological features of chronic gastritis in Iranian patients.**

***Methods:* In this cross-sectional study, gastric biopsy was taken from 166 patients with non-ulcer dyspepsia. The specimens were processed for DNA extraction and identification of glmM gene. The vacA subtypes and cagA gene were tested by PCR. Histopathological features were recorded and graded according to updated Sydney system.**

***Results:* 76.7% of the *H.pylori* strains were cagA gene positive. The proportions of vacA gene subtypes s1, s2, m1 and m2 in the 78 strains isolated were 70.5%, 29.5%, 37.2% and 62.8%, respectively. 83.3% of the vacA-positive strains had s1 allele. Twenty-six strains (33.3%) were positive for both cagA and m1 allele. Positive cagA status and vacA subtypes were not associated significantly with presence of neutrophil infiltration, intestinal metaplasia or *H.pylori* density. Only vacA s1 was significantly associated with more severe inflammation (P=0.02). The dominant genotype of *H.pylori* was vacA-positive s1/m2. CagA gene positivity rate was not closely associated with severity of the disease.**

***Conclusion:* *H.pylori* strains showing vacA s1 genotype were associated with more severe gastritis. These findings show that vacA genotyping may have clinical relevance in Iran.**

Keywords: *Helicobecter pylori*, cagA status, vacA subtypes, Chronic gastritis

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Introduction

Helicobacter pylori is a gram-negative rod associated with gastroduodenal disease. It is estimated that *H.pylori* infects more than 50% of the world's population. Seroepidemiologic study in different parts of Iran revealed near 90% prevalence of *H.pylori* infection in adults older than 35 years (1). There is evidence for the existence of different strains of *H.pylori* with different degrees of virulence (2,3).

Two major important virulence factors of *H.pylori*, cytotoxin-associated protein (cagA) and vacuolating cytotoxin (vacA) encoded by cagA and vacA genes, respectively, have been well described. It has been reported that cagA gene is present in approximately 60% of *H.pylori* strains from Western populations but over 90% of the strains from Southeast Asian populations (4-7).

Gene cagA is considered a marker for the presence of cag PAI, which is associated with the most virulent *H.pylori* strains and with a more severe clinical outcome of the infection (8). Clinically, infection with the cagA positive *H.pylori* strain has been associated with higher grades of gastric mucosal inflammation as well as severe atrophic gastritis and has been suggested to play an important role in the development of gastric carcinoma (9-11). The *H.pylori* vacA gene encodes a secreted protein (vacA) that has been reported to exhibit a pleiotropic activity on gastric epithelial cells as well as on T lymphocytes. The vacuolating cytotoxin gene (vacA) is present in nearly all *H.pylori* strains. Alignment of the vacA nucleotide sequences of toxigenic and non-toxigenic *H.pylori* strains has revealed two distinct families, namely s1 (s1a; s1b) and s2, with one of two different vacA alleles in the middle region (m1 and m2). Analysis of different isolates of *H.pylori* has revealed all possible combinations of these alleles, with the exception of s2m1, suggesting that recombination events occur in nature between different vacA alleles (12). Studies conducted in several countries have shown that vacA-type s1 and cagA-positive *H.pylori* strains are associated with severe *H.pylori*-induced peptic ulcer disease (13-15). Thus, existing data is contradictory and cannot explain the pathogenic role of *H.pylori* in the development of different gastric diseases. Consequently, it might be useful to know the genetic diversity of *H.pylori* strains in Iran, a region with a high frequency of *H.pylori*-induced gastric disease.

In this study, we established various polymerase chain reactions (PCRs) to detect cagA gene and vacA subtypes of *H.pylori* strains isolated from patients with chronic gastritis and then determined local dominant cagA/vacA genotypes of the organism.

Materials and Methods

Patients

One hundred sixty six patients who had non-ulcer dyspepsia and performed upper GI-endoscopy during February to December 2006 in Taleghani Hospital, Tehran-Iran were enrolled in this cross-sectional study. Clinical data collected for each patient including age, gender, chief complaint, associated medical history, medication use and family history of gastric polyp/cancer. The following exclusion criteria were considered at baseline: peptic ulcer disease, bleeding complications, attempts to eradicate *H.pylori*, previous gastric resection, use of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs), or antibiotics two weeks before the study.

All patients gave informed consent and the study was approved by the Ethical Committee of Research Center for Gastroenterology and Liver Disease.

Histopathology

During endoscopy two biopsy specimens were taken from the antrum for histological evaluation. These specimens were fixed in 10% buffered formalin, embedded in paraffin, cut in sequential 4μm sections and stained with hematoxylin and eosin (H&E) and modified Giemsa stain. Multiple high powered fields were examined by two pathologists blinded to the characteristics of *H.pylori* strains. Histological severity of gastritis was graded using the criteria as described in the updated Sydney classification system (16). *H.pylori* density, atrophy, intestinal metaplasia, polymorphonuclear cell infiltration and mononuclear cell infiltration were determined and scored as normal (score=0), mild (score=1), moderate (score=2) and marked (score=3).

Isolation and identification

Two biopsy specimens were taken from the greater curvature of the antrum for histological examination and one biopsy for *H.pylori* culture. Gastric biopsy specimens for culture were kept in transport medium consisting of thyoglycolate with 1.3 g/L Agar (Merck Co, Hamburg, Germany) with 3% yeast extract (Oxoid Ltd., Basingstoke,. UK) and brought to the laboratory on the day of endoscopy. In each case, the gastric biopsy specimens were cultured on *Brucella*

Agar with 7% sheep blood and supplements with different antibiotics, incubated under microaerophilic conditions at 37° C for 3-10 days.

Preparation of Genomic DNA and Polymerase Chain Reaction (PCR)

DNA from each *H.pylori* isolates was extracted from the multiple colony sweeps by using QIAamp tissue method (Qiagen, Hilden, Germany). The genotypes of vacA single sequences (s1 or s2) and middle regions (m1 or m2), the presence of cagA and glmM (ureC) were determined by PCR. Primers sequences are listed in Table 1. All PCR mixtures were prepared in a volume of 25 µl containing 1 × PCR buffer, 500 nM of each primer, 1.5 mM $MgCl_2$; 200µM from each dNTP, 1.5U Taq DNA polymerase, and 300ng DNA sample. The mixtures were placed in a thermocycler (Eppendorf AG 22331, Hamburg, Germany). PCR products were visualized by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and examined under UV illumination.

Table 1: Oligonucleotide primers used for cagA, glmM (ureC) and vacA alleles

Gene	Primer designation	Sequence	PCR product size
cagA	CagA F1	AACAGGACAAGTAGCTAGCC	701
	CagA R1	TATTAATGCGTGTGTGGCTG	349
vacA s1s2	VAIF	ATGAAAAAAACCCTTTTAC	259 (s1)
	VAIXR	CGAATTGCAAGTGATGGT	286(s2)
glmM	GlmM1-R	GCTTACTTTCTAACACTAACGCGC	296
	GlmM2-F	GGATAAGCCTTTTAGGGGTGTTAGGG	
vacAm1a	VA3-F	GGTCAAAATGCGGTCATGG	300 (m1a)
	VA3-R	CCATTGGTACCTGTAGAAAC	
vacAm1b	VAm-F3	GGCCCCAATGCAGTCATGGAT	300 (m1b)
	VAm-R	GCTGTTAGTGCCTAAAGAAGCAT	
vacAm2	VA4-F	CATAACTAGCGCCTTGCAC	400 (m2)
	VA4-R	GGAGCCCCAGGAAACATTG	

Data Analysis

Chi-Square and Fisher's exact tests were used for analysis of categorical data. The Mann-Whitney Rank Sum test was used for assessing differences between ordered categories such as histological grade or cytotoxin production. Data discussed and analyzed in the discussion was analyzed using Fisher's exact test or Chi-Square which ever was appropriate. Analyses were done using Sigma Stat for Windows V2.03 (SPSS, Chicago, IL). A P value of <0.05 was accepted as statistically significant.

Results

There were 80 (48.2%) female and 86 (51.8%) male patients with the mean age of 47.78 (20-82) years. Eighty six of 166 patients who had positive product for glmM gene were included in this study.

vacA and cagA status of isolates

Of the 86 strains, 66 strains (76.7%) were cagA positive. Complete vacA s- and m-region genotype was obtained in 78 out of 86 cases (90.7%). Repeated experiments failed to yield PCR product for the other eight patients. Typing of the signal region and middle region of the vacA gene from 78 strains showed that the s1 allele was present in 55 of the 78 isolates (70.5%). Another twenty-three strains (29.5%) were positive for s2 allele. 83.3% of the cagA-positive strains had s1 allele and 72.2% of the 28 cagA-negative strains had s2 allele ($P < 0.001$) (Table 2).

Table 2: cagA and vacA association

	vacA					
	s1	s2	*P*	m1	m2	*p*
Cag A +	50	10	<0.001	26	34	0.04
Cag A -	5	13		3	15	
Total	55	23		29	49	

The presence of the m1 allele of the middle region was detected in 29 strains (37.2%) while 49 strains (62.8%) had positive PCR product for the m2 allele. Twenty-six strains (33.3%) were positive for both cagA and m1 allele while 34 strains (43.6%) were positive for both cagA and m2 allele ($P = 0.04$).

There was intestinal metaplasia in 11 (12.8%) of antral specimens. Eight (72%) of the *H.pylori* isolates obtained from these patients were cagA positive (P=0.7). No difference was seen between various types of vacA genotypes and intestinal metaplasia.

***H.pylori* density and histopathological findings**

In all of the 86 subjects, chronic gastritis with different severities was confirmed by histopathology. Antral biopsies of seven patients were negative for *H.pylori* in histopathology report but they were positive for expression of glmM gene.

The degree of *H.pylori* density was mild in 24.4%, moderate in 33.7% and severe in 41.9%. As shown in Table 3, the degree of *H.pylori* colonization was significantly correlated with the degree of gastric neutrophil infiltration (P< 0.001) and the degree of mononuclear cell infiltration (P= 0.003) but not with the presence of intestinal metaplasia or dysplasia.

Table 3: Association of *H.pylori* colonization and histopathological findings

	***H.pylori* density**						
	Score	0	1	2	3	P^a	P^b
Neutrophilic infiltration	0 (neg)	7	10	8	1	<0.001	<0.001
	1	0	1	8	8		
	2	0	2	11	19		
	3	0	1	2	8		
Mononuclear cell infiltration	0 (neg)	0	0	0	0		<0.001
	1	4	2	2	0		
	2	3	9	24	23		
	3	0	3	3	13		
Intestinal metaplasia	Neg.	3	14	25	33		0.08
	Pos.	4	1	4	3		

Score: normal, 0; mild, 1; moderate, 2; marked, 3.
p^a compared presence or absence of histopathological parameters with *H.pylori* density
p^b compared severity of the histopathological parameter with *H.pylori* density

***H.pylori* density and cagA status and vacA subtypes**

Fifty of the 65 subjects (76.9%), who had a denser (moderate or severe) *H.pylori* colonization, were cagA positive. There was no statistically significant association between cagA presence and density of *H.pylori* (P=0.94). The severity of *H.pylori* colonization was independent from vacA signal region type s1 or s2 ($P = 0.99$), and middle region m1 or m2 ($P = 0.85$).

cagA status and histopathological findings

Positive cagA status was not associated significantly with presence of neutrophil infiltration, intestinal metaplasia, *H.pylori* density or degree of mononuclear cell infiltration.

vacA alleles and histopathological findings

The presence of specific vacA signal region type (s1 or s2) or middle region type (m1 or m2) had no significant association with the degree of *H.pylori* colonization, or neutrophil infiltration. Only vacA s1 was significantly associated with more severe mononuclear cell infiltration (P=0.02) (Table 4).

Table 4: Association of cagA status and vacA alleles and histopathological findings

		cagA			vacA					
	Score	+	-	*P*	s1	s2	*P*	m1	m2[a]	*P*
***H.pylori* density**	0 (neg)	6	1	0.67	3	1	0.71	2	2	0.69
	1	10	4		9	4		4	9	
	2	24	5		17	10		12	15	
	3	20	10		26	8		11	23	
Neutrophil infiltration	0 (neg)	18	8	0.52	13	9	0.51	8	14	0.65
	1	15	2		12	4		6	10	
	2	25	7		24	7		10	21	
	3	8	3		6	3		5	4	
Mononuclear cell infiltration	0 (neg)	0	0	0.47	0	0	0.02	0	0	0.29
	1	5	3		2	3		1	4	
	2	45	14		37	19		19	37	
	3	16	3		16	1		9	8	

Score: normal, 0; mild, 1; moderate, 2; marked, 3.

Discussion

The gastric pathogen *H.pylori* is one of the most genetically diverse bacterial species which may be involved in the complex variety of diseases in infected patients (1). There are geographic genetic variations among *H.pylori* strains. Studies in several countries have demonstrated different distributions of vacA alleles and the presence of cagA gene in genotypes of *H.pylori* strains and their association with the development of gastroduodenal diseases (17-20).

In European and North American populations, prevalence of cagA-positive *H.pylori* varied between 64% and 79% (12,14) whereas in Asian countries such as Japan, Korea, China, Turkey and India, the proportion of cagA+ *H.pylori* strains was usually over 90% in all isolates (14,21-23). There are few data available about cagA status in Iran. In previous investigation done by Siavoshi *et al*. cagA was present in 44% of the patients (24). Our results revealed that 76.7% of our patients are positive for cagA gene which is near to European and North American populations. In many studies the presence of cagA has been shown to have a significant association with increased severity of chronic gastritis and the presence of atrophic gastritis; although we found no statistically significant difference among strains with or without cagA gene. It is likely that as the majority of *H.pylori*-infected individuals in Asian countries harbor cagA-positive strains, associations of cagA status and diseases are not observed in these regions (14,15). Surprisingly, the previous study from Iran reported that cagA presence was more frequent only in patients with gastric cancer (24).

The vacA genotypes show considerable variability in different geographic regions. In this study, genotyping of the vacA gene showed that the signal sequence of the vacA gene was s1 type in 70.5% isolates, and the mid-region of the gene was m2 type in 62.8% isolates. A recent study reported the same results on the signal sequence in Iranian isolates

(78%) (24). This observation is similar to the results obtained from China and Turkey (21,22) but in the middle region, they were substantially different from the results in Brazil and Portugal in which m1 was the dominant type (25,26). In addition Aydin *et al*. from Turkey and the large, multicenter study of van Doorn *et al*. reported an equal prevalence of m1 and m2 subtypes (27,28).

As in other reports (29), we also found that cagA and vacA s1 were highly significantly associated with each other ($P < 0.001$) and vacA s2 genotype was more frequent in cagA negative patients ($P<0.001$). We also found that patients with type s1 strains had more severe degrees of mononuclear cell infiltration as compared with type s2 strains ($P=0.02$). Similarly, Martins *et al.* (25) and Nogueira *et al.* (26) observed that allele s1 is associated with high degrees of gastric tissue inflammation and this correlation was in companion with cagA positivity and m1 allele; however we did not find the latter correlation. However, the current study does not rule out any association between the expression of vacA or cagA protein and the virulence of *H.pylori*.

Conclusion

From our experiment, we conclude that the dominant genotypes of *H.pylori* in Iranian patients with chronic gastritis may be cagA-positive s1/m2. cagA gene positivity rate is probably not closely associated with severity of the disease. *H.pylori* strains showing vacA s1 genotype are associated with more severe gastritis but this virulence factor does not appear to determine the overall pattern. Therefore, it is likely that the nature of the disease complicating chronic infection is determined by host, environmental and bacterial factors and perhaps new virulence factors should be described with more power to discriminate among *H.pylori* strains.

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