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High Frequency of *vacA s1m2* Genotypes Among *Helicobacter pylori* Isolates From Patients With Gastroduodenal Disorders in Kermanshah, Iran

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Abstract

Background: *Helicobacter pylori* infection and related diseases outcome are mediated by a complex interplay between bacterial, host and environmental factors. Several distinct virulence factors of *H. pylori* have been shown to be associated with different clinical outcomes. Here we focused on *vacA* and *cagA* genotypes of *H. pylori* strains isolated from patients with gastric disorder.

Objectives: The aim of this study was to determine the frequency of two toxins and genotypes of VacA toxin in patients referred to a central hospital in the west of Iran (Imam Reza hospital, Kermanshah) during 2011 - 2012.

Patients and Methods: Samples were collected from patients infected with *H. pylori*. Gastric biopsy specimens from the stomach antrum and corpus were cultured. PCR analysis was performed for genotyping *H. pylori vacA* and *cagA* genes.

Results: *Helicobacter pylori* was isolated from 48% (96/200) of patients with gastroduodenal disorders. In 81/96 (84%) cases, the *cagA* gene was present. Among different genotypes of *vacA*, two *s1m2* and *s2m2* genotypes were dominant with frequency of 39.5% and 50%, respectively. The frequency of the *s1m1* genotype was 7.2% (7/96), which is much lower than elsewhere. *H. pylori* isolates with positive results for *cagA* gene and *vacA s1m2* genotypes showed statistically significant correlation with peptic ulcer (*s1m2* 13/34 [38.2%] P = 0.003). However, isolates of *H. pylori* infection with *cagA* gene and *vacA s2m2* genotypes were significantly associated with development of gastritis (*s2m2* 41/42 [97.6%] P = 0.000).

Conclusions: About 90% of *H. pylori* strains potentially contained *vacA s2m2* and *s1m2* genotypes. Infection with *H. pylori* strain containing the *cagA* gene or the *vacA s1m1* and *s1m2* genotypes was associated with increased incidence of peptic ulcer disease (PUD).

Keywords: Peptic Ulcer, *Helicobacter pylori*, *cagA*, *vacA*

1. Background

Helicobacter pylori is a Gram-negative comma shaped bacterium, which can cause chronic or acute gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and mucosa associated lymphoid tissue (MALT) lymphoma. Although most infected individuals may remain asymptomatic (1), *H. pylori* is colonized in more than 50% of the world's population (2, 3). The reasons for variation of pathogenesis and prevalence of bacterium might be due to differences in race, health and living standards and more importantly, the virulence factors of the predominant type of the isolated bacteria in the population (4). Among the most important virulence factors we focused on Vacuolating cytotoxin A (VacA) and Cytotoxin-associated gene A (CagA) (5, 6).

VacA is an 88-KD toxin processed from a 140 KD precursor protein (7, 8). The toxin can be found in all *H. pylori* isolates. The protein constitutes of two variable regions; the signal region (s) located at the N-terminal end of the protein and the middle region (m). There are two major types of s; *s1* and *s2* and three minor *s1* segments; *s1a*, *s1b* and *s1c*. The m region is also known to have two major genotypes: *m1* and *m2* and two minor subtypes: *m1a* and *m1b*. Therefore, the toxin has 4 major and several minor subtypes (9, 10).

CagA is a 125 to 145 kD protein and present in 60 to 90 % of isolates. It is assumed that presence of CagA toxin in a colonized strain implies higher risk of gastritis and gastric cancer (11, 12). In addition to presence and absence of toxin, another important factor in virulence indicator of toxin is structure of protein. The protein consists

of several EPIYA motives like EPIYA-ABC or EPIYA-ABD. In western countries with predominant EPIYA-ABC motif isolates, the frequency of gastritis, gastric and duodenal ulcers and gastric cancer is much lower than Far East countries with EPIYA-ABD predominant strains (13). According to published data, the prevalence of *H. pylori* among Iranians may reach up to 90%, so it is important to predict the outcome of infection within Iranian population (14). As mentioned before, the presence of two toxins and genotypes of *VacA* is relevant with the appearance of gastric disorders.

2. Objectives

Although there are several reports concerning the prevalence of toxins and genotypes in the Capital and some parts of Iran, we still lack reliable data concerning the aforementioned subjects in the western region of Iran. Therefore, the aim of this study was to determine the frequency of two toxins and genotypes of VacA toxin in patients referred to a central hospital in the west of Iran (Imam Reza hospital, Kermanshah) during 2011 - 2012.

3. Patients and Methods

In this study, 200 patients with gastroduodenal disorders including gastric ulcer, duodenal ulcer and gastritis were enrolled. Every patient with gastroduodenal symptoms referred to endoscopy department of Imam Reza hospital, Kermanshah and patients hospitalized in Internal Medicine (Gastroenterology) were examined by a gastroenterologist. The patients were considered as groups with symptoms of dysphagia, difficulty swallowing, stomach ulcers, reflux, indigestion, and atrophy. The patients who received bismuth drugs, antibiotics and proton pump inhibitors two weeks before sampling and patients with gastrointestinal bleeding were excluded.

3.1. Helicobacter pylori Isolation and DNA Extraction

Biopsy samples were transferred to the laboratory in 1 mL brain heart infusion broth with 25% glycerol within 3 hours. Homogenized samples were cultured on Columbia agar (Merk, Germany) containing 10% egg yolk, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and the plates incubated at 37°C under microaerophilic atmosphere (10% carbon dioxide, 5% oxygen and 85% nitrogen) for 3 to 5 days. Presumptive colonies were confirmed as *H. pylori* by biochemical tests including catalase, oxidase and urease, and also Gram staining. Confirmed colonies were subcultured on new medium and after incubation the colonies were subjected to DNA extraction by kit according to the manufacturer's protocol (DNPTM kit, Sinaclon, Iran).

3.2. Polymerase Chain Reaction (PCR)

First *H. pylori* density was confirmed by PCR analysis using genus and species specific primers targeted 16SrRNA and *ureC* respectively (Table 1). PCR analyses were performed for determination of *vacA* s (*s1*, *s2*) m (*m1*, *m2*), genotypes and also *cagA* gene using the specific primers (Table 1). *cag* empty site specific primers were used to confirm the absence of entire cag PAI. PCR reaction was performed in 15 μL volume including 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 1X PCR buffer, 1 U Taq DNA polymerase and 100 ng of *H. pylori* chromosomal DNA. After heating at 95°C for 5 minutes, amplification was performed over 30 cycles of 95°C for 30 seconds, 30 seconds at specific annealing temperature for each primer and 72°C for 30 seconds followed by 72°C for 5 minutes. Annealing temperature were 58°C for gene *cagA*, 60°C for alleles of *vacA* and 54°C for cag empty site primers. PCR products were subjected to electrophoresis, stained by ethidium bromide and photography (gel Documentation system, BioRad. Singapore).

3.3. Statistical Analysis

Statistical analysis of data was performed using logistic regression, chi-square test and Fisher's exact test, with significance set at a P value of < 0.05. Genotypes with mixed status for *vacA* were excluded from the calculations of association.

4. Results

In this study, 200 patients with gastroduodenal difficulties were enrolled; only 96 cases were infected with *H. pylori*. Age of patients was between 16 and 76 years. Of 96 patients, 41 (42.7%) were female and 55 (47.3%) male. Based on clinical diagnosis, of 96 cases, 20 (20.8%) had peptic ulcer (7 duodenal ulcer and 13 gastric ulcer) and 76 (79.2%) had gastritis. All 96 *H. pylori* isolates were confirmed by *ureC* (16) and 16SrRNA PCR (Figure 1).

4.1. cagA

All genotypes were determined by PCR according to specific primers. Figure 2 shows gel electrophoresis of *H. pylori* genotyping by *cagA*, *vacA* s and m alleles. Overall detection rate of *cagA* gene in *H. pylori* isolates was 84.3% (81/96). From 81 *cagA* positive patients, 45 were male and 36 female. Based on clinical diagnosis, 64 patients were implicated with gastritis, 6 with duodenal ulcers and 11 stomach ulcer. The complete pattern of all positive and negative patients is shown in Table 2.

All *H. pylori* isolates were tested with cag empty site specific primers to confirm the absence of cag PAI. *cag* empty site PCR positive result found in 21 isolates. Among 21 samples, 6 had positive results for *cagA* gene and only 15 isolates had true negative findings for *cagA* gene.

4.2. vacA

vacA gene has variation regions including signal and middle regions classified as *s1*, *s2* and *m1*, *m2* alleles,

respectively. However, alleles divided into sub-alleles including *s1a*, *s1b*, *m1a* and *m1b*. *H. Pylori* isolates were screened for presence of all sub-alleles by PCR technique. From 96 *H. pylori* isolates, 47(48.9%) isolates had positive results for s1 allele and 49 (51.1%) for s2. S1a subtype was identified in 45 isolates and *s1b* subtype in 2 isolates. The presence of m1 was confirmed in 10 (10.5%) isolates, while 86 (89.5%) isolates had positive findings for m2. m1a subtype was identified in 8 isolates and *m1b* subtype in 2 isolates. Combined forms of suballeles *s1m1* genotype were identified in 7.3% of the isolates, *s1m2* in 39.5% of the isolates, *s2m1* in 3.2% and *s2m2* in 50% of the isolates. Presence of *cagA* gene among *s1m1*, *s1m2*, *s2m1* and *s2m2* positive isolates were 42.8%, 89%, 100%, and 87.5%, respectively.

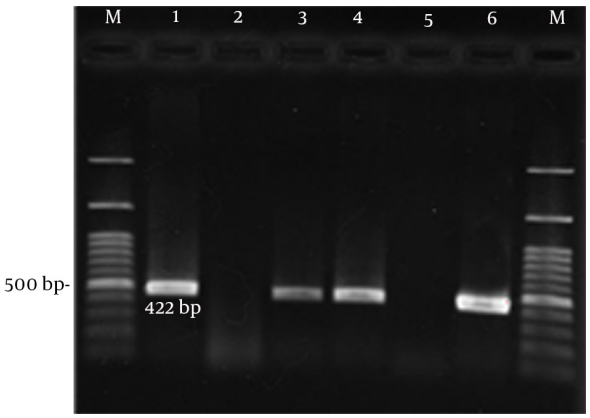
4.3. Helicobacter pylori Genotypes and Their Association With Gastrointestinal Diseases

Helicobacter pylori isolates with positive results for *cagA* gene and *vacA s1m2* genotypes were significantly correlated with peptic ulcer disease (*s1m2* 13/34 [38.2%] P = 0.003). However, development of gastritis was significantly associated with *H. pylori* infection in isolates with *cagA* gene and *vacA s2m2* genotypes (*s2m2* 41/42 [97.6%] P = 0.000) (Table 3).

Table 1. List and Characteristic of Oligonucleotide Primers Used in the Study

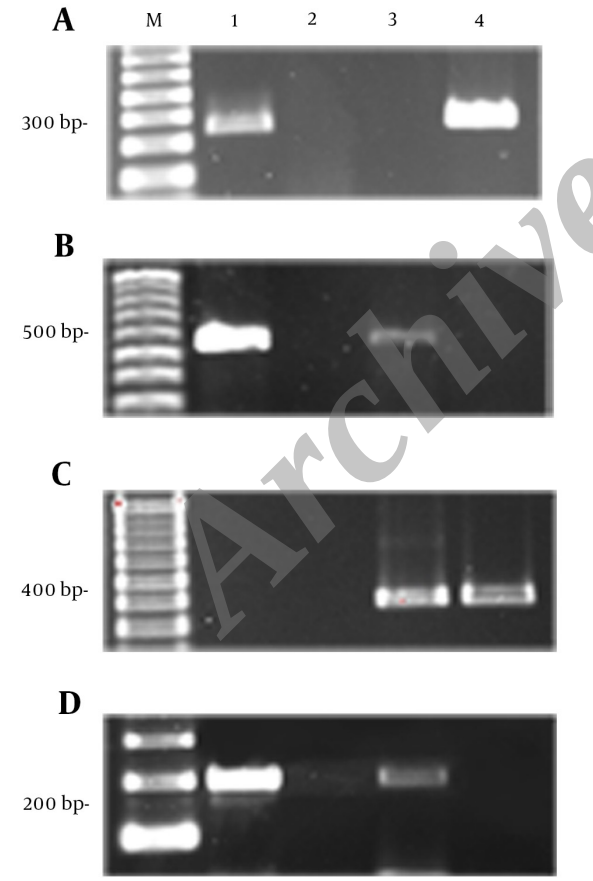
Primer Name		Primer Sequence (5′ to 3′)	Product Size, bp	References
16SrRNA	F	GAT TTT ACC CCT ACA CCA	422	(15)
	R	GCT ATG ACG GGT ATC C		
Hp-ureC	F	CAT CGC CAT CAA AAG CAA AG	214	(16)
	R	CAG AGT TTA AGG ATC GTG TTA G		
cagA	F	GAT AAC AGG CAA GCT TTT GAG G	349	(17)
	R	CTG CAA AAG ATT GTT TGG CAG A		
vacAS1a	F	GTC AGC ATC ACA CCG CAA C	190	
	R	CTG CTT GAA TGC GCC AAA C		
vacAS1b	F	AGC GCC ATA CCG CAA GAG	187	
	R	CTG CTT GAA TGC GCC AAA C		
vacAS2	F	AGCGCCATACCGCAAGAG	199	
	R	CTG CTT GAA TGC GCC AAA C		
vacAm1	F	GGT CAA AAT GCG GTC ATG G	290	
	R	CCA TTG GTA CCT GTA GAA AC		
vacAm2	F	GGA GCC CCA GGA AAC ATT G	352	
	R	CAT AAC TAG CGC CTT GCA C		
vacAm1a	F	GGT CAA AAT GCG GTC ATG G	290	
	R	CTG TTA GTG CCC GCA GAA AC		
vacAm1b	F	GGCCCCAATGCAGTCATGGAT	291	
	R	GCTGTTAGTGCCTAAAGAAGCAT		
***cag PAI* empty site**	F	ACA TTT TGG CTA AAT AAA CGC TG	550	(18)
	R	GGT TGC ACG CAT TTT CCC TTA ATC		

Figure 1. Gel Electrophoresis of Genus Specific 16SrRNA PCR Products From *H. pylori* Isolates



M; 100 bp marker, 1; positive control, 2; negative control, 3,4; positive samples for *16srRNA* PCR.

Figure 2. Gel Electrophoresis of *H. pylori* Genotyping by *cagA*, *vacA* s and m Alleles



(A) M; 100 bp marker, 1; positive control, 2; negative control, 3,4; negative and positive isolates for *cagA*. (B) M; 100 bp marker, 1; positive control, 2; negative control, 3,4; negative and positive isolates for cag empty site. (C) M; 100 bp marker, 1; negative control, 2, 3; negative and positive isolates 3; positive control for *vacA* m alleles. (D) M; 100 bp marker, 1; positive control, 2; negative control, 3,4; positive and negative isolates for *vacA* s alleles.

Table 2. Patient Characteristics and Distribution of *H. pylori* *vacA* and *cagA* Genotypes According to the Diseases[a]

Genotypes	Gastritis (n = 76)	Peptic Ulcer (n = 20)	
		Gastric ulcer (n = 13)	Duodenal ulcer (n = 7)
***vacA*s2**	48 (63.2)	1 (7.7)	-
***vacA*s1**	28 (36.8)	12 (93.3)	7 (100)
***vacA*s1a**	26 (34.2)	12 (92.3)	7 (100)
***vacA*s1b**	2 (2.6)	-	-
***vacA*m2**	69 (90.8)	10 (76.9)	7 (100)
***vacA*m1**	7 (9.2)	3 (23.1)	-
***vacA*m1a**	5 (6.6)	3 (23.1)	-
***vacA*m1b**	2 (2.6)	-	-
cagA			
Positive	64 (84.2)	11 (84.61)	6 (85.75)
Negative	12 (15.8)	2 (15.49)	1 (14.3)

[a]Data are presented as No. (%).

Table 3. Frequency of *Helicobacter Pylori vacA s1m2* and *s2m2* Alleles and *cagA* Genotypes According to the Diseases

vacA Alleles	Gastritis (n = 72)		Peptic Ulcer (n = 17)	
	cagA$^+$	*cagA*$^-$	*cagA*$^+$	*cagA*$^-$
s1m2	21	1	13	3
s2m2	41	6	1	-
Total	62	10	14	3

5. Discussion

The rate of infection by *H. pylori* is averagely 50% in the world population (19). While, the frequency in western countries is approximately 30%, the infection rate in Asia is about 60 to 80% (20). In 0.1 to 1 % of the population, the infection progresses to stomach cancer. In this study, pathogenic properties of *H. pylori* strains collected from patients with upper gastrointestinal diseases living in the west of Iran were presented. *Helicobacter pylori* infection is common in Iran, but we lack any information concerning the prevalence of the infection in the west of Iran (21, 22). However, available data suggest that the prevalence is as high as other cities in Iran. The prevalence of *vacA* genotypes and *cagA* gene in *H. pylori* isolates from different parts of the world are different, and there is a direct association between specific genotypes and certain clinical manifestations (23). Our data showed that the prevalence of *cagA* gene in *H. pylori* isolates was 84% (81/96). According to published data, the prevalence of *cagA* in Iranian isolates were 62%, 92% and 68.7% in Tehran, Jahrom and Tabriz, respectively (22, 24, 25). It is obvious that *cagA* gene frequency is almost the same in different geographic areas in Iran.

Although the prevalence of *cagA* harboring strains in Iran is similar, the pattern varies among different geographical areas from less than 50% in the central Asia to more than 99% in East Asian countries. This variation could be related to different methodologies like the use of different *cagA* specific PCR primers and genetic variation of isolates. *cagA* gene frequency is varied (2, 26, 27). The prevalence of *H. pylori cagA* positive strains in this study is similar to the reports from Europe and North America with prevalence of 74% and 88%, respectively (28, 29). Many studies reported a higher prevalence of *cagA* gene in patients with peptic ulcer disease. However, prevalence of *cagA* positive strains was higher in isolates from patients with peptic ulcer compared with *cagA* negative strains, even though the difference was not statistically significant.

There are plenty of reports showing a significant association between the prevalence of *cagA* gene with disease progression; also, the pathogenicity is due to the number of EPIYA motives in *CagA* protein. The number of EPIYA motives in *CagA* determines the phosphorylation capability of the protein and higher level of phosphorylation increases inflammatory activity of the toxin. Essentially, all *H. pylori* strains carry *vacA* gene, which in certain strains allows in vitro expression of a protein toxin that induces vacuolation of a wide variety of eukaryotic cells. The gene contains both conserved and variable regions, forming a mosaic gene. There are two major regions, called the s (signal sequence-encoding) and m (for mid region-encoding). The VacA toxins with *S1* motif are more potent than *S2* types (30).

The results of this study showed that the prevalence of s2 and *s1* harboring strains are similar. The frequency of *s1* positive *H. pylori* was 40% (19/47) in patients with peptic ulcer disease (PUD) and 60% (28/47) in those with non-ulcer disease (NUD). This is contradictory to other reports which showed that the frequency of this allele in PUD is higher than NUD. For example, in the USA, 90% of patients with duodenal ulcer had *s1* allele and the authors concluded that infection with *H. pylori* strains harboring this allele has an increased risk of developing PUD (31, 32). While, s2 allele frequency was 98% (48/49) in patients with gastritis and only 2% (1/49) in those with PUD.

Among different genotypes of *vacA*, *s1m2* and *s2m2* genotypes were dominanted with frequency of (39.5%) s1m2 and (50%) s2m2. In this study, *s1m1* genotype frequency was 7.3% (7/96), which is much lower than any other report. The frequency of *s1m1* genotype is 24% to 84% worldwide; 26% in Hong Kong, 24% in Nigeria, 48% in Ethiopia, 78% in Korea, 80% in Brazil and 33% in Iran (23, 33-37). There is a direct correlation between toxin activity of different *vacA* genotypes and pathogenicity of *H. pylori*. Moreover, the *s1m1* genotype has a higher toxicity and can increase gastric atrophy and play an important role in creating gastric ulcer, while it may reduce gastric acid secretion. However, *s1m2* and *s2m2* genotypes are less potent (38).

The frequency of *H. pylori* strains harboring *s1m1* isolated from patients with gastric ulcer *s1m1* genotype was very low in our study. The *s2m2* genotypes were more prevalent in our study (50%, 48/96), while it was contrary to other publications from Iran (27%) and also around the world (0% - 57%) (23, 34, 37). So far, the high prevalence of *s2m2* has been reported only in North Africa (57%) (30). In this study, *s2m2* genotype frequency was 97.9% (47/48) in patients with gastritis and 2.1% (1/48) in those with peptic ulcer. These rates are different with two other published studies reporting 11.9% and 38% (39, 40). Most studies found a significant association between genotype $cagA^+$ and *s2m2 vacA*, but in this study, there was no significant association between $cagA^+$ and *s2m2* or $cagA^-$ with *s2m2*. All the s2m1 positive strains had positive results for *cagA* gene. Moreover, 87.5%, 89% and 42.8% of *H. pylori* strains with the *vacA s2m2*, *s1m2* and *s1m1* genotypes contained the *cagA* gene as well. Interestingly, among *cagA* negative strains, three *s1m2* harboring strains caused peptic ulcer and three *s1m1* harboring strains were related to gastritis.

In conclusion, in patients with gastric disorders in Kermanshah, we found high rates of *H. pylori* infection, gastritis and PUD. Over 85% of *H. pylori* organisms carry *cagA* gene, indicating the presence of putative cag PAI virulence marker. In addition, about 90% of *H. pylori* strains potentially contained *vacA s2m2* and *s1m2* genotypes. Infection with *H. pylori* strain containing the *cagA* gene or the *vacA s1m1* and *s1m2* genotypes was associated with increased incidence of PUD. We showed that association between *cagA* gene and *s1m1* genotype was more significant than *s1m2* genotype in development of gastric ulcer disease. It seems that *s1m1*$cagA^+$ strains could predispose development of gastric ulcer in Kermanshah.

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Footnotes

Authors' Contribution: Study concept and design: Ramin Abiri, Hamid Pajavand; acquisition of data: Homayoon Bashiri, Hamid Pajavand; analysis and interpretation of data: Hamid Pajavand, Parviz Mohajeri, Ramin Abiri, Amirhooshang Alvandi; drafting of the manuscript: Hamid Pajavand; critical revision of the manuscript for important intellectual content: Behnam Kalali, Markus Gerhard; statistical analysis: Farid Najafi; administrative, technical, and material support: Hamid Pajavand, Somaye Bakhtyari; study supervision: Ramin Abiri.

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