Brief Report

Prevalence of Helicobacter pylori vacuolating cytotoxin A gene as a predictive marker for different gastrodoudenal diseases

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Abstract

Objectives: *Helicobacter pylori* (*H. pylori*) has several virulence factors such as *vacA* and *cagA* genes. Mosaicism in *vacA* alleles with two distinct families of *vacA* signal sequences (*s1*, *s2*) and two distinct families of middle region alleles (*m1*, *m2*) is reported. The aim of this study was determination of *H. pylori vacA* allelic types in Chaharmahal and Bakhtiyari province, Iran and their correlation with six different gastroduodenal diseases.

Patients and Methods: This cross-sectional descriptive study was performed on 200 antral gastric biopsy specimens that were obtained from patients undergoing upper gastrointestinal tract endoscopy in Hajar Hospital of Shahrekord. Initially, *H. pylori* strains were identified by rapid urease test (RUT) and *ureC* primers, and thereafter, we used seven specific primers for detection of *vacA* genotypes. Statistical analyses were used to find their relationship to gastric disorders.

Results: The frequency of the *vacA* alleles, *s1a/m1a*, *s1a/m1b*, *s1a/m2*, *s1b/m1a*, *s1b/m1b*, *s1b/m2*, *s1c/m1a*, *s1c/m1b*, *s1c/m2*, *s2/m1a*, *s2/m1b* and *s2/m2* were respectively, 27(16.46%), 8(4.87%), 45(28.43%), 7(4.26%), 5(3.04%), 10(6.09%), 12(7.31%), 4(2.43%), 18(10.97%), 6(3.65%), 0 and 22(13.41%).

Conclusion: *s1a/m2* strains were the most prevalent strains in this region and there was a considerable relationship between *s1a/m1a*, *s1a/m2*, *s2/m2* and *s1c/m1a* with some gastric disorders. As the findings are different from other regions of the world, extended molecular epidemiologic investigations are recommended in other cities of Iran.

***Keywords**:* Helicobacter pylori, vacA, Chaharmahal and Bakhtiari, Gastroduodenal diseases.

Introduction

Helicobacter pylori is a major cause of human gastro duodenal tract all over the world (1), and is associated with many diseases such as gastric ulcer, duodenal ulcer, gastritis, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and distal gastric cancer (2,3). There are reports describing a significant difference in the prevalence of *H. pylori* infection, between and within countries due to variation in geographical locations and ethnicity of each population (4). The reason for such a clinically diverse outcome of infection remains uncertain, but many include host and bacterial virulence factors are postulated to be responsible(5).

Bacterial virulence strongly depends on genes structure and expression of the relevant proteins. There are two *H. pylori* bacterium recognized as most virulent that are crucial in the formation of gastric mucosal lesions: VacA (vacuolating cytotoxin A) and CagA (cytotoxin associated gene A) both of which take part in colonization and modulation of the host body (1).The *vacA* gene is present in most *H. pylori* strains, but VacA product may not be expressed in all cases. There has been an attempt to characterize and classify differences in the *vacA* gene and to associate specific genotypes with different *H. pylori* associated disease (6). *vacA* gene contains areas of conservative and variable nucleotide sequences characteristics for mosaic structure. In all *H. pylori* strains, a segment of *vacA* gene, encoding C-terminal domain of the protein and a segment situated near the N-terminal are strongly conservative. On the other hand, the so-called middle region (m region) and the fragment encoding a protein signal sequence present high level of variability (1).

There are two main types of signal sequences, *s1* (subtype *s1a*, *s1b*, *s1c*) and *s2* and two middle region *m1* (subtype *m1a*, *m1b*) and *m2*. The existence of various genotypes of *s* and *m* regions enable the formation of numerous genotypic combination of *vacA* gene (7). The current consensus is that *s1/m1* strains showed a high level of vacuolating cytotoxin activity, whereas *s2/m2* strains do not exhibit vacuolating cytotoxin. It remains unclear whether the *vacA* genotypes were useful markers for clinical outcomes (6).

Initial reports indicated that the *s1* genotypes would be found in close association with clinical outcomes in

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Received for Publication: January 3, 2011
Revision Received: February 28, 2011
Revision Accepted: March 8, 2011

Western countries, however, the prevalence of this genotype is extremely high (almost 100%) in East Asian countries irrespective of clinical outcomes (6). There is little information about the virulence factors of *H. pylori* in Iran in relation to gastroduodenal diseases. The aim of this study was to determine the frequency of *vacA* genotypic alleles in Chaharmahal and Bakhtiari Province and evaluate the relationship between these alleles and six gastrointestinal diseases.

Patients and Methods

A total number of 200 patients who had received upper-endoscopy treatments in Hajar hospital, during June and November 2009 in Shahrekord, Iran, were enrolled in this study. Each patient's history sheet was examined in detail and findings were recorded on standard Performa including demographic data. All patients read and signed an 'informed consent' form at the beginning of endoscopy and declared their willingness for the application of their anonymous data for research purposes. For each patient and with the use of a disinfected endoscope two biopsy specimens from the antrum were taken. One piece of each specimen was examined by rapid urease test (RUT) for detection of *H. pylori*. RUT was performed with a Gastric Urease kit (Bahar-afshan Co, Iran). A second piece from positive samples in RUT was used in PCR. DNA was isolated from biopsy specimens using Genomic DNA purification kit (DNP^{TM}, CinnaGen, Iran) according to manufacturer's instructions. DNA preparations from biopsy specimens of 164 *H. pylori* positive patients were subjected to PCR. Primers used for PCR detection of *H. pylori* were ET-2U (5'-CCC TCA CGC CAT CAG TCCCAA AAA-3') and ET-2L (5'-AAG AAG TCA AAA ACG CCC CAA AAC-3') (8). Primers used for PCR assays of *vacA* gene are shown in Table 1. DNAs from *H. pylori* (D0008, Genekam, Germany) were used as positive control of *vacA* genes, and sterile distilled water was used as negative control. PCR was done in 20 µl (for ET2) or 25 µl (for *vacA*) of total reaction volume containing: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM dNTPs each (Fermentas), 0.4 µM primers, 0.25 U of Taq DNA polymerase (Fermentas), and 2 µl (40-260 ng/µl) of DNA. The PCR was performed in a DNA Thermal Cycler (Eppendrof, Mastercycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany Co.), with 40 cycles for ET2 primer and 35 cycles for *vacA* primers. Each cycle consists of denaturation at 95°C/45 sec; annealing at 59°C/30 sec for ET2, 54°C/45 sec for *vacA s1a*, *s1c*, 58°C/1min for *vacA s1b, s2* and 56°C/1min for *vacA m1a, m1b and* 60°C/1min for *vacA m2*; extension at 72°C/45 sec. There was another longer extension of 6 min at 72°C. For identification of the amplified products, 10 µl of the PCR reaction mixture was analyzed by electrophoresis on 1% agarose gel under ultraviolet light.

The data were then analyzed by SPSS software (Version16 *SPSS.* Inc, USA) and *p* value was calculated using Chi-square and Fisher's exact tests to find any significant relationship. *P* value less than 0.05 was considered statistically significant.

Results

Among 200 patients, based on RUT and PCR for *ureC* using DNA from samples, 164 (82%) patients were infected with *H. pylori*, 79 individuals (48.2%) were men and 85 (51.8%) were women with an average age of 47 years (range, 15 to 88 years). Moreover, 16 (9.8%) of patients had gastric ulcers, 22 (13.4%) had duodenal ulcers, 3 (1.8%) had gastric cancer, 160 (97.6%) had gastritis, 3(1.8%) had duodenitis, 34 (20.7%) had gastric nodularity and 52 (31.7%) had gastric erosions. It should be noted that some patients had several diseases together.

Possible combinations of *vacA* alleles were determined in this population. All the 164 samples were positive with *ureC* too and were amplified with both *vacA s* and *m* regions.

Table1: Oligonucleotide primers used for *vacA* alleles.(7)

Region	Primer designation	Sequence	PCR product size
s1a	vacA s1a-F	5'-CTC TCG CTT TAG TAG GAG C-3'	213 bp (843-1055)
	VA1-R	5'-CTG CTT GAA TGC GCC AAA C-3'	
s1b	SS3-F	5'-AGC GCC ATA CCG CAA GAG-3'	187 bp (869-1055)
	VA1-R		
s1c	vacA s1c-F	5'-CTC TCG CTT TAG TGG GGY T-3'	213 bp (843-1055)
	VA1-R		
s2	SS2-F	5'-GCT AAC ACG CCA AAT GAT CC-3'	199 bp (433-631)
	VA1-R		
m1a	VA3-F	5'-GGT CAA AAT GCG GTC ATG G-3'	290 bp (2741-3030)
	VA3-R	5'-CCA TTG GTA CCT GTA GAA AC-3'	
m1b	VAm-F3	5'-GGC CCC AAT GCA GTC ATG GAT-3'	
	VAm-R3	5'-GCT GTT AGT GCC TAA AGA AGC AT-3'	
m2	VA4-F	5'-GGA GCC CCA GGA AAC ATT G-3'	352 bp (976-1327)
	VA4-R	5'-CAT AAC TAG CGC CTT GCA C-3'	(2284-2635)

Overall, for *s* region, 135 (82.3%) samples were classified as *s1* and 29 (17.7%) as *s2*. Out of 135 *s1* strains, 79 (48.1%) samples were *s1a*, 21 (12.8%) were *s1b* and 35 (21.3%) were *s1c* positive. For *m* region, 67 (40.7%) were classified as *m1* and 97 (59.1%) were classified as *m2*. In the case of *m1* subtyping, the distribution of *m1a* and *m1b* was 52 (31.7%) and 15 (9.14%) respectively. As the results show in table 2, the frequency of genotype *s1a/m2* was the highest and we did not observe any *s2/m1b* genotype in this region.

Furthermore, there was significant relationship between duodenal ulcer and *s1a/m1a* (*P*=0.04), *s1a/m2* (*P*=0.02) and *s2m2* (*P*=0.05). Additionally, we observed association between nodularity and *s1c/m1a* (*P*=0.03) and moreover, gastric erosion and *s1a/m1a* (*P*=0.01) genotypes, however, there was no relationship between gastric ulcer, gastric cancer, duodenitis and gastritis with *vacA* genotypes (Table 3).

Table2: Relationship between Mid-region and signal sequence typing of *vacA* alleles for 164 samples.

	Signal sequence			
Mid region type	*S1a* (%)	*S1b* (%)	*S1c* (%)	*S2* (%)
M1a (%)	27(16.5)	7(4.3)	12(7.3)	6(3.7)
M1b (%)	8(4.9)	5(3)	4(2.4)	0
M2 (%)	45(27. 4)	10(6.1)	18(11)	22(13.4)

Discussion

H. pylori is one of the most genetically diverse bacterial species, with any given isolates easily distinguished from most others by DNA fingerprinting or sequencing of representative gene segments. This mutational diversity has been enhanced by extensive interstrain gene transfer and recombination (10). The geographic distribution of distinct *H. pylori* genotypes and the prevalence of virulent bacterial genotypes in several regions have remained unknown. (6) In the current evaluation, we studied 200 random patients of whom 164 were infected with *H. pylori*, and then we analyzed the *vacA* gene alleles in *H. pylori* positive samples.

Many studies are published regarding *vacA* genotypes all over the world. In Tehran, Iran Mohammadi et al and Jafari et al. introduced *s1a/m2* as a dominant strain of the region. (6, 11). In addition, Nahaei et al. from Tabriz, Iran, Farshad et al. and Kamali-sarvestani et al. from Shiraz, Iran reported that the prevalence of *s1/m2* genotype was high in their cities (12,13,14). We have found *s1a/m2* genotype dominant in Shahrekord, Iran, as well and therefore, we can suggest that this strain is predominant in Iran.

On the contrary, in other countries the results are different. The majority of Portuguese strain (72%) comprised type *s1a* (15), however, *s1a/s1b* were equally present in France, Italy and North America (16). The predominant *vacA* subtype in East Asian e.g in Korea (80.4%) and Hong Kong (95.8%) was *s1c* allele (17, 18). Furthermore, in Northern Thailan *s1a* and *s1c/m1* are equally common (17) and in Lithuania, Cuba and Lebanon *vacA s1a*, but in Japan *s1c/m1b* were common. (10, 20, 21, 22).

Investigator's opinion about the association between *vacA* genotypes and gastric disorders are different, for example in Iran Jafari et al. found no correlation (6), whereas Mohammadi et al and Molaei et al found *s1a* allele associated with more severe inflammation (11,23). As we compared the *vacA* genotypes with patient's clinical manifestations, there was significant association between some diseases and some genotypes.

Table3: Correlation between *vacA* subtypes and clinical outcomes.

***vacA* genotypes**	**G.U n=16 no.(%)**	**D.U n=22 no.(%)**	**G.C n=3 no.(%)**	**DUO n=3 no.(%)**	**NOD n=34 no(%)**	**G.E n=52 no.(%)**	**GAS n=160 no.(%)**	**Total n=164 no.(%)**
S1a	9(56.2)	11(50)	1(33.3)	1(33.3)	19(55.8)	18(34.6)	76(47.5)	79(48.1)
S1b	3(18.7)	1(4.5)	1(33.3)	1(33.3)	3(8.8)	9(17.3)	20(12.5)	21(12.8)
S1c	3(18.7)	3(13.6)	0	0	4(11.7)	12(23.07)	34(21.2)	35(21.3)
S2	1(6.2)	7(31.8)	1(33.3)	1(33.3)	8(23.5)	10(19.2)	28(17.5)	29(17.6)
M1a	4(25)	9(40.9)	2(66.6)	0	7(20.5)	10(19.2)	52(32.5)	52(31.7)
M1b	3(18.7)	3(13.6)	0	0	2(5.8)	6(11.5)	15(9.3)	15(9.1)
M2	9(56.2)	10(45.4)	1(33.3)	3(100)	25(73.5)	33(63.4)	92(57.5)	97(59.1)
S1a/m1a	1(6.2)	4(18.1)	1(33.3)	0	6(17.6)	3(5.7)	27(16.8)	27(16.4)
S1a/m1b	2(12.5)	2(9.09)	0	0	2(5.8)	2(3.8)	7(4.3)	8(4.8)
S1a/m2	6(37.5)	2(9.09)	0	1(33.3)	11(32.3)	13(25)	41(25.6)	45(27.4)
S1b/m1a	2(12.5)	0	0	0	0	3(5.7)	6(3.7)	7(4.2)
S1b/m1b	0	0	0	0	0	3(5.7)	4(2.5)	5(3.04)
S1b/m2	1(6.2)	1(4.5)	1(33.3)	1(33.3)	3(8.8)	3(5.7)	10(6.2)	10(6.09)
S1c/m1a	1(6.2)	1(4.5)	0	0	0	3(5.7)	12(7.5)	12(7.3)
S1c/m1b	1(6.2)	1(4.5)	0	0	0	1(1.9)	4(2.5)	4(2.4)
S1c/m2	1(6.2)	1(4.5)	0	0	4(11.7)	8(15.3)	18(11.2)	18(10.9)
S2/m1a	0	1(4.5)	1(33.3)	0	1(2.9)	1(1.9)	6(3.7)	6(3.6)
S2/m1b	0	0	0	0	0	0	0	0
S2/m2	1(6.2)	6(27.2)	0	1(33.3)	7(20.5)	9(17.3)	22(13.7)	22(13.4)

G.U: Gastric ulcer; D.U, Duodenal ulcer; G.C: Gastric cancer; GST: Gastritis; DUO: Duodenitis; NOD: Gastric nodularity; G.E: Gastric erosion .

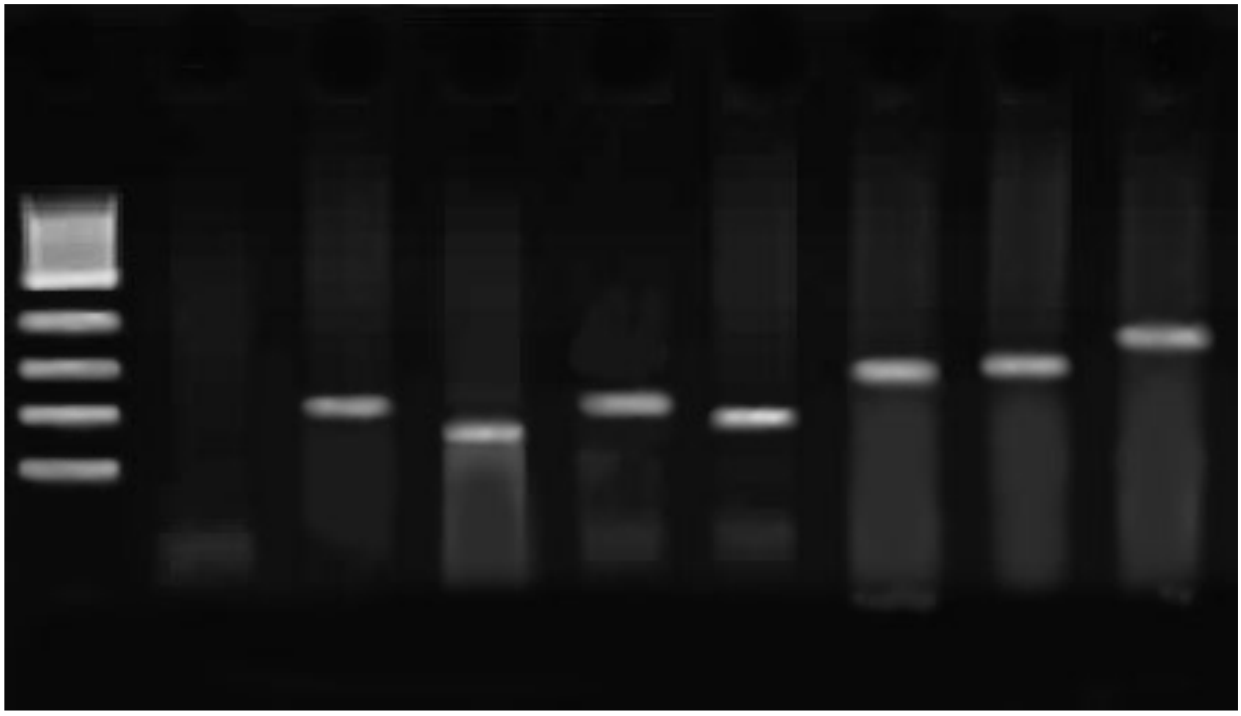

Figure 1: Electrophorestic identification of amplified products of *Helicobacter pylori* vacuolating cytotoxin A gene:

1) 100 bp marker 2) Negative control 3) S1a :213bp 4) S1b: 187bp 5) S1c: 213bp
6) S2: 199bp 7) M1a: 290bp 8) M1b: 291bp 9) M2: 352bp

In Thailand, Japan, Korea, Colombia and America, they found no relationship (24) whereas in Cuba, Lebanon, Hong Kong, England and most of the European countries, a significant association between *s1* allele and PUD diseases are reported (18, 20, 22). The reason for this discrepancy was unclear but we are sure that the sequence diversity exists in different geographic locations.

We conclude that *vacA s1a/m2* strains are predominant in strains isolated from Chaharmahal and Bakhtiari Province, Iran. We also found *s2/m1* genotype, which was reported to be rare but 3.7% in this region. Surprisingly, *s2m2* genotype, which was non-toxigenic in most countries, was associated with many gastroduodenal diseases such as duodenal ulcer in this study. Further and extended molecular epidemiology researches in other regions of Iran are recommended.

Acknowledgments

The authors would like to thank Miss M. Ghodsi for statistical advises, Mrs. R. Ardalan for editing the manuscript and Mr. Gh. Ramezani at the Endoscopy Unit of Hajar Hospital, Shahrekord, for his sincere technical and clinical support. We are also grateful to Islamic Azad University, Shahrekord and Jahrom Branches for their executive support of this project.

Conflict of interest

None declared

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