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Prevalence of *Helicobacter pylori vacA*, *cagA*, *cagE*, *iceA*, *babA2*, and *oipA* genotypes in patients with upper gastrointestinal diseases

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ABSTRACT

Background and Objectives: *Helicobacter pylori* has been strongly associated with peptic ulcer diseases, chronic gastritis, ulcers, and reported as a risk factor for gastric cancer, too. The vaculating cytotoxin (*vacA*), the cytotoxin associated genes (*cagA*), the induced by contact with epithelium factor antigen (*iceA* gene), blood adhesion binding antigen (*babA2*), and outer membrane protein *oipA* have been described as different virulence factors of *H. pylori*. The aim of this study was to investigate the prevalence of the *vacA, cagA, cagE, iceA, babA2* and *oipA* genotypes of *H. pylori* isolates from patients with upper gasterointestinal problem or dyspepsia.

Material and Methods: *H. pylori* isolated from endoscopic biopsies obtained from 222 studied patients. PCR was done only on cultured positive samples. The *vacA* alleles, cagA, cagE, iceA, babA2 and *oipA* genotypes were determined by PCR.

Results: The isolation rate of *H. pylori* strains from culture of gastric biopsies was 16.7%. The *vacA* alleles *s1*, *s2*, *m1* and *m2* were detected in 20 (54.1%), 14 (37.8%), 9 (24.3%) and 23 (62.2%) isolates, respectively. *VacA s1c* genotype was detected in 70.3% of isolates. *s1m2* was the most frequent *vacA* allelic combination in the examined *H. pylori* strains. The *cagA* gene was detected in 62.2%, *cagE* in 40.5%, *iceA1* in 48.6%, *iceA2* in 16.2%, *oipA* in 81.1% (95% CI: 0.0902-0.1798) and *babA2* in 94.6% (95% CI: 0.113- 0.207). A significant correlation was observed between *vacAs1* and *cagA* genotypes ($P < 0.008$), *vacAs1*/*cagE* ($P = 0.001$), *vacAs2/cagA* ($P < 0.047$), and *vacAs2/cagE* ($P = 0.016$) with Non-ulcer dyspepsia; but there were not observed any correlation between other virulence markers.

Conclusion: No significant correlation was found between the existence of *vacA, cagA, cagE, iceA, babA2,* and *oipA* genes with peptic ulcer diseases and non-ulcer dyspepsia groups of studied patients.

Keywords: *Helicobacter pylori*, Prevalence, genotypes, gastrointestinal diseases

INTRODUCTION

Helicobacter pylori infection is one of the most common infectious diseases all over the world. It is responsible for a remarkable number of illness and abdominal pain (1). More than half of the world's population is infected with this organism. *H. pylori* plays role in occurrence of gastric and duodenal cancers and intestinal lymphoma. Numerous genes

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such as *vacA, cagA, cagE, iceA, babA* and *oipA*, have been recognized as an important cause of pathogenesis of *H. pylori* infection (2-5). The cytotoxin-associated gene product (*cagA*), the vacuolating toxin (*vacA*), and the adhesion protein *babA2* are major virulence factors of *H. pylori* that have been described (4). The severity of diseases caused by strains which express *babA* is greater than diseases by strains that do not express the gene. The presence of the *cagE* gene has also been associated with more severe clinical outcomes (5). The induced by contact with epithelium (*iceA*) gene has two main allelic variants *iceA1* and *iceA2*. The expression of *iceA1* is up-regulated on contact between *H. pylori* and human epithelial cells, and may be related with peptic ulcer disease. The expression of the outer inflammatory protein A (*oipA*) associated with IL-8 induction and is related with severe clinical outcomes (5). Even though *H. pylori* infection is common in Iran, there is only a few information about the genotyping of *H. pylori* strains (6, 7). The genotype determination of *H. pylori* isolates from infected individuals with higher risk for severe diseases may lead to further knowledge about the relationship between supposed virulence genes and clinical signs. The aim of this study was to investigate the *vacA*, *cagA*, *cagE*, *iceA*, *babA2*, and *oipA* genotypes of *H. pylori* and their correlation with clinical diseases in patients referred to endoscopy ward of the Beheshti hospital in Kashan, Iran.

MATERIALS AND METHODS

Study populations. Two hundred and twenty two patients with signs of abdominal pain or burning, nausea, vomiting, frequent burping, bloating and weight loss with an average age of 44.69 ± 18 years (range from 16 to 88) had undergone endoscopic investigation at Beheshti hospital in Kashan, Iran, from July 2010 through Jun 2012. *H. pylori* strains were isolated from the gastric mucosa biopsies specimens of *H. pylori* infected patients. Patients who received *H. pylori* eradication therapy protocol or treatment with antibiotics, bismuth-containing compounds, H2-receptor blockers, or proton pump inhibitors within 4 weeks prior to the study were excluded from the study. Informed consent was obtained from all participants, and the study was approved by the ethics committees of Kashan University of Medical Sciences.

***H. pylori* culture.** Three gastric mucosal biopsy specimens were obtained from each patient. Specimens were used for culture, the rapid urease test, and pathological examination. One antral and one corpus specimen were directly inoculated onto the agar gel to perform the rapid urease test (RUT). The results were recorded within 24 hours. A positive RUT was indicated when the color changed from yellow to pink. The culture positive and/or positive RUTs specimens were used for chromosomal DNA extraction if the culture was negative.

Each specimen was immediately placed into Stuart's transport medium and sent to the laboratory within 2hrs at 4°C. The biopsy specimens were smeared on the surface of Columbia agar plates supplemented with 10% horse serum and a set of antibiotics including 5 mg/l trimethoprim, 10 mg/l vancomycin, 5mg/l cefsulodin, and 5 mg/l amphotericin B. Then plates were incubated at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2), and examined after 7 days of incubation. The isolates were identified by Gram staining of the colonies, typical cell morphology, and testing for the presence of urease, oxidase, and catalase.

Chromosomal DNA extraction The genotype profiles of *H. pylori* isolates were determined by PCR. Chromosomal DNA was extracted from confluent plate cultures expanded from a single colony using a commercially available kit (QIAGEN Inc., Valencia, CA, USA). Primer sequences, sizes, conditions of PCR amplifications of the *glmM* gene for detection and confirmation of *H. pylori*, the virulence genes which were designed based on published papers with a modification of PCR mixtures, and PCR conditions are summarized in Table 1. Each PCR of *glmM, vacA, cagA, cagE, iceA, babA2*, and *oipA* was performed in a total volume of 50µl containing 100ng genomic DNA from *H. pylori* culture, 200 µM each of dNTP, 1×PCR buffer (20 mM Tris-HCl, pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$ (2 mM $MgCl_2$ for *CagA*), 0.5µM of each primer (0.2 µM for babA2 and 0.3 µM for *CagA*), and 1.5 units of Taq polymerase. Negative controls were added to each PCR run including all reagents except template DNA which was substituted with ultrapure water. Aliquots of amplified samples (10 µl) were electrophoresed on 1.5-2% agarose gel in TAE buffer. The gel was stained with ethidium bromide 0.5 µg/ml. The amplified bands were visualized under ultraviolet light and photographed.

Table 1. Primer sets used for genotyping *H. pylori* by PCR.

Genes	Primer sequence (5´ à3´)	PCR product (bp)	PCR conditions	References
glmM	AAGCTTTTAGGGGTGTTAGGGGTTT AAGCTTACTTTCTAACACTAACGC	294	93°C, 1 min; 55°C, 1 min; 72°C, 1 min (35 cycles)	10
vacA s1/s2	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	259/286	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)	10
s1a	GTCAGCATCACACCGCAAC CTGCTTGAATGCGCCAAAC	190	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)	10
s1b	AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)	10
s1c	CTCTCGCTTTAGTGGGGYT CTGCTTGAATGCGCCAAAC	213	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)	10
m1/m2	CAATCTGTCCAATCAAGCGAG GCGTCAAAATAATTCCAAGG	567/642	94°C, 1 min; 52°C, 1 min; 72°C, 1 min (35 cycles)	10
cagA	ATAATGCTAAATTAGACAACTTGAGCGA TTAGAATAATCAACAAACATCACGCCAT	298	94°C, 1 min; 60°C, 1 min; 72°C, 1 min (45 cycles)	10
cagE	TTGAAAACTTCAAGGATAGGATAGAGC GCCTAGCGTAATATCACCATTACCC	508	94°C, 1 min; 53°C, 45 s; 72°C, 45 s (35 cycles)	10
iceA1	GTGTTTTTAACCAAAGTATC CTATAGCCATTATCTTTGCA	247	95°C 1 min; 57°C, 1 s; 72°C, 1 min (35 cycles)	10
iceA2	GTTGGGTATATCACAATTTAT TTTCCCTATTTTCTAGTAGGT	229	95°C 1 min; 57°C, 1 s; 72°C, 1 min (35 cycles)	10
babA2	CCAAACGAAACAAAAAGCGT GCTTGTGTAAAAGCCGTCGT	271	94°C, 1 min; 45°C, 1 min; 72°C, 1 min (30 cycles)	10
oipA	GTTTTTGATGCATGGGATTT GTGCATCTCTTATGGCTTT	401	94°C, 1 min; 56°C, 1 min; 72°C, 1 min (35 cycles)	5

Statistical analysis. The Chi square and Fischer's exact tests were applied to estimate the statistical differences between disease and various genotypes. The P-value < 0.05 considered as significant statistical differences.

RESULTS

The studied patients were 99 (44.6%) males and 123 (55.4%) females with age range of 16 to 88 years old (mean age 44.6 ± 18.01 years). Clinical data revealed that from 222 patients with gastric complaints investigated by gastric endoscopy, 129 (58.1%) had gastritis, 47(21.2%) diagnosed as non-ulcer dyspepsia (NUD), 31 (13.9%) and 12 (5.4%) had gastric and duodenal ulcers, respectively. Three (1.4%) of patients had gastric carcinoma. The isolation rate of *H. pylori* strains from gastric biopsies was 37 out of 222 (16.7%), (95% CI: 0.118-0.216) in 12 males (32.4%), and 25 females patients (67.6%). The most common clinical diagnoses were non-ulcer dyspepsia in 29 patients (78.4%), followed by peptic ulcer diseases in 8 patients (21.6%). *H. pylori* DNA were extracted from 37 strains. DNA reliability and specificity was confirmed by *glm*M amplification and all of our isolates were positive for the *glmM*. The predominant genotype in strains by PCR was the *babA2* (94.6%) followed by the *oipA* (81.1%), *cagA* (62.2%), the *iceA1* (48.6%), and *cagE* in 40.5%, whereas the *iceA2* was detected only in 16.2% and of strains.

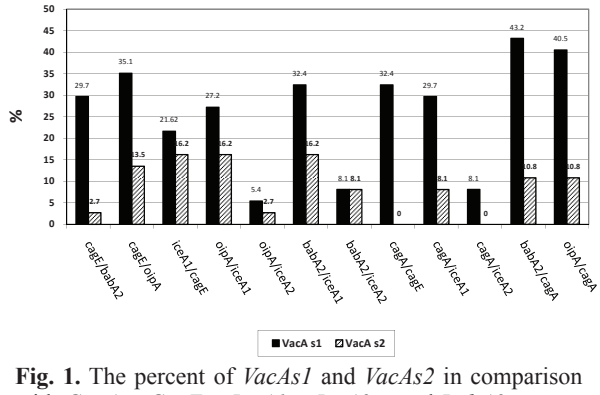

Fig. 1. The percent of *VacAs1* and *VacAs2* in comparison with *CagA+, CagE+, IceA1+, IceA2+*, and *BabA2+* genes of *H. pylori*; the bars represent the percent of persons infected with *H. pylori* having *vacA* genotype.

The *vacA* alleles *s1*, *s2*, *m1* and *m2* were detected in 20 (54.1%), 13 (35.1%), 9 (24.3%) and 23 (62.2%) isolates, respectively. The allele *s1m2* was the most frequent *vacA* allelic combination in *H. pylori* strains examined, followed by *s2 m2*, *s1m1* and *s2m1*. The

Table 2. The percent of *vacA* with *cagA*, *cagE*, *iceA1*, *iceA2*, *babA2*, and *oipA* genotypes in 37 strains of *H. pylori*.

vacA	No. (%)	cagA+ (%)	CagE+ (%)	iceA1+ (%)	iceA2+ (%)	BabA+ (%)	OipA+ (%)
s-region							
S1	20 (54.1)	18 (90)	16 (80)	12 (60)	3 (15)	18 (90)	17 (85)
S1a	20 (54.1)	18 (90)	13 (65)	13 (65)	3 (15)	18 (90)	16 (80)
S1c	26 (70.3)	15 (57.7)	10 (38.5)	15 (57.7)	6 (23.1)	25 (96.2)	21 (80.8)
S2	13 (35.1)	4 (30.8)	1 (7.7)	6 (46.2)	3 (23.1)	13 (100)	10 (76.9)
S1a+s1c	13 (35.1)	12 (92.3)	9 (69.2)	10 (76.9)	3 (23.1)	12 (92.3)	10 (76.9)
S1c + S2	11 (29.7)	3 (27.3)	0 (0)	6 (54.5)	3 (27.3)	11 (100)	9 (81.8)
m-*region*							
m1	9 (24.3)	7 (77.8)	5 (55.6)	6 (66.7)	3 (33.3)	9 (100)	8 (88.9)
m2	23 (62.2)	16 (69.6)	9 (39.1)	12 (52.2)	3 (13)	21 (91.3)	19 (82.6)
m1m2	1 (2.7)	1 (100)	1 (100)	1 (100)	0 (0)	1 (100)	1 (100)
s\m region							
s1m1	6 (16.2)	6 (100)	5 (83.3)	6 (100)	2 (33.3)	6 (100)	6 (100)
s1m2	15 (40.5)	13 (86.7)	9 (60)	7 (46.7)	1 (6.7)	13 (86.7)	12 (80)
s2m1	2 (5.4)	1 (50)	0 (0)	0 (0)	1 (50)	2 (100)	1 (50)
s2m2	8 (21.6)	3 (37.5)	0 (0)	5 (62.5)	2 (25)	8 (100)	7 (87.5)

vacA s1a subtype was identified in *H.pylori* strains from 54.1% of patients (20 out of 37). The *vacA s1b* subtype was not recognized in this study, while the *VacA s1c* subtype was identified in 70.3% patients (26

Table 3. The clinical presentation of *H. pylori* infection according to *H. Pylori* strains genotype in studied patients.

Clinical presentation / *H. Pylori* genotype	Peptic ulcer (n = 8) NO.	non-ulcer dyspepsia (n = 29) NO.	Total NO. (%)
Vac A			
m1	1	8	9 (24.3)
m2	6	17	23 (62.2)
m1m2	0	1	1 (2.7)
s1	4	16	20 (54.1)
s1a	5	15	20 (54.1)
s1c	6	20	26 (70.3)
s2	3	10	13 (35.1)
s1m1	0	6	6 (16.2)
s1m2	4	11	15 (40.5)
s2m1	1	1	2 (5.4)
s2m2	2	6	8 (21.6)
s1a +s1c	3	10	13 (35.1)
s1c + s2	3	8	11 (29.7)
+ cagA	4	19	23 (62.2)
+ cagE	3	12	15 (40.5)
+ iceA1	2	16	18 (48.6)
+ iceA2	2	4	6 (16.2)
iceA1+ iceA2	0	3	3 (8.1)
+ babA2	7	28	35 (94.6)
oipA	5	25	30 (81.1)

Table 4. Distribution of *H. pylori genotype* and risk of upper gastrointestinal diseases.

Genotypes	Peptic ulcer NO. (%)	Non ulcer dyspepsia NO. (%)	P value	Odds Ratio	Confidence Interval 95% Lower-Upper
m1	1 (11.1)	8 (88.9)	0.357	0.375	0.40-3.551
m2	6 (26.1)	17 (73.9)	0.340	2.118	0.363-12.342
s1	4 (20)	16 (80)	0.553	0.813	0.169-3.895
s1a	5 (25)	15 (75)	0.447	1.556	0.312-7.551
s1c	6 (23.1)	20 (76.9)	0.556	1.350	0.227-8.031
s2	3 (21.4)	11 (78.6)	0.635	1.036	0.207-5.198
cagA	4 (17.4)	19 (82.6)	0.343	0.526	0.108-2.564
cagE	3 (20)	12 (80)	0.588	0.850	0.170-4.256
babA2	7 (20)	28 (80)	0.390	0.250	0.014-4.511
oipA	5 (16.7)	25 (83.3)	0.156	0.267	0.045-1.579
iceA1	2 (11.1)	16 (88.9)	0.133	0.271	0.047-1.576
iceA2	2 (33.3)	4 (66.7)	0.387	2.083	0.306-14.168

out of 37), and the *VacA s2* type was identified in 13 patients (35.1%). Mixed *H. pylori VacA s1a* and *vacA s1c* subtypes were present in 35.1%, and combination of *vacA s2* and *vacA s1c* subtypes in 29.7% of patients (Table 2). There was no statistical association between *vacAs1m1* and *vacAs1m2* with clinical presentation. The *iceA1* and *iceA2* subtypes were detected in 48.6% and 16.2% of *H. pylori* infected patients, respectively. The *iceA1* was found in 2 out of 8 (25%) of the peptic ulcer patients, while *IceA2* was found in 55.2% (16 out of 29) of the non-ulcer dyspeptic patients. The *babA2* gene was detected in 94.6% (35 out of 37) of the *H. pylori* infected patients. However, there was no statistically significant difference in each individual genes among the patient groups ($p>0.05$). The association of *vacA* with *cagA*, *cagE*, *iceA1*, *iceA2*, *babA2*, and *oipA* genotypes in 37 strains of *H. pylori* is presented in Table 2. The strains typed as *vacAs1/cagA/baba2* detected in 43.2% and *vacAs1/cagA/oipA* in 40.5% of isolates (Fig. 1). A significant correlation was observed between *vacAs1* and *cagA* genotypes ($P < 0.008$), *vacAs1*/*cagE* ($P = 0.001$), *vacAs2/cagA* ($P < 0.047$), and *vacAs2/cagE* ($P = 0.016$) with non-ulcer dyspepsia; but there were not observed any correlation between other virulence markers. The majority (96.6%) of patients with non-ulcer dyspepsia possessing the *babA2* genotype and 86.2% defined as *oipA* genotype (Table 3). Distribution of *H. pylori* genotypes and risk for upper gastrointestinal diseases is showed in Table 4.

DISCUSSION

It is known that more than half of the world's human population is colonized by *H. pylori* (8). The predominant genotypes in this study were the *babA2* followed by the *oipA*, *cagA*, *iceA1*, *cagE* and *iceA2*. Previous studies reported that the *vacA* genotype and occurrence of the *cagA* gene varied in *H. pylori* isolates collected from different parts of the world. These genotype variations affect the clinical presentation in *H. pylori* infected patients. The presence of the *cagA* gene varies from a minimum of 50% in some Middle East (9) to a maximum of 99% in many East Asian countries (10, 11). The percentage of *cagA*-positive *H. pylori* strains found in our study is less than reported data from European and North American studies (74% to 88%) (12-14). Many studies have proposed that *cagA* is a useful marker for the most virulent strains that are associated with peptic ulcer diseases, atrophic gastritis and adenocarcinoma (15). The *vacA* is an important virulence factor in nearly half of *H. pylori* isolates that encoding the vacuolating cytotoxin in various mammalian cell lines in-vitro. The *H. pylori* isolates classified according to presence of different families of *vacA* signal sequences *(s1a, s1b, s2*), and middle region alleles *(m1, m2)* (16). In this study *vacA s1* was seen more than *vacAs2*. The study from Middle East showed that *vacA s1* and *s2* genotypes were similarly expected to be present in patients. African Arabs mainly were infected with *s2* and South-Asians with the *s1* genotypes (9). In the present study the *vacA s1m2* genotype was found in 40.5% and *vacA s2m2* in 21.6% of *H. Pylori* infected patients. According to previous studies, the most frequent genotype *vacA s1m2,* isolated in this study had lower vacuole formation activity than the *vacAs1m1*, which might accomplished by less severe pathological

effects as well as less clinical consequences (40.5% vs. 16.2%), while those with *vacAs2* (35.1%) fails to induce cell vacuolation in-vitro. Strains carrying the *s1ml* mosaic combination of the gene *vacA* show higher levels of cytotoxic activity than *s1m2* strains, whereas *s2m2* strains do not secret the vacuolating cytotoxin. The *m1vacA* and *m2vacA*, which are mostly formed by isolates containing the *s1/m1vacA* and *s1/m2vacA* genes, respectively; have different cell type specificities in cytotoxicity study (16). The *vacAs1c* genotype was dominant in this study (70.3%), but the *vacA s1b* subtype was not recognized. The presence of multiple organisms within a host may occur as a result of recombination procedures leading to genetic shift, however ongoing mutation inside a strain may leads to the formation of quasi species by genetic drift. Several genotypic markers such as *cagA, vacA, s1a* and *iceA1* are related with an increased risk of disease (17). The *iceA* gene may be related with peptic ulcer disease (18) while some studies have recommended a contrary association (19). The *iceA1* genotype detected in 48.6% of our patients. This finding agrees with previous reports that the *IceA1* allele was found more frequent than the *IceA2* allele in Chinese, Japanese, Korean, Dutch and Thai Patients (10, 20-22). The *iceA2* has been found to be main allele among American and Brazilian patients (19, 22). The prevalence rate for *BabA2* in this study was 94.6%, which is higher than reports from Colombia 57% and Costa Rica 73.7%, but it is similar to results from Chilie 97.4% and Japan 96.8% (23-25). In a study from Isfahan, Iran, the incidence rate of *babA2* was 71.6%. They reported there is no relationship between genotype and clinical outcomes (gastritis and PUD) (25). Most of the *H. pylori* strains in Asia are *babA2* positive, surprisingly unrelated to clinical outcome (26). This study revealed a high prevalence of *oipA* genes (81.1%), which is in agreement with the prevalence of *oipA* genes strains in Bulgarian patients (27), but is far less than the data reported from Tunisia (90.8%) (5). In present survey the *oipA* gene was found in 62.5% of peptic ulcer patients and 86.2% of non-ulcer dyspepsia. Significant correlations were observed between *vacAs1*/*vacAs2* with *cagA* and *cagE* genotypes. On the other hand, we did not observe any correlation between *vacAs1* and *iceA1*, *iceA2*, *oipA* and *babA2* genotypes. No significant relationship was observed between *vacA* genotypes and the manifestations of peptic ulcer diseases, which is in agreement with previous reports (28). Ribeiro *et al.* showed that *vacAs1* was the only prognostic factor for peptic ulcer disease (29). Molaei *et al.* reported that the frequencies of *vacA* gene subtypes *s1*, *s2*, *m1* and *m2* in 78 isolated strains were 70.5%, 29.5%, 37.2% and 62.8%, respectively. They showed that *vacAs1* was significantly associated with more severe gastritis and 83.3% of the *vacA*-positive strains had *s1* allele (30). Dabiri *et al.* reported that there was no significant association between *cagA* and *cagE* status or *vacA* genotypes and clinical outcomes. The *oipA*-positive strains were more common in non-ulcer dyspepsia than in peptic ulcer patients (6). The present study showed that patients with peptic ulcer disease and non-ulcer dyspeptic patients nearly were infected equally by multiple strains of *H. pylori*. The strains typed as *vacA s1*/ *cagA*+/ *iceA*+/ *oipA*+, and *babA2* + were more prevalent than those typed as *vacAs2*/ *cagA*+/ *iceA*+/ *oipA*+, and *babA2*+. With close concern to distribution of virulence genes to sex, educational status, and smoking habit there were no statistically significant difference among *vacA, cagA, cagE, iceA, babA2,* and *oipA* genes. No significant correlation were found between the existence of *vacA, cagA, cagE, iceA, babA2,* and *oipA* genes with peptic ulcer diseases groups in studied patients.

In conclusion, this is the first study that reveals a high prevalence of *baba2, oipA, vacA, cagA, iceA1* and *cagE* genes *in H. pylori* isolates in Kashan. The *s1m2* genotype was the most prevalent among all patients, but there was no correlation to peptic ulcer disease. Statistically significant correlations were observed between *vacAs1/cagA, vacAs1/cagE, vacAs2/cagA* and *vacAs2/cagE* virulence markers with Non-ulcer dyspepsia.

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