Restriction fragment length polymorphism of virulence genes *cagA, vacA* and *ureAB* of *Helicobacter pylori* strains isolated from Iranian patients with gastric ulcer and nonulcer disease

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ABSTRACT

Objective: To investigate the distribution of different genotypes of major virulence factors *cagA, vacA* and *ureAB* among *Helicobacter pylori (H. pylori)* strains isolated from patients with ulcerative and nonulcerative diseases.

Methods: This study was performed in Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, during November 2004 to October 2005. Sixty-five *H. pylori* strains, 30 from patients with gastric ulcer (ulcerative disease) and 35 from patients with gastritis (nonulcerative disease) were analyzed by polymerase chain reaction (PCR) to investigate the presence of *cagA, vacA* and *ureAB* genes. The amplified fragments were then digested with the restriction enzymes *HaeIII* (for *ureAB*) *HinfI* (for *cagA*) and *HphI* (for *vacA*).

Results: We found a significantly higher prevalence of *vacA*-positive strains in ulcerative disease (UD) than that in nonulcerative disease (NUD) patients ($p<0.05$). Restriction fragment length polymorphism (RFLP) analysis revealed 2 different patterns for *cagA* gene. The prevalence of pattern β with 3 bands was significantly higher in both groups of patients. *HaeIII* digestion resulted in a strictly homogeneous pattern for 83.33% of the *vacA*+ strains isolated from the patients with UD. This pattern was significantly associated with UD status ($p<0.05$). The *ureAB* polymorphism analysis revealed 10 distinguishable DNA banding patterns among them the pattern named *ureAB* 5a was the most prevalent (47.61%) in all isolates. No association between a specific DNA pattern and clinical disease was observed for *cagA* and *ureAB* ($p>0.05$).

Conclusion: It seems that in our patients, the presence of *cagA* gene may not necessarily be a risk factor for ulcer disease, while a homologous genotype of *vacA* appears to be associated with an increase risk of UD development. Lastly, despite the existence of a high degree of genomic variability within *ureAB*, conserved DNA banding profiles are distributed in our areas.

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Helicobacter pylori (H. pylori) associated gastritis is today recognized as the major cause of duodenal and gastric ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma.[1] The reasons for such a clinically diverse outcome of infection may include host and environmental factors as well as differences in the prevalence or expression of bacterial virulence factors.[2,3] Individual *H. pylori* isolates demonstrate a high level of genomic diversity as defined by different techniques.[4-7] Genomic differences may affect virulence factors, altering their function and antigenicity. Antigenic variation of certain gene products may represent an immune escape mechanism for *H. pylori* strains in the host organism.

A large number of studies have attempted to identify virulence markers genotypes in *H. pylori* allowing the disease outcome of an infection to be predicted. These studies were mainly based on analysis of *vacA*,[3,8,9] *cagA*,[10-12] and urease gene.[13,14] According to the literature, virulent cytotoxin *vacA*, *ureAB* and *cagA* producing strains are more common among patients with a variety of clinical symptoms of gastritis, gastric ulcer, duodenal ulcer, and reflux esophagitis.[15,16] However, discrepancies on the association of different genotypes with increased virulence and ulcer or nonulcer disease or gastric carcinoma development, have been described in reports from diverse geographical regions worldwide.[1]

Helicobacter pylori infection is common in Iran with approximately 82-92% of individuals infected.[17,18] Therefore, the present study is aimed at investigating

vacA, *cagA* and *ureAB* status present in *H. pylori* isolates recovered from Iranian patients, as well as the relevance of genotyping these virulence factors to define our strain genetic diversity and correlation between genotypes and gastric ulcer and nonulcer diseases.

Methods. A total of 114 patients undergoing endoscopy, at the Endoscopy Ward of Nemazee Hospital of Shiraz University of Medical Sciences in Shiraz, Southern Iran, were included in this study (mean age 41.3 ± 14 years, range 16-80 years, 60 males and 54 females). The diagnosis of *H. pylori* infection and the confirmation of gastric disease by histology were established by a central study pathologist. Another antral biopsy was taken from each patient and transferred to the lab in appropriate transfer media (brain heart infusion broth supplemented with 20% glucose). General exclusion criteria for patients' recruitment to the study were previous attempts to eradicate *H. pylori*, use of antibiotics, proton pump inhibitors or bismuth compounds within the last 2 weeks prior to endoscopy, and previous gastric surgery. Formal permissions from all patients under the study were provided before sampling.

Isolation of H. pylori strains. Biopsy samples from patients were gently homogenized and cultured on rapid urease test media and Brucella agar base (Merek, Germany) supplemented with 10% lysed horse blood and antibiotics of amphotericin B (2 µg/lit), trimethoprim (5 µg/lit) and nalidixic acid (10 µg/lit). The cultures were kept in a microaerophilic atmosphere (6% O_2, 7.1% CO_2, 7.1% H_2, 79.8% N_2) provided by Anoxomate (Mark II, Mart Microbiology BV, Netherlands) at 37°C for 5-10 days. The samples were also evaluated for presence of *H. pylori* by positive oxides, catalase and rapid urease tests.

DNA extraction. *Helicobacter pylori* isolates were pelleted, resuspended in 383 µl of Tris-EDTA buffer [10 mM Tris-HCl and 1mM EDTA, PH=8.0), 15 µl of 10% Sodium Dodecyl Sulfate] and 2 µl of 20 µg/ml solution of proteinase K, and incubated at 56°C for 2 hours in a hot block. The DNA was extracted with an equal volume of phenol-chloroform-isopropanol (25:25:1), precipitated with 2 volume of 97% ice cold ethanol followed by washing with 70% ethanol, and redissolved in 50 µl of TE buffer, as described in detail elsewhere[19] and used for polymerase chain reaction (PCR) assay.

The PCR examination. The primers sequences were previously reported[9] and obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). Descriptions and sequences of the PCR primers used in this study are given in **Table 1**. All PCR mixtures contained 1 x PCR buffer, 200 µM each deoxynucleoside triphosphate, 25 pmol of each primer, 1.5 mM $MgCl_2$, 5U of Taq polymerase and 10 µl of DNA extracted from *H. pylori* isolates. The PCR amplification included an initial denaturation step at 94°C for 2 min followed by 35 cycles with the following profiles: for *vacA*, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min; for *cagA*, 94°C for 45s, 50°C for 45s, and 72°C for 45s; for *ureAB,* 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Amplifications were carried out in a gradient thermal cycler (Eppendorf, Germany). Individual PCR products were electrophoresed on agarose gels, stained with ethidium bromide, and were photographed.

The PCR-RFLP analysis. The PCR amplified *vacA*, *cagA* or *ureAB* fragments were digested with *HaeIII* (for *ureAB*) *HinfI* (for *cagA*) and *HphI* (for *vacA*), for 4 h at 37°C in the appropriate buffer recommended by the supplier (MBI, Fermentas, Lithuania). The digests were analyzed by elecrtophoresis in a 2% agarose gel with 1x Tris-Acetate-ethylenediaminetetraacetic acid (EDTA) buffer followed by ethidium bromide staining.

Statistical analysis. Fishers exact test was used for statistical evaluation of data derived from the results of the procedures mentioned above. An amount of <0.05 was accepted for P value as statistically significant.

Results. ***Patient groups and prevalence of H. pylori infection.*** According to endoscopic and pathologic findings the patients were categorized to 2 groups: ulcerative (gastric ulcer) (37) and nonulcerative (gastritis) (77). Totally from antrum of ulcerative and nonulcerative patients 30 (81.08%) and 35 (45.45%) *H. pylori* strains were isolated respectively.

Prevalence of cagA, vacA and ureAB among H. pylori positive patients. In polymerase chain reaction analysis from the total of 65 *H. pylori* isolates, 31 strains (47.69%) were *cagA*+, 37 strains (56.92%) *vacA*+ and 42 (64.61%) strains *ureAB*+. The *cagA*, *vacA* and *ureAB* positivity were higher in patients with ulcerative disease (UD) (60%, 80% and 73.3% respectively) than that in patients with nonulcerative disease (NUD) (37.14%, 37.14 % and 57.14% respectively), but the difference between the groups was statistically significant (p<0.05) only for *vacA* gene (data not shown).

The PCR-RFLP analysis. Following digestion of the amplified *cagA* gene with *HinfI* enzyme, 2 different patterns were revealed, α and β (**Table 2**). The prevalence of pattern β was significantly higher in both groups of patients, compare with the pattern of α (p<0.05). However, the statistical analysis had shown no significant association between this pattern and the clinical outcomes (p>0.05).

The *HphI* digestion of the 1.162-bp *vacA* fragment resulted in a strictly homogeneous profile for 83.33% of the *vacA*+ strains (20 of 24) isolated from the

Table 1 - Polymerase chain reaction (PCR) primers for amplification of *CagA, VacA* and *UreAB* sequences

Gene and DNA region amplified	Primer	Primer sequence (5'-3')	Size (bp) of PCR product
vacA	Forward Reverse	GCTTCTCTTACCACCAATGC TGTCAGGGTTGTTCACCATG	1,162
cagA	Forward Reverse	AGTAAGGAGAAACAATGA AATAAGCCTTAGAGTCTTTTTGGAAATC	1,320
ureAB	Forward Reverse	AGGAGAATGAGATGA ACTTTATTGGCTGGT	2,420

patients with UD. However, 46.15% (6 from 13) of the *vacA*+ strains isolated from patients with NUD showed a strictly homogeneous profile. This pattern was significantly associated with UD status ($p<0.05$) (**Figure 1**).

All 42 *H. pylori ureAB*+ isolates under the study were classified into 10 distinguishable DNA banding patterns by *ureAB* polymorphism analysis (**Table 3**). This finding suggests a great genetic diversity of urease genes among *H. pylori* clinical isolates. The patterns have 3 to 6 bands with different sizes. Twenty out of the 42 isolates (47.6%) belonged to the pattern named *ureAB* 5a with 5 bands from them 65% (13 from 20) belonged to UD isolates suggesting that strains from patients with more severe forms of gastroduodenal disease were more homogeneous than strains carried by patients with milder disease. No association between a specific DNA pattern and clinical disease was observed ($p>0.05$) for *ureAB*.

Discussion. According to the different reports, overall *H. pylori* infection prevalence in Iran is 82-90%.[17,18] Although these data indicate the occurrence of *H. pylori* infection in our country, little is known about the genetic features of isolates that cause infectious disease. In the present study, we addressed the investigation of *cagA*, *vacA* and *ureAB* status and genotypes of *H. pylori* isolates recovered from an Iranian population resident in South of Iran with 2 forms of gastric diseases, ulcerative and nonulcerative. On analyzing 65 *H. pylori* isolates, it was found that 31 strains (47.69%) were *cagA*+, 37 strains (56.92%) *vacA*+ and 42 (64.61%) strains *ureAB*+. *cagA*, *vacA* and *ureAB* positivity was higher in patients with UD (60%, 80% and 73.3% respectively) than that in the patients with NUD (37.14%, 37.14 % and 57.14% respectively), but the difference between the groups was statistically significant ($p<0.05$) only for *vacA* gene (data not shown). It was reported from different centers that in patients with ulcer diseases, the positivity rates of *cagA* and *vacA*, and both *cagA*, *vacA* were 71-100%, 47.5-92%, 37-75%, respectively.[20,21] In all of these studies, the positivity of *cagA* and *vacA* was higher in the patients with UD, however, some was statistically significant[22,23] and some were not when it was compared to patients without ulcer.[24,25] Such differences in the prevalence of *cagA* and *vacA* positivity could not be explained precisely; however, they have been attributed to the genetic heterogeneity or to differences in the geographic location.[1,26,27] Adherence of *H. pylori* to the gastric epithelium and secretion of interleukins are believed to be an important step in the induction of active inflammation of the mucosal layer, which then can result in ulceration. Vacuolating cytotoxin *vacA* aid in colonization of the gastric mucosa and subsequently, seem to modulate the host's immune system.[28] Host cytokines gene polymorphisms may be as important as exogenous stimuli in influencing the number of cytokines produced and, consequently, the pattern and severity of inflammation.[29] These polymorphisms may differ from one geographical population to another, based on genetic diversity, which can explain the possibilities for certain genotypes in Iranian population that prone them to *H. pylori vacA*+ infection.

Using *HinfI* restriction enzyme, we found 2 genotypes for *cagA* gene. The genotype β was significantly more prevalent among all our isolates in compare with genotype α (92.31% vs. 7.69%, respectively) but with no significant association with a specific clinical outcome. This finding is in accordance with Saribasak et al[12] study stating that they could find only one genotype 2a for *H. pylori cagA* positive strains, which were typical

Table 2 - Restriction fragment length polymorphism - polymerase chain reaction patterns of *cagA* gene in a representative group of patients

Pattern	No. of patients (*cagA*+)				Total	
	UD		NUD			
α (no cut)	2	(11.1)	1	(7.7)	3	(9.7)
β (3 bands)	16	(88.9)	12	(92.3)	28	(90.3)
Total	**18**		**13**		**31**	

UD - ulcerative disease, NUD - non-ulcerative disease

genotypes in strains from Western countries. Therefore, it seems that the evaluation of genetic diversity in *H. pylori*-associated *cagA* gene can be attributable to the colonial relationshiop and epidemiology of *H. pylori* in defined population. On the other hand, although *cagA* positivity was higher in the patients with ulcer than that in the patients without ulcer, this was not statistically significant and did not seem to be an important risk factor for the development of ulcer in our patients. It can be explained by the absence of sequence divergence in *cagA* genes of our isolates, when divergence within portions of the *cagA* product may impact on the structure, antigenicity, function and consequently on the role of that in a specific clinical feature.

The RFLP-PCR analysis of the PCR products with several restriction enzymes confirmed the high degree of diversity of the genomic structure of the *vacA* gene among *H. pylori* strains isolated from gastric biopsy specimens.[30,31] The digestion of PCR products with *HaeIII* enzyme allowed us to identify a genetic correlation for 26 of 37 *H. pylori* strains examined, thus, resulting in a homogeneous group of strains with identical *vacA* gene restriction patterns. In addition, these strains were strongly associated with the presence of the *ureAB* gene rather than *cagA* gene and occurred more frequently in the patients with ulcerative disease. In fact, the genetically related strains were isolated from 83.33% of ulcerative patients but significantly ($p<0.05$) less frequently (46.15%) from nonulcerative patients. Therefore, our results support the finding that *cagA* gene status may not necessarily be a universal virulent marker as claimed by Yamaoka et al.[15] However, in disagreement with these authors, this study validates that *vacA* genotype may well predict clinical outcome as shown by van Doorn et at.[32]

As it has been shown in **Table 3** all 42 *H. pylori ureAB*+ isolates under the study were classified into 10 distinguishable DNA banding patterns by *ureAB* polymorphism analysis. These results showed considerable genetic divergence among *H. pylori* isolates that circulate in our community. Foxall et al[33] found 10 distinct patterns among 22 clinical isolates when *HaeIII* restriction enzyme digested the 2.4-kb PCR product amplified from *ureAB* gene. Akopyanz et al[4] demonstrated that 27 *HaeIII* RFLP patterns were obtained from the same PCR products for *ureAB* gene. In a similar study performed by Catalano et al[14] 90 *H. pylori* isolates were classified into 33 distinguishable DNA banding patterns by *ureAB* polymorphism analysis. In our study, although 10 different patterns were obtained for *ureAB*, almost 48% of the isolates belonged to only one pattern named *ureAB* 5a with 5 bands. This finding corresponds with the results of Catalano et al,[14] which showed one fingerprint named *ureAB* 4 was the most prevalent among 33 patterns. Then, although it seems generally that a large and very genetically diverse population of *H. pylori* circulates in

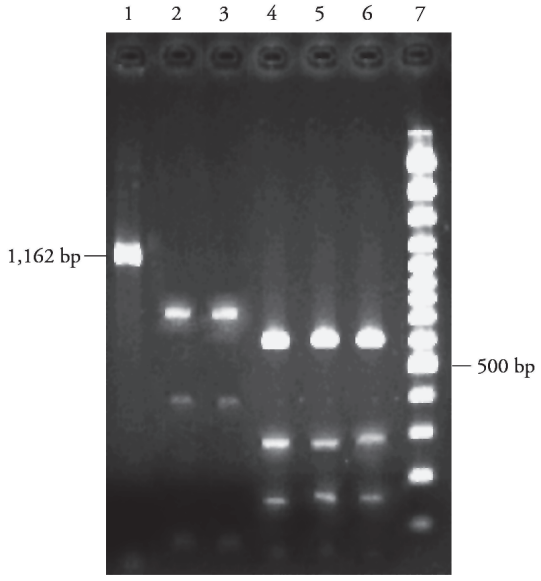

Figure 1 - Representative restriction fragment length polymorphism - polymerase chain reaction results for *HphI* digestion of the 1.162-bp *vacA* fragment in *H. pylori* strains isolated from patients with ulcerative diseases (lanes 2,3,4) and nonulcerative diseases (lanes 5,6). Lane 1: no digested *vacA* fragment, Lane 7: molecular size marker.

Table 3 - Restriction fragment length polymorphism - polymerase chain reaction patterns of *ureAB* gene in a representative group of patients.

Pattern	No. of patients (*ureAB*+)				Total	
	UD		NUD			
ureAB 3a	1	(4.5)	1	(5)	**2**	**(4.8)**
ureAB 3b	1	(4.5)	1	(5)	**2**	**(4.8)**
ureAB 4a	1	(4.5)	2	(10)	**3**	**(7.1)**
ureAB 4b	2	(9.1)	1	(5)	**3**	**(7.1)**
ureAB 4c	1	(4.5)	2	(10)	**3**	**(7.1)**
ureAB 5a	12	(54.5)	8	(40)	**20**	**(47.6)**
ureAB 5b	1	(4.5)	2	(10)	**3**	**(7.1)**
ureAB 5c	1	(4.5)	1	(5)	**2**	**(4.8)**
ureAB 6a	1	(4.5)	1	(5)	**2**	**(4.8)**
ureAB 6b	1	(4.5)	1	(5)	**2**	**(4.8)**
Total	22		20		**42**	

UD - ulcerative disease, NUD - non-ulcerative disease

the community, certain genomic markers, such as *ureAB* are widely distributed and partially conserved.

In conclusion, the results of this study by PCR-RFLP pattern analysis suggest that the presence of *cagA* gene may not necessarily be a risk factor for ulcer disease, while a homologous genotype of *vacA* appears to be associated with an increase risk of UD development in our area. Lastly, the existence of a high degree of genomic variability within *H. pylori ureAB* gene may be of practical significance when considering the effectiveness of urease-based vaccines in diverse and heterogeneous human population.

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