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Analysis of *vacA*/*cagA* genotypes/status in *Helicobacter* pylori isolates from Iranian children and their association with clinical outcome

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Background/aim: More than 50% of Iranian children are infected with *Helicobacter pylori*; however, no data exist about the association of *vacA*/*cagA* genotype/status with disease outcomes in them. We analyzed association of *vacA*/*cagA* genotypes/status of children's isolates with gastric inflammation status as the first step in *H. pylori* pathogenesis.

Materials and methods: Antral biopsies for culture and histopathology were taken from 328 children in 1997–2009. *vacA* (*s*, *m*) alleles and *cagA* statuses of the isolates were determined by PCR. Histopathology was performed according to the Sydney system; gastritis was scored as normal, mild, moderate, severe, and follicular.

Results: A total of 159 culture-positive cases, with no mixed infections, were enrolled in the study. Of them, 60% were *cagA*-positive; 21.4%, 37.1%, 16.3%, and 25.2% cases were *s1m1*, *s1m2*, *s2m1*, and *s2m2*, respectively. Histopathology showed normal (4.4%), mild-chronic (31.4%), moderate-chronic (38.4%), severe-chronic (10.7%), and follicular gastritis (15.1%) cases. Thirty-four (21.4%) of the children had ulcers. Correlation ($P < 0.05$) was observed between more severe (moderate, severe, follicular) status and both *vacAs1* allele and *cagA*-positive status. No significant relation was observed between genotype/status of *vacA*/*cagA* and ulcers ($P > 0.05$).

Conclusion: *vacA*s1 and *cagA* are associated with more severe gastric inflammation in Iranian children. Association of *vacA*s1 and *cagA* with more severe pathology in Iran may be similar to that of other parts of the world.

Key words: *Helicobacter pylori*, children, gastritis, *cagA*, *vacA*, ulcer, Iran

1. Introduction

Helicobacter pylori infection occurs all over the world, and more than 50% of adults are infected with this pathogen (1). In Iran, *H. pylori* infection is present in nearly 90% of adults and appears to occur early in childhood with >50% of infections occurring before 15 years of age (2,3).

H. pylori is defined by its high level of genetic diversity; the strains residing in an individual can change over time to adapt to the host stomach (4,5). However, genetic diversification of strains may be more limited in infected children due to the shorter time of infection.

Among the most important virulence factors of *H. pylori*, the Cag pathogenicity island (Cag-PAI) and VacA toxin have been proposed to play a crucial role in *H. pylori*-induced pathogenesis (6–9). For this reason, numerous studies have been performed on the relationship between *cagA* status, as well as *vacA* genotypes and severity of *H. pylori*-related diseases. In Western countries, investigators have observed an association between *cagA*/*vacA* status/genotypes and disease outcome (10–13). However, reports from some other regions indicated no relationship between *cagA*-positive status or *vacA* genotype and disease outcome (14–17).

Concerning the reports from Iran, analysis of 159 studies has shown no consistency among their results regarding association with disease outcome, and therefore further investigations have been suggested (18). Furthermore, limited data are available on Iranian children regarding epidemiology, characteristics, and relationship with disease of *cagA*/*vacA* status/genotypes.

Clinically, the first pathological manifestation of *H. pylori* infection is usually an active antral chronic gastritis that can be classified into mild, moderate, and severe categories (19). Although all conditions required for the progression of chronic gastritis to severe pathology including gastric cancer are still not clear, the degree or severity of gastric inflammation may be important in predisposing to the development of a more severe outcome.

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By evaluating the first step in gastric injury, we undertook this study aiming to investigate *H. pylori cagA*/*vacA* status/genotype distribution in Iranian children and their relationships with various degree of gastritis.

2. Materials and methods

2.1. Patients and clinical specimens

Patients were 328 nonconsecutive children admitted to the Children's Medical Center of Tehran in 1997–2009 who sought medical attention because of clinical symptoms. Admitted children had manifestations such as recurrent abdominal pain, vomiting, nausea, hematemesis, and melena. Informed consent was obtained before endoscopy. Their clinical signs and endoscopic alterations of the gastric mucosa and presence or absence of ulcers were recorded. Based on endoscopic features, including nodular gastritis, ulcer, and/or a positive rapid urease test (RUT) in the endoscopy room, 2 similar antral biopsies for culture and pathology were taken. RUT was performed using urea broth as previously described (20).

2.2. Histological examination

Histological examination was performed after H&E and Giemsa staining. *H. pylori* density, gastritis, and inflammation were graded according to a modified Sydney system. Gastritis was scored as absent (normal), mild, moderate, or severe; the cases of gastritis with follicular formation were classified as follicular gastritis that was either associated with activity or was without activity (19,21).

2.3. *H. pylori* isolation and identification

Biopsy materials were placed within a thioglycolate base medium (Merck, Germany), 10% sheep blood, and antibiotics (Fluka) including 8 mg of polymyxin B, 2 mg of amphotericin B, and 6 mg of vancomycin (per liter) and incubated at 37 °C under a microaerophilic atmosphere. After 2 to 3 days, 20 μL was used to streak the plates containing a *Brucella* agar base (Merck), 10% sheep blood, and antibiotics. Identification of isolates was performed by Gram staining, biochemical tests, and PCR using the primers for *H. pylori*-specific *16sRNA* and *UreC*. *H. pylori* isolates were stored at –70 °C in skim milk containing 15% glycerol and 10% fetal calf serum (Gibson).

2.4. DNA isolation and PCR

PCR for the *ureC* gene was performed using a *Helicobacter* PCR kit (Cinaclon BioScience, Iran, Cat. No. PR7843C) according to manufacturer's recommendations. The presence of 492-bp fragments in 1.5% agarose gel indicated a positive test.

Identification of the *16sRNA* gene, *vacA* alleles, and *cagA* gene was performed according to a protocol previously adopted (22). For this purpose, DNA was extracted using phenol-chloroform and boiling methods according to previously described protocols (22). PCR primers for *16srRNA*, *vacA*, and *cagA* (Faza Biotech Inc., Iran) were designed on the basis of published sequences of *H. pylori* (23,24), as demonstrated in Table 1.

H. pylori ATCC 26695 and 43504 strains were used as controls. PCR reaction was performed in a 25-mL mixture

Table 1. PCR primers used in this study.

Primer	Sequences	Size
vacA (*s1*)	for: 5' ATGGAAATACAACAAACACAC 3' rev: 5' CTGCTTGAATGCGCCAAAC 3'	259
vacA (*s2*)	for: 5' ATGGAAATACAACAAACACAC 3' rev: 5' CTGCTTGAATGCGCCAAAC 3'	286
vacA (*m1*)	for: 5' CAATCTGTCCAATCAAGCGAG 3' rev: 5' GCGTCTAAATAATTCCAAGG 3'	570
vacA (*m2*)	for: 5' CAATCTGTCCAATCAAGCGAG 3' rev: 5' GCGTCTAAATAATTCCAAGG 3'	642
cagA	for: 5' AATACACCAACGCCTCCA 3' rev: 5' TTGTTGCCGCTTTTGCTCTC 3'	400
16*srRNA*	for: 5' GCTAAGAGATCAGCCTATGTCC 3' rev: 5' TGGCAATCAGCGTCAGGTAATG 3'	700

containing 0.5 µg of extracted DNA, 0.2 mM (each) deoxynucleoside triphosphates, 0.2–0.4 µM (each) primer, 1.5–2 mM $MgCl_2$, and 5 U of Taq polymerase in PCR buffer (Cinna Gen Co., Iran). Following denaturation at 95 °C (5 min), the *16srRNA* fragment was amplified through 39 cycles as follows: 94 °C (1 min), 55 °C (1 min), and 72 °C (2 min); extension was continued at 72 °C for 7 min. After denaturation at 95 °C (4 min), the *vacA* (*s1*, *s2*, *m1*, *m2*) alleles were amplified through 35 cycles as follows: 95 °C (1 min), 52 °C (1 min), and 72 °C (1 min); extension was continued at 72 °C for 10 min. For *cagA*, following denaturation at 94 °C (4 min), it was amplified through 35 cycles as follows: 94 °C (1 min), 59 °C (1 min), and 72 °C (1 min); extension was continued at 72 °C for 10 min. Aliquots of each PCR product were electrophoresed in a 1.5% (w/v) agarose gel in Tris-borate-EDTA buffer (90 mM Tris-HCl, 90 mM boric acid, 0.002 M EDTA) and stained in ethidium bromide at 0.5 mg/mL.

2.5. Statistics

All data were entered into SPSS 15 and were analyzed by chi-square and Fisher's exact tests to determine the effect of *cagA* status and *vacA* allelic groups on pathological and histological features.

3. Results

By analyzing 328 biopsy samples, 172 (52.44%) were recognized as having *H. pylori*, from which 13 biopsies (7.56%) had more than 1 *H. pylori* strain regarding *vacA* genotypes and were omitted from statistical analysis. Ten (69%) of these mixed-infection cases corresponded to children older than 9 years old.

The average age of the 159 children taken for this study was 9.5 ± 2.6 years. Eighty-seven (54.7%) of them were boys and 72 (45.3%) were girls. Endoscopic observations showed nodular gastritis, erosive gastritis, erosive duodenitis, gastric ulcers, and duodenal ulcer pathologies.

Histological results showed 7 (4.4%) with normal histology, 50 (31.4%) with mild chronic gastritis, 61 (38.4%) with moderate chronic gastritis, 17 (10.7%) with severe chronic gastritis, and 24 with (15.1%) follicular gastritis with or without activity. Figure 1 demonstrates a typical case of follicular gastritis, corresponding to children with a high score of *H. pylori* in histopathology.

Of the 159 isolates, 95 (60%) were *cagA*-positive and 64 (40%) were *cagA*-negative (Table 2). According to PCR analysis of *vacA* alleles, 34 (21.4%) were *s1m1*, 59 (37.1%) were *s1m2*, 26 (16.3%) were *s2m1*, and 40 (25.2%) were *s2m2*. Prevalence of *vacAs1* was 58.5% (93 cases) and that of *vacAm1* was 37.7% (60 cases). Figure 2 represents PCR products of *16sRNA*, *cagA*, and *vacA* (*s1*, *s2*, *m1*, *m2*) genotypes in 1.5% agarose gel.

Comparison of histological features between *vacA* groups showed a significant association ($P < 0.05$) between more severe (moderate, severe, and follicular) pathological status and *s* alleles of *vacA* but did not show a relation with *m* alleles of *vacA* (Table 3). *H. pylori i*solates with *vacA*s1

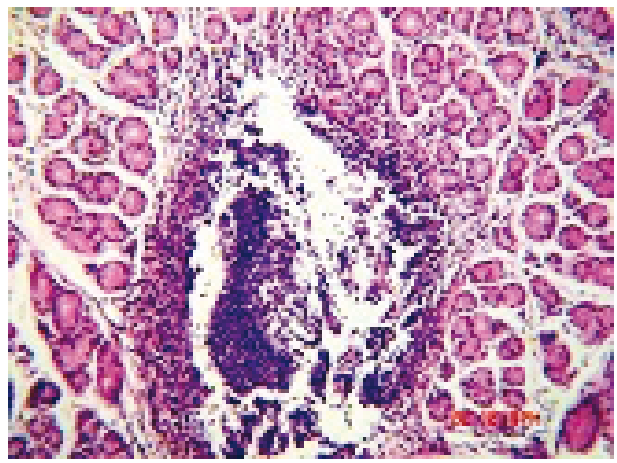

Figure 1. Antral mucosa with presence of a large lymphoid follicle in lamina propria (follicular gastritis), hematoxylin and eosin stain, 400×.

Table 2. Relationship between histopathological status and *cagA* status of *H. pylori* isolates (percentage: according to total).

Histopathological status	*cagA* status	
	cagA-positive	*cagA*-negative
Normal	3 (3.2%)	4 (6.25%)
Mild	23 (24.2%)	27 (42.2%)
Moderate	40 (42.1%)	21 (32.8%)
Severe	9 (9.5%)	8 (12.5%)
Follicular	20 (21.0%)	4 (6.25%)
Total	95 (60%)	64 (40%)

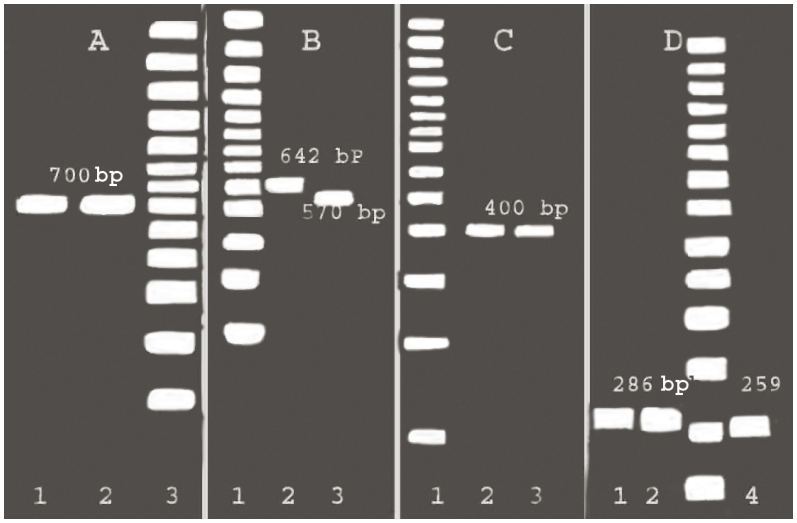

Figure 2. Representative figure of PCR products in 1.5% agarose gel. **A)** Lanes 1 and 2: 700-bp PCR products from 26695 strain, a clinical strain; lane 3: 100-bp DNA ladder. **B)** Lane 1: 100-bp DNA ladder; lanes 2 and 3: 642-bp and 570-bp products of *vacAm2* and *vacAm1*, respectively. **C)** Lane 1: 100-bp DNA ladder; lanes 2 and 3: 400-bp products of *cagA* gene for ATCC 43504 standard strain and a clinical *H. pylori* strain. **D)** Lanes 1 and 2: 286-bp products of *vacAs1* for ATCC 43504 standard and a clinical *H. pylori* strain, respectively; lane 4: 259-bp product of *vacAs2* for ATCC 43504 strain.

Table 3. Relationship between histopathological status and *vacA* genotypes of *Helicobacter pylori* isolates (percentage: according to total).

Histopathological status	*vacA* genotype			
	vacAs1m1	*vacAs1m2*	*vacAs2m1*	*vacAs2m2*
Normal	0 (0%)	2 (3.4%)	3 (11.5%)	2 (5%)
Mild	10 (29.4%)	14 (23.7%)	11 (42.3%)	15 (37.5%)
Moderate	16 (47.0%)	24 (40.7%)	8 (30.8%)	13 (32.5%)
Severe	4 (11.8%)	9 (15.3%)	0 (0%)	4 (10%)
Follicular	4 (11.8%)	10 (16.9%)	4 (15.4%)	6 (15%)
Total	34 (21.4%)	59 (37.1%)	26 (16.3%)	40 (25.2%)

alleles (with *m1* or *m2*) were related significantly to more severe histopathological status.

Sixty-nine out of 102 cases with moderate, severe, or follicular gastritis were *cagA*-positive and 33 were *cagA*-negative. There was a significant association ($P < 0.05$) between the severity of histopathological status and the presence of *cagA*. Association of both *cagA* status and *vacA* alleles with pathology is demonstrated in Table 4. There was a significant correlation ($P < 0.05$) between occurrence of both *cagA* and *vacA*s1 and more severe pathology.

In endoscopic evaluation, 34 children (21.4%) presented with ulcers, but 125 children (78.6%) had no ulcers. The isolates from 65% of the ulcer-positive patients were *cagA*-positive and 35% were *cagA*-negative (Table 5). Statistical analysis showed no significant relationship between genotype/status of *vacA*/*cagA* and the presence of ulcers ($P > 0.05$). No significant association was observed according to sex concerning ulcer development ($P > 0.05$).

4. Discussion

To avoid any controversy related to presence of mixed infections, only 159 isolates recognized as nonmixed were used in this investigation. To minimize the risk of in

Table 4. Relationship between histopathological status and *cagA*/*vacA* status/genotypes of their *H. pylori* isolates (percentage: according to total).

cagA/*vacA* status/genotype		Histopathological status					
		Normal	Mild	Moderate	Severe	Follicular	Total
cagA+	*s1m1*	0 (0.0)	4 (4.2)	11 (11.6)	4 (4.2)	3 (3.2)	22 (23.2)
	s1m2	2 (2.1)	7 (7.4)	15 (15.8)	3 (3.2)	10 (10.5)	37 (38.9)
	s2m1	1 (1.1)	5 (5.3)	6 (6.3)	0 (0.0)	2 (2.1)	14 (14.7)
	s2m2	0 (0.0)	7 (7.4)	8 (8.4)	2 (2.1)	2 (2.1)	22 (23.2)
cagA-	*s1m1*	0 (0.0)	6 (9.4)	5 (7.8)	0 (0.0)	1 (1.6)	12 (18.8)
	s1m2	0 (0.0)	7 (10.9)	9 (14.1)	6 (9.4)	0 (0.0)	22 (34.4)
	s2m1	2 (3.1)	6 (9.4)	2 (3.1)	0 (0.0)	2 (3.1)	12 (18.8)
	s2m2	2 (3.1)	8 (12.5)	5 (7.8)	2 (3.1)	1 (1.6)	18 (28.1)

Table 5. Relationship between presence of ulcers and *vacA*/*cagA* genotypes/status (percentage: according to total).

Endoscopic observation	*cagA*-positive				*cagA*-negative			
	s1m1	*s1m2*	*s2m1*	*s2m2*	*s1m1*	*s1m2*	*s2m1*	*s2m2*
Ulcer	6 (6.3%)	9 (9.4%)	3 (3.2%)	4 (4.2%)	2 (3.1%)	5 (7.8%)	2 (3.1%)	3 (4.7%)
No ulcers	16 (16.8%)	28 (29.5%)	11 (11.6%)	18 (18.9%)	10 (15.6%)	17 (26.6%)	10 (15.6%)	15 (23.4%)

vitro genetic change, the strains used in this work did not undergo more than 2 laboratory subcultures.

PCR screening demonstrated that *cagA*-positive status was dominant among the children's isolates. This higher prevalence was consistent with that of adults in Iran (25–28). We found that *cagA*-positive status was significantly ($P < 0.05$) associated with a greater inflammatory response in histopathology, since 67.6% of strains isolated from patients with moderate, severe, and follicular pathology were *cagA*-positive (Table 2). Regarding the previous studies performed on adults, controversial results have been obtained in Iran. While a great number of studies in Iran indicated no influence of *cagA* status or its 3' variable region on the progression of disease (26–34), correlation between positive *cagA* status and severe clinical outcomes was reported in some other studies (35–37). Consistent with the cases of Iranian adult populations (27,28,38–41), *vacA s1* was the most prevalent in the present investigation (Table 3). This dominance was also consistent with the cases of other countries in the northern part of the Middle East (42,43). We found that the mosaic genotype of *vacAs1m2* was the most prevalent, which was similar to the cases of adults in Iran (26,38,39). The *vacAs2m1* genotype that was the least prevalent in this work (Table 3) was also the least prevalent in Iranian adults (25–27,38,39). However, data on prevalence of other combinations of *s* and *m* alleles in Iranian adults have been controversial (25,38–41,44).

By evaluation of the first step of gastric injury in pathology, we observed a significant association between both *vacAs1* genotype and *cagA*-positive status and more serious (moderate, severe, and follicular gastritis) pathology (Table 4). This association was statistically significant ($P < 0.05$) and was consistent with the results of the only 2 reports concerning adult cases in Iran (35,37).

Presence of controversial results on the association of *H. pylori cagA*/*vacA* status/genotype with disease state in Iranian adults remains unexplained. However, several causes may be in relation with these controversies. In the majority of the studies performed in Iran, the sample size was not large enough to obtain a statistically significant result with respect to clinical symptoms and genotyping. In the present study, no significant association was observed between *cagA* status or *vacA* genotype and ulcers among 34 children (Table 5). Absence of such association may be due to the lower number of ulcer cases. The geographic origin and therefore the genotype of patients (residing in various regions of Iran) could also play a role in these controversies. Another problem may be generation of mixed populations of bacteria either in the stomach (during long-term infection) or in the laboratory after multiple

subcultures. In these conditions, the strains submitted to genotyping may not be the same as those involved in the development of disease. We observed that 77% of 13 cases of mixed infections corresponded to children older than 9 years, suggesting that in the younger ones, the rate of mixed infections would be lower. In addition, most studies in Iran have compared the *vacA*/*cagA* genotype/status of the isolates obtained from patients with gastric cancer (in general in low numbers) with those obtained from patients with peptic ulcer disease or nonulcer dyspepsia. This comparison may have been done without taking into consideration the multifactorial nature of gastric cancer, where bacterial factors interact with host susceptibility and environmental and dietary factors.

Although major reasons for the endoscopy referral of children residing in developing regions may be the higher rate of infection, globally the number of studies concerning association of *cagA*/*vacA* status/genotype with disease outcome for children is much lower than that for adults. While antral nodularity is a well-described endoscopic feature of *H. pylori*-infected children, the frequency of severe states of infection in children is lower than adults. A few reports from various regions of the world including Greek, Brazilian, Japanese, and Chinese children have shown the discrepancies concerning *cagA*- and *vacA*-dependent pathogenicity (45–48). Such divergence may be attributed to their low sample sizes and differences in their genetic and geographic locations.

The present work has provided useful data on Iranian children by evaluating the first step of gastric injury among a statistically sufficient number of subjects. Furthermore, the results have demonstrated that association of both *vacA*s1 and *cagA* with more severe pathology in this region may be similar to the majority of results obtained worldwide.

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