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Prevalence of Virulence Genes and Antigen Pattern in *Helicobacter pylori*-Infected Patients and the Level of Some Inflammatory Cytokines Compared with Non-infected Individuals

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Abstract

Background: The worldwide prevalence of *Helicobacter pylori* is about 50%. This bacterium needs a number of virulence factors for pathogenesis.

Objectives: This study aimed to determine the prevalence of virulence genes (*ureB*, cytotoxin-associated gene A [*cagA*], and vacuolating cytotoxin [*vacA*]), as well as the antigenic profile in *H. pylori* strains.

Methods: Eighty-five patients with abdominal pain, including 46 *H. pylori*-positive and 39 *H. pylori*-negative cases, were enrolled in this study. The serum levels of interleukin (IL)-17F, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) cytokines were measured by multiplex kits and flow cytometry. After molecular identification by the *ureC* gene, *vacA*, *cagA*, and *ureB* genes were detected by polymerase chain reaction (PCR). Finally, after antigenic extraction, the whole-cell protein was exhibited by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE).

Results: The prevalence of *vacA*, *ureB*, and *cagA* genes were 91.3%, 67.39%, and 50%, respectively. The frequency of genes and cell surface antigens were not significantly different based on the gastritis severity ($P > 0.05$). IL-17F significantly ($P = 0.046$) increased in the presence of 19.5 kDa (outer membrane protein [OMP]). Moreover, the OMP antigen significantly enhanced immunoglobulin A (IgA; $P = 0.013$). In the presence of the 66-kDa (ureB) antigen, the serum level of IFN-γ increased ($p = 0.041$). Finally, the CagA protein led to increased IgG antibody levels ($p = 0.027$).

Conclusions: Early detection of *H. pylori* infection can play a crucial role in managing it. Our results suggest that IL-17F, TNF-α, and IFN-γ cytokines could be diagnostic markers. However, further studies are required to fully investigate this suggestion.

Keywords: *Helicobacter pylori*, Cytokines, Gastritis, Antigenic Profile, Virulence Gene

1. Background

The common microorganism of the human stomach is a Gram-negative bacterium, *Helicobacter pylori*, that colonizes the gastric mucosa of more than half the world population (1, 2). The prevalence of *H. pylori* is 20% and > 90% in developed and developing countries, respectively (3). This bacterium was detected in a gastric biopsy for the first time in Australia. Urease activity lets the bacterium survive on the gastric epithelium (4). *Helicobacter pylori* colonization occurs in the gastric mucosa through some virulence genes, such as cytotoxin-associated gene A (*cagA*) and vacuolating cytotoxin (*vacA*) (5). The colonization, adhesion, and invasion of *H. pylori* strains into the gastric epithelial cells is facilitated by different virulence genes (6).

Once *H. pylori* is located on the gastric lumen, a permanent infection develops, and its long-term presence (if left untreated) leads to several gastro-duodenal diseases, such as gastric ulcer, chronic gastritis, duodenal ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric cancer (7). Antibody response to *H. pylori* has indicated that the level of antibody response provides information beyond the detection of infection. The relationship between high antibody levels was found to be associated with the grade of histological gastritis, gastric mucosal inflammation, mucosal bacterial density, and gastric cancer risk (8). After *H. pylori* colonization in the stom-

ach, inflammatory responses (production of cytokines) by host immune system cells occur in the gastric mucosa (9).

The virulence of *H. pylori* varies in different geographical regions. Previous studies have clearly defined that *H. pylori* virulence factors have a strong effect on pathogenicity and treatment results (10). Some *H. pylori* virulence factors, including *CagA*, *VacA*, *H. pyloricag* pathogenicity island (*cagPAI*), and adhesion proteins, are vital in gastric disease pathogenesis (11). Studies have shown that extremely virulent *H. pylori* strains carry *cagPAI*, including 31 genes (40 kb region) involved in the host's inflammatory response and *cagA* translocation (12). *CagA* is an extremely studied *H. pylori* virulence gene. It is placed at the end of *cagPAI* and encodes an immunodominant protein with a molecular weight of 120 kDa (13).

It has been reported that severe inflammation, gastric cancer, and gastric ulcer are intensely associated with *cagA* gene expression (14, 15). The presence of *cagA* in *H. pylori* strains usually accompanies other virulence factors, including *vacA* (16). Primary studies on *vacA* have discovered 2 main polymorphic families of s-region and m-region (17, 18). The m-region and s-region encoded the *vacA* m1 or m2 and *vacA* s1 or *vacA* s2 allele, respectively. According to the literature, *vacA* m1 strains are more likely to cause gastric epithelial damage than *vacA* m2 strains (19). In Northeast Asia (such as South Korea and Japan), *vacA* m1 type strains are more common. In contrast, *vacA* m2 type strains dominate Southeast Asia (such as Vietnam and Taiwan) (20). The s1 type is further divided into s1a, s1b, and s1c, and the m1 type is divided into m1a and m1b alleles (21).

The majority of prior studies of *H. pylori*-induced cytokines have focused on cytokine messenger RNA (mRNA) detection or protein quantification in supernatants from *in vitro* cultures of gastric biopsy specimens, isolated gastric lymphocytes, or gastric epithelial cell lines (22-25). However, in this study, we assessed the levels of cytokines in the blood of patients. In addition, the association between the antigenic profile of bacterial strains and cytokines in the peripheral blood of infected individuals was explored in the current study, which has never been done before. One of the major problems with *H. pylori* infections is the late diagnosis. We can employ inflammatory cytokines as early detection indicators by measuring their levels in people infected with *H. pylori* strains having pathogenic antigen patterns.

2. Objectives

This study aimed to evaluate virulence genes, antigen patterns of *H. pylori*, and levels of some inflammatory cytokines in *H. pylori*-infected patients.

3. Methods

3.1. Sample Collection

Eighty-five patients with gastritis and dyspepsia were enrolled in this study. Sampling was carried out in Imam Khomeini Hospital, Ahvaz, Iran. Inclusion criteria were patients older than 21 years with dyspepsia and *H. pylori* infection agreed to participate in the study and signed the consent form. Exclusion criteria were patients who received therapy with nonsteroidal anti-inflammatory drugs (NSAIDs) or antibiotics within 4 weeks of study entrance, patients who reported upper gastrointestinal bleeding, and pregnant females. The rapid urease and histopathological test made the initial diagnosis of *H. pylori* infection in these patients. The gastritis level was also defined in the samples. After taking the stomach mucus biopsy, 1 part of the biopsy was used for the urease test and culture to approve *H. pylori* infection. The other part was used in histopathological studies (kept in 10% phosphate-buffered formalin). In addition, 10 mL of blood was taken from the patients, and the serum was instantly extracted using centrifugation at 3,200 rpm (15 min). The extracted serum samples were stored at -70°C. Immunoglobulin G (IgG) and IgA antibodies against *H. pylori* were measured using ELISA kits (Roche-Germany) (26). Besides the relatively small sample size, the limitation of our study was the lack of previous studies on the topic.

3.2. Helicobacter pylori Isolation and Detection

The biopsy samples were cultured on Brucella agar (Merck, Germany) containing vancomycin (10 μg/mL), trimethoprim (5 μg/mL), amphotericin B (2.5 μg/mL), and sheep blood (5%). After incubation under 10% CO_2 and temperature of 37°C conditions for 3 - 5 days, the colony appears, and the usual microbiological tests (such as Gram staining, catalase, urease, and oxidase tests) were performed for *H. pylori* detection (27-29). Genomic DNA was extracted according to a standard protocol, which was placed in the Pooyagen Azma Company Kit (Tehran, Iran). Then, the purity (A260/A280) and concentration of the extracted DNA were determined using a NanoDrop ND-1000 spectrophotometer.

The quality of the extracted DNA samples was determined using a 2% agarose gel stained with SYBR Green (30). Espinoza et al. advocated using the *glmM* gene to detect *H. pylori* in 2011 (31). Indeed, the *glmM* housekeeping gene is required for bacterial proliferation and cell wall formation, making it unique to *H. pylori* (32). Therefore, the *glmM* (*ureC*) gene was used for the molecular identification of strains; *ureC* primers are displayed in Table 1. Polymerase chain reaction (PCR) was performed in the final volume of 25 μL containing 12.5 μL master mix 2X (SinaClon, Iran), 1

μL of each primer, 1 μL genomic DNA, and 9.5 μL deionized water. The extracted DNA of *Escherichia coli* ATCC 25922 was used as a negative control instead of *H. pylori* genomic DNA. DNA from isolates with known genes was used as a positive control. PCR was accomplished using a thermocycler (Bio-Rad, USA) under initial denaturation conditions of 4 min at 94°C, followed by 30 cycles of denaturation (60 s, 94°C), primer binding (30 s, 51°C), elongation (90 s, 72°C), and final elongation (4 min, 72°C). Ten microliters of the PCR product was subjected to electrophoresis on a 2% agarose gel in 1X TBE buffer at 80 V for 30 min and stained with SYBR Green (30, 33).

3.3. Virulence Genes Detection and vacA Genotyping

The systems of PCR were the same as mentioned above except for the primers. The amplification condition is illustrated in Table 2. Also, the necessary volume of compounds required in PCR is listed in Table 3. Primers used for *cagA*, *ureB*, and genotyping of *vacA* are presented in Table 1. In the end, the reaction product was evaluated by 2% agarose gel electrophoresis (30, 35).

3.4. Whole Helicobacter pylori Cell Protein Profile Analysis

As previously described by Huang et al. (36) and Sheykhian et al. (37), whole *H. pylori* cell protein profiles were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The bacterial cell was washed by cold phosphate-buffered saline (PBS). After centrifugation, the bacterial pellet was suspended in 10 mL of Tris-HCl buffer (pH 7.8) containing EDTA (1 mM/L) and phenylmethylsulfonylfluoride (PMSF; 1 mM/L). The tube containing bacterial suspension was sonicated for 8 cycles (MSE ultrasonicator). Debris was removed by centrifugation at 1,500g for 15 min. The pellet was resuspended in 10 mL of Tris.HCl (pH 7.8), and then ribonuclease (Rnase) and deoxyribonuclease (DNase; Sigma-Aldrich, USA) were added (0.1 mg/mL). After incubation at 37°C for 2 h, the supernatant was centrifuged at 150,000g and 4°C for 45 min. The *H. pylori* cell wall pellet was dissolved in 10 mL of sarcosine (2%). After 30 min incubation at room temperature, the outer membrane was pelleted, the supernatant was removed, and the pellet was dissolved in 1 mL of PBS containing 1 mM PMSF (pH 7.8).

3.5. Serum Level of IL-17F, TNF-α, and IFN-γ by Flow Cytometry

The serum level of interleukin (IL)-17F, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were evaluated by flow cytometry using the 13-plex LEGENDplex™ Human Th Cytokine Panel (BioLegend, USA). First, the serum samples were diluted to 1: 6; then, they were tested according to the kit manufacturer's instructions. Finally, the contents of the well plate were transferred to fluorescence-activated cell sorting (FACS) tubes (Abcam, United Kingdom) to be read by the flow cytometry (BioRad, USA).

3.6. Statistical Analysis

The Mann-Whitney U test and independent sample *t*-test were used based on the data normality. The Kolmogorov-Smirnov test determined the normality of the data. P values < 0.05 were considered statistically significant.

4. Results

4.1. Study Population

Histopathology, fast urease, and PCR demonstrated that 46 samples were infected with *H. pylori* (Hp+), while the remaining 39 cases were not infected (Hp-). The participants' mean age was 43.83 $\pm$ 13.41 and 41.28 $\pm$ 12.65 years for the Hp+ and HP-, respectively. The Hp+ group included 17 females and 29 males, and the Hp- group included 22 females and 17 males. There was no significant relationship between age and sex in both groups (Table 4). The gastritis severity in the Hp+ group was mild in 23.91% (11/46) and moderate in 76.09% (35/46) of samples. There was also a significant difference between the 2 groups in IgG and IgA levels (Table 4).

4.2. Prevalence of Virulence Genes

The prevalence rates of virulence genes *cagA*, *vacA*, and *ureB* in the Hp+ group are summarized in Figure 1A. The highest prevalence was related to the *vacA* gene, with a frequency of 91.3%. *VacA* gene genotyping was performed (Figure 1B), and the frequency of *vacA* m1 and *vacA* m2 was 32.6% and 78.26%, respectively. The abundance of 3 genes and *vacA* alleles by gastritis severity is also shown in Figure 2. Based on these results, the frequency of genes is not significantly different in patients with mild and moderate gastritis ($P > 0.05$).

4.3. Antigenic Profile Analysis

After extraction of whole-cell antigens and analysis by SDS-PAGE (Figure 3), 7 well-known antigens were evaluated in the strains based on the molecular weight (kDa). Figure 4A shows the prevalence of antigen expression in the strains. Also, the relationship between antigens and gastritis severity is shown in Figure 4B. The presence of antigens was not significantly different based on the gastritis severity ($P > 0.05$).

Table 1. The Primer Used in the Study

Gene	Primer Sequence	Amp. Size (bp)	Reference
***ureC*-F**	5′- GGATAGACGATGTGATAGG -3′	224 bp	(33)
***ureC*-R**	5′- TTGGTTAGGGTGTAAAGC -3′		
***cagA*-F**	5′- ATAATGCTAAATTAGACAACTTGAG-3′	298 bp	This study
***cagA* -R**	5′- TTAGAATAATCAACAAACATCACGC-3′		
***vacAm1/m2*-F**	5′- CAATCTGTCCAATCAAGCGAG-3′	567 - 642 bp	(34)
***vacAm1/m2* -R**	5′- GCGTCAAAATAATTGAAGG-3′		
***ureB*-F**	5′- AGTAGCCCGGTAGAACACAACATCCT-3′		This study
***ureB*-R**	5′- AGTCCTTTGTCATAAGCCGCTTGG-3′		

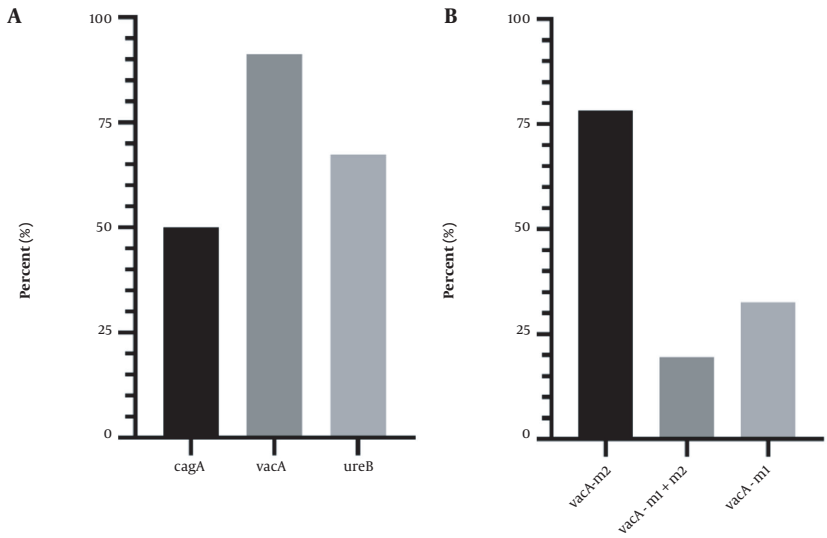

Figure 1. Frequency of studied virulence genes (A) and frequency of *vacA* gene alleles (B).

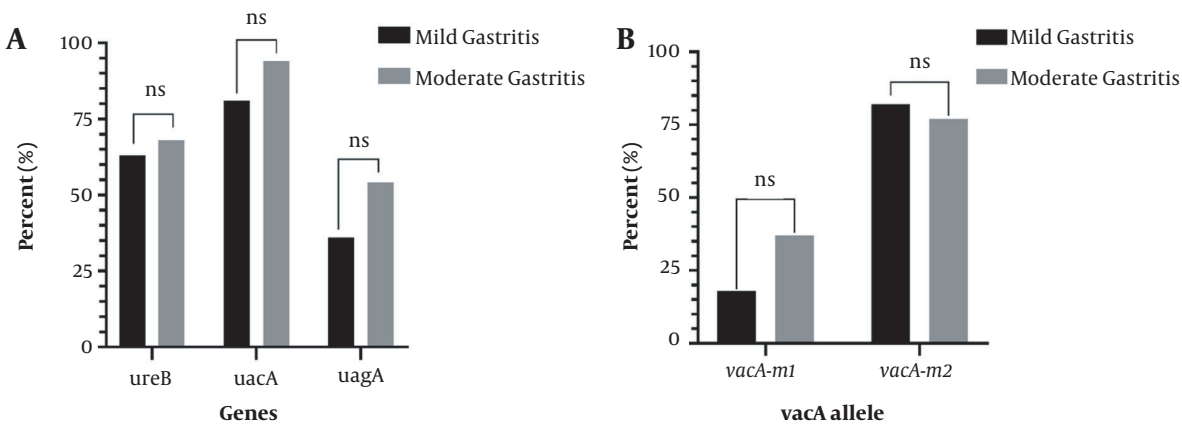

Figure 2. Frequency of studied genes (A) and *vacA* alleles based on the gastritis severity (B) (ns, not significant).

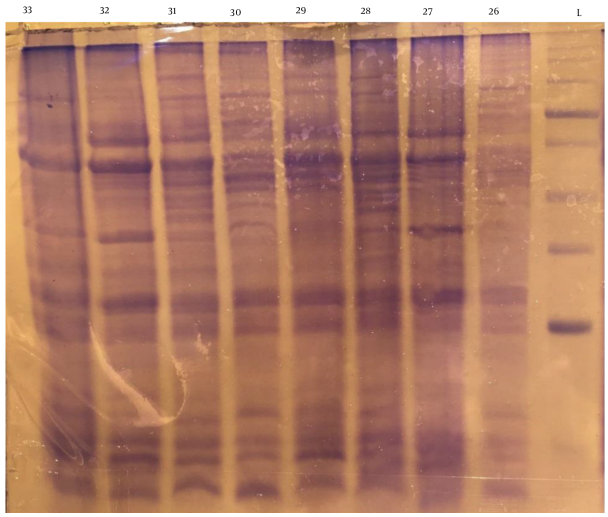

Figure 3. SDS-PAGE of whole-cell antigens of *Helicobacter pylori* (L, ladder and 26 - 33, *H. pylori* strains).

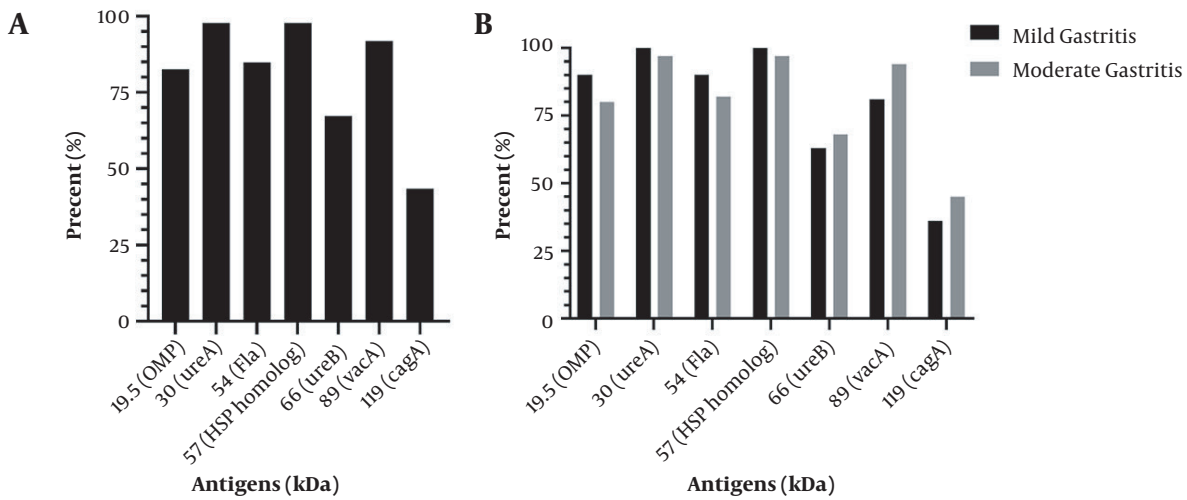

Figure 4. Antigen profile in *Helicobacter pylori* strains (A) and antigen profile based on gastritis severity (B) (ns, not significant).

Table 2. Amplification Conditions for *cagA*, *ureB*, and *vacA* Genes

Step	Time
Initial denaturation	94°C, 4 min
Cycling (30 cycles)	
Denaturation	94°C, 1 min
Annealing (*cagA*)	60, 30 s
Annealing (*ureB*)	60, 30 s
Annealing (*vacAm1/m2*)	52, 30 s
Extension	72°C, 90 s
Final extension	72°C, 5 min

Table 3. The Volume of Compounds Required in PCR

Compounds	Volume (μL)
Distilled water	7.5
Master mix	12.5
DNA samples	3
Round primer mixture	2
Total	25

4.4. Correlation Between Antibodies, Cytokines' Levels, and Antigen Profiles

As demonstrated in Table 5, the 3 studied cytokines' serum levels differ significantly in the Hp+ and Hp- groups

Table 4. Demographic Information of the *Helicobacter pylori* Positive (Hp+) and *H. pylori* Negative (Hp-) Groups

Variables	Groups [a]		P Value [b]
	Hp-	Hp+	
Age (y)	41.28 ± 12.65	43.83 ± 13.41	0.41
Sex No. (%)			0.07 [c]
Female	22 (56.41)	17 (36.95)	
Male	17 (43.58)	29 (63.05)	
Gastritis, No. (%)			
Mild	None	11 (23.91)	< 0.001 [c]
Moderate	None	35 (76.09)	< 0.001 [c]
Antibodies, (%)			
IgG	9.95 ± 2.14	99.11 ± 57.2	< 0.001 [c]
IgA	6.02 ± 2.13	74.21 ± 47.63	< 0.001 [c]

[a] Values are presented as No. (%) or mean ± SD.
[b] P values were considered statistically significant at the level of 0.05.
[c] Significant P values.

(P < 0.05). Also, the effect of *H. pylori* antigens on the level of antibodies and cytokines' serum levels are shown in Table 6. According to the results, IL-17F significantly (P = 0.046) increased in the presence of 19.5 kDa [outer membrane protein (OMP)]. Moreover, the OMP antigen enhanced IgA (P = 0.013) significantly. In the presence of the 66-kDa (ureB) antigen, the serum level of IFN-γ increased (P = 0.041). Finally, the CagA protein increased IgG antibody levels (P = 0.027).

Table 5. IL-17F, TNF-α, and IFN-γ Levels in the *Helicobacter pylori* Positive (Hp+) and *H. pylori* Negative (Hp-) Groups

Cytokines	Groups [a]		P Value [b]
	Hp- (pg/mL)	Hp+ (pg/mL)	
IL-17F	30.93 ± 12.47	142 ± 199.5	< 0.001
TNF-α	40.16 ± 14.98	80 ± 95.9	0.003
IFN-γ	35.11 ± 15.68	248.7 ± 808.8	< 0.001

[a] Values are presented as mean ± SD.
[b] P values were considered statistically significant at the level of 0.05.

5. Discussion

The *ureC* gene is highly conserved and has been applied to identify *H. pylori* strains. A previous study reported that the sensitivity and specificity of *ureC* is more than 90% (38). The present study showed that the prevalence of *vacA*, *ureB*, and *cagA* genes was 91.3%, 67.39%, and 50%, respectively. According to previous studies, *cagA* has a frequency of 71.4% in Turkey (39), 54% in Sudan (40), 62% in South Africa (41), and 77.27% in India (42). One of the most important virulence factors in *H. pylori* is an 89-kDa protein, VacA, which can cause cell depletion. In Pandya et al.'s study (42), the prevalence of the *vacA* gene was 4.54% in India, which is consistent with our results. However, the prevalence of *vacA* in South Africa (2019) was similar to the present study (90.6%) (41). The *vacA* polymorphic gene encodes the VacA protein. The m1 genotype of the middle part of the gene is associated with high cytotoxicity. The m2 genotype is found in non-cytotoxic strains (43). Among all *vacA* positive strains, 78.26% had the m2 allele, and 32.6% had the m1 allele. Another virulence gene in this study was the *ureB*, which is present in 67.39% of the strains. Urease consists of the main subunits UreA and UreB (44). This enzyme plays a vital role in the colonization of *H. pylori*. In addition, urea stimulates the production of inflammatory cytokines by mononuclear phagocytes (45).

Surface antigens on the *H. pylori* cell or secreted from the cell include CagA, VacA, urease subunits (UreA and UreB), heat shock protein (HspA and HspB), subunits of flagellin, catalase, lipopolysaccharide, OMP, and several unknown antigens (46, 47). In this study, known antigens were evaluated in whole isolated strains. In the present study, the CagA antigen was found to be associated with high levels of IgG antibodies. CagA has been identified as a vital virulence factor in *H. pylori* (48), and CagA antibodies have been observed in patients with gastritis, gastric ulcer, and gastric cancer (49-51). In 2016, Seo et al. showed an association between the CagA antigen and high levels of IgG and IgA antibodies (52). However, in the present study, no association was found between IgA and CagA antigen. In the 2000s, 80% of *H. pylori* strains carried the *cagA* gene in Japan and Hong Kong (53, 54), and 94% of 33 Korean children had the *cagA* gene (55). In Japan, CagA was the most reactive antigen found in all *H. pylori*-infected serum samples (even from children under 3 years of age) (56). Therefore, the CagA antigen in each region will be important to detect *H. pylori* infection, and the prevalence of this antigen in strains can be important for diagnosis (56). In Korean

Table 6. The Antibodies and Cytokines' Serum Levels in the Presence of *Helicobacter pylori* Antigens [a]

Antigens (kDa)	IL-17F (pg/mL)	TNF-α (pg/mL)	IFN-γ (pg/mL)	IgG (pg/mL)	IgA (pg/mL)
19.5 (OMP)					
Positive	164.88	76.96	266.97	94	104
Negative	137.28	94.5	162	123	68
30 (UreA)					
Positive	141.83	79.32	253.46	100	74
Negative	153	111	35	53	69
54 (Fla)					
Positive	145.87	82.63	266.73	102	74
Negative	120.94	65.43	148.37	85	76
57 (HSP homolog)					
Positive	141.59	80.65	251.78	100	75
Negative	164	51	111	81	59
66 (UreB)					
Positive	211.47	123.4	520.67	100	79
Negative	108.5	59	117.13	99	72
89 (VacA)					
Positive	143.85	80.18	263.34	101	76
Negative	123.5	78.25	95.13	78	53
119 (CagA)					
Positive	175.86	103.39	386.33	117	63
Negative	116.08	62	142.86	76	83

[a] Significant correlations are shown in bold.

studies, the result of a positive serological test for the CagA antibody was considered as *H. pylori* infection (57). Also, we demonstrated that IgA increased significantly in the presence of OMP (19.5 kDa).

Serum levels of IL-17F, TNF-α, and IFN-γ cytokines were assessed in the study population. There was a significant difference in cytokine levels between patients with and without *H. pylori*. Thus, the colonization of *H. pylori* in the stomach can lead to an inflammatory response in the absorption of immune system cells in the gastric mucosa (58). IL-17 mediates the activation of polymorphonuclear neutrophils and leads to gastritis (59). It has been previously reported that there is a significant increase in IL-17 and IFN-γ in the early stages of *H. pylori* infection (59). IL-17 can stimulate immune cells to release inflammatory mediators, including IL-1, IL-6, and TNF-α (59). All the IL-17F, TNF-α, and IFN-γ cytokines involved in gastritis increased in our studied population. More studies are required to clarify the role of *H. pylori* virulence factors in the production of cytokines. CagA antigens are more effective in stimulating dendritic cells (DCs) to induce IL-23/IL-17 expression. Also, IL-17 activation by the ERK1/2 MAP kinase pathway is more associated with the CagA antigen (60). We demonstrated that the level of IL-17F in patients with CagA-positive *H. pylori* was increased (not significantly). Also, the presence of OMP (19.5 kDa) and UreB (66 kDa) antigen caused significant changes in IL-17F and IFN-γ, respectively.

5.1. Conclusions

In conclusion, the antigen profile of *H. pylori* isolated from Ahwaz, Iran, was shown in the present study. However, for further investigations, we suggest using western blotting and ELISA techniques in addition to SDS-PAGE. As it is clear, early detection of *H. pylori* infection can play a crucial role in reducing the risks of this bacterium. Therefore, by investigating the levels of inflammatory cytokines and their relationship with bacterial antigen profile, a suitable cytokine can be identified for the rapid diagnosis of *H. pylori* infection. Our results suggest that IL-17F, TNF-α, and IFN-γ cytokines could be used as a diagnostic marker. However, further investigations are required to approve this suggestion.

Footnotes

Authors' Contribution: Study concept and design, A. S. M., E. M., S.A. S.; and A. G.A. K.; Acquisition of data, A. S. M., E. M., and S.A. S.; Analysis and interpretation of data, A. S. M., E. M., and S.A. S.; Drafting of the manuscript, A. S. M., E. M., S.A. S., and A. G.A. K.; Critical revision of the manuscript for important intellectual content, A. S. M.; E. M., S.A. S., and A. G.A. K.; Statistical analysis, A. S. M., and S.A. S.; Administrative, technical, and material support, E. M., S.A. S., and A. G.A. K.; Study supervision, E. M., S.A. S., and A. G.A. K. All authors read and approved the final manuscript.

Conflict of Interests: There is no conflict of interests.

Data Reproducibility: The data presented in this study will be available on request from the corresponding author by this journal representative at any time during submission or after publication. Otherwise, all consequences of possible withdrawal or future retraction will be with the corresponding author.

Ethical Approval: This study was approved by the Ethics Committee of Islamic Azad University, Shiraz, Iran (code: IR.IAU.SHIRAZ.REC.1400.003), (Link: ethics.research.ac.ir/EthicsProposalViewEn.php?id=196383).

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Informed Consent: All patients were admitted to the study with written informed consent.

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