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FECAL *HELICOBACTER PYLORI GLMM* AND *16S rRNA* GENES CORRELATE WITH SERUM TNF-α AND IL-1β CYTOKINE FLUCTUATIONS

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The proinflammatory cytokines of TNF-α and IL-1β have been reported to be increased in gastric mucosal surfaces in people with *Helicobacter pylori* infection. Accordingly, this study was conducted to investigate the relationship between the presence of *H. pylori* genes and the serum oscillations of these cytokines. In this study, DNA was first extracted from the stool samples of infected individuals and used as DNA template to investigate the presence of *glmM* and *16S rRNA* genes in PCR. The ELISA assay was employed to examine serum levels of TNF-α and IL-1β cytokines. According to statistical analysis, there was a significant correlation between the presence of *glmM* and *16S rRNA* genes in the stool samples of infected persons and the serum oscillations of TNF-α and IL-1β cytokines. At the end of study and analysis of the data in case group with HPSAg+, 47.6% of the *glmM* gene and 23.6% of the *16S rRNA* gene were positive. In addition, a significant correlation was observed between the presence of *glmM* and *16S rRNA* genes in the stool specimens of infected individuals and the serum levels of TNF-α and IL-1β cytokines ($p < 0.05$). Considering the results, it can be concluded that fluctuations in the amount of HPSA, TNF-α, and IL-1β in *H. pylori* infection depend on the presence of *glmM* and *16S rRNA* genes. The presence of *glmM* and *16S rRNA* in the stool sample increases by boosting the response level to stool antigen (HPSA), IL-1β, and TNF-α, suggesting the prognosis of the disease with a bacterial virulence form using stool tests.

Keywords: *H. pylori*, IL-1β, TNF-α, *16S rRNA*, *glmM*

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Introduction

Helicobacter pylori are spiral-shaped, Gram-negative, microaerophilic bacteria with flagella that develop gastritis, gastric ulcer, and gastric adenocarcinoma, as well as are associated with primary gastric lymphoma and expansion within the glandular epithelium [1, 2]. *H. pylori* infection often constantly causes chronic swelling, but only some of the Helicobacter strains can create ulcer more than the others. However, the nature of immune response may indicate different consequences of the infection by this organism [3]. Several previous studies on the patient with *H. pylori* infection reported an increase in the proinflammatory cytokines of TNF-α, IL-1β, IL-1RN, IL-8, and IL-10 in gastric mucosal surfaces [4, 5].

TNF-α is the main mediator of acute inflammatory responses to Gram-negative bacteria and other infectious microbes and responsible for many common complications of acute generalized infections. The lipopolysaccharide (LPS) in the cell wall of the Gram-negative bacteria is the strongest stimulant of TNF-α production by macrophages. The key physiological action of TNF is to recruit the neutrophils, monocytes, and macrophages toward the infection site and activation of these cells [6]. TNF, similar to IL-1, affects the cells that regulate the body temperature in the hypothalamus [6].

IL-1β is a proinflammatory cytokine caused by *H. pylori* infection and a strong inhibitor of gastric acid secretion. This proinflammatory cytokine has an affinity for colonization of *H. pylori* and consequently exacerbation of gastric inflammation [7]. Studies have shown that IL-1β-31 and IL-1β-511 polymorphisms are associated with high production of IL-1β and develop gastric cancer [8, 9].

The main function of IL-1β, like TNF-α, is to participate in host inflammatory responses to infection and other inflammatory stimuli. The IL-1β secretion in large amounts and then penetration into the bloodstream result in endocrine action and generally along with TNF induce fever, synthesize the acute-phase proteins in the liver, and lead to metabolic weakness [6].

In the process of developing the disease by *H. pylori*, this bacterium can be established for many years in the stomach, and it affects the patient's immune system, resulting in the induction of inflammation in the gastric mucosa. This chronic inflammatory response recruits neutrophil, followed by T- and B-lymphocytes and macrophages to the inflammatory site. The immune response stimulated in the affected area causes inflammation and tissue damage due to the release of inflammatory mediators, such as TNF and IL-1 cytokines, as well as the free radicals of NO and O [10]. With continued process, the chronic gastritis is

created. These conditions in different patients may lead to gastric atrophy, intestinal metaplasia, or gastric cancer, depending on virulence factors, host genetic factors and, as previously stated, the host immune system that differs in various races [11].

Considering the extent of the financial and life-threatening losses associated with this bacterium, particularly the major cause of gastrointestinal cancers, the development of the study will be very valuable in this regard. Considering the number of patients with gastrointestinal problems in Ilam province (Iran), as well as the fact that gastritis is one of the stages prior to cancer in people with gastrointestinal problems, it seems that the information on the secretion of cytokines and their relationship with bacterial virulence and antigenic factors (LPS) could be exploited to diagnose and to accurately understand the relevant mechanisms and factors associated with the disease for screening and preventing at the higher levels. Therefore, this study was designed to investigate the presence of *glmM* and *16S rRNA* genes of *H. pylori* in the infected patients and their relation with serum levels of TNF-α and IL-1β cytokines.

Materials and Methods

This case–control study was performed on 84 patients with gastrointestinal complaints referring to medical diagnostic laboratories in Ilam. The case group consisted of the subjects with positive rapid *H. pylori* stool antigen (HPSAg+) and the control group had negative HPSAg test (HPSAg). Demographic characteristics of the subjects (age, sex, occupation, and education) were recorded in the designed questionnaire. The study objectives were explained and the consent of the individuals was drawn to participate in the study, then the blood and stool samples were taken and sent to the Immunology Laboratory of Ilam University of Medical Sciences for conducting the tests and studies.

DNA extraction and polymerase chain reaction (PCR)

The DNA was extracted from all stool specimens using DNA extraction kit (CinnaGen Co., Iran), concentrated in ethanol, and stored at −20 °C in freezer. PCR was used to investigate the presence of *glmM* and *16S rRNA* genes. For this purpose, specific primers were designed for these genes using IDALLEL version 5 Software (Table I).

Table I. Specific primers used for polymorphism analyses

Gene	Primer	Sequence (5′→3′)	Product PCR (bp)
glmM	F	ACCCGATATTGTTCGAC	224
	R	GCGAATATACGCGGTTA	
16s rRNA	F	CAATGGCTATACCTAC	521
	R	GATGGAATTAGCCTTACG	

Note: F: forward; R: reverse; PCR: polymerase chain reaction.

The target DNA amplification with a final volume of 25 µl contained 12 µl of prepared master mix (Ampliqune, Finland), 7 µl of sterilized double-distilled water, 1.5 µl of each of the forward and reverse primers, and finally, 3 µl of the DNA template (DNA extracted from the stool samples), which was poured into 0.2-micron microtubes, vortexed, and then transferred into a thermal cycler device. The specific temperature conditions for each of these genes were adjusted after complete setup (Table II).

At the end of the PCR process, electrophoresis was performed to observe the results and determine the presence of *glmM* and *16S rRNA* genes in the PCR product. In this way, 7 µl of the PCR product was transferred into the wells of 1% agarose gel containing DNA safe stain immersed in an electrophoresis tank with TAE (TAE buffer is a buffer solution containing a mixture of Tris base, acetic acid and EDTA) 1×, which was then exposed to electric current at 85 V for 85 min. Then, Gel Doc was used to observe and take images (Figure 1).

Enzyme-linked immunosorbent assay (ELISA)

The serum of all blood samples from the participants was separated with the help of specific kits (Bosterbio Company: Antibody and ELISA Experts, Pleasanton, CA) to measure the levels of TNF-α and IL-1β cytokines using ELISA reader device, according to the manufacturer's instructions.

All the results were analyzed by SPSS software using Kolmogorov–Smirnov test, one-way analysis of variance, Kruskal–Wallis test, and logistic regression. A p value of <0.05 was considered as a significance level.

Table II. Specific temperature schedule for primers

Gene	Hot start	Denaturation	Annealing	Extension	Final extension
glmM	95 °C, 5 min	94 °C, 1 min	51 °C, 1 min	72 °C, 1 min	72 °C, 10 min
16s rRNA	95 °C, 5 min	94 °C, 1 min	55 °C, 1 min	72 °C, 1 min	72 °C, 10 min

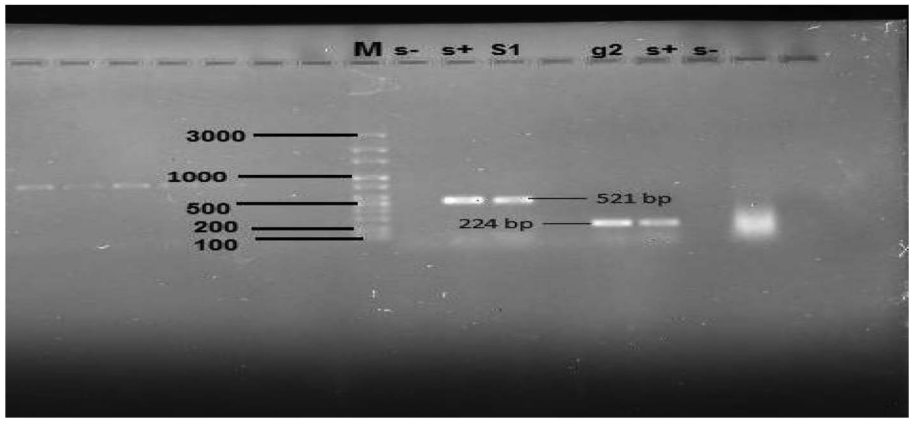

Figure 1. Image of 1% agarose gel with 521 bp bands of *16s rRNA* gene and 224 bp bands of *glmM* gene. M: Marker 100–3,000 bp; S1 and g2 are samples

Results

At the end of the study and analysis of the data in the case group with HPSAg+, 47.6% of the *glmM* gene and 23.6% of the *16S rRNA* gene were positive. In this study, 45.3% of the subjects were women with the mean age of 44.3 years and 54.7% were men with the mean age of 50.2 years. The frequency of *glmM* gene in the stool sample of the men and women groups was 23.8%, but the frequency of *16S rRNA* gene was observed to be 14.3% in men and 9.5% in women.

To evaluate the mean age, the subjects were divided into four age groups between 4–90 years, such as 4–10, 11–20, 21–49, and 50–90 years; the most frequent presence of *glmM* and *16S rRNA* genes in stool DNA samples was observed in the age group of 21–49 years (Table III).

It should also be noted that the frequency of people infected with *H. pylori* and subsequently the frequency of *glmM* and *16S rRNA* genes were higher in the

Table III. Study of the age group of the subjects

	Age group			
Genes	4–10 years	11–20 years	21–49 years	50–90 years
glmM (%)	–	24	40.5	4.8
16s rRNA (%)	2.4	–	19	2.4

Table IV. Evaluation of serum levels of IL-1β and TNF-α cytokines with the presence of *glmM* and *16s rRNA* genes

	glmM		*16s rRNA*	
Variable	−	+	−	+
TNF-α Cutoff 81	140.8	465.3	109.8	387.4
IL-1β Cutoff 9	18.2	74.4	19.1	43.6

illiterate and semiliterate groups than in other groups with higher educational level.

Studying the serum levels of TNF-α and IL-1β proinflammatory cytokines showed that the most frequency of individuals with serum IL-β1 higher than the cutoff point (9 pg/ml) was observed in the samples positive for *glmM* and *16S rRNA* genes in stool DNA.

According to statistical analysis, there was a significant correlation between the presence of *glmM* and *16S rRNA* genes in the stool of infected persons and the serum oscillations of TNF-α and IL-1β cytokines ($p < 0.05$).

As seen at first glance, people who were positive in stool DNA for each of these two genes, the level of these cytokines was significantly higher than those in other study group (Table IV).

Discussion

According to the World Health Organization, approximately 50% of adults in developed countries and about 90% of adults in developing countries are suffering from *H. pylori* infection [2]. In developing countries, 70%–80% of children under the age of 15 years are infected with this bacterium. Low socioeconomic areas have pivotal role in transmitting this infection [12].

The results of many studies suggest that the prevalence of *H. pylori* is more common in men than in women [13–15]. In other studies, the contradictory findings are also evident [16, 17]. In this study, the frequency of female patients was more than that of male patients.

The mean age group was between 40 and 60 years old [14, 17–19]. Based on our analysis, the mean age of infected individuals was 42.3 years. However, there was no significant relationship between *glmM* gene and age.

Measurement of serum levels of TNF-α and IL-1β cytokines in the study population revealed that the secretion of these cytokines in individuals positive for *glmM* and *16S rRNA* genes was significantly higher than the negative ones in terms

of the presence of the gene in the stool DNA specimen, suggesting importance of the role of these genes in stimulating the immune system.

In the previous studies, the stool antigen test was introduced as a low-cost and rapid diagnostic method with high sensitivity and specificity for the diagnosis of *H. pylori* infection in children [18, 20–22]. We used this non-invasive diagnostic test as a golden standard for detecting the presence of infection in this study.

Reports have shown that increased proinflammatory cytokines may affect the pathological process of *H. pylori* infection [23, 24]. TNF-α can be found in chronic inflammatory conditions. It has been indicated that *H. pylori* secretes a 19-kDa protein (Tip) inducing TNF-α that can bind to DNA to enhance the expression of the TNF-α cytokine in the stomach [25].

In this study, there was no statistically significant difference in the serum levels of IL-1β and TNF-α with demographic variables, but the mean difference of these immune factors was significant in case and control groups. There was no statistically significant relationship between sex and these cytokines, but the means of IL-1β and TNF-α were higher in men than in women.

In a study on serum levels of circulating IL-6 and TNF-α in the patients with *H. pylori* infection, no statistically significant differences were observed between serum levels of TNF-α and the sex and age in positive and negative *H. pylori* groups [26], which is consistent with this study. The results of this study and a study by Van demonstrated that this pathogen does not significantly alter the level of TNF-α [26], while the data from this study indicated an increase in serum levels of IL-1β and TNF-α cytokines in *H. pylori*-infected individuals. In another study, the mean level of these two cytokines in the *H. pylori*-infected group was significantly higher than in non-infected individuals [25].

According to the reported data on IL-1β and TNF-α cytokines, it is expected that in people with high levels of HPSA response, especially in chronic conditions, the rate of these cytokines or their mean, as well as the frequency of people with high levels of IL-1β and TNF-α in the case group, is greater than the control group. This is well documented in this research, because the mean level of these cytokines in the case group was about 1.5–2 times more than the mean in the control group. However, it is also worth pointing out that increased levels of these cytokines have been observed in the ages of 21–50 years.

PCR is known as a high-sensitivity method, which can detect the low count of *H. pylori* present in the sample, including stool specimens (with a success rate of 25%–100%) [27]. In general, the difference in the detection rate of *H. pylori* in the stool sample is due to the destruction of the bacteria in the intestinal tract or the presence of inhibitors such as complex polysaccharides as well as the low

concentration of bacteria in the stool. In recent studies, the sensitivity of this method varied from 21% to 65.22% [28, 29].

One of the interesting findings in this research is the association between the PCR results of stool having *glmM* and *16S rRNA* genes and TNF-α and IL-1β variables and HPSAg test, which is of high clinical significance in diagnosis. This suggests that the presence of *glmM* gene in the stool elevates by 2.8 times more with the increase of each unit of the HPSA test and enhances 1.1 times more with the increase of each unit of IL-1β cytokine in the serum of patients infected with *H. pylori* (Table V).

Moreover, the probability of the presence of *16S rRNA* gene in the stool increases to 2.98 times more with the increase of each unit of the HPSA test and up to 1.01 times more with the increase in each unit of TNF-α cytokine in the serum of patients infected with *H. pylori* (Table VI).

Table V. Correlation of *glmM* gene with variables HPSA, TNF-α, and IL-1β

		B	SE	Wald	*df*	Sig.	Exp(*B*)
Step 1[a]	HpSA	1.032	0.310	11.054	1	0.001	2.806
	IL-1β	0.095	0.032	8.618	1	0.003	1.100
	TNF-α	0.004	0.004	1.323	1	0.250	1.004
	Constant	−5.253	1.228	18.307	1	0.000	0.005

Note: Assuming meaningful sig. <0.05 or $p < 0.05$. *B*: coefficient for the constant (also called the "intercept") in the null model; SE: standard error around the coefficient for the constant; Wald: the Wald χ^2 test that tests the null hypothesis that the constant equals 0; *df*: degrees of freedom for the Wald χ^2 test; Sig: sig is the *p* value that measures the significance of the coefficient; Exp(*B*): the exponentiation of the *B* coefficient, which is an odds ratio.

[a]Variables entered on Step 1: HpSA, IL-1β, and TNF-α.

Table VI. Correlation of *16s rRNA* gene with variables HPSA, TNF-α, and IL-1β

		B	SE	Wald	*df*	Sig.	Exp(*B*)
Step 1[a]	HPSA	1.094	0.366	8.941	1	0.003	2.987
	IL-1β	−0.035	0.027	1.722	1	0.189	0.965
	TNF-α	0.010	0.004	6.235	1	0.013	1.010
	Constant	−5.493	1.400	15.406	1	0.000	0.004

Note: Assuming meaningful sig. <0.05 or $p < 0.05$. *B*: coefficient for the constant (also called the "intercept") in the null model; SE: the standard error around the coefficient for the constant; Wald: the Wald χ^2 test that tests the null hypothesis that the constant equals 0; *df*: degrees of freedom for the Wald χ^2 test; Sig: sig is the *p* value that measures the significance of the coefficient; Exp(*B*): the exponentiation of the *B* coefficient, which is an odds ratio.

[a]Variables entered on Step 1: HpSA, IL-1β, and TNF-α.

Conclusions

According to the findings, we can say that the oscillations of HPSA, TNF-α, and IL-1β tests depend on the presence of *H. pylori* infection and thus *glmM* and *16S rRNA* genes. The presence of *glmM* and *16S rRNA* in the stool increases by boosting the response level to stool antigen (HPSA), IL-1β, and TNF-α. This suggests the prognosis of the disease with the bacterial virulence form using stool tests.

Conflict of Interest

The authors declare no conflict of interest.

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