Correlation of *16s rRNA* with serum levels of the cytokines, TNF-α and IL-1β, in subjects with a positive *Helicobacter pylori* Stool Antigen test (HPSA)

Ayat Moradipour[1], Afra Khosravi[2*], Mohammad Reza Mehrabi[3], Sara Faryadian[4]

1. Department of Microbiology, Borujerd Branch, Islamic Azad University, Borujerd, Iran
2. Department of Immunology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
3. Department of Laboratory Sciences, Borujerd Branch, Islamic Azad University, Borujerd, Iran
4. Department of Physiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran

***Corresponding author:** Tel: +98 9126223958 Fax: +98 8432235746
Address: Department of Immunology, Faculty of Medicine, Ilam University of Medical Sciences
E-mail: afrakhosravi@yahoo.co.uk
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Abstract

Introduction: *Helicobacter pylori (H. pylori)*, is a bacterium responsible for upper gastrointestinal tract diseases. The *16s rRNA* is a common *H. pylori* gene which are usually preferred for diagnosis purpose. The aim of this study was to determine the prevalence and abundance of *16s rRNA* in fecal samples and also evaluate correlation between the level of *16s rRNA* and activities of the cytokines, TNF-α and IL-1β, in serum.
Materials and methods: The present study was performed on 84 subjects with digestion problems. Fecal and blood samples were collected and 16s rRNA gene was assayed using PCR. The serum levels of TNF-α and IL-1β levels were measured by enzyme-linked immunosorbent assay (ELISA).
Results: The results of the study revealed that there was a positive correlation between the 16s rRNA gene, *H. pylori* stool antigen (HPSA) and TNF-α cytokine. The study also noted that with every unit of increase in either of the quantified parameters of HPSA and IL-1β, the presence of 16s rRNA in fecal samples, showed a 2.98 and 1.01 times rise, respectively.
Conclusion: According to the obtained results, it may be concluded that activities of cytokine TNF-a correlated well with the presence of HPSA and 16srRNA gene in the stomach's lining. Increase in the activities of HPSA and TNF-a cytokine could be associated with the presence of *16s rRNA* in feces.

Keywords: *Helicobacter pylori*, 16s rRNA, TNF-α, IL- 1β, HPSA

Introduction

Helicobacter pylori is a gram-negative, spiral shaped, motile and microaerophilic bacillus with a length of 3.5μ and a width of0.5μ, which is associated with peptic/grastic ulcers (1). Although bacterial infection is asymptomatic in most cases, symptoms are found in some people and in few cases it leads to malignancies including adenocarcinoma and gastric lymphoma and other peptic disorders (2, 3). In recent years many studies have been done to determine the exact prevalence of *H. pylori*, including molecular and serological methods. For example, cagA is a gene in *H. pylori* which codes 128-145 kDa protein, of the same name. The gene is found in 50-70% of strains of the bacteria, and the product of this gene after entering the host cell cytosol and phosphorylation, becomes active and causes many functional and morphological changes in the host cell (4, 5). Other common *H. pylori* genes include glmM and 16srRNA which are usually preferred

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for diagnosing infection. The accuracy of diagnosis of *H. pylori* infection with the use of stool PCR varies from 25 to 100% due to stool inhibitors or organism decomposition in the intestines, but since this is a non-invasive method, it is preferable to invasive procedures (6). In addition to pathogenicity of the bacterium and its impact on the disease, recent studies have shown that host genetic and environmental factors are significantly involved in the incidence of *H. pylori* infection (7, 8). For example, in a study by Oba-Shinjo and Zhang in 2004 and 2008, it was observed that the presence of cytokines such as TNF-α and IL-1β is important in predicting susceptibility to *H. pylori* infection (9). Today, the use of stool antigens in affected patients is one of the most common methods of diagnosing the infection in the clinic. *H. pylori* infection leads to increase in the secretion of the cytokines and also since the cytokine activities are associated with the spread of microbes, the present study aims to investigate the prevalence of *16s rRNA* gene in the feces of infected people and correlate it with the levels of serum cytokines of TNF-α and IL-1β and stool antigen (HPSA).

Materials and methods

Sample collection: The study included 84 patients referred to medical diagnostic laboratories in Ilam, Iran. Among the study subjects included were 42 patients showing positive for *H. pylori* stool antigen (HPSA) test and 42 with negative HPSA test result. Demographic characteristics of study population (such as gender, age, education, occupation) was collected using a questionnaire after explaining the objectives of the study, and an informed consent was obtained from individuals for participation in the study. Blood and stool samples were collected and transferred to immunology laboratory of Ilam University of Medical Sciences to perform experiments and studies.

DNA extraction and PCR: DNA was extracted from all stool samples using a DNA isolation kit manufactured by Sina Clon Company, and samples of extracted DNA were kept at -20°C after concentrated by ethanol. PCR was used to assess the presence of *16srRNA* genes in extracted DNA samples, for this purpose, specific primers were developed for the gene by IDALLEL software Version 5 as follows:

F: GCAATCAGCGTCAGTAATGTTC and R: GCTAAGAGATCAGCCTATGTCC,

based on this paired primer in the case of the presence of *16s rRNA* gene in PCR product; bands with a length of 521bp were identified with the help of markers. PCR was performed on a Termocycler manufactured by EPENDORF Company at temperature conditions as follow; 95°C for 5 minutes (Hot Start), followed by 35 cycles, each consisting of 94°C for 1 minute (Denaturation), 51°C for 1 minute(Annealing), 72°C for 1 minute(Extention), and at the end 72°C for 10 minutes(Final Extension). Target DNA was replicated at a final volume of 25 µL, including 12 µL of Mastermix prepared by Ampliqone Company in Finland, 7 µL sterile distilled water, 1.5 µL of each of forward and reverse primers, and at the end 3 µL of template DNA i.e. DNA extracted from samples of stool which were poured into 0.2-mL microtubes and after a short shaking in Vortex, entered into the thermocycler. In the final step of the process of PCR, electrophoresis of PCR product was performed to determine the presence of *16s rRNA* gene, so that 7µL of the PCR product were immersed into wells of agarose gel 1% containing DNA Safe Stain dye and transferred to electrophoresis tank containing TAE-1X buffer, and an electrical current was passed through the gel with a voltage of 85 for an hour. Gel was photographed and examined using GeL Dock manufactured by12q Biorrad Company (Figure 1). ELISA was performed using serum samples for

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measuring the activities of serum TNF-α and of IL-1β cytokines.

Statistical analysis

The results of the study were statistically analyzed using Kolmogorov–Smirnov test, one-way ANOVA and Kruskal–Wallis analysis, and logistic regression by SPSS software. P<0.05 was considered as significant level.

Results

Among the control group who had tested negative HPSA test, none were positive for *16s rRNA* gene. However, in case group who had shown a positive HPSA test, 10 of 42 samples had *16s rRNA* gene in stool DNA samples (Table 1).

In this study, 45.2% of study subjects was female with a mean age 44.3 and 54.8% were male with an average age of 50.2 years. *16s rRNA* gene frequency in stool of both males and females was equal to 11.9%. To evaluate mean age, subjects were divided into five groups aged from 1 to 90 years with the highest frequency of gene in stool DNA samples was from the age group of 21 to 49 year with a frequency of 19%. Also in this study, in terms of education, the highest frequency of infected subjects with *H. Pylori* was in group of subjects with low educational level or even illiterate, but there was no statistically significant relationship in terms of the presence of *16s rRNA* gene in stool DNA samples of these subjects.

The rate of the studied cytokines the highest frequency of subjects with TNF-α above the cut-off rate was found in samples that were positive in terms of *16s rRNA* with a mean value of 125 pg / ml.

There was no significant relationship between the presence of *16s rRNA* gene in stool DNA samples and age, (P>0.05), but there was a significant relationship between the presence of this gene and quantitative variables WBC, RBC, TNF-α, IL-1β and HPSA (P<0.05); as seen in the first look for the subjects who have this gene in their stool DNA, levels of these variables were reported as compared to other subjects.

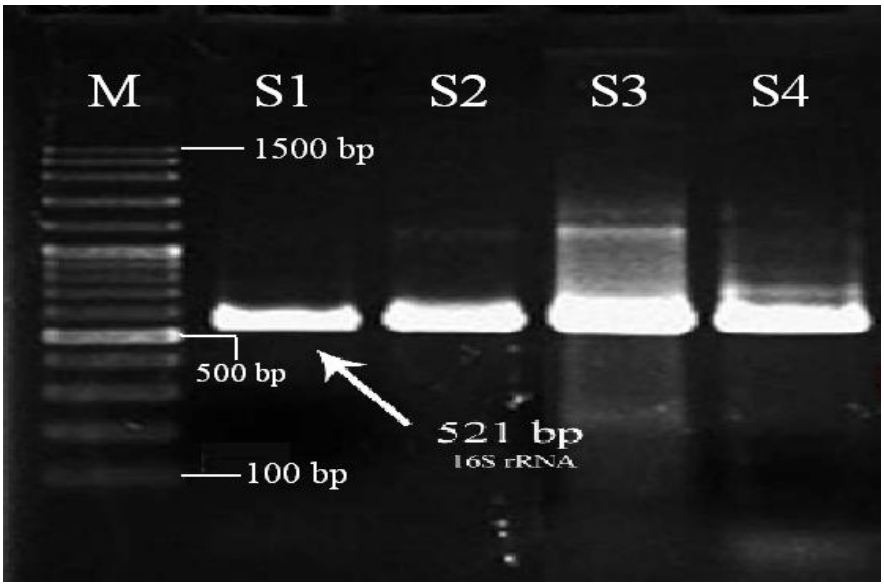


Figure 1. Photo of 1% agarose gel with 521 bp bands of 16s rRNA gen. M, Marker 100-1500bp; S, Samples.

Table 1. The results of the study & PCR for *16s rRNA* gene.

Group	Number of subjects	Female	Male	Age (years)	*16s rRNA* Frequency

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Case (HPSA+)	42	26.2%	23.8%	42.3	23.8%
Control (HPSA-)	42	19%	31%	52.5	0
Total	84	45.2%	54.8%	48	11.9%

Discussion

H. pylori is the most common cause of chronic bacterial infection and the major cause of peptic ulcers in the world. According to the World Health Organization, approximately 50% of adults in developed countries and almost 90% of adults in developing countries are infected with *H. pylori* (10). Colonization often takes place in early childhood and before the age of 10 (11). In developing countries, 70 to 80 percent of children become infected with the bacteria up to the age of 15. Living in areas with low socio-economic level has primary role in the transmission of the infection (12).

Previous studies show that the prevalence of *H. pylori* is more in men than women (13, 14, and 15). The reverse is also evident in other studies results (16, 17). In the present study, the prevalence of female patients was higher than that of male patients.

The mean age of patients was between 40 and 60 years among previous studies (14, 17, 18). The average age of those infected with the bacteria in present study was 42.3; however there was no significant relationship between *16s rRNA* gene and age of the subjects.

However, by comparison of serum levels of proinflammatory cytokines TNF-α and IL-1β in patients with the gene and negative subjects, the importance of *16s rRNA* gene can be further understood in the severity of inflammation in the body of infected people.

In previous studies, stool antigen test was introduced as a low-cost and rapid diagnostic method with high sensitivity and specificity for the detection of *H. pylori* infection in children (19-21). We also used this non-invasive diagnostic test as the gold standard in our study.

Previous reports have shown that increased proinflammatory cytokines may also affect the pathological process of *H. pylori* infection. TNF-α can be found in a state of chronic inflammation. It has been shown that *H. pylori* releases a protein inducing a 19 kDa necrosis factor alpha (Tip) that can increase TNF-α gene expression in the stomach by DNA-binding activity. Some researchers have shown that serum levels of TNF -α in patient with *H. pylori* infection and over-expression of the cytokine lead to tumor induction and promotion of peptic cancer (22). In this study, serum levels of IL-1β and TNF-α cytokines did not show a significant difference with variables, but the difference in the mean values of these immune factors between the case and control groups was significant. Although there was not a statistically significant relationship between gender and these cytokines, the average IL-1β and TNF-α were higher in males than in females.

In a study on circulating serum levels of IL-6, IL-8 and TNF-α in patients with *H. pylori* infection, there was not a significant difference in serum levels of TNF-α and age and gender between positive and negative *H. Pylori* groups (23), which is consistent with the results of our study. The results of this study and the study by Fan showed that the pathogen makes no significant change in serum levels of TNF-α (23, 24). However, data from our study showed an increase in serum levels of IL-1β and TNF-α cytokines in people infected with *H. pylori*, in another study, the average of these two cytokines was significantly higher in infected group with *H. pylori* than non-infected individuals (22, 25).

According to the information cited about IL-1β and TNF-α cytokines it is expected that for individuals with high HPSA response especially in chronic conditions, levels of these cytokines, or average of the two, as well as frequency of subjects with high levels of IL- 1β and TNF-α to be

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higher in the case group than the control group and this has been well demonstrated in our research, because the average of the cytokines in the case group is about one and a half to two times more than the average in the control group. However, it is also useful noting that increased levels of the cytokines have been observed in the ages from 21 to 50 years.

PCR is a powerful technique with high sensitivity, which can detect small numbers of *H. pylori* present in a sample including stool samples (with a success rate of 25 to 100%). In general, the difference in the detection rate of *H. pylori* in stool samples is due to destruction of the bacteria in the digestive tract or the presence of inhibitors such as complex polysaccharides and also low concentration of bacteria in the stool. The sensitivity of this method in recent studies has varied from 21 to 65.22 percent (4).

Conclusion

According to the obtained results, it may be concluded that activities of cytokines TNF-α and IL-1β positively correlated with the presence of HPSA antigen and the *16s rRNA* gene in the stomach's lining. An increase in responses to fecal antigens and TNF-α is associated with an increased chance of presence of *16s rRNA* in feces.

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