

# Antigenic and immunogenic evaluation of *Helicobacter pylori* FlaA epitopes

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## ABSTRACT

**Objective(s):** *Helicobacter pylori* are among most common human pathogens affecting at least half of the world's population. Mobility is one of the important primary factors in bacterial colonization and invasion. The purpose of this research is cloning, expression, and purification of FlaA protein specific epitopes in order to evaluate their antigenicity and immunogenicity.

**Materials and Methods:** The antigenic region of the *flaA* gene was bioinformatically predicted using Epitope mapping software's and the predicted epitopes were expressed in a prokaryotic expression vector. The antigen was injected into the animal model (mice BALB/c) and some indicators including IgG1, IgG2a, IgA, IFN- $\gamma$ , and IL 5 were measured.

**Results:** The immunogenicity studies in animal models by measuring serum antibodies (IgG1, IgG2a, and IgA) and cytokines (IFN- $\gamma$  and IL5) revealed that the rFlaA induces a proper immune response in animal models.

**Conclusion:** The recombinant FlaA protein is antigenic and immunogenic. Therefore, it might be used in order to design of specific diagnostic kits and recombinant vaccines against *H. pylori*.

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## Introduction

*Helicobacter pylori* is a Gram-negative, microaerophilic, spiral bacterium colonized in the human gastric mucosa that are associated with several disorders such as atrophic gastritis, gastric adenocarcinoma, peptic ulcers, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma (1, 2). Many studies have shown that *H. pylori* is a facultative intracellular microorganism (3, 4) being able to reproduce in the epithelial cell (5), macrophages (6), and dendritic cells (7). More than 50% of world's populations are infected by this bacterium. Indeed, approximately 20-30% of people in developed countries and up to 70- 80% in developing countries are affected by *H. pylori* (8). The infection rate in Iran, as a developing country, is about 80% (9). The fourth most prevalent type of cancer in the world is gastric cancer that is the second cause of cancer-related fatality with approximately 700,000 deaths per year (10). The International Agency for Research on Cancer attributed 92% of stomach cancers to *H. pylori* and classified it as a Group 1 carcinogen (11). The high prevalence of infections, high expenses of antibiotic treatment and

the increasing rates of antibiotic resistance have necessitated developing vaccines against *H. pylori* (12). Mobility is one of the important essential factors in invasion and colonization of bacteria. In this regard, nonmotile mutants are either less efficient or unable to create infection in the host (13). At the beginning of entering the stomach area, *H. pylori* use lophotrichous flagella as a driving force to reach the region with a low acidity (antrum) and penetrate to epithelial layers for colonization (14, 15). There are 3-7 flagella in *H. pylori*, which consist of two proteins called FlaA (53kDa) and FlaB (54kDa)(16). FlaA is the dominant protein in the whole and surface structure of flagella (17). The genes encoding these proteins are conserved among different strains, therefore, they can be good choices for the production of vaccines and diagnostic kits (18). Identification of the conserved microbial antigens to stimulate host immunity responses is one of the main approaches in order to achieve efficient recombinant vaccines. Therefore, to prevent cross-reactions, easy manipulation, increasing the diagnostic kits' specificity, prevention of carcinogenesis potential, and the use of immunodominant epitopes or combination of multiple

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epitopes instead of full antigens is a more acceptable strategy (19, 20). Therefore, in the current research, the specific region of FlaA protein with appropriate immunodominant epitope was predicted by bioinformatics software and the recombinant protein was evaluated in terms of antigenicity and immunogenicity potential.

## Materials and Methods

### Cultivation and isolation of genomic DNA

Endoscopic biopsy of *H. pylori* positive patients were cultured on Brucella agar medium (Merck, Germany) containing trimetoprim (5 µg/ml), vancomycin (10 µg/ml), amphotericin B (2.5 µg/ml), enriched with 5% of sheep blood. Three days after incubation under microaerophilic conditions. The presence of *H. pylori* were confirmed using standard microbiological and biochemical assays and the genomic DNA was extracted using standard CTAB/NaCl method. To determine the purity, the bacterial chromosome was assessed by 0.8% of horizontal agarose gel electrophoresis in 1XTBE buffer. The quantity of DNA sample was confirmed by spectrophotometry at 260 nm (21).

### Identification of antigenic regions and gene amplification

The FlaA protein sequence of *H. pylori* strain J99 (AAD06133) with a length of 510 amino acids was obtained from NCBI and the antigenic part 214-353 (138 aa) was identified using bioinformatics programs including IEDB and IgPred software's. The appropriate primers for the target region of *flaA* gene sequence (AE001439) with a length of 414 bp were designed by Alell ID software and their specificity was confirmed by Blast program. The forward primer:

(5'-TATGGATTTCGGCGTGTAGCAGAAGTGAT-3')

contains restriction site for *Bam*HI and Reverse primer:

(5'-ATTCTCGAGGACATTGATGCTCTTAGCGT C-3')

contains *Xho*I, underlined. Polymerase chain reaction were performed in 50 µl total volume contained the following : 3 µl of template DNA, 1 µl of each primers (10 picomole), 1 µl of dNTP, 4 µl of MgCl<sub>2</sub> (25 mM), 5 µl of PCR buffer (10X) and 1 µl of *Expand DNA polymerase* (Roche, Germany) mixed with 34 µl deionized water.

The following profile were used for amplification: an initial denaturation step at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min , extension at 72 °C for 1 min and a final extension for 5 min at 72 °C. The PCR products were evaluated by electrophoresis on 1% agarose gel and a 414 bp band was observed expectantly. The PCR product was sequenced by MWG Company (Germany) to confirm that the desired product had been obtained.

### Cloning and transformation

The PCR product was purified by purification kit (Roche, Germany) from the agarose gel. Subsequently the bacterial expression vector pET32a (Novagene, USA) and PCR product were digested with the same restriction enzymes, *Bam*HI and *Xho*I. The ligation process was performed by T4 DNA ligase (Cinaclon, Iran) at 22 °C for overnight. For transformation of recombinant pET32a, DH5α and BL21 (DE3) pLysS (Stratagene, USA) strains of competent *E. coli* cells were prepared by calcium chloride method. Because of having protease enzymes, the *Escherichia coli* DH5α was selected only for isolation and characterization of recombinant plasmids (jumping and PCR tests ) and the *E. coli* BL21 (DE3) pLysS was used as the expression host for production of recombinant protein (22).

### Expression and purification of recombinant FlaA

The resulting suspension (*E. coli* BL21 (DE3) pLysS) of transformation procedure was cultured on nutrient agar containing chloramphenicol (35 mg/ml) and ampicillin (100 mg/ml). The single colonies were cultured in 1.5 ml nutrient broth medium at 37 °C for overnight. The LB medium containing yeast extract 0.5 g, Bactopepton 1 g, glucose 0.1 g, NaCl 0.5 g, KCl 0.05 g, MgCl<sub>2</sub>.6H<sub>2</sub>O 0.025 g, CaCl<sub>2</sub> 0.025 g, nutrient broth 0.25 g, ampicillin (100 mg/ml), chloramphenicol (35 mg/ml) was prepared as induction medium in a volume of 50 ml. The next day, 500 µl of cultured bacteria injected to the medium and incubation at 37 °C was carried along by shaking until optical density at 600 nm reached to 0.6. Finally, the induction of protein expression was performed by IPTG (final concentration 1 mM) and sediments were collected in 2, 4, and 6 hr after induction. The resulting protein product was analyzed by SDS-PAGE and Coomassie Blue staining. Eventually, the expressed protein was purified by affinity chromatography (Ni-NTA kit Qiagen, USA). Due to the attachment of 6xHis tag to C terminal of protein product, it could bind to the resins. Subsequently, the purified protein was dialyzed by phosphate buffered saline (PBS) (pH= 7.2) at 4 °C overnight. The quality and quantity of the purified recombinant FlaA protein were analyzed by SDS-PAGE and spectrophotometry (260/280 nm) methods.

### Western blotting

The purified antigenic fragment of FlaA protein were transferred on PVDF membranes and bands visualized by Ponceau S staining. After washing with the distilled water, the blotted membranes were blocked by 2.5% (w/v) BSA in TBS buffer for 1 hr. Subsequently, the membranes were incubated for 1.5 hr at room temperature with 1: 100 dilution of antibodies (serums of patients with *H. pylori* active infection and healthy individuals' serum as a negative control) on a rocker. Washing repeated and

the membranes were incubated for 2 hr at room temperature with a second antibody HRP-conjugated anti-mice IgG (ABcam, UK) with 1:1000 dilution in 1X TBS buffer. The reaction was developed by diaminobenzidine (DAB) (Roche, Germany) as a substrate solution and bands were visualized (11).

### **Immunogenicity assay**

To evaluate the immunogenicity, six-week old BALB/c mice in 2 groups (5 per group) were selected. In order to adaptation and confirm health, animal models were kept for a week in lab environment. Afterwards, the injections were performed subcutaneously in the groin area in accordance to the time table in the early, fourteenth and twenty-first days. The mixture of 150 µg rFlaA antigen and 100 µl of complete Freund's adjuvant for the first injection and the mixture of 75 µg of rFlaA antigen and 100 µl of incomplete Freund's adjuvant for the second and third injections were used for each mouse (total volume of the all injections were 200 µl). At the end of twenty-eighth day, the blood serums and the spleen cells were obtained to evaluate serum antibodies IgG1, IgG2a, IgA and cytokines IFN-γ, IL5. Furthermore, the MTT assay was performed (13).

### **Preparation of sera and cell suspensions**

The mice were anesthetized and blood samples were collected. To separate the sera, centrifuging at 3000 rpm for 10 min at room temperature were performed and the samples were stored at 4 °C. Immediately for each mouse, the spleen was isolated and perfused with RPMI 1640. The resulting suspensions were poured on 4 ml ficoll gradient separately and centrifuged at 1,500 rpm for 15 min at 20 °C. The buffy coats were isolated by Pasteur pipette and cells washed three times with PBS solution (centrifuged at 3000 rpm for 15 min at 4 °C). Finally every sample mixed by RPMI1640 medium containing 10% FCS and the dilutions were reached to  $1-2 \times 10^6$  cell/ml. The obtained cell suspensions were used to assess cytokines and MTT assay (23).

### **Determination of serum IgG1, IgG2a and IgA**

Standard ELISA plate (MaxiSorp, Nunc A/S, and Denmark) was coated overnight at 4 °C with the rFlaA protein (50 µl per well) at a final concentration of 5 µg/ml in PBS (pH: 7.4). After 3 washing cycles (PBS with 0.1% of Tween 20) each well was saturated with 150 µl of blocking buffer (PBS with 1% BSA) for 2 hr at room temperature. Three washing cycles were repeated and 50 µl of the diluted sera (every group 5 samples) with PBS (1:5) added to related wells and

incubated for 1 hr at 37 °C. The sera of nonimmunized mice were used as a negative control. After the 3 washing cycles, 50 µl of anti-mouse IgG1, IgG2a and IgA (AB-Cam USA) (1:1000) were added to each wells and incubated for 1 hr at 37 °C. Three washing cycles were repeated and 100 µl of OPD(O-phenyldiamin, Daku Denmark) added to wells as substrate of the reaction and incubated 10 min in dark situation. The reaction was stopped by addition 100 µl of 2M H<sub>2</sub>SO<sub>4</sub> and optical density measured at 490 nm (13).

### **MTT test**

Proliferation was checked by 3-(4,5-dimethyl- thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay method. A total of  $2 \times 10^6$  cell/ml in RPMI -1640 supplemented with 10% FBS were stimulated with 1 µg/ml PHA or 10 µg/ml of rFlaA. The plates were incubated in a 5% CO<sub>2</sub> at 37 °C for 72 hr. Twenty micro liters of MTT (5 mg/ml) were added to the cells, following by incubation for 4 hr. After centrifugation, the medium was removed and 200 µl of dimethyl sulfoxide (DMSO) were added to each well. The optical density (OD) values of stimulated and non-stimulated cells were measured at 540 nm using a micro-titer plate reader (Stat Fax2100,USA). All experiments were performed in triplicates. Proliferation responses for MTT assay were expressed in terms of the mean stimulation index (SI) and obtained by dividing the optical density values of stimulated cells by the respective OD values of the non-stimulated ones (13).

### **Cytokine assay (IFN-γ and IL-5)**

In order to measure IFN-γ and IL-5, 1 ml of the cell suspensions ( $1-2 \times 10^6$  cell/ml) were added to each wells of the microplate (24 wells) and the samples sorted to control (Ag<sup>-</sup>) and Ag<sup>+</sup> as ternary. 10 µg/ml of rFlaA was added to the related wells and Incubation for 72 hr at 37 °C with 5% CO<sub>2</sub> performed. The supernatant of the wells were collected and the level of IFN-γ and IL-5 measured by R&D Quantikine Mouse sets (USA). In this way, the optical densities of the standard samples were measured and according to the standard curves amount of cytokines (pg/ml) obtained. The sensitivity of test for IL-5 and IFN-γ was 7 pg/ml and 2 pg/ml, respectively (13).

## **Results**

### **Amino acids sequence and DNA amplification**

The amino acid sequence of the antigenic region of FlaA (138aa) and its corresponding DNA sequence (414 bp) were predicted by epitope mapping softwares such as: IEDB Analysis Resource and IgPred softwares (Figure 1).

MAFQVNTNINAMNAHVQSALTQNALKTSLERLSSGLRINKAADDASGMTVADSLRSQASSLGQAIANT  
NDGMGIIQVADKAMDEQLKIDTVKVKATQAAQDGGTTESRKAIQSDIVRUQGLDNINNTTYNGQALL  
SGQFTNKEFQVGAYSNOIKASIGSTTSKIGQVRIATGAUTASGDISLTKQVDGVNDVTLESVKVSSSAG  
TGI **GVLAEMNKNSNRTGVKAYASVITTSQVAVQSGSLNLTNGIHLGNIAIKKNDSDGRIVAANAVTS**  
**ETGVEAYTDQKGRNLRSIDGRGIEKTDSSVNGPSALTMVNGGQDLTKGSTNYGRISLTRDAKSINVS**  
ASDSQHLGFTAIGFGESQVAETTVNLRDVTGNFNANVKSASGANYNAVASGNQSLGSGVTLRGAMVV  
IDIAESAMKMLDKVRSDLGSVQNMISTVNNISITQVNVKAAESQIRDVDFEESANFNKNILAQSGSY  
AMSQANTVQQNLRLLT

Figure 1. The result of antigenic fragments of FlaA protein

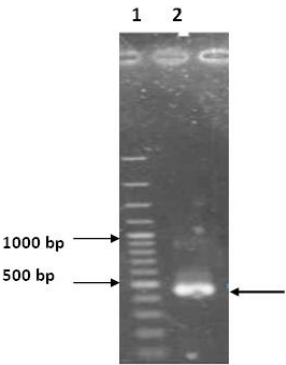


Figure 2. Antigenic region of FlaA gene amplification by PCR. Lane 1, molecular weight marker (100 bp ladder); Lane 2, amplified gene

The specific primers were designed and DNA amplification showed the expected size of 414 base pair (Figure 2) comparing to 100 bp DNA ladder 321 (Fermentas). The sequencing result of the PCR product fragment revealed complete homology at the nucleo-tide level to FlaA gene in NCBI.

Expression and purification of recombinant protein

The pET32a containing rFlaA epitope was transformed into the *E. coli* BL21 (DE3) pLysS and expression of the recombinant protein induced by IPTG (1 mM). SDS-PAGE analysis of the induced cells showed the band with a desired molecular weight of 35 kDa (Figure 1). Because of adding 6 amino acids (6xHis- tag) to the end of products by pET32a expression system, the purified rFlaA protein was obtained using Ni-NTA resin by affinity chromatography (Figure 3).

Immunoblotting analysis

The Western blot analysis was used to investigate the antigenicity of the recombinant FlaA protein. In this test, 4 blood serum samples from patients with *H. pylori* acute infection and a serum sample from a healthy individual examined and bands were observed in the range of 35 KDa. The results shown in the Figure 4.

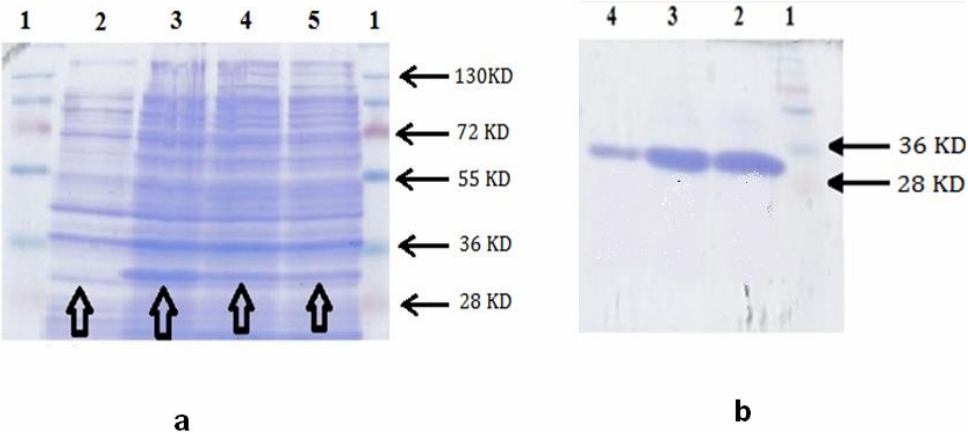
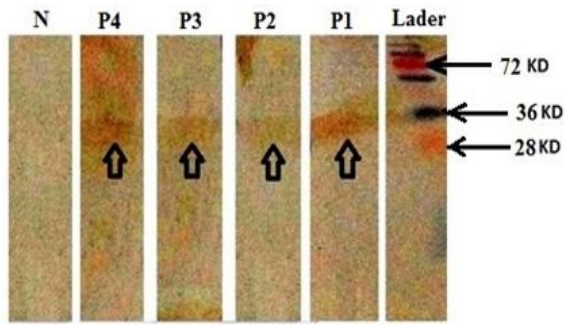


Figure 3. Expression and purification of rFlaA protein. 1a: expression of rFlaA protein, Lane 1: protein marker, Lane 2: pET32a-FlaA before induction, Lanes 3: pET32a-FlaA 2hr after induction, Lane 4: pET32a-FlaA 4hr after induction, Lane 5: pET32a-FlaA 6hr after induction 1b: purification of rFlaA protein by Ni-NTA kit. Lane1: protein marker, Lane2-4: elution of FlaA protein through the Ni-NTA column





**Figure 4.** Western blot analysis of FlaA antigenic fragment  
N: Western blotting by *Helicobacter pylori* negative sera (negative control), P1-P4: Western blotting by *Helicobacter pylori* positive sera from patients

#### Humoral response to recombinant FlaA protein

Sera collected one week after the last injection were assayed for the presence of recombinant FlaA protein specific antibodies (IgG2a, IgG1 and IgA) by ELISA. The optical densities of antibodies at 490 nm were measured (Figure 5).

The antigenic region FlaA immunizations induced antibody IgG2a responses ( $1.9 \pm 0.26$ ). This antigen also induced IgG1 titers which were lower than the IgG2a titers ( $1.716 \pm 0.13$ ). The level of specific IgA against antigenic region FlaA was higher than PBS treated group ( $0.5 \pm 0.13$ ).

Statistical analysis (ANOVA,  $P < 0.05$ ) showed a significant difference between the means of immunized and control groups ( $P < 0.05$ ).

#### The results of MTT test

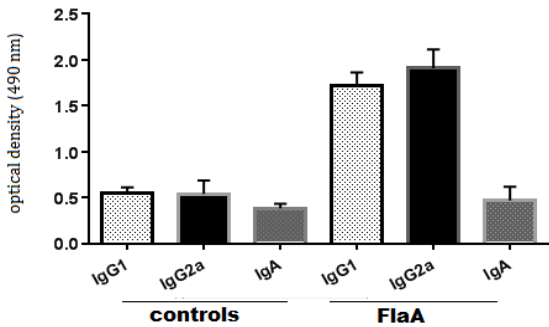
The specific Tcell proliferative response by rFlaA was obtained 1 week after the last injection and the PHA mitogen used as positive control. By comparing the optical density of results at 570 nm, it was found that rFlaA protein has the ability to induce specific cell proliferation. Statistical analyses were showed a significant difference between the means of immunized and control groups (data not show).

#### Cytokine profile of immunization by rFlaA

The production of IFN- $\gamma$  and IL-5 in the supernatants of splenic lymphocytes cultures was measured by ELISA. One week after the last injection, the optical density of cytokines IFN- $\gamma$  and IL-5 were measured according to the related standard curves at 492 nm.

Stimulation of splenic lymphocytes from mice after vaccination with antigenic region of FlaA resulted in significantly higher levels of the IFN- $\gamma$  and IL5 than stimulation of cells from PBS-immunized mice.

Splenic lymphocytes of immunized mice, on average released up to 135 pg/ml of IFN- $\gamma$  (Figure 6a). Nevertheless, few amounts of IL-5 (8 pg/ml) was detected (Figure 6b). High levels of IFN- $\gamma$  compared



**Figure 5.** The optical density of sermic IgG2a, IgG1, IgA at 490 nm

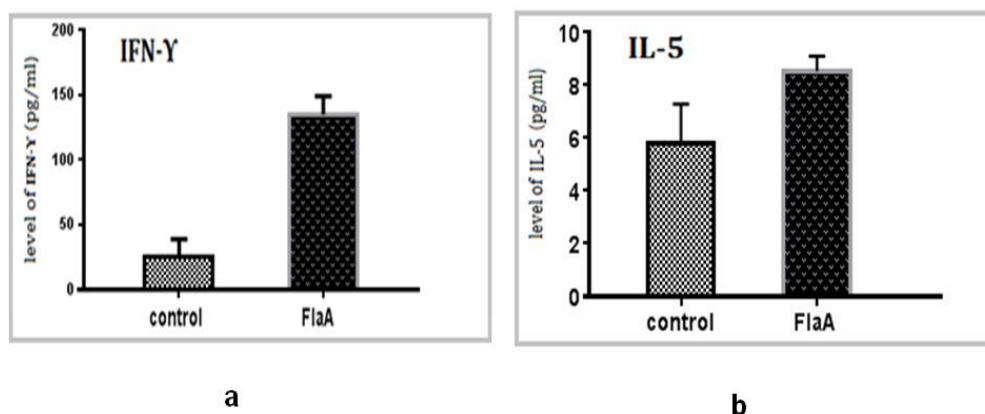
with IL-5 indicated that there was a significant Th1 based response. Statistical analysis was showed a significant difference between the means of immunized and control groups. ( $P < 0.05$ ).

#### Discussion

In this study, immunization with an antigenic fragment of *H. pylori* flagella induced a significant level of immuno response in mice.

Protective immunity against *H. pylori* is not fully understood. Data obtained from transgenic mice suggest that MHC-II restricted CD4+ T lymphocytes play an essential role in immunity, much more than humoral immunity (24). However, many studies show that cell immunity in infection is caused by *H. pylori*. The activation of Th1 results in the formation of pro-inflammatory response that kills the pathogens (25). Other studies showed that the T lymphocytes play a main role in protection against *H. pylori* infection (13). For many years, it has been accepted that Th2 gives protection against *H. pylori* (26); but studies with outstanding Th1 pathways revealed that by IL-5 and IFN- $\gamma$  production, disclosed that protection is due to Th1 responses (27). According to information obtained from experiments based on IFN- $\gamma$  and IL-5 deficient, mice showed that the increased inflammation is connected with *H. pylori* infection, which signifies the vitality of Th1 pathway (25). However, other studies suggested that both Th1 and Th2 responses might have a crucial role in the protection against *H. pylori* infection (28). In this regard, IFN- $\gamma$  also acts on B cells to stimulate the production of certain subclasses of IgG antibodies, especially IgG2a.

The flagellum is a key virulence factor related to *H. pylori* motility, evasion, and persistent colonization (29). Initially, *H. pylori* cause a noninvasive superficial infection of the gastric epithelial surface. Other studies have shown that *Vibrio cholerae* flagellin is a candidate for stimulating immune responses (13). Thus, we evaluated immunologic responses to an anti- *H. pylori* vaccine containing antigenic epitope of FlaA.



**Figure 6.** Quantitative ELISA analysis of IFN-γ (a) and IL5 (b) measured in the lymphocyte culture of mice immunized with specific recombinant protein

In the present study, we detected antigenic region (an epitope) of FlaA, which can induce specific neutralizing antibodies and cellular immune response against FlaA subunit as a potential candidate for controlling *H. pylori* infection. We hypothesized that there are multiple mechanisms for activating protective immunity against *H. pylori* due to the complexity of the immune system network. Therefore, we selected Th-epitope from FlaA for activation of the immune response. The lymphocyte cell proliferation assay showed that the Th-epitope could stimulate high proliferative responses and analysis of the cytokines, suggesting that IFN-γ and IL-5 were all significantly induced by an epitope of FlaA. These results indicate that epitope of FlaA induced a mixed Th1-Th2 immune response, which might contribute to the reduction of bacteria in the stomach. Besides, immunization with FlaA recombinant protein significantly increased the levels of specific IgG1, IgG2a, and IgA compared with PBS group. For mice, it is generally accepted that the IgG1 and IgA response reflects helper activity of Th2 CD4+ T cells, where IgG2a results from Th1 activity (30). Therefore, it was also speculated that antigenic region of FlaA induced a mixed Th1-Th2 response.

In this study, evaluation of IFNγ and IL5 responses after stimulation of spleen cells with the recombinant antigenic region of FlaA exhibited the ability of this protein to produce these cytokines. The results showed that the IFN-γ level in the supernatant of PBMCs from immunized mice significantly increased ( $P=0.001$ ). The level of IL-5 in the vaccinated group showed a high production of a cytokine in mice immunized with the recombinant antigenic region of FlaA. The level of IL-5 in cell culture in the vaccinated group was significantly increased ( $P=0.029$ ). In addition, the results show that IgA and IgG antibodies response in mice immunized by antigenic region of FlaA are detectable in the serum. According to the results, it can be concluded that recombinant antigenic region of FlaA

stimulates immune responses toward Th1. But, high levels of IgG1 and IgA titers and IL5 are observed in the group vaccinated with the recombinant antigenic region of FlaA. Furthermore, consistent with other studies, we demonstrated that the antigen is capable of stimulating effective immune responses. The results of Le Guo and colleagues confirm the data of this study. In a study conducted by Guo, humoral and cellular immunity was stimulated in mice, which prevented infection in vaccinated mice. They showed stimulated humoral and cellular immunity in mice vaccinated with *H. pylori* urease and *V. cholerae* toxin (25). In contrast, in our research, cellular and humoral immune responses were stimulated in mice with flagella antigenic regions.

The results show that antigenic region of flagella of *H. pylori* can cause strong humoral and cellular immune responses after mice immunization. Evaluation of cytokine responses after stimulation of spleen cells with recombinant proteins FlaA exhibited the ability of these proteins to produce IFN-gamma and IL5. Assessing the levels of IgG1 and IgG2a antibody titers also confirmed the cytokine results.

## Conclusion

It can be concluded that FlaA stimulates immune responses toward Th1 and Th2. Therefore, stimulation of cellular and humoral immune responses can cause restricted bacteria in mucosal and infested tissue. However, since using vaccines consisting of pathogen subunits is more efficient when two or more subunits are used, applying FlaA and other antigens in *H. pylori* vaccination can cause higher immunogenicity.

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## Conflict of interest

The authors declare that no conflict of interest exists.

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