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Antigenicity Identification of a Novel Recombinant Multi-Epitope Antigen Based on *FlaA* and *UreB* Antigens of *Helicobacter pylori*

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Abstract

Background: *Helicobacter pylori* is the main cause of stomach ulcers and gastric cancer. Hence, the diagnosis, treatment, and prevention of *H. pylori* infection can considerably reduce the fatality.
Objectives: This study aimed to construct a dual-antigen protein by combining the antigenic regions of *UreB* and *FlaA* of *H. pylori* and determine its antigenicity as a promising vaccine and serodiagnosis candidate.
Methods: The antigenic regions of *FlaA* and *UreB* were detected by immunological bioinformatics, amplified and joined together by polymerase chain reaction (PCR) with special primers containing linker sequences. Then, it was cloned into *pET-32a* and after expression and purification of the recombinant multi-epitope protein (*rFlaA-UreB*), its antigenicity was evaluated by immunoblotting using the sera of infected patients.
Results: DNA sequencing and enzyme digestion analysis showed the *rFlaA-UreB* gene was successfully inserted into pET32a. The recombinant protein was produced and purified via affinity chromatography and its molecular weight was similar to what had been predicted. Moreover, data indicated that *rFlaA-UreB* was recognized by all patients' sera and its sensitivity and specificity were high.
Conclusions: Although the developed recombinant multi-epitope protein was very smaller and lighter than the natural forms of these two critical antigens, they all had close antigenic properties. Therefore, this recombinant protein can be an important antigen in the diagnosis and vaccination against *H. pylori*.

Keywords: Flagellum, Recombinant Protein, Urease, *Helicobacter pylori*

1. Background

Most people in the world have a history of infection with *Helicobacter pylori*, a Gram-negative, spiral-shaped, and microaerophilic bacillus that resides the human gastric and duodenal mucosa. Persistent, chronic infection with *H. pylori* induces stomach ulcers and gastric cancer (1-4). *Helicobacter pylori* is recognized as a class I human carcinogenic agent and one of the etiological agents in human gastric adenocarcinoma (5). Thus, it seems that prevention, early detection, and treatment of this infection can decrease morbidity and mortality rates in infected patients. Unfortunately, current treatment against *H. pylori* infection is associated with some drawbacks such as reinfection, increased antibiotic resistance, side effects, patient compliance, and high cost (6, 7). Therefore, vaccination against this bacterium could be a potential way to control the *H. pylori* infection.

Epitope-based vaccines are a new strategy for increasing immune response to pathogens. Some advantages of an epitope vaccine include (i) increased safety, (ii) increased immunogenicity of predefined epitopes, and (iii) ability to focus immune responses on conserved epitopes (8). However, research has proven that immunity created against *H. pylori* using a single antigen does not lead to effective protection, rather effective immunity against this infection is achieved via a combination of various antigens (9-11). Some commercial diagnostic kits used to detect *H. pylori* infection utilize a combination of antigens because this strategy provides high sensitivity and specificity compared to a single antigen (12-15).

Based on previous studies, *UreB* and *FlaA* are excellent candidates for the production of vaccines and diagnostic antigens. *UreB* (the B subunit of urease and one of the four subunits of this enzyme) elicits an immune response;

it acts as a portion of whole urease to metabolize urea to ammonia and generate a neutral environment (16-20). *FlaA* (the predominant subunit of the flagella) is an important pathogenic factor responsible for *H. pylori* colonization and persistent infection (7, 21-23).

2. Objectives

In this study, we designed, constructed, cloned, and expressed a recombinant multi-epitope gene based on the combined immunodominant regions of *UreB* and *FlaA* (*rFlaA-UreB*) genes of *H. pylori*. Moreover, we detected its antigenicity as a vaccine candidate and diagnostic antigen by using infected human sera in western blot analysis.

3. Methods

3.1. Chromosomal DNA of H. pylori, Strains, Plasmids, and Media

Chromosomal DNA of *H. pylori* was purchased from Imam Khomeini Hospital (a teaching university hospital in Tehran, Iran). *Escherichia coli* strain *DH5α* (Stratagene, USA) was used for cloning. The recombinant protein was produced by *pET32a* plasmid (Novagene, USA). The *FlaA-UreB* recombinant protein was produced using *pET32a* in *E. coli BL21 (DE3) pLysS*.

3.2. Identification of Antigenic Regions

We used *UreB* (NC-018939.1) and *FlaA* (WP-000885496.1) gene sequences for epitope mapping. B-cell epitope prediction was performed using IEDB, ElliPro, DiscoTope, and IgPred (24).

3.3. Gene Replication

Specific primers required for polymerase chain reaction (PCR) were designed using Allele ID software. The *FlaA* gene of *H. pylori* was amplified by primers 5'CGGGATCCGGCGTGTTAGCAGAAGTGAT3' (forward primer) and 5'CGCCGCCGCCAGGACATTGAGCTCTTAGCGTC3' (reverse primer) with a linker sequence. The *UreB* gene was amplified by 5'CTGGCGGCGGCGGCTATGGGTCGTGTGGGT3' (forward primer) and 5'CCGCTCGAGGAAAATGCTAAAGAGTTGCG3' (reverse primer) with a linker sequence. The replication of the *FlaA* gene was done in a 25-μL volume, containing 1 μL of template, 10 pmol of each primer, 50 mM of $MgCl_2$, 0.5 μL of dNTP, 2.5 μL of buffer (10×), and 1 unit of Taq polymerase. Proliferation of the *FlaA* gene was done under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 56°C for 20 seconds, extension at 72°C for 40 seconds, and a final extension at 72°C for 5 minutes.

The PCR of the *UreB* gene was performed in a total volume of 25 μL under the same PCR conditions as the *FlaA* PCR amplification (except for annealing at 62°C for 20 seconds) (25). Then, for making the conjugated gene as a template for last PCR amplification, we prepared a tube in a total volume of 25 μL, containing 1.5 μL of *FlaA* PCR product, 1.5 μL of *UreB* PCR product, 1.5 μL of $MgCl_2$ (50 mM), 0.5 μL of dNTP, 2.5 μL of buffer (10×), and 0.5 μL of the Taq polymerase (neither of primers was used) and amplified by 20 cycles in the same PCR conditions as the *FlaA* and *UreB* PCR amplification (except for annealing at 55°C for 20 seconds). The final PCR was done in a 25-μL volume containing 2 μL of template (1 to 6 diluted previous PCR product in double distilled water), 0.5 μL of forward primer of *FlaA*, 0.5 μL of reverse primer of *UreB* (10 pmol), 1.5 μL of $MgCl_2$ (50 mM), 0.5 μL of dNTP, 2.5 μL of buffer (10×), and 0.5 μL of the Taq polymerase. The final duplication was performed at an annealing temperature of 58°C.

3.4. Recombinant Plasmid Construction and Purification

The PCR product, *rFlaA-UreB*, was purified by the high-pure PCR product purification kit (Roche, Germany). Both *pET32a* and PCR product were digested with *BamHI* and *XhoI* and ligated by T4 DNA ligase (Cinagene, Iran). Moreover, *E. coli BL21* (*DE3*) *pLysS* (Stratagene, USA) and *E. coli DH5α* (Stratagene, USA) component cells were prepared using calcium chloride ($CaCl_2$) method (25) and transformed by plasmids. The *pET32a-FlaA-UreB* was transformed into *E. coli BL21 (DE3) pLysS* cells as the host for the expression of recombinant protein production (25). To confirm the transformation of *pET32a-FlaA-UreB* into *E. coli BL21 (DE3) pLysS*, the PCR and enzymatic digestion with *BamHI* and *XhoI* were performed. Finally, the nucleotide sequences of recombinant plasmid *pET32a-FlaA-UreB* were analyzed using the dideoxy chain termination method by standard primers, according to a procedure described by Sanger et al. (26).

3.5. Expression of Recombinant Dual Antigen Multi-Epitope Protein rFlaA-UreB

The *pET32a-FlaA-UreB* plasmids were transformed into *E. coli BL21 (DE3) pLysS* grown in 50 mL of nutrient broth supplemented with ampicillin (100 μ/mL) and chloramphenicol (35 μg/mL) at 37°C with vigorous agitation at 220 rpm. The cultured cells were grown until OD600 nm of 0.6. The production of *rFlaA-UreB* recombinant protein was induced by adding 50 μg of Isopropyl-β-D thiogalactopyranoside (IPTG) to the final concentration of 1 mM, followed by 4 hours of incubation. The recombinant protein was purified with Ni-NTA Agarose resin (Qiagen, USA) based on

the manufacturer's instruction. The purified recombinant protein was analyzed by SDS-PAGE (15%) and spectrophotometry (260/280 nm) (25).

3.6. Immunoblot Analysis

Immunoblot analysis was done (25) using the sera obtained from 15 seropositive *H. pylori* patients and 15 negative human sera detected by culture, urease test, and pathological analysis. For immunoblot analysis, 0.5 μg of the purified recombinant protein was used per well. Then, the recombinant protein was blotted on polyvinylidene difluoride (PVDF) membrane (Roche, Germany) using transfer buffer containing 25 mM of Tris (pH = 8.3), 192 mM of glycine, and 20% methanol at 90 V for 3 hours at 4°C. The blotted PVDF was blocked with 2.5% (w/v) BSA in TBS buffer for one hour at room temperature. Then, PVDF was incubated for 2 hours at room temperature with diluted (1:100) and normal patients sera. After reaction with the primary antibody, the blot was washed three times with Tris-buffered saline (TBS; 0.5 M of NaCl, 0.02 M of Tris, and 0.05% Tween-20, pH = 8.5) and incubated with anti-human IgG (Abcam, United Kingdom) in 1:1000 dilution. The blots were developed by diaminobenzidine (DAB) solution (Sigma, USA).

3.7. Statistical Analysis

Statistical analysis was performed using *t* test to evaluate differences in variables between the groups. Comparison between groups was made based on the Mann-Whitney U test. Data were analyzed by SPSS version 16.0 software.

4. Results

4.1. Detection of Antigenic Region

In this study, we predicted antigenic fragments of *FlaA* and *UreB* protein sequences through epitope mapping. We used IEDB software to predict the B-cell epitope. According to the results, amino acid sequences of 215 to 352 in *FlaA* (Figure 1) and 365 to 568 in *UreB* (Figure 2) were selected as regions with the most pronounced antigenic properties.

4.2. DNA Amplification, Multi-Epitope Gene Construction, and Molecular Characterization of rFlaA-UreB

The amplified genes had the expected size of 414 base pair (bp) in *FlaA* and 609 bp in *UreB* compared to a 100-bp DNA ladder (Figure 3). The *rFlaA-UreB* gene was constructed with 1035 bp compared to a 100-bp DNA ladder (Figure 4). The results of PCR and enzymatic digestion of the recombinant plasmid (*pET32a-FlaA-UreB*) with restriction enzymes confirmed the transformation and showed that the target gene was inserted into the vector correctly. Then, *pET32a-FlaA-UreB* was sequenced and the results were confirmed. Data showed that the target gene had 100% homology with the reported original sequences (data not shown).

GVLAEVINKNSNRTGVKAYASVITTSDVA

VQSGSLSNLTLNGIHLGNIADIKKNDSDG

RLVAAINAVTSETGVEAYTDQKGRLNLRS

IDGRGIEIKTDSVSNGPSALTMVNGGQD

LTKGSTNYGRLSLTRLDAKSINV

Figure 1. The result of *FlaA* antigenic fragment prediction

AMGRVGEVITRTWQTADKNKKEFG

RLKEEKGDNDNFRIKRYLSKYTINPAIA

HGISEYVGSVEVGKVADLVLWSPAFF

GVKPNMIIKGGFIALSQMGDANASIP

TPQPVYYREMFAHHGKAKYDANITF

VSQAAYDKGIKEELGLERQVLPVKNC

RNITKKDMQFNDTTAHIEVNPETYHV

FVDGKEVTSKPANKVSLAQLFSI

Figure 2. The result of *UreB* antigenic fragment prediction

4.3. Expression and Purification of Recombinant Protein

The molecular weight of the recombinant protein was estimated at 38.045 kDa. In the presence of 6xHis-tag in the N-terminal region of *rFlaA-UreB* added by the *pET-32a* vector, the protein molecular weight, purified using Ni-NTA resin affinity chromatography, was 57.62 kDa. The SDS-PAGE analysis of induced *E. coli BL21 (DE3) pLysS* showed a band similar to what that had been predicted with desired molecular weight (Figure 5).

4.4. Immunoblotting Analysis

In this study, the antigenicity of the recombinant protein was determined by Western blotting via the sera of humans who suffered *H. pylori* infection. Our data showed the desired band with an estimated molecular weight of 57.62

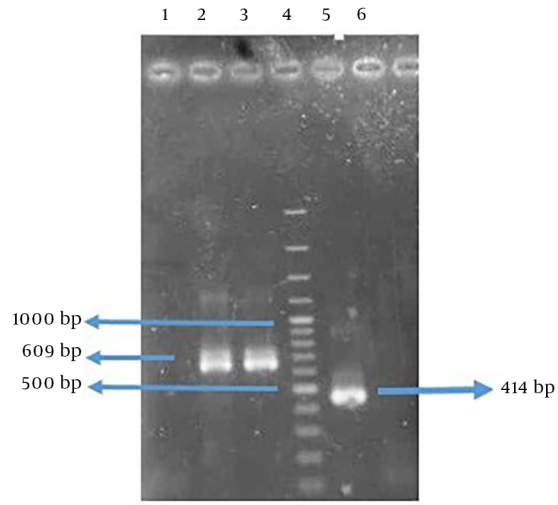

Figure 3. *FlaA* and *UreB* genes from *Helicobacter pylori* amplified by PCR. Lane 1: negative control of *UreB*, lane 2: positive control of *UreB*, lane 3: *UreB* gene, lane 4: 100-bp DNA marker, lane 5: *FlaA* gene, lane 6: negative control.

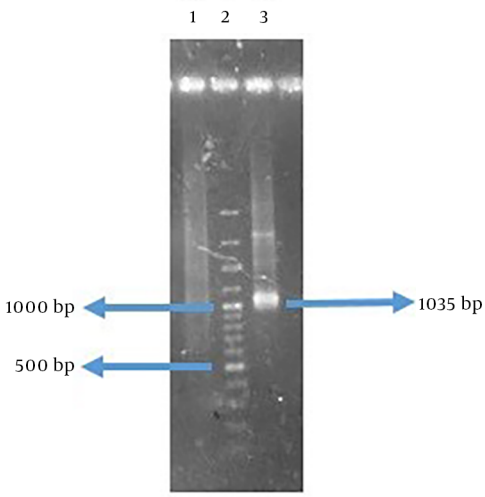

Figure 4. *rFlaA-UreB* recombinant multi-epitope gene construction and amplification by PCR. Lane 1: negative control of *rFlaA-UreB*, lane 2: 100-bp DNA marker, lane 3: *rFlaA-UreB* PCR product.

kDa. In fact, the recombinant protein reacted with the patients' sera whereas all 15 human normal sera used as negative controls failed to act similarly. Therefore, based on Western blot analysis results, the produced recombinant protein had an antigenicity enabling it to react with the sera of patients (Figure 6).

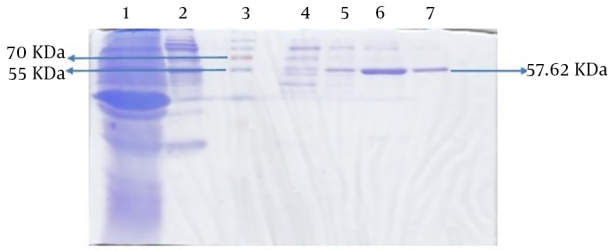

Figure 5. Expression and purification of *rFlaA-UreB* protein. Lane 1: *Escherichia coli BL21 (DE3) pLysS* destruction pellet, lane 2: *pET-32a-FlaA-UreB* after 2-h induction, lane 3: protein marker, lane 4 - 7: purified *rFlaA-UreB* protein.

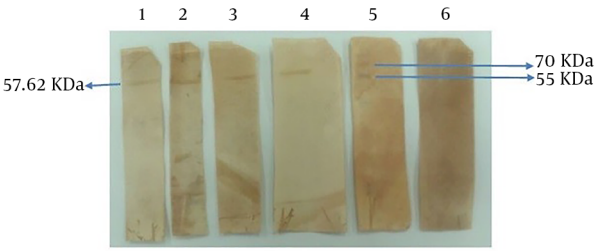

Figure 6. Western Blot analysis of *rFlaA-UreB* by using infected human sera. Lanes 1 - 4: Western Blotting by infected human sera, lane 5: protein marker, lane 6: Western Blotting by normal sera (negative control).

5. Discussion

Nowadays, the technology of multi-epitope antigens is widespread. Design and construction of multi-epitope DNA and protein antigens can serve as (i) a vaccine candidate against different cancers like breast cancer and cervical cancer (27, 28), (ii) a vaccine candidate for the prevention and control of some infectious diseases like Toxoplasmosis, Influenza, and HIV-1 (29-31), and (iii) an antigen in diagnostic kits for *Trypanosoma cruzi*, *Toxoplasma gondii*, *Hepatitis C*, and Tuberculosis (32-35). Several virulence genes of *H. pylori* have been identified, some of which including UreB, HpaA, NapA, FlaA, and FlaB have been investigated as vaccines and serologic diagnostic candidates for *H. pylori* infection (36-39).

The prevalence of these antigens in *H. pylori* isolated strains is 100%, 100%, 93.6%, 100%, and 99%, respectively. Therefore, among proteins, *UreB* is the optimal antigen and others can be potential antigens for developing *H. pylori* diagnostic kits and vaccines (40). The benefits of this protein in stimulating immune responses include the same nucleotide sequence and high prevalence among strains, high molecular weight, high expression rate, and strong antigenicity of urea protein (41). Previous research on this antigen showed despite these benefits, oral immunization

with a dual antigen was achieved using a combination of *H. pylori* heat-shock protein and urease in *H. felis*-infected mice models and induced a strong immune response.

Similarly, in another study, immunization with *HpaA* or *UreB* alone induced weak immune responses; however, when both antigens were used together, a stronger immune response was produced (42-45). These results indicate that combining antigens can be a good strategy for developing vaccines against *H. pylori* infection. Moreover, a mixture of antigens in diagnostic kits provides higher sensitivity and specificity than a single antigen (12-15). The 100% *FlaA* expression rate among different *H. pylori* isolates and 98.4% of its specific antibody positive rate in infected patients (with the highest expression rate and specific antibody positive rate in the second rank after *UreB*) make it appropriate for selection compared to other antigens of *H. pylori* (40).

The construction of many epitope proteins involves several proteins or domains of proteins. The linker sequence is particularly important for the construction of functional fusion proteins. Several studies have investigated the linker sequence, showing that the flexibility and hydrophilicity of the linker were important in the function of domains. For this reason, we chose LAAA as a peptide linker for making a recombinant multi-epitope protein (46).

Therefore, in this study, we decided to design and construct a multi-epitope antigen via combining the hyperantigenic regions of *UreB* with the hyperantigenic regions of *FlaA* in order to increase the specific immune response and safety and decrease side effects of unfavorable epitopes associated with complete antigens. Conserved epitopes of *UreB* and *FlaA* with appropriate antigen properties were determined by bioinformatics methods and used instead of whole antigens (11). These regions were confirmed by four software applications, as mentioned earlier. Based on the results, the recombinant protein had high antigenicity and could stimulate the immune response.

In this study, the antigenicity of the specified regions was evaluated through the production of recombinant *rFlaA-UreB*. Then, the antigenicity of the protein was examined by immunoblotting, performed on the patients' sera with *H. pylori* infection. Therefore, it can be concluded although *rFlaA-UreB* is much smaller than normal *UreB* and *FlaA*, it has the same antigenic properties. Thus, it seems this recombinant protein can be effectively used for vaccines. Moreover, it could be applied as a diagnostic antigen for *H. pylori* infections in different kits.

5.1. Conclusions

In summary, the study identified the antigenic areas of recombinant *rFlaA-UreB* protein by human sera infected with *H. pylori*. It can be concluded that the antigenic areas of this multi-epitope protein have an antigenic property that can be further used for the development of *H. pylori* vaccines and diagnostic kits.

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Footnotes

Authors' Contribution: Study design: Hamid Abtahi and Ghasem Mosayebi. Literature review: Behzad Khansarinejad. Data analysis: Zeinab Hamzehloo and Mina Zolfaghari. Manuscript preparation: Hamid Abtahi, Ghasem Mosayebi, and Zeinab Hamzehloo.

Conflict of Interests: The authors declare no conflict of interest.

Ethical Considerations: This study is a part of the thesis proposal of M.Sc (No.: 2025) and its ethical code from the Ethical Committee of Arak University of Medical Sciences, Arak, Iran is 93-171-19.

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Patient Consent: It is not declared by the authors.

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