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* **Corresponding author.**
sm-mousavi@stu.scu.ac.ir

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Developing a Multiepitope Vaccine Against Helicobacter pylori using the CTLA-4 Extracellular Domain

Seyedeh Maryam Mousavi[1*], Banafsheh Sadat Torabi[2], Amin Hashemi[3], Somayeh Mohammadi[4], Masoud Faraji[5], Mohsen Sadeghi[6]

1 Department of Biology, Faculty of Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran.
2 Department of Biology, Faculty of Sciences, Islamic Azad University, Damghan, Iran.
3 Department of Microbiology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran.
4 Department of Microbiology, Faculty of Basic Sciences, Islamic Azad University, Lahijan, Iran.
5 Department of Microbiology, Faculty of Sciences, Islamic Azad University, Karaj, Iran.
6 Department of Microbiology, Faculty of Advanced Sciences and Technology, Islamic Azad University, Tehran Medical Branch, Tehran, Iran.

Abstract

Helicobacter pylori is a pathogen that has been consistently linked to gastrointestinal cancer, particularly gastric cancer. Reports indicate that this pathogen is resistant to antibiotic treatments, necessitating the development of new treatment strategies. Here, to design a vaccine against *H. pylori* immunoinformatic tools were used. For this purpose, five virulence proteins including Flagellin B (FlaB), Flagellin A (FlaA), Urease subunit beta (UreB), CagA and Vacuolating cytotoxin autotransporter (VacA) were selected. Antigenicity and allergenicity were evaluated, and the epitopes with the highest scores were chosen. Linkers were used to connect the epitopes and an extracellular domain of CTLA-4 was positioned on the N-terminal. Biochemical features were predicted with the ProtParam server, and the second structure was predicted with the Prabi server, while the third structure was predicted with the Robetta, Alphafold and I-TASSER servers. VaxiJen and AllerTOP servers were used to evaluate the vaccine's antigenicity and allergenicity, respectively. After confirming the structure of the designed vaccine, molecular docking was performed with the TLR5 molecule using AutoDock Vina software. The SnapGene tool was utilized to in silico cloning of the vaccine in pET-3a vector. To evaluate the efficacy of the recombinant vaccine, the multi-epitope gene of *H. pylori* was cloned into the pCDNA3.1 vector, and its expression was analyzed in the spleen tissue of BALB/c mice using RT-qPCR. It has been shown in the results that the vaccine designed can bind to the TLR5 molecule on the surface of immune cells. Despite being an antigen, this vaccine didn't have any allergenic properties. Immunoinformatic is a promising tool for designing various drugs and vaccines. The results demonstrated that the expression levels of TNF-α and IFN-γ were significantly increased in the vaccine-treated group compared to the control group ($P < 0.05$). The elevated expression of pro-inflammatory cytokines indicates that the designed DNA vaccine successfully induced a cellular immune response against *H. pylori*.

Keywords: *H. pylori*; Vaccine design; CTLA-4; Immunoinformatic; Gastric cancer

1 Introduction

H. pylori was first identified by Warren and Marshall in 1982 [1], a milestone that marked a new era. Despite immune responses, this pathogen is able to survive within the stomach mucosa. Cell receptors are attached to microorganisms by adhesion molecules. Mechanical damage is prevented by their acid tolerance. Adhesins are connected to carbohydrate areas on the stomach epithelium by both hosts and pathogens. Inflammation of the gut occurs due to this attachment [2]. There is an overlap between acute and chronic inflammation. The duration of acute inflammation can vary from minutes to hours. The healing and recovery of damage is impacted by chronic inflammation. Complement, cytokines, acute phase proteins, and phagocytic cells are necessary for inflammation. The pathogen triggers pattern recognition receptors to activate innate immune responses and cause acute inflammation [3].

The host is protected by powerful immune responses against *H. pylori*, which can colonize the stomach for years, but inflammation can cause damage. Hematological, cardiovascular, dermatological, and neurological problems can be caused by significant gastrointestinal diseases [4]. To comprehend disease etiology and treatment, it is crucial to research both adaptive immune response mechanisms and innate immunity [5].

H. pylori is a parasite bacterium that resides in the stomach and duodenum of humans. If not eliminated, it can cause infection [6]. The elimination of *H. pylori* substantially decreases stomach inflammation and facilitates the healing of ulcers. The main treatment for *H. pylori* is clarithromycin (CLA) because it has a low minimal inhibitory concentration, effectively absorbs through the mucosal lining, and can reduce stomach acid production. CLA, amoxicillin or metronidazole were administered for an extended duration of treatment, along with a proton pump inhibitor. CLA reduces the effectiveness of triple therapy and enhances the use of multiple drugs in *H. pylori*-resistant gram-negative infections [7]. In 2017, the WHO prioritized clarithromycin-resistant *H. pylori* antimicrobial research. Some nations have antibiotic eradication rates below 60%. Extended antibiotic use can cause resistance [8]. Due to the growing antibiotic resistance of *H. pylori*, the challenge of eliminating the bacteria is becoming more difficult, and there is a possibility of reinfection even after treatment. Consequently, there is an immediate requirement to produce a viable vaccination for *H. pylori*. Due to the constraints and deficiencies of conventional vaccine development methods, which are time-consuming and have low efficacy, numerous scientists are employing immunoinformatic methodologies to create a vaccine for preventing *H. pylori* infection [9].

The development of vaccines has slowed down as a result of inconsistencies in study results and the abandonment of clinical trials. Increasing infection eradication challenges and global infection rates require additional research on *H. pylori* treatments. In recent years, numerous studies have investigated bacterial antigens and epitopes that could aid in the development of a *H. pylori* vaccine. Many *H. pylori* proteins, including UreB, BabA, VacA, Hsp60, HpaA, NAP, and CagA, were found to be vaccine antigens [10]. Here in this study, a multi-epitope vaccine against H. *pylori* was designed and investigated using CagA, VacA, and FlaB epitopes.

2 Materials and Methods

2.1 Protein Selection

The selection of five proteins, which have characteristics listed in Table. 1, was based on previous articles on the design of the vaccine against *H. pylori*. The UniProt database (https://www.uniprot.org) was utilized to obtain the sequences for the proteins. The antigenicity of the protein sequences was checked in the VaxiJen v2.0 database (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). The threshold was deemed to be above 0.4. In addition, the UniProt database provided us with the amino acid sequence of human CTLA-4 extracellular domain (UniProt ID: P16410). Fig. 1 **A** demonstrates the process of this study.

Table 1: The proteins used in epitope screening

No.	Protein	Gene	UniProt ID	VaxiJen Threshold
1	Flagellin B (FlaB)	flaB	Q07911	0.7346
2	Flagellin A (FlaA)	flaA	P0A0S1	0.7128
3	Urease subunit beta (UreB)	ureB	P69996	0.6783
4	CagA	cagA	Q9RF15	0.4346
5	Vacuolating cytotoxin autotransporter (VacA)	vacA	P55981	0.5713

2.2 B-Cell epitopes prediction

Linear epitopes were determined using the BepiPred-3.0 server in the DTU Health Tech database (https://services.healthtech.dtu.dk/services/BepiPred-3.0/). At this stage, Threshold>0.5 and Top epitope percentage cutoff was considered to be higher confidence (top 20%). The IEDB conformational B cell prediction tool ElliPro (http://tools.iedb.org/ellipro) was utilized to select epitopes with exposed amino acids. Threshold>0.5 and maximum distance was selected. The selection was made for epitopes that had a score of above 0.9.

2.3 T-cell epitopes prediction

Various T Cell Epitope Prediction servers in the IEBD database (https://www.iedb.org) were used to determine the binding of antigens to MHC-I and MHC-II, as well as MHC-I Immunogenicity. In the evaluation of binding to MHC-I, 9 mer antigens were considered and in the evaluation of binding to MHC-II, 12-18 mer antigens were considered. In both cases, SMM was the prediction method used. The prediction score is determined by the IC50 value, and the antigens with the smallest IC50 values were chosen. Subsequently, by examining MHC-I immunogenicity, antigens with scores above 0 were selected.

2.4 Allergenicity

The allergenicity filter declined the epitopes with the highest score in the previous steps using AllerTOP v.2.0 server (https://www.ddg-pharmfac.net/AllerTOP/). For further work, epitopes that were not allergenic were selected.

2.5 Multi-epitope vaccine sequence construction

The AAY and KK linkers were used to connect the epitopes of B and T cells identified in earlier steps and included in the creation of the multi-epitope vaccine. The vaccine's affinity was improved by connecting the amino acid sequence of the CTLA-4 extracellular domain to the combined antigen epitopes with the EAAAK linker Fig. 1**B**.

2.6 Biochemical characteristic evaluation

An effective vaccine is capable of triggering a robust immune response while also having appropriate biochemical properties. To assess the physicochemical characteristics of vaccines, we employed ProtParam (https://web.expasy.org/protparam/). This tool calculates various parameters including aliphatic index, theoretical isoelectric point (pI), molecular weight, half-life prediction, amino acid composition, instability index, and grand average of hydropathicity (GRAVY) [11].

2.7 Antigenicity and Allergenicity evaluation of vaccine

Besides the antigenicity and allergenicity of epitopes were evaluated separately, both indicators were also evaluated for the final vaccine. For this purpose, servers VaxiJen 2.0 and AllerTOP2.0 were used respectively.

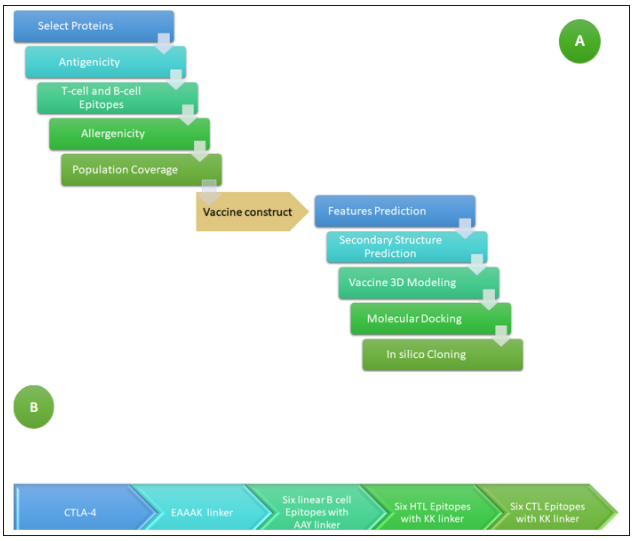

Fig. 1: A) The workflow of the study, B) Overview of the designed vaccine structure

The sequence of vaccines is presented below:

KAMHVAQPAVVLASSRGIASFVCEYASPGKATEVRVTVLRQADSQVTEVCAATYM
MGNELTFLDDSICTGTSSGNQVNLTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGT
QIYVIDPEPCPDSDEAAAKGQVRIATGAAAYDVAVQSGSLAAYDQQPQTEAAAAYD
PDQKPIVDAAYNGNSFTSYKAAYKDKPKDKPSAAYVQSGTVRELKKVASAAGANA
KKMAKATGDFSKKEAKAGFFSNKKPDKVWRIQAKKFAFFRNALVKKQSGSFAMAQ
ANAVQKKSGSFAMAQANAVQQKKGDWLDIFLSFVFKKGGDWLDIFLSFVFNKKKL
NSRLVNLSRRHTKKILNSRLVNLSRRHT

2.8 Secondary structure prediction

The secondary structure of proteins describes how the polypeptide chain folds and wraps. The primary types of secondary structures include α-helix, β-sheet, β-turn, and random coil. The Prabi server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html), is a widely used software

for predicting secondary protein structures. The default window width and similarity threshold values were followed [12].

2.9 Vaccine tertiary structure prediction

To draw the three-dimensional structure of the designed peptide, the I-TASSER (https://zhanggroup.org/I-TASSER/), the AlphaFold 18 (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=mbaIO9pWjaN0) and the Robetta (https://robetta.bakerlab.org/) servers were used. In the next step, the validation of the 3D structure was evaluated using the VAST tool (https://structure.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html). The models provided by the mentioned servers were compared using SAVES v6.0 server (https://saves.mbi.ucla.edu/). The Robetta model received higher scores than the other model.

2.10 Molecular docking

The study of molecular docking has shown a high level of promise and fundamentality in characterizing the interaction and binding affinity between vaccine constructs and the human Toll-like receptor 5 (TLR5). Molecular docking was performed by obtaining the crystal structure of human TLR5, which was obtained from the RCSB protein data bank (PDB ID: 3J0A). The precision of molecular docking was evaluated using AutoDock Vina 1.1.2 software based on RMSD criteria. Discovery Studio 4.5 software was used to draw the three-dimensional structure of the vaccine with the target protein.

2.11 In silico cloning

The Gene Infinity server (http://www.geneinfinity.org/sms/sms_backtranslation.html) was utilized to perform back-translation of the amino acid sequence of the multi-epitope vaccine. Subsequently, the GenScript server (https://www.genscript.com/tools/rare-codon-analysis) was employed to assess the codon adaptation index (CAI) and GC value of the optimized gene sequence. Ultimately, the genetic sequence of the vaccine was incorporated into the pET-3a vector utilizing the SnapGene tool.

2.12 Construction and Amplification of Recombinant Plasmid

A synthetic gene encoding the selected *Helicobacter pylori* antigens was cloned into the pET-3a expression vector using restriction sites EcoRI and XhoI. The ligated vector was introduced into chemically competent *E. coli* DH5α cells prepared using calcium chloride. A heat-shock step at 42°C for 45 seconds facilitated plasmid uptake. The transformed bacteria were plated on LB agar containing 100 µg/ml ampicillin and incubated overnight at 37°C. Single colonies were selected and cultured in LB broth with ampicillin for large-scale plasmid production.

2.13 Confirmation of Plasmid Construction

Plasmid DNA was extracted using the Qiagen Plasmid Mini Kit, according to the manufacturer's instructions. The integrity and size of the plasmids were assessed using 1% agarose gel electrophoresis. Concentration and purity were determined via NanoDrop spectrophotometry. To confirm the presence of the inserted antigen gene, restriction digestion was performed using EcoRI and XhoI. The digested fragments were visualized on agarose gel to confirm successful cloning. PCR analysis was also employed to amplify the target region using gene-specific primers. The thermocycling protocol included an initial denaturation (95°C, 5 min), followed by 35 cycles of denaturation (95°C, 30 sec), annealing (58°C, 45 sec), extension (72°C, 60 sec), and a final elongation (72°C, 5 min).

2.14 Immunization of Mice with DNA Vaccine

Twenty-four BALB/c female mice (6–8 weeks old) were purchased from the Pasteur Institute of Iran. Animals were kept under standard laboratory conditions and randomly divided into two groups (n=12 per group). Group A received 100 µg of recombinant plasmid DNA encoding *H. pylori* antigens suspended in 100 µl of sterile PBS via intramuscular injection on days 0, 14, and 28. Group B (control group) received the same volume of PBS alone. All animal experiments were performed in accordance with ethical guidelines for animal care.

2.15 Analysis of Antigen Gene Expression

At day 35, mice were euthanized, and spleen tissues were collected for gene expression analysis. Total RNA was extracted using the RiboEx total RNA kit (GeneAll, Korea). RNA integrity was evaluated by agarose gel electrophoresis, and concentrations were determined by NanoDrop 2000. First-strand cDNA synthesis was performed using a commercial reverse transcription kit (Yekta Tajhiz Azma, Iran). Quantitative real-time PCR was carried out using SYBR Green-based detection and primers specific to the inserted gene. The thermal profile involved initial denaturation (95°C, 3 min), followed by 45 cycles of

denaturation (95°C, 5 sec), annealing/extension (60°C, 30 sec), and a final melting curve from 60°C to 95°C. The 2^−ΔΔCt method was used to calculate relative gene expression.

2.16 Statistical Analysis

Experimental data were analyzed using GraphPad Prism software (version 9). All values were expressed as mean ± standard deviation (SD). Differences between groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Epitopes prediction

The selection of epitopes was made based on IC50 values and their scores. Their allergenicity was assessed after that. Table. 2 displays the epitopes related to every protein that are highly antigenic but not allergenic. The results were used to screen for conformational epitopes with a score greater than 0.9. None of them had a score above 0.9.

3.2 Vaccine sequence construction

The screening was based on the high affinity of the CTLA-4 extracellular domain and the dominant epitopes of FlaB, VacA, and CagA. After combining the overlapping sequences, linkers were used to link the CTLA-4 extracellular domain to the epitopes. There were four domains in the final multi-epitope vaccine structure, which included an extracellular domain of CTLA-4, B cell epitopes, CTL, and HTL epitopes. To keep it from degrading, the CTLA-4 extracellular domain was attached to the N-terminus of the vaccine and bonded to B cell epitopes through the EAAAK linker. In addition, AAY and KK linkers were used to connect B cells and T cell (HTL and CTL) epitopes, respectively, and the junction of B cell and T cell epitopes was connected by a KK linker.

3.3 Evaluation of physical and chemical properties

An online software was utilized to determine the biochemical characteristics, immune response, and allergenicity of the multi-epitope vaccine. Protparam results indicated that the vaccine consisted of 361 amino acids with a molecular weight of 39.21 kDa, a theoretical pI value of 9.63, and an estimated half-life of 16.3 h in mammalian reticulocytes, >20 h in yeast, and 2 h in *Escherichia coli*. The vaccine had an instability index of 24.97, which indicates that it was stable. The aliphatic index and GRAVY index were 73.63 and -0.300, respectively. VaxiJen 2.0 predicted an antigen fraction of 0.5545 for the vaccine sequence, higher than the 0.4 threshold, suggesting that the vaccine might be antigenic. According to AllerTOP 2.0, the multi-epitope vaccine was non-allergenic.

3.4 Secondary structure prediction

Using the Prabi server, the secondary structure of the multi-epitope vaccine was predicted Fig. 2. As shown in Fig. 3, alpha helix, extended strand, beta turn, and random coil accounted for 37.40%, 25.76%, 13.02%, and 23.82%, respectively.

3.5 Modeling and refining the tertiary structure of the vaccine

The I-TASSER, AlphaFold, and Robetta servers were used to construct the tertiary structure of the multi-epitope vaccine. In I-TASSER results model 4 Fig. 3 was selected as the optimized model based on C-score (C-score=-4.21, Estimated TM-score = 0.27±0.08, Estimated RMSD = 16.9±2.8Å). In AlphaFold results model 3 Fig. 4 was selected according to pLDDT (pLDDT=53.8 pTM=0.371 tol=5.23). As can be seen, the first 126 residues are related to CTLA-4 and it has complete homology with the data in the database, but the epitopes used showed low homology. Also, the predicted ligand binding sites was CTLA-4 domain. These three structures were compared by the SAVES server and the model provided by Robetta was selected for the next steps Fig. 5**A**.

3.6 Molecular docking

The results of molecular docking are shown in the Fig. 5**B**. Vaccine docking was carried out with all target protein, and the docked energy was at an acceptable level. The most stable model was selected according to the docking energy of_6.9 kcal/mol.

Table 2: Prediction of dominant Tand B cell epitopes of each protein

Protein	Epitope	Sequence
FlaB	CTL	VQSGTVREL
		VASAAGANA
		TNSQGIGAG
		EASLDIQGR
	HTL	QSGSFAMAQANAVQ
		SGSFAMAQANAVQQ
		AQSGSFAMAQANAVQ
		SGSFAMAQANAVQQN
		GSFAMAQANAVQQN
	Linear B cell	GSTSSDKIG
		SGAGMLASA
		NDYKIETVR
FlaA	CTL	SIDGRGIEI
		GAMVVIDIA
		DSDGRLVAA
	HTL	SRKAIQSDIVRLIQ
		RKAIQSDIVRLIQ
		ESRKAIQSDIVRLI
		SRKAIQSDIVRLI
	Linear B cell	GQVRIATGA
		DVAVQSGSL
		SNLTLNGIH
		RSIDGRGIE
UreB	CTLu	KYDANITFV
		NKKEFGRLK
		GIKEELGLE
		KADIGIKDG
	HTL	NFRIKRYLSKYTIN
		FRIKRYLSKYTINP
		GDNDNFRIKRYLSKYT
	Linear B cell	GDANASIPT
CagA	CTL	MAKATGDFS
		EAKAGFFSN
	HTL	GDWLDIFLSFVF
		GGDWLDIFLSFVFNK
		GDWLDIFLSFVFNKE
		GGDWLDIFLSFVF
	Linear B cell	DQQPQTEAA
		DPDQKPIVD
		KNPTKKNQY
		KERQEAEKN
		SEKEKEKFQ

Protein	Epitope	Sequence
VacA	CTL	PDKVWRIQA
		FAFFRNALV
		AGKGFNEFP
		NIKNVEITR
	HTL	LNSRLVNLSRRHT
		ILNSRLVNLSRRHT
		LNSRLVNLSRRHTN
		SRLVNLSRRHTNNI
	Linear B cell	NGNSFTSYK
		KDKPKDKPS
		NTTQNNANN

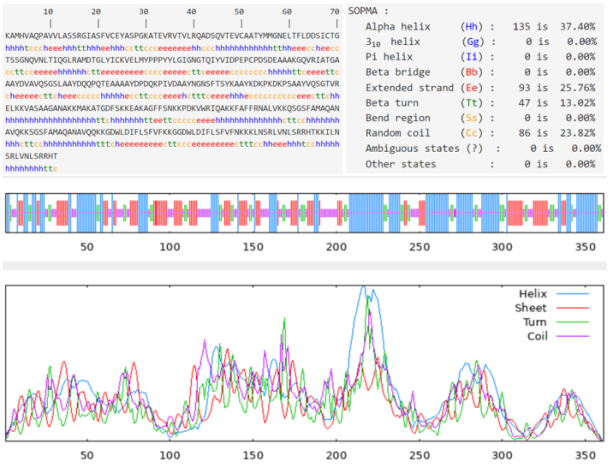

Fig. 2: Secondary structure of vaccines obtained from Prabi database

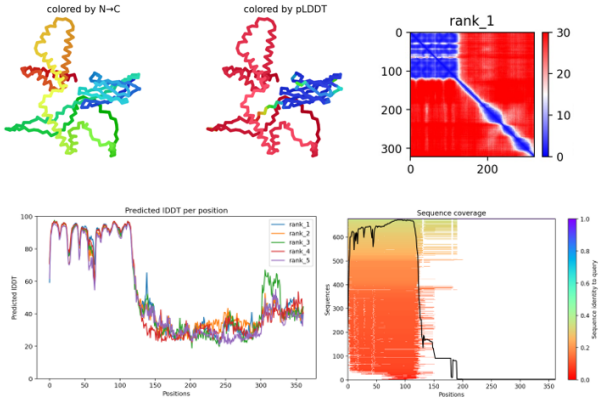

Fig. 4: Tertiary structure of vaccines obtained from AlphaFold database

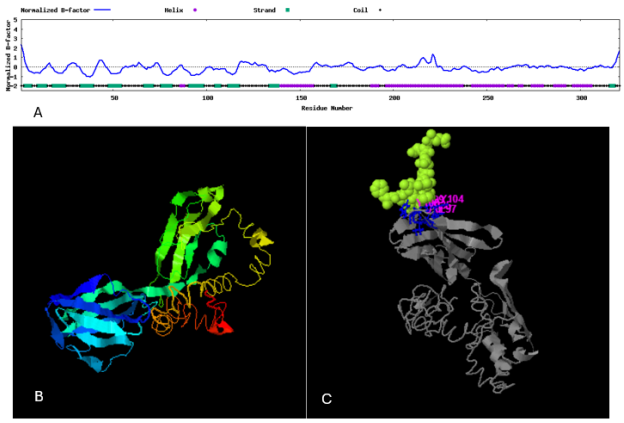

Fig. 3: Tertiary structure of vaccines obtained from I-TASSER database (A: Predicted normalized B-factor, B: The final model predicted by I-TASSER, C: Ligand binding sites)

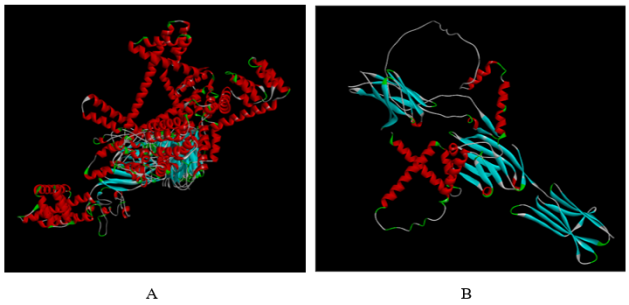

Fig. 5: A)Tertiary structure of vaccines obtained from Robetta database, B) Molecular docking of vaccine (CTLA-4) with TLR-4

3.7 In silico cloning

The vaccine structure was back translated using the Gene Infinity server, and the codon usage table was designated as *E. coli* K12. Using 1083 nucleotides as

inputs, the GenScript tool was used to evaluate the CAI and GC values of the gene sequence. The optimized sequence had a CAI value of 1 (the ideal value was 0.8–1.0), and a GC content of 56% (the ideal value was 30%–70%). Finally, a double-enzyme digestion method was adopted. NsiI and PflMI which did not exist in the target gene in the multiple cloning site, were selected as restriction sites and added to the 5′ and 3′ ends of the optimized gene. The modified gene sequence was inserted into the pET-3a vector using SnapGene software, and the results can be seen in Fig. 6.

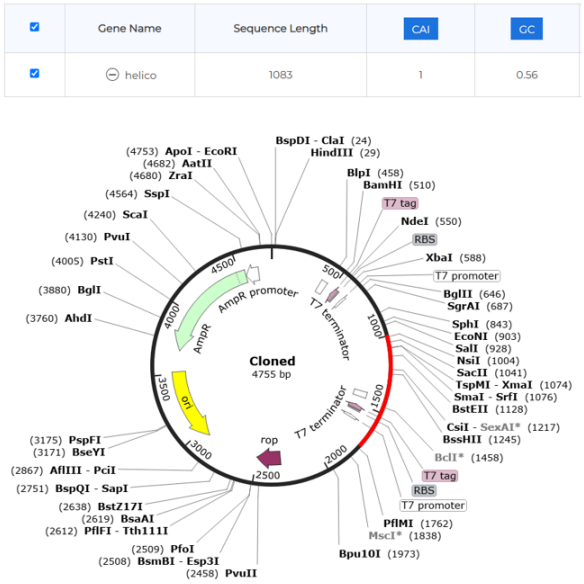

Fig. 6: Structure of the in-silico vector pET-3a. The vaccine, vector, and restriction sites are included. The red region represents the vaccine encoding gene

3.8 Cloning Assessment

The growth of *E. coli* DH5α colonies on LB agar supplemented with ampicillin provided preliminary evidence of successful transformation, as the recombinant plasmid carried an ampicillin resistance gene Fig. 7**A**. To validate the proper insertion of the *H. pylori*-derived multi-epitope gene, restriction enzyme digestion using EcoRI and XhoI was performed. The extracted plasmid DNA was assessed for purity via spectrophotometric analysis and for integrity by 1% agarose gel electrophoresis. Clear separation of bands corresponding to the vector backbone and insert fragment confirmed accurate cloning Fig. 7**B**.

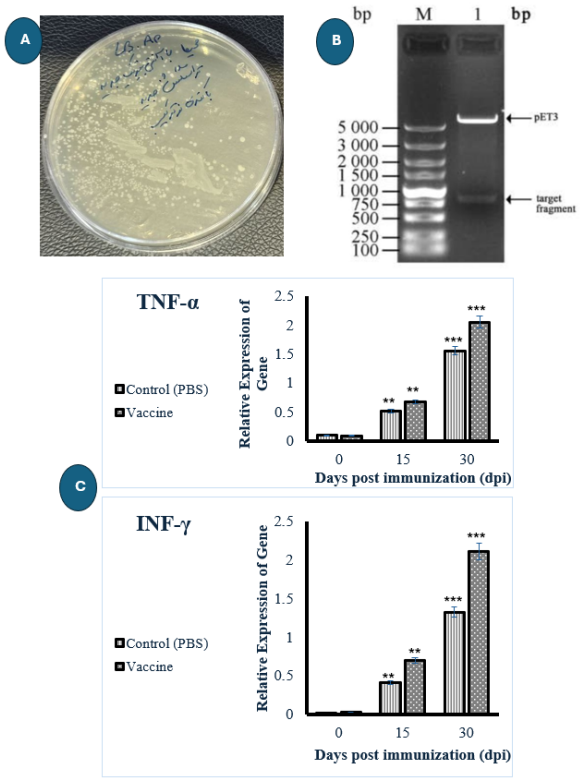

Fig. 7: A,B) Confirmation of cloning, C) Analysis of TNF-α and INF-γ gene expression in mouse spleen after receiving the vaccine (P<0.01, ***P<0.001)**

3.9 Gene Expression Analysis

Quantitative PCR analysis revealed differential gene expression profiles between the experimental and control groups. Notably, the expression of **TNF-α** and **IFN-γ** was significantly elevated in spleen tissues of mice immunized with the vaccine candidate, in comparison to the PBS-treated controls ($P < 0.05$). These results suggest that the DNA vaccine elicited a strong Th1-type immune response, consistent with the intended immunostimulatory role of the designed construct Fig. 7**B**.

4 Discussion

Here, five significant proteins involved in the pathogenesis of *H. pylori* were examined for vaccine design. Antigenicity and allergenicity were evaluated, and the epitopes with the highest scores were chosen, which were associated with proteins FlaB, CagA, and VacA. Linkers were used to connect the epitopes and an extracellular domain of CTLA-4 was positioned on the N-terminal. The goal of choosing this domain was to protect the vaccine from degradation and bind to target molecules.

Biochemical features were predicted with the ProtParam server, and the second structure was predicted with the Prabi server, while the third structure was predicted with the Robetta, Alphafold and I-TASSER servers. VaxiJen and AllerTOP servers were used to evaluate the vaccine's antigenicity and allergenicity, respectively. Finally, after confirming the structure of the designed vaccine, molecular docking was performed with the TLR5 molecule using AutoDock Vina software. The SnapGene tool was utilized to in silico cloning of the vaccine in pET-3a vector. It has been shown in the results that the vaccine designed can bind to the TLR5 molecule on the surface of immune cells. Despite being an antigen, this vaccine didn't have any allergenic properties.

H. pylori colonization causes the stomach to accumulate dendritic cells (DCs), macrophages, neutrophils, and T and B lymphocytes, causing a complex inflammatory response [13]. CD4 T helper cells, particularly Th1 cells, which form host-secreted cytokines, are more important than thought [14]. Cytotoxin-associated gene A (CagA) impacts Th17 differentiation via STAT3 and NF-κβ pathways [15, 16]. *H. pylori*-specific active Th1 and Th17 cells are capable of eliminating pathogens, but bacteria that pass through them can result in gastritis and peptic ulcers due to their high activity [16]. Th2 is responsible for defending against exogenous bacteria and helminths, even though it is also involved with *H. pylori* immunity is unclear [17, 18]. The colony size of bacteria in mice increased when IL-4 was deficient, but a Th2 response decreased it and prevented infection [19]. Different studies have demonstrated that antibodies and the Th2 response are not required for protection without IL-4 [20, 21].

FlaA, which is considered the major flagellin, has an immunogenicity that is significant due to its highly conserved nucleotide sequence. Flagella are produced by the FlaB dimer [22]. Clinical vaccine development could be improved by FlaA and FlaB [23, 24]. Urase is an important factor in the colonization and virulence of *H. pylori*, which is located on the surface of the bacteria, and its pathogenic role is to hydrolyze urea to produce CO_2 and ammonia and adjust the pH value of the environment around the bacteria. The hexameric form of Urease is made up of UreA and UreB. UreB is recognized as the most promising vaccine antigen due to its excellent immunogenicity and low toxicity compared to UreA, and is commonly used as a candidate antigen for clinical vaccine studies [25, 26].

One of the major toxins secreted by *H. pylori* is the Vacuolating cytotoxin A (VacA) named after its ability to induce the formation of “vacuole”-like membrane vesicles in the cytoplasm of gastric cells. VacA has been associated with the disruption of mitochondrial functions, stimulation of apoptosis, blockade of T cell proliferation and promotion of regulatory T cells, thereby making it a promising vaccine target. The evaluation of detoxified VacA has shown that it is a vaccine antigen [27].

CagA and VacA, encourage the production of inflammatory factors, which has a direct impact on the outcomes of patients [28]. A meta-analysis demonstrates that strains of bacteria that are positive for CagA increase the risk of gastric cancer, which confirms previous research indicating that CagA antibodies elevate the chance of developing cancer. The procedure appears intricate. *H. pylori* delivers CagA into gastric epithelial cells by means of integrin [29]. Vacuolating toxin A predicts the pathogenicity of *H. pylori* following CagA. VacA is associated with vacuolation, membrane-channel formation, apoptosis, proinflammatory response, and tumorigenesis [30].

The glycoprotein CTLA-4, is part of the immunoglobulin superfamily CD28-B7. It is found on the surface of activated clusters of specialized CD4+ and CD8+ T lymphocytes [31]. Three parts make up the CTLA-4 monomer molecule, which includes an intracellular domain, a transmembrane domain, and an extracellular domain. The combination of the CTLA-4 extracellular domain with an antigen can increase the immune response to the antigen, resulting in a very effective vaccine [32]. By using the stronger attraction between the extracellular domain of CTLA-4 and the receptor molecules on antigen-presenting cells (APCs), we may eliminate the intracellular domain and the transmembrane domain, while still preserving the high affinity of the CTLA-4 extracellular domain. APCs are targeted specifically by the vaccine because it has a strong binding affinity for the receptor molecules on their surface. Toll-like receptors (TLRs) are pattern recognition receptors located on the surface of APCs. They are capable of recognizing pathogen-associated molecular patterns derived from microbes, as well as damage-associated molecular patterns originating from injured tissues [33]. Research has indicated that TLR-2 significantly contributes to the inflammatory

response caused by *H. pylori* [5]. Furthermore, it has been shown that epithelial cells exert a significant impact on the innate immune system through the activation of TLR-4 in cases of *H. pylori*–associated gastritis [34]. Here we used CTLA-4 extracellular domain to increase the affinity of the vaccine to APCs. In the molecular docking section, we observed that the binding of this domain to TLR-4 occurred well and with appropriate energy, which shows that the vaccine may bind to its target. Furthermore, the extracellular domain of CTLA-4 does not possess a negative immune regulation function due to the absence of a transmembrane and intracellular domain. This limits the transmission of immunological signals into the cell. However, it still maintains a strong affinity with receptors on the surface of APCs [35].

5 Conclusion

According to the role of *H. pylori* in the occurrence of digestive problems and especially gastric cancer, it seems more logical to provide preventive solutions than therapeutic solutions. Vaccines are a suitable option for managing this pathogen due to antibiotic resistance. Here, using important proteins in the pathogenesis of *H. pylori*, a multi-epitope vaccine was designed and also the CTLA-4 domain was used to increase the stability and efficacy of the vaccine. Based on the results, the designed vaccine was able to bind to the receptors on the surface of immune cells, which indicates that the vaccine has the ability to bind to the target. Bioinformatics can be a promising tool for designing various drugs and vaccines. The elevated expression of pro-inflammatory cytokines indicates that the designed DNA vaccine successfully induced a cellular immune response against *H. pylori*.

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