RESEARCH PAPER

Immunoinformatic based Identification of Virulent Outer Membrane Proteins and Rational Multi-Epitope Vaccine Design against *Helicobacter pylori*

Ilangovan Karthiga[1], Sambandam Ravikumar[2], S. Shankar[3], Kumar Rangarajalu[1] Thiyagarajan Sanjeevi, Manju M[1]

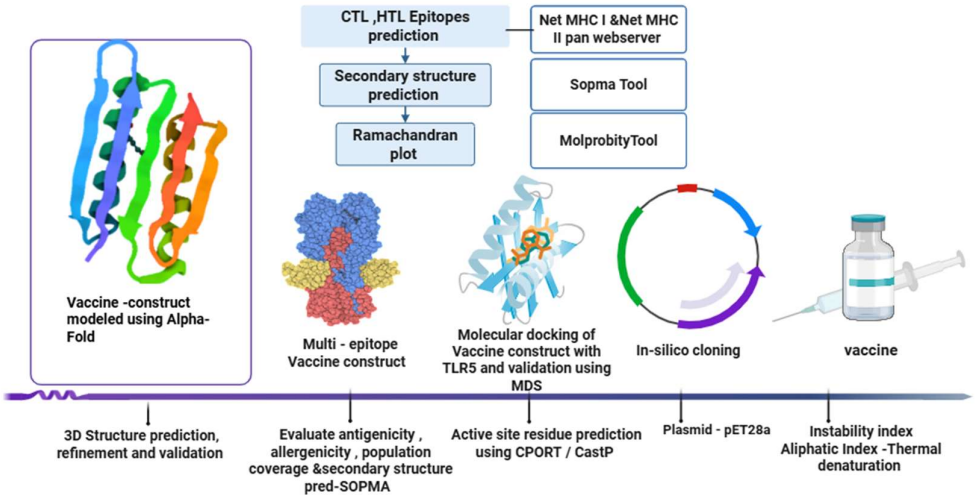
[1]Department of Biochemistry, Aarupadai Veedu Medical College & Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Puducherry, India.*Ilangovan Karthiga* - https://orcid.org/0009-0004-4872-Karthigasaiba@gmail.com Kumar.rangarajalu@avmc.edu.in thiyagarajan.sanjeevi@avmc.edu.in 1023*Thiyagarajan Sanjeevi* - https://orcid.org/0000-0002-4006-7184 *Manju M* - https://orcid.org/0000-0003-3315-1174
[2]Department of Medical Biotechnology, Aarupadai Veedu Medical College & Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Puducherry, India. *Sambandam Ravikumar* - https://orcid.org/0000-0001-7351-0421Sambasivamshankar76@gmail.com Sambandam@avmc.edu.in
[3]Department of General Medicine /Medical Gastroenterology, Aarupadai Veedu Medical College & Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Puducherry, India.*Kumar Rangarajalu* – https://orcid.org/0000-0002-9848-030X drmanjumay1@gmail.com

*Correspondence; Sambandam Ravikumar,
*Department of Medical Biotechnology, Aarupadai Veedu Medical College & Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Kirumampakkam, Puducherry, 607402, India.
Email: ravikumar.sambandam@avmc.edu.in

co-correspondence; Kumar rangarajalu,
Department of Biochemistry, Aarupadai Veedu Medical College & Hospital, Vinayaka Mission's Research Foundation (Deemed to be University), Kirumampakkam, Puducherry, 607402, India.
Email: kumar.rangarajalu@avmc.edu.in

Abstract

Helicobacter pylori, is responsible for a variety of gastrointestinal illnesses in the human body This bacterial strain has now become a significant health threat globally. Addressing it with existing medications is challenging because of the lack of suitable common targets for genetically varied strains. The current scenario of *H. pylori* demands the identification of potential virulent protein for the development of effective vaccine candidate. Moreover, few total proteomes have been reviewed to date, which also necessitates the characterization of virulent protein to unravel its pathogenesis. As the conserved OMPs that are expressed during the infection are more exposed to the host immune responses, they are found to be promising targets with a significant impact in the host pathogen interactions. Consequently, developing effective targeted vaccines necessitates alternative methods to eradicate *H. pylori* infection. In



this research, we created an innovative vaccine design utilizing B-cell derived T-cell epitopes from five key virulent outer membrane proteins (Hop, Ton B, Hypothetical protein 1, Hypothetical protein 2, Hypothetical protein 3) These proteins were finalized based on its antigenicity and non-allergenicity score using in-silico tools. In this study we have designed structure for the hypothetical protein, since its structural and functional characterization is still unknown, potentially it

*Author for Corresponden ; ravikumar.sambandam@avmc.edu.in

could contribute to increase in pathogenicity and observed the potential induction of an immune response through advanced immunoinformatic techniques. The protein-protein docking analysis between the human Toll-like receptor 5 (TLR5) and the vaccine construct aids in predicting the signalling pathways that stimulate an immune response. The calculated negative score (−88.8, ± 1.8) of the molecular docking complex indicates a highly favourable binding interface. Moreover, Molecular dynamics simulation research has validated the appropriate docking of TLR5 with the vaccine candidate. Root mean square deviation (RMSD) analysis demonstrated that the complexes attained thermodynamic equilibrium, stabilizing around 1.5 nm. Overall MDS results substantiate the structural integrity of the designed vaccine constructs under physiological conditions. Normal mode analysis (NMA) calculates the molecular motion of the docking complex. The low eigenvalue ($3.857e{-}0^{7}$) indicates the stable and flexible molecular motion in the binding interaction side. Finally, in-silico cloning of vaccine candidate was performed using expression vector pET28a (+) with the optimized restriction sites.

Keywords *H. pylori*, Outer membrane protein, Multi epitope vaccine. Hypothetical protein, Anti-microbial resistance

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1.Introduction

Helicobacter pylori is a gram-negative, flagellated, microaerophilic bacterium that colonizes the epithelial lining of the stomach[1]. The prevalence of this infection is estimated to affect approximately 50% of the global population, with variations observed based on geographic location, age, socioeconomic status. Typically, the infection is acquired during early childhood and is likely to persist until effective treatment is administered. *Helicobacter pylori* infection is associated with a wide range of diseases, including gastritis, gastric ulcers, and stomach cancer[2]. Notably, stomach cancer ranks as the third leading cause of cancer-related mortality globally, with 90% of cases attributable to *H. pylori* infection[3,4]. The eradication of *Helicobacter pylori* involves various treatment regimens, including triple, sequential, quadruple, and bismuth-containing therapies consisting of proton pump inhibitors (PPIs), clarithromycin (CAM), and amoxicillin (AMPCs) and metronidazole[5,6]. Due to high resistance to metronidazole and clarithromycin with the widespread use of STT regimen, *Helicobacter pylori* eradication rates have declined to unacceptable levels[7]. The selection of antibiotics is contingent upon the resistance profiles of the strains and the regional resistance rates[6,8]. The World Health Organization (WHO) has identified clarithromycin-resistant *H. pylori* strains as a high priority for research and development in the field of novel antimicrobial agents[8,9]. Helicobacter pylori's distinctive outer membrane features contribute significantly to its capacity to live in the harsh acidic environment of the stomach and develop long-term colonization of the gastric mucosa [10]. One of the critical challenges in understanding the pathophysiology of *H. pylori* is the identification and characterization of virulent molecules related to its pathogenesis [11].Outer Membrane Proteins (OMPs) of *H . pylori* play a crucial role in host-pathogen interactions, mediating adhesion, invasion, immune evasion, and dissemination of the organism [12]. Additionally, the ability of *H. pylori* to adapt to the host environment and avoid the host immune responses adds complexity to the pathogenesis [13]. The creation of a powerful chimera vaccine serves as a proactive approach to address drug abuse and the issues related to multidrug-resistant strains of *Helicobacter pylori* (Figure 1). Vaccine research is currently underway; however, this development is still in its early stages as potential vaccine targets may not be effective[14]. Therefore, a linear perspective suggests that there may be a need for effective variants of subunit vaccines. In this analysis, membrane proteins that are exposed on the surface were selected from a total of 38 outer membrane proteins found in *H. Pylori* verified strain from which five such antigenic proteins are selected for in-silico design of vaccine model. A computational framework was used to determine antigenic common B-cell and T-cell epitope to construct a vaccine construct against *H. pylori* Ton- B and Hop proteins are involved in host interaction and adaption to acid environment these proteins are highly immunogenic and easily recognized by the host's antibodies and immune cells similarly ,3 hypothetical proteins of *H. pylori* also having virulence property. In this research, we utilized functional annotation and extensive in silico techniques to discover new therapeutic targets and potential lead compounds. Our specific focus was on hypothetical proteins (HPs), which are an underexplored source of potential vaccine targets. Hypothetical Proteins are those whose biological roles remain uncharacterized but are crucial for comprehending physiological and biochemical processes. Numerous HPs are part of functional groups and may be involved in human diseases, presenting unique chances for the identification of new structures and functions[15]. Computational methods have previously been effective in predicting the functions of Hypothetical Proteins from various species, such as *Streptomyces coelicolor* [16] Mycobacterium tuberculosis[17]. By investigating HPs, our goal was to uncover new therapeutic targets that could aid in the creation of more efficient treatments. To do this, we

employed a variety of computational tools, servers, databases, and software to annotate and verify the functions of HPs and to identify potential vaccine candidates against *H. pylori.*

In this work we selected five antigenic proteins (Ton-B, Hop, Hypo1,Hypo2, Hypo3) from *H. Pylori* .Utilizing these antigenic protein common B-cell and T-cell epitopes was predicted and vaccine construct was developed .We also characterized different properties of vaccine candidates such as secondary structure, antigenicity, allergenicity and physiochemical properties .we also designed a structure for hypothetical protein using robertta and validated the structure and performed tertiary structure modelling and model validation of vaccine construct. The vaccine construct was docked with Human TLR5 to understand the binding affinity and finally validated using Molecular dynamic simulation study. Additionally in-silico cloning was done to optimize the vaccine candidate.

2. Materials and method

2.1 Retrieval of protein sequence and antigenicity, allergenicity prediction

The protein sequences were retrieved in FASTA format from the NCBI database for sequence analysis[18]. We then identified orthologues using the OrthoVenn3 tool, and a pan-genome analysis was performed to identify the core proteomes. Subcellular localization was predicted using PSORTbv.3.0 and the CELLO v2.0 webserver to determine if the proteins were of outer membrane, cytoplasmic, extracellular, or periplasmic origin; we strictly adhered to outer membrane proteins. We selectively took *H. pylori* unique sequences. Signal peptides were predicted using SignalP 6.0, a deep learning-based tool, to assess secretion and surface exposure and TMHMM v2.0, which predicts transmembrane helices in proteins using a hidden Markov model approach, was used to identify transmembrane helices, ensuring the selection of accessible, non-buried epitopes and proteins were then checked for their virulence properties based on the virulent score[18]. The proteins were evaluated for antigenicity using ANTIGENpro, which employs machine learning models trained on protein microarray data to predict protein antigenicity without relying on sequence alignment, and VaxiJen v2.0, which predicts protective antigens based on an alignment-independent method using the physicochemical properties of proteins[18]. Proteins with VaxiJen scores above 0.4 were considered antigenic[19]. Allergenicity was assessed using AllergenFP v1.0, which predicts protein allergenicity using descriptor-based fingerprinting and a support vector machine algorithm, also a descriptor-based classifier that compares physicochemical properties with known allergens[20]. BLASTp, which finds regions of local similarity between protein sequences to confirm specificity and avoid cross-reactivity screening, was performed against the human proteome to minimize the risk of autoimmunity, and sequences with significant similarity were excluded[18].

2.2 Structural modelling and validation of Hypothetical protein

The three-dimensional (3D) structure of 3 hypo protein was modelled using robetta (Figure 2) Robetta integrates comparative (homology) modelling, which utilizes homologous template structures, with de novo modelling for regions where no suitable templates are available[21]. The predicted structure of Hypothetical protein was validated using a Ramachandran plot, a tool that assesses the stereochemical quality of the protein by examining the geometry of each residue and overall structural features (Figure2). This thorough validation confirmed that the hypothetical protein structural model is reliable for further analyses.

2.3 Secondary structure prediction

The secondary structure of the identified hypothetical protein was predicted using the SOPMA tool, a popular bioinformatics server that utilizes a two-stage neural network algorithm. This algorithm analyses position-specific scoring matrices (PSSMs) produced by PSI-BLAST to forecast the presence of α-helices, β-sheets, and coils within the protein sequence, offering insights into its structural arrangement[22].

2.4 Evaluation of physiochemical properties

The physicochemical parameters of five proteins were assessed using ExPASy ProtParam , based on the sequences and pKa values of the amino acids present in the protein [23]. ProtParam calculates several parameters including molecular weight (MW), theoretical isoelectric point (pI), amino acid composition, estimated half-life, instability index (considered stable if it is <40), aliphatic index, and grand average of hydrophobicity (GRAVY). ProtParam employs the "N-end rule," which links a protein's half-life to the characteristics of its N-terminal residue [24]. The aliphatic index is derived from the volume filled by aliphatic side chains, which positively impacts the thermal stability of globular proteins. The GRAVY value is determined by calculating the total hydropathy of all amino acids and dividing it by the total number of amino acids in the protein, reflecting whether the protein is more hydrophobic or hydrophilic[20].

2.5 B cell epitope prediction

B lymphocyte cells produce antibodies and memory cells, thereby initiating long-lasting immune responses against pathogens. To predict sequence-based linear B-cell epitopes of the four target proteins, we used ABC pred online server. The FASTA sequences of the target proteins were submitted as input, and all default settings were applied.

2.6 MHC epitope prediction

T lymphocytes are essential in initiating immune responses through cytotoxic and helper T-cell activities [25]. MHC-I and MHC-II epitopes were chosen based on prior research. Utilizing the T-cell epitope prediction

tool on Net MHC I pan server, B-cell epitopes of target proteins were identified. The 4.1 method endorsed by Net MHC pan was employed to identify MHC-I and MHC-II epitopes from all available datasets. Following the successful determination of T-cell epitopes, a shared epitope for B-cells, T-cells, and both MHC molecules was selected, potentially serving as a vaccine capable of eliciting humoral and cellular immunity. Overlapping epitopes originating from the same protein region were carefully filtered to avoid redundancy. only epitopes with high antigenicity and broad allele coverage, were selected for inclusion of in the final vaccine construct.

2.7 Vaccine construction

To develop a Multi epitope peptide vaccine, epitopes from CTL, HTL, and B-cells that exhibited high immunogenicity, antigenicity, and minimal allergenicity and toxicity were chosen. The ultimate vaccine construct was created by connecting these epitopes with suitable spacer sequences: AAY linkers were employed to link CTL epitopes, promoting proteasomal processing; GPGPG linkers were utilized to connect HTL epitopes, ensuring effective MHC-II binding. and 50s ribosomal protein was linked to the N terminus of the vaccine construct to enhance immune response.

2.8 Characterization and validation of the design model

To validate the enhanced tertiary structure obtained from the Galaxy refine server, we utilized two open-source online resources: PROSA-web for summarizing error scores and Molbrobity for examining the Ramachandran plot. we performed further assessments concerning biological (toxicity) and immunological (antigenicity, allergenicity) properties utilizing the Toxin-Pred server, respectively.

2.9 Discontinuous B cell epitope

Discontinuous B-cell epitopes were predicted using Ellipro-online tool. A score of 0.9 signifies that 90% of the protein residues are located within the ellipsoids, while the remaining 10% lie outside. The clustering of discontinuous epitopes is determined by the distance parameter R, measured in angstroms (Å); a greater value of R corresponds to a larger discontinuous epitope.

2.10 Molecular docking

Molecular docking serves as a foundational and highly promising technique essential for elucidating the interaction mechanisms and binding strength between vaccine constructs and the human Toll-like receptor 5 (TLR5)[26]. In this study, the crystal structure of human TLR5, sourced from the RCSB Protein Data Bank under the PDB ID: 3J0A, the structure was prepared and edited using pymol[26]. The HADDOCK web server, accessed through a user-friendly interface, facilitated the execution of the molecular docking procedures as described by De Vries et al[27]. Prior to the docking process, identification of active residues (those directly engaging in the interaction) and passive residues (neighbouring residues) for both TLR5 and the vaccine construct was performed using the CPORT server, following the methodology outlined by de Vries and Bonvin [28]. The binding affinity of the docking complex, reflected by the Gibbs free energy (ΔG), determines the nature of the interaction pattern, while the dissociation constant (KD) values at 37°C were predicted using the PRODIGY web server[29].

2.11 Molecular dynamic simulation

To further investigate the stability of the peptide-MHC complexes, molecular dynamics simulations (MDS) were conducted with GROMACS v2025. The OPLS force field was utilized, and the system was solvated with a SPC water model within a cubic simulation box. Counterions (Na+/Cl-) were introduced to neutralize the system.

2.12 Normal mode analysis (NMA)

iMODs server was used to study the protein flexibility and was performed on docked complex to check the molecular motion and structural stability of the docked complex This tool generates molecular deformability, B-factor, variance, co-variance map, eigenvalues and elastic network.

2.13 In-silico cloning

In silico cloning was conducted to investigate the expression of our developed multi-epitopic vaccine candidate in *H. pylori.* The Java Codon Adaptation (JCat) tool was utilized to optimize our construct for expression within the *H. pylori* strain. Furthermore, we chose two filtering parameters to minimize unnecessary errors: (a) avoiding rho-independent transcription terminators, and (b) excluding prokaryotic ribosome binding sites. *E. coli* K12 strain was used as a host and was cloned in pET 28a vector. The codon adaptation index (CAI) value for the optimized vaccine construct was (within the range of 0.8 to 1.0), and the optimal GC content was (falling between 40 - 60%), indicating a high likelihood of successful protein expression. In the next step, we added two restriction enzymes to both ends.

3 Result

3.1 Identification of Target protein

H. pylori verified protein sequences were retrieved from the NCBI database in FASTA format. Orthologues proteins were identified and selectively taken *H. pylori* unique sequence (Total cluster- 74) (Table1) and then subcellular localization was done to identify outer membrane proteins where we happened to find 38 OMP (Table 2). In this study five proteins of *H. pylori* were selected after fine refinement for the possible vaccine construction. We got 5 proteins where in which 3 hypothetical proteins were identified and designed a structure using rosetta modelling (Figure2) and validated using Ramachandran plot Other such proteins such as Ton-B and Hop gene where the structure of Ton-B and Hop protein structures were modelled using Alpha fold

server SignalP 6.0 analysis revealed no signal peptides in either protein (Table 3). TMHMM v2.0 analysis confirmed the absence of transmembrane helices in the selected regions (Table 4). To confirm that the epitopes are accessible, the structures of the TonB and Hop proteins were verified by comparing their sequences with existing template structures. A high sequence similarity was found (97.17% for TonB and 90.41% for Hop) demonstrating significant structural conservation and validating the appropriateness of the predicted models using Ramachandran plot for further computational studies.[18]. Although hypothetical protein lacks a resolved crystal structure, its high sequence conservation and strong antigenicity support its inclusion in the vaccine design. Virulence of the protein was determined and selected the proteins with high virulence score >0.9(Table 5) Antigenicity analysis showed that both proteins had scores above the 0.4 threshold, indicating strong immunogenic potential (Table 6). ANTIGEN-pro predictions further supported their antigenic nature. Allergenicity assessment using AllergenFP v1.0 identified all the proteins were non-allergenic. (Table 7)[18]. In addition, BLASTp analysis against the human proteome revealed no significant similarities, indicating a low potential for causing autoimmune reactions. These results supported using and as promising candidates for multi epitope vaccine design (Table 8)[18]. Furthermore, the physiochemical properties (Table 9) and secondary structure prediction of the selected antigenic proteins were done (Table 10).

3.2 Epitopes prediction

To enhance prediction accuracy, ABCpre were employed to identify linear B-cell epitopes. For ABCpred results, selection was based on the score. Preference was given to peptides that scored highly in ABCpred. especially when both methods identified overlapping peptides. To confirm antigenicity, the VaxiJen tool was used to assess the selected peptides, and those with scores above 0.4 were chosen as dominant linear B-cell (LBL) epitopes (see Table 11). Cytotoxic T lymphocyte (CTL) epitopes were predicted using the Net MHC I pan 4.1 Major Histocompatibility Complex I (MHC I) binding prediction tool, with high-scoring and antigenic epitopes selected for inclusion in the multi-epitope vaccine design (Table 12). Helper T lymphocyte (HTL) epitopes were predicted using the Net MHC II pan 4.1 binding prediction tool, and sequences with higher percentile ranks and strong antigenicity were selected as dominant HTL epitopes.

3.3 Development of Vaccine construct

The vaccine design incorporated a selection of 20 MHC class I epitopes and 20 MHC class II epitopes, which were connected via AAY and GPGPG linkers, respectively. Additionally, the 50S ribosomal protein L7/L12, identified with NCBI accession no. P0A7K2, functioned as an adjuvant and was attached to the N-terminus of the vaccine construct using the EAAAK linker. The integrated MHC-I, MHC-II epitopes, and adjuvant collectively constituted the final vaccine construct, comprising 461 amino acids, equating to approximately 48.56 kDa in size, with an Extinction coefficient of 37250 M−1 cm−1, as illustrated in Figure 3.

3.4 Secondary structure prediction

The secondary structure perdition of the following vaccine construct was done using SOPMA. The analysis of the secondary structure of the multi-epitope vaccine construct indicated that the model was primarily composed of coil regions, which include 371 residues (80.4%). This suggests a significant level of structural flexibility that was crucial for the presentation of epitopes and the recognition by the immune system. Alpha helices made up 81 residues (17.57%), which play a role in maintaining the structural stability of the protein. No beta-sheet structures were detected in the predicted model. The substantial presence of coil regions is a defining characteristic of multi-epitope vaccine constructs, promoting appropriate folding, flexibility, and efficient interaction with immune receptors.

3.5 Evaluation of antigenicity and allergenicity of the designed vaccine construct

Antigenic propensity is a crucial factor in the design of vaccine constructs that typically elicit both humoral and cell-mediated immune responses against *H. pylori*. The vaccine's predicted antigenicity score was 1.0221, which exceeds the threshold of 0.4, suggesting a strong potential to trigger an immune response. Moreover, the vaccine was anticipated to be non-allergenic (Table 12). The Allergen-FP v.1.0 server concluded that the vaccine constructs are non-allergenic sequences with score (0.6816), while the Aller-TOP v.2.0 also supported the non-allergenic nature of the vaccine. Therefore, the vaccine construct may proceed to further investigation. According to the Protein-Sol server, the vaccine was predicted to be soluble in water, with a solubility score of 0.6022. Additionally, the toxicity was predicted using toxi-pred tool which predicted the proteins as non-toxic.

3.6 Vaccine analysis and refinement of vaccine construct

The multi-epitope vaccine developed in this study consists of 461 amino acid residues, with a calculated molecular weight of 48.56kDa and a theoretical isoelectric point (pI) of 6.73, reflecting the basic characteristics of the protein. Its instability index was estimated at 26.15, indicating that the construct was stable. The vaccine construct has 6726 number of atoms. The aliphatic index was 62.00, implying that the vaccine has good thermostability. The GRAVY score was -0.673, showing that the vaccine was hydrophilic, which promotes better interaction with other proteins. The estimated half-life of the vaccine was 30 hours in mammalian reticulocytes, over 20 hours in yeast, and more than 10 hours in Escherichia coli. The ExPASy-ProtParam results for all other physicochemical properties of the vaccine construct are listed in Table 13.

3.7 Structure of vaccine construct and validation

The initial vaccine construct comprised 461 amino acids. Following sequence optimization and the elimination of repetitive regions, the final construct was condensed to 180 amino acids. This refined sequence was subsequently employed for structural modelling, docking studies, and molecular dynamics simulations. The three-dimensional structure of vaccine construct was constructed utilizing Alpha-fold (refer to Figure 4) and subsequently refined with Galaxy-Refiner server. The optimal model, chosen based on structural quality metrics, underwent validation through various tools. Analysis of the Ramachandran plot using Mol-probity indicated that 96.63% of residues were located in the most favoured regions, 99.4% in additionally allowed regions, 0.4% in generously allowed regions, and merely 0.56% in disallowed regions (Table14) (Figure5a). The ProSA-web tool produced a Z-score of -3.16, which aligns with the characteristics of native protein structures (illustrated in Figure 5).

3.8 Discontinuous B-cell epitope prediction

The Elli-Pro online web server was used to predict discontinuous B-cell epitopes based on the tertiary structure of the vaccine construct. twelve epitopes were identified by the Elli-Pro server, and the epitope with the highest prediction score of 0.97 was selected as the discontinuous epitope. The 3D structures of the predicted discontinuous epitopes are displayed in Figure 6.

3.9 Molecular docking study

HADDOCK grouped 143 structures into 6 clusters, accounting for 71% of the water-refined HADDOCK-generated models. The highest-ranked cluster, with the lowest HADDOCK score, is considered the most important for docking analysis[26]. The HADDOCK score of −88.8 ± 1.8. suggests a strong interaction between the vaccine construct and TLR5. The buried surface area (BSA) score was 1218.2 ± 50.6 Å², indicating close contact and reduced water exposure at the protein binding interface. Additionally, RMSD plots were used to assess the docking interactions, as shown in (Figure7) The small RMSD value of the docked complex indicates the formation of a high-quality docked structure. The HADDOCK-calculated values for electrostatic energy, desolvation energy, restraint violation energy, van der Waals energy, and Z-Score are presented in Table 15[26]. (Figure 7) illustrates the molecular docking between Human TLR5 and the vaccine construct, while Table 14 shows the hydrogen bonds formed between the two protein molecules. PRODIGY provided the binding affinity in terms of Gibbs free energy (ΔG) and the dissociation constant (Kd) for the docked complex, which were −8.9 kcal/mol and 2.9 e-07 M, respectively (Table 15). The negative value of the docking complex indicates that the docking structure was thermodynamically stable[26].

3.10 Molecular dynamic simulation

To assess the atomic-level stability and dynamic behaviour of the vaccine construct –TLR complexes, 100 ns MDS were performed. To assess the stability of the docked complex, molecular dynamics (MD) simulations were conducted. The Root Mean Square Deviation (RMSD) analysis revealed an initial increase during the equilibration phase, followed by stabilization at approximately 1.5 nm, suggesting that the complex attained structural stability over the course of the simulation (Figure8a). The Root Mean Square Fluctuation (RMSF) profile indicated that the majority of residues exhibited minimal fluctuations, with slightly elevated flexibility observed at the terminal regions, consistent with typical protein behaviour (Figure8b). Analysis of the Radius of Gyration (Rg) demonstrated a reduction during the initial phase, subsequently stabilizing around 2.0 nm, implying that the complex preserved a compact conformation throughout the simulation period (Figure8c). Additionally, hydrogen bond analysis confirmed the presence of a consistent number of hydrogen bonds, reflecting stable intermolecular interactions within the complex (Figure8d). The SASA value initially dropped from about 140 nm² to roughly 110–115 nm² in the early stage of the simulation, reflecting structural changes and greater compactness of the protein. After around 15–20 ns, the SASA values levelled off, showing only slight variations around 110–115 nm² for the rest of the simulation(Figure8e). This relatively steady SASA pattern indicates that the protein complex preserved a consistent degree of solvent exposure and structural stability throughout the simulation.

3.11 Normal mode analysis

The flexibility of biological macromolecules is an essential characteristic for engaging with substrates or the machinery involved in protein–protein interactions[30]. Consequently, iMODs assesses molecular motion and structural flexibility via a Normal Mode Analysis (NMA) study, which utilizes the coordinates of the docked complex. Chain deformability measures the capability of a given molecule to deform at each residue and it's represented as chain hinges. (Figure9a) B-factor column gives an averaged RMS value (Figure9b). An Eigenvalue associated to each normal mode represented. The tool has generated Eigenvalue of $3.8574e^{-07}$(Figure9C). The variance associated to each normal mode was inversely related to the eigenvalue (Figure9d). Covariance matrix indicates coupling between pairs of residues, it's represented with a coloured graph as correlated (red) and uncorrelated (blue) motions (Figure9e). The elastic network model defines which pair of atoms are connected by springs. Each dot in the graph represents one spring between the corresponding pair of atoms. darker grey indicates the stiffer springs and vice versa (Figure 9f).

3.12 In-silico cloning

In this study, Jcat was employed to enhance the expression of the multi-epitope vaccine in *E. coli* K12 strain. A total of 461 amino acids were provided. Following codon optimization, the Codon Adaptation Index (CAI) might achieve 1.0 with GC contents of 48.46%, which was comparable to E. coli K12's GC contents of 50.7%. This suggested that E. coli K12 would be the primary host for the final multi-epitope vaccination with the optimized sequence. The optimized vaccine construct's CAI value, which ranges from 0.8 to 1.0, was 0.3463577653, indicating a high likelihood of protein expression. Then the codon optimized DNA sequence was then cloned into pET28a plasmid. The next step involves adding two restricted endonucleases (Hind III and Not I) to both ends of the vaccine design that was cloned inside the pET28a (+) vector using benchling software (Figure10). The length of the clone was 6969kb.

4. Discussion

This research highlights the pressing need for innovative vaccine strategies aimed at *H. pylori*, a bacterium that affects nearly half of the global population and is a key factor in many gastrointestinal and extra-gastrointestinal diseases[31]. The lack of a commercially available vaccine, coupled with the increasing issue of antibiotic resistance, complicates existing treatment options[32]. Based on the immunogenicity score, 37 protein sequences from 74 protein sequences—including *H. pylori* outer membrane protein alone were selected for this investigation. The examination of protein epitopes taken consideration of B cell linear epitope prediction, MHC-I binding prediction, and MHC-II binding prediction. Based on their distribution in clinical strains and immunogenicity score, Hypo1, Hypo2, Hypo 3, Ton-B, hop genes were chosen. The five screened antigens were chosen based on score., we anticipate that the hypothetical proteins might have some promising candidate antigens that could produce a better immunological response. We designed and validated the structure of hypothetical protein and the analysis indicated that this hypothetical protein processes potential virulence property and and c-Subunit vaccine development is no longer limited to just one antigen. A multi-epitope vaccination is more effective than a single antigen because it can block many pathogenic channels and elicit a wider neutralizing antibody response. The activation of both humoral and cell-mediated immune responses is crucial for the effective design of vaccines. Rahman et al. conducted a study focused on the identification of therapeutic targets mediated by the core proteome and the design of a multi-epitope vaccine against Helicobacter pylori. They identified overlapping B-cell and T-cell epitopes to develop nine distinct vaccine constructs, incorporating linkers and adjuvants. Among these, the construct designated C-8, characterized by the lowest allergenicity and highest antigenicity, was selected as a promiscuous vaccine candidate intended to effectively stimulate the host immune response[33]. In this research, T-cell epitopes were identified based on the anticipated B-cell epitopes to guarantee the stimulation of both antibody-mediated and cytotoxic immune responses. Following this, MHC class I and MHC class II binding epitopes were predicted utilizing various immunoinformatic tools to improve the precision and dependability of epitope selection. Additionally, overlapping epitopes that are shared by both B-cell and T-cell responses were chosen and assembled with suitable peptide linkers to create a stable and immunogenic multi-epitope vaccine candidate. Urrutia et al. developed an innovative epitope-based oral vaccine targeting Helicobacter pylori. This multiepitope vaccine incorporated the cholera toxin subunit B (CTB), which served as a mucosal adjuvant to enhance the immunogenicity of the vaccine when administered orally [34].another study conducted by Meza et al focused on the development of an innovative vaccine targeting Helicobacter pylori, characterized by its multi-antigenic, multistage, and multi-epitope properties. In their research, in silico methodologies were employed to design a chimeric construct comprising the cholera toxin B subunit fused with multiple epitopes derived from urease B, vacA, cagA, and hspA proteins[35]. In our study the50s Ribosome protein adjuvant was used at N-terminal to enhance the immunogenicity at cellular level for the construction of multi-epitope vaccine. The 3D structure of the vaccine construct was designed using alpha fold web server and the structure was validated using Ramachandran plot through molprobity and z score with PROSA web tool. HADDOCK server was utilized to estimate the interaction between vaccine construct and TLR5 complex which demonstrated a positive interaction, with a HADDOCK score of −88.8 ± 1.8, suggesting the formation of a stable complex Next, the binding affinity between the designed vaccine and TLR5 was evaluated using the PRODIGY server, which calculated an exact binding affinity score with a Gibbs free energy (ΔG) of −8.9 kcal/mol and a dissociation constant (KD) of 2.9E-07 M. The stability of the vaccine structure was assessed through molecular dynamics simulations using the Gromacs software package, which produced plots illustrating molecular flexibility and stability. Additionally, a Normal Mode Analysis (NMA) was conducted to examine molecular mobility and comparative deformability. The eigenvalue obtained was $3.8574e^{-07}$, indicating good flexibility of the docking complex. Finally, the benchling tool was used to clone the desired vaccine sequence within the pET28a (+) expression vector (*E. coli*).

In conclusion, the evaluation of the *H. pylori* multi-epitope vaccine is promising, underscoring its potential use and providing new perspectives for the progress of *H. pylori* vaccine development. Subsequent studies will necessitate additional experimental validation to determine the druggability of the developed multi-epitope vaccine. Furthermore, the approach utilized in this research demonstrates considerable promise for the creation of multi-epitope vaccines and ought to be taken into account for the advancement of vaccines targeting various infectious agents.

4. Conclusion

We have made efforts to develop a chimaera vaccine for *H. pylori* by targeting outer membrane proteins. This vaccine includes epitopes that stimulate IFN-γ production in T-cells, along with B-cell epitopes that can elicit strong cell-mediated immunity and humoral responses. It holds the potential to provide protection for various population groups worldwide. The vaccine has been thoroughly characterized regarding its physicochemical properties, as well as its antigenic and allergenic profiles. Docking studies and immune simulations indicated effective binding with TLR5 and the generation of both cell-mediated and humoral immune responses. MD simulation of vaccine construct with TLR-5 exhibit biophysical stability and acts as the promising candidate for experimental validation. Additionally, we conducted in-silico cloning to confirm the efficient expression of the vaccine construct in an expression vector for future applications. However, in-vitro validation is necessary to ensure effective control of *H. pylori* infection. We anticipate that this vaccine will demonstrate promising outcomes in managing *H. pylori* infections.

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Institutional Review Board Statement: Not applicable

Availability of Data and Materials The data supporting the findings of this study are available upon reasonable request from the corresponding author.

Conflict of Interest The authors have no conflicts of interest to declare.

Acknowledgments None.

Patient Consent for Publication Not applicable.

Figure 1 Comparison of conventional antibiotic treatment and in silico vaccine design against *Helicobacter pylori*, showing antibiotic regimens with variable outcomes and computational vaccine development aiming for improved immune protection.

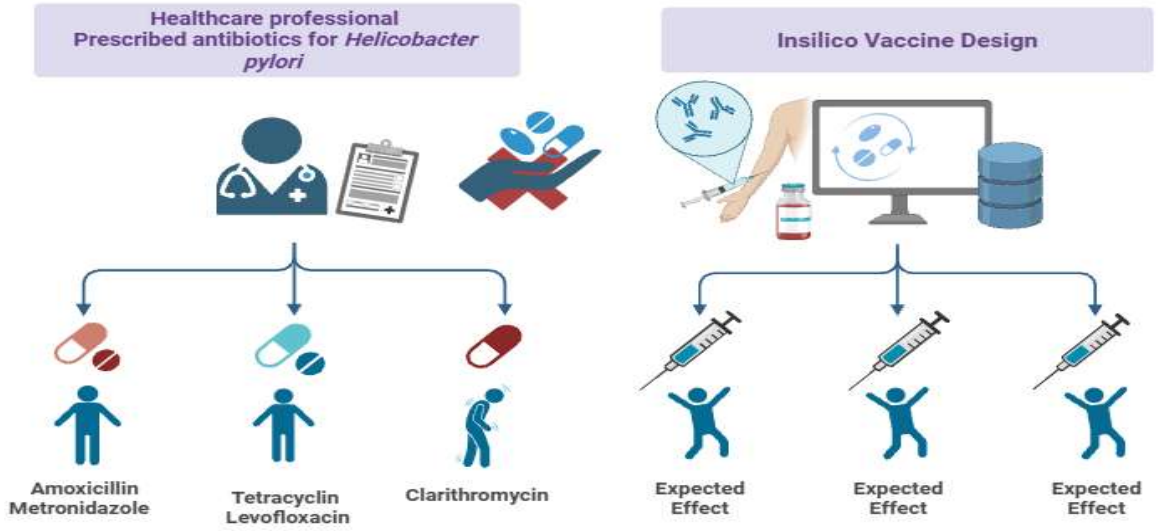


Figure 2 Predicted 3D structures of three *Helicobacter pylori* proteins—WP_120824222.1 (417 aa), WP_267285939.1 (376 aa), and WP_267286003.1 (418 aa)—with corresponding Ramachandran plots demonstrating the stereochemical quality of each model.

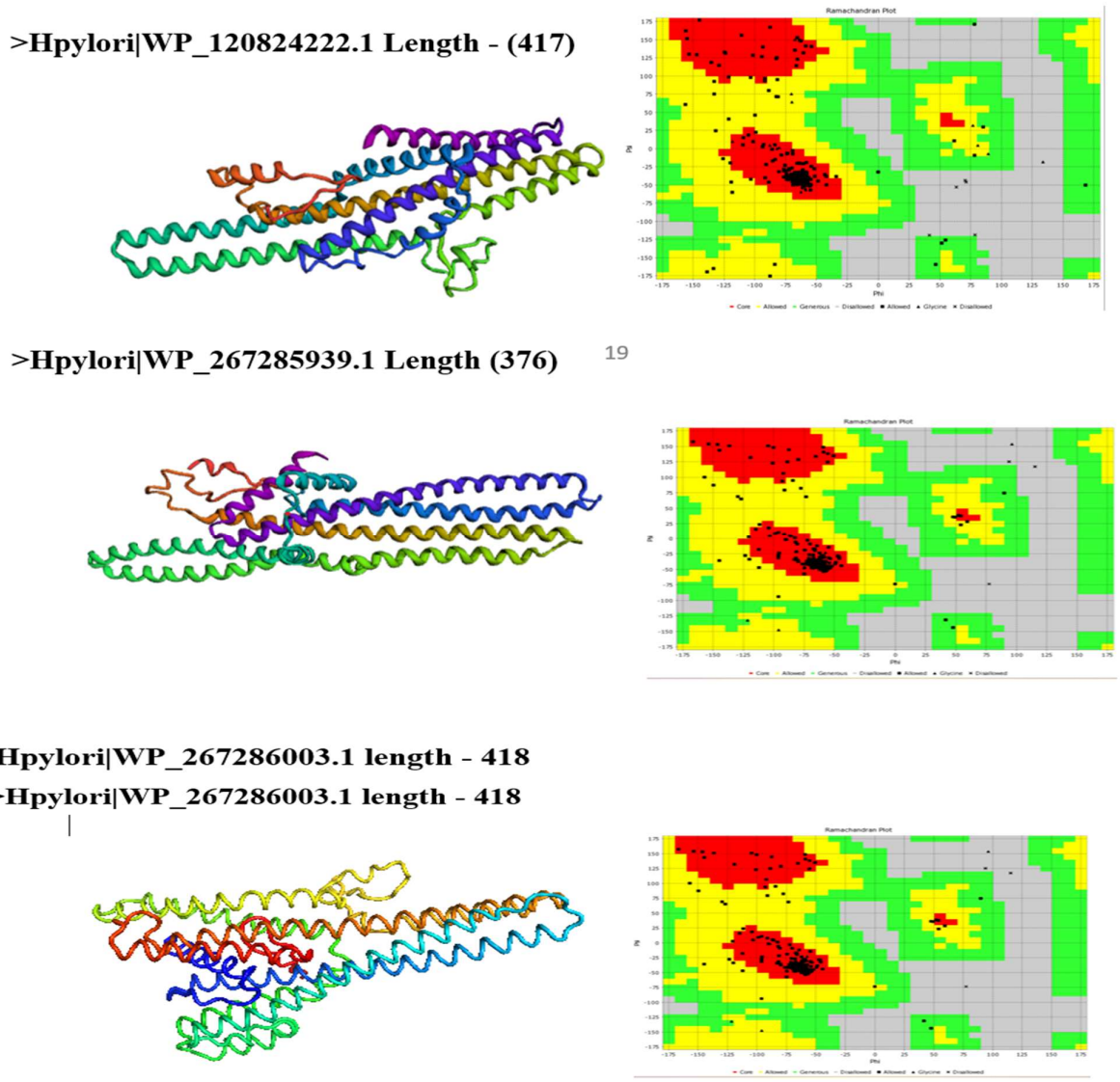


Figure 3 The multi-epitope vaccine construct is depicted schematically, with adjuvant, cytotoxic T lymphocyte (CTL), and helper T lymphocyte (HTL) epitopes derived from hypothetical Helicobacter pylori proteins (Hypo1, Hypo2, Hypo3), Ton-B dependent receptor, and Hop protein, all designed to enhance cellular and humoral immunity.

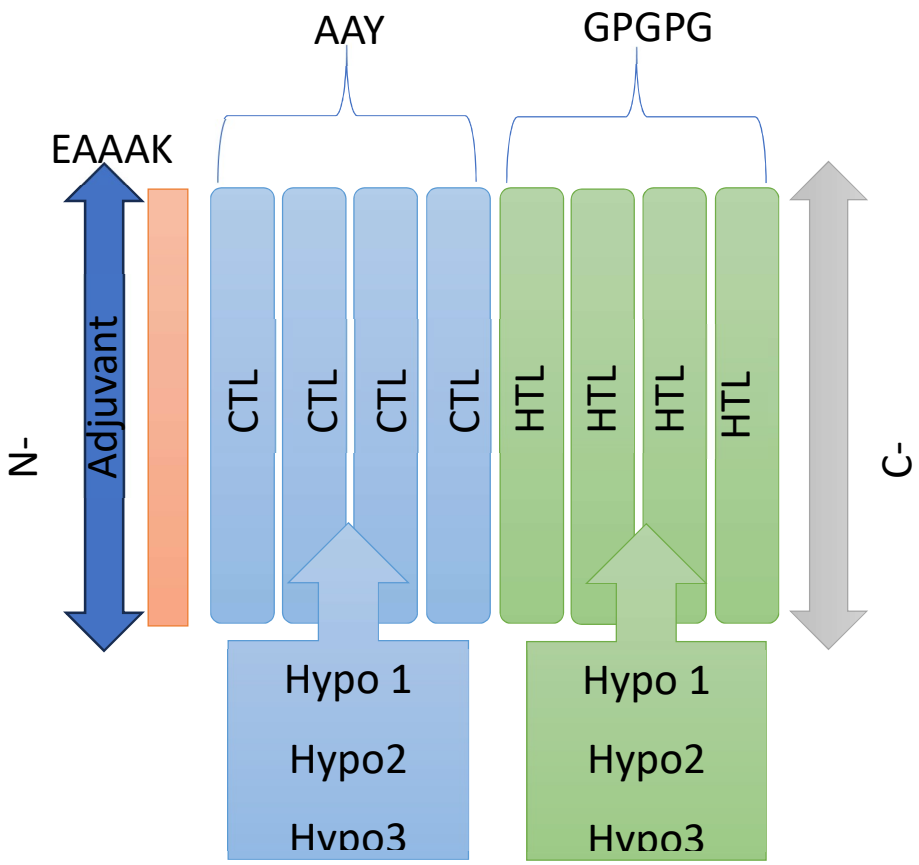


Figure 4 AlphaFold predicted the tertiary structure of the multi-epitope vaccination (461 amino acids). The model is represented as a ribbon, with colors ranging from N-terminus (blue) to C-terminus (red). The vaccine construct is structurally stable, including visible α-helices, β-sheets, and loop regions.

a) Refined three-dimensional structure of the multi-epitope vaccine construct for Toll like receptor-5(TLR5) docking

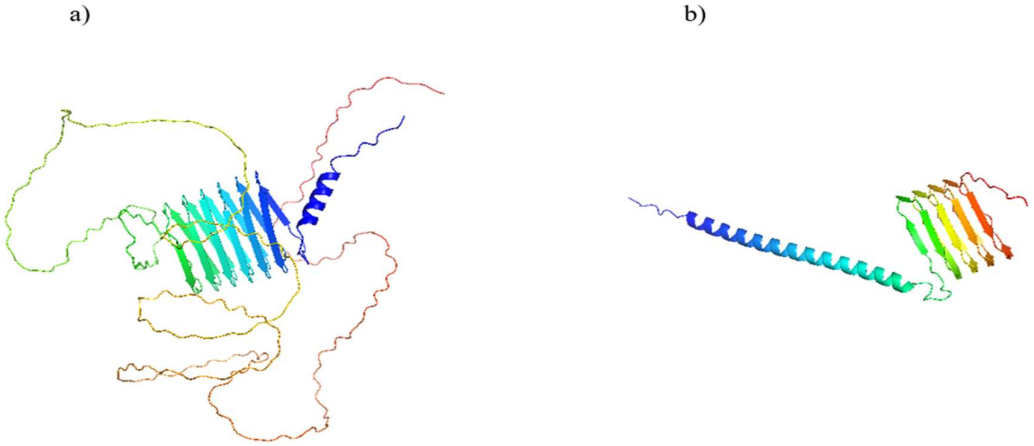


Figure 5 (a) Ramachandran plot illustrating the distribution of backbone dihedral angles (φ and ψ) for residues in the refined vaccine model.
(b) Z-score graph produced by PROSA-web representing the overall quality of the predicted structure.

(c) Residue energy graph from PROSA-web displaying the local quality of the model throughout the protein sequence.

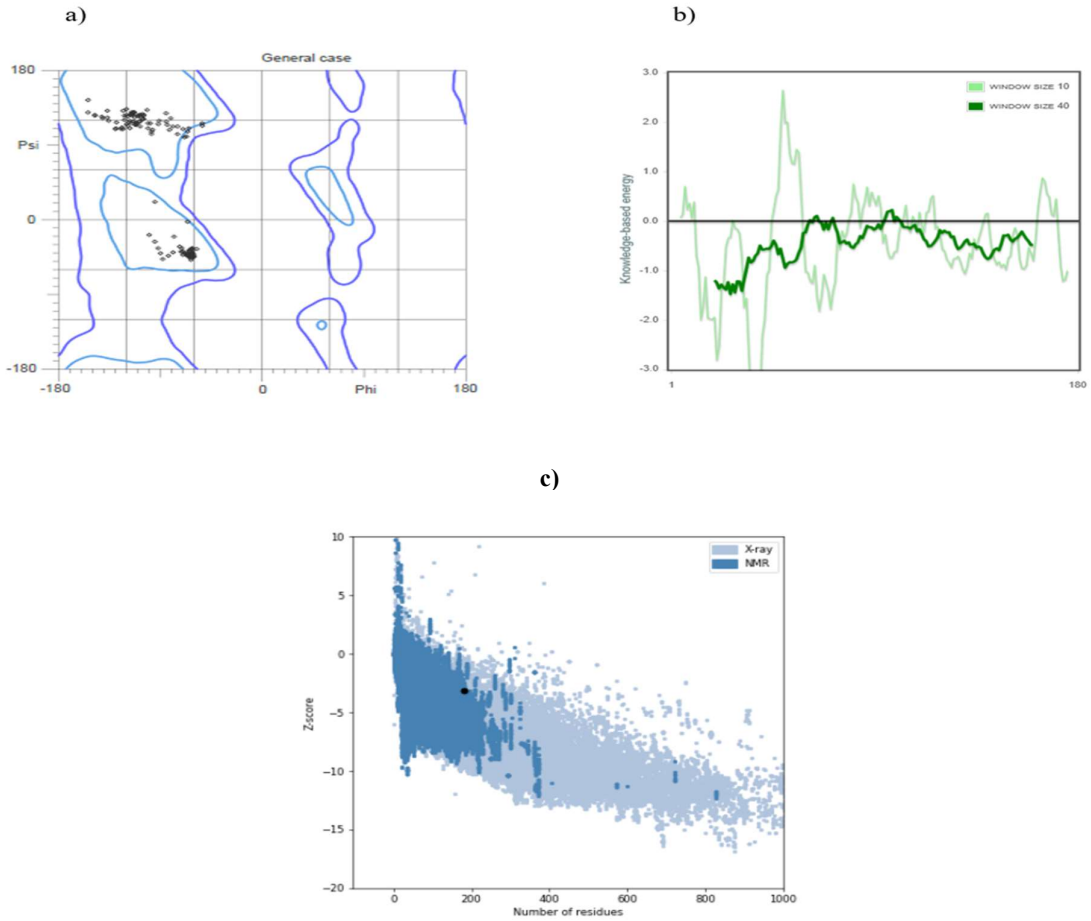


Figure 6 Yellow color sphere structure showed discontinuous B cell epitope in 3D structure of multi-epitope vaccine

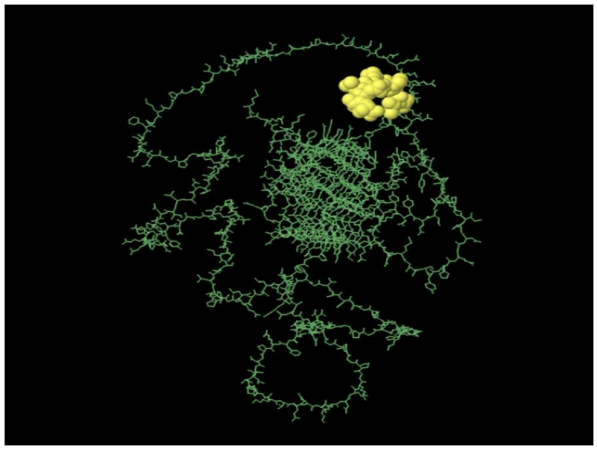


Figure 7 3D structure of the docked complex showing the interaction between the vaccine construct and the TLR5 receptor protein, visualized in ribbon representation with different colors indicating individual chains.

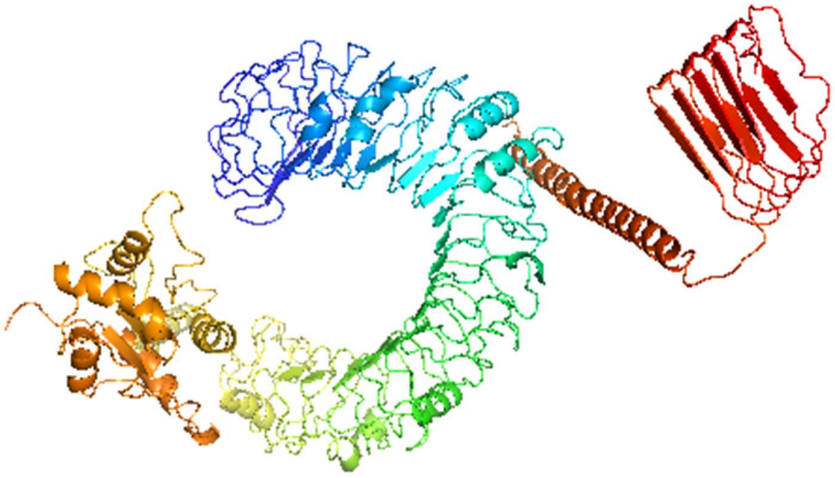
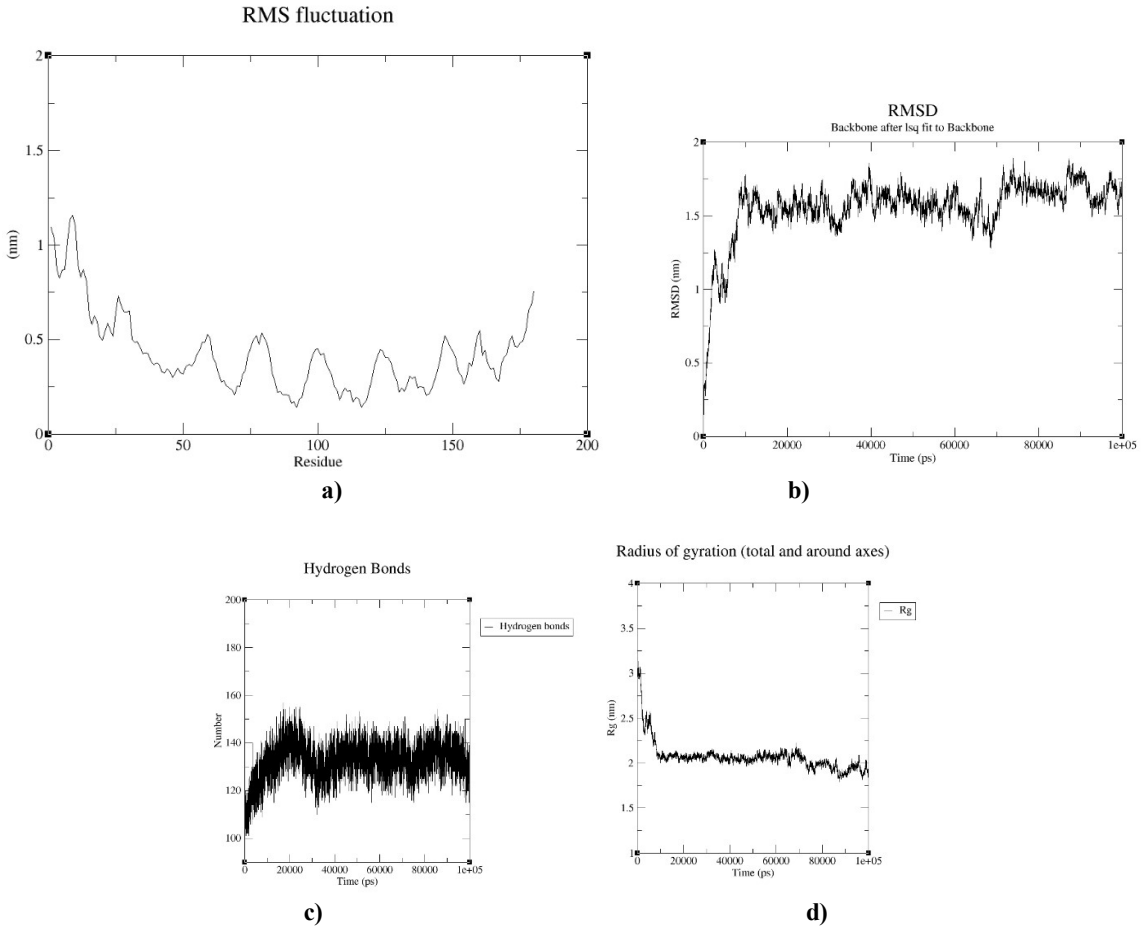
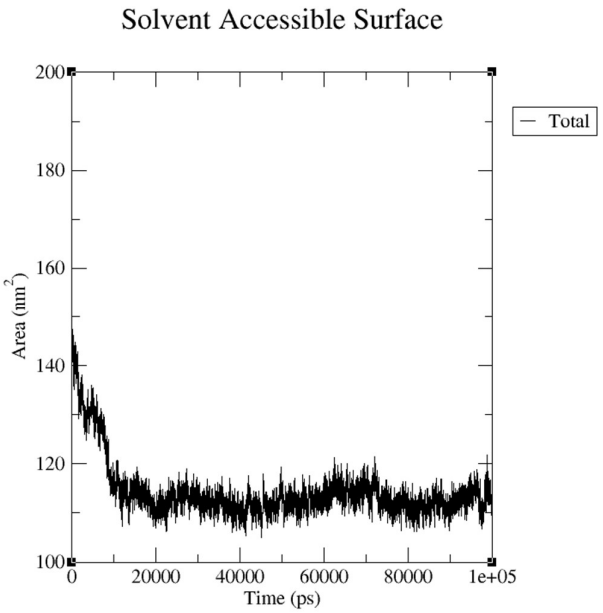


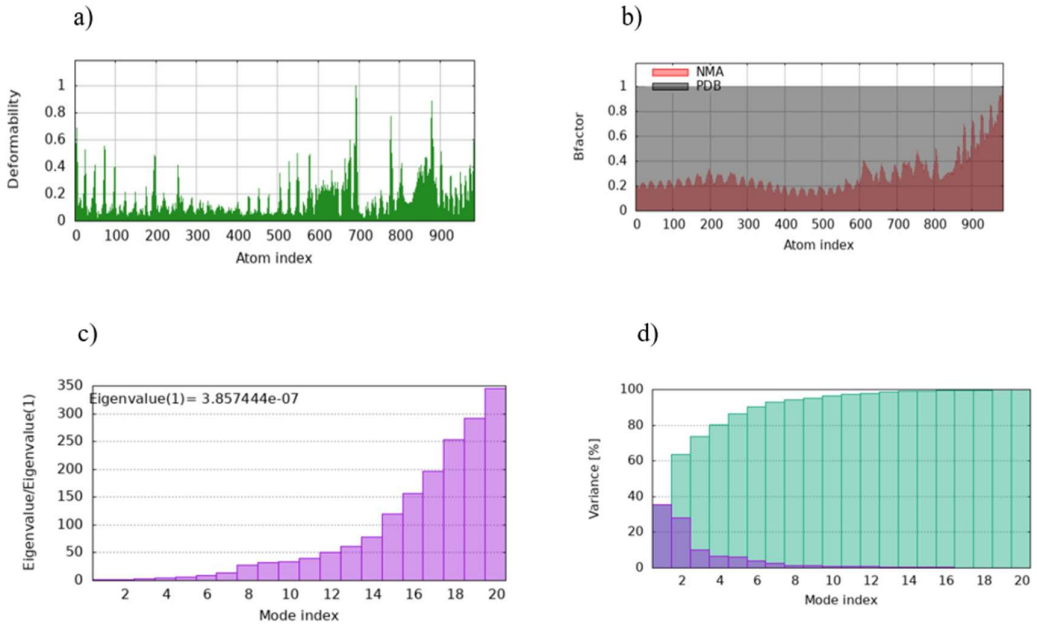
Figure 8 Molecular dynamics simulation of the vaccine construct-TLR5 complex showing a) Root mean square deviation (RMSD) b) Root mean square fluctuation (RMSF), c) Radius of gyration, d) Hydrogen bond analysis e) Solvent accessible surface area (SASA)





e)

Figure 9 This figure shows the results of iMODS (NMA) a) Deformability plot, b) B-factor, c) Eigenvalue, d) variance plot, e) Co-variance plot, f) Elastic network



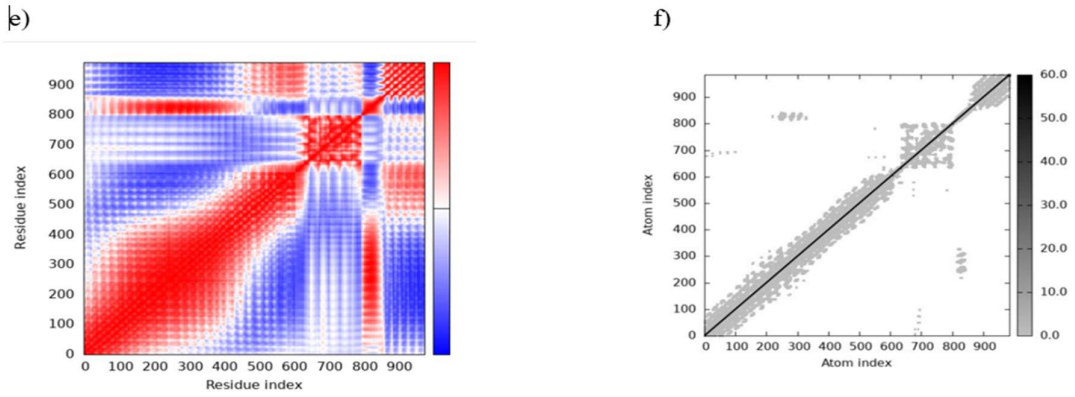


Figure 10 Circular plasmid map of the recombinant pET-28a(+) vector (6969 bp) showing the inserted vaccine construct along with key features including T7 promoter, T7 tag, lacI, kanamycin resistance gene, origin of replication, and restriction enzyme sites.

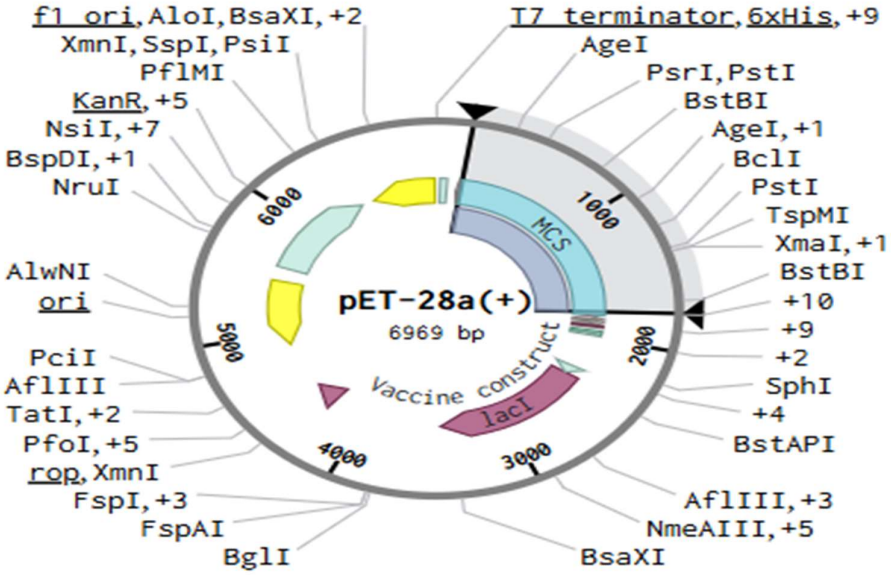


Table 1 List of Non -Human Homologues *H. pylori* unique sequence from Verified NCBI database

Sl. No	NCBI Protein ID
1.	WP_000395385.1
2.	WP_000750185.1
3.	WP_000919408.1
4.	WP_120856486.1
5.	WP_120856649.1
6.	WP_120857136.1
7.	WP_120857650.1
8.	WP_120857856.1
9.	WP_120857978.1
10.	WP_220839659.1
11.	WP_267285856.1
12.	WP_267285864.1
13.	WP_267285865.1

14.	WP_267285872.1
15.	WP_267285930.1
16.	WP_267285951.1
17.	WP_267285961.1
18.	WP_267285967.1
19.	WP_267286085.1
20.	WP_267286086.1
21.	WP_276206525.1
22.	WP_096423885.1
23.	WP_120856774.1
24.	WP_120857150.1
25.	WP_120857994.1
26.	WP_267285827.1
27.	WP_267285866.1
28.	WP_267286072.1
29.	WP_108303707.1
30.	WP_120856672.1
31.	WP_120856705.1
32.	WP_120858311.1
33.	WP_267286036.1
34.	WP_120857910.1
35.	WP_267285879.1
36.	WP_267285980.1
37.	WP_267286021.1
38.	WP_001868682.1
39.	WP_120858083.1
40.	WP_120858118.1
41.	WP_267286006.1
42.	WP_120857152.1
43.	WP_267285850.1
44.	WP_267286159.1
45.	WP_267286202.1
46.	WP_120856691.1
47.	WP_267285854.1
48.	WP_267285871.1
49.	WP_267286141.1
50.	WP_120857110.1
51.	WP_120857663.1
52.	WP_120857665.1
53.	WP_267285867.1
54.	WP_120857021.1
55.	WP_120857876.1
56.	WP_267285892.1
57.	WP_120824223.1
58.	WP_267286002.1
59.	WP_267286196.1
60.	WP_000892777.1
61.	WP_240446566.1
62.	WP_245059111.1
63.	WP_267285890.1
64.	WP_267285896.1
65.	WP_267286149.1
66.	WP_120857733.1
67.	WP_120858157.1
68.	WP_267286070.1
69.	WP_120857695.1
70.	WP_267285846.1

71.	WP_267286114.1
72.	WP_120824222.1
73.	WP_267285939.1
74.	WP_267286003.1

Table 2 The table shows the predicted subcellular localization of outer membrane, Extracellular, Periplasmic, cytoplasm regions.

NCBI Protein ID	Location
WP_000395385.1	OM,Ext
WP_000750185.1	OM
WP_000919408.1	OM
WP_120856486.1	Peri, OM
WP_120857856.1	OM
WP_120857978.1	OM
WP_220839659.1	OM. Ext
WP_267285864.1	OM
WP_267285872.1	OM
WP_267286085.1	OM
WP_267286086.1	OM
WP_096423885.1	OM
WP_120856774.1	OM
WP_120857150.1	OM
WP_120857994.1	OM
WP_267285827.1	OM
WP_267285866.1	OM
WP_267286072.1	OM
WP_120856672.1	Cyto, OM
WP_120856705.1	Cyto, OM
WP_120857910.1	OM
WP_267285879.1	OM
WP_267285980.1	OM
WP_267286021.1	OM
WP_267286006.1	Ext, OM, Cyto
WP_267286159.1	Ext, OM
WP_120857110.1	OM
WP_120857665.1	Ext,OM
WP_267285867.1	Extr ,OM
WP_120857021.1	OM
WP_120857876.1	OM
WP_267285892.1	OM
WP_267285846.1	Cyto,OM
WP_267286114.1	OM,Cyto,Extra
WP_120824222.1	OM
WP_267285939.1	OM
WP_267286003.1	OM

TABLE 3 The table presents the signal peptide prediction of selected *H. pylori* proteins

Sl. No	NCBI Accession No.	Signal Peptide Box	Score
1.	WP_000395385.1	Sec/SPI	0.9992
2.	WP_000750185.1	Sec/SPI	0.9989
3.	WP_000919408.1	Sec/SPI	0.9799
4.	WP_120856486.1	Sec/SPI	0.9869
5.	WP_120857136.1	Sec/SPI	0.9931
6.	WP_120857856.1	Sec/SPI	0.999
7.	WP_120857978.1	Sec/SPI	0.9805
8.	WP_220839659.1	Sec/SPI	0.9991

9.	WP_267285864.1	Sec/SPI	0.9987
10.	WP_267285872.1	Sec/SPI	0.7601
11.	WP_267286085.1	Sec/SPI	0.6119
12.	WP_267286086.1	Sec/SPI	0.971
13.	WP_096423885.1	Sec/SPI	0.9974
14.	WP_120856774.1	Sec/SPI	0.9961
15.	WP_120857150.1	Sec/SPI	0.7384
16.	WP_120857994.1	Sec/SPI	0.9013
17.	WP_267285827.1	Sec/SPI	0.9986
18.	WP_267285866.1	Sec/SPI	0.9947
19.	WP_120857910.1	Sec/SPI	0.9769
20.	WP_267285879.1	Sec/SPI	0.9858
21.	WP_267285980.1	Sec/SPI	0.6712
22.	WP_120857665.1	Sec/SPI	0.9844
23.	WP_267285867.1	Sec/SPI	0.6601
24.	WP_120857021.1	Sec/SPI	0.5745
25.	WP_120857876.1	Sec/SPI	0.9986
26.	WP_267285892.1	Sec/SPI	0.9984
27.	WP_120824222.1	Sec/SPI	0.9263
28.	WP_267285939.1	Sec/SPI	0.9988
29.	WP_267286003.1	Sec/SPI	0.7954

Table 4 The table shows the prediction of transmembrane helices in selected *H. pylori* proteins

Sl. No	NCBI Protein Accession No.	No. of Helix
1.	WP_000395385.1	0
2.	WP_000750185.1	0
3.	WP_000919408.1	0
4.	WP_120856486.1	0
5.	WP_120857136.1	1
6.	WP_120857856.1	0
7.	WP_120857978.1	0
8.	WP_220839659.1	0
9.	WP_267285864.1	0
10.	WP_267285872.1	0
11.	WP_267286085.1	0
12.	WP_267286086.1	0
13.	WP_096423885.1	0
14.	WP_120856774.1	0
15.	WP_120857150.1	1
16.	WP_120857994.1	0
17.	WP_267285827.1	0
18.	WP_267285866.1	0
19.	WP_120857910.1	0
20.	WP_267285879.1	0
21.	WP_267285980.1	0
22.	WP_120857665.1	0
23.	WP_267285867.1	0
24.	WP_120857021.1	0
25.	WP_120857876.1	0
26.	WP_267285892.1	0
27.	WP_120824222.1	0
28.	WP_267285939.1	0
29.	WP_267286003.1	0

Table 5 The table represents the virulence prediction of selected *H. pylori* proteins with significant scores were considered potential targets

Sl. No	NCBI Protein Accession No.	Virulence Result	Score

1.	WP_000395385.1	Virulent	1.0215
2.	WP_000750185.1	Virulent	1.0221
3.	WP_000919408.1	Virulent	1.0206
4.	WP_120856486.1	Virulent	1.0392
5.	WP_120857136.1	Virulent	0.923
6.	WP_120857856.1	Virulent	0.9265
7.	WP_120857978.1	Virulent	1.0049
8.	WP_220839659.1	Virulent	0.9918
9.	WP_267285864.1	Virulent	1.0016
10.	WP_267285872.1	Virulent	1.0647
11.	WP_267286085.1	Virulent	1.0005
12.	WP_267286086.1	Virulent	1.0025
13.	WP_096423885.1	Virulent	1.0024
14.	WP_120856774.1	Virulent	0.9
15.	WP_120857150.1	Virulent	0.0303
16.	WP_120857994.1	Virulent	0.9157
17.	WP_267285827.1	Virulent	0.8639
18.	WP_267285866.1	Virulent	1.0217
19.	WP_120857910.1	Virulent	1.0743
20.	WP_267285879.1	Virulent	1.0874
21.	WP_267285980.1	Virulent	1.0132
22.	WP_120857665.1	Virulent	1.0009
23.	WP_267285867.1	Virulent	0.9988
24.	WP_120857021.1	Virulent	1.0065
25.	WP_120857876.1	Virulent	1.0107
26.	WP_267285892.1	Virulent	1.0005
27.	WP_120824222.1	Virulent	1.0739
28.	WP_267285939.1	Virulent	1.0055
29.	WP_267286003.1	Virulent	0.9722

Table 6 The table presents the antigenicity prediction of selected *H. pylori* proteins

Sl. No	NCBI Protein Accession ID	Antigenicity Result	Score
1.	WP_000395385.1	Antigen	0.516
2.	WP_000750185.1	Antigen	0.6887
3.	WP_000919408.1	Antigen	0.4698
4.	WP_120856486.1	Antigen	0.5584
5.	WP_120857136.1	Antigen	0.6395
6.	WP_120857856.1	Antigen	0.51
7.	WP_120857978.1	Antigen	0.4646
8.	WP_220839659.1	Antigen	0.4822
9.	WP_267285864.1	Antigen	0.6874
10.	WP_267285872.1	Antigen	0.3923
11.	WP_267286085.1	Antigen	0.4191
12.	WP_267286086.1	Antigen	0.5357
13.	WP_096423885.1	Antigen	0.5338
14.	WP_120856774.1	Antigen	0.4681
15.	WP_120857150.1	Antigen	0.3302
16.	WP_120857994.1	Antigen	0.4854
17.	WP_267285827.1	Antigen	0.5846
18.	WP_267285866.1	Antigen	0.4677
19.	WP_120857910.1	Antigen	0.6065
20.	WP_267285879.1	Antigen	0.4988
21.	WP_267285980.1	Antigen	0.4919
22.	WP_120857665.1	Antigen	0.6038
23.	WP_267285867.1	Antigen	0.5776
24.	WP_120857021.1	Antigen	0.576
25.	WP_120857876.1	Antigen	0.546

26.	WP_267285892.1	Antigen	0.558
27.	WP_120824222.1	Antigen	0.4221
28.	WP_267285939.1	Antigen	0.4782
29.	WP_267286003.1	Antigen	0.3948

Table 7 This table shows allergenicity prediction of selected *H. pylori* proteins (Proteins predicted as non-allergens were considered safe)

Sl. No	NCBI Protein Accession ID	Allergenicity Result	Score
1.	WP_000395385.1	Non-Allergen	0.5182
2.	WP_000919408.1	Non-Allergen	0.4822
3.	WP_120856486.1	Non-Allergen	0.5463
4.	WP_120857136.1	Non-Allergen	0.5120
5.	WP_120857856.1	Non-Allergen	0.5151
6.	WP_120857978.1	Non-Allergen	0.6059
7.	WP_220839659.1	Non-Allergen	0.4970
8.	WP_267285872.1	Non-Allergen	0.4940
9.	WP_096423885.1	Non-Allergen	0.4940
10.	WP_120856774.1	Non-Allergen	0.5151
11.	WP_120857150.1	Non-Allergen	0.4970
12.	WP_120857994.1	Non-Allergen	0.5060
13.	WP_267285827.1	Non-Allergen	0.4880
14.	WP_267285866.1	Non-Allergen	0.4677
15.	WP_267285879.1	Non-Allergen	0.5690
16.	WP_267285980.1	Non-Allergen	0.5822
17.	WP_120857665.1	Non-Allergen	0.5822
18.	WP_267285867.1	Non-Allergen	0.5690
19.	WP_120824222.1	Non-Allergen	0.5337
20.	WP_267285939.1	Non-Allergen	0.7361
21.	WP_267286003.1	Non-Allergen	0.5306

Table 8 Functional annotation of selected outer membrane proteins of *H. pylori*

Sl. No	NCBI Protein Accession ID	Protein Name
1.	WP_000395385.1	outer membrane protein
2.	WP_000919408.1	outer membrane protein
3.	WP_120856486.1	outer membrane protein
4.	WP_120857136.1	outer membrane protein
5.	WP_120857856.1	outer membrane protein
6.	WP_120857978.1	Hop family outer membrane protein HopI
7.	WP_220839659.1	outer membrane protein
8.	WP_267285872.1	Hop family outer membrane protein HopJ/HopK
9.	WP_096423885.1	outer membrane beta-barrel protein HofF
10.	WP_120856774.1	outer membrane beta-barrel protein HofB
11.	WP_120857150.1	outer membrane family protein
12.	WP_120857994.1	outer membrane beta-barrel protein HofH
13.	WP_267285827.1	outer membrane beta-barrel protein HofE
14.	WP_267285866.1	outer membrane beta-barrel protein HofG
15.	WP_267285879.1	outer membrane beta-barrel protein
16.	WP_267285980.1	outer membrane beta-barrel protein
17.	WP_120857665.1	TonB-dependent receptor
18.	WP_267285867.1	TonB-dependent receptor domain
19.	WP_120824222.1	Hypothetical protein
20.	WP_267285939.1	Hypothetical protein
21.	WP_267286003.1	Hypothetical protein

Table 9 The table shows physiochemical properties of selected *H. pylori* outer membrane proteins.

Sl. No	Protein Name	Protein ID	Molecular weight	Theoretical pI	Instability Index	Aliphatic Index	GRAVY	No. of Amino Acids
1	Hypothetical protein 1	WP_120824222.1	47128.7	8.75	31.49	82.95	-0.6024	413
2	Hypothetical protein 2	WP_267285939.1	43667.3	9.11	44.40	92.71	-0.5425	376
3	Hypothetical protein 3	WP_267286003.1	48190.6	8.90	32.22	79.83	-0.6944	418
4	Ton B-dependent receptor domain	WP_267285867.1	90846.34	8.99	27.63	72.60	-0.4460	813
5	Hop family outer membrane protein HopJ/HopK	WP_267285872.1	41621.8	7.13	29.92	79.43	-0.2730	367

Table 10 Secondary structure composition of selected *H. pylori* proteins

Protein ID	Protein Name	Alpha helix (%)	Beta bridge (%)	Random coil (%)	Extended Strand (%)
WP_120824222.1	Hypothetical protein	83.54%	-	14.77%	1.69%
WP_267285939.1	Hypothetical protein	88.56%	-	9.84%	1.60%
WP_267286003.1	Hypothetical protein	76.32%	-	20.81%	2.87%
WP_267285867.1	Ton B-dependent receptor domain	18.57%	-	66.91%	14.51%
WP_267285872.1	Hop family outer membrane protein HopJ/HopK	40.05%	-	42.78%	17.17%

Table 11: A Selection of MHC-I epitopes (derived from B-cell epitopes) based on Vaxijen score > 0.7

Protein	B cell epitopes	MHC I epitopes	HLA allele	Vaxigen score
Hypo 1	AQYQIESIRYNYENLK	QIESIRYNY	HLA-A*01:01 HLA-A*26:01 HLA-B*58:01 HLA-B*15:01	1.1480
	TPLIVNDPELHAGQAT	IVNDPELHA	HLA-A*01:01 HLA-A*02:01 HLA-A*26:01 HLA-B*07:02 HLA-B*58:01	0.9045
	TKTGEQTTRMRDMQNL	KTGEQTTRM	HLA-A*01:01 HLA-A*03:01 HLA-A*26:01 HLA-B*07:02 HLA-B*40:01	**1.9995**
	NQARYNQLSAEASLDI	NQLSAEASL	HLA-B*15:01 HLA-A*02:01	**1.0322**

			HLA-A*24:02 HLA-B*07:02 HLA-B*08:01	
Hypo 2	FSNAQIMMTFDSQTNA	MTFDSQTNA	HLA-A*01:01 HLA-A*02:01 HLA-A*03:01 HLA-A*26:01 HLA-B*58:01	**1.0857**
	QQSHNKTLEYNALINH	QSHNKTLEY	HLA-A*01:01 HLA-A*03:01 HLA-A*26:01 HLA-B*07:02 HLA-B*08:01	**1.1882**
	NLKSCTKELRQRYQNA	ELRQRYQN	HLA-A*26:01 HLA-B*08:01 HLA-B*07:02	1.2461
	HTLKTLNIEFLSELSE	TLKTLNIEF	HLA-B*07:02 HLA-A*01:01 HLA-A*02:01 HLA-A*03:01 HLA-A*24:02 HLA-A*26:01	**1.6508**
Hypo 3	SKRLDALQTNFGVTDP	RLDALQTNF	HLA-A*01:01 HLA-A*02:01 HLA-A*03:01 HLA-A*24:02 HLA-A*26:01 HLA-B*07:02	**0.9864**
	NFGVTDPTANHNKQGI	TANHNKQGI	HLA-B*07:02 HLA-B*08:01 HLA-B*40:01 HLA-B*07:02 HLA-B*58:01	**1.0096**
	RARRYFQNPNVKFDQF	YFQNPNVKF	HLA-A*01:01 HLA-A*02:01 HLA-A*24:02 HLA-A*26:01 HLA-B*07:02	**1.0063**
	RARRYFQNPNVKFDQF	NPNVKFDQF	HLA-A*01:01 HLA-A*24:02 HLA-A*26:01 HLA-B*07:02 HLA-B*08:01	**1.6425**
Ton-B	IRNVQIDPLFRPNDIA	NVQIDPLFR	HLA-A*01:01 HLA-A*03:01 HLA-A*26:01 HLA-A*01:01 HLA-A*03:01	1.3614
	EEEKEERKTERKKDQK	KTERKKDQK	HLA-A*03:01	2.2939

			HLA-A*01:01 HLA-A*03:01 HLA-A*01:01	
	YVRGIEDRLARVTVDG	GIEDRLARV	HLA-A*02:01 HLA-A*26:01 HLA-B*08:01 HLA-A*01:01	1.4452
	GAAQMGASYGHQGNTI	GAAQMGASY	HLA-A*01:01 HLA-A*26:01 HLA-B*07:02 HLA-B*58:01 HLA-B*15:01	0.9747
Hop	AEENGAYASVGFEYSI	AYASVGFEY	HLA-A*01:01 HLA-A*03:01 HLA-A*24:02 HLA-B*07:02 HLA-B*27:05	0.8546
	YQAKMHTSFFQIPLNF	YQAKMHTSF	HLA-B*08:01 HLA-A*01:01 HLA-A*02:01 HLA-A*24:02 HLA-A*26:01 HLA-B*07:02	0.7160
	HGKGLNTSLFFKRLVV	GLNTSLFFK	HLA-A*03:01 HLA-B*27:05 HLA-A*01:01	0.7568
	NLEVQFNQSQNRMLSS	VQFNQSQNR	HLA-A*03:01 HLA-A*26:01 HLA-B*27:05 HLA-B*15:01	0.9357

Table 11: A Selection of MHC-II epitopes (derived from B-cell epitopes) based on Vaxijen score > 0.5

Protein	B cell epitopes	MHC II epitopes	HLA allele	Vaxigen score
Hypo 1	AQYQIESIRYNYENLK	QYQIESIRYNYENLK	DRB1_0101 DRB1_0102 DRB1_0103 DRB1_0301 DRB1_0305 DRB1_0401	0.8384
	TPLIVNDPELHAGQAT	TPLIVNDPELHAGQA	DRB1_0102 DRB1_0103 DRB1_0305 DRB1_0401 DRB1_0404 DRB1_0405	0.8280
	TKTGEQTTRMRDMQNL	KTGEQTTRMRDMQNL	DRB1_0101	1.1687

			DRB1_0102 DRB1_0701 DRB1_0801 DRB1_0803 DRB1_0901	
	NQARYNQLSAEASLDI	QARYNQLSAEASLDI	DRB1_0101 DRB1_1401 DRB1_1454 DRB1_1601 DRB3_0202	1.1780
Hypo 2	FSNAQIMMTFDSQTNA	SNAQIMMTFDSQTNA	DRB1_0101 DRB1_0901 DRB1_1001 DRB1_1101 DRB3_0202 DRB1_1501	0.7988
	QQSHNKTLEYNALINH	QSHNKTLEYNALINH	DRB1_0901 DRB1_1001 DRB1_1104 DRB3_0202 DRB1_1454	0.8248
	NLKSCTKELRQRYQNA	LKSCTKELRQRYQNA	DRB1_0901 DRB1_1501 DRB5_0101 DRB1_1601 DRB1_0301	0.9246
	HTLKTLNIEFLSELSE	HTLKTLNIEFLSELS	DRB4_0101 DRB1_1402 DRB5_0202 DRB4_0103 DRB3_0202	0.5015
Hypo 3	SKRLDALQTNFGVTDP	KRLDALQTNFGVTDP	DRB1_0101 DRB1_1101 DRB1_1104 DRB4_0103 DRB3_0101	0.9399
	NFGVTDPTANHNKQGI	FGVTDPTANHNKQGI	DRB1_0901 DRB1_1001 DRB1_1104 DRB4_0101 DRB5_0101	0.9097
	RARRYFQNPNVKFDQF	ARRYFQNPNVKFDQF	DRB3_0202 DRB1_1501 DRB1_1454 DRB1_1402 DRB4_0101	0.6669
	RARRYFQNPNVKFDQF	RARRYFQNPNVKFDQ	DRB1_0101 DRB1_0901 DRB5_0202 DRB4_0103	0.6767

			DRB1_1601	
Ton-B	IRNVQIDPLFRPNDIA	RNVQIDPLFRPNDIA	DRB1_1001 DRB3_0202 DRB1_1454 DRB1_1402 DRB4_0101	0.8676
	EEEKEERKTERKKDQK	EEKEERKTERKKDQK	DRB1_1101 DRB1_1104 DRB4_0101 DRB4_0103 DRB1_1601	2.5670
	YVRGIEDRLARVTVDG	VRGIEDRLARVTVDG	DRB1_1501 DRB1_1454 DRB5_0202 DRB3_0101 DRB1_1402	1.0126
	GAAQMGASYGHQGNTI	AAQMGASYGHQGNTI	DRB5_0101 DRB4_0103 DRB1_1601 DRB1_1503 DRB4_0101	0.8824
Hop	AEENGAYASVGFEYSI	EENGAYASVGFEYSI	DRB1_1001 DRB1_1101 DRB3_0202 DRB4_0101 DRB5_0202	1.1879
	YQAKMHTSFFQIPLNF	QAKMHTSFFQIPLNF	DRB1_0901 DRB3_0202 DRB1_1402 DRB4_0103 DRB3_0101	1.1842
	HGKGLNTSLFFKRLVV	HGKGLNTSLFFKRLV	DRB1_1001 DRB1_1104 DRB4_0101 DRB1_1601 DRB1_1503	0.9348
	NLEVQFNQSQNRMLSS	LEVQFNQSQNRMLSS	DRB1_1001 DRB1_1101 DRB1_1454 DRB4_0103 DRB3_0101	0.8988

Table 12 Secondary structure composition of predicted vaccine construct

Name	Alpha helix	Beta sheets	Coil structure
Vaccine construct	81 (17.57%)	0	371 (80.4%)

Table 13 Antigenicity, allergenicity and physiochemical property assessment of the multi-epitope-based vaccine construct

Features	**Assessment**

Virulence	1.0595 (Virulent)
Antigenicity	**1.0221** (Antigen)
Allergenicity	0.6816 (Non- allergen)
Solubility	0.6022(Soluble)
Molecular weight	48561.79
Number of amino acid	461
Theoretical Isoelectric point (PI)	6.73
Number of atoms	6726
Formula	$C_{2126}H_{3300}N_{616}O_{676}S_8$
Half life	30 hour- mammalian reticulocyte, >20hours- yeast > 10 hours (*E. coli*)
Aliphatic index	62.0
Instability index	26.15 (Stable)
Grand average of hydropathicity (GRAVY)	0.673

Table 14 Ramachandran plot analysis of the refined vaccine construct model

S.No	Type of Amino acid residue	No of Amino acid residue	Percentage
1	Favoured region	172	96.63%
2	Allowed region	177	99.8%
3	Generously allowed region	100	2.81%
4	Disallowed region	1	0.56%
5	Number of glycine residues	1	0.56%
6	Number of proline residues	12	-
7	Number of non-glycine and non-proline residues	165	-
8	Total number of residues	178	-

Table 15 HADDOCK docking statistics and binding affinity analysis of the vaccine construct-TLR5 complex

Vaccine construct -TLR5	**Score**
HADDOCK Score	-88.8 +/-1.8
Cluster size	13
RMSD from the overall lowest -energy structure	1.6 +/-0.9
Van der walls energy (kcal mol)	-29.5 +/-5.1
Electrostatic energy (kcal mol)	-291.7 +/-50.6
Desolvation energy (kcal mol)	-9.0 +/-2.0
Restraints violation energy (kcal mol)	80.4 +/-38.1
Buried surface area (A)	1218.2 +/-43.2
Z-score	-2.3
PRODIGY binding score	Gibbs free energy (ΔG) − 8.9 kcal mol−1 and dissociation constant (Kd) 2.9 e-07M

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