Received: 21 February 2024 | Revised: 6 May 2024 | Accepted: 20 June 2024 | Published online: 27 June 2024

RESEARCH ARTICLE

Medinformatics
2024, Vol. 00(00) 1–13
DOI: 10.47852/bonviewMEDIN42022655

BON VIEW PUBLISHING

Designing Lsa46-Based Multi-Epitope Peptide Vaccine Against Leptospirosis: Immunoinformatic Approach

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Abstract: Leptospirosis represents a significant global public health problem due to its epidemiological impact, complex pathogen biology, and diverse clinical manifestations. Effective treatment, management, and preventive measures, including vaccination, are vital for addressing this disease. Lsa46, a surface-exposed outer membrane protein in leptospires, plays a crucial role in pathogenesis. In this study, we utilized multiple epitopes predicted from Lsa46, validated in previous study, to design a potential vaccine candidate construct. The antigenicity, allergenicity, autoimmunity, population coverage, immune simulation, molecular interactions, etc., were assessed using various computational tools. 3D structure modeling revealed a stable and high-quality model for epitope constructs. Immune simulation confirmed a robust immune response induced by the Designer Epitope construct, including IgG, IgM, MHC-I, MHC-II, cytokines, and interleukin production. Interaction analysis with immune cells and receptors, such as TCRαβ, TCRγδ, and TLRs, provided insights into the epitope construct's potential immunogenic response. Docking studies with TLR2 and TLR4 indicated their interaction with the epitope, suggesting their involvement in the immune response against leptospirosis. Further experimental validations are required to confirm these prediction results.

Keywords: leptospirosis, Lsa46, multi-epitope, peptide vaccine, immune simulation

1. Introduction

The rapid advancement of computational tools has transformed biology and medicine, particularly in the fields of reverse vaccinology and immunoinformatics. This approach has successfully led to the development of vaccines against bacterial pathogens like *Neisseria meningitidis* and *Streptococcus pneumonia* [1]. Immunoinformatics has also played a vital role in discovering antiviral drug targets and identifying vaccine candidates for emerging viral diseases such as influenza, Ebola, and COVID-19 [2].

Leptospirosis, a global zoonotic disease caused by leptospira bacteria, primarily *Leptospira interrogans*, affects both humans and animals, posing a significant health burden in tropical regions with poor sanitation and dense reservoir host populations. Despite approximately one million severe cases and 60,000 annual deaths globally, underreporting and misdiagnosis likely lead to underestimated prevalence [3]. Outbreaks linked to environmental factors, such as flooding, expose individuals to contaminated water and soil, with higher-risk groups including farmers, sewer workers, and veterinarians. Pathogenesis involves intricate interactions between host immune responses and bacterial virulence factors, resulting in a spectrum of clinical manifestations from mild flu-like symptoms to severe multi-organ dysfunction and fatal outcomes [4].

Early diagnosis and prompt antibiotic therapy are vital for managing leptospirosis. Considering the impact of leptospirosis on public health, preventive strategies are of paramount importance. Vaccination plays a crucial role in preventing leptospirosis, particularly in high-risk populations. Although vaccines are available, they are often limited in their coverage and effectiveness. Ongoing research aims to develop improved vaccines with broader serovar coverage and long-lasting immunity; thus, it helps in mitigating the burden of leptospirosis and reducing its impact on global health [5].

Multi-epitope-based subunit vaccines, composed of selected antigenic epitopes based on the computational predictions, offer several advantages including improved safety, scalability, and potential for broad-spectrum protection. The incorporation of multiple epitopes in a single vaccine construct holds the promise of enhancing immune response specificity and effectiveness [6].

This research study aims to explore the rational design of a multi-epitope-based subunit vaccine for leptospirosis, highlighting its potential in addressing challenges associated with traditional vaccine development against the disease. The previous *in silico* study on Lsa proteins demonstrated that Lsa46 consists of potent B- and T-cell epitopes. It also exhibited stable interactions with relevant immunological ligands, establishing it as a potential

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vaccine candidate. This was achieved through molecular docking, molecular dynamic simulation, and binding energy calculations. Building upon this, the present study focuses on *in silico* prediction of Lsa46 epitopes which were identified in the previous study, to design an alternative multi-epitope-based subunit vaccine candidate that offers cross-protection for disease management. The investigation employed several online servers and tools for sequential and structural analyses of multi-epitope designed to characterize the vaccine candidate construct.

2. Materials and Methods

The selected B-cell and T-cell epitopes of Lsa46 which showed desired interaction in docking and displayed structural and binding stability in our previous *in silico* study were taken for the multi-epitope-based vaccine design against leptospirosis [7].

2.1. Multi-epitope antigen design

The B-cell epitopes (398-418, 397-413, 90-106, and 356-372) and T-cell epitopes (MHC-I/CTL: 181-189 (HLA-A*32:01) and 133-144 (HLA-A*02:06) and MHC-II/HTL: 216-230 (HLA-DRB1*01:01), 341-358 (HLA-DRB1*13:02) were used in vaccine construct designing. These T- and B-cell epitopes were arranged based on their antigenicity scores.

2.1.1. Characterization of predicted epitopes

The primary sequence-based prediction analysis including antigenicity, allergenicity, toxicity, physicochemical parameter prediction, conservation analysis, population coverage, and autoimmunity prediction of the 105mer epitope was carried out to find whether the epitope is potential and safe as a vaccine candidate or not.

2.1.2. Antigenicity and allergenicity prediction

An online prediction server Vaxijen v2.0 [8] was utilized to determine the potential antigenicity of the epitope. The antigenicity was assessed using the default parameters of the server, with a threshold value of ≥ 0.4. This prediction method relied solely on the physicochemical properties of proteins, disregarding sequence alignment features. VirulentPred tool [9] was used to determine whether the peptide selected was virulent or not.

The AlgPred2 server and AllerTop v.2.0 [10, 11] were employed to assess the allergic/non-allergic nature of the epitope sequence. The allergenicity of vaccine sequences by AlgPred2 server was predicted using a hybrid approach (SVMc + IgEepitope + ARPsBLAST + MAST) with the highest accuracy and sensitivity. Furthermore, the selected epitopes were assessed for toxicity prediction using the ToxinPred2 [12] server. This tool predicts toxicity by considering both peptide toxicity and the toxicity of entire protein sequences.

2.1.3. Conservation and population coverage prediction

The epitope was submitted for analysis with the IEDB conservancy analysis tool to determine the level of conservancy in the peptide sequences. Those exhibiting conservancy were then chosen for subsequent analysis. Additionally, the IEDB population coverage analysis tool was utilized to predict the immunogenic response to the T-cell epitopes among the human population based on their respective HLA genotype frequencies. The calculation performed by this tool determines the proportion of individuals expected to react to a specific epitope set. It relies on HLA genotypic frequencies and data regarding MHC binding and/or T-cell restriction. The population/area selected was the entire "world" which includes allelic frequencies for 115 countries and 21 different ethnicities grouped into 16 different geographical areas. Epitopes with a population coverage of 50% or higher were taken for additional analysis.

2.1.4. Autoimmunity prediction

In order to minimize the risk of autoimmunity, each of the chosen individual B- and T-cell epitopes, along with the combined epitope as a single peptide, underwent BLASTP search analysis against the Human proteome. An effective vaccine ideally should not share sequence similarity with human proteins.

2.1.5. Subcellular localization of the epitope

The protein subcellular localization prediction was done to predict where the Designer Epitope resides in a cell or subcellular site. For the localization prediction, pSORTb 3.0 [13] and CELLO v.2.5 [14] online servers were used.

2.1.6. Physicochemical parameter prediction

Different physicochemical parameters ranging from instability index, *in vitro* and *in vivo* half-life, theoretical isoelectric point (pI), amino acid composition, GRAVY, molecular weight, etc, of the epitopes were assessed by Expasy. To evaluate the *in vivo* half-life of proteins, the "N-end rule" principle was employed, which revolves around recognizing the N-terminal residue of the proteins to determine their degradation rate. Moreover, the hydrophobicity of the protein is evaluated using GRAVY, which computes the average hydropathy of all amino acids within the protein sequence.

2.1.7. Secondary structure properties

The secondary structure conformational states, e.g., helix, sheet, turn, and coil of the epitope, were predicted using an online secondary structure analysis tool PSIPRED 4.0 (Protein Structure Prediction Server) [15] and SOPMA (Self-Optimized Prediction Method with Alignment) [16] with default parameters.

2.1.8. 3D structure modeling and validation

The 3D structure of the multi-epitope peptide was modeled using *ab-initio* modeling tool, I-TASSER [17]. The modeled 3D structure of the epitope was visualized using PymoL visualization tool. The modeled structures underwent quality check and validation using PROCHECK [18] server, which includes Ramachandran plot analysis.

2.1.9. Immune simulation of the epitope construct

Computational immune simulations were performed to determine immune response profile of the multi-epitope construct by the C-ImmSim online server. The Celada-Seiden model is employed by C-ImmSim to describe the humoral and cellular profiles of a mammalian immune system responding to the epitope. A single dose of antigen injection was administered. The simulation was performed with default parameters. The simulation volume was kept at 50 µl, simulation steps selected were 1,000, random seed was 12,345, and the epitope injection with no LPS was selected. The immune simulation of the four modeled epitope-based vaccine construct was analyzed on the basis of the literature [19], and three injections of the multi-epitope vaccine construct were simulated at different intervals of 1 month. Also, as a control the whole Lsa46 protein itself was injected as antigen to understand and compare the immune response profile.

2.2. Multi-epitope subunit vaccine design

Distinct linkers facilitated the assembly of epitopes identified by various immunoinformatics software to design the final vaccine construct. CTL (MHC-I binding) epitopes were connected using the AAY linker, HTL (MHC-II binding) epitopes using the GPGPG linker, and B cells using the KK linker. The vaccine's immunogenicity was enhanced by fusing the N-terminal of the vaccine with a 45-mer amino acid sequence of β-defensin, utilizing the EAAAK linker. The β-defensin peptides trigger the activation of innate immunity cells and attract naive T cells via the chemokine receptor-6 (CCR-6). To facilitate intracellular delivery of the designed vaccine, a TAT sequence (11 amino acids, YGRKKRRQRRR) was added to the C-terminal. Linkers (AYY, KK, and GPGPG) are crucial for inducing an extended conformation, improving the protein's flexibility, aiding in proper protein folding, and facilitating the separation of functional domains, thereby increasing the overall stability of the protein structure.

2.2.1. Molecular interaction studies

The epitope-based vaccine construct was used as a ligand for docking in four unique patterns to evaluate how changes in epitope design affected binding efficiency. Therefore, the epitope alone, epitope with linkers and adjuvants, repeat of the epitope, and repeat of the epitope with linkers and adjuvants were processed for interaction analysis. To achieve this, the 3D structures of all the ligands were modeled using the I-TASSER server, and the quality of the structures was validated using the Ramachandran plot through the PROCHECK [18] server. The molecular interaction between the modeled epitope-based vaccine constructs with the immunoglobulins – IgG and IgM(Fab), human TCRαβ, human TCRγδ, MHC-I, MHC-II, human TLR2, and human TLR4 was analyzed by molecular docking method using BIOVIA Discovery Studio 2021.

2.2.2. Quality assessment of the 3D structure of vaccine construct

The secondary structure of the final selected vaccine construct was predicted using the PSIPRED and SOPMA tools. Antigenicity/Allergenicity/Toxicity/Autoimmunity was also predicted. Frustratometer 2 [20] online server was employed for quantifying the degree of local frustration manifested in the vaccine construct peptide molecules. DISOPRED v.3 [21] was used to predict the disorders of the final vaccine construct.

3. Results

3.1. Multi-epitope vaccine development

The epitopes utilized for vaccine design are the predicted B- and T-cell epitopes of the Lsa46 protein, which were confirmed through *in silico* analysis in the previous study. The epitopes 398–418 and 397–413 have overlapping regions and when individually analyzed, both showed structurally and energetically stable interactions [7] and thus were combined together. The combined total B-cell epitope is TDGKGSSEPIVPMKNPETDYKTKDEW TQGEEIEVLNSKITGHTDLNGDSQDNL (length 53), and the combined T-cell epitope is RLYFTRYPFFQFANGKIGVPINT VYSEIAATISKDNLNSYLKENLNIKIKIT (length 52). The 105mer long B- and T-cell epitopes are TDGKGSSEPIVPM KNPETDYKTKDEWTQGEEIEVLNSKITGHTDLNGDSQDNL RLYFTRYPFFQFANGKIGVPINTVYSEIAATISKDNLNSYLK ENLNIKIKIT. This combined single epitope representing an antigen is used for the present study. This epitope is mentioned as "Designer Epitope."

Table 1. Antigenicity and toxicity prediction

Sequence	Antigenicity	Virulence	Toxicity
DGKGSSEPIVPMKNPET DYK	1.03	0.65	−1.27
TKDEWTQGEEIEVLNS	1.07	0.65	−0.34
KITGHTDLNGDSQDNL	1.06	0.65	0.14
TDGKGSSEPIVPMKNP	1.2	0.65	−0.99
RLYFTRYPF	1.3	0.65	−1.41
FQFANGKIGV	0.8	0.65	−1.30
PINTVYSEIAATISK	0.4	0.65	−1.27
DNLNSYLKENLNIKIKIT	1.05	0.65	−0.34
TDGKGSSEPIVPMKNP ETDYKTKDEWTQGE EIEVLNSKITGHTDL NGDSQDNLRLYFTR YPFFQFANGKIGVPI NTVYSEIAATISKDN LNSYLKENLNIKIKIT	1.05	0.65	<0.5

Note: The antigenicity, virulence, and toxicity prediction of all the individual B- and T-cell epitopes and the Designer Epitope were done using the VaxiJen, VirulentPred, and ToxinPred online servers. The predicted antigenic value above default threshold of 0.4 is regarded as antigen, and the positive virulence score is regarded as the virulent peptide. The antigenicity of Designer Epitope is higher than 1, which is a good antigenic score enough to produce an immune response in the host. The virulence prediction score for all the epitopes was same, indicating all the epitopes were potent for inducing immunogenic response. The individual epitope toxicity was predicted using batch analysis, and Designer Epitope analysis was done using protein scanning analysis of ToxinPred server. All the predictions showed a non-toxic prediction threshold value below 0.5, making it a safer peptide.

3.2. Evaluation of antigenicity/virulence/ allergenicity/autoimmunity

The antigenicity score of the Designer Epitope was predicted to be 0.99, indicating it as a probable antigen enough to activate immunity against leptospiral pathogens. The predicted antigenicity score of the whole Lsa46 protein was 0.51. The VirulentPred server gave a positive virulence prediction score value above the threshold for all the selected individual epitopes, indicating the selected epitope can induce immunogenic response in the host (Table 1).

Since allergenic proteins or peptides often elicit adverse immune reactions, it is crucial for the vaccine to be non-allergenic. The allergenicity prediction of the Designer Epitope by servers, AlgPred2, and AllerTop v.2.0 showed it as non-allergen, and it does not contain experimentally proven IgE epitope. Also, the prediction gives no hits when BLAST was done against ARP (Allergen Representative Peptides) database. The results of individual epitope predictions from the ToxinPred server batch submission are displayed in Table 1 indicating that all the individual epitopes are non-toxic. Also, the protein scanning analysis of the Designer Epitope using the ToxinPred server for the identification of highly toxic regions within the epitope revealed that the Designer Epitope regions were also non-toxic. Additionally, the selected individual epitopes and the Designer

Epitope did not show any homology with any human protein, thus making it safe for human use.

3.3. Conservation analysis

Utilizing conserved epitopes in an epitope-based vaccine approach would provide broader protection against multiple strains or species, in contrast to epitopes derived from highly variable genome regions. The percentage conservancy range was determined by analyzing all the epitopes (both B and T cell) matches within a set of 93 non-redundant Lsa46 sequences from different strains/serovars available in the NCBI database. This analysis revealed a conservation range of 35% to 95% across the targeted structural proteins.

3.4. Population coverage

MHC molecules have many variations, with over a thousand known types in humans, called HLA alleles. To ensure broader coverage of the population for peptide-based vaccines or diagnostics, it is important to choose multiple peptides that bind to different HLA types.

The worldwide population coverage prediction of the T-cell epitopes showed that all the four epitopes of Lsa46 covered 100% of the combined MHC-I and MHC-II worldwide population. When the MHC-I and MHC-II class epitopes were individually analyzed, 91.9% of the world population were covered by the MHC-I, and the MHC –II epitopes covered the major population with 100%. The combined T-cell epitope showed 100% coverage for both MHCs. The population coverage analysis of the epitopes confirmed that the Designer Epitope is effective for most of the population throughout the world (Supplementary Figure 1).

3.5. Analysis of physicochemical properties

The physicochemical properties of the Designer Epitope were analyzed. Its molecular weight was computed to be of 11.87 KDa. Theoretical estimation of isoelectric point (pI) was 5.08, indicating that the peptide is acidic. Extinction coefficient of the peptide at $A^{0.1\%}280$ nm measured in water was 1.09 (12950 $M^{-1}cm^{-1}$). The GRAVY value of −0.696 and instability index of 26.52 indicated that the epitope can interact with water molecules and is a stable peptide. A protein's instability index indirectly indicates its stability, with a value less than 40, indicating stability and greater than 40 indicating instability. The predicted half-life of the peptide was 7.2 h in mammalian reticulocytes (*in vitro*), over 20 h in yeast (*in vivo*), and more than 10 h in *E. coli* (*in vivo*).

3.6. Subcellular localization of the epitope

The subcellular localization prediction revealed the Designer Epitope as an extracellular outer membrane peptide when analyzed using pSORTb v.3.0 and CELLO v.2.5 servers. CELLO and PSORTb predict the subcellular localization based on its amino acid sequence and dipeptide composition.

3.7. Secondary structure prediction

The secondary structure of the Designer Epitope was predicted with the use of online servers SOPMA and PSIPRED. The SOPMA analysis of secondary structure showed that the Designer Epitope is composed of alpha-helix (22%), random coil (44.1%), extended strand (25.5%), and beta turn (9.52%). The SOPMA prediction image of Designer Epitope is given in Supplementary Figure 2.

3.8. 3-D structure modeling and validation

I-TASSER server was used to model the 3-D structure of the Designer Epitope using thread modeling method. The 3D structure modeled using I-TASSER server was visualized using PyMol (Figure 3). The analysis of the Ramachandran plot with the PROCHECK server confirmed that 89.8% of amino acids reside within the favorable region, while 5.5% are within the allowed region, with only 4.6% falling into the outlier region. The structure analysis of the modeled 3D structure showed that it is stereochemically stable with negligible outliers. The ProSA web online server was used to analyze the standard of the structure and gave a Z-score value of −1.07, and its Ramachandran plot showed that most of the amino acid in the structure are in favorable region. The ProsA web server evaluates both the overall and local quality of the predicted model using the Z-score. Typically, a positive Z-score indicates the presence of an irregular section within the generated 3D protein model. The Ramachandran plot and ProSA server structure validation plot are shown in Figure 1.

3.9. Immune simulation

Immune simulation of the Designer Epitope construct was done to assess the immune response in the body. Both IgG and IgM individual response and IgG + IgM combined response were predicted. The combined IgG + IgM response showed the highest titer, and it lasted for about 35 days with one injection followed by the IgM and followed by the IgG. The cell-mediated immune response by T helper and T cytotoxic cells was also evident with a single-dose administration. T helper cells, mainly Th1 cells, were more predominant than Th17 and TR cells, and it was actively present for 1 month. The cytokine and interleukin production also increased along with the antibody production. The simulation results suggested that the single injection was good enough to potentially induce first-line immunoglobulins-IgG1, IgG2, IgM, Th, Tc, cytokine, and interleukins. The immune simulation result is shown graphically for total antibodies, lymphocytes, and cytokine counts (Figure 2).

3.10. Extended *in silico* analysis of the Designer Epitope

In order to examine whether the immunogenicity of the designed peptide could be enhanced, further extensive studies have been carried out using the Designer Epitope on the basis of previous *in silico* results analysis. For this, the Designer Epitope used in the previous analysis has been modified to check whether the immunogenicity can be increased or not. Thus, the "Designer Epitope" (AgI), "Designer Epitope Repeat" (AgII), "Designer Epitope Vaccine Construct" (AgIII), and "Designer Epitope Repeat Vaccine Construct" (AgIV) were taken for further analysis. The Designer Epitope Repeat is the Designer Epitope repeated twice. The Designer Epitope Vaccine Construct is the modification of the Designer Epitope with addition of the linkers and adjuvant in the appropriate positions. The Designer Epitope Repeat Vaccine Construct is the Designer Epitope Repeat with linkers and adjuvant. The respective sequence and codes used to represent each designed epitope are given in Supplementary Table 1.

The 3D structures of the modified epitope constructs were modeled using the I-TASSER server and validated using the Ramachandran plot analysis. The Ramachandran plot of the modified epitope constructs indicates that the structures are of good stereochemical quality. The Ramachandran plots of the

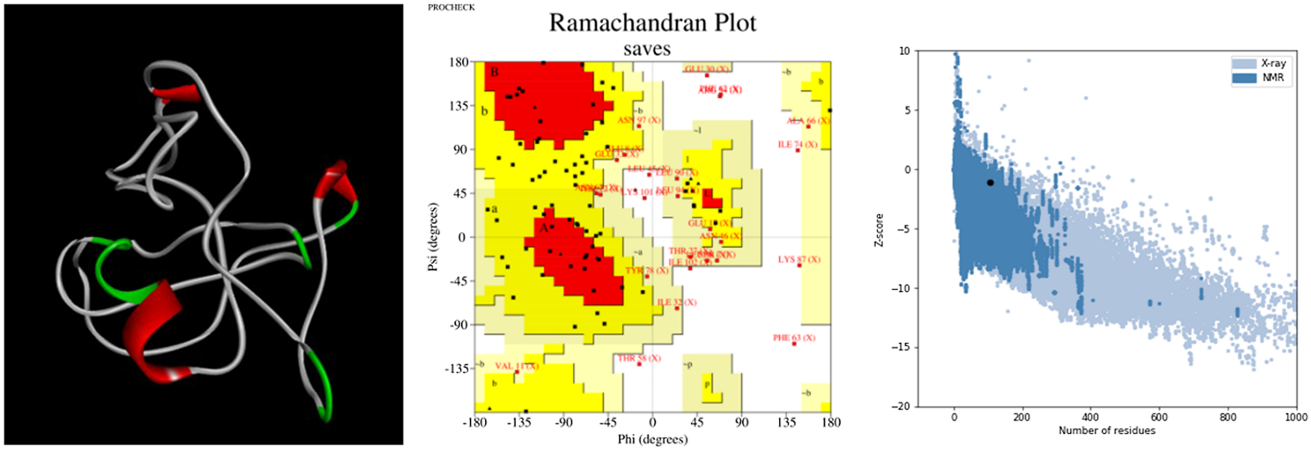


Figure 1. 3D structure and structure validation by Ramachandran plot and ProSA web server. The 3D structure of Designer Epitope modeled using I-TASSER server visualized using PyMol and the Ramachandran plot of the modeled 3D structure validated by PROCHECK server. The Ramachandran plot shows that the modeled 3D structure is stable. The modeled Designer Epitope 3D structure validation by ProSA web server gives a Z-score plot. In this plot, groups of structures from X-ray and NMR sources are distinguished by different colors. This analysis has been done to check whether the z-score of the Designer Epitope structure is within the range of scores typically found for native proteins of similar size. The Z-score of −1.07 (displayed as a black dot) indicates an overall good quality of the model

Designer Epitope Repeat, Designer Epitope Vaccine Construct, and Designer Epitope Repeat Vaccine Construct showed that about 94.6%, 95.4%, and 94% residues of structures respectively fall in the allowed region. Among the modified structures, the Designer Epitope Vaccine Construct exhibited a greater stereochemical stability, with a slightly higher percentage of amino acid residues falling in favorable regions. The modeled 3D structures visualized in PyMol tool are shown in Figure 3, and the Ramachandran plot of the structures is given in Supplementary Figure 3.

3.11. Immune simulation of all the epitope vaccine constructs

Immune simulation was also performed with three doses of antigen constructs. Graphical representations of the simulation results are provided for the total number of B lymphocytes, TH, and TC cells (Supplementary Figure 4–7), as well as for the total number of antibodies, cytokines, and interleukins (Figures 4 and 5).

3.11.1. Immune simulation of AgI/AgII/AgIII and AgIV

The simulation for AgI revealed a strong secondary and tertiary immune response, emphasizing their prominence over the primary response. Administering the vaccine through three injections was found necessary to trigger first-line immunoglobulins (IgG1, IgG2, and IgM). Both primary and secondary responses showcased heightened levels of various immune markers, including IgM, IgM + IgG, IgG1 + IgG2, IgG1, IgG2, T cells, cytokines, and interleukins. Subsequent exposures to AgI epitope injections resulted in decreased antigen levels alongside increased responses from memory T-cell populations (CTL and HTL), confirming the immunogenicity of T-cell epitopes. Macrophage activity consistently increased with each exposure, while NK cell activity remained elevated throughout. Significant elevation in IFN-gamma, IL-10, IL-23, and IL-12 levels was observed upon subsequent exposures. After three injections at monthly intervals, antigen levels peaked similarly (reaching zero titer), with notable increases in IgM + IgG and IgG1 + IgG2 levels. Memory B- and T-cell populations surged throughout the exposure period, while IFN-gamma levels remained consistently high. These results underscore the robust immune responses induced by AgI epitope constructs, even with short exposure and repeated exposures.

The immune simulation of Designer Epitope Repeat (AgII) showed a pattern similar to Designer Epitope (AgI), with minor differences in initial antibody titers but consistent antigen levels. Both AgI and AgII displayed an initial antibody spike followed by steady concentrations. Concentrations of interleukins, IFN-gamma, B cells, and TH cells, including memory cells, were comparable for both constructs, with more prominent secondary and tertiary immune responses. These findings suggest that a single epitope peptide can induce relevant immune responses, including first-line and memory responses.

The immune simulation of Designer Epitope Vaccine (AgIII) exhibited an initial decrease in antibody titer followed by stabilization, similar to AgI and AgII. IFN-gamma peaked similarly, but IL-23 concentration was lower in AgIII compared to AgI and AgII. B-cell dynamics showed changes over time, with a decrease in B memory cells after 250 days and a rise in non-memory B cells, including IgM, IgG1, and IgG2, after 150 days. TH cell populations remained constant across all constructs.

For Designer Epitope Repeat Vaccine Construct (AgIV), all immunoglobulins except IgG2 showed a slight increase compared to AgIII, with consistent antigen concentration. AgIV displayed a similar cytokine and interleukin response as AgIII. B-cell populations resembled AgIII, with a small increase observed after 150 days. TH cell populations showed consistent patterns across all constructs.

Across all epitope constructs and the whole protein antigens, a consistent observation emerges: after the initial immune response differences, all immune factors stabilize for approximately one year. The simulation results underscore the prevalence of secondary and tertiary immune responses over the primary response. Administering the vaccine through three injections is recommended to potentially induce first-line immunoglobulins (IgG1, IgG2, and IgM) and cell-mediated immune responses. Overall, the immune simulation study highlights the epitope regions' capacity to elicit immune-mediated responses. Compared to the Lsa46 whole protein antigen, similar immune response

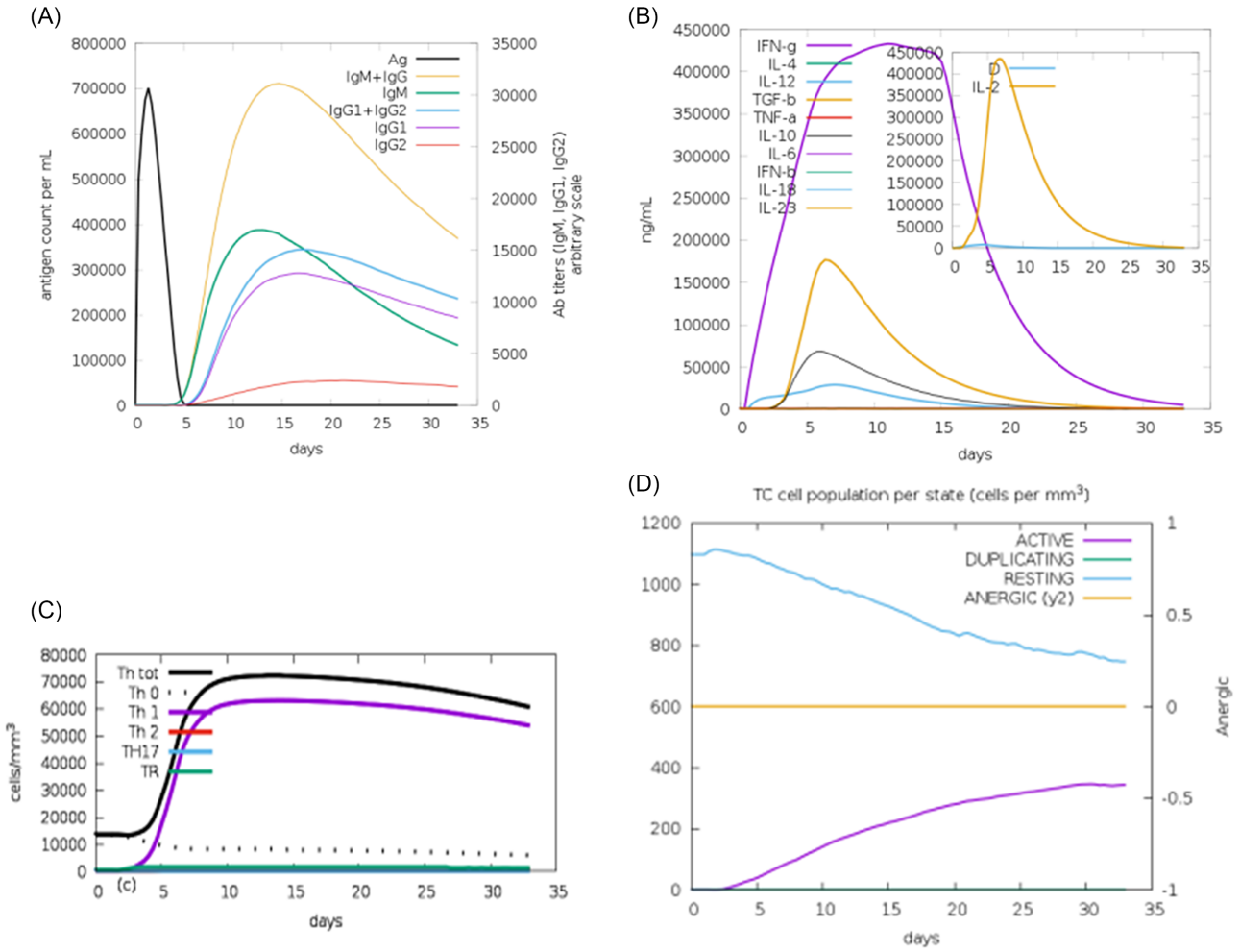
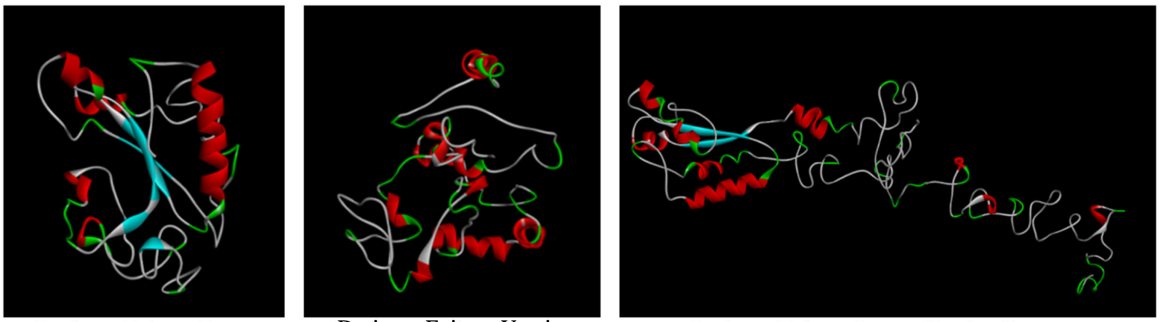


Figure 2. Immune simulation of the Designer Epitope. The immune simulation of the Designer Epitope was performed using C-ImmSImm online server. The simulation figure shows that both the antibody-mediated and cell-mediated immune response can be triggered with single-dose administration of the epitope. The antigen titer has been reduced to zero by the fifth day after the administration, and later on, the immunoglobulin, lymphocytes, cytokines, and interleukin concentration increased, and the response is stable for up to one month with single injection. Figure A represents the antibody response, B represents the cytokines and interleukin counts, and C and D represent the TH and TC cell population count, respectively



Designer Epitope Repeat | Designer Epitope Vaccine Construct | Designer Epitope Repeat Vaccine Construct

Figure 3. The 3D structures of modified Designer Epitope. The figure represents the 3D modeled structures of the modified Designer Epitope visualized in PyMol visualization tool. These 3D structures were modeled using I-TASSER online server

patterns were observed, indicating that small peptides alone can trigger immune responses effectively. Although AgIV displayed considerable immune responses, its structural characteristics suggest potential instability and susceptibility to mutations, warranting further analysis. Consequently, AgI, AgII, and AgIII were chosen for further studies due to their structural stability and ability to elicit robust immunogenic responses comparable to the Lsa46 whole protein.

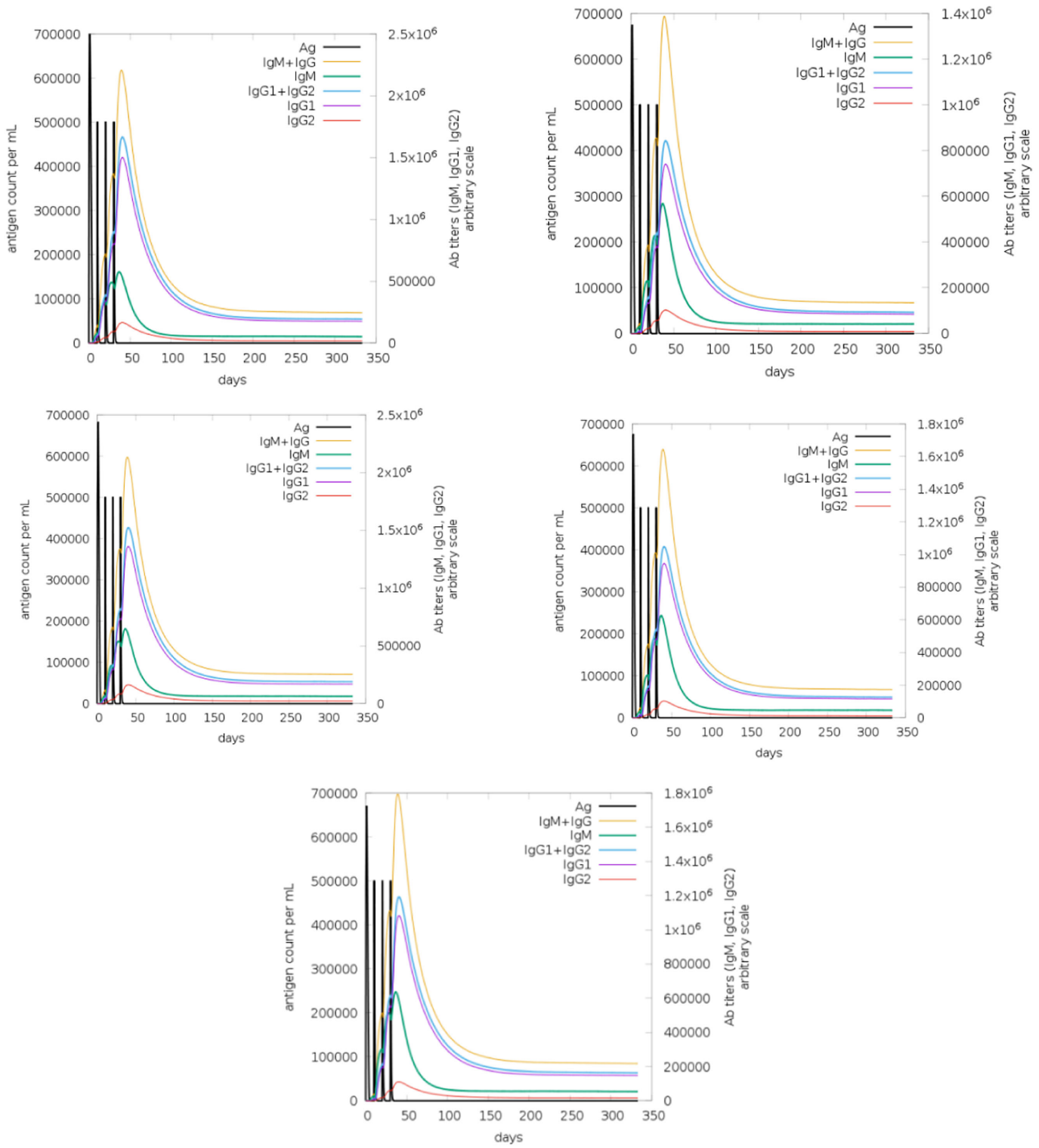


Figure 4. Immune simulation of Ag – Ab response prediction. Antibody response of all the constructs when predicted using C-ImmSIm server, starting from the AgI, AgII, AgIII, and AgIV and Lsa46 whole protein. The overall antibody and antigen concentration are in almost similar pattern

3.12. Molecular interaction studies

The previous studies show that the humoral immunity as well as cell-mediated immunity confers protection against leptospirosis in humans and cattles. For understanding binding and molecular interaction, differences between the immunogenic peptide with IgG, IgM, TCRαβ, TCRγδ, MHC-I, MHC-II, human TLR2, human TLR4 were analyzed using molecular docking studies using Z-docking (protein-protein). The AgI, AgII, and AgIII were taken as the immunogenic vaccine construct for the molecular interaction studies.

3.12.1. Molecular interaction study of the Designer Epitopes – AgI, AgII and AgIII

In the molecular interaction studies of the Designer Epitopes (AgI, AgII, and AgIII), Blind docking was employed to analyze their interactions with different immune ligands. For AgI, a total

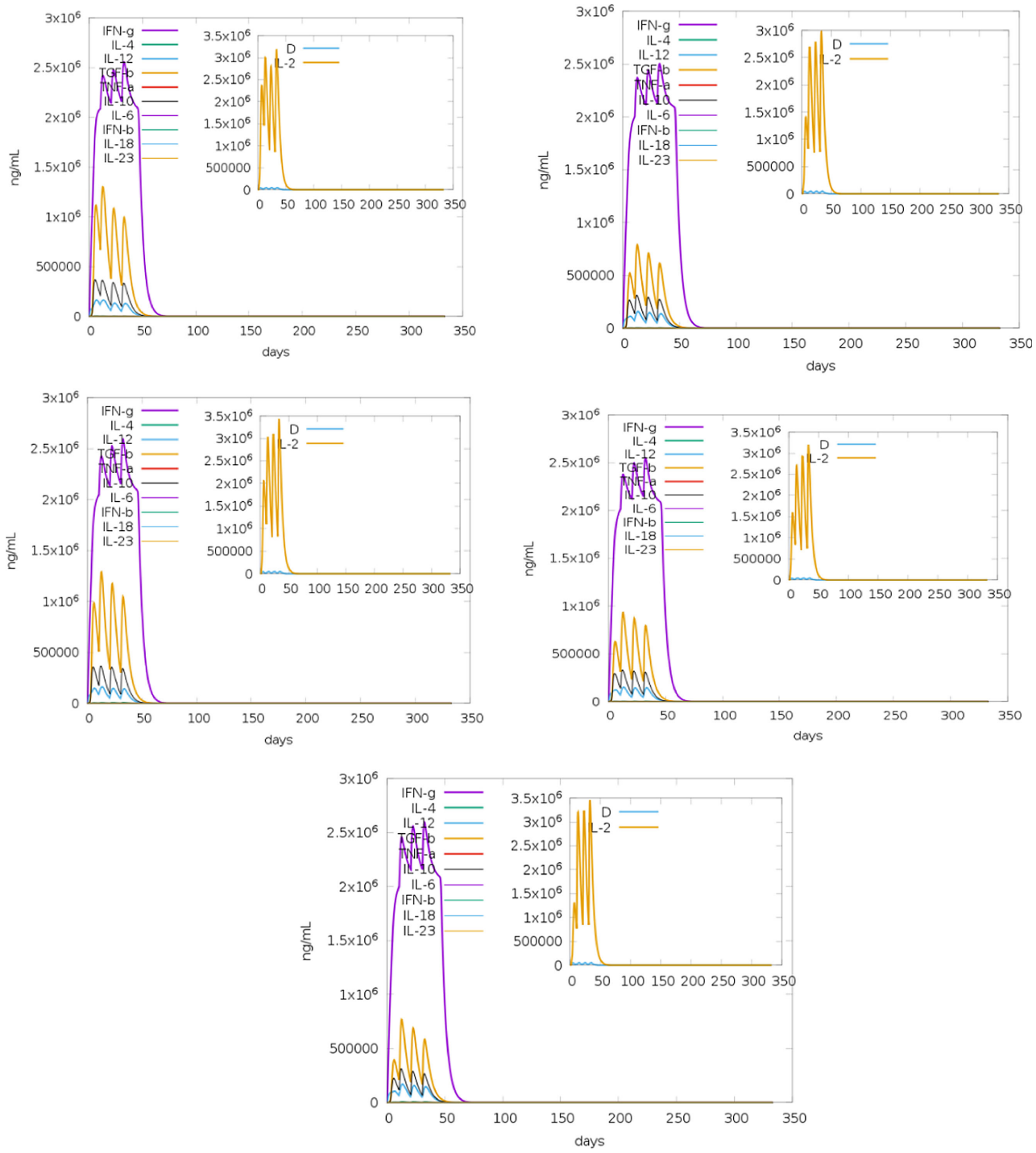


Figure 5. Immune simulation of cytokines and interleukin response plot. Cytokine and interleukin response of all the constructs starting from the AgI, AgII, AgIII, and AgIV and Lsa46 whole protein

of 2000 poses were generated in the Z-dock, and the best pose, which exhibited best Z-rank scores, was selected. The AgI peptide demonstrated favorable binding with all the docked ligands, displaying desirable hydrogen bond interactions. The docking of AgI with IgG gave the highest Z-rank score of −124.62, followed by human TLR2 (−121.24), TCR αβ (−107.61), MHC-I (−106.13), human TLR4 (−105.52), IgM (−105.36), and MHC-II (−91.98). The Z-rank score is typically indicative of the predicted affinity or binding strength between a ligand and a target protein. The docking interactions of the AgI with the selected ligands have indicated its potential as a peptide capable of eliciting various immune responses, including TLR4-mediated response, which plays a crucial role in pathogen elimination. The hydrogen bond interacting residues, corresponding Z-rank scores, and bond distances of pose 1 in the docked complexes are presented in Supplementary Table 2.

AgII docking generates a total of 2000 poses, and it was observed that the first pose displayed best Z-rank scores. The

Table 2. Molecular interaction study of AgIII vaccine construct

Ligands	Z-rank score	H-bond interacting residues	H-bond distance (Å)
IgG	−141.57	IgG:GLY341 – LSA46:ASP191	1.97
IgM	−115.48	LSA46:SER142 – IgM:GLY2200	2.4
		LSA46:THR160 – IgM:SER2642	2.39
		LSA46:GLN190 – IgM:TYR2664	2.52
		IgM:ASN2138 – LSA46:GLU153	2.98
TCRαβ	−118.48	LSA46:ASN86 – TCRαβ:SER138	2.29
		TCRαβ:SER137 – LSA46:PHE84	2.89
		TCRαβ:SER189 – LSA46:ASN86	2.74
TCRγδ	−116.38	LSA46:ALA131 – TCRγδ:GLY138	2.81
		TCRγδ:TYR134 – LSA46:PRO122	2.41
		TCRγδ:TYR134 – LSA46:ASN124	2.31
		TCRγδ:TRP271 – LSA46:ASN283	2.72
MHC1	−123.88	LSA46:THR100 – MHC-I:THR271	2.66
		LSA46:LYS150 – MHC-I:GLN180	2.75
		MHC-I:LYS268 – LSA46:THR100	2.38
MHC-II	−134.5	–	–
Human TLR2	−115.85	LSA46:GLN83 – huTLR2:ASP384	2.09
		LSA46:ASN128 – huTLR2:GLU383	3.01
		LSA46:ASN128 – huTLR2:ASP384	2.56
Human TLR4	−126.94	LSA46:GLU153 – huTLR4:GLN436	2.44
		huTLR4:ARG382 – LSA46:VAL147	2.22
		huTLR4:GLN430 – LSA46:GLY141	2.54

Note: The molecular interaction between the AgIII and the immunologically significant ligands was done by molecular docking using BIOVIA Discovery Studio. The H-bond interacting residues and their Distance are given. The Designer Epitope Repeat shows good interactions with all the selected ligands except for MHC-II.

AgII exhibited favorable hydrogen bond interactions with all the ligands that were docked. However, the Z-rank scores of the AgII with the ligands were comparatively lower when compared to the AgI and ligand dockings. This difference in Z-rank scores could potentially be attributed to conformational changes in the 3D structure resulting from the presence of repeat sequence. The docking of AgII with MHC-I yielded the highest Z-rank score of −124.63, followed by IgM (−117.9), human TLR2 (−111.02), IgG (−112.7), MHC-II (−108.92), human TLR4 (−109.6), and TCRαβ (−92.51). The hydrogen bond interacting residues, corresponding Z-rank scores, and bond distances of pose 1 in the docked complexes are presented in Supplementary Table 3.

The AgIII exhibited desired hydrogen bond interactions with all the docked ligands, except for MHC-II docking. Even though there were no epitope interacting residues in the MHC-II docking, it still showed hydrogen bond interactions with the selected adjuvant. In terms of Z-rank scores, the AgIII–IgG docking showed the highest score of −141.57, followed by human TLR4 (−126.94), MHC-I (−123.88), TCRαβ (−118.48), TCRγδ (−116.38), human TLR2 (−115.85), and IgM (−115.48). The docking interactions of the AgIII with the selected ligands, except for MHC-II, indicate its potential as a peptide-based vaccine construct capable of triggering an immune response against pathogen attacks. While the AgI showed binding interactions with MHC-II, these interactions appeared to be lacking after incorporating other vaccine construct residues, possibly due to changes in the 3D structural conformation.

The inclusion of human beta defensin as a component demonstrated interactions with MHC-II, which could compensate for the MHC-II binding and subsequent enhancement in the immune cascade. The hydrogen bond interacting residues, corresponding Z-rank scores, and bond distances of pose 1 in the docked complexes are presented in Table 2, and the H-bond interactions of adjuvant are shown in Table 3. The docked complexes images are shown in Supplementary Figure 8, and docked complex H-bond interaction images are shown in Figure 6.

Table 3. Adjuvant and MHC-II docking H-bond interactions of AgIII

H-bond interactions	H-bond distance (Å)
β – defensin: GLY23 – MHC-II:GLU46	2.9
β – defensin:CYS43 – MHC-II:GLU96	2.2
MHC-II:GLU46 – β – defensin:ILE13	2.9
MHC-II:ARG50 – β – defensin:VAL21	2.4

Note: The molecular interaction between the AgIII adjuvant regions and the MHC-II is shown with the H-bond interacting residues and their distance.

Based on the immune simulation, docking, and 3D structure analysis, the AgIII is selected as the final vaccine construct which can be used as a potential vaccine candidate against leptospirosis. The AgIII showed good immunogenic primary and secondary response and molecular docking analysis also gave a desirable binding interaction with the immune ligands selected.

3.13. Modeling and quality assessment of the 3D structure of final vaccine construct

The final multi-epitope vaccine construct (Designer Epitope Vaccine Construct – AgIII) comprising 213 amino acid residues was subjected to validation for antigenicity, allergenicity, physicochemical properties, and various other *in silico* analysis.

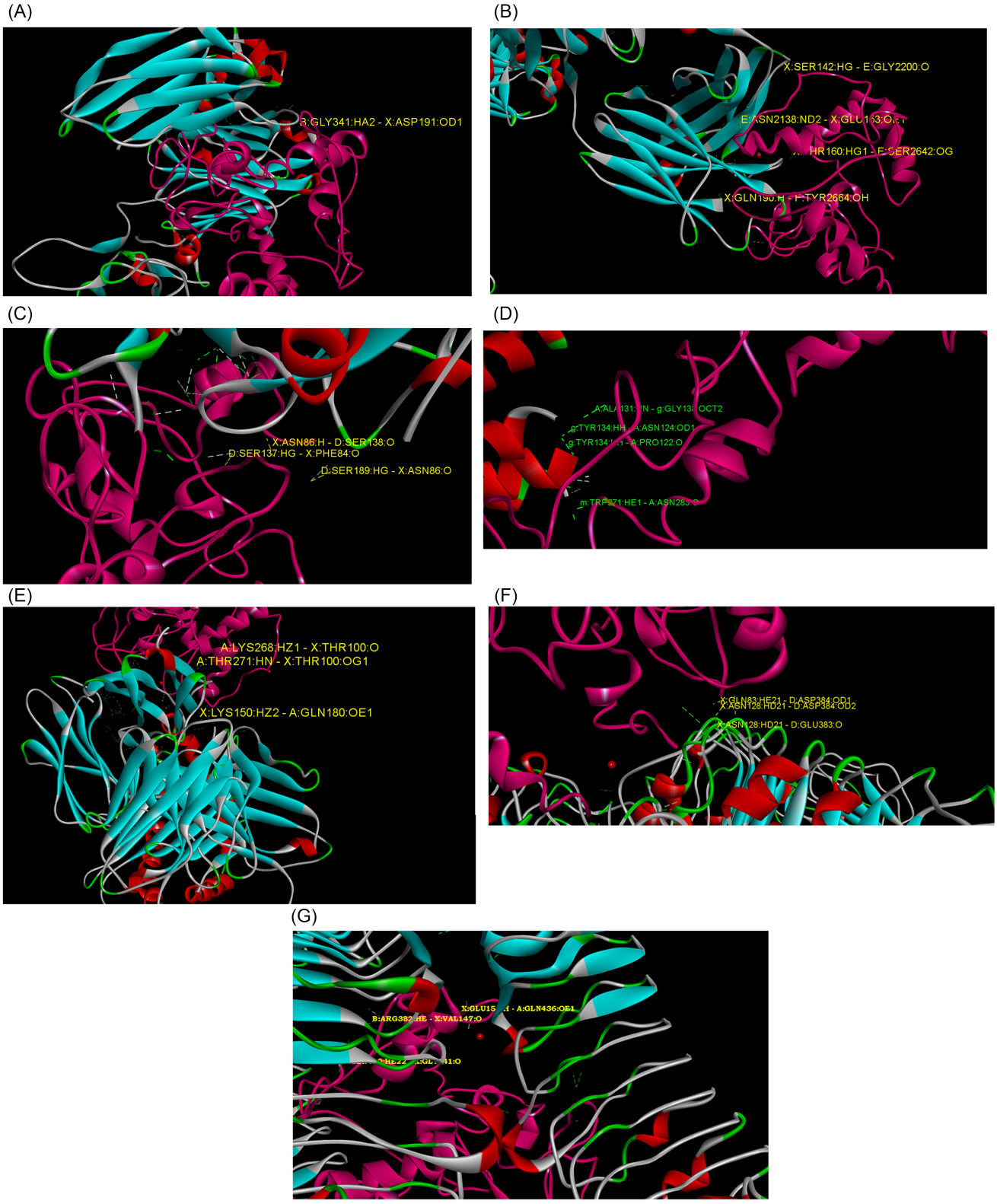


Figure 6. Molecular interaction images of Designer Epitope Vaccine Construct (AgIII). The molecular docking images of the H-bond interactions involving the AgIII are presented. Figures A to G depict the Designer Epitope Vaccine Construct with IgG, IgM, TCR$\alpha\beta$, TCR$\gamma\delta$, MHC-I, human TLR2, and human TLR4, respectively. In each figure, both the ligand and the vaccine construct are represented as cartoon shapes, with the vaccine construct highlighted in magenta color. The interaction images for the AgIII with MHC-II are not shown as there were no H-bond interactions. The H-bond interacting residues are labeled and highlighted as yellow

The analysis of secondary structure was employed to predict important factors for protein stability and vaccine efficacy, including solvent accessibility, transmembrane helices, globular regions, and β-turn regions. The SOPMA secondary structure prediction server revealed that the peptide consists of 21.6% alpha-helix, 22.54% of extended strand, 11.27% of beta turn, and 44.6% random coil regions. PSIPRED predicted that the peptide's secondary structure comprised of 42.33% alpha-helix, 22.09% extended strand, 4.42% beta turn, and 31.16% random coil. PSIPRED is more accurate and reliable than methods like SOPMA and employs a robust neural network architecture, capturing long-range interactions and context while continually updating with new algorithms and datasets for improved accuracy in secondary structure prediction. The sequence and graphical representation of the predicted secondary features of the peptide are given in Supplementary Figure 9.

3.14. Characterization of the final vaccine construct

The antigenicity of the vaccine construct was predicted as antigenic with a score of 0.95. The peptide sequence allergenicity prediction showed that it does not contain experimentally proven IgE epitope. The physicochemical parameter of the peptide was computed positively charged peptide with 28 kDa molecular weight, pI of 9.73, GRAVY value of −0.578, instability index value of 38.02 (stable), estimated half-life is 30 h (mammalian reticulocytes, *in vitro*), >20 h (yeast, *in vivo*), >10 h (*E. coli, in vivo*). The estimated half-life in mammalian reticulocytes has been increased and in yeast and *E. coli* remains the same. The extinction coefficient of the peptide at $A^{0.1\%}280$ nm measured in water was 0.871 (20775 $M^{-1}cm^{-1}$).

The sites of high local frustration of AgIII have been evaluated by Frustratometer online server (Supplementary Figure 10). Minimally frustrated linkages form the stable folding core of the molecule, often indicating biologically significant regions like binding or allosteric sites. The localization of frustration of final vaccine construct showed that the highly frustrated domains are strongly cross-linked by minimally frustrated contact networks. The minimally frustrated regions are found more than the highly frustrated regions.

DISOPRED v3 online server was used to predict the disordered regions of the final vaccine construct, which is shown in Supplementary Figure 11. The prediction result indicates that there is no disorder in the epitope region of the sequences.

The overall *in silico* assessment of the Lsa46 epitope peptide as a vaccine candidate revealed that the AgIII can be the optimal candidate which satisfies immunological factors needed as a potential vaccine.

4. Discussion

Leptospirosis necessitates an enhanced vaccine due to limitations in current whole-cell vaccines, such as incomplete coverage against various Leptospira strains, the need for frequent booster doses, and safety concerns. This study aimed to design an effective vaccine targeting leptospiral pathogens, focusing on the 'Designer Epitope Vaccine Construct (AgIII).' This 213-mer epitope is predicted to show antigenic, immunogenic properties, non-allergenic/non-autoimmune nature, compatibility with HLA alleles, activation of diverse immune cells, wide population applicability, and localization on the pathogen's cell surface, making it a promising vaccine candidate. With both T- and B-cell epitopes and stable interactions with immunologically relevant ligands, this epitope shows potential for developing a highly effective and broad-spectrum leptospirosis vaccine.

Immunogenicity and safety were considered in selecting AgIII, which exhibited higher antigenicity scores than the whole Lsa46 protein, indicating its potential as a core immunogenic region capable of activating immunity against Leptospiral pathogens. Virulence prediction also supported this. By excluding potential allergenic components and avoiding overlap with human genes, adverse reactions, autoimmune responses, and unintended side effects can be minimized [22]. The allergenicity, toxin, and homology analyses with human proteins further confirmed the safety profile of the selected final vaccine candidate.

For effective immune responses, vaccine constructs should include surface-exposed epitopes on the target protein, typically found on the outer membrane with conserved regions across pathogen strains. These epitopes interact directly with antibodies, B cells, and antigen-presenting cells, facilitating efficient recognition and immune response initiation without antigen processing [23]. Identifying and characterizing a surface protein associated with modulating the host's innate immune response will assist in devising an improved strategy to address this bacterial zoonosis. The selected epitopes in this study are surface-exposed, ensuring accessibility and facilitating antibody-mediated immune responses. Subcellular localization predictions of AgIII using two widely used tools, CELLO and pSORTb, identified AgIII as an extracellular outer membrane peptide.

In this study, the selected multi-epitopes demonstrated binding capability to 100% of MHC class I and class II alleles, offering broader protection against different strains/serovars of leptospiral pathogens. The whole Lsa46 protein also exhibited greater conservation among various strains and serovars. Prioritizing epitopes with at least 50% population coverage is crucial for maximizing vaccine impact. Effective vaccine constructs should activate both cytotoxic T lymphocytes (CTLs) and helper T lymphocytes (HTLs) for cell-mediated immunity, as well as B cells for humoral immunity [24]. The final vaccine construct (AgIII) comprised T and B-cell epitopes, exhibiting desirable vaccine properties. Leveraging the HLA system is vital for achieving broad vaccine coverage, including epitopes that bind to both HLA class I and class II alleles, thereby expanding the range of immune responses and ensuring recognition and response in individuals with diverse HLA profiles. Selecting multiple epitopes with different HLA binding specificities enhances population coverage, resulting in an unbiased vaccine across ethnicities [25].

The designed peptide vaccine candidates with longer peptides and repetitive epitopes were considered to enhance immunogenicity and sustain immune responses. These strategies stimulate CD8 cytotoxic T lymphocytes and induce robust immune reactions. Linkers play a vital role in multi-epitope vaccines, providing flexibility and spacing between epitopes for proper conformation and immunogenicity [26]. In our study, we combined prominent T- and B-cell epitopes from the Lsa46 protein with β-defensin as an adjuvant and appropriate linkers. Our findings align with immune simulations, showing changes in the concentration of immunological components produced, particularly immunoglobulins, comparing epitope regions alone to those linked with adjuvants and linkers. The populations of T cells, cytokines, interleukins, macrophages, NK cells, and dendritic cells remained consistent across both scenarios. Furthermore, both candidates exhibited sustained responses in a similar pattern without any delayed response observed at later stages.

Th1 cells and humoral immunity are pivotal for Type 1 immunity and protection against leptospirosis, with T-cell antigen

receptors and Toll-like receptors (TLR2 and TLR4) playing crucial roles. Interaction analysis confirmed the immunogenic response of the epitope toward all immunological ligands, including TLR2 and TLR4, which are vital for controlling leptospirosis in mice. *L. interrogans* infection in double TLR2/TLR4 knockout mice led to a swift organ failure, highlighting the crucial role of these receptors in disease management. The absence of TLR4 activation, coupled with virulent leptospires cytosolic localization and induction of macrophage apoptosis, may contribute to human susceptibility to leptospirosis, compared to mouse resistance [27]. Immunogenic peptide interaction analysis was conducted to evaluate the vaccine construct immunogenic response toward both human TLR2 and TLR4. The AgIV exhibited a similar immune response to the AgIII, indicating that a single repetition of the epitope is sufficient.

In this study, we employed various immunoinformatics approaches to design a multi-epitope-based vaccine, offering safety and cost-effectiveness, compared to traditional vaccines. These peptide vaccines are being developed for a range of diseases, including HIV, HCV, foot and mouth disease, malaria, influenza, swine fever, HPV, and anthrax. Efforts are also ongoing to create therapeutic anti-cancer vaccines for various cancers, though most are in early phases of development [28]. Multi-epitope vaccines have shown effectiveness against viruses like influenza A, hepatitis B, and hepatitis C, as well as zoonotic viruses such as Ebola virus, Nipah Virus, Marburg virus, Crimean Congo Hemorrhagic Fever virus, and Monkeypox virus [29]. These findings help in optimizing the design of a vaccine against leptospirosis and may contribute to the development of effective immunization strategies.

5. Conclusion

Continual advancements in computational techniques present significant potential for addressing biological and medical challenges, particularly in combating infectious diseases. Immunoinformatics analysis of the Lsa46 multi-epitopes has led to the design of a peptide construct with stability, conservation, antigenicity, and non-allergic properties, making it a promising vaccine candidate. Through *in silico* tools and servers, the 2D and 3D structures of the selected Designer Epitope Vaccine Construct have been studied and validated using standard Ramachandran plot analysis, confirming its reliability for further investigations. Immune simulation and molecular docking with key immunological ligands indicate that the vaccine construct possesses a stable and stereochemically ideal structure, supporting its potential as a vaccine candidate. However, additional wet lab studies, including the synthesis of the final vaccine construct and experimental validation, are necessary to advance this research toward final vaccine development.

Acknowledgement

The authors express their gratitude for the support from SIUCEB at the Department of Computational Biology and Bioinformatics, University of Kerala, India, which provided the essential facilities for conducting this study. We also thank the University of Kerala for providing a fellowship to Junaida M. Ibrahim, while Perumana R. Sudhakaran received support from ISCA, Kolkata, through the Asutosh Mookerjee Fellowship.

Ethical Statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Data Availability Statement

All the data discussed have been included in the manuscript and with its supplementary figures and tables.

Author Contribution Statement

Junaida M. Ibrahim: Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Padikara K. Satheeshkumar:** Investigation, Writing – review & editing, Project administration. **Achuthsankar S. Nair:** Methodology, Resources, Writing – review & editing, Project administration. **Oommen V. Oommen:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Project administration. **Perumana R. Sudhakaran:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Project administration.

Supplementary Information

The supplementary files are available at https://doi.org/10.47852/bonviewMEDIN42022655

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How to Cite: Ibrahim, J. M., Satheeshkumar, P. K., Nair, A. S., Oommen, O. V., & Sudhakaran, P. R. (2024). Designing Lsa46-Based Multi-Epitope Peptide Vaccine Against Leptospirosis: Immunoinformatic Approach. *Medinformatics*. https://doi.org/10.47852/bonviewMEDIN42022655