Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
www.all-imm.com

ORIGINAL ARTICLE

OPEN ACCESS

Revolutionizing leishmaniasis control: leveraging immunoinformatics for precision-driven *in-silico* vaccine design

Meeral Ovais[a†], Munazza Kanwal[b†], Shumaila Naz[b], Tariq Aziz[c*], Nawal Al-Hoshani[d], Maher S. Alwethaynani[e], Sahar Abduljawad[f], Jamal I. Asseri[g], Hanan Abdulrahman Sagini[h], Shaza N. Alkhatib[i], Fakhria A. Al-Joufi[j], Syed Babar Jamal[b*]

[a]*Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA, USA*
[b]*Department of Biological Sciences, National University of Medical Sciences, Rawalpindi, Pakistan*
[c]*Laboratory of Animal Health, Hygiene and Food Quality, University of Ioannina Arta Greece*
[d]*Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia*
[e]*Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Shaqra University, Riyadh, Saudi Arabia*
[f]*College of Health Sciences, Umm Al-Qura University, Al-Lith 28425, Saudi Arabia*
[g]*Department of Biological Sciences, College of Science and Humanities, Shaqra University, Shaqra, Saudi Arabia*
[h]*Department of Biological Sciences, College of Sciences, University of Jeddah, Jeddah, Saudi Arabia*
[i]*Department of Biological Sciences, Collage of Sciences and Arts Khulais, University of Jeddah, Jeddah, Saudi Arabia*
[j]*Department of Pharmacology, College of Pharmacy, Jouf University, Aljouf, Saudi Arabia*

Received 21 July 2025; Accepted 19 October 2025
Available online 1 January 2026

KEYWORDS
immunoinformatics;
leishmaniasis;
multi-epitope
vaccine pteridine
reductase

Abstract
Leishmaniasis is an endemic disease in many countries that affects vulnerable populations of humans, dogs, and cats. This study employs immunoinformatics to design a multi-epitope vaccine construct for leishmaniasis by identifying vaccine targets on the protein Pteridine Reductase. T-cell and B-cell epitopes were screened for antigenicity, toxicity, and allergenicity. Shortlisted T-cell targets were confirmed to have appropriate IC values (≤ 50 nM and ≤ 500 nM for MHC I and MHC II, respectively). One B-cell epitope, six MHC class I epitopes, and 25 MHC class II epitopes were selected for inclusion in the vaccine construct, which was linked with EAAAK, CPGPG, and AAY linkers, along with a CPG Oligodeoxynucleotide adjuvant. The vaccine construct had a Ramachandran score of 89.9% and an Errat score of 98.4615. HLA alleles were predicted to produce an immune response in 81.8% of the global population, indicating encouraging potential for broad immunogenicity. Successful binding of the vaccine construct with TLR-9 was confirmed through molecular docking, and the docked complex exhibited a low eigenvalue of 2.06e-0.6 and a ΔG of -11.8 kcal mol-1, indicating stable binding and a high level of flexibility. Codon optimization was carried out, followed by in-silico cloning

***Corresponding authors**: Syed Babar Jamal, Department of Biological Sciences, National University of Medical Sciences, Rawalpindi, 46000, Pakistan. *Email address*: babar.jamal@numspak.edu.pk and Tariq Aziz, Laboratory of Animal Health, Hygiene and Food Quality, University of Ioannina Arta Greece. *Email address*: iwockd@gmail.com

https://doi.org/10.15586/aei.v54i1.1519

of the vaccine in Escherichia coli K12 strain using the vector pET-21a (+). These results suggest that the vaccine is stable and capable of eliciting a promising immune response against Leishmania, making it a favorable candidate for experimental trials.

Introduction

Leishmaniasis is a vector-borne disease caused by the protozoan *Leishmania*, which can be zoonotic or anthroponotic.[1,2] The disease is transmitted through female sandflies (*Phlebotomus* and *Lutzomyia*),[3] with humans and animals as hosts, and it primarily affects dogs and cats.[2,4,5] Leishmaniasis can lead to renal disease, epistaxis, and several other clinical abnormalities,[4,6] particularly in vulnerable populations such as immunocompromised, malnourished, and poor communities. The World Health Organization classifies leishmaniasis as a neglected tropical disease (NTD), with a global prevalence of 12 million cases and an incidence of up to 1.2 million cases per year across 98 countries.[1,3] Its prevalence and distribution are strongly influenced by factors such as climate and environment, contributing to widespread endemicity in regions including Asia, Africa, Europe, and the Americas.[2,7]

Different types of leishmaniasis have been managed using various treatment approaches. Clinically simpler lesions are treated with thermotherapy, photo- or laser therapy, and intralesional injections, whereas more complex lesions are managed with amphotericin, pentamide, and oral therapies. Pentavalent antimonials are considered the standard treatment for severe cases of leishmaniasis; however, resistance to these drugs has developed in many *Leishmania* species, making them a less favorable option. Currently, immunocompromised patients are treated with liposomal amphotericin B in combination with other therapies.[1,8] Despite these strategies, treatment of leishmaniasis remains in its nascent stages and frequently results in relapse in dogs.[9]

There is currently no successful human vaccine for leishmaniasis; however, some vaccine candidates for dogs have shown promise. The lack of effective human vaccines is most likely due to economic pressures and the absence of a suitable human adjuvant system for vaccine testing. Studies have also suggested that the use of whole *Leishmania* parasites in vaccine candidates may impair antigen presentation, thereby reducing vaccine efficacy.[10] At present, several vaccine candidates are undergoing clinical trials, including one developed by the Infectious Disease Research Institute (IDRI, Washington, Seattle), which uses recombinant *Leishmania* proteins, *L. infantum*, and *L. donovani*. The Sabin Vaccine Institute Product Development Partnership (Sabin PDP) is also developing a recombinant *L. donovani* vaccine, and numerous other vaccine strategies are under investigation.[1]

In this study, Pteridine Reductase was selected as a potential vaccine candidate because of its role in folate metabolism in the *Leishmania* parasite. The pteridine pathway reduces folate and pteridines, which are essential for nucleotide acquisition during DNA synthesis and are necessary for parasite growth and metacyclogenesis. Therefore, inhibition of Pteridine Reductase would impair DNA replication and lead to cell death.[11,12] Previous studies have suggested that it is a suitable drug-target candidate *in vitro* when tested against Iranian Lizard Leishmania.[13] In this study, T-cell and B-cell epitopes were filtered, and vaccinomics was applied to design a multi-epitope vaccine candidate. Docking was performed with TLR-9, an intracellular pattern recognition receptor primarily localized in endosomes, where it recognizes unmethylated CpG motifs commonly found in microbial DNA. TLR-9 is expressed in immune cells such as plasmacytoid dendritic cells and macrophages, both of which play critical roles in anti-*Leishmania* responses. Activation of TLR-9 triggers the MyD88-dependent pathway, leading to NF-κB activation and the production of pro-inflammatory cytokines and type I interferons. TLR-9 expression is upregulated during *Leishmania* infection, and stimulation with synthetic CpG oligodeoxynucleotides (ODNs) has been shown to enhance protective Th1 responses, making it a promising immunological target in vaccine design. For this reason, TLR-9 was chosen as the docking receptor to evaluate the potential innate immune activation.[14]

The structural and immunological properties of the candidate were analyzed to validate its potential as an effective and safe vaccine against leishmaniasis. The methods applied in this study are illustrated in Figure 1.

Materials and Methods

Retrieval and analysis of protein sequences for pteridine reductase

UniProt (https://www.uniprot.org/) was used to identify and download the sequence of the selected protein, Pteridine Reductase. The retrieved sequence was analyzed to confirm that its characteristics were suitable for vaccine viability. ExPasy ProtParam (https://web.expasy.org/protparam/) was used to calculate the GRAVY score and TMHMM (https://services.healthtech.dtu.dk/services/TMHMM-2.0/) was applied to predict transmembrane helices in the protein. Antigenicity was assessed using VaxiJen v2.0 (https://ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), allergenicity using AllerTop v2.0 (https://www.ddg-pharmfac.net/AllerTOP/), and toxicity using ToxinPred 3.0 (https://webs.iiitd.edu.in/raghava/toxinpred/). AllerTop is an in-silico prediction server that outperforms other allergen prediction tools with 94% sensitivity. All sequences used in this study were obtained from public, non-sensitive repositories.

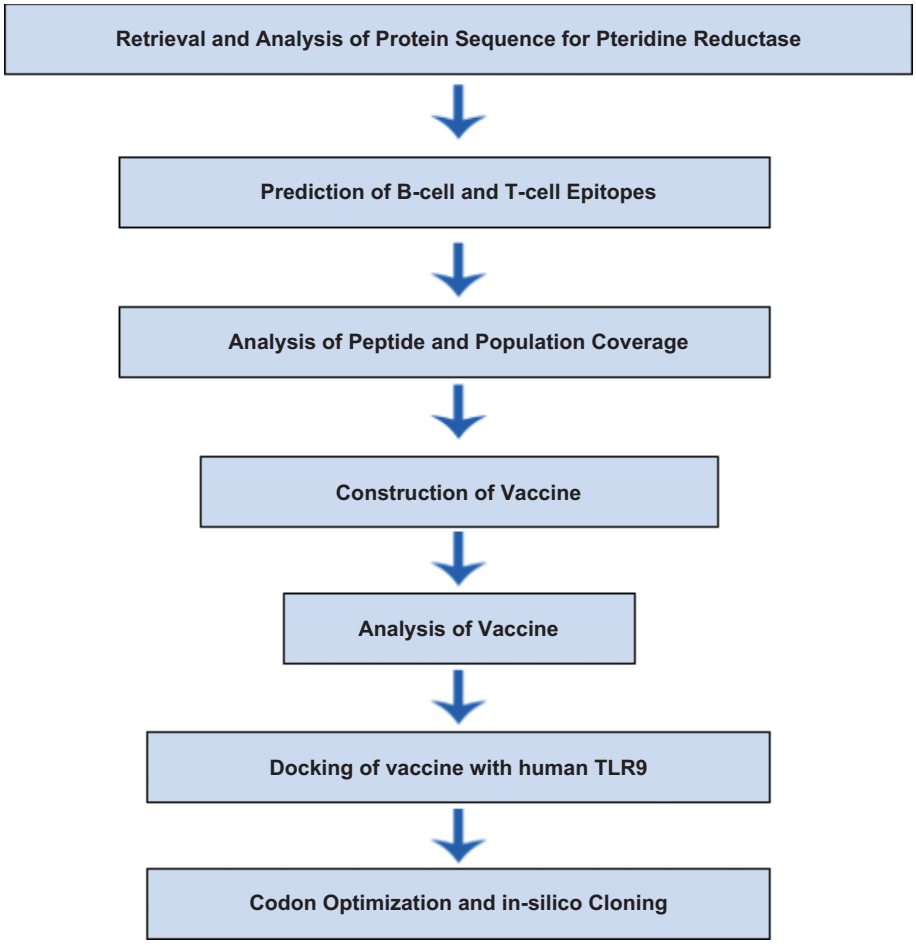

Figure 1 Methods of study to design in-silico Leishmania vaccine candidate.

Epitope prediction

Identifying B-cell epitopes is crucial because of their role in antibody production, which is essential for humoral immunity.[10,15] The selected sequence was analyzed using the Immune Epitope Database and Analysis Resource (IEDB)v2.28 (https://www.iedb.org/) to predict the linear B-cell epitopes of the conserved domains. B-cell epitopes that were non-allergenic, antigenic, and non-toxic were selected as the predicted epitopes.

T-cell epitopes were also identified because of their crucial role in the adaptive immune system.[10,16] For T-cell prediction, IEDB was used to forecast epitopes for MHC class I and MHC class II. MHC class I epitopes with a predicted IC value ≤ 50 were selected for further screening, whereas MHC class II epitopes with a predicted IC value ≤ 500 were shortlisted for further analysis based on antigenicity, allergenicity, and toxicity.

Peptide analysis

Additional analysis of the predicted peptides was carried out to identify antigenic, non-allergenic, and non-toxic candidates. To assess antigenicity, the peptide sequences of the epitopes were subjected to VaxiJen v2.0, and those with antigenicity scores greater than 0.4 were selected for evaluation. AllerTop v2.0 was then used to determine allergenic properties, and ToxinPred was applied to identify non-toxic peptides. Epitopes that fulfilled all criteria were prioritized.

Population coverage analysis

Population coverage analysis was conducted to ensure the compatibility of the multipeptide vaccine with diverse HLA alleles across different ethnic groups and populations. The selected epitopes were analyzed using the IEDB population coverage web tool to determine the percentage of the global population with HLA alleles compatible with the vaccine.

Construction of vaccine construct

Vaccine construction was carried out by compiling all prioritized epitopes with adjuvants and linkers previously used in Leishmania candidate vaccines. Synthetic oligodeoxynucleotide (CpG ODN) was selected as an adjuvant because its stability was confirmed in a previous study.[11,16] Another study suggested that CpG ODN provides sustained cellular

protection against immune challenges through dendritic cells, which are critical for effective vaccination against Leishmania.[17] CpG ODN has also been reported as an effective and economical option, and it is a common adjuvant in vaccine development, making it an optimal choice over other adjuvants.[18] The AAY linker was chosen to connect MHC I and MHC II epitopes because of its flexibility and neutrality, which preserve functionality and presentation. AAY linkers bind CTL epitopes to one another while maintaining their separation and alignment.[19] The adjuvant was connected using the EAAAK linker, whereas the CPGPG linker was employed between B-cell epitopes and MHC I epitopes. Finally, a 6X-His tag was added to the C-terminus of the vaccine sequence.

Analysis of vaccine

Vaccine efficacy was evaluated through multiple analyses. Physicochemical properties were determined using ProtParam, while the Errat score and Ramachandran plot were assessed using UCLA DOE (https://saves.mbi.ucla.edu/). Antigenicity was confirmed with VaxiJen v2.0, allergenicity was assessed with AllerTop v2.0, and toxicity was evaluated using ToxinPred 3.0. A BLASTp search was also performed to identify potential autoimmune cross-reactivity and allergen mimicry. To gain a more detailed understanding of the vaccine structure, the Protein Structure Prediction Server (PSIPRED) 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/) was used to visualize loops and helices in the secondary structure, and trRosetta (https://yanglab.qd.sdu.edu.cn/trRosetta/) was employed to predict the tertiary structure. trRosetta generated five structures in the form of Protein Data Bank (PDB) files, the most suitable of which was uploaded into CHIMERA for tertiary structure visualization. NetOGlyc 4.0 and NetPhos 3.1 were then used to screen the final vaccine construct for post-translational modifications.

Docking of vaccine with human TLR9

TLR9 was selected as a receptor because of its ability to activate dendritic cells (DCs) and induce host protection against *Leishmania* species.[20,21] DCs activate TLR9 through its ligand, CpG-ODN, thereby initiating the innate immune response. TLR9 is expressed on the endosomal membranes of B-cells and DCs, where it mediates proinflammatory cytokine responses and contributes to infection control.[22] PDB files for the vaccine were generated using trRosetta 2, while the RCSB Protein Data Bank v4.2 (https://www.rcsb.org/) w was used to retrieve the PDB file for human TLR9. The active and passive sites of each structure were identified using castp 3.0 (http://sts.bioe.uic.edu/castp/indusing?3igg), and docking was performed with HDOCK (http://hdock.phys.hust.edu.cn/). The resulting complexes were visualized using UCSF Chimera 1.18 and BIOVIA Discovery Studio before proceeding to in-silico cloning. iMODS (https://imods.iqf.csic.es/) was then used to perform Normal Mode Analysis (NMA) in Internal Coordinates (IC) to derive eigenvalues, elastic networks, deformability plots, and covariance matrices. PRODIGY 2.2.2 (https://rascar.science.uu.nl/prodigy/) was used to predict Kd and Binding affinity.

Codon optimization and in-silico cloning

Codon optimization and in-silico cloning of the vaccine were performed to ensure efficient expression.[14,23] The Integrated DNA Technologies Codon Optimization tool (https://www.idtdna.com/pages/tools/codon-optimization-tool) was used to adapt codons for the selected expression host. *Escherichia coli* K12 was chosen as the host strain because of its availability, ease of growth, and economic advantages.[24] The optimized sequence obtained from the codon optimization tool was then input into SnapGene software and inserted into the vector pET-21a (+), enabling visualization of the cloning process.

Molecular dynamic simulation

This study utilized GROMACS 2024.02 to simulate a vaccine-docked protein complex under biologically relevant conditions. The vaccine structure was converted into a GRO file compatible with the OPLS-AA force field, while simulations were performed using the CHARMM36 force field for a 50 ns molecular dynamics (MD) run. The optimal ligand pose, selected based on docking scores, was prepared using an NVT ensemble with the V-rescale thermostat set at 303.15 K and equilibrated for 1 ns. A TIP3P water model was used to define a cubic box (100 × 100 × 100 Å) with a 10 Å buffer surrounding the protein, and physiological ionization states at pH 7.0 were maintained using K^+ and Cl^- ions placed via Monte Carlo methods.[25]

The system underwent steepest descent energy minimization (5,000 steps) followed by two equilibration phases (100 ps each) under NVT and NPT ensembles with Berendsen and Parrinello-Rahman barostats, respectively. The MD production phase employed periodic boundary conditions (PBC) for realistic molecular interactions, a 2 fs time step, and the Particle Mesh Ewald (PME) method for long-range electrostatics. Non-bonded interactions were truncated at 1.2 nm, and the LINCS algorithm was applied to constrain bond lengths. CHARMM36m force field parameters were used, and analyses included Root Mean Square Deviation (RMSD), radius of gyration (RoG), and binding energy calculations using the “gmmpbsa” module. Interaction distances were assessed with VMD, and visualizations were generated in PyMOL. A 1 ns NVT equilibration at 303.15 K was also performed. This comprehensive workflow accurately captured ligand-protein dynamics and energetics.[26]

Results

Protein analysis

The selected protein, Pteridine Reductase, was evaluated and found to be stable and hydrophobic, as indicated by its positive GRAVY index. Furthermore, the protein was predicted to be non-allergenic and non-toxic (Table 1).

Table 1 Properties of pteridine reductase.

Protein	Amino Acid	Instability Index	Estimated Half-Life	Aliphatic Index	Grand Average of Hydropathicity (GRAVY)	Theoretical PI	Antigenicity Score
Pteridine reductase Uniprot ID: Q9NR09 BIRC6_HUMAN	288	31.88 (stable)	30 hours (mammalian reticulocytes, in vitro) >20 hours (yeast, in vivo) >10 hours (Escherichia coli, in vivo)	87.85	0.059	6.70	0.5405

B-cell prediction

A list of predicted B-cell epitopes was obtained from IEDB and assessed for selection. The shortlisted B-cell epitopes were determined to be antigenic, non-allergenic, non-toxic, and soluble in water. Only one B-cell epitope, consisting of nine amino acids, fulfilled these criteria (Table 2).

T-cell epitope prediction

Epitopes for both MHC class I and class II were predicted, and the results were shortlisted to include only antigenic, non-toxic, water-soluble, and non-allergenic epitopes. Six epitopes were identified for MHC class I (Table 3), and 25 epitopes were identified for MHC class II (Table S1).

Population coverage analysis

To evaluate the global applicability of the selected epitopes, population coverage analysis was performed for T-cell epitopes across both MHC class I and MHC class II. The analysis indicated that the HLA alleles used to predict T cells could elicit an immune response in 81.8% of the global population, demonstrating encouraging potential for broad immunogenicity (Figure 2). Achieving high population coverage with a vaccine is essential for maximizing its effectiveness and ensuring widespread protection.

Table 2 Predicted B-cell epitopes.

Peptide	Antigenicity Score
GTPAKHRGT	0.9482

Vaccine construction

The selected epitopes were used to construct an in-silico vaccine by combining the peptides with appropriate linkers and adjuvants. The construct refers to the selected targets, epitopes, adjuvants, and linkers that may serve as potential models for vaccines.[27,28] CpG Oligodeoxynucleotide (CpG ODN) was selected as an adjuvant and added to the N-terminus, while the AAY linker was chosen to connect MHC I and MHC II epitopes because of its ability to enhance vaccine functionality and structural integrity. The adjuvant was linked using the EAAAK linker, whereas the CPGPG linker was inserted between B-cell epitopes and MHC I epitopes. The CPGPG linker was used because of its ability to enhance the immune response elicited by a vaccine.[29] This combination of linkers between B-cell epitopes is known to intensify immune responses.[30] Finally, a 6X-His tag was added to the C-terminus of the vaccine sequence. The proposed construct consists of 484 amino acids (Figure 3). These linkers and adjuvants were incorporated with one B-cell epitope,

Table 3 MHC class I epitope prediction.

Allele	Seq_num	Start	End	Length	Peptide	IC50	Rank
HLA-A*68:02	4	31	39	9	ELAPLQIRV	9.43	0.09
HLA-A*68:02	3	17	26	10	ETATADLFGS	55.7	0.4
HLA-A*30:01	5	30	38	9	KYITGTCVK	20.71	0.1
HLA-B*07:02	1	52	60	9	NARRPNSAI	71	0.24
HLA-A*02:06	4	47	56	10	SVLVDDMPPA	29.6	0.32
HLA-A*02:03	4	47	56	10		59.93	0.85
HLA-A*02:06	5	8	17	10	YQRDSSAAEV	29.07	0.32
HLA-A*02:03	5	8	17	10		48.49	0.75

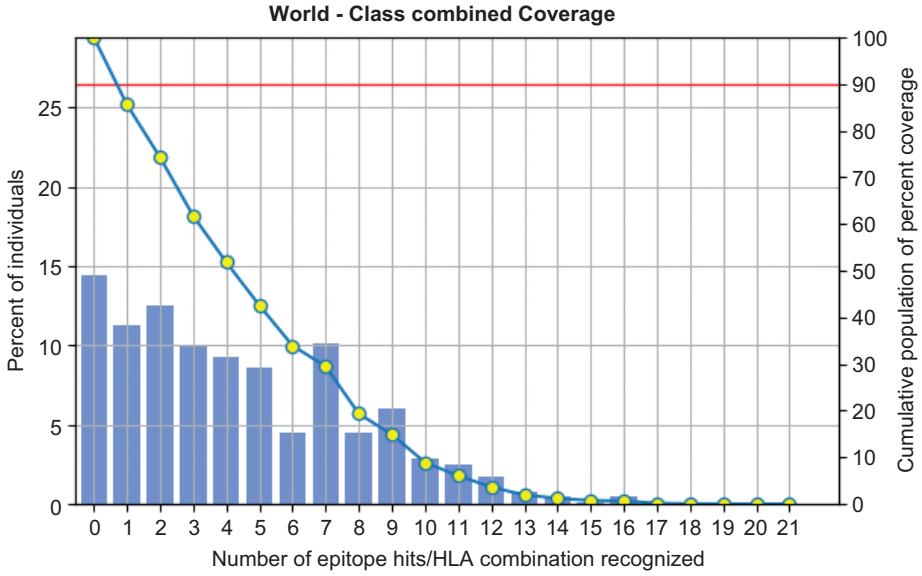

Figure 2 Combined coverage of T cell epitopes of combined MHC classes using IEDB.

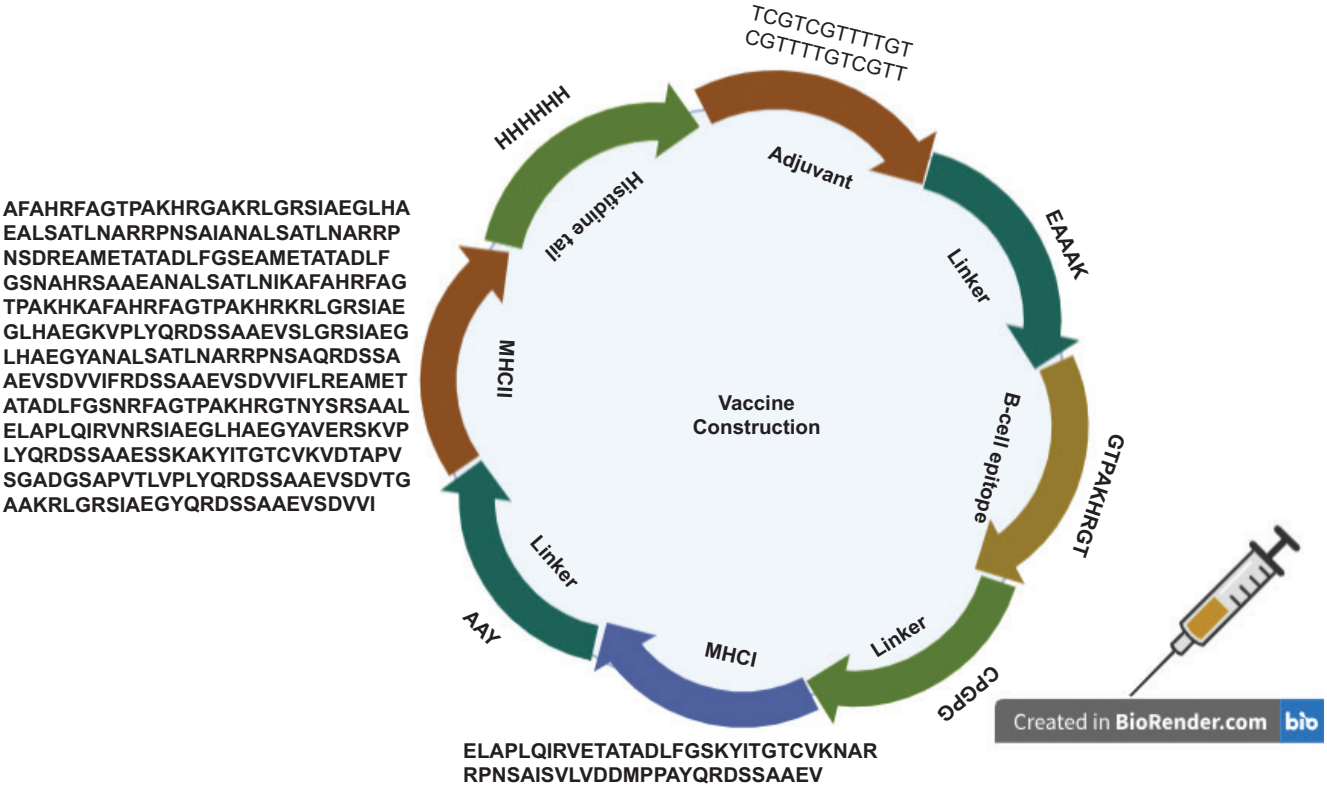

Figure 3 Constructed vaccine consisting of linkers, adjuvants and peptides of B cells and T cells.

six MHC class I epitopes, and 25 MHC class II epitopes, resulting in a potentially robust and stable vaccine.

Prediction of secondary and tertiary structure of vaccine construct

The positions and percentages of helices, strands, and coils were predicted using PSIPRED 4.0 (Figure 4). Likewise, trRosetta 3.14 was employed to visualize the tertiary structure of the vaccine (Figure 5).

Analysis of vaccine

Vaccine potency was confirmed by verifying that the construct met specific quality criteria. UCLA DOE analysis of the Ramachandran plot showed that 89.9% of residues were

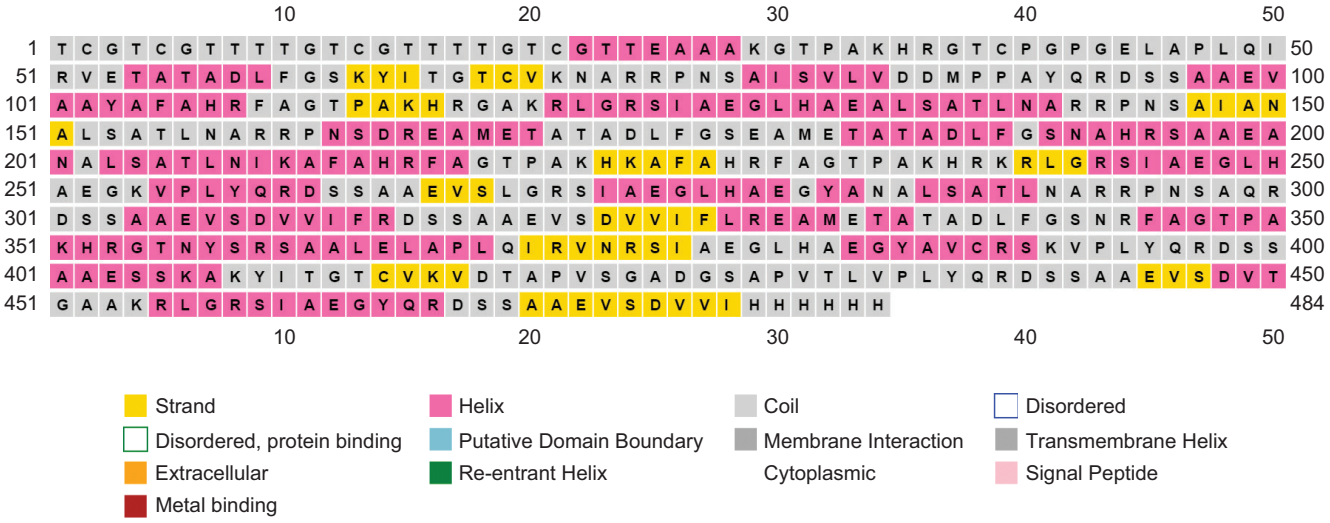

Figure 4 Graphical representation of the secondary structure features of the vaccine predicted using PSIPRED 4.0. Coils are shown in gray, helices in pink, and strands in white.

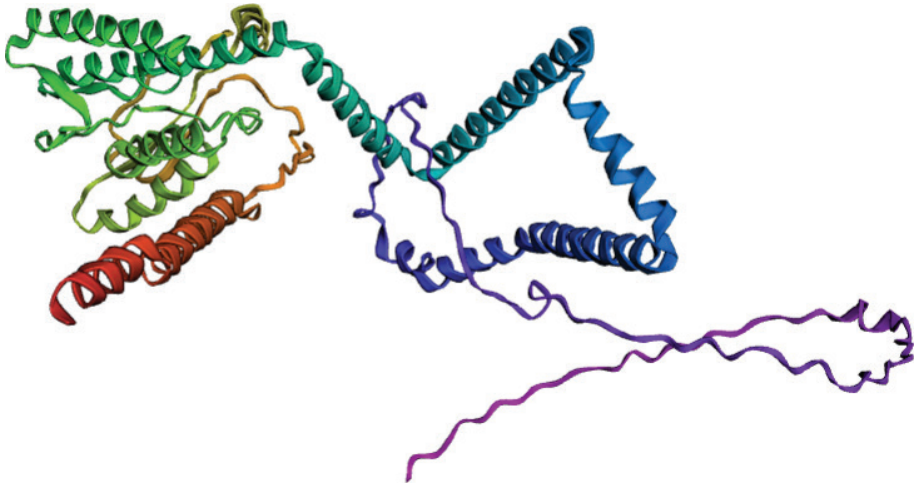

Figure 5 Visualization of the tertiary structure of vaccine construct on trRosetta.

present in the most favorable region, while 8.9% of residues were present in the overall allowed region (Figure 6). Similarly, UCLA DOE analysis revealed an Errat score of 98.4615, indicating a high overall quality factor (Figure 7). The ProSA Z-score server reported a score of -5.56, and the local quality model indicated that most residues were located within the acceptable region. Furthermore, the vaccine was predicted to be antigenic, non-toxic, and non-allergenic, confirming that the construct was stable and potentially effective. BLASTp analysis against the human proteome (taxid:9606) revealed no significant similarity (defined as >35% identity over ≥80 amino acids), suggesting a minimal risk of autoimmune cross-reactivity. The vaccine construct was visualized using UCSF Chimera (Figure 8). NetOGlyc 4.0 identified 23 residues as positive glycosylation candidates, with scores exceeding the 0.5 threshold, the majority of which were located in the N-terminal or non-epitopic regions. Similarly, NetPhos 3.1 predicted phosphorylation sites primarily in non-epitopic or linker regions, reducing the risk of glycosylation masking epitopes.

Docking of vaccine construct with human TLR-9

Molecular docking was performed to evaluate interactions between the TLR-9 ligand and the vaccine construct.[23] Human TLR-9, a pattern recognition receptor (PRR), was selected because of its critical role in activating the innate immune system.[31] The active sites of TLR-9 and the vaccine construct were identified using CASTp, and docking was carried out with HDOCK to analyze the interactions between the two structures. The results indicated that the vaccine construct could bind to TLR-9 and was expected to trigger a strong immune response, as supported by the docking score and confidence score (Table 4). PRODIGY

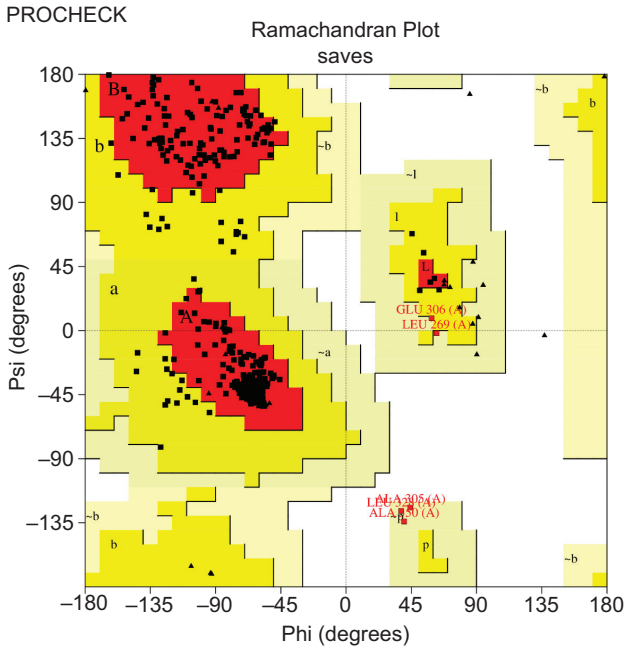

Figure 6 Ramachandran plot showing the allowed and favored regions of dihedral angles in the vaccine construct, generated using UCLA DOE.

1.15.8 predicted a Gibbs free energy (ΔG) of -11.8 kcal mol^{-1} and a KD of 2.2e-09. These low values suggest that the vaccine construct was stable and exhibited strong binding affinity, supporting its potential as a successful vaccine candidate. The docked complex was visualized using UCSF Chimera (Figure 9).

Normal mode analysis

The docked complex was analyzed using iMODS to reproduce the collective functional motions of biological molecules.[32] The individual distortion of each residue determined the deformability of the complex, represented by green hinges along the chain (Figure 10A). The eigenvalue of the complex was 2.06e-0.6, indicating a high degree of flexibility (Figure 10B). Similarly, the elastic network model was applied to assess flexibility by representing atoms as nodes connected through virtual springs (Figure 10C). Darker regions in the graph indicated greater stiffness between atoms. The covariance matrix illustrated the coupling between residue pairs, where red regions indicated correlated motions, blue regions indicated anti-correlated motions, and white regions indicated uncorrelated motions (Figure 10D).[33]

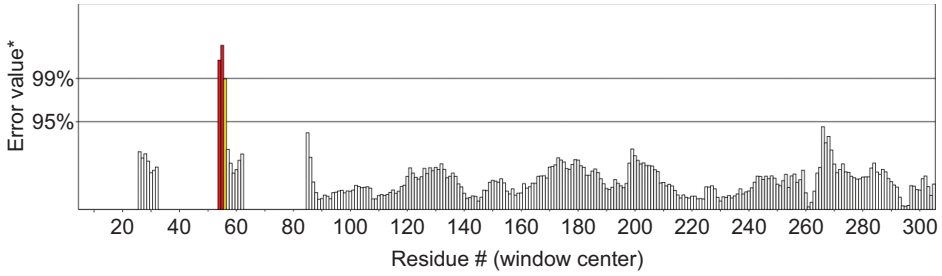

Figure 7 ERRAT score plot showing ERRAT value of 98.4615 using UCLA DOE.

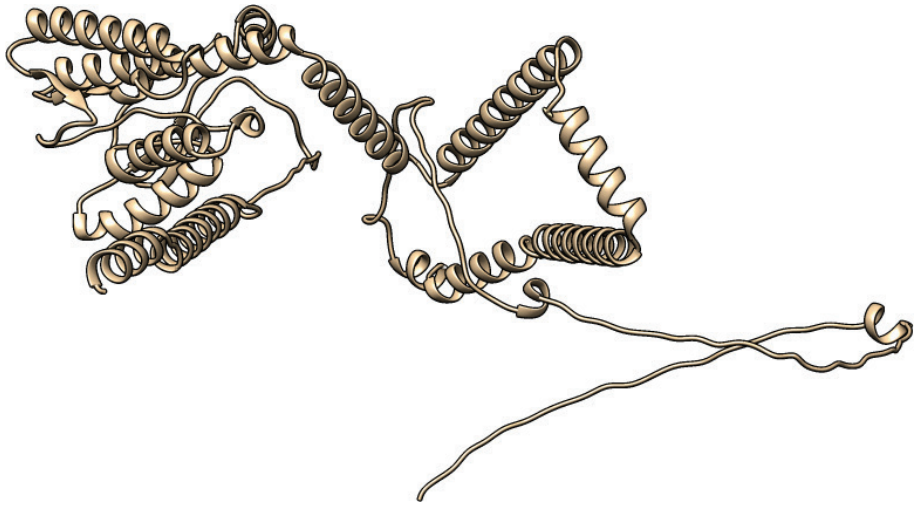

Figure 8 Vaccine construct visualized through UCSF CHIMERA.

Codon optimization and in-silico cloning

The IDTDNA web tool optimized codons and generated 1,452 nucleotides with improved expression levels[34] in the selected vector host. The pET plasmid system is commonly used to produce recombinant proteins in *Escherichia coli*.[5] Among these, pET21a(+) is widely employed because its T7

Table 4 Statistical Analysis of docked complex from HDOCK

Docking score	-36.1.39	
Confidence score	0.9856	
Ligand RMSD (Å)		2.1 Å

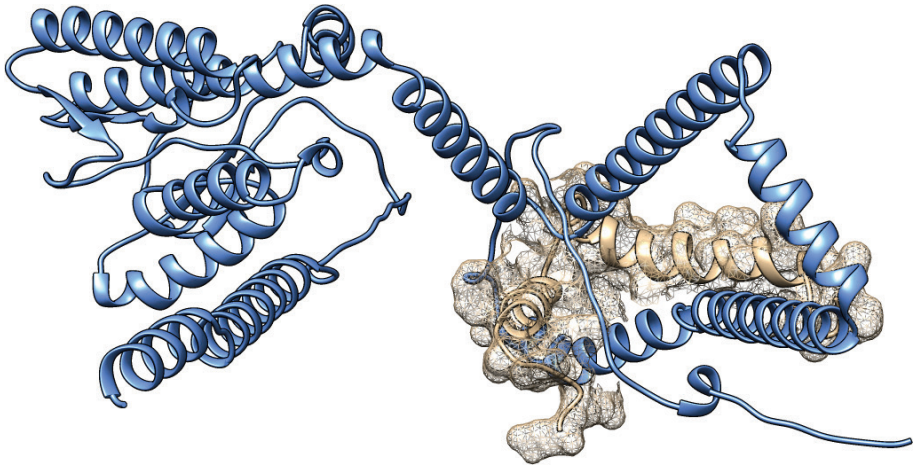

Figure 9 Docked complex of vaccine construct (blue) with human TLR9 (yellowish-white) on UCSF Chimera.

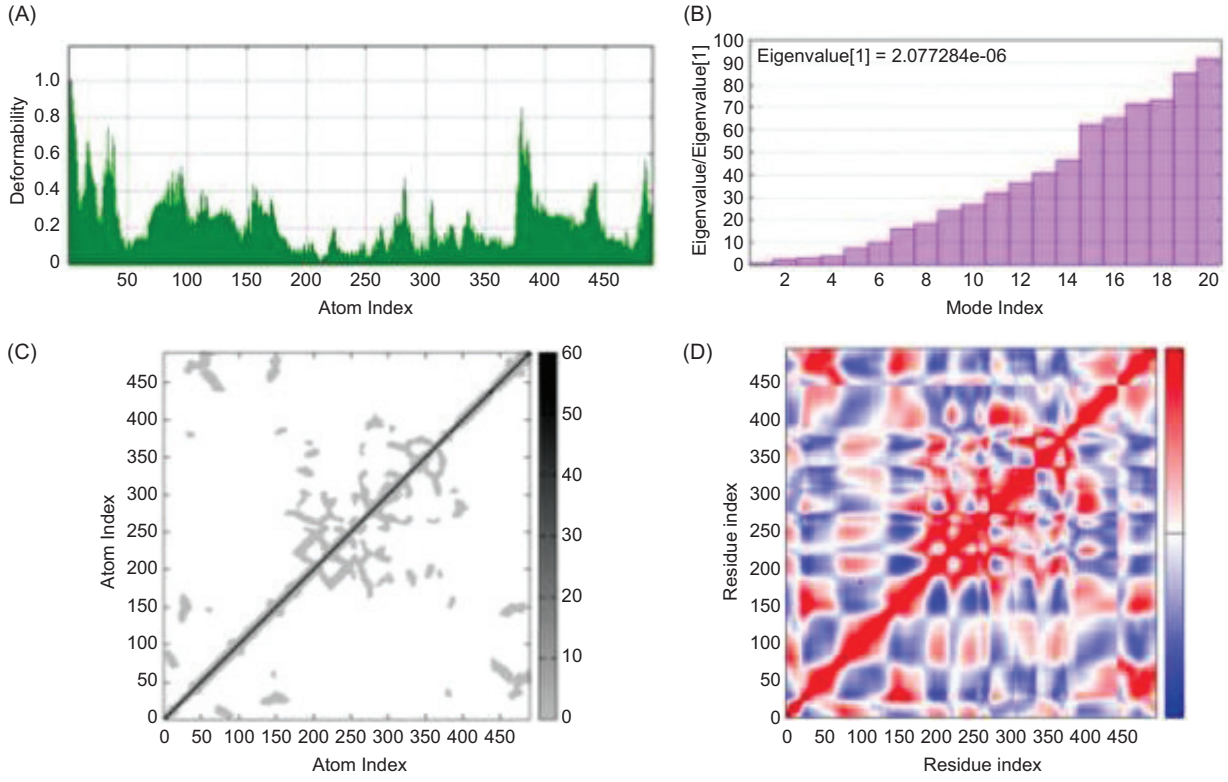

Figure 10 iMODS analysis of the docked vaccine complex. (A) Residue-wise distortion representing deformability. (B) Eigenvalue plotted against mode index. (C) Elastic network model. (D) Covariance matrix.

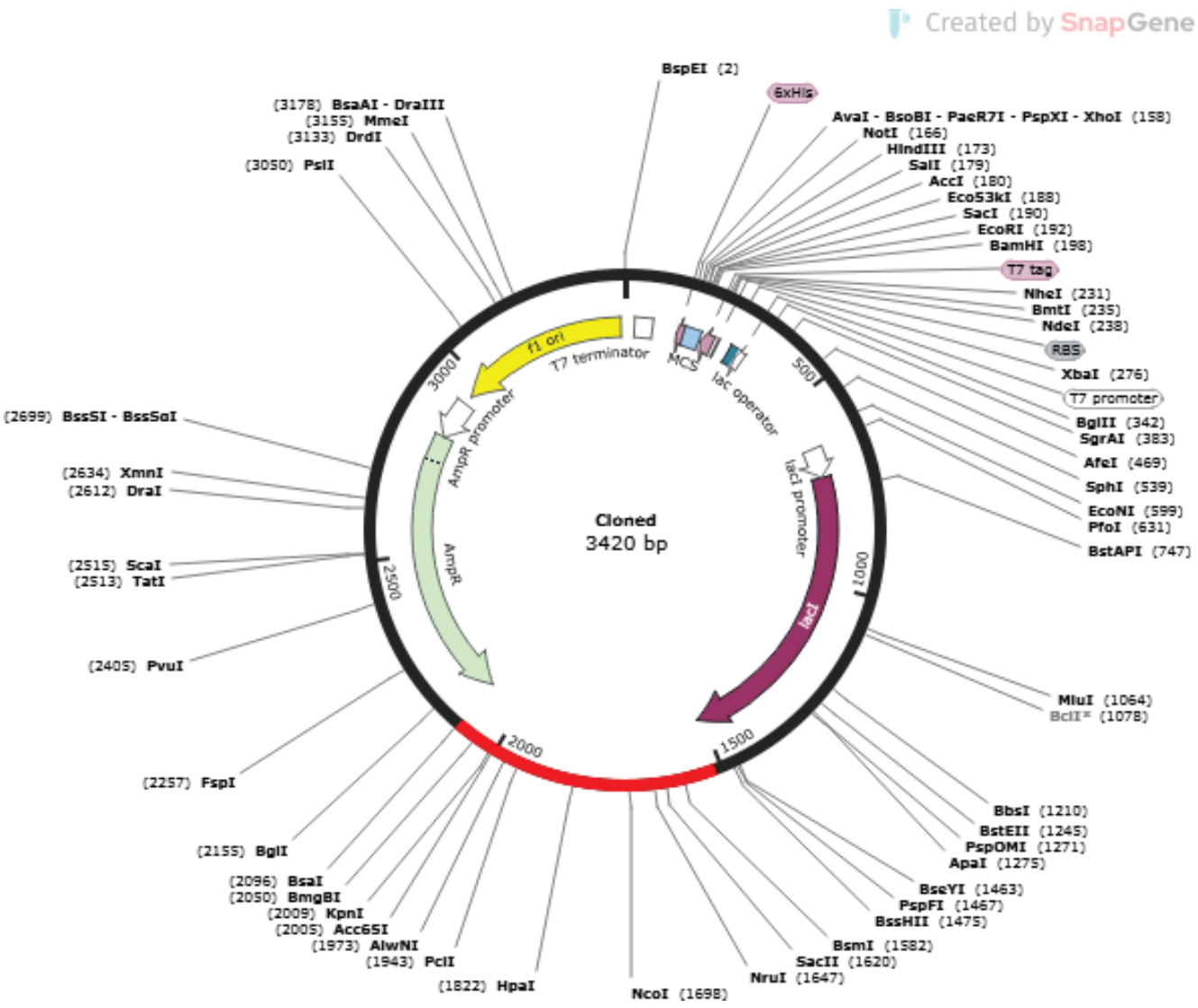

Figure 11 Vaccine construct cloned for expression in *E.coli* using pET-21a (+) vector.

promoter sequence maximizes protein expression in bacterial systems.[24,35] SnapGene was used to insert the vaccine fragment into pET-21a(+) and perform cloning, resulting in a complete clone of 3.420 kb (Figure 11).

Molecular dynamic simulation

Molecular dynamics simulation was performed to analyze the behavior of the protein target in a biological system. In Figure 12A, a 50 ns simulation was conducted, yielding a maximum RMSD of 1.93 nm at 42 ns, with a mean RMSD of 1.3 nm observed at 21 ns. The RMSD initially started low (~0.5 nm) and gradually increased, indicating that the protein complex underwent structural adjustments before reaching stability.

Between 35 and 40 ns, the RMSD reached a plateau (~1.5–1.7 nm), suggesting that the system had stabilized and achieved equilibrium. Similarly, the RMSF plot in Figure 12B highlighted dynamic regions that may represent potential binding sites or regions contributing to interaction flexibility. Regions with higher fluctuations (~2.5–3.0 nm) corresponded to flexible loops, termini, or exposed regions of the protein complex, whereas lower RMSF values (~0.5–1.0 nm) indicated more rigid or structured regions, such as the protein core or binding interface.

Figure 12C illustrates the compactness and structural changes of the molecule over time, as determined by molecular dynamics simulation through the radius of gyration (Rg). The Rg was approximately 3.8–4.0 nm at the beginning, indicating an extended structure, but it dropped to about 3.0 nm during the simulation, suggesting folding or compaction. Minor variations were observed throughout, consistent with thermal fluctuations; however, the Rg stabilized at 2.8–3.0 nm after 40,000 ps, indicating that the system had reached a stable equilibrium state. This pattern reflects structural alterations such as folding or collapse, with the molecule ultimately stabilizing into a potentially native-like conformation. During the equilibration stage, the Parrinello–Rahman barostat was applied at a pressure of 1 atm and a temperature of 303.15 K using a constant NPT ensemble

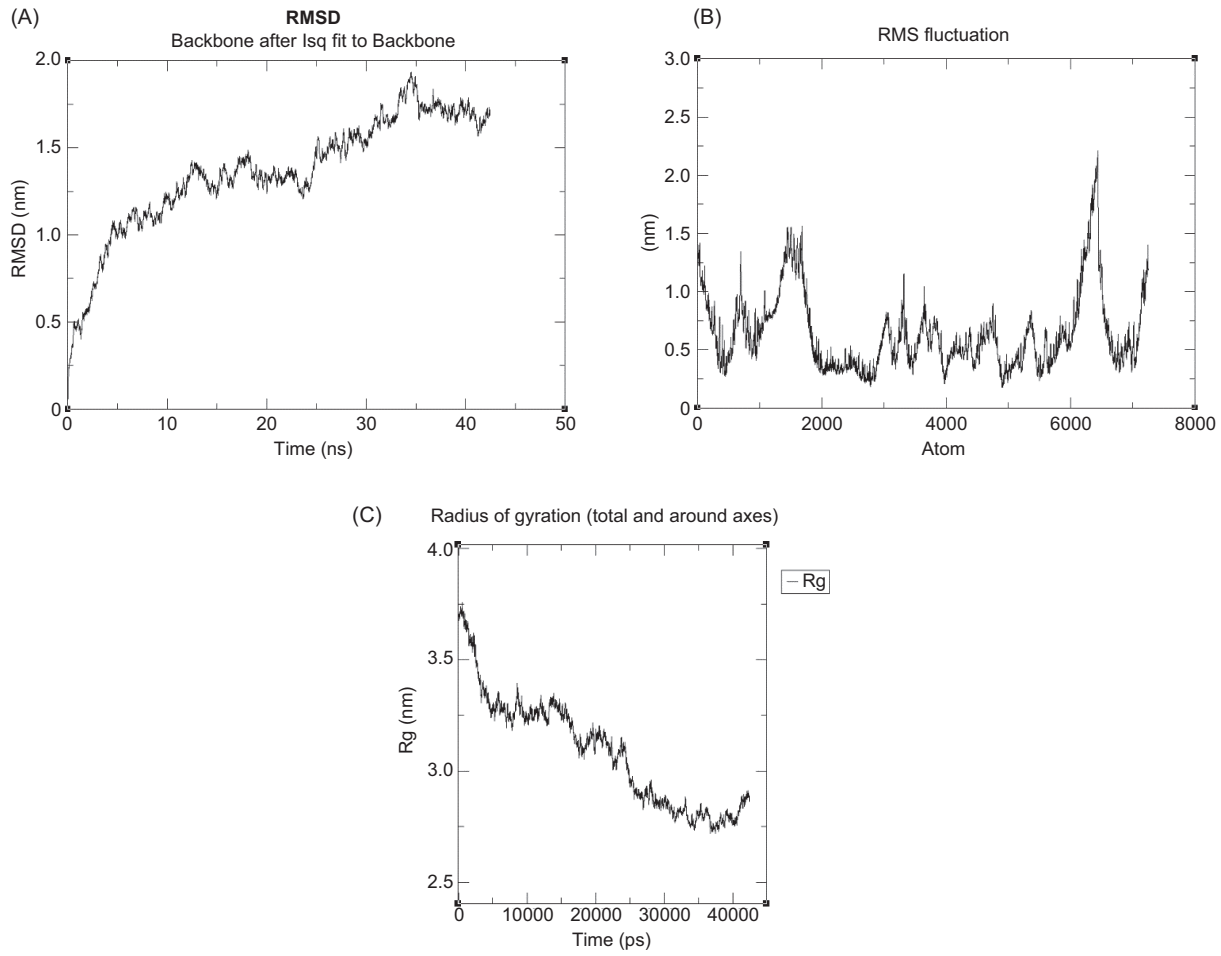

Figure 12 Molecular dynamics simulation analysis of the vaccine–TLR4 docked complex. (A) RMSD graph showing a steady increase in fluctuations, reaching a maximum at ~43 ns before stabilizing at equilibrium. (B) RMSF plot with the highest fluctuations observed between residues 1000–2000, reaching ~1.5 nm. (C) Radius of gyration (Rg) plot showing overall compactness of the complex throughout the simulation.

Discussion

Leishmania, the parasite responsible for leishmaniasis, is the second leading cause of parasitic death worldwide after malaria, comprising more than 20 species that cause human disease across the globe. High levels of genetic variability among these species, combined with variable clinical manifestations and rapid adaptation in culture, complicate the development of standardized treatments, highlighting the urgent need for new therapies and preventive vaccines.[36] With 350 million people at risk, this neglected tropical disease poses a major global public health challenge.[37] *Leishmania* parasites have evolved mechanisms to subvert or inhibit the host immune response, making efficient treatment or prevention critically important. A thorough understanding of parasite biology and host immunology is essential for the development of effective vaccines. Immunoinformatics enables the generation of dynamic protein profiles, providing insights into interactions between the vaccine and the host immune system. This approach allows testing and analysis of immunogenic data to ensure vaccine safety and efficacy. Furthermore, in-silico methods enhance epitope specificity by selecting antigens predicted to elicit strong immune responses, while offering a cost- and time-effective strategy for the early stages of vaccine design.[38]

The present study employed an immunoinformatics approach to design a vaccine construct targeting the Pteridine Reductase protein to induce an immune response. Previous studies have applied molecular docking and visualization to identify potential inhibitors of *Leishmania* as drug targets;[11,21] however, none have explored its potential as a vaccine target. Immunoinformatics has accelerated vaccine development by reducing both time and cost compared to traditional methods.[38] This approach enables accurate prediction of vaccine targets, stability, and efficacy. The antigenicity of Pteridine Reductase 1 was evaluated, with ideal antigenicity scores ranging from 0.4000 to 2.000.[39] The protein was selected based on its stability, estimated half-life, aliphatic index, GRAVY, theoretical pI, and antigenicity score.

B cells are responsible for the creation of antibody-secreting plasma cells and memory B cells that can recognize and respond to antigens in immediate and long-term immune responses, respectively. The B-cell and T-cell epitopes of the antigen were further prioritized based on

their antigenicity, allergenicity, and toxicity. Epitopes with an immunogenicity score of less than 0.00 on the VaxiJen server indicated positive antigenicity and immunogenicity scores, which would be highly antigenic and exhibit a high affinity for interacting with MHC complexes and enhancing immune responses. B-cell epitopes with high affinity and antigenic properties were identified, whereas T-cell epitopes with appropriate IC values were considered to have high affinity.[40] One B-cell peptide and 31 T-cell peptides were identified. Thereafter, the peptides were combined with adjuvants and linkers to develop the vaccine construct. CpG ODN was utilized as an adjuvant and has been applied in other multi-epitope vaccines as well, including in a Mycobacterium tuberculosis vaccine designed by Wang et al.,[41] where CpG ODN was used due to its ability to stimulate dendritic cells to produce cytokines such as TNF-a, IL-6, and IL-12, and to trigger T lymphocytes and IFN secretion as a Th1 response. CpG ODN has also been tested as an adjuvant in mice immunized with Leishmania, resulting in improved survival. The AAY linker was used between MHC I and MHC II, and a CPGPG linker was employed between B-cell epitopes and MHC I, as these linkers improve the presentation of epitopes by promoting their natural formation.[29] The EAAAK linker reduced the interaction of the vaccine with other protein regions, thereby increasing its stability. These linkers were also used in a study by Ramprasad et al.[42] EAAAK is used to separate functional domains and maintain functional properties of epitopes in vaccine constructs. This is achieved through a peptide linker with a rigid alpha-helix structure, making EAAAK a popular choice as a linker in vaccines.[29] The peptides were synthesized using linkers and adjuvants to produce the final vaccine construct, which consisted of 484 amino acids.

The vaccine construct was inspected to determine allergenicity patterns, antigenicity, toxicity, physiochemical properties, and structural stability, and was found to meet the necessary criteria for an effective immune response with minimal adverse effects.[43] The Ramachandran plot revealed that 89.9% of the residues were present in the most favorable region and 8.9% of the residues were present in the overall allowed region, indicating that the structure was of high quality and stability. This score is on the borderline of the ideal threshold (< 90%) for acceptable stereochemical quality, which may result in minor but overall insignificant deviations.[44] A previously proposed Leishmania vaccine construct had a Ramachandran score of 81.5% in the most favored regions,[45] which is lower than the construct presented in this study, indicating superior structural stability and stereochemical quality. The Errat score was 98.4615, which further verified the dependability of the model. This was higher than another immunoinformatics study that reported an Errat score of 84.90.[46] Post-translational modifications were rarely observed in epitopic regions, making the vaccine less likely to contain masked epitopes or altered antigen processing.[47] Furthermore, population coverage of 81.8% was higher than that of a previously proposed Leishmania vaccine, which had a population coverage of 69.14%.[45]

Visualization of the structures enabled the examination of the vaccine construct, and molecular docking was carried out using the human Toll-like receptor (TLR-9). A study found that TLR-9 signaling results in protective immunity against Leishmania due to its ability to induce proinflammatory cytokines such as IL-12 by dendritic cells, concluding that TLR-9 stimulation is essential for NK cell activation.[48] TLR-9 has been used in other studies and has been hypothesized to improve the efficacy of a vaccine against cutaneous leishmaniasis and to increase the level of T-cell response when used in a mouse model as an immunotherapeutic treatment against cutaneous leishmaniasis.[49] TLR-9 functions as an immune enhancer due to its ability to promote nuclear translocation and activation of nuclear factor-κB (NF-κB), resulting in an enhanced proinflammatory response, activation, and T-cell proliferation of DCs.[50] TLR-9 knockout mice experience a loss of protective immunity against the *Leishmania* parasite.

NMA is a computational method used for molecular refinement or replacement, capable of characterizing flexible conformations available to molecules around an equilibrium position. The eigenvalue, which represents the motion stiffness of the complex, was 2.06e-0.6. This low score indicates that the construct can easily undergo deformation and exhibits a high level of flexibility.[21,33] This analysis confirmed that the docked molecule achieved a successful interaction and that the resulting structure demonstrated high flexibility, making it an encouraging candidate. Codon optimization resulted in the generation of 1452 nucleotides. In-silico cloning into *E. coli* was performed, and the resulting clone was 3.420 kb long. The results of the study were consistent with the hypothesis that interactions between the vaccine and its targets were dynamic and stable. Additionally, Molecular Dynamic Simulation conducted using GROMACS 2024.2 revealed a stable configuration for the vaccine and TLR-9 docked complex.

While analysis has suggested promising results, the reliability of immunoinformatics is limited by the availability of datasets and remains challenged by epitope prediction errors, the complexity of the immune system, and genetic variability among individuals. These limitations make in vivo and in vitro validation crucial.[51,52] This study suggests that the vaccine candidate is stable and safe, making it a promising option against Leishmania. However, this candidate must be tested in vivo and in experimental trials using appropriate model organisms to validate the safety and efficacy of the vaccine.

Conclusion

Leishmania is a growing threat, with high mortality and morbidity rates in more than 98 countries. The development of an effective human vaccine is imperative for managing disease transmission, particularly given the absence of an existing vaccine. Using an immunoinformatics approach, a multi-epitope vaccine construct was designed to target both innate and adaptive immune responses. Potential vaccine targets were identified and investigated for allergenicity, immunogenicity, and antigenicity to ensure their safety and performance as vaccine candidates. Further analysis confirmed that the vaccine elicited antibody- and cell-mediated immune responses. The construct was then docked with human TLR-9 and visualized to verify that the interaction was stable and had high

binding affinity. Finally, in-silico cloning was performed to determine vaccine expression in *E. coli*. Evaluation of the construct indicated that the vaccine is capable of inducing an immune response. However, in vivo and in vitro experiments are essential to confirm the safety and efficacy of the vaccine construct.

Data Availability Statement

All the data generated in this research work has been included in this manuscript.

Acknowledgement

The authors are thankful to Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2026R437), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Author's Contribution

Conceptualization, Syed Babar Jamal; methodology, Meeral Ovais and Munazza Kanwal; software, Shumaila Naz; validation, Nawal Al-Hoshani and Sahar Abduljawad; formal analysis, Fakhria A. Al-Joufi; investigation, Munazza Kanwal and Meeral Ovais; resources, Tariq Aziz; data curation, Hanan Abdulrahman Sagini.; writing—original draft preparation, Munazza Kanwal and Meeral Ovais.; writing—review and editing, Shaza N. Alkhatib and Jamal I Asseri; visualization, Maher S Alwethaynani; supervision, Tariq Aziz and Syed Babar Jamal.; project administration, Syed Babar Jamal; funding acquisition, Tariq Aziz.

Conflict of Interest

The authors declare no conflict of interest.

References

1. Soltan MA, Elbassiouny N, Gamal H, Elkaeed EB, Eid RA, Eldeen MA, et al. In Silico Prediction of a Multitope Vaccine against Moraxella catarrhalis: Reverse Vaccinology and Immunoinformatics. Vaccines. 2021;9(6):669. https://doi.org/10.3390/vaccines9060669
2. Baneth G, Solano-Gallego L. Leishmaniasis. Vet Clin North Am Small Anim Pract. 2022 Nov; 52(6):1359-1375. https://doi.org/10.1016/j.cvsm.2022.06.012
3. de Vries HJC, Schallig HD. Cutaneous Leishmaniasis: A 2022 Updated Narrative Review into Diagnosis and Management Developments. Am J Clin Dermatol. 2022;23(6):823-840. https://doi.org/10.1007/s40257-022-00726-8
4. Mann S, Frasca K, Scherrer S, Henao-Martínez AF, Newman S, Ramanan P, et al. A Review of Leishmaniasis: Current Knowledge and Future Directions. Curr Trop Med Rep. 2021;8(2):121-132. https://doi.org/10.1007/s40475-021-00232-7
5. Montaner-Angoiti E, Llobat L. Is Leishmaniasis the New Emerging Zoonosis in the World? Vet Res Commun. 2023;47:1777-1799. https://doi.org/10.1007/s11259-023-10171-5
6. Pradhan S, Schwartz RA, Patil A, Grabbe S, Goldust M. Treatment options for leishmaniasis. Clin Exp Dermatol. 2022;47(3):516-521. https://doi.org/10.1111/ced.14919
7. Chen X, Shi Y, Zhou S, Geng M, Tu H, Song J, et al. Risk factors of visceral leishmaniasis in the world: a review. Chinese J Schistosomiasis Control. 2024;36(4):412-421. https://doi.org/10.16250/j.32.1374.2024079
8. Husein-ElAhmed H, Gieler U, Steinhoff M. Evidence supporting the enhanced efficacy of pentavalent antimonials with adjuvant therapy for cutaneous leishmaniasis: a systematic review and meta-analysis. J Eur Acad Dermatol Venereol. 2020;34(10):2216-2228. https://doi.org/10.1111/jdv.16333
9. Abdellahi L, Iraji F, Mahmoudabadi A, Hejazi SH. Vaccination in Leishmaniasis: A Review Article. Iran Biomed J. 2022;26(1):1-35.
10. Qureshi H, Basheer A, Faheem M, Arshad MW, Rai SK, Jamal SB. Designing a multi-epitope vaccine against *Shigella dysenteriae* using immuno-informatics approach. Front Genet. 2024;15:1361610. https://doi.org/10.3389/fgene.2024.1361610
11. Kayraklioglu N, Horuluoglu B, Klinman DM. CpG Oligonucleotides as Vaccine Adjuvants. Methods Mol Biol. 2021;2197:51-85. https://doi.org/10.1007/978-1-0716-0872-2_4
12. Molteni M, Gemma S, Rossetti C. The Role of Toll-Like Receptor 4 in Infectious and Noninfectious Inflammation. Mediators Inflamm. 2016;2016:6978936. https://doi.org/10.1155/2016/6978936
13. Kazemi B, Tohidi F, Bandehpour M, Yarian F. Molecular cloning, expression and enzymatic assay of pteridine reductase 1 from Iranian lizard *Leishmania*. Iran Biomed J. 2010 Jul;14(3):97-102. PMID: 21079660 (PMCID: PMC3904060)
14. López-Blanco JR, Aliaga JI, Quintana-Ortí ES, Chacón P. iMODS: Internal coordinates normal mode analysis server. Nucleic Acids Res. 2014;42(W1):W271-W276. https://doi.org/10.1093/nar/gku339
15. Biogene C. pET-21a-d(+). [Internet]. Available from: https://www.creative-biogene.com/pet-21a-item-vet1482-441351.html
16. Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Schoenian G, et al. Leishmania infections: Molecular targets and diagnosis. Mol Aspects Med. 2017;57:1-29. https://doi.org/10.1016/j.mam.2016.11.012
17. Shah JA, Darrah PA, Ambrozak DR, Turon TN, Mendez S, Kirman J, et al. Dendritic cells are responsible for the capacity of CpG oligodeoxynucleotides to act as an adjuvant for protective vaccine immunity against *Leishmania major* in mice. J Exp Med. 2003 Jul 21;198(2):281-291. https://doi.org/10.1084/jem.20030645
18. das Neves GM, Kagami LP, Gonçalves IL, Eifler-Lima VL. Targeting pteridine reductase 1 and dihydrofolate reductase: the old is a new trend for leishmaniasis drug discovery. Future Med Chem. 2019;11(16):2107-2130. https://doi.org/10.4155/fmc-2018-0512
19. Dashti F, Raisi A, Pourali G, Razavi ZS, Ravaei F, Sadri Nahand J, et al. A computational approach to design a multiepitope vaccine against H5N1 virus. Virol J. 2024;21(1):67. https://doi.org/10.1186/s12985-024-02337-7
20. Wang H, Wang S, Fang R, Li X, Xing J, Li Z, et al. Enhancing TB vaccine efficacy: current progress on vaccines, adjuvants and immunization strategies. Vaccines. 2023;12(1):38. https://doi.org/10.3390/vaccines12010038
21. Bhattacharya P, Gannavaram S, Ismail N, Saxena A, Dagur PK, Akue A, et al. Toll-like Receptor-9 (TLR-9) Signaling Is Crucial for Inducing Protective Immunity following Immunization with Genetically Modified Live Attenuated Leishmania Parasites. Pathogens. 2023;12(4):534. https://doi.org/10.3390/pathogens12040534
22. Melisi D, Frizziero M, Tamburrino A, Zanotto M, Carbone C, Piro G, et al. Toll-Like Receptor 9 Agonists for Cancer

Therapy. Biomedicines. 2014;2(3):211-228. https://doi.org/10.3390/biomedicines2030211
23. Bauer JA, Pavlović J, Bauerová-Hlinková V. Normal Mode Analysis as a Routine Part of a Structural Investigation. *Molecules*. 2019;24(18):3293. https://doi.org/10.3390/molecules24183293
24. Adhikari UK, Tayebi M, Rahman MM. Immunoinformatics Approach for Epitope-Based Peptide Vaccine Design and Active Site Prediction against Polyprotein of Emerging Oropouche Virus. *J Immunol Res*. 2018;2018:6718083. https://doi.org/10.1155/2018/6718083
25. Naz A, Awan FM, Obaid S, Rasool N, Parveen Z, Ahmed A, et al. Identification of putative vaccine candidates against *Helicobacter pylori* exploiting exoproteome and secretome: a reverse vaccinology based approach. Infect Genet Evol. 2015;32:280-291. https://doi.org/10.1016/j.meegid.2015.03.027
26. Basheer A, Jamal SB, Alzahrani B, Faheem M. Development of a tetravalent subunit vaccine against dengue virus through a vaccinomics approach. *Front Immunol*. 2023;14:1273838. https://doi.org/10.3389/fimmu.2023.1273838
27. Pappalardo F, Flower D, Russo G, Pennisi M, Motta S. Computational modelling approaches to vaccinology. *Pharmacol Res*. 2015;92:40-45. https://doi.org/10.1016/j.phrs.2014.08.006
28. Brown F, Hoey EM, Martin SJ, Rima BK, Trudgett A. Vaccine Design. Trends Biotechnol. 1994;12(6):251.
29. Kanwal M, Basheer A, Bilal M, Faheem M, Aziz T, Alamri AS, et al. In silico vaccine design for *Yersinia enterocolitica*: a comprehensive approach to enhanced immunogenicity, efficacy and protection. Int Immunopharmacol. 2024;143:113241. https://doi.org/10.1016/j.intimp.2024.113241
30. Bello AR, Nare B, Freedman D, Hardy L, Beverley SM. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A. 1994;91(24):11442-11446. https://doi.org/10.1073/pnas.91.24.11442
31. Boakye A, Gasu EN, Mensah JO, Borquaye LS. Computational studies on potential small molecule inhibitors of *Leishmania* pteridine reductase. J Biomol Struct Dyn. 2023;41(21):12128-12141. https://doi.org/10.1080/07391102.2023.2166119
32. Faria MS, Reis FCG, Lima APC. Toll-Like Receptors in Leishmania Infections: Guardians or Promoters? J Parasitol Res. 2012;2012:930257. https://doi.org/10.1155/2012/930257
33. Fu H, Liang Y, Zhong X, Pan Z, Huang L, Zhang H, et al. Codon optimization with deep learning to enhance protein expression. Sci Rep. 2020;10(1):17617. https://doi.org/10.1038/s41598-020-74091-z
34. Jalal K, Abu-Izneid T, Khan K, Abbas M, Hayat A, Bawazeer S, et al. Identification of vaccine and drug targets in *Shigella dysenteriae* sd197 using reverse vaccinology approach. Sci Rep. 2022;12:251. https://doi.org/10.1038/s41598-021-03988-0
35. Sacramento L, Trevelin SC, Silva Júnior DS, Costa DL, Almeida RP, Pereira C, et al. Toll-Like Receptor 9 signaling in dendritic cells regulates neutrophil recruitment to inflammatory foci following Leishmania infantum infection. Infect Immun. 2015;83(12):4604-4616. https://doi.org/10.1128/IAI.00975-15
36. Sasidharan S, Saudagar P. Leishmaniasis: Where Are We and Where Are We Heading? *Parasitology Res*. 2021;120(5):1541-1554. https://doi.org/10.1007/s00436-021-07139-2
37. Stacey KJ, Blackwell JM. Immunostimulatory DNA as an adjuvant in vaccination against Leishmania major. Infect Immun. 1999;67(8):3719-3726. https://doi.org/10.1128/IAI.67.8.3719-3726.1999
38. Tarrahimofrad H, Rahimnahal S, Zamani J, Jahangirian E, Aminzadeh S. Designing a multi-epitope vaccine to provoke the robust immune response against influenza A H7N9. *Sci Rep*. 2021;11(1):24485. https://doi.org/10.1038/s41598-021-03932-2
39. Yang Z, Bogdan P, Nazarian S. An in silico deep learning approach to multi-epitope vaccine design: a SARS-CoV-2 case study. Sci Rep. 2021;11(1):3238. https://doi.org/10.1038/s41598-021-81749-9
40. Fakruddin M, Mazumdar RM, Mannan KS, Chowdhury A, Hossain MN. Critical factors affecting the success of cloning, expression, and mass production of enzymes by recombinant *E. coli*. ISRN Biotechnol. 2013;2013:1-7. https://doi.org/10.5402/2013/590587
41. Liese J, Schleicher U, Bogdan C. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. Eur J Immunol. 2007 Dec;37(12):3424-3434. https://doi.org/10.1002/eji.200737182
42. Kaye PM, Matlashewski G, Mohan S, Le Rutte E, Mondal D, Khamesipour A, et al. Vaccine value profile for leishmaniasis. Vaccine. 2023;41(S2):S153-S175. https://doi.org/10.1016/j.vaccine.2023.01.057
43. Shilling PJ, Mirzadeh K, Cumming AJ, Widesheim M, Köck Z, Daley DO. Improved designs for pET expression plasmids increase protein production yield in *Escherichia coli*. *Commun Biol*. 2020;3:214. https://doi.org/10.1038/s42003-020-0939-8
44. Park SW, Lee BH, Song SH, Kim MK. Revisiting the Ramachandran plot based on statistical analysis of static and dynamic characteristics of protein structures. J Struct Biol. 2023 Mar;215(1):107939. https://doi.org/10.1016/j.jsb.2023.107939
45. Kumar P, Kumar P, Shrivastava A, Dar MA, Lokhande KB, Singh N, et al. Immunoinformatics-based multi-epitope containing fused polypeptide vaccine design against visceral leishmaniasis with high immunogenicity and TLR binding. *Int J Biol Macromol*. 2023 Dec;253(Pt 8):127567. https://doi.org/10.1016/j.ijbiomac.2023.127567
46. Naz S, Aroosh A, Caner Ş, Atalay EŞ, Toz S, Ozbel Y, et al. Immunoinformatics approach to design a multi-epitope vaccine against cutaneous leishmaniasis. *Vaccines*. 2023;11(2):304. https://doi.org/10.3390/vaccines11020304
47. Begum M, Khurshid R, Saleem A, Nagra SA. MUC1 based breast cancer vaccines: role of post translational modifications. J Ayub Med Coll Abbottabad. 2008 Oct-Dec;20(4):130-133
48. eBioMedicine, "Leishmania: An Urgent Need for New Treatments," eBioMedicine, 2023.
49. Ramprasadh SV, Rajakumar S, Srinivasan S, Susha D, Sharma S, Chourasiya R. Computer-Aided Multi-Epitope Based Vaccine Design Against Monkeypox Virus Surface Protein A30L: An Immunoinformatics Approach. Protein J. 2023 Dec;42(6):645-663. https://doi.org/10.1007/s10930-023-10150-4
50. Srivastava S, Saudagar P, Bhowmick S, Khadem S, Jha BA, Saha B, et al. Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin Exp Immunol. 2013;172(3):403-409. https://doi.org/10.1111/cei.12084
51. Dhanushkumar T, Santhosh ME, Prasanna K Selvam, Rambabu M, Dasegowda KR, Vasudevan K, et al. Advancements and hurdles in the development of a vaccine for triple-negative breast cancer: a comprehensive review of multi-omics and immunomics strategies. Life Sci. 2024 Jan 15;337:122360. https://doi.org/10.1016/j.lfs.2023.122360
52. Mandal S, Khushi, Waribam PC, Srivastava R, Patgiri SJ, Natarajaseenivasan K. Development of a multi-epitope vaccine candidate targeting blood-stage of malaria through immunoinformatics approach. Hum Immunol. 2025;86(4):111346. https://doi.org/10.1016/j.humimm.2025.111346