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In Silico Epitope Mapping and Structural Modelling of Tp0751 for Multi-Epitope Vaccine Design Against Treponema pallidum

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Abstract

Syphilis is a serious and life threatening sexually transmitted infectious disease caused by treponema pallidum. It is a gram-negative spiral-shaped bacterium that can affect almost any organ and spread to the whole body. This infection can pass from one to another through sexual contact or from mother to child during pregnancy. There is an urgent need for an effective vaccine against this infection to provide long-lasting protection as it has been ranked the second deadliest sexually transmitted disease after AIDS. This study involves vaccine designing using the predicted T cell and B cell epitopes. Out of the several membrane protein Tp07510 (pdb id: 5JK2) was selected as it is a surface exposed protein which is highly accessible to immune system, therefore they can be good vaccine candidate. It also plays role immune envasion and pathogen host interaction. It consists of several chains (A, B, C, D, E, F, I) among with chain A was selected because it showed immunogenicity and potentially triggered T cell responses, making it a strong candidate for B cell and T cell epitope mapping. Antigenicity, allergenicity, toxicity, solubility was checked of the selected epitopes. The final vaccine candidate sequence was checked for several parametres on protparam. The vaccine candidate was docked with the Toll Like Receptor 2,3,4 which showed high binding affinity which can as a potential candidate.

Keywords: *Treponema Pallidum, Syphilis, Immunoinformatic, Epitopes, Vaccine, Tp0751, Immune Simulation.*

1. INTRODUCTION

Treponema is a spiral shaped bacteria that lives in a low oxygen environment which belongs to spirochaetaceae family. Species including T. pallidum, T. endemicum and T. pertenue are responsible for diseases like syphilis, endemic

syphilis, and yaws. Only three stains of T. pallidum have been fully sequenced. These bacteria spread through human contact only. Syphilis is the most deadly STD after AIDS which can cause damage to multiple organs **(Khan, 2023)**. The WHO reported 12 million new syphilis cases, mostly in developing countries, where innate syphilis led to many miscarriages and infant deaths in 1999. Even though T. pallidum is responding to antibiotics, syphilis is remerging in recent years .

Syphilis and AIDS have a similar mode of transmission. Syphilis can persist for many years if left untreated. It progresses through different stages: early stage (includes primary, secondary and early latent syphilis) and late stage (includes latent syphilis and tertiary forms). Syphilis has reemerged in several developed countries even with the availability of advanced diagnostic tools and effective treatment. Widespread outbreaks occurred in Russia and China, mainly affecting heterosexual populations during 1990s. smaller outbreaks were reported among homosexual men in many countries. A rise in syphilis cases among pregnant women has been reported more recently in the U.S. Globally, there are about 2million new syphilis cases reported annually.

Syphilis in its early stages (primary and secondary) significantly increases the risk of HIV transmission, making it a key factor in the spread of HIV and contributing to the rising number of syphilis cases. Research from the 1960s and 1970s showed that erythromycin was not effective in treating syphilis during pregnancy, likely because it doesn't cross the placenta well. Despite being used for over 70 years, penicillin is still very effective against Treponema pallidum, the bacteria that causes syphilis. Azithromycin, an oral antibiotic, has become more widely used due to challenges with other treatments, but resistance to this drug has been reported in many parts of the world. Recent reviews indicate a growing number of syphilis cases in developed countries, highlighting the urgent need for improved diagnostic methods and the development of an effective vaccine.

Around 20 outer membrane protein(OMP) antigens have of T. pallidum have been identified so far. Among which Gpd antigen, Tp92 antigen, and Tpr family antigens have been extensively studied for their cellular locations, structures, functions, and gene conservation. These OMPs are believed to stimulate animals to produce opsonic antibodies, which help the immune system recognize and eliminate T. pallidum through phagocytosis. Tp92 antigen stands out as a promising vaccine candidate due to its strong similarity across different strains, good ability to stimulate the immune system, and potential for protection. The Tp92 gene from the Nichols strain shows 95.5–100% sequence similarity with other Treponema species, making it a strong option for creating vaccines that could offer broad protection against the Nichols strain and other T. pallidum variants.

Introducing a new drug to the market still typically takes around 10-15 years and requires substantial financial investment, even though there are major advancements in high throughput screening and synthetic chemistry speeding up drug development. In silico approaches have been increasingly used to study bacterial pathogens, leading to the discovery of many drug-resistant targets and cases where effective vaccines are lacking recently. Reverse vaccinology has been widely used and efficient strategy for rapidly identifying new vaccine candidates, in the post genomic era. This study applies reverse vaccinology along with subtractive genomics to identify vaccine candidate and potential therapeutic targets for treating syphilis.

2. METHODS AND MATERIALS

2.1. Target protein sequence extraction

The amino acid sequence of the protein Pallilysin from Treponema pallidum (Nichols strain) was retrieved from the NCBI database. Treponema pallidum outer membrane protein was searched and the protein Tp07510 (pdb id: 5JK2) was selected. Its FASTA sequence was downloaded. BLAST was performed for the sequence to check the presence of the non-homologous sequence in humans. It turned out to be non-homologous which means it can be an ideal vaccine candidate. Further its properties were analysed using Protparam tool which proved that the protein was stable.

2.2. Epitope prediction

- **B cell epitope prediction:** B-cell epitopes are regions of a protein that stimulate antibody production. We will predict linear B-cell epitopes using IEDB (Immune Epitope Database). To predict B-cell epitopes, the selected protein's FASTA

sequence is submitted to the IEDB Analysis Resource **(https://www.iedb.org/)**, using the B-cell epitope prediction tool. The Bepipred Linear Epitope Prediction method is then used to analyze the sequence. After running the tool, regions with scores equal to or above the threshold of 0.5 are identified as potential linear B-cell epitopes. These high-scoring epitope sequences are then saved for further analysis, such as immunogenicity assessment, structural mapping, or vaccine design.

- **T cell epitope prediction:** T-cell epitopes bind to MHC (Major Histocompatibility Complex) molecules, activating the immune response. MHC1 and MHC11 epitopes were predicted using IEDB. To identify potential T-cell epitopes, the FASTA sequence of the target protein is uploaded to the IEDB**(https://www.iedb.org/)** MHC-I and MHC-II prediction tools. Common HLA alleles are selected to ensure broad population coverage. The prediction is then run, and epitopes with strong binding affinity—indicated by IC50 values less than 500 nM—are identified as strong binders. These top-scoring epitopes are saved for further analysis, such as immunogenicity testing, population coverage studies, or incorporation into multi-epitope vaccine constructs.

2.3. Evaluation of Predicted T and B cells Epitopes for Antigenicity,

Allergenicity, and solubility and toxicity: The selected epitopes should be highly antigenic and non-allergenic to be considered good vaccine candidates.to assess the antigenicity of predicted epitopes, the sequences are submitted to the VaxiJen server **(http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html)**. By selecting the bacterial model, the tool evaluates the epitope's potential to act as an antigen based on its physicochemical properties. A VaxiJen score greater than 0.4 is considered indicative of strong antigenicity, suggesting that the epitope is likely to elicit an immune response. High-scoring epitopes are selected for further validation and potential inclusion in vaccine design. To evaluate the allergenicity of the selected epitopes, their sequences are uploaded to the AllerTOP server **(https://www.ddg-pharmfac.net/AllerTOP/)**. This tool predicts whether an epitope is likely to cause an allergic reaction based on its physicochemical properties and known allergen data. Epitopes classified as non-allergenic are preferred, as they are safer and more suitable for vaccine development. Only those predicted to be non-allergenic are selected for further immunological and structural analysis.

The solubility of the selected epitope sequences was analyzed using PepCalc **(https://pepcalc.com)**. Each epitope sequence was entered into the tool in the one-letter amino acid format, and the "Calculate Peptide Properties" option was selected. The tool evaluated various properties, including net charge and hydrophobicity, and provided a solubility prediction. Peptides classified as "likely soluble" were considered suitable, while those predicted to be insoluble were excluded from further analysis. The solubility data was recorded for each epitope to assist in selecting the most promising vaccine candidates. The toxicity of the selected epitope sequences was analyzed using the ToxinPred server **(https://webs.iiitd.edu.in/raghava/toxinpred/)**. The "Designing and Prediction of Toxic Peptides" module was used, where each epitope sequence was entered in one-letter amino acid format. The SVM (Swiss-Prot based) method was selected for toxicity prediction, and the analysis was performed using the "Only prediction of toxicity" option. After submission, the server classified each epitope as either toxic or non-toxic based on its physicochemical properties. Epitopes predicted to be non-toxic were shortlisted for further vaccine development, while toxic peptides were excluded from the study.

2.4. Population Coverage Analysis

The global distribution of HLA alleles was considered to assess the potential reach of the predicted T-cell epitopes. Population coverage analysis was carried out using the IEDB Population Coverage Tool **(http://tools.iedb.org/population/)**. All shortlisted epitopes along with their corresponding HLA alleles were submitted to the server. The tool estimates the percentage of individuals within different ethnic groups that

would be able to present at least one of the predicted epitopes, based on documented HLA allele frequencies. The output included three key parameters: (i) population coverage (%), representing the fraction of individuals predicted to respond; (ii) average hit, the mean number of epitope–HLA combinations recognized per individual; and (iii) PC90, the minimum number of epitope–HLA combinations recognized by 90% of the population. Coverage was analyzed separately for MHC class I and MHC class II epitopes, as well as in a combined analysis.

2.5. Final vaccine candidate (final vaccine sequence).

Epitopes that satisfied all predefined criteria were concatenated using linker peptides to create the final vaccine construct. These linkers played a crucial role, as they not only enabled the structural assembly of the vaccine as an independent immunogen but also enhanced the production of higher antibody titers compared to isolated epitopes. Furthermore, the use of specialized peptide linkers facilitated the selection of epitopes for fusion applications. For instance, the GPGPG linker sequence was shown to effectively inhibit the formation of junctional epitopes and optimize the antigen presentation pathway, a critical process for engaging helper T lymphocyte (HTL) epitopes **(Alexander et al., 1998)**. To augment immune stimulation, an appropriate adjuvant was conjugated with EAAAK at the N-terminal of the vaccine. This integrative strategy ensured a robust and precisely targeted immune response.

2.6. Vaccine properties

The vaccine sequence was analyzed for allergenicity and antigenicity using the AllerTOP and VaxiJen v 2.0 servers, respectively. Subsequently, the ProtParam tool was utilized to predict the physicochemical properties of the sequence. Parameters such as molecular weight, theoretical isoelectric point (pI), total number of negatively and positively charged residues, instability index, aliphatic index, extinction coefficient, and the grand average of hydropathicity (GRAVY) were assessed to inform and support the experimental study.

2.7. Secondary and tertiary structure analysis

To determine the proportions of secondary structures in the final designed vaccine, the PSIPRED v3.3 and SOPMA servers were used. The amino acid sequence of the vaccine construct was submitted to the trRosetta server to predict its three-dimensional structure. The predicted tertiary structure was subsequently refined using the GalaxyRefine server to improve its overall quality and stability. The structural integrity of the refined model was then evaluated using the PDBsum server, with particular emphasis on the

Ramachandran plot. This diagram provided a graphical assessment of the model by analyzing the distribution of residues across different conformational zones: outliers, allowed, and favored. These zones corresponded to various backbone dihedral angles (phi Φ and psi ψ), with residues in the fevered region indicating the most stable and commonly observed conformations. Finally, Discovery Studio was employed to visualize the three-dimensional structure of the vaccine construct.

2.8. Immune simulations

To advance our comprehension of the immune response elicited by the engineered vaccine, we utilized the C-IMMSIM server, a distinguished bioinformatics platform known for its proficiency in simulating and assessing the vaccine's potential to activate a wide spectrum of immune cells. This analysis included B cells, T cells, macrophages, natural killer (NK) cells, dendritic cells, as well as the production of cytokines, which are critical mediators in immune responses. In our in-silico simulations, we administered three distinct doses of the vaccine at four-week intervals. All the parameters, including simulation volume, HLA molecules, random seed, vaccine injections, and adjuvants, were maintained at their default settings. The objective of conducting the simulation under these specified conditions was to obtain an unaltered representation of the immune response to the multi-epitope vaccine.

2.9. Molecular docking

Molecular docking has become an advanced computational method for assessing the complex

interactions between proteins and their corresponding receptors. Within the framework of inflammatory responses induced by the bacterium Treponema pallidum, Toll-like receptors TLR4, TLR2, and TLR3 have been identified as playing crucial roles. The structures of TLR4 (PDB ID: 4G8A), TLR3 (PDB ID: 1ZIW), and TLR2(PDB ID: 6NIG) were retrieved from the Protein Data Bank in PDB format. The HDock server, which specializes in High Ambiguity Driven protein–protein Docking, was employed for docking studies between the vaccine and the receptor. The HDock server was employed to calculate the binding free energy of vaccine–TLR complexes using the molecular mechanics/generalized Born surface area (MM-GBSA) method. This combined computational approach improved the accuracy and reliability of the analysis, allowing for a deeper understanding of the molecular interactions within the complexes. The binding affinity, was estimated through MM-GBSA to evaluate the strength of the vaccine–TLR interactions. To further explore the specific residue interactions within the complexes, the PDBsum server was used. Additionally, the three-dimensional structures of the vaccine–TLR complexes were visualized using discovery studio software.

2.10. Molecular dynamics simulations:

Molecular dynamics (MD) simulation is a computational approach used to study the behaviour of molecular systems over time at the atomic level. It enables the exploration of intricate atomic and molecular interactions and movements. In this study, MD simulations were conducted to evaluate the dynamic behavior of atoms involved in the interactions and to assess the binding affinity of the vaccine–TLR complexes. The simulations were carried out using iMODS server this server evaluate the structural stability and flexibility of the protein-ligand complex

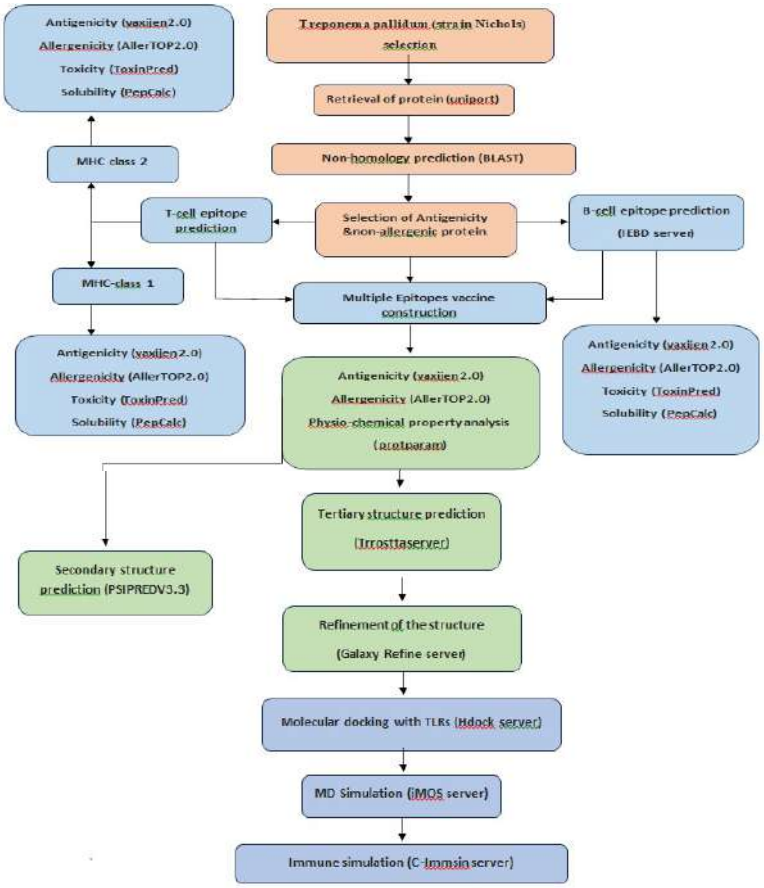

Fig-1: The flowchart represents the overall methodology for developing a multi-epitope subunit vaccine construct.

ISSN: 2583-7354

Table-1: Physicochemical Properties of the Selected Protein (TP0751)

No	Property	Value
1	Number of Amino Acids	167
2	Molecular Weight	18,130.39 Da
3	Theoretical pI	8.50
4	Extinction Coefficient ($M^{-1}cm^{-1}$)	19,480
5	Absorbance at 0.1% (1 g/L)	1.074
6	Estimated Half-life	30 hrs (mammalian, in vitro); >20 hrs (yeast); >10 hrs (E. coli)
7	Instability Index (II)	49.30 (Classified as unstable)
8	Aliphatic Index	80.18
9	GRAVY (Grand Average of Hydropathicity)	-0.231 (Hydrophilic)
10	Total Atoms	2,531
11	Molecular Formula	$C_{800}H_{1250}N_{242}O_{236}S_3$
12	Positively Charged Residues (Arg + Lys)	14
13	Negatively Charged Residues (Asp + Glu)	13

3. RESULTS

The anticipated characteristics and properties of Tp0751 protein are detailed in Table 1. These characteristics provide advantage for constructing multi-epitope vaccines, making this protein particularly favorable for vaccine expression.

Further, the B cell and T cell epitope are selected from the protein sequence.

The B cell epitope of these protein was predicted using the IEDB server. The Antigenicity, Allergenicity, solubility and toxicity were evaluated. In the context of vaccine development, the B cell epitope should be non-allergenic, high antigenicity, non-toxic properties and good water soluble. A detailed summary of the results is given in Table 2, where three B cell epitope were selected.

3.1. Epitope prediction and screening

Table-2: The linear B cell epitope of T. pallidum (Tp0751) protein.

no	start	end	peptide	Antigenicity	Solubility	Toxicity	Allergenicity	length
1	5	27	SHGNAPPAPV GGAAQTHTQ PPVQ	yes	Good water solubility.	non-toxin	NON-ALLERGEN	23
2	56	66	QTEISPNSGDI	yes	Good water solubility.	non-toxin	NON-ALLERGEN	11
3	89	99	IFLVDSAHTH R	yes	Good water solubility.	non-toxin	NON-ALLERGEN	11

3.2. Identification of T cell epitopes

The T cell epitope (MHC class 1 and MHC class 2) of Tp0751 protein utilizing the IEDB MHC I and MHC 2 binding servers. These predicted epitopes were subsequently evaluated for the antigenicity, allergenicity, solubility and toxicity analysis to exclude those not meeting the established criteria. After that the selection of five MHC class 1 and seventeen MHC class 2 epitope was identified for vaccine development, as in Table 3,4.

Table-3: The MHC class 1 epitope of Tp0751 protein.

Peptide	Antigenicity	Solubility	Toxicity	Allergenicity	Rank score
EHAEITFSR	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.1
HTHRKTVSF	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.03
LVDSAHTHRK	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.37
SAHTHRKTV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.2
SSIRRRLEV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.25

Table-4: The MHC class 2 epitope of Tp0751 protein.

Peptide	Antigenicity	Solubility	Toxicity	Allergenicity	Rank score
DGQYTRYHAGPASAP	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.03
AVIHVRAVEDVARLK	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.87
DGQYTRYHAGPASAP	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.03
FESHAVIHVRAVEDV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	4.3
IRRRLEVTFESHAVI	yes	Good water solubility.	non-toxin	NON-ALLERGEN	5.1
ISSIRRRLEVTFESH	yes	Good water solubility.	non-toxin	NON-ALLERGEN	3.5
NTAISSIRRRLEVTF	yes	Good water solubility.	non-toxin	NON-ALLERGEN	3.1
RIALWNRATHGEQGA	yes	Good water solubility.	non-toxin	NON-ALLERGEN	4.4
RLEVTFESHAVIHVR	yes	Good water solubility.	non-toxin	NON-ALLERGEN	1.7
RNTAISSIRRRLEVT	yes	Good water solubility.	non-toxin	NON-ALLERGEN	2.8
RRLEVTFESHAVIHV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	1.3
RRRLEVTFESHAVIH	yes	Good water solubility.	non-toxin	NON-ALLERGEN	1.6
SIRRRLEVTFESHAV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	1.6
SSIRRRLEVTFESHA	yes	Good water solubility.	non-toxin	NON-ALLERGEN	1.7
TAMRIALWNRATHGE	yes	Good water solubility.	non-toxin	NON-ALLERGEN	3.4
TRNTAISSIRRRLEV	yes	Good water solubility.	non-toxin	NON-ALLERGEN	5.4
VQEIFLVDSAHTHRK	yes	Good water solubility.	non-toxin	NON-ALLERGEN	0.18

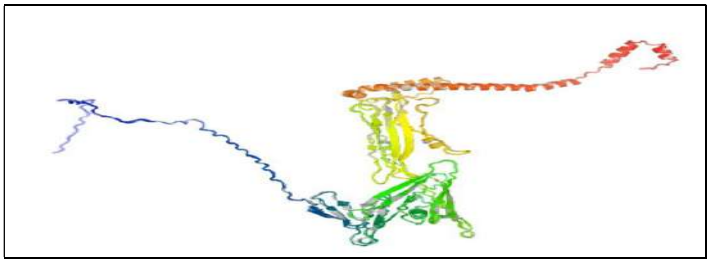

Fig-2: Predicted 3D structure of T. pallidum vaccine constructs, visualized by discovery studio.

3.3. Population Coverage Analysis

The population coverage analysis demonstrated that the selected epitopes provide broad global representation. For MHC class I, the epitopes showed 98.55% worldwide coverage, with an average hit of 10.85 and a PC90 value of 5.76 (Figure X). For MHC class II, the coverage was slightly lower at 81.81%, with an average hit of 6.71 and PC90 of 2.20 (Figure Y). When epitopes from both classes were combined, the global coverage increased markedly to 99.74%, with an average hit of 17.56 and a PC90 value of 10.06 (Figure Z). These results indicate that the designed multi-epitope construct has the potential to provide nearly universal population coverage, supporting its suitability as a broad-spectrum vaccine candidate.

ISSN: 2583-7354

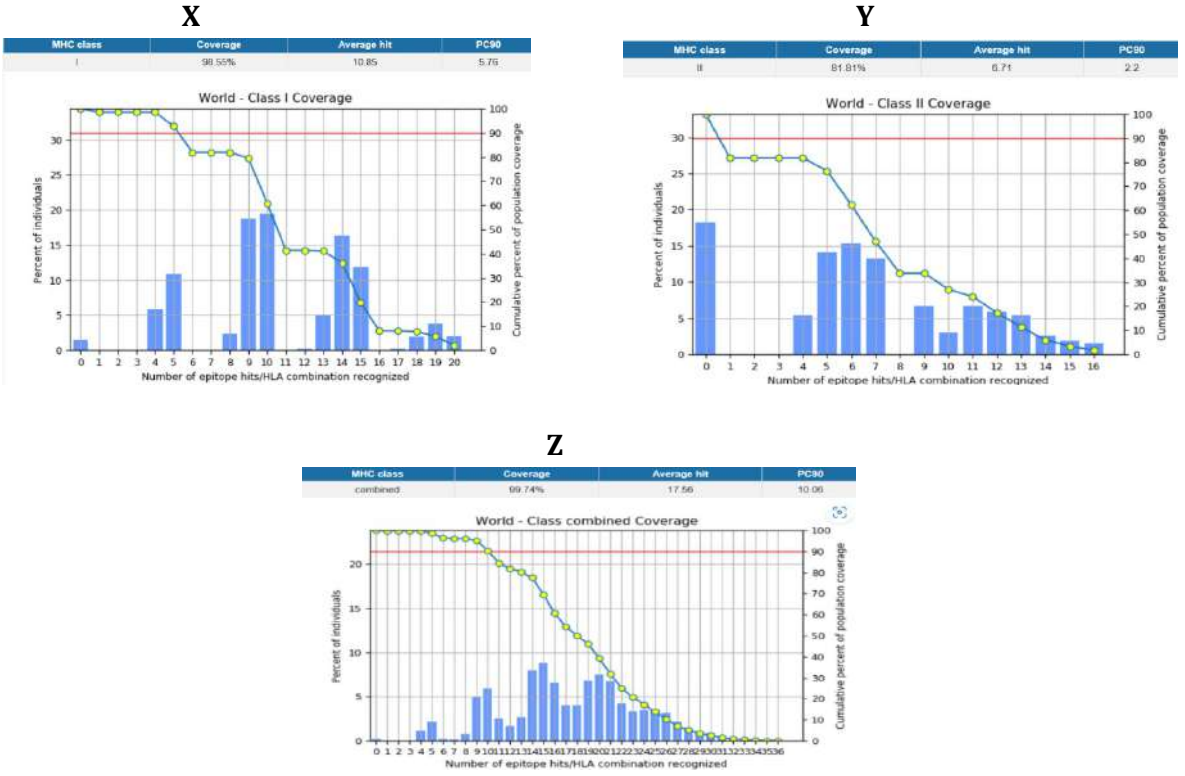

Fig-3: Figure X. Global population coverage of predicted MHC class I epitopes (98.55%). Figure Y. Global population coverage of predicted MHC class II epitopes (81.81%). Figure Z. Combined MHC class I and II population coverage (99.74%).

3.4. Final Constructed vaccine Antigenicity / Allergenicity / Solubility / Toxicity Validation:

In silico validation of the designed multi-epitope vaccine construct. The antigenicity score (0.9226) indicates that the construct is a probable antigen. Allergenicity analysis classified the construct as a non-allergen, showing similarity with ARID1A from Homo sapiens. The vaccine also demonstrated good water solubility and was predicted to be non-toxic. These properties collectively suggest that the vaccine candidate is safe, soluble, and capable of eliciting an immune response.

3.5. Construction of epitope-based vaccine

Multi-epitope vaccine construction. The ultimate vaccine design comprised epitopes, specifically the L7/L12 ribosomal protein used as a adjuvant for construction because The L7/L12 ribosomal protein was used as an adjuvant due to its strong immunogenicity, ability to activate TLR4, and enhancement of antigen presentation through increased MHC expression and cytokine production. Its non-toxic and stable nature made it suitable. The L7/L12 ribosomal protein as adjuvant at the N-terminal to enhance the immunogenicity.

An EAAAK linker was used to separate the adjuvant from the epitope region, ensuring structural stability. Multiple T cell and B cell epitopes were incorporated and linked using GPGPG linker to facilitate proper antigen processing. After that using the protparam server the property of constructed vaccine were studied Table 5.

Table-5: Constructed vaccine physio-chemical property.

N0	Parameter	Value
1	Number of Amino Acids	615
2	Molecular Weight	63,428.21 Da
3	Theoretical pI	9.80

4	Total Atoms	8,907
5	Molecular Formula	$C_{2778}H_{4423}N_{873}O_{830}S_3$
6	Extinction Coefficient (280 nm)	16,960 $M^{-1}cm^{-1}$
7	Absorbance (0.1% solution)	0.267
8	Instability Index (II)	36.21 (Stable)
9	Aliphatic Index	70.16
10	GRAVY (Hydropathicity)	-0.439 (Hydrophilic)

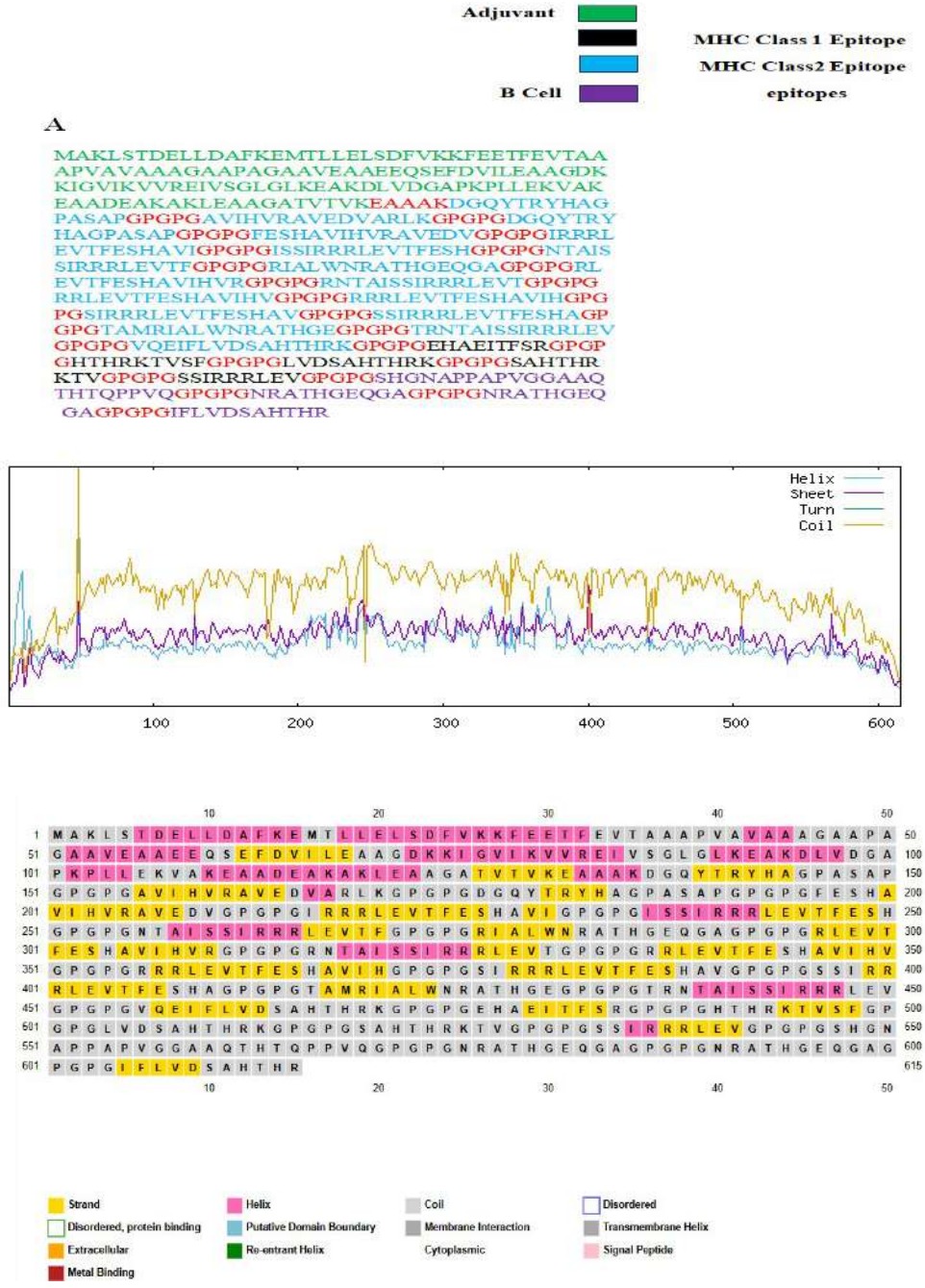

Fig-4: The secondary model of the designed vaccine. (A) the amino acid composition of the vaccines. (B) The secondary structure prediction results of SOPMA server. (c) The secondary structure prediction result of PSIPREDV3.3 server.

3.6. Secondary structure prediction

The secondary structural characteristics of the constructed vaccine, as analyzed by the SOPMA server and PSIPREDV server, are presented in figure 3. The sequence comprises 2.60% alpha helix, 0.81%extended strand, beta turns and 50%random coils.

3.7. Tertiary structure prediction

Following the prediction by the trRosetta server, the tertiary structure model was submitted to the galaxy refine server for structural refinement. The tertiary structure was visualized using discovery studio. The galaxy refines model Figure 4, underwent validation using the pdbsum server and ERRAT server. The analysis conducted using the ERRAT server score is 85.78.

3.8. Immune simulations

The Immsin server was used for immune simulations. the simulation showed that the immune system mounted an effective and well-organized response following antigen exposure. B cell were quickly activated, resulting in the formation of memory B cell and strong production of IgG1 antibodies, which help in the long-term immunity. Plasma B cell peaked around day 10, while TH1 cells dominated the T cell response. the immune activity over time, indicating proper regulation and long-term immunity Figure 5(A).

The simulation showed that cytotoxic T cell maintained a fairly stable population, with the active and duplicating cells decreasing over time while anergic cell increased. NK cells fluctuated slightly but remained with a stable rang. Macrophages become more active and engaged in antigen processing and presentation. Dendritic cells were initially active in antigen presentation and then stabilized. epithelial cells remained constant with minimal change Figure 5(B)

In the graph the antigen and antibody response over 35 days post infection. antigen levels increased very quickly around in 3 days. IgM appeared first, peaking by day 10, followed by a gradual increase in IgG which persisted longer. The IgM+IgG+IgG1+IgG2 Immunocomplex level this indicating an effective and sustained immune response Figure 5(C).

IL-2 and IFN-α increased the most, with IL-2 rising quickly around day 5 and then dropping sharply. Others like IL-6, IL-10, and IL-23 rose more slowly and stayed at lower levels. IL-4 and TGF-β remained low throughout. The inset graph highlighted the strong and early response of IL-2 and IFN-α Figure 5(D).

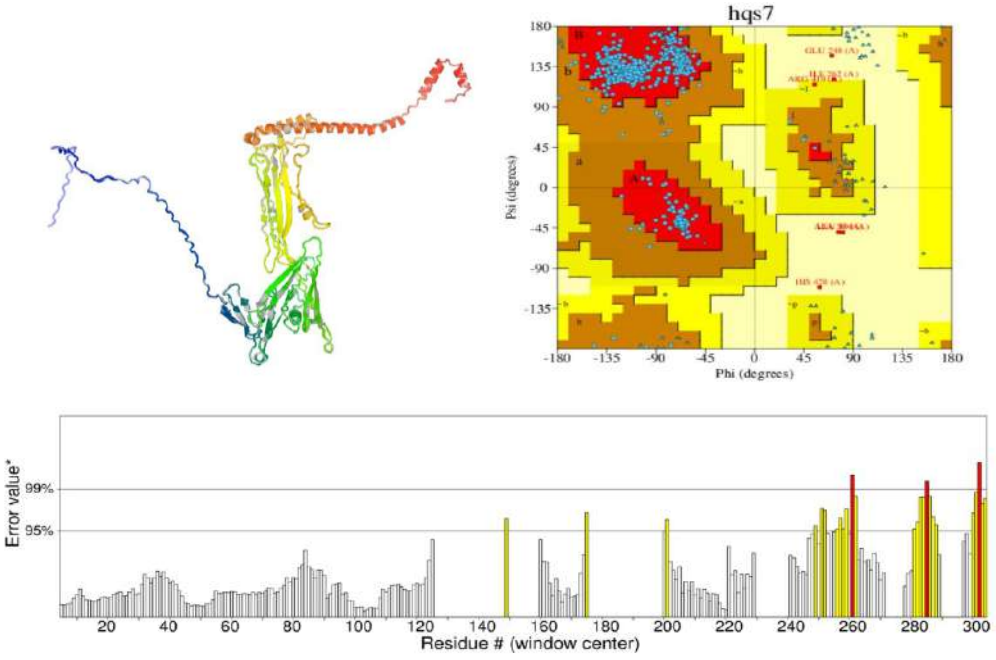

Fig-5: The prediction and refinement of the tertiary structure model. The 3D structure, pdbsum server Ramachandran plot, ERRAT overall quality of the trRosetta model.

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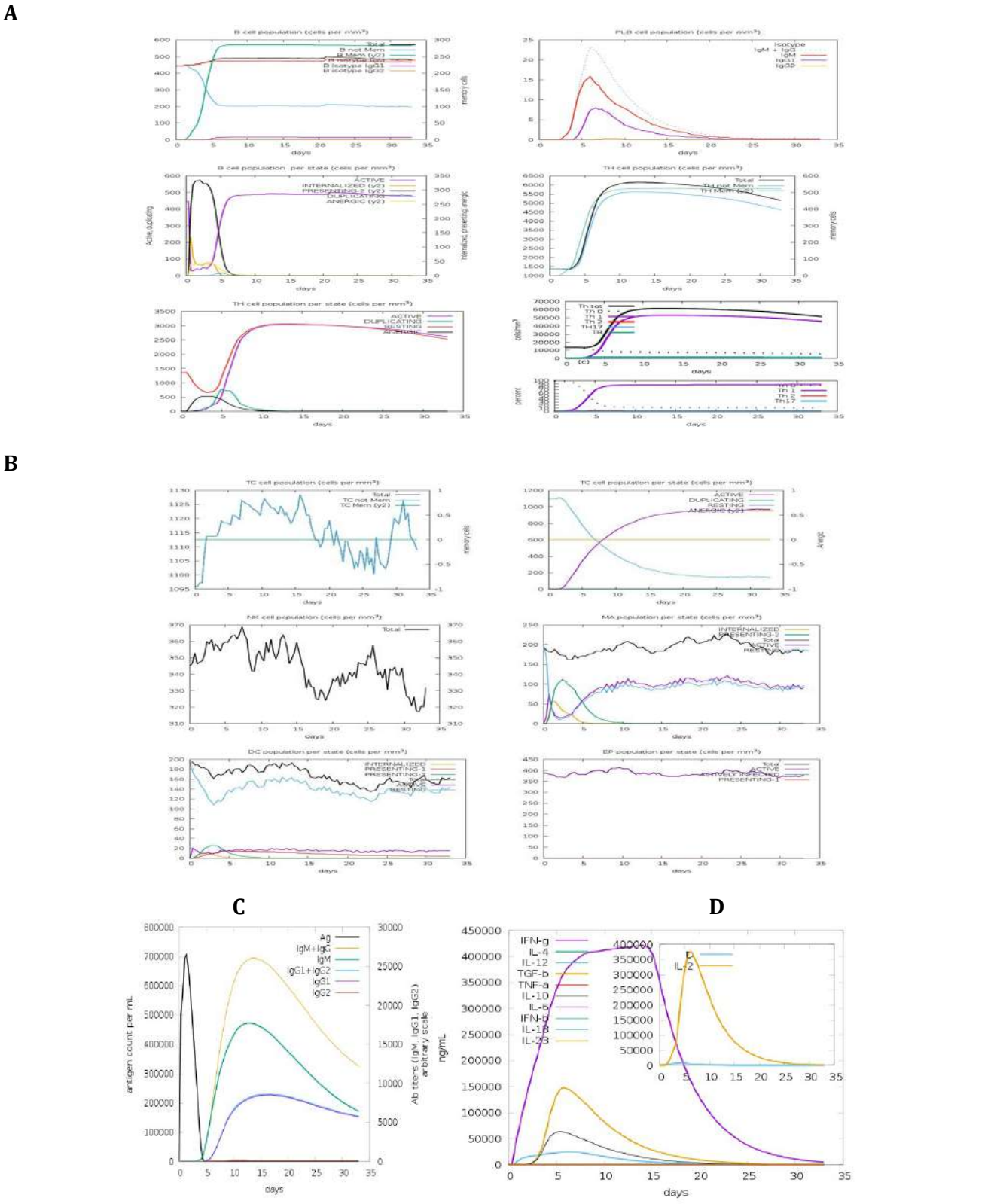
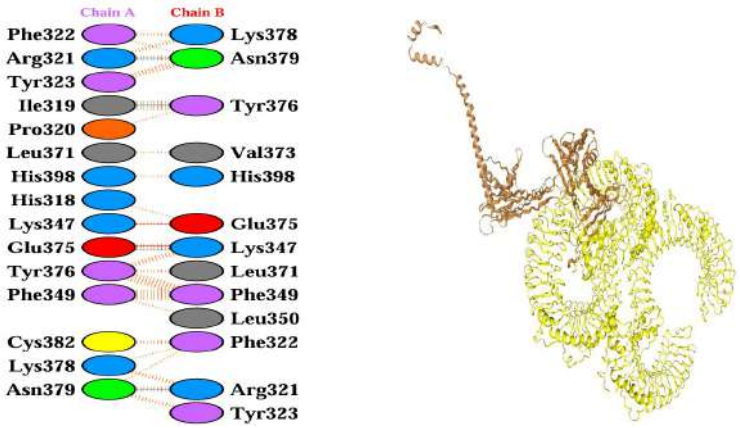


Fig-6: Simulated immune responses generated in response to the designed vaccine. (A) (i)–(iii) B cell responses, (iv)–(viii) T-cell responses. (B) (i) Natural Killer cells responses, (ii) Dendritic cells responses, (iii) Macrophages responses, (iv) Epithelial cells responses. (C) Immunoglobulin responses with respect to the vaccine exposure. (D) Cytokine responses.

3.9. Molecular docking

TLR2, TLR3 and TLR4 were chosen as the immune receptors due to their critical role in the immune respone against T. pallidum. molecular docking analyses were performed between the multi-epitope vaccine and the immune receptors TLR2 (PDB ID: 6NIG), TLR3(PDB ID: 1ZIW), TLR4(PDB ID: 4G8A). the Hdock server are used for molecular docking and binding free energy calculation. The Hdock scores for the vaccine -TLR complexs were -285.69, -324.10, -299.50 Table 6.

TLR2



TLR4

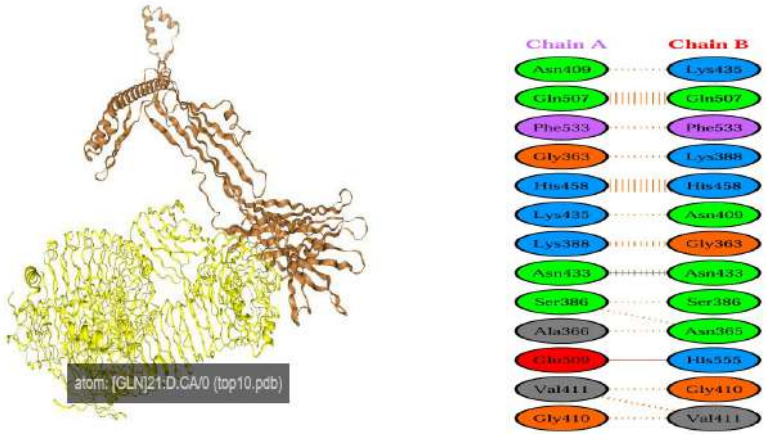


Fig-7: The molecular docking structure in the HDock server and interaction in the PDBsum server. (A) The 3D structure and interaction of vaccine-TLR 2 complex; (B) The 3D structure and interaction of vaccine-TLR 4 complex.

Table-6: Molecular docking between vaccine and TLR.

NO.	TLR-2	TLR-3	TLR-4
Docking score	-285.69	-324.10	-299.50
Confidence score	0.9378	0.9702	0.9521
Ligand rmsd	306.31	119.57	114.18

3.10. Molecular dynamics simulations

Molecular dynamics (MD) simulation offers a detailed, temporal insight into system dynamics over a broad range, enabling a comparative assessment of the conformational integrity of the three vaccine-TLR complexes. The iMODS server

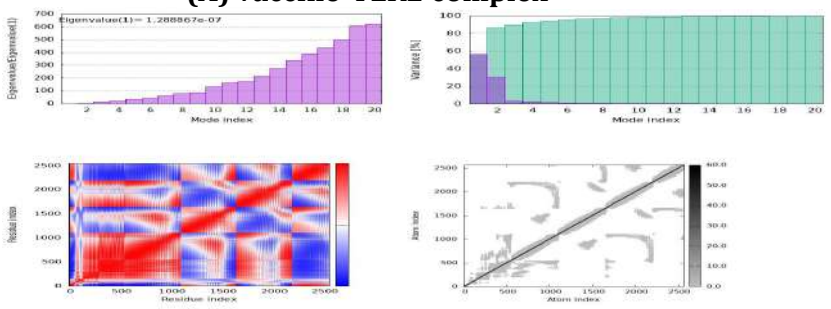
used to evaluate the structural stability and flexibility of the protein-ligand complex. Parameter such as deformability, evgenvalues, B-facter, and convariance maps were analysed which helps in to confirm the stability and potential efficacy of the design vaccine candidate. Table 7 show the vaccine-TLR complex different feature among them and it also show the Overall Stability.

Table-7: Molecular dynamics simulations between vaccine-TLR complex.

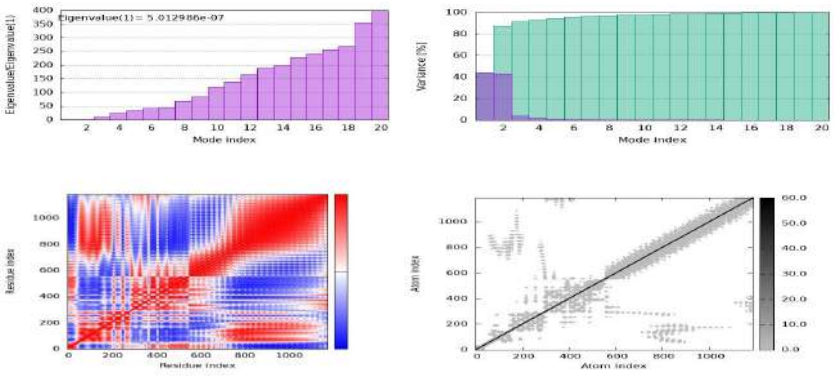
Feature	**Vaccine-TLR2 complex**	**Vaccine-TLR3 complex**	**Vaccine-TLR4 complex**
Deformability	High peaks at specific regions indicating flexible hinge areas	Peaks showing hinge points; moderate flexibility	Peaks mostly at terminal regions; flexible hinge points
B-factor Match (NMA vs PDB)	Good match between predicted and experimental values	Good match between NMA and PDB values	Close match with experimental B-factors
First Eigenvalue	1.288867e-07 Lowest (most flexible, least stable)	5.012986e-07 Highest (least flexible, most stable)	1.511050e-07 Moderate (balanced flexibility & stability)
Variance Contribution	First few modes explain majority of motion	First few modes dominate the motion	Initial modes contribute most to motion
Covariance Map	Clear red/blue zones: correlated and anticorrelated motion	Strong red/blue patterns indicating communication	Coordinated structural motions with clear red/blue patterns
Elastic Network	Darker gray spots show stiffer connections in some regions	Denser dark gray areas indicating stronger/stiffer structure	Mix of stiff and flexible connections
Overall Flexibility	High	Low	Moderate
Overall Stability	Low	High	Moderate

It was analysed that the first structure was highly flexible that included various region that that behave like hinges. the predicted and experimented B factors matched having the lowest eigenvalue. That meant it was most flexible but the least stable one. Few modes explained the motion, and clear patterns of movement was shown by the covariance map. The elastic network showed stiffness, but the structure showed flexibility but less stability. The second structure was the most stable but least flexible, with strong connections and a higher eigenvalue. The third structure had a mix of stiff and flexible parts, showing moderate flexibility and stability.

(A) vaccine-TLR2 complex



(B) vaccine-TLR3 complex



(C) vaccine-TLR4 complex

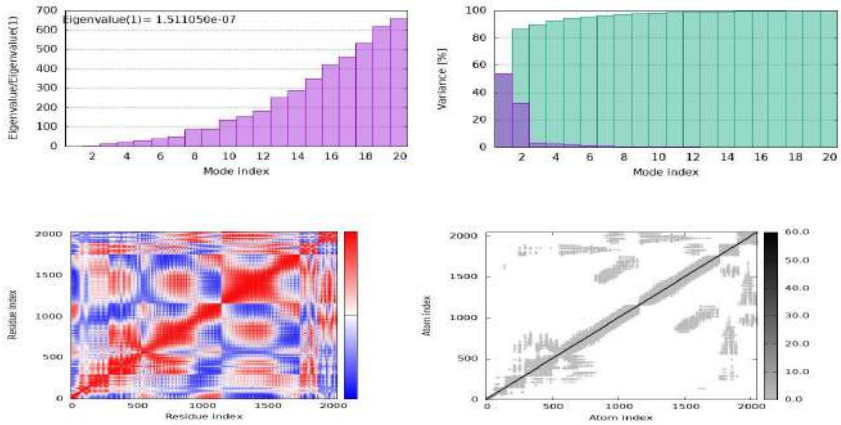


Fig-8: Molecular dynamics simulation of the vaccine-TLR complex in the iMODS server. (A) MD simulation of the vaccine-TLR2. (B) MD simulation of the vaccine-TLR3. (c) MD simulation of the vaccine-TLR4.

4. COMPARATIVE ANALYSIS WITH PREVIOUS STUDIES:

The findings of the present study are consistent with and extend previous efforts aimed at vaccine development against Treponema pallidum. Cameron et al. (2000) highlighted Tp92 as a promising candidate due to its sequence conservation and ability to elicit opsonic antibodies, establishing a foundation for surface-exposed proteins as potential immunogens **(Cameron, 2000)**. Molini et al. (2022) further advanced this approach by mapping B-cell epitopes of TprC and TprD, demonstrating their capacity to stimulate cross-protective immune responses in treponematoses **(Molini, 2022)**. More recently, **Khan et al. (2023)** utilized subtractive proteomics and reverse vaccinology pipelines to identify additional antigenic targets, underscoring the power of computational approaches for systematic antigen prioritization **(Khan, 2023)**. While **Jiang et al. (2025)** proposed a multi-epitope construct integrating heat shock proteins to enhance immunogenicity **(Jiang, 2025)**, the present study uniquely focuses on Tp0751 (Pallilysin), a protein with critical roles in adhesion, tissue invasion, and immune evasion. By integrating B- and T-cell epitope prediction with antigenicity, allergenicity, and toxicity profiling, followed by structural refinement, docking with TLR2/3/4, and molecular dynamics simulations, this study provides a more comprehensive validation pipeline. In comparison with earlier works, the proposed Tp0751-based vaccine design not only supports the feasibility of multi-epitope constructs but also advances the field by emphasizing structural stability, receptor

engagement, and immune simulation analyses, thereby offering a stronger preclinical rationale for its consideration as a vaccine candidate against T. pallidum.

5. DISCUSSION

Treponema pallidum is a highly invasive pathogen responsible for syphilis, a disease of major public health concern due to its ability to cross critical protective barriers, including the blood–brain barrier and the placental barrier. Transmission occurs primarily through sexual contact, where the bacterium penetrates via mucosal membranes or microabrasions in the skin. Although penicillin remains the standard and highly effective treatment, syphilis cases continue to rise globally, and reinfections are common. These trends underscore the urgent need for a prophylactic vaccine to curb transmission and reinfection rates. Recent advances in syphilis vaccine research have shown promise, with experimental models such as C57BL/6 mice being employed for preclinical evaluation; however, no vaccine is yet available for public use.

Among the surface-exposed antigens of T. pallidum, TP0751 (pallilysin) has emerged as a particularly attractive vaccine target. This protein plays a crucial role in host–pathogen interactions by binding to extracellular matrix components such as laminin and fibronectin, thereby facilitating tissue colonization, vascular penetration, and dissemination across protective barriers. TP0751 can also disrupt intercellular junctions, enhancing bacterial invasion. Previous animal studies have demonstrated its immunogenic potential, supporting its inclusion as a candidate antigen in rational vaccine design.

In the present study, a multi-epitope vaccine construct was designed using TP0751 as the core immunogen. Through reverse vaccinology, highly antigenic and non-allergenic B-cell and T-cell epitopes were identified, followed by screening for solubility and toxicity to ensure construct safety. The addition of an L7/L12 ribosomal protein adjuvant and linkers (EAAAK and GPGPG) was incorporated to enhance immunogenicity and maintain structural stability. Computational analyses revealed favorable physicochemical properties, including stability, solubility, and suitable molecular weight, supporting the feasibility of downstream expression and purification. Immune simulations predicted strong humoral and cellular immune responses, characterized by robust IgG production and cytotoxic T cell activation. Molecular docking demonstrated strong binding interactions with TLR2, TLR3, and TLR4, with TLR3 showing the highest affinity. Molecular dynamics simulations further confirmed the stability of the vaccine–TLR3 complex, highlighting its potential as a potent immune stimulator.

Importantly, the predicted interactions with TLRs can be linked to well-established signaling pathways. Engagement of TLR2 is known to activate the MyD88-dependent pathway, leading to NF-κB translocation and secretion of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, which promote innate immune activation. Binding to TLR3 activates the TRIF-dependent pathway, resulting in production of type I interferons (IFN-α and IFN-β) and enhanced antigen presentation, which are critical for bridging innate and adaptive immunity. Interaction with TLR4 can trigger both MyD88- and TRIF-dependent cascades, thereby inducing a broad cytokine response that balances pro-inflammatory and antiviral signaling. The strong and stable association of the construct with TLR3, in particular, suggests that interferon-mediated immunity may be central to its protective potential against T. pallidum.

Additionally, population coverage analysis revealed that the combined set of MHC class I and class II epitopes achieved 99.74% worldwide coverage. This nearly universal distribution across diverse HLA alleles suggests that the designed vaccine construct could elicit protective immune responses in individuals from most ethnic and geographic backgrounds, further supporting its potential as a globally applicable vaccine candidate.

Despite these encouraging findings, the present study has several limitations. The predictions are entirely computational and require validation through in vitro and in vivo experiments. Other potential antigens with lower expression levels may have been overlooked, and the immunogenicity and safety of the adjuvant and linkers should be experimentally verified. Additionally, population coverage and epitope conservancy analyses across multiple T. pallidum strains will further strengthen the construct's applicability.

This study provides a computationally validated multi-epitope vaccine design targeting TP0751 of T. pallidum. By engaging multiple TLR signaling pathways and eliciting both humoral and cellular immune responses in silico, the construct represents a promising candidate for experimental evaluation. The reverse vaccinology pipeline employed here may also serve as a framework for the design of vaccines against other bacterial pathogens with similar challenges.

6. LIMITATIONS & FUTURE WORK

Despite promising outcomes, this study has several limitations. First, as an entirely in silico investigation, the predicted epitopes, structural models, and immune responses require experimental validation in vitro and in vivo. Computational tools, although highly reliable, may not fully capture the complexity of host–pathogen interactions or potential off-target effects. Second, the focus on Tp0751, while justified by its critical pathogenic role, may overlook other potentially synergistic antigens that could improve vaccine efficacy. Third, although adjuvants and linkers were incorporated into the construct, their immunogenicity and safety must be confirmed through laboratory and animal studies. Finally, this study did not assess potential variations in immune response across diverse human populations, which may limit global vaccine applicability.

Future work should aim to validate the predicted epitopes and vaccine construct experimentally through immunological assays, including antigenicity testing, cytokine profiling, and antibody titration in appropriate animal models. Further exploration of additional outer membrane proteins in combination with Tp0751 may lead to broader and more durable protection. In addition, population coverage analyses across different HLA alleles should be expanded to ensure global applicability of the proposed vaccine candidate **(Bui, 2006)**. Incorporation of advanced simulation methods, such as molecular dynamics with longer trajectories and machine-learning–assisted epitope prediction, could further refine the construct. Ultimately, translational studies including preclinical animal trials and phase I clinical evaluations will be crucial to establish the safety, immunogenicity, and protective efficacy of this vaccine against T. pallidum.

7. CONCLUSION

Treponema pallidum, a sexually transmitted pathogen, is frequently misdiagnosed, particularly among men who have sex with men and are coinfected with HIV, posing a significant threat to global health. Previous studies have indicated that individuals with late latent syphilis demonstrate resistance to symptomatic reinfection by heterologous strains of T. pallidum. Additionally, repeated inoculation with gamma-irradiated T. pallidum has been shown to induce protective immunity in rabbits. These findings suggest that the development of protective vaccines is a viable possibility. In the present study, we present the development of an innovative multi-epitope vaccine designed to target outer membrane (Tp0751) proteins in T. pallidum. Through the systematic screening of B and T cell epitopes within the pathogen's proteins, we engineered a vaccine characterized by its safety, non-allergenic nature, water soluble, Non-toxic and enhanced antigenicity and specificity. Comprehensive evaluations, including protein structure assessment, immunoinformatics analysis, physicochemical prediction, and molecular dynamics simulations, confirmed that our vaccine possesses appropriate structural, physicochemical, and immunological properties. The development of this vaccine holds the potential to serve as a crucial foundation for initiatives aimed at curbing the transmission of T. pallidum, thereby representing a substantial advancement in mitigating its prevalence and impact on public health.

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