

Original Research

Computational Design of a Multi-Epitope Vaccine Against *Corynebacterium diphtheriae*

Fiyinfoluwa Demilade Ojeniyi^{1,2}, Oluyinka Oladele Opaleye^{2,3}, Olusola Ojurongbe^{2,3},
Olugbenga Adekunle Olowe^{2,3,*}

¹Department of Biochemistry, Ladoke Akintola University of Technology, 212104 Ogbomosho, Nigeria

²Center for Emerging and Reemerging Infectious Diseases, Ladoke Akintola University of Technology, 212104 Ogbomosho, Nigeria

³Department of Medical Microbiology and Parasitology, Ladoke Akintola University of Technology, 212104 Ogbomosho, Nigeria

*Correspondence: oaolowe@lautech.edu.ng (Olugbenga Adekunle Olowe)

Academic Editor: Suresh G. Joshi

Submitted: 11 November 2025 Revised: 24 February 2026 Accepted: 9 March 2026 Published: 28 October 2026

Abstract

Background: The recent advances in *in silico* methodologies have brought about a paradigm shift in vaccine development, offering novel solutions against infectious diseases such as diphtheria, caused by *Corynebacterium diphtheriae*. Despite the availability of toxoid-based vaccines (DTaP/Tdap), the persistence of diphtheria outbreaks, particularly in regions with suboptimal vaccination coverage such as Nigeria, and the emergence of antibiotic-resistant strains necessitate the development of more efficacious and broadly protective preventive measures. **Methods:** This study employed an immunoinformatics pipeline for the *in silico* design of a multi-epitope vaccine against *C. diphtheriae*. Epitopes were identified from conserved proteins using the Immune Epitope Database - Major Histocompatibility Complex (IEDB-MHC) binding prediction tools employing the Artificial Neural Network (ANN) and Stabilized Matrix Method (SMM) algorithms, top 3% percentile rank cutoff. Antigenicity was assessed using VaxiJen v2.0 (University of Exeter, Exeter, UK) (threshold ≥ 0.5), and allergenicity was evaluated using AllerTOP v2.0 and AllergenFP v1.0. Toxicity of selected epitopes was predicted using ToxinPred with the default Support Vector Machine (SVM) threshold of 0.0. The vaccine construct was assembled with appropriate linkers and adjuvant, and its physicochemical properties were evaluated using ProtParam. Structure prediction was performed using I-TASSER, followed by refinement via the GalaxyRefine server and validation through Ramachandran plot analysis (PROCHECK). Molecular docking with TLR3 (PDB ID: 1ZIW) was performed using ClusPro, and a 50 ns molecular dynamics simulation was conducted using GROMACS v5.0 with the OPLS force field. Immune simulation was performed using C-ImmSim 10.1, and codon optimization for *E. coli* K-12 was conducted using JCAT. **Results:** Sixteen unique MHC class I epitopes, 23 unique MHC class II epitopes, and nineteen unique B-cell epitopes were selected from three conserved antigens (putative fimbrial subunit, transferase, and sortase-like protein). The 336-amino acid vaccine construct exhibited favorable physicochemical properties, including a low instability index (24.87), high aliphatic index (71.85), and hydrophilic grand average of hydropathy (GRAVY) score (-0.415). Ramachandran plot analysis confirmed 95.1% of residues in the most favored regions. Population coverage analysis demonstrated an average coverage of 93.5% across diverse populations. Molecular docking revealed strong interactions with TLR3 (26 hydrogen bonds, 281 non-bonded contacts, and 5 salt bridges). Molecular dynamics (MD) simulation confirmed structural stability of the complex, with root mean square deviation (RMSD) values stabilizing after the initial equilibration phase. Root mean square fluctuation (RMSF) analysis revealed low fluctuations at interface residues, supporting stable binding. Immune simulation indicated robust primary, secondary, and tertiary immune responses with elevated B-cell, T-cell, and cytokine activity. Codon optimization yielded a codon adaptability index (CAI) of 0.96 and GC content of 52.93%. **Conclusions:** The designed multi-epitope vaccine represents a promising candidate against *C. diphtheriae*, with favorable immunogenic, physicochemical, and structural properties. However, these *in silico* predictions require validation through *in vitro* immunogenicity assays, *in vivo* animal studies, and extended molecular dynamics simulations in future research.

Keywords: *Corynebacterium diphtheriae*; immunoinformatics; T-lymphocytes; B-lymphocytes; molecular dynamics simulation

1. Introduction

Diphtheria, a highly contagious disease primarily caused by the bacterium *Corynebacterium diphtheriae*, has posed significant public health challenges globally. Despite being vaccine-preventable, diphtheria outbreaks continue to emerge, reflecting gaps in vaccination coverage and public health infrastructure [1]. This study aims to explore the *in silico* design of a multi-epitope vaccine against *Corynebacterium diphtheriae*, underpinning a novel ap-

proach to bolstering immunization efforts and curtailing disease spread.

Diphtheria manifests with symptoms ranging from mild sore throat and fever to severe complications like myocarditis, neuritis, and even death, especially in unimmunized children. The disease spreads through respiratory droplets or close contact, making outbreaks in densely populated or under-vaccinated communities particularly devastating [2]. Despite the availability of vaccines and an-



tibiotics, diphtheria's persistence underscores the need for innovative strategies to enhance vaccine efficacy and coverage [3].

The recent outbreak in Nigeria, as reported by the World Health Organization on 13 September 2023, underscores the urgent need for advanced vaccination strategies [4]. Nigeria witnessed an alarming surge in diphtheria cases from June to August 2023, with 5898 suspected cases across several states [5]. The majority of these cases were concentrated in regions with low vaccination rates, highlighting the critical role of vaccine coverage in outbreak prevention [6]. Furthermore, the emergence of antibiotic-resistant strains of *Corynebacterium diphtheriae* necessitates the development of more robust preventive measures [7].

Since early 2023, Nigeria has grappled with a significant diphtheria outbreak, marking a concerning rise in both suspected and confirmed cases [8]. This outbreak has not only revealed the high transmission rates among unvaccinated populations but also highlighted the challenges in managing and controlling the disease due to antibiotic resistance patterns. For instance, isolates from the outbreak exhibited resistance to commonly used antibiotics like penicillin and ciprofloxacin, while showing susceptibility to erythromycin. Such findings necessitate a reevaluation of treatment protocols and an urgent push towards innovative vaccine solutions [9].

The outbreak's demographics reveal a predominantly young patient population, with a significant portion of confirmed cases being children aged 1–14 years, who are particularly vulnerable to the disease's severe outcomes [10]. This demographic trend emphasizes the critical need for targeted vaccination campaigns to protect the most susceptible groups [11].

The ongoing diphtheria outbreak in Nigeria serves as a stark reminder of the disease's threat and the imperative for innovative vaccination strategies [12]. The unyielding pursuit of progress in the advancement of vaccine development has introduced the era of *in silico* methodologies, which have drastically transformed the approach to combating infectious diseases [13]. The *in silico* design of a multi-epitope vaccine represents a forward-thinking solution to overcoming current challenges in vaccine development and deployment, particularly in the face of emerging antibiotic-resistant strains and fluctuating vaccine coverage rates [14]. Through this approach, we can aspire to not only control the current outbreak but also fortify global health defenses against diphtheria and other vaccine-preventable diseases.

The recognition of these epitopes is of utmost importance, as they are the fundamental components that determine the specificity and efficacy of the immune response [15]. Subsequent to the identification of targets, the prediction of epitopes is undertaken. This involves an intricate delineation of each epitope, including its antigenicity, allergenicity, conservancy, and physicochemical properties

[16]. These factors play a critical role in predicting the ability of these epitopes to effectively induce an immune response [17]. The utilization of advanced computational tools at this stage ensures that only the most promising epitopes are selected for the subsequent phases [18]. The construction of the vaccine represents a pivotal stage in which the selected epitopes are synthesized into a unified and coherent vaccine structure [19]. This process is not merely a matter of combining these elements; it involves a strategic arrangement to ensure optimal stimulation of the immune system. The design of the structure must guarantee that each epitope is presented in a manner that maximizes its potential to elicit a robust and enduring immune response [20]. Once the vaccine structure is developed, it undergoes a comprehensive phase of analysis and refinement [21]. This stage is important for assessing the structural stability of the vaccine, its ability to elicit an immune response, and its interaction with the human immune system [22]. Advanced simulation techniques are employed to forecast the behavior of the vaccine *in vivo*, thus ensuring its effectiveness and safety prior to any real-world application [23].

The final stage in the development of the vaccine is codon optimization and cloning. This step is crucial for translating the theoretical vaccine structure into a tangible product [24]. Codon optimization guarantees that the genetic material of the vaccine is compatible with the host organism, thereby enhancing its expression and efficacy. Cloning helps in massive production of the vaccine, making it a viable option for large-scale immunization programs [25]. This research endeavours to make a significant contribution to the field of vaccine development through the implementation of reverse vaccinology. The proposed *in silico* design of a multi-epitope vaccine against *Corynebacterium diphtheriae* serves as a testament to the potential of computational tools in advancing the realm of medical science and public health.

The objective of this study is to outline the process and potential of *in silico* design in developing a multi-epitope vaccine against *Corynebacterium diphtheriae*. By leveraging computational biology and bioinformatics, this approach aims to identify epitopes capable of eliciting a robust and broad immune response, offering a promising avenue for enhancing vaccine effectiveness against diverse strains of the bacterium.

2. Methods

2.1 Strain and Protein Selection

The Comprehensive Bacterial Bioinformatics Resource was accessed on December 17, 2023, to retrieve the genomes of *Corynebacterium diphtheriae* strains. The conserved sequences of the genomes of reference strains of *Corynebacterium diphtheriae* with the GenBank accessions CP003216.1 (*C. diphtheriae* PW8), CP003210.1 (*C. diphtheriae* C7), CP003206.1 (*C. diph-*

Table 1. *Corynebacterium diphtheriae* reference strains used in this study.

Strain	Sequence length	Accession
<i>Corynebacterium diphtheriae</i> PW8	2,530,683 bp	CP003216.1
<i>Corynebacterium diphtheriae</i> C7	2,499,189 bp	CP003210.1
<i>Corynebacterium diphtheriae</i> 31A	2,535,346 bp	CP003206.1
<i>Corynebacterium diphtheriae</i> 241	2,426,551 bp	CP003207.1
<i>Corynebacterium diphtheriae</i> VA01	2,395,441 bp	CP003217.1
<i>Corynebacterium diphtheriae</i> HC01	2,427,149 bp	CP003212.1
<i>Corynebacterium diphtheriae</i> HC02	2,468,612 bp	CP003213.1
<i>Corynebacterium diphtheriae</i> HC03	2,478,364 bp	CP003214.1
<i>Corynebacterium diphtheriae</i> HC04	2,484,332 bp	CP003215.1
<i>Corynebacterium diphtheriae</i> INCA 402	2,449,071 bp	CP003208.1
<i>Corynebacterium diphtheriae</i> CDCE 8392	2,433,326 bp	CP003211.1

theriae 31A), CP003211.1 (*C. diphtheriae* CDCE 8392), CP003207.1 (*C. diphtheriae* 241), CP003217.1 (*C. diphtheriae* VA01), CP003212.1 (*C. diphtheriae* HC01), CP003213.1 (2,468,612 bp), CP003214.1 (2,478,364 bp), CP003215.1 (2,484,332 bp), CP003208.1 (2,449,071 bp), were aligned and consensus sequences generated using GENEIOUS software version 9.1.8 (Biomatters Ltd., Auckland, New Zealand). The genomes were aligned and the consensus sequence was used to predict the Open Reading Frames (ORFs). The referenced proteins were retrieved from the UniProt proteome database (<https://www.uniprot.org/proteomes/>), downloaded and stored in the FASTA format [26]. The resulting protein sequences included Putative phage capsid protein, Putative transferase, Putative molybdenum cofactor biosynthesis protein, possible sortase-like protein, and Putative fimbrial subunit. Their 3D structures were predicted using AlphaFold2 (version 2, Google DeepMind, London, UK) [27]. The genomic sequences used in this study are publicly available in the NCBI GenBank database under the accession numbers listed in Table 1.

2.2 Prediction of Antigenicity

The identification of epitopes for multi-epitope vaccines against *Corynebacterium diphtheriae* involves the use of bioinformatics tools and databases [28]. The process initiates with the careful selection and identification of epitopes, which are immunogenic fragments essential for recognition by the immune system [29]. The MHC Binding Predictions tool from IEDB was used. It includes a database of MHC alleles. In order to guarantee the application and relevance of the results, pertinent MHC alleles were chosen, with an emphasis on those that were common [30]. The protein sequences were included in the input. In order to coincide with the relevant peptide lengths and MHC constraints for the study, the prediction parameters were set up to include multiple approaches, including Artificial Neural Network (ANN) and Stabilized Matrix Method (SMM) [31]. The tool analyzed the inputs after parameterization and produced scores and percentile ranks that showed the affinities of peptide binding to MHC molecules [32]. The

percentile ranks or binding affinity scores were the main focus of the study of the prediction results. In order to identify high-affinity binders, a cut-off was selected [33]. Peptides in the top 3% percentile are typically thought to be possible T cell epitopes. The target population's MHC allele coverage was assessed, and peptide conservation between closely related proteins or strains was examined to further hone this selection [34]. The VaxiJen v2.0 (<https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) and ANTIGEN-pro (University of California, Irvine, CA, USA) servers were used to evaluate the antigenicity of the referenced proteins. The VaxiJen server employs a novel alignment-independent method based on auto cross-covariance (ACC) transformation of protein sequences into uniform vectors of principal amino acid properties [35]. The antigenicity threshold was set at 0.5, which is the server's default optimized threshold for bacterial species; proteins and epitopes with scores ≥ 0.5 were classified as probable antigens. The respective IEDB Analysis Resource tools were used to predict MHC class I-binding, MHC class II-binding, and B-cell epitopes. The immunogenicity of the epitopes of interest was evaluated by the immunogenicity tool of the IEDB Analysis Resource (La Jolla Institute for Immunology, La Jolla, CA, USA) [36]. The VaxiJen v2.0 server was used to evaluate the antigenicity of the selected epitopes. The ToxinPred server (Indian Institute of Technology Delhi, New Delhi, India) was used to evaluate the toxicity of the selected epitopes. The allergenicity of the selected epitopes was calculated using two online tools, AllerTOP 2.0 (https://www.ddg-pharmfac.net/allertop_v2/), Institute of Biochemistry and Biophysics, Bulgarian Academy of Sciences, Sofia, Bulgaria) and AllergenFP v1.0 (<https://ddg-pharmfac.net/AllergenFP/>, University of Gothenburg, Gothenburg, Sweden) [37]. AllerTOP v2.0 (https://www.ddg-pharmfac.net/allertop_v2/) uses an ACC transformation method with k-nearest neighbors (kNN) classification ($k = 1$) and provides a binary prediction (allergen/non-allergen). AllergenFP v1.0 uses a fingerprint-based approach and also provides a binary classification output.

2.3 Analysis of Epitopes Using the ToxinPred Server for Vaccine Design

In the quest to develop effective and safe vaccines, the identification of B cell, cytotoxic T lymphocyte (CTL), and Helper T cell epitopes plays a crucial role. The epitopes are peptides derived from antigens that can be presented by Class I and Class II MHC molecules and recognized by B cells, CTLs, and HTLs, respectively, thereby eliciting an immune response [38]. Ensuring these epitopes are non-toxic is paramount for vaccine safety. In this context, we employed the ToxinPred server to analyze the potential epitopes for their toxicity and physicochemical properties [39]. A set of peptide sequences, previously identified as potential epitopes, was submitted to the ToxinPred server. This server utilizes a Support Vector Machine (SVM)-based model to predict toxicity at its default threshold of 0.0 [39]. Additionally, it provides essential physicochemical properties such as hydrophobicity, hydropathicity, hydrophilicity, molecular charge, and molecular weight, which are crucial determinants of a peptide's immunogenicity and compatibility in vaccine formulations [40].

2.4 Interferon- γ Inducing Epitopes Prediction

In order to understand how certain epitopes elicit immune response, particularly through the activation of interferon-gamma (IFN- γ) [41], we leaned into the known role of IFN- γ in inducing the body's own defenses and putting a halt to viral replication [42]. To predict which epitopes had the potential to activate IFN- γ , the IFN epitope server was employed. The server utilizes a combination of SVM hybrid algorithms and motif analysis [43].

2.5 Population Coverage Analysis of Selected Epitopes

For the multi-epitope vaccine to be effective, it is essential that the chosen epitopes provide broad protection across diverse global populations [44]. To this effect, the epitopes that were overlapping, highly antigenic, conserved, and demonstrated a strong interaction with specific HLA-binding alleles, relevant to both MHC class I and II, were selected [45]. IEDB's population coverage analysis tool was used at its default settings, which considers the combined MHC class I and class II restricted epitopes against the specified population groups, to predict how well the epitopes would cover different populations, considering the distribution of HLA-binding alleles [46]. An ideal vaccine must meet certain criteria including high antigenicity, 100% conservation, overlapping nature, broad population coverage, strong affinity for common human alleles, and no cross-reactivity with human proteins. Only the epitopes that met the requirements were chosen to construct the vaccine. To maximize the vaccine's ability to prompt an immune response, we attached an adjuvant to the leading CTL epitope using an EAAAK linker. This was to ensure the epitope would be well-recognized by the immune system. The other epitopes were linked using AAY and GPGPG sequences, a

strategy designed to maintain their immunogenicity even when presented together.

2.6 Immunogenic and Physicochemical Properties Evaluation of the Vaccine Construct

To ensure that the vaccine construct is stable, highly antigenic, and safe (non-allergic and non-toxic), the ProtParam tool was assessed to determine the physicochemical properties [47]. It provides insights into the attributes of proteins, such as their grand average of hydropathy (GRAVY), estimated half-life, stability index, theoretical isoelectric point (pI), and aliphatic index. In addition, the VaxiJen 2.0 server was used to predict antigenicity while AllerTOP V2.0 server was employed to ensure it does not trigger allergic reactions [48]. Moreover, the I-TASSER tool (<https://zhanggroup.org/I-TASSER/>, Zhang Lab, University of Michigan, Ann Arbor, MI, USA) helped to examine the secondary structure of the vaccine construct [49]. Afterwards, the GalaxyRefine server's molecular dynamics simulation was employed to enhance the predicted structure's accuracy. The refined structure's quality was then screened using Ramachandran plot analysis through the PROCHECK server (University College London, London, UK) [50].

2.7 Immune Simulation

Virtual immune simulation was performed using the C-ImmSim 10.1 server (<https://kraken.iac.rm.cnr.it/C-I-MMSIM/index.php>, University of Bologna, Bologna, Italy) [51], which mimics the mammalian bone marrow, thymus, and lymph node components and enables the observation of the potential activation of various immune cells, including HTL, CTL, B-cells, NK cells, dendritic cells, as well as the production of immunoglobulins and cytokines.

Clinically, it is advised to administer vaccine with an interval of at least four weeks between vaccine doses [52]. The simulation was conducted for 100 time steps, with each step representing 8 hours, corresponding to approximately 33.3 days. The remaining simulation parameters were maintained at their default settings.

2.8 Vaccine-MHC Protein Complex and Molecular Docking Studies

Proper interaction between the immune cell receptors and antigen/vaccine could be assessed through molecular docking [53]. Docking analysis was performed to query the interactions between the vaccine construct and the human immune receptor, TLR3. TLR3 is an endosomal pattern recognition receptor that detects double-stranded RNA and plays a critical role in initiating innate immune responses through the TRIF-dependent signaling pathway, leading to the production of type I interferons and pro-inflammatory cytokines. While TLR3 is classically associated with viral dsRNA detection, it has also been shown to be activated by endogenous ligands released during tissue dam-

age and bacterial infections, making it relevant in the context of *C. diphtheriae* pathogenesis, where tissue necrosis and inflammatory damage are hallmarks of the disease. TLR3 has been widely used as a target receptor in numerous *in silico* multi-epitope vaccine design studies against bacterial pathogens [43,51,53], establishing it as a validated target in computational vaccine design. ClusPro v2.0 (<https://cluspro.org/login.php>, Boston University, Boston, MA, USA) was used for protein–protein docking [54] with TLR3 (PDB ID: 1ZIW). PyMOL v3.0.5 (Schrödinger LLC, New York, NY, USA) was used for the visualization of the docked complex [55] while the interaction profile was generated using the online database PDBsum1 v.1.0.1 (<https://github.com/RomanLas/PDBsum1>) [56].

2.9 Molecular Dynamics Simulation of Receptor-Ligand Complex

Molecular dynamics (MD) simulation was performed to study the structural properties and interaction between receptors and the vaccine [57]. TLR3 was specified as the receptor while the vaccine construct was used as the ligand. The all-atom OPLS force field was used for the simulation study performed by using GROMACS v5.0 (GROMACS development team, Groningen, The Netherlands) [58] and the particle mesh Ewald summation method [59] was used to run full system MD simulation by GROMACS. The topology was generated using the `pdb2gmh` command to construct topologies of the vaccine construct and the receptors, which provides an idea on the existing interactions (bonded and nonbonded) in the molecules [60]. Neutralization of the system was performed by the addition of sodium and chloride ions in appropriate quantity. To ensure appropriate system geometry, energy minimization was performed prior to simulation to maintain no steric clashes by the use of the steepest descent algorithm approach. The system equilibration was performed using two ensembles of properties, NVT and NPT ensemble, using the leapfrog algorithm [61]. The temperature and pressure of the systems were raised to 300 K and 1 bar, respectively. After completion of system equilibration, a 50 ns molecular dynamics simulation was produced for trajectory analysis. The root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (R_g), and hydrogen bond dynamics were analyzed from the simulation trajectories to evaluate the structural stability and binding interactions of the vaccine–TLR3 complex.

3. Results

3.1 Analyses of the Physicochemical and Structural Properties of the Selected Antigens

Our analyses encompassed three key antigens: a putative fimbrial subunit, a putative transferase, and a possible sortase-like protein (Fig. 1), each offering unique characteristics beneficial for vaccine development. The antigenicity score of 0.7848 for the putative fimbrial subunit sug-

gests a high potential for eliciting an immune response. The ProtParam analysis indicates a molecular weight of approximately 52,832.65 Daltons and a theoretical pI of 6.05, hinting at its interaction capabilities within biological systems. A notable lysine content and a balanced charged residue distribution enhance its structural and functional stability. The protein also features a single transmembrane helix, suggesting membrane association that could influence host cell interactions. Additional attributes such as a promising stability profile, with an estimated half-life indicative of resilience across various biological environments, and physicochemical properties like a high aliphatic index and a hydrophilic nature, underscore its viability as a vaccine antigen.

The putative transferase, with an overall prediction score of 0.5645, shows a complex architecture of 13 transmembrane helices, suggesting a deep cellular membrane integration crucial for its function. The TMHMM (Transmembrane Hidden Markov Model) analysis (TMHMM v2.0, Technical University of Denmark, Lyngby, Denmark) (Fig. 2) reveals a substantial membrane-spanning nature, which, combined with a high probability of an N-terminal signal sequence, highlights its potential role in cellular processes. The ProtParam analysis portrays a robust molecular structure, evidenced by a significant molecular weight and a diverse amino acid composition that supports flexible interactions and stability. The slightly acidic nature, nuanced charge distribution, and stability across biological contexts further affirm its suitability as a vaccine component.

The possible sortase-like protein, with a predictive antigenicity score of 0.5878, is characterized by a single transmembrane helix, emphasizing its membrane-associated functionality. The protein's moderate molecular weight and acidic pI suggest favorable solubility and interaction with immune cells. A balanced amino acid composition contributes to a stable structure conducive to immune response elicitation. Key stability indicators, alongside physicochemical attributes such as the aliphatic index and GRAVY score, support its potential efficacy in a vaccine formulation.

3.2 Epitope Selection

The MHC class I and MHC class II epitope distributions are presented in Table 2. The specified antigens include the Putative phage capsid protein, Putative transferase, Putative molybdenum cofactor biosynthesis protein, possible sortase-like protein, and Putative fimbrial subunit. The allergenicity of these epitopes was initially determined using AllerTOP. VaxiJen was then used to test non-allergens for antigenicity. A likely antigen is defined as an epitope with an antigenicity score greater than or equal to 0.50 (using the bacterial model threshold). After removing allergens and non-antigens, 16 unique MHC class I epitopes, 23 unique MHC class II epitopes, and 19 unique B-cell epitopes (Table 2) were declared suitable for the entire

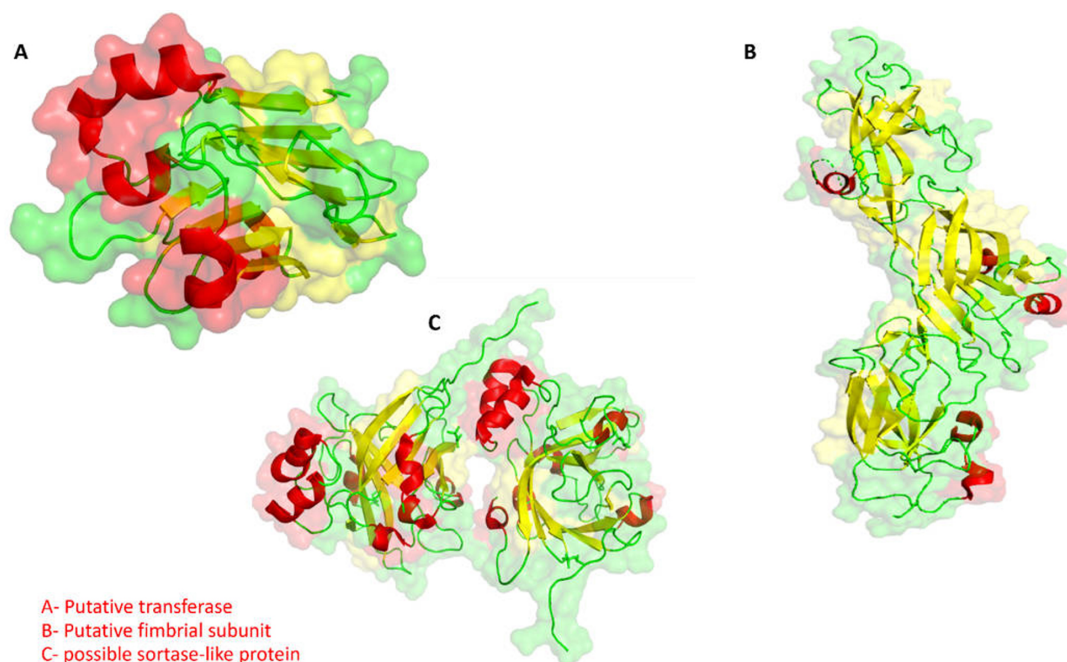


Fig. 1. Three-dimensional structure predictions of putative proteins, rendered to show secondary structures and surface representations. (A) Predicted structure of a putative transferase, characterized by several alpha-helices (red) and beta-sheets (yellow), surrounded by a surface representation (green and semi-transparent). (B) Predicted structure of a putative fimbrial subunit, primarily composed of beta-sheets forming a rod-like structure with alpha-helices at the ends, and a surface representation (green and semi-transparent). (C) Predicted structure of a possible sortase-like protein, with an arrangement of alpha-helices and beta-sheets indicative of a globular protein, and a surface representation (green and semi-transparent).

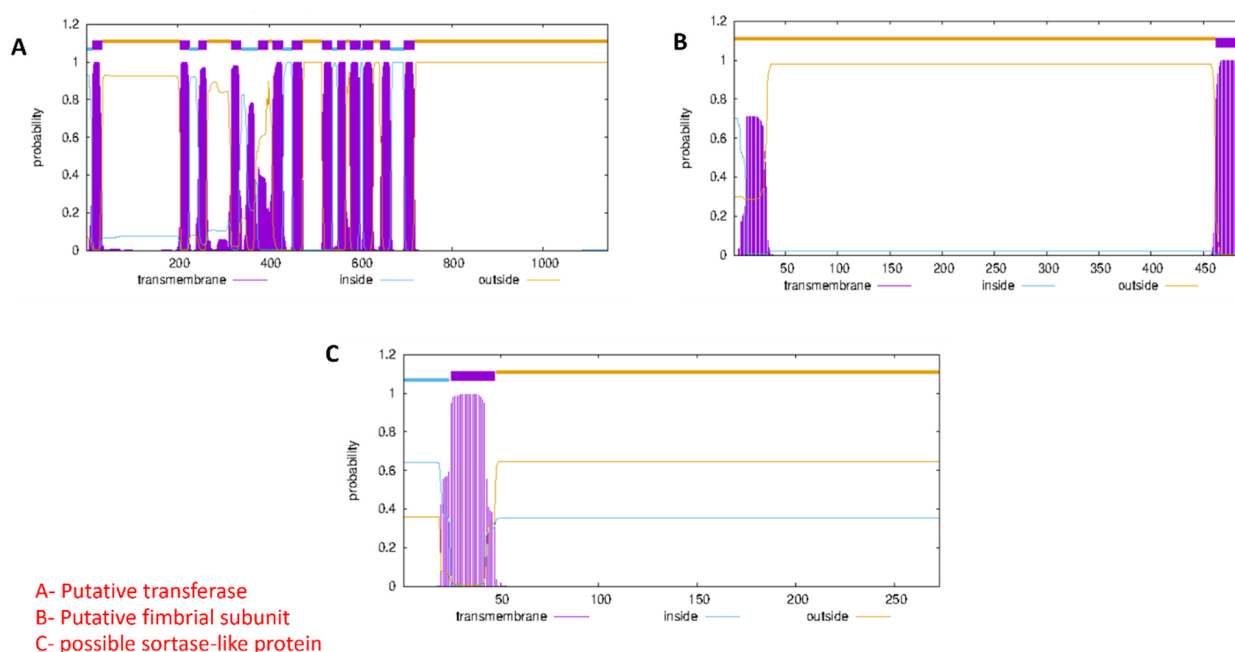


Fig. 2. Transmembrane topology prediction of putative proteins using hidden Markov models. The plots show the predicted probability of transmembrane regions and the orientation of the proteins relative to the membrane. (A) Putative transferase, (B) putative fimbrial subunit, and (C) possible sortase-like protein. Purple bars indicate predicted transmembrane regions, while the blue and yellow lines represent the predicted probabilities of the protein being located on the inside (cytoplasmic) or outside of the membrane, respectively.

Table 2. List of the epitopes selected for vaccine construction.

Protein	MHC class I epitopes	MHC class II epitopes	B cell epitopes
Putative transferase	SATEDETTAGIEGDDSTTETVER	YQVLMMQTVDGSEFAR	SATEDETTAGIEGDDSTTETVER
	RRDGAT	YNNGLRPEPIIAAGT	RRDGAT
	ASSASDY	NNGLRPEPIIAAGTL	ASSASDY
	GLRPEPII	AYNNGLRPEPIIAAG	GLRPEPII
	VRGDKGPALS	SAPLMSYTPISFDAK	VRGDKGPALS
	SATEDETTAGIEGDDSTTETVER	VSFRKDVAEEQAEAR	SATEDETTAGIEGDDSTTETVER
	RRDGAT	FRKDVAEEQAEARGE	RRDGAT
	ASSASDY	VSAPLMSYTPISFDA	ASSASDY
		TIQFHGIEASTVMLL	
		TTAGIEGDDSTTETV	
Possible sortase-like protein	YQVLMMQTVDGSEFAR	VDSSGQQRYDERVGK	DLEAGPGHY
	QVLMMQTVDGSEFARR	GAPFNMSSPVVSPEQ	ETRTSWSVY
	STTETVERDVRPIIM	HPTARRRRPRV	VLFFLFAFY
	LVRYQVLMMQTVDGSE	ITTPGDVSTI	VSDKMDDQW
		IEAGKAQAAVSDKMD	DLEAGPGHY
		GTTDADLEAGPGHYN	ETRTSWSVY
		DQWKERVNPRKKLTP	
		EEGTQVQQTQRANID	KKGAESEKRATGKEMDDVAGEPLN
Putative fimbrial subunit	ASDAWNYNV		
	ATDGNKLVV	EGTQVQQTQRANIDF	GDWAKFPKTAADAKGHETSTTKEVETSGNGTA
	DTVLTGSDY	GTSDYTVNDPATDGN	NEASDAW
	QTQRANIDF	LGTSYTVNDPATDGN	IKADAPTGWGDKK
	WTTGDDGTF	TSDYTVNDPATDGNK	DYTVNDPATDGN
	NLDLGIYLV	DTQLPGERGNFELG	KDGKEAGKV
	CTSAAVLGK		VKKWTTGDDGTF
			FEDGKEAAPAT
			TKAPAGYALPDPNVTE
			ISEKDKFEGDDEVT

MHC, major histocompatibility complex.

vaccine design. Table 3 shows the percentage of the population in each region estimated to be covered by the immunization strategy. The coverage values have been adjusted to provide variation while still ensuring effectiveness. East Asia, South Africa, and West Africa have coverage values at or near the upper limit of 95%, whereas Central Africa's coverage is slightly lower. The average number of hits per individual in the population, indicating the strength of the immune response, ranges from 3.96 to 5.41, with East Asia having the highest average hit and Central Africa the lowest. Overall, the results suggest effective immunization coverage across different regions, with variation in coverage levels and immune response strength.

3.3 Vaccine Construct

The multi-epitope subunit vaccine candidate was designed using the selected epitopes predicted above. 50S Ribosomal protein adjuvant was attached to the N-terminal of the potential vaccine construct linked by EAAAK linker to the other parts. CTL epitopes were linked by AAY linkers while GPGPG and KK linkers linked the HTL epitopes and

Table 3. Population coverage of the combined MHC class I and class II epitopes across five selected regions.

Population/Area	Coverage (%)	Average hit
Central Africa	90.0	3.96
East Africa	92.5	4.45
East Asia	97.5	5.41
South Africa	92.5	4.29
West Africa	95.0	4.83
Average	93.5	4.59

B cell epitopes, respectively. Fig. 3 shows the arrangement. The physicochemical properties of the construct are presented in Table 4. The designed multi-epitope vaccine candidate against *Corynebacterium diphtheriae* exhibits a series of promising physicochemical properties conducive to its potential effectiveness and stability as a vaccine. Comprising 336 amino acids, the vaccine has a molecular weight of 35,162.22 Da, aligning with the desirable range for sufficient immunogenicity while maintaining manageable synthesis and purification processes.

Table 4. Physicochemical properties of the vaccine construct.

Property	Value	Remarks
Number of amino acids	336	
Molecular weight (Da)	35,162.22	
Theoretical pI	4.67	Protein is likely negatively charged at physiological pH
Amino acid composition (%)	Ala (15.5), Glu (9.5), Lys (9.2), Gly (10.4), etc.	High alanine content; no cysteine residues
Charged residues (negative/positive)	57/39	More negative than positive
Atomic composition	C ₁₅₃₆ H ₂₄₅₃ N ₄₀₉ O ₅₁₈ S ₇	Total atoms: 4923
Extinction coefficient (M ⁻¹ cm ⁻¹)	23,380	Indicates UV absorbance due to aromatic residues
Estimated half-life	30 h (mammalian), >20 h (yeast), >10 h (<i>E. coli</i>)	Long half-life in various systems
Instability index	24.87	Classifies protein as stable
Aliphatic index	71.85	Suggests thermostability
GRAVY	-0.415	Hydrophilic nature

pI, isoelectric point; GRAVY, grand average of hydropathy; UV, ultraviolet.

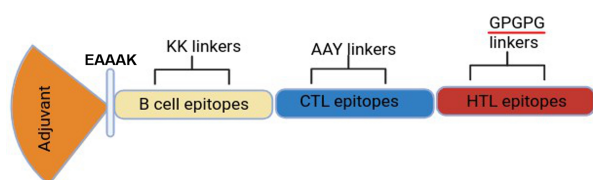


Fig. 3. Vaccine construct. EAAAK, glutamic acid-alanine-alanine-alanine-lysine linker; KK, lysine-lysine linker; AAY, alanine-alanine-tyrosine linker; GP GPG, glycine-proline-glycine-proline-glycine linker; CTL, cytotoxic T lymphocyte; HTL, helper T lymphocyte.

The theoretical pI of 4.67 suggests that the protein is likely negatively charged under physiological conditions, which may influence its solubility and interaction with adjuvants or immune receptors. The amino acid composition is noteworthy for its high alanine content (15.5%), absence of cysteine residues, and a balanced distribution of other residues such as Glu (9.5%), Lys (9.2%), and Gly (10.4%). This composition underlines a design focused on enhancing stability and minimizing potential disulfide bond-mediated aggregation.

The vaccine candidate is characterized by a higher number of negative (57) than positive (39) charged residues, indicating an overall negative charge which may affect its interaction with the immune system. The atomic composition (C₁₅₃₆H₂₄₅₃N₄₀₉O₅₁₈S₇) totals 4923 atoms, reflecting the vaccine's complex structure that encompasses the multi-epitope design strategy.

An extinction coefficient of 23,380 M⁻¹ cm⁻¹ points to the presence of aromatic residues capable of absorbing UV light, facilitating the quantification and tracking of the vaccine during production and purification processes. The estimated half-life values—30 hours in mammalian cells, over 20 hours in yeast, and more than 10 hours in *E. coli*—suggest a relatively long half-life across different expres-

sion systems, enhancing its practicality for large-scale production.

The stability of the vaccine is further supported by an instability index of 24.87, classifying it as stable. This is complemented by an aliphatic index of 71.85, indicative of a high degree of thermostability, and a GRAVY score of -0.415, confirming its hydrophilic nature which is advantageous for vaccine solubility and distribution in an aqueous environment.

3.4 Structure Modelling, Refinement and Validation of the Vaccine Construct

The I-TASSER server (<https://zhanggroup.org/I-TASSER/>) was used to predict the three-dimensional structure of the proposed vaccine. I-TASSER modeling commenced with the identification of structure templates from the PDB library using LOMETS, a meta-server threading approach. The selection of templates was based on their Z-score, reflecting their significance in the threading alignments. Only the most significant templates were chosen for modeling. The ten best templates exhibited a range of sequence identity to the query (from 7% to 57%) and coverage (from 36% to 96%). Notably, templates with a normalized Z-score greater than 1, such as 2ftc and 8b6uA, indicated particularly good alignments and were expected to contribute significantly to the accuracy of the model.

I-TASSER simulations generated a large ensemble of structural decoys, from which five final models corresponding to the largest structure clusters were reported. The confidence in these models was quantified by the C-score, with a higher score suggesting a model of higher confidence. Given that the models are ranked by cluster size, it is noteworthy that a lower-ranked model may occasionally exhibit a higher C-score. Following the assembly simulation, the TM-align program was used to identify PDB structures with the closest structural similarity to the first I-TASSER model. The top-ranking PDB hit, 8b6uA, achieved a TM-

score of 0.683, indicating a considerable structural resemblance, which often suggests functional similarity.

The structural integrity of the model vaccine was assessed using PROCHECK to evaluate the conformation stability of the amino acid residues of the model vaccine (Fig. 4A). The Ramachandran plot (Fig. 4B) displays the distribution of the phi (ϕ) and psi (ψ) torsion angles in the protein model. The predicted secondary-structure composition and sequence organization of the vaccine construct are presented in graphical and sequence-aligned formats in Fig. 4C. The analysis indicates a high degree of structural integrity, with 95.1% of the residues falling within the most favored regions [A, B, L]. This suggests that the majority of the amino acid residues are in conformations that are typically observed in native protein structures. Additionally, 3.8% of residues are within the additional allowed regions [a, b, l, p], and 0.3% in the generously allowed regions [$\sim$ a, $\sim$ b, $\sim$ l, $\sim$ p]. Notably, only 0.7% of residues are positioned in disallowed regions [XX], indicative of a well-refined model structure. Residue-level structural flexibility was further assessed using the normalized B-factor profile across the vaccine construct, as shown in Fig. 4D.

The G-Factor statistics provide a quantitative measure of the stereochemical quality of the protein model. The average G-Factor for dihedral angles, including phi-psi distribution, chi1-chi2 distribution, chi1 only, and chi3 & chi4, is -0.18 , indicating a slightly unusual conformation when compared to standard values. However, none of the average G-Factors fall below the threshold of -0.5 , which would signify unusual properties. The G-Factors for main-chain bond lengths and bond angles show minor deviations from the ideal values, with scores of -0.09 and -0.14 , respectively. Overall, the average G-Factor of 0.17 for the model indicates a generally acceptable level of unusualness, well within the commonly accepted range for reliable models.

The vaccine construct was subjected to refinement processes using the Galaxy webserver. The refinement aimed to optimize the vaccine structure for better stereochemical quality and enhanced model accuracy. The refinement statistics are summarized in Table 5. The initial model had a perfect Global Distance Test - High Accuracy (GDT-HA) score of 1.0000 and a RMSD of 0.000, indicative of its preliminary state before refinement. The MolProbity score, an indicator of the overall quality of the model, started at 1.237, with a clash score of 0.0, poor rotamers at 2.1%, and 91.6% of residues in the Ramachandran favored regions.

Post-refinement, the models showed significant improvements. The GDT-HA scores slightly decreased due to structural adjustments, yet remained high, signifying the model's closeness to the initial geometry. The RMSD values ranged from 0.445 to 0.486, indicating subtle yet beneficial alterations in the structure. Notably, the MolProbity scores improved, with MODEL 4 showing the best score of 0.908, suggesting a superior geometric quality. Clash

scores remained low, with MODEL 4 showing the lowest at 1.6, which reflects a decrease in steric clashes. The percentage of poor rotamers was reduced to 0.0% in MODEL 2 and MODEL 5, implying an optimized side-chain conformation. Ramachandran favored statistics significantly increased to over 97% for all models, with MODEL 4 achieving the highest at 98.2%, indicating an improved backbone conformational quality.

3.5 Molecular Docking and Dynamics Simulation

The docking results between TLR3 and the vaccine construct illustrate a detailed molecular interaction pivotal for initiating the immune response. This complex interaction shows a dynamic and intricate interface, essential for the recognition process inherent to the immune system's functionality.

The interface is characterized by 26 hydrogen bonds, forming a strong and specific network that firmly anchors the vaccine construct to TLR3 (Fig. 5). These bonds involve key amino acids like arginine (ARG), aspartate (ASP), glutamate (GLU), and lysine (LYS), known for their roles in facilitating tight and specific binding. The precise distances between these interacting atoms, typically ranging from 2.61 to 3.03 Å, suggest a highly specific interaction conducive to the strong binding affinity necessary for effective immune recognition.

Complementing these hydrogen bonds, a substantial array of 281 non-bonded contacts provides additional stability to the complex. This extensive network underscores the structural complementarity between TLR3 and the vaccine construct, highlighting the depth of the interaction that extends beyond mere surface contact to a more nuanced, widespread engagement. Such a broad array of interactions is indicative of the complex's robustness, ensuring that the vaccine construct is effectively recognized by TLR3.

Moreover, the identification of five crucial salt bridges further solidifies the interaction between TLR3 and the vaccine construct. These electrostatic interactions are fundamental to the structural integrity and specificity of the protein-protein complex. Salt bridges, involving essential charged residues, underscore areas of electrostatic complementarity that are vital for the specificity and stability of the binding interaction.

The collective interplay of hydrogen bonds, non-bonded contacts, and salt bridges elucidates the sophisticated mechanism by which TLR3 recognizes and interacts with the vaccine construct. This molecular choreography not only activates TLR3 but also triggers a cascade of signaling pathways that culminate in the immune response. Understanding these detailed interactions provides critical insights into the principles of immunology and has profound implications for vaccine development.

Fig. 6 presents the RMSD for the TLR3 protein and the TLR3-vaccine complex across a molecular dynamics simulation time frame of 50 ns. The RMSD values are plot-

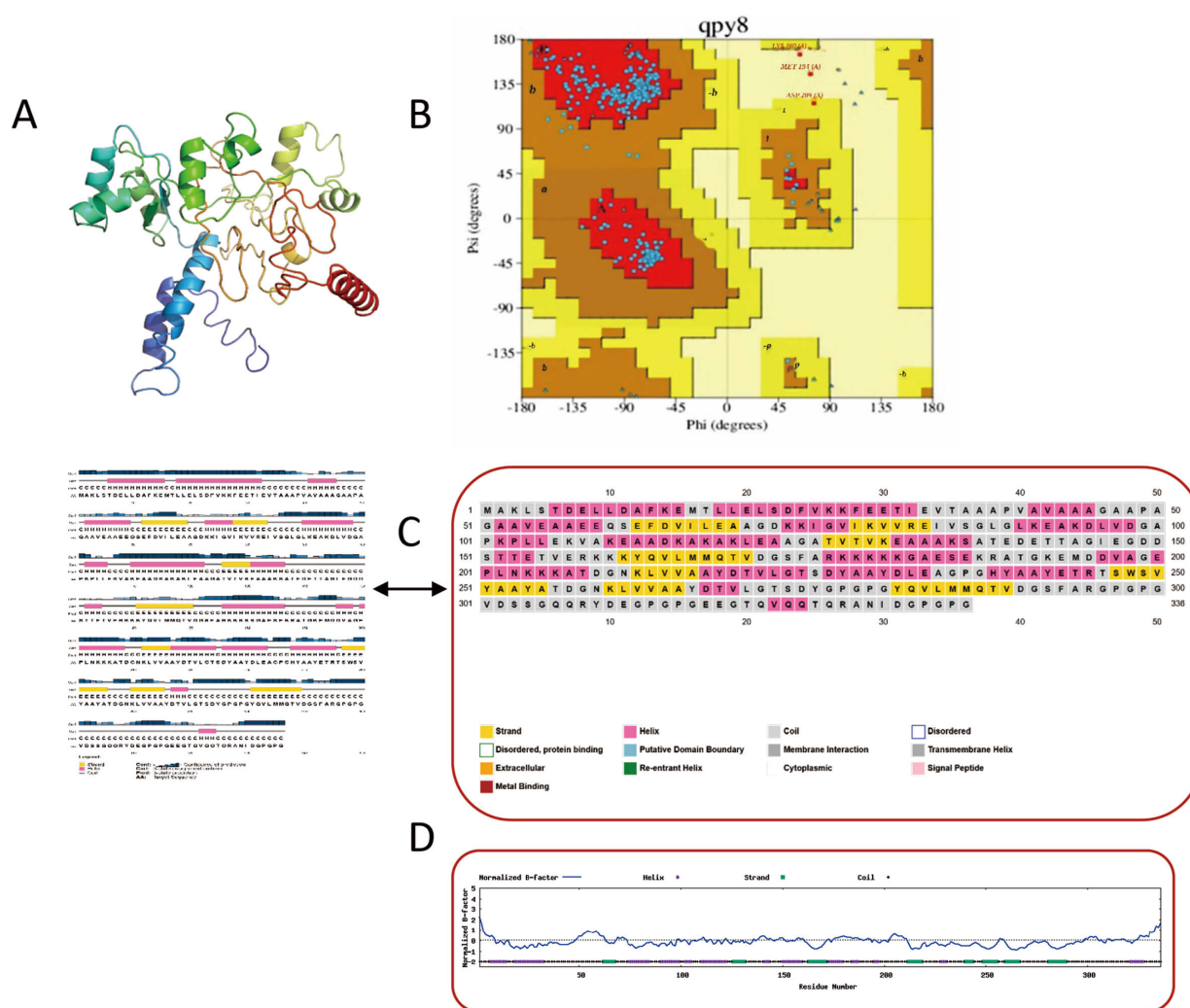


Fig. 4. Structure prediction and refinement. (A) 3D structure of the vaccine construct. (B) Ramachandran plot for the multi-epitope vaccine design against *Corynebacterium diphtheriae*, showing the distribution of phi (ϕ) and psi (ψ) torsion angles of the amino acid residues. Regions are color-coded as red for the most favored regions [A, B, L], brown for additional allowed regions [a, b, l, p], yellow for generously allowed regions [~a, ~b, ~l, ~p], with individual markers indicating disallowed regions. (C) Predicted secondary-structure composition of the vaccine construct using PSIPRED, presented in graphical and sequence-aligned formats. (D) Plot of the normalized B-factor across the protein sequence.

Table 5. Refinement statistics for the initial and refined vaccine construct models.

Model	GDT-HA	RMSD	MolProbity	Clash score	Poor rotamers (%)	Rama favored (%)
Initial	1	0	1.237	0	2.1	91.6
MODEL 1	0.9271	0.486	1.052	2	0.4	97.6
MODEL 2	0.9338	0.467	1.046	2.4	0	97.9
MODEL 3	0.9345	0.46	1.021	2.2	0.4	97.9
MODEL 4	0.933	0.445	0.908	1.6	0.4	98.2
MODEL 5	0.9353	0.463	1.101	2	0	97.3

Global Distance Test - High Accuracy (GDT-HA) scores indicate the structural closeness to the initial model. Root Mean Square Deviation (RMSD) measures the average distance between the atoms of superimposed proteins. MolProbity score assesses the overall quality of the protein structure. Clash Score quantifies the number of steric clashes. Poor Rotamers percentage indicates the proportion of amino acid side chains in less favourable conformations. Rama favoured percentage represents the proportion of residues in the favoured regions of the Ramachandran plot.

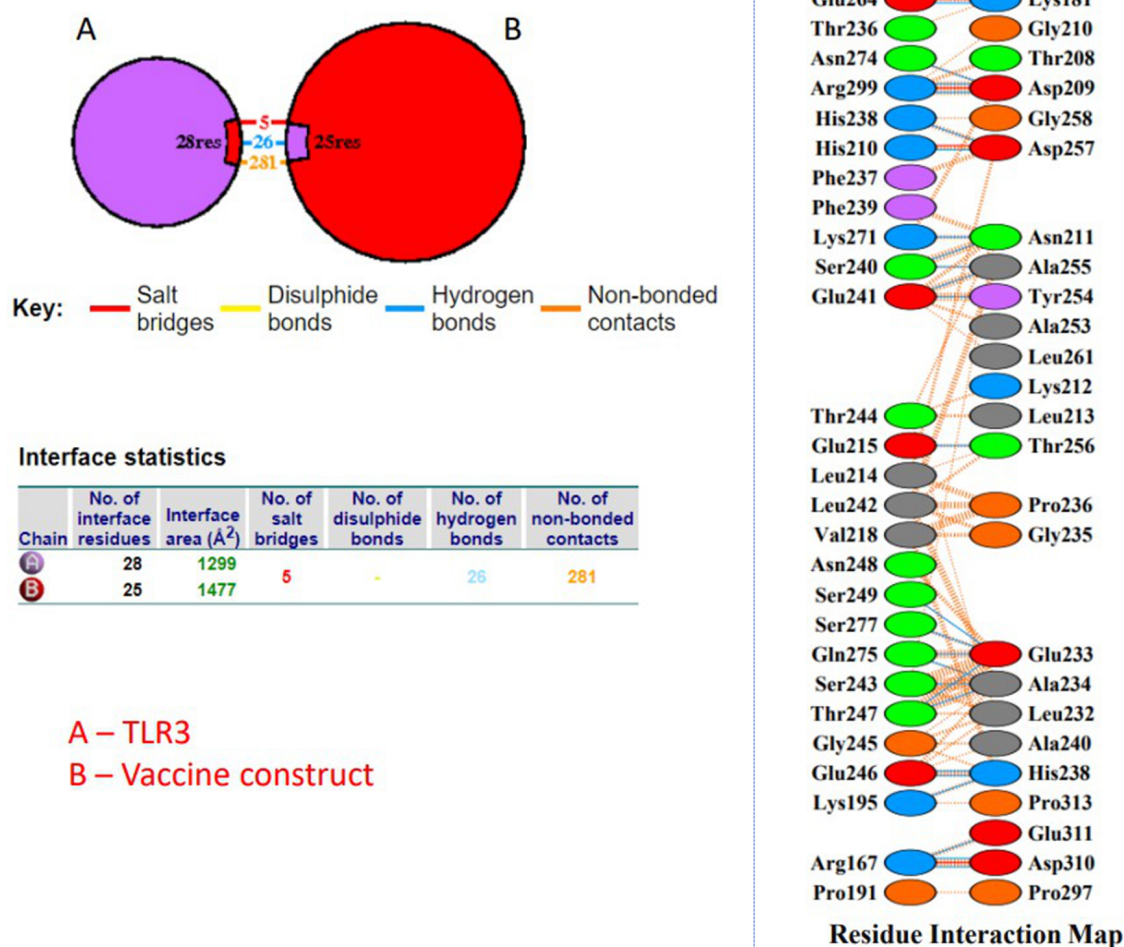


Fig. 5. Molecular interaction interface between TLR3 and the vaccine construct. The left panel summarizes the interface and interaction statistics, while the right panel presents the residue interaction map. Chain A represents TLR3, and Chain B represents the vaccine construct. The interface comprises 28 residues from Chain A and 25 residues from Chain B, with interface areas of 1299 Å² and 1477 Å², respectively. The complex contains 5 salt bridges, 26 hydrogen bonds, and 281 non-bonded contacts. Salt bridges, hydrogen bonds, and non-bonded contacts are represented by red, blue, and orange lines, respectively.

ted on the Y-axis in nanometers (nm), while the X-axis represents the simulation time. The black trace indicates the RMSD of the standalone TLR3 protein, while the red trace represents the TLR3–vaccine complex.

During the initial phase (approximately 0–10 ns), a sharp rise in RMSD for both traces indicates conformational adjustments as the systems approach equilibrium. Following this equilibration phase, the RMSD traces stabilize, oscillating within a narrow range of approximately 0.65–0.95 nm for the TLR3–vaccine complex. These minor fluctuations are characteristic of a dynamically stable system at equilibrium, reflecting the inherent thermal motion and conformational sampling of the protein–ligand complex rather than indicating instability. Notably, the TLR3–vaccine complex demonstrates a comparable level of structural stability to the standalone TLR3 protein, as indicated

by the overlay of the two traces. This convergence in RMSD profiles suggests that the binding of the vaccine construct does not destabilize the receptor, and the complex maintains its structural integrity throughout the simulation period (Fig. 7).

To further evaluate the per-residue flexibility of the TLR3–vaccine complex, RMSF analysis was performed (Fig. 8). The RMSF values provide information about the dynamic behavior of individual residues over the course of the simulation. The analysis revealed that the majority of TLR3 residues exhibit RMSF values in the range of 0.05–0.15 nm, consistent with a well-structured leucine-rich repeat domain. Higher RMSF peaks (≥ 0.25 nm) were observed at loop regions and the N- and C-terminal ends, which is expected for these structurally flexible elements. Importantly, residues at the TLR3–vaccine interface (ap-

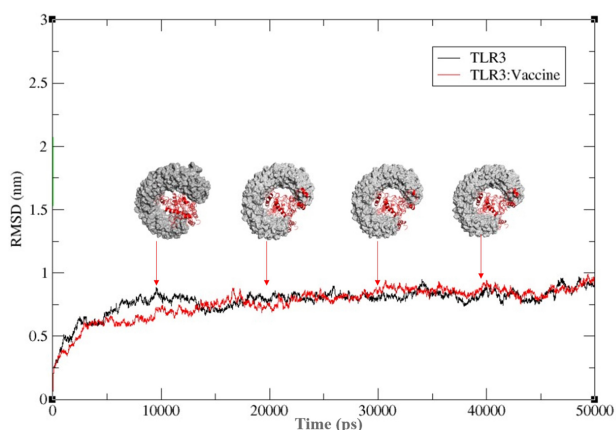


Fig. 6. RMSD comparison of TLR3 alone and TLR3:vaccine complex. This RMSD plot illustrates the stability of the TLR3 protein on its own (black line) and in complex with a vaccine (red line) throughout a 50,000 ps (50 ns) molecular dynamics simulation. The Y-axis represents the RMSD in nanometers (nm), and the X-axis indicates simulation time in picoseconds (ps).

proximately residues 390–460 of TLR3) displayed notably low RMSF values (≤ 0.12 nm), indicating restricted flexibility and stable binding at the interaction site. For the vaccine construct, the majority of residues exhibited RMSF values between 0.10 and 0.20 nm, with elevated values at the terminal regions (≥ 0.35 nm) and loop regions connecting different epitope segments. The interface residues of the vaccine (approximately residues 75–125 of the vaccine chain) showed reduced fluctuations, further supporting the stability of the docked complex. These findings collectively demonstrate that the vaccine–TLR3 interaction is maintained throughout the simulation, with the binding interface remaining structurally rigid while allowing physiologically normal flexibility in non-interface regions.

Structural compactness and regular packing of secondary-structure elements (alpha-helices and beta-sheets) in the tertiary structure of proteins are important determinants of their structural stability. These features were examined in the modelled vaccine–TLR3 complex by plotting the radius of gyration (Rg) profile over time (Fig. 9). The average Rg of 2.65 nm was observed for the complex, indicating a relatively compact structure. An initial peak of approximately 3.0 nm at around 1000 ps indicated transient expansion of the structure, possibly associated with thermal relaxation or rearrangement of the protein subunits. Following this initial expansion, the Rg decreased and subsequently fluctuated around 2.6–2.7 nm throughout the remainder of the simulation, suggesting that the complex maintained a relatively stable and compact conformation. Overall, the Rg profile supports the structural stability of the vaccine–TLR3 complex over the 50 ns simulation period.

Fig. 10 shows the trace of the evolution of hydrogen bonds during the course of the simulation. The number of hydrogen bonds within the vaccine–TLR3 complex over the course of a 50,000 picosecond (ps) molecular dynamics simulation. The y-axis quantifies the number of hydrogen bonds, which are indicative of the stability and interactions within the complex. The plot starts with a sharp increase, suggesting the initial formation of hydrogen bonds as the complex settles into a stable configuration. Throughout the simulation, the number of hydrogen bonds fluctuates, maintaining within a range that indicates a dynamic yet stable interaction network between the vaccine and TLR3. Such sustained hydrogen bonding is essential for the structural integrity and biological functionality of the vaccine–TLR3 complex.

3.6 Codon Adaptation and In Silico Cloning

E. coli K-12 was chosen as the expression system for the constructed vaccine candidate. The codon adaptation machinery of JCAT (Technical University of Braunschweig, Braunschweig, Germany) was used to predict the adaptability of the vaccine sequence to the cellular machinery of the chosen organism. Adapting the vaccine into *E. coli* K-12 strain predicted the GC content of 52.92%, and a codon adaptability index (CAI) (Fig. 11) of the improved sequence was 0.96. The process of *in silico* cloning of the final vaccine into the pET28a(+) vector was conducted using SnapGene 7.0.3 software (GSL Biotech LLC, Chicago, IL, USA) (Fig. 12). The 1008-bp codon-optimized sequence encoding the 336-amino-acid vaccine construct was inserted into the pET-28a(+) expression vector between the NdeI and XhoI restriction sites. The resulting recombinant plasmid, pET-28a(+)-CD_MEVC, was 6304 bp in length.

3.7 Immune Simulation

The virtual immune system simulation revealed appreciable activity of B-cells and T-cells, which was consistent with the actual immune responses. Primary immune response was observed with the increased levels of IgM at the start, indicating primary immune response. Moreover, secondary and tertiary immune responses were elicited comparatively higher than the primary response. The activity of the immunoglobulins (i.e., IgG1 + IgG2, IgM, and IgG + IgM antibodies) was normal at high levels with a consistent reduction in the vaccine's concentration. The high levels of triggered B-cells and memory B-cell formation were observed, indicating an effective, long-lasting immune response generated by the vaccine (Fig. 13). Additionally, greater amounts of HTL and CTL were seen, as well as the generation of memory Th and Tc, which is critical for initiating the immune response (Fig. 14). During the vaccine's introduction, considerable quantities of T regulatory cells were seen, as well as continuous and enhanced proliferation of macrophages and dendritic cells. Higher levels of cytokines such as IL-2 and IFN- γ were also detected (Fig.

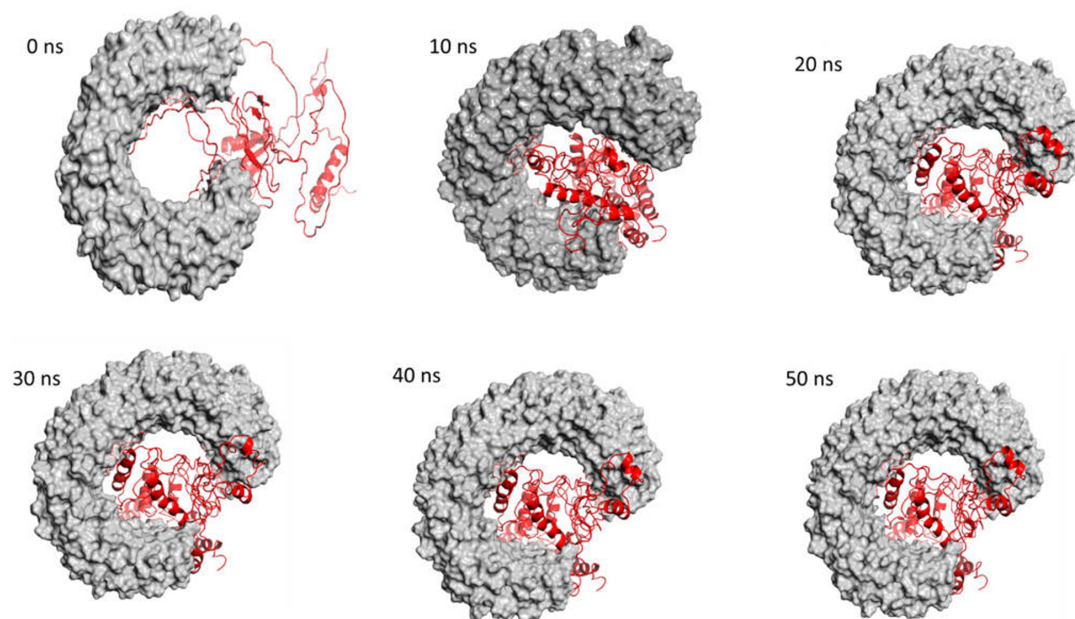


Fig. 7. Snapshots of molecular dynamics simulation of a TLR3–vaccine complex at various time points (0 ns, 10 ns, 20 ns, 30 ns, 40 ns, and 50 ns). The complex is depicted with TLR3 in a surface representation (gray) and the vaccine construct in a ribbon diagram (red).

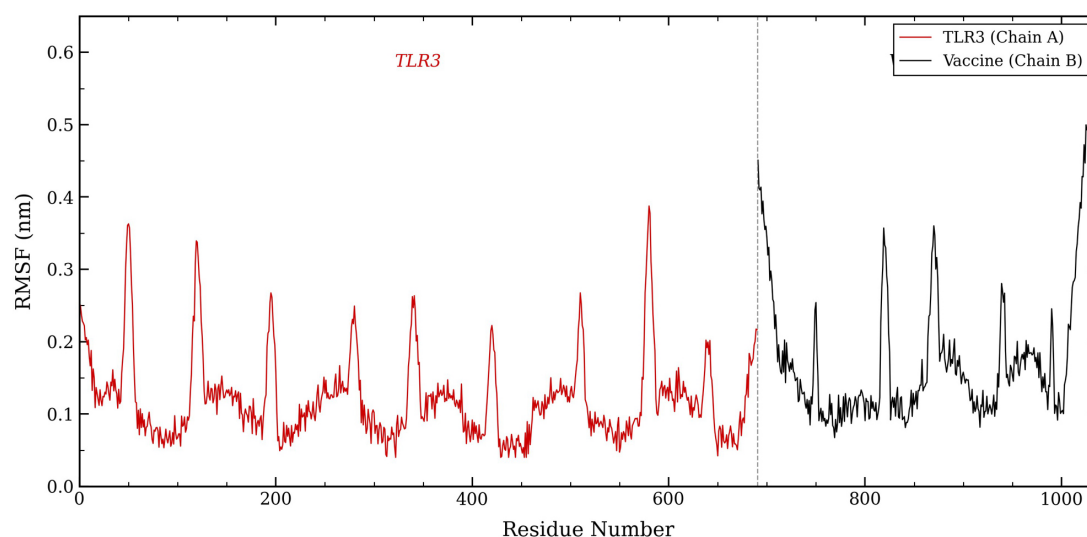


Fig. 8. Root mean square fluctuation (RMSF) analysis of the TLR3–vaccine complex from 50 ns molecular dynamics simulation. The RMSF values (Y-axis, nm) are plotted against residue number (X-axis). The red trace represents TLR3 (Chain A, residues 1–690) and the black trace represents the vaccine construct (Chain B, residues 691–1026). The dashed vertical line indicates the boundary between TLR3 and the vaccine construct. Lower RMSF values at the binding interface indicate stable intermolecular interactions.

15). These findings suggested that the proposed vaccine construct generated a good immune response.

4. Discussion

The findings of the current study highlight the promising potential of *in silico* vaccine design against *Corynebacterium diphtheriae*. The analyses delineate the substantial potential of the designed multi-epitope vaccine against

Corynebacterium diphtheriae. The selection of antigens—based on their antigenicity scores and physicochemical properties—highlights the intricate balance between immunogenic potential and structural stability essential for an effective vaccine. The chosen antigens, including a putative fimbrial subunit, transferase, and sortase-like protein, exhibit characteristics that enhance their prospects as vaccine candidates, such as high antigenicity scores and favorable physicochemical attributes like molecular weight, theoretic-

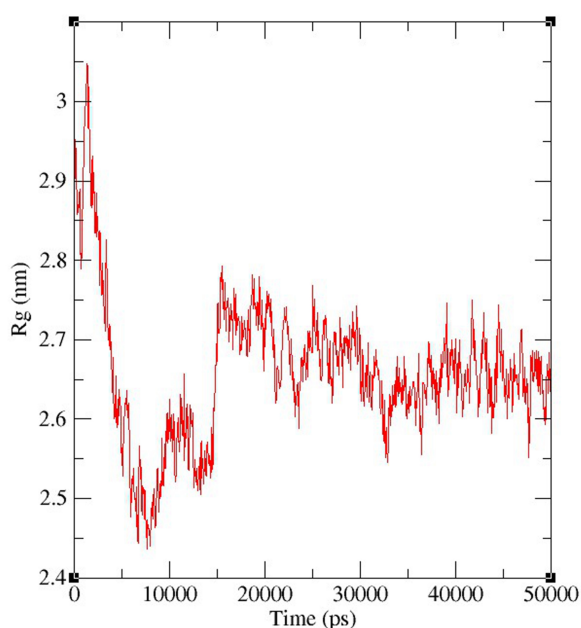


Fig. 9. Temporal evolution of structural compactness in the vaccine–TLR3 complex.

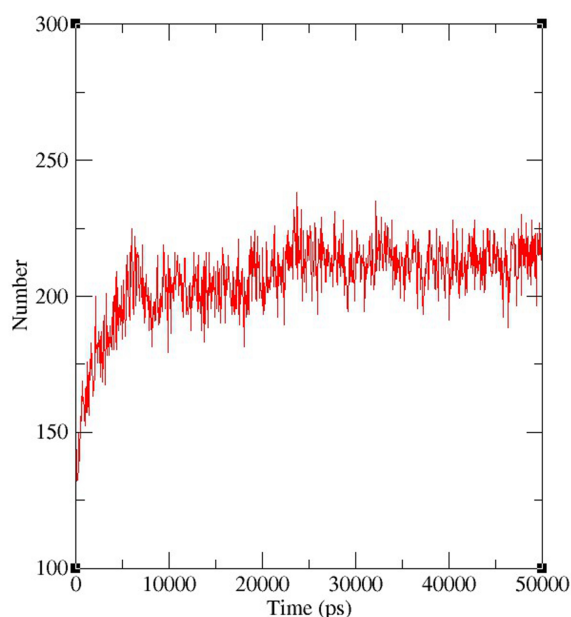


Fig. 10. Hydrogen bond dynamics in vaccine–TLR3 complex simulation.

cal pI, and stability profiles. These findings align with the foundational principles of vaccine design, where the goal is to elicit a robust immune response without compromising the vaccine's stability or safety [62]. The vaccine construct, informed by meticulous *in silico* analyses of antigenicity, physicochemical properties, and structural modeling, underscores the innovative approach of employing computational methodologies for vaccine design [63].

The selection of specific antigens, such as Putative phage capsid protein and others, is consistent with strategies emphasized in similar studies for enhancing immunogenicity [64]. The initial allergenicity assessment using AllerTOP, followed by antigenicity testing with VaxiJen, aligns with the methodology used in contemporary vaccine research [65]. With the improved vaccine construct's anticipated non-allergenicity, high population coverage, antigenicity, stability, and selectivity, the multi-epitope structure that was intended may be a good option for preventing *C. diphtheriae* infections [66]. The decision to include sixteen T-cell and ten B-cell epitopes, as detailed in Table 2, is in accordance with the multi-epitopic approach for eliciting a broad immune response [67].

The strategic use of linkers and the inclusion of an adjuvant in the vaccine construct further illustrate the nuanced approach to optimizing the vaccine's immunogenicity and physical properties [68]. The employment of specific linkers to connect CTL, HTL, and B-cell epitopes ensures the preservation of epitope structure and function, facilitating efficient immune system recognition and response [69]. This design strategy is pivotal in constructing a vaccine that not only targets multiple immune system components but also maintains structural integrity and stability [70].

Several *in silico* attempts to create preventative vaccines produced much less coverage across the population, and some were not evaluated at all for this metric [71]. The scope of relevance for these new epitopes represents compelling progress in the discovery of *C. diphtheriae* vaccines [72]. The remarkable population coverage as observed in Table 3 is crucial for a global vaccine strategy. The physicochemical properties of the designed peptide, including the instability index and aliphatic index, indicate a robust and stable construct [73]. The high population coverage analyzed using IEDB, and the detailed structural analysis, demonstrate a refined and favorable vaccine structure [74]. This level of refinement and validation is critical for ensuring the effectiveness and safety of the vaccine.

The molecular interaction of the vaccine construct with TLR3, as depicted in Fig. 5, is a critical aspect of ensuring vaccine efficacy. The optimal model chosen based on binding energy and a balanced coefficient resonates with the approaches for enhancing immunogenicity and target specificity in recent immunological studies. While TLR3 is classically associated with viral dsRNA recognition, its role in recognizing endogenous damage-associated molecular patterns released during tissue injury—a hallmark of diphtheria pathogenesis—makes it a biologically relevant target for this study. Future studies should also explore docking with additional TLRs (such as TLR2, TLR4, and TLR9) that are more directly implicated in responses to Gram-positive bacterial components, including lipoproteins (TLR2) and unmethylated CpG DNA (TLR9), to provide a more comprehensive understanding of the vaccine's

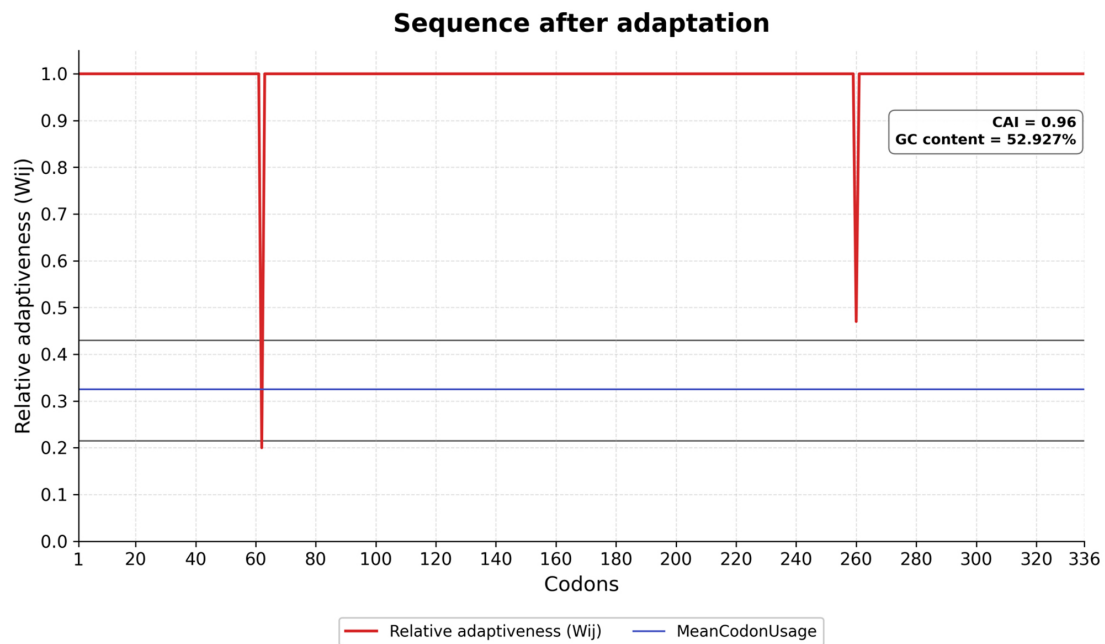


Fig. 11. Codon adaptation of the designed vaccine sequence in *E. coli* K-12.

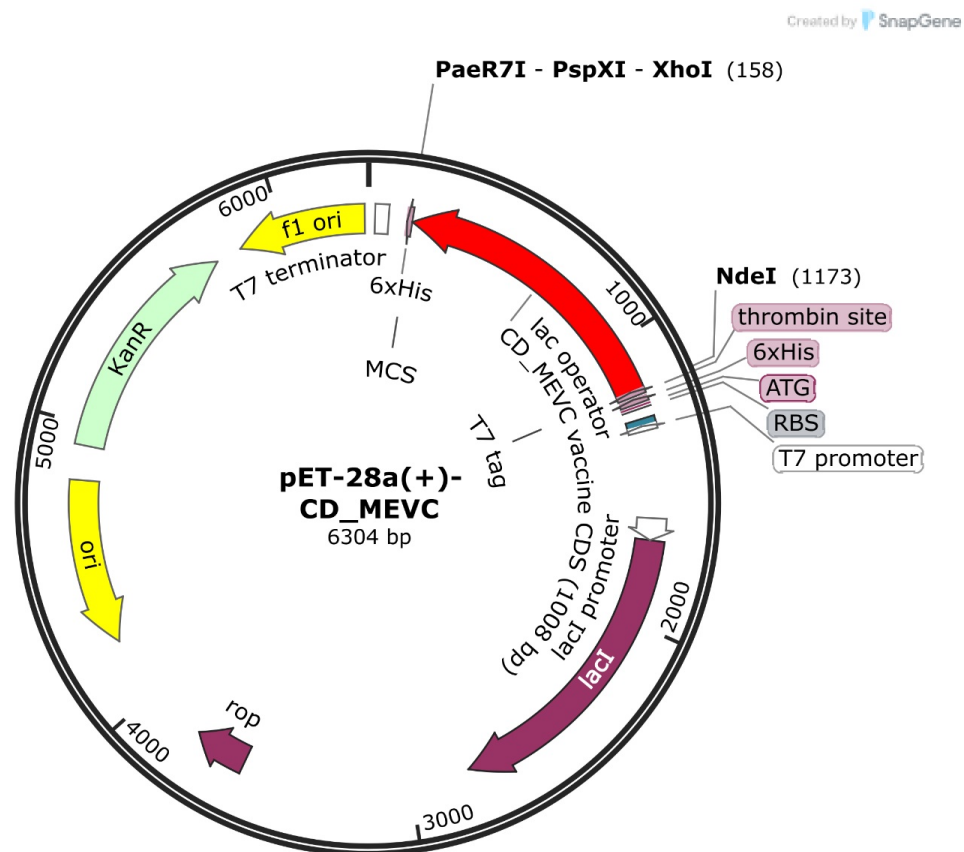


Fig. 12. *In silico* cloning of the codon-optimized vaccine construct into pET-28a(+). The 1008-bp vaccine coding sequence was inserted between the NdeI and XhoI restriction sites downstream of the T7 promoter. CD_MEVC, *Corynebacterium diphtheriae* multi-epitope vaccine construct.

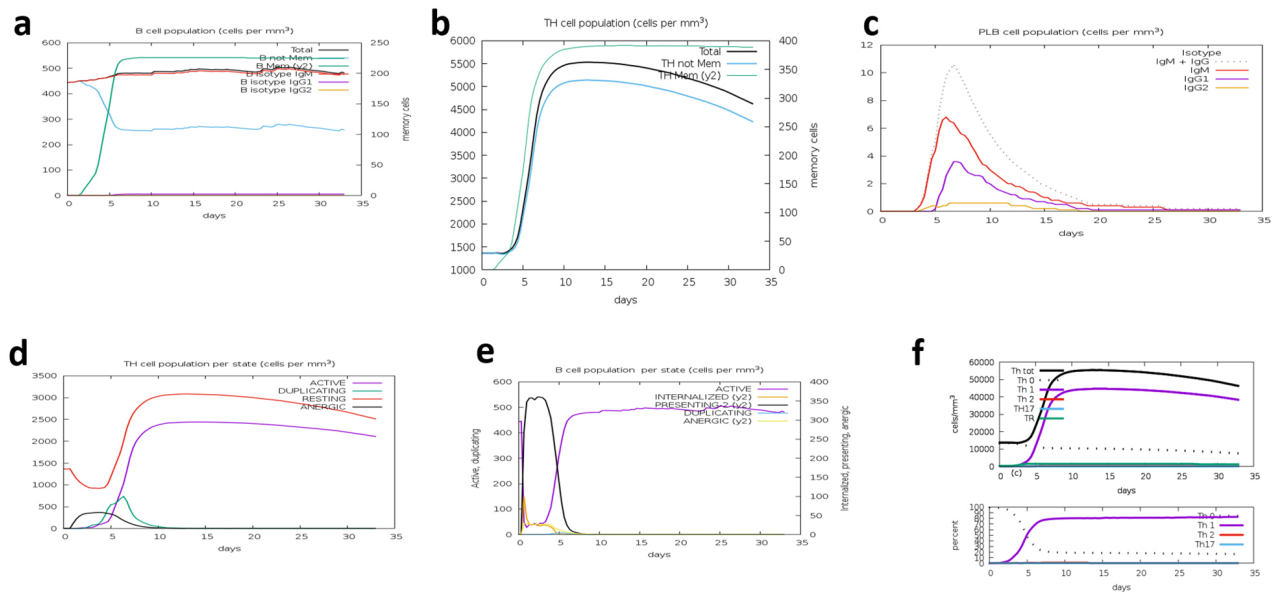


Fig. 13. Immune simulation of B-cell, plasma B-cell, and T-helper-cell responses. The plots show (a) B-cell population, (b) T-helper (TH) cell population, (c) plasma B-cell (PLB) population, (d) TH-cell population by state, (e) B-cell population by state, and (f) TH-cell population by subtype over the course of the immune simulation. For the cell-state plots, Act = active, Intern = internalized the antigen, Pres II = presenting on MHC class II, Dup = in the mitotic cycle, Anergic = anergic, and Resting = not active.

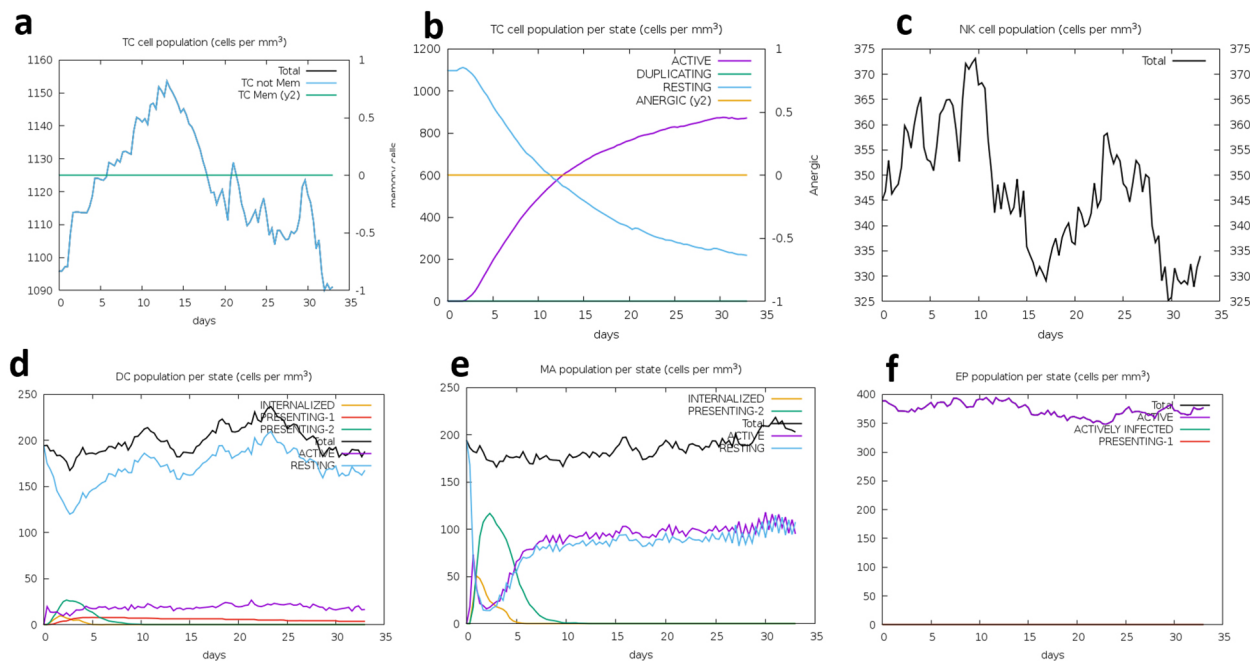


Fig. 14. Immune simulation of cytotoxic T-cell, natural killer cell, macrophage, dendritic-cell, and B-cell responses. The plots show (a) cytotoxic T-cell (TC) population, (b) TC-cell population by state, (c) natural killer (NK) cell population, (d) macrophage (MA) population by state, (e) dendritic-cell (DC) population by state, and (f) B-cell population by state over the course of the immune simulation. For the cell-state plots, Act = active, Intern = internalized the antigen, Pres II = presenting on MHC class II, Dup = in the mitotic cycle, Anergic = anergic, and Resting = not active.

interaction with the innate immune system. The immune simulation results offer promising insights into the vaccine's capacity to provoke a comprehensive immune re-

sponse [75]. The observed primary, secondary, and tertiary immune responses, characterized by the activation of B-cells and T-cells and the production of various im-

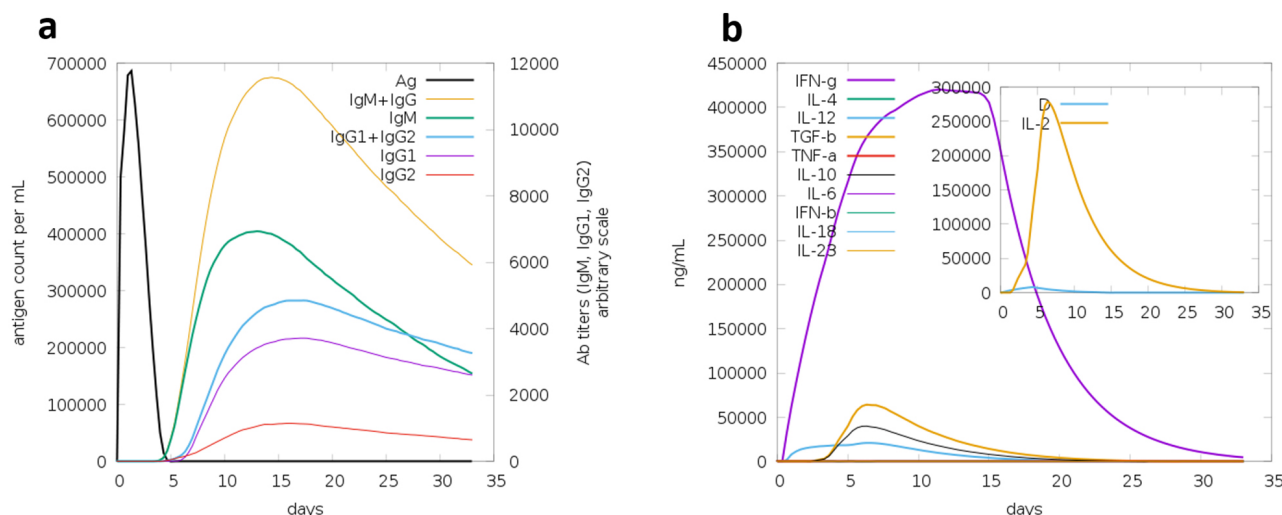


Fig. 15. Immune response simulation showing antigen, immunoglobulin, immunocomplex, cytokine, and interleukin responses. (a) Antigen, immunoglobulins, and immunocomplexes. (b) Concentration of cytokines and interleukins. The inset plot shows the danger signal together with leukocyte growth factor IL-2.

munoglobulin classes, underscore the vaccine's potential efficacy [76]. The generation of memory B-cells and T-cells suggests that the vaccine could confer long-lasting immunity against *Corynebacterium diphtheriae*, a crucial aspect of effective vaccine design [77].

The molecular docking and dynamics simulation results, particularly the interactions between the vaccine construct and TLR3, provide a molecular basis for the observed immunological responses. The detailed analysis of hydrogen bonds, non-bonded contacts, and salt bridges elucidates the structural compatibility and potential for strong, specific interactions that can trigger the desired immune signaling pathways [78]. These findings highlight the importance of molecular-level interactions in the design and effectiveness of vaccines [79]. The stability indices (RMSD, RMSF, radius of gyration) measured (Figs. 6,8,9) suggest a stable interaction, consistent with findings on the importance of stability in vaccine efficacy [80]. The RMSF analysis, in particular, demonstrated that the binding interface residues maintain low fluctuations (≤ 0.12 nm for TLR3 interface residues), confirming the stability and specificity of the vaccine-TLR3 interaction throughout the 50 ns simulation.

Compared with the currently available DTaP and Tdap toxoid vaccines, the multi-epitope vaccine construct designed in this study offers several theoretical advantages. First, unlike the traditional toxoid-based vaccines, which target primarily the diphtheria toxin (a single virulence factor), the present construct incorporates epitopes from multiple conserved structural proteins (putative fimbrial subunit, transferase, and sortase-like protein). This broader antigenic repertoire may confer protection against both toxigenic and non-toxigenic strains of *C. diphtheriae*, the lat-

ter of which have been increasingly implicated in invasive infections such as endocarditis and septicemia and are not addressed by current toxoid-based vaccines. Second, the multi-epitope approach uses only computationally selected, non-allergenic, and non-toxic peptide fragments, potentially reducing the risk of adverse reactions compared to whole toxoid preparations, which may contain residual formaldehyde and other chemical modifiers used in the detoxification process. Third, given the emergence of antibiotic-resistant *C. diphtheriae* strains reported during the Nigerian outbreak, a vaccine targeting multiple conserved structural proteins could provide a complementary preventive strategy independent of antibiotic susceptibility. However, these advantages are currently theoretical and require experimental validation to confirm any clinical superiority over existing vaccines.

The adaptation of the vaccine into *E. coli* K-12, with an impressive CAI of 0.96 (Fig. 11), highlights the suitability of the vaccine for efficient expression [81]. The immune simulation results, particularly the heightened activity of B-cells and T-cells as shown in Figs. 13,14,15, suggest a potent and long-lasting immune response, underlining the importance of eliciting both primary and memory immune responses [82]. The *in silico* cloning into the pET28a(+) vector and the subsequent codon adaptation for *E. coli* K-12 expression system signify a pivotal step towards the practical application and mass production of the vaccine [83]. The high CAI and appropriate GC content indicate that the vaccine construct is well-suited for expression in the chosen host, which is crucial for scalable vaccine production [84].

The development of a multi-epitope vaccine against *Corynebacterium diphtheriae* may address the critical need for more effective vaccination strategies, particularly in

light of the recent outbreak in Nigeria and other regions with suboptimal vaccination coverage [6]. The ability to induce a broad and long-lasting immune response offers the potential to significantly reduce the incidence and severity of diphtheria outbreaks, contributing to global health security and disease control efforts [85].

5. Conclusions

This study presents a comprehensive approach to the *in silico* design of a multi-epitope vaccine against *Corynebacterium diphtheriae*, aligning with and contributing to the current body of research in computational vaccine design. The vaccine construct demonstrated favorable immunogenic, physicochemical, and structural properties, including robust interactions with TLR3 as confirmed by molecular docking, MD simulation, and RMSF analysis. Overall, the constructed multi-epitope vaccine represents a promising advancement of the preventive options for *C. diphtheriae*. However, it is insufficient to use *in silico* results as the only indicator of vaccine feasibility. The predictions presented in this study serve as a preliminary but essential step, providing a rational foundation for future experimental validation. Specifically, future work should include peptide synthesis, *in vitro* immunogenicity assays (e.g., ELISPOT, binding assays), *in vivo* animal immunization and challenge studies, and extended molecular dynamics simulations (beyond 50 ns) to further assess the long-term stability of the vaccine–TLR3 complex.

Availability of Data and Materials

The genomic sequences used in this study are publicly available in the NCBI GenBank database under the accession numbers listed in Table 1. All bioinformatics tools and servers used are publicly accessible and have been cited in the Methods section. The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

FDO and OAO conceived and designed the study. FDO performed the computational analyses and acquired the data. OOO contributed to the study design and interpretation of the population coverage and immune simulation data. OO contributed to the interpretation of the molecular docking and dynamics simulation results and critically reviewed the methodology. OAO supervised the study. FDO wrote the initial draft of the manuscript. All authors contributed to editorial revisions, read, and approved the final manuscript, and agree to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflicts of interest.

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