Original Article

In silico design of a multi-epitope rabies vaccine candidate incorporating African HLA diversity: A reverse vaccinology approach

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Abstract

Rabies remains a neglected zoonotic disease with disproportionately high incidence and mortality across African regions, highlighting the need for improved and population-tailored preventive strategies. The aim of this study was to design and evaluate a multi-epitope rabies vaccine candidate targeting the rabies virus glycoprotein using a reverse vaccinology and immunoinformatics approach, with consideration of African human leukocyte antigen (HLA) allele diversity. A total of eleven cytotoxic T-lymphocyte (CTL), nine helper T-lymphocyte (HTL), and nine B-cell linear epitopes were predicted and subsequently filtered based on immunogenicity, interferon-gamma (IFN-γ) induction potential, antigenicity, allergenicity, and toxicity. The selected epitopes were assembled into a vaccine construct using appropriate adjuvants and immunostimulatory linkers. Population coverage analysis demonstrated a high theoretical coverage of 99.95% across five African subregions, underscoring the advantage of region-specific vaccine design. The final construct exhibited favorable physicochemical properties, including an instability index of 29.00 and a Grand Average of Hydropathy (GRAVY) score of -0.304, indicating stability and hydrophilicity. Structural validation showed 95.6% residues in favored regions of the Ramachandran plot, with an ERRAT score of 98.86 and a ProSA Z-score of -3.26. Molecular docking with toll-like receptor 4 (TLR4) revealed strong binding interactions, including 35 hydrogen bonds and ten salt bridges. Immune simulation predicted robust humoral and cellular responses with memory cell formation, while normal mode analysis supported structural stability and flexibility. Furthermore, the construct was successfully codon-optimized (codon adaptation index: 0.97; GC content: 51%) and in silico cloned into the pET-28a(+) vector, indicating potential for expression in *Escherichia coli*. These findings support the theoretical feasibility of a population-specific rabies vaccine candidate and warrant further experimental validation.

Keywords: Rabies, multi-epitope vaccine, immunoinformatics, African HLA diversity, reverse vaccinology

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Introduction

Rabies is a fatal zoonotic disease caused by a single-stranded RNA virus belonging to the genus *Lyssavirus* within the family *Rhabdoviridae*, which induces progressive inflammation of the central nervous system and ultimately leads to death [1]. Rabies remains a significant global public health concern, causing an estimated 59,000 deaths annually worldwide, with approximately 98% of cases occurring in Africa and Asia [2]. In Africa, rabies is estimated to cause 21,000–25,000 deaths annually across 32 countries [3,4]. Human infection predominantly results from bites of infected domestic dogs, which serve as the principal reservoir. The sustained burden of rabies in Africa is exacerbated by limited public awareness, inadequate epidemiological surveillance, and suboptimal implementation of mass vaccination programs [5,6].

Modern cell culture–based rabies vaccines have demonstrated high protective efficacy when administered according to recommended protocols. However, their widespread utilization remains limited in low-income settings due to relatively high production costs, the requirement for multiple doses, dependence on cold-chain logistics, and limited access to post-exposure prophylaxis (PEP) services [1,7,8]. Beyond these logistical barriers, biological factors may also influence vaccine effectiveness. In particular, host genetic variability in human leukocyte antigen (HLA) alleles plays a central role in antigen presentation and adaptive immune activation [9]. Variations in HLA alleles can affect the magnitude of immune responses and contribute to differences in cellular immune activation across populations.

At the mechanistic level, HLA molecules determine the repertoire of peptide epitopes presented on the surface of antigen-presenting cells [10]. Variations in HLA binding grooves influence peptide-binding specificity and affinity, thereby shaping the pool of presented epitopes [11,12]. This process directly affects T-cell receptor recognition and activation of $CD4^+$ and $CD8^+$ T lymphocytes. Consequently, differences in HLA allele distribution across populations may lead to variability in epitope presentation efficiency and downstream immune responses. Therefore, incorporating population-specific HLA profiles into epitope-based vaccine design is biologically justified.

Multi-epitope vaccines developed using immunoinformatics approaches have emerged as a promising strategy for systematic prediction of T-cell and B-cell epitopes and their interactions with HLA molecules [13]. This approach facilitates early assessment of antigenicity, allergenicity, and toxicity, thereby reducing development time and cost [14,15], and has been successfully applied to multiple pathogens [16]. Multi-epitope vaccines also provide improved safety and flexibility by incorporating selected epitopes rather than whole pathogens, and can be tailored to population-specific HLA profiles [17].

African populations exhibit high genetic diversity at HLA loci due to complex evolutionary and selective pressures [18], resulting in distinct allele frequency distributions compared to non-African populations [19-21]. However, current vaccine design often relies on global HLA datasets derived predominantly from European and Asian populations, which may not accurately reflect African allele distributions [22-24]. This mismatch may limit the inclusivity of vaccine-induced immune responses.

Although several immunoinformatics-based rabies vaccine studies have been reported, most have not incorporated population-specific HLA profiles. Therefore, the aim of this study was to design a multi-epitope rabies vaccine candidate by integrating African HLA allele frequency data within a reverse vaccinology and immunoinformatics framework. Conserved $CD8^+$ T-cell, $CD4^+$ T-cell, and B-cell epitopes were identified and evaluated for their interactions with prevalent African HLA alleles, followed by the construction of a vaccine candidate with favorable predicted immunogenicity and structural properties.

Methods

Study design and approaches

This study was designed as an in silico reverse vaccinology and immunoinformatics approach to develop a multi-epitope rabies vaccine candidate incorporating African HLA diversity. The overall workflow of the study is illustrated in **Figure 1**. Briefly, the study began with the identification of rabies virus antigenic proteins, followed by the analysis of HLA allele distribution across African populations to establish a relevant immunogenetic framework. Subsequently, cytotoxic T lymphocyte (CTL), helper T lymphocyte (HTL), and linear B-cell epitopes were predicted and screened based on multiple immunological and safety criteria. The selected epitopes were then used to construct a multi-epitope vaccine incorporating appropriate adjuvants and linkers. The designed construct was further evaluated through physicochemical characterization, structural modeling, and validation analyses. In addition, immune simulation, molecular docking, and normal mode analysis were performed to assess the theoretical immunological behavior and structural stability of the construct. Finally, codon optimization and in silico cloning were conducted to evaluate the potential expression of the vaccine in a suitable host system. Detailed methodologies for each step are described in the subsequent sections.

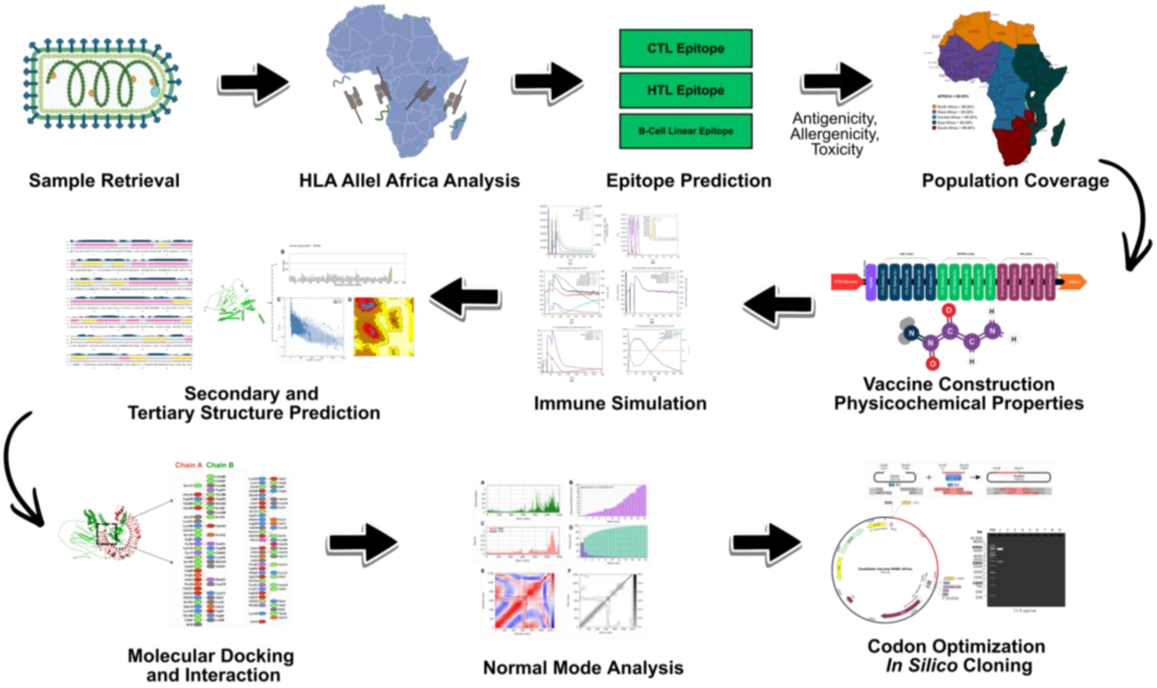

Figure 1. Workflow of the vaccine design strategy using immunoinformatics and reverse vaccinology pipeline.

HLA allele frequency analysis

HLA allele frequency data were obtained from the Allele Frequency Net Database (AFND) (http://allelefrequencies.net/) [25]. Records corresponding to African populations were screened, and alleles were selected based on their recurrence across multiple independent datasets and studies [18,21,26]. Rather than applying a fixed frequency threshold, alleles consistently reported with relatively high frequencies across different African population groups were prioritized to capture broadly representative HLA diversity. Due to heterogeneity in dataset size and reporting formats within AFND, weighting by population size was not performed. This approach was intended to minimize bias from single-population datasets while retaining commonly distributed alleles across the continent.

T-cell epitope prediction

T-cell epitope prediction was carried out to identify putative peptides capable of binding to MHC class I and class II molecules using a validated in silico framework. using the next-generation T-cell epitope prediction pipeline available via the Immune Epitope Database (IEDB) (https://nextgen-tools.iedb.org/), employing the NetMHCpan 4.1 EL algorithm, which integrates peptide–major histocompatibility complex (MHC) binding affinity with eluted ligand data [27,28]. The Immune Epitope Database (IEDB) is a comprehensive, experimentally curated repository of immune epitopes and provides validated computational tools for epitope prediction. The prediction algorithms implemented in IEDB, including NetMHCpan, are trained on large datasets of experimentally determined peptide–MHC binding interactions and have been widely benchmarked for predictive performance. Nevertheless, these predictions remain probabilistic and may include false positives or false negatives, as computational models cannot fully capture the complexity of antigen processing, epitope presentation, and immunodominance in vivo [29]. For MHC class I, nonameric (9-mer) peptides were predicted, whereas MHC class II predictions focused on 15-mer peptides, consistent with canonical peptide lengths presented by each MHC class [30,31]. To retain high-confidence binders, predicted epitopes were filtered using a stringent threshold, with only the top 1% of peptides selected. Binding prediction was followed by an initial screening of predicted immunogenicity using the IEDB tool (https://tools.iedb.org/immunogenicity/) and interferon-gamma (IFN-γ) induction potential responses using the IFNepitope server (https://webs.iiitd.edu.in/raghava/ifnepitope/), where epitopes yielding positive predictive scores were selected for further analysis [29,32].

Linear B-cell epitope prediction

Linear B-cell epitope prediction was performed to identify antigenic regions with the potential to be recognized by humoral immune components. Analysis was conducted using the BepiPred-2.0 algorithm, available through the B-cell epitope prediction tool of the IEDB B-cell epitope prediction tool (https://tools.iedb.org/bcell/) [33,34]. This method combines a random forest machine-learning approach trained on experimentally validated epitope data with amino acid propensity scales to predict linear B-cell epitopes. Peptide regions with predicted epitope scores above the 0.500 threshold were selected. Peptides ranging from 9 to 30 amino acids were retained to reflect commonly reported linear B-cell epitope lengths [35].

Antigenicity, allergenicity, and toxicity

The antigenic potential of the predicted epitopes was evaluated using VaxiJen v2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), an alignment-independent method based on auto cross-covariance transformation of amino acid properties. Peptides exceeding the threshold value of 0.400 were considered potentially antigenic [36]. Further epitope selection was refined by assessing allergenicity using AllerTOP v2.1 (https://www.ddg-pharmfac.net/allertop_test/). This tool employs a machine-learning approach to classify peptides as allergenic or non-allergenic based on physicochemical descriptors [37,38]. Additionally, the potential toxicity of selected epitopes was evaluated using the ToxinPred server (http://crdd.osdd.net/raghava/toxinpred/), which predicts toxic profiles using quantitative matrix and machine-learning-based models [39]. Only epitopes predicted to be antigenic, non-allergenic, and non-toxic were retained for subsequent in silico analyses.

Population coverage analysis

Population coverage analysis was performed to estimate the theoretical distribution of the selected T-cell epitopes across diverse African populations using the population coverage tool provided by the IEDB (https://tools.iedb.org/population/), which integrates epitope–HLA binding data with population-specific HLA allele frequencies [40,41]. The selected MHC class I– and class II–restricted epitopes and their corresponding HLA alleles were evaluated across five major African regions: North Africa, West Africa, Central Africa, East Africa, and South Africa. This regional stratification was applied to capture broad immunogenetic variability across the continent [42].

Vaccine construction and physicochemical properties

The multi-epitope vaccine construct was designed by assembling the selected T-cell and B-cell epitopes into a single chimeric sequence using defined linker strategies to support theoretical epitope processing and structural independence. Cholera toxin B (CTB) subunit was incorporated as an adjuvant due to its reported ability to enhance antigen uptake and immune stimulation. A pan–HLA-DR–binding epitope (PADRE) sequence was included to promote broad CD4$^+$ T-cell responses across diverse HLA class II alleles [43,44]. Linkers (AAY, GPGPG, and KK) were employed to support appropriate epitope processing and presentation by maintaining structural separation between functional elements. Specifically, AAY linkers support proteasomal cleavage and MHC class I presentation, whereas GPGPG linkers promote MHC class II epitope processing by reducing junctional immunogenicity. KK linkers were incorporated to improve flexibility and minimize structural interference between adjacent B-cell epitopes. An invasin-derived sequence was additionally incorporated to enhance host–cell interaction and antigen delivery within the constraints of an in silico design framework [45,46]. The physicochemical properties of the final construct, including molecular weight, theoretical isoelectric point, instability index, aliphatic index, and grand average of hydropathicity (GRAVY), were analyzed using the ExPASy ProtParam (https://web.expasy.org/protparam/) as a preliminary assessment of its predicted biochemical characteristics, acknowledging the purely computational nature of these analyses [47,48].

Immune simulation

Immune simulation was performed using the C-ImmSim server (https://kraken.iac.rm.cnr.it/C-IMMSIM/) to explore the theoretical immunological profile of the proposed multi-epitope vaccine construct [49,50]. The simulation modeled repeated antigen exposure with vaccine administrations at time steps 1, 84, and 170, corresponding to four-week intervals. The simulation was conducted for 1,050 time steps to assess predicted immune responses over time [51,52].

Secondary and tertiary structure prediction

Secondary structure prediction of the finalized vaccine construct was performed using PSIPRED (https://bioinf.cs.ucl.ac.uk/psipred/). PSIPRED utilizes position-specific scoring matrices derived from PSI-BLAST to estimate the distribution of α-helices, β-strands, and random coils along the amino acid sequence [53]. Further folding tendencies of this construct were understood at the sequence level. Subsequently, the three-dimensional (3D) structure of the vaccine construct was predicted using the AlphaFold Server (https://alphafoldserver.com/), a deep learning–based framework designed to generate structural models from primary amino acid sequences [54,55]. 3D model validation was carried out using ProSA (https://prosa.services.came.sbg.ac.at/prosa.php) to evaluate the overall model quality through Z-score analysis [56]. In addition, stereochemical properties were further examined using structural analysis and verification server (SAVES)(https://saves.mbi.ucla.edu/), which integrates multiple validation tools, including ERRAT and Ramachandran plot analysis. ERAAT was used to assess non-bonded atomic interactions and Ramachandran plot to evaluate backbone dihedral angle distributions [57].

Molecular docking and interaction

Molecular docking analysis was conducted to explore the potential interaction between the designed multi-epitope vaccine construct and TLR4. TLR4 was selected as a representative innate immune receptor due to its role in pathogen recognition and immune signaling, particularly its involvement in initiating downstream signaling pathways that contribute to antigen presentation and adaptive immune priming [58,59]. The docking analysis was conducted as an exploratory computational approach to evaluate potential receptor-level interactions of the designed construct, without implying direct biological engagement [60,61]. Docking simulations were performed using the ClusPro 2.0 server (https://cluspro.org/), an automated protein–protein docking platform that clusters docked conformations based on energetically favorable interactions [62]. The docked complex representing the most populated cluster was selected for further interaction analysis. Structural visualization and analysis of intermolecular interactions were carried out using PDBsum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/), focusing on hydrogen bonds and salt bridges between the vaccine construct and TLR4 [54].

Normal mode analysis

Intrinsic structural flexibility and theoretical stability of the docked vaccine–TLR4 complex were investigated by Normal mode analysis (NMA), which examines its collective molecular motions under in silico conditions. The analysis was performed using the iMODS platform (https://imods.iqf.csic.es/). This platform utilizes an elastic network model and internal coordinate framework to evaluate low-frequency normal modes associated with large-scale conformational changes [63,64]. Deformability patterns, motion stiffness, and dynamic correlations between residues were evaluated properly without relying on time-dependent molecular dynamics simulations.

Codon optimization and in silico cloning

Codon optimization of the final vaccine construct was performed using the Java Codon Adaptation Tool (JCAT) server (https://www.jcat.de/). The optimization process helps in codon usage to the host-specific preference while avoiding potential transcriptional and translational constraints, such as premature termination signals and unwanted restriction sites [65,66]. The optimized nucleotide sequence was subsequently subjected to in silico cloning analysis using SnapGene software (version 8.2; GSL Biotech LLC, San Diego, CA, USA) to evaluate cloning feasibility and expression compatibility. The optimized gene sequence was virtually inserted into the pET-28a(+) expression vector using *EcoRI* and *BamHI* restriction sites. Subsequently, agarose gel electrophoresis (1.5% agarose) was performed to validate the cloning outcome [67,68].

Results

HLA allele frequency in Africa

Analysis of HLA allele distribution across African populations identified a representative set of prevalent HLA class I and class II alleles. Alleles from the HLA-A, HLA-B, and HLA-C loci, as well as the HLA-DRB1 locus, were selected based on their recurrence across multiple African population studies [25,26]. The resulting allele panel reflects the broad immunogenetic diversity observed within the African continent. This curated HLA dataset was subsequently used for T-cell epitope prediction and population coverage analysis. The selected alleles are presented in **Table 1**.

Table 1. Representative HLA class I and class II alleles in African populations based on allele frequency data from the Allele Frequency Net Database

Locus	Allele
A	A*02:01, A*01:01, A*02:05, A*24:02, A*29:02, A*30:01, A*03:01, A*23:01, A*68:02, A*02:04, A*11:01, A*30:02, A*74:01, A*29:01
B	B*44:02, B*50:01, B*35:01, B*51:01, B*45:01, B*41:01, B*08:01, B*42:01, B*52:01, B*53:01, B*08:02, B*07:02, B*07:05, B*08:03, B*07:06, B*07:07, B*07:08, B*07:12, B*07:17
C	C*06:02, C*04:01, C*07:01, C*12:03, C*08:02, C*02:02, C*16:01, C*15:02, C*17:01, C*07:02, C*02:10, C*03:04
DRB	DRB1*01:02, DRB1*03:01, DRB1*04:02, DRB1*07:01, DRB1*08:04, DRB1*11:01, DRB1*13:01, DRB1*13:02, DRB1*13:03, DRB1*15:01, DRB1*03:02, DRB1*03:04, DRB1*11:02, DRB1*13:04, DRB1*15:03

T-cell epitope prediction

Eleven putative $CD8^+$ T-cell epitopes were identified against MHC class I molecules, derived from multiple rabies virus structural proteins, including G, L, M, N, and P [69,70]. These epitopes were selected based on stringent binding criteria and subsequent computational screening. The identified epitopes represent peptides with high predicted binding affinity to African-relevant HLA class I alleles and are presented in **Table 2**.

Table 2. Predicted major histocompatibility complex (MHC) class I–restricted $CD8^+$ T-cell epitopes derived from rabies virus structural proteins

Protein	Epitope	Immunogenicity	IFN-γ	Antigenicity	Allergenicity	Toxicity
G	GFVDERGLY	0.2040	Positive	0.7808	Non-allergen	Non-toxin
	LSAGALTAL	0.1300	Positive	0.8271	Non-allergen	Non-toxin
	ALLFVPLLV	0.1281	Positive	0.4827	Non-allergen	Non-toxin
L	HRYNRWITL	0.3862	Positive	0.4445	Non-allergen	Non-toxin
	RLYFVITEK	0.3733	Positive	1.5114	Non-allergen	Non-toxin
M	GLQIRVSAK	0.0688	Positive	1.6487	Non-allergen	Non-toxin
N	WALTGGMEL	0.0106	Positive	1.2192	Non-allergen	Non-toxin
	ETAPFVKIV	0.0501	Positive	0.4845	Non-allergen	Non-toxin
	SAIRVGTVV	0.2242	Positive	0.8708	Non-allergen	Non-toxin
P	HLQGEPIEV	0.2543	Positive	0.7991	Non-allergen	Non-toxin
	RAGLADLEM	0.1079	Positive	1.7867	Non-allergen	Non-toxin

IFN-γ: interferon-gamma

Similarly, MHC class II analysis identified nine putative $CD4^+$ T-cell epitopes with high predicted binding affinity to the selected HLA-DRB1 alleles [71]. These epitopes were derived from conserved regions of the rabies virus proteins and were retained based on consistent performance across the applied computational selection criteria. The predicted MHC class II epitopes are presented in **Table 3**.

Table 3. Predicted MHC class II–restricted $CD4^+$ T-cell epitopes derived from rabies virus proteins

Protein	Epitope	Immunogenicity	IFN-γ	Antigenicity	Allergenicity	Toxicity
G	ELKVGYISAIKVNGF	0.0196	Positive	0.7527	Non-allergen	Non-toxin
L	KESINWFITRDSNLA	0.4753	Positive	0.6164	Non-allergen	Non-toxin
M	GMNWVYKLRRTLIFQ	0.3454	Positive	0.6340	Non-allergen	Non-toxin
	DDNTEFVGLQIRVSA	0.3145	Positive	1.0003	Non-allergen	Non-toxin
	MNWVYKLRRTLIFQW	0.1732	Positive	0.7665	Non-allergen	Non-toxin
N	HSYFIHFRSLGLSGK	0.1729	Positive	1.6066	Non-allergen	Non-toxin
	AVPHSYFIHFRSLGL	0.1584	Positive	1.1069	Non-allergen	Non-toxin
	VPHSYFIHFRSLGLS	0.1305	Positive	1.2348	Non-allergen	Non-toxin
P	SGERFLKIWSQTVEE	0.1790	Positive	0.6848	Non-allergen	Non-toxin

IFN-γ: interferon-gamma

Linear B-cell epitope prediction

Linear B-cell epitope prediction identified nine putative epitopes distributed across multiple Rabies virus structural proteins, including G, L, N, M, and P. These epitopes were selected based on predicted antigenic propensity and peptide length [34]. All retained peptides exhibited favorable antigenicity and were predicted to be non-allergenic and non-toxic, supporting their potential as humoral immune targets. The identified linear B-cell epitopes and their corresponding properties are presented in **Table 4**.

Table 4. Predicted linear B-cell epitopes derived from rabies virus structural proteins

Protein	Epitope	Length	Antigenicity	Allergenicity	Toxicity
G	IPDKLGPWSPIDIH	14	1.3729	Non-allergen	Non-toxin
	NFVGYVTTTFKRKHFRPTPDA	21	1.1161	Non-allergen	Non-toxin
L	GATGGGNPRV	10	0.8421	Non-allergen	Non-toxin
	TMTGDYSPNKKVS	13	0.7130	Non-allergen	Non-toxin
N	LSGKSPYSSN	10	0.6487	Non-allergen	Non-toxin
	RTDVEGNWALTGGMELTRDPTVP	23	0.8035	Non-allergen	Non-toxin
M	ADSRGPLEGEELEYS	15	0.6459	Non-allergen	Non-toxin
	SRAGQLWSDMSLQTQRSEEDKDSSL	25	0.5485	Non-allergen	Non-toxin
P	SKKFQLLVESNK	12	0.4679	Non-allergen	Non-toxin

Population coverage

Population coverage analysis demonstrated that the selected MHC class I- and class II- restricted T-cell epitopes provided broad theoretical coverage across African populations. Regional and overall coverage estimates are presented in **Figure 2**. The highest predicted coverage was observed in West and East Africa (approximately 99.99%), followed by North and Southern Africa (99.95%), while Central Africa showed slightly lower but substantial coverage of 99.90%. These findings reflect the inclusion of commonly distributed HLA alleles across African population.

Multi-epitope vaccine construct

The multi-epitope vaccine construct was assembled by integrating the selected MHC class I, MHC class II, and linear B-cell epitopes into a single chimeric sequence using predefined linker arrangements. The construct comprised an N-terminal cholera toxin B subunit adjuvant, followed by the PADRE sequence and the ordered epitope segments connected via AAY, GPGPG, and KK linkers [43,63]. A schematic representation of the overall vaccine construct and epitope organization is illustrated in **Figure 3**.

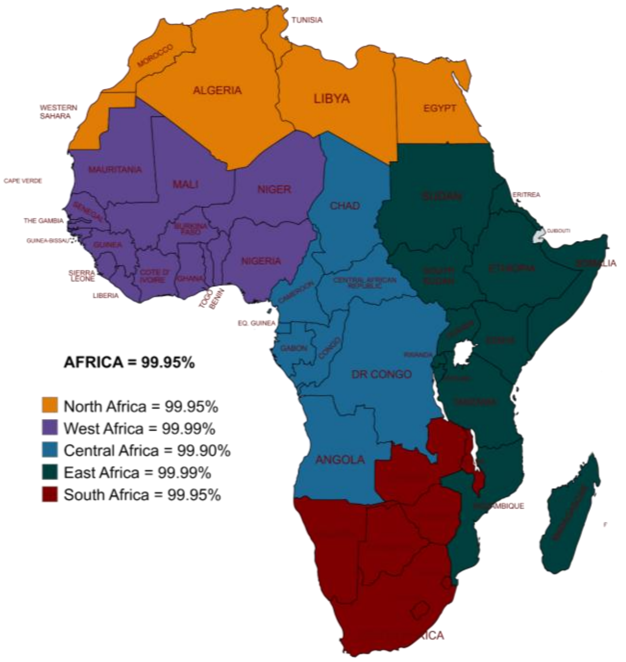

Figure 2. Predicted population coverage of the selected MHC class I- and class II-restricted T-cell epitopes across African regions.

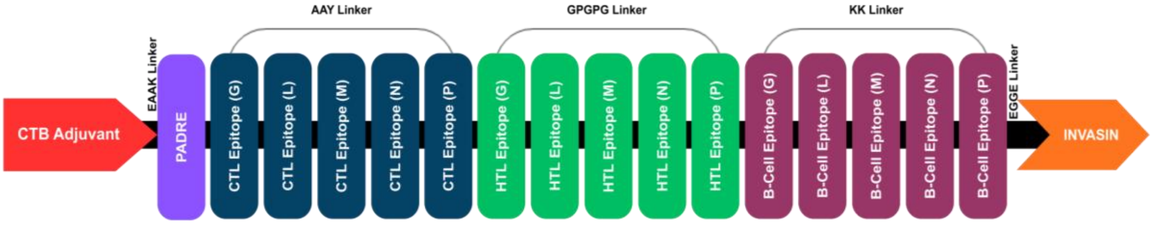

Figure 3. Schematic representation of the multi-epitope vaccine construct.

Physiochemical evaluation results

The physicochemical characteristics of the vaccine construct consisted of 633 amino acids with a molecular weight of approximately 69.38 kDa and a basic isoelectric point (**Table 5**). The instability index indicated that the construct is stable, while the aliphatic index suggested favorable thermostability. The negative GRAVY value reflects an overall hydrophilic profile. The construct was also predicted to be antigenic and non-allergenic.

Table 5. Predicted physicochemical properties of the multi-epitope vaccine construct

Property	Compound value	Remark
Number of amino acids	633	Suitable
Molecular weight	69379.65	Suitable
Theoretical isoelectric point	9.72	Basic
Chemical formula	$C_{3163}H_{4900}N_{850}O_{883}S_{13}$	-
Instability Index	29.00	Stable
Estimated half-life	>30 hours (mammalian reticulocytes, in vitro) >20 hours (yeast, in vivo) >10 hours (*Escherichia coli*)	-
Aliphatic Index	76.21	Thermostable
Grand average of hydropathicity (GRAVY)	-0.304	Hydrophilic
Antigenicity	0.6371	Antigenic
Allergenicity	Non-allergen	Non-allergen

Immune simulation

Antigen levels exhibited transient peaks after each administration followed by gradual clearance (**Figure 4A**), while antibody titers increased progressively with a shift toward IgG-dominated responses (**Figure 4B**). Expansion of B-cell populations and the formation of memory B cells were observed over time (**Figure 4C**), alongside activation of CD4$^+$ T-helper (**Figure 4D**) and CD8$^+$ cytotoxic T-cell populations (**Figure 4E**). Natural killer (NK) cell levels showed moderate fluctuations throughout the simulation period (**Figure 4F**).

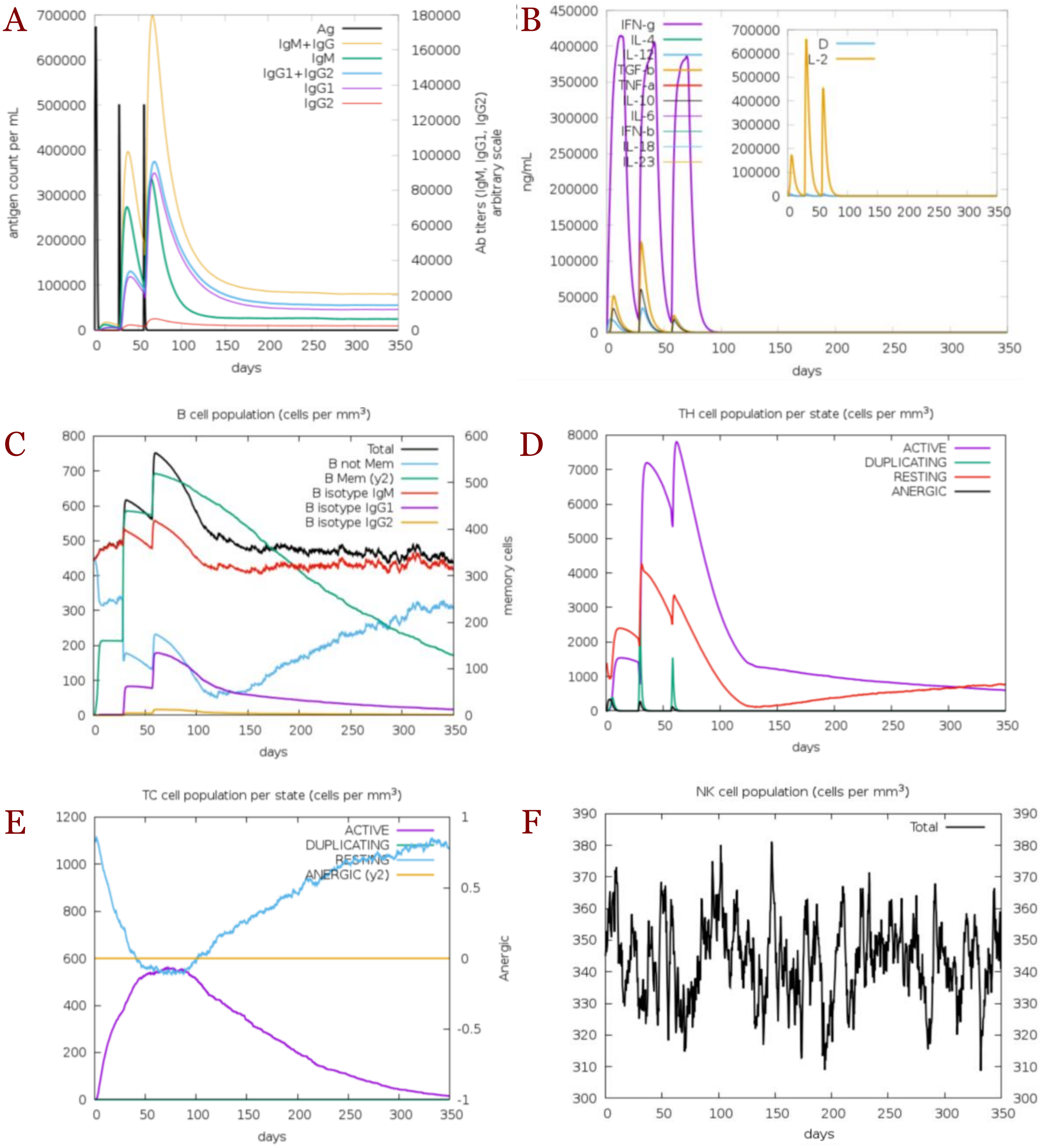

Figure 4. Simulated immune response profile of the multi-epitope vaccine construct using the C-ImmSim server, with antigen administrations at time steps 1, 84, and 170 over 1,050 simulation steps. (A) Antigen concentration dynamics, (B) antibody titers (IgM and IgG subclasses), (C) B-cell and memory B-cell populations, (D) CD4$^+$ T-helper cell populations, (E) CD8$^+$ cytotoxic T-cell populations, and (F) natural killer (NK) cell population trends.

Predicted secondary and tertiary structures of the multi-epitope vaccine construct

The predicted secondary structural elements and their positional distribution are presented in **Figure 5**. The construct exhibited a heterogeneous composition dominated by α-helices and random coils, with a smaller proportion of β-strands distributed along the sequence. This distribution reflects the composite nature of the construct, comprising multiple epitopes, linkers, and functional sequences.

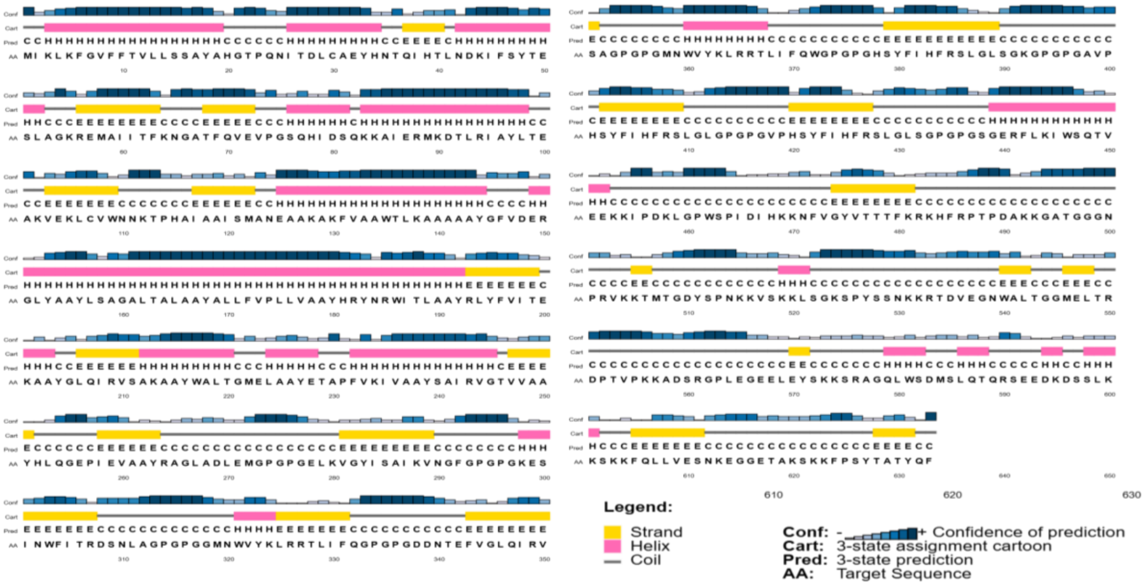

Figure 5. Predicted secondary structure of the multi-epitope vaccine construct, illustrating the distribution of α-helices, β-strands, and random coils along the amino acid sequence (PSIPRED).

The predicted 3D structure and validation results are presented in **Figure 6**. The modeled tertiary structure provides a representation of the spatial conformation of the vaccine construct (**Figure 6A**). Structural analyses indicated favorable quality metrics. ERRAT analysis yielded an overall quality factor of 98.86 (**Figure 6B**), suggesting a probable high proportion of acceptable non-bonded atomic interactions [72]. ProSA analysis produced a Z-score of -3.26 (**Figure 6C**). Ramachandran plot analysis demonstrated that 95.6% of residues were located in the most favored regions (**Figure 6D**).

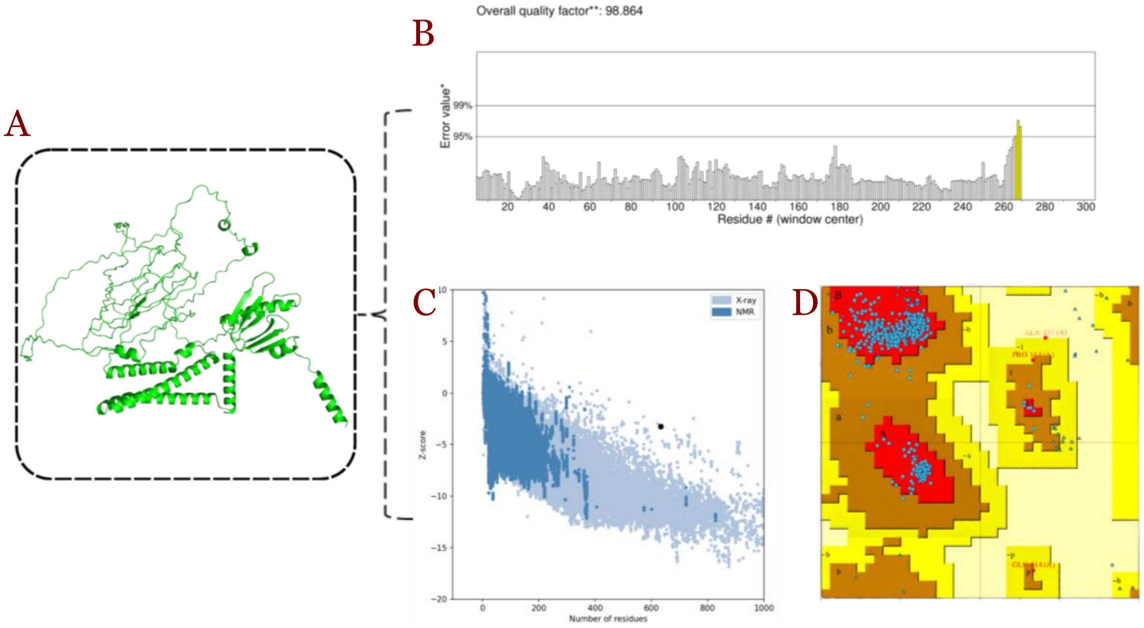

Figure 6. Tertiary structure prediction and validation of the designed multi-epitope vaccine construct. (A) Predicted 3D model of the vaccine construct generated using AlphaFold; (B) ERRAT analysis showing an overall quality factor of 98.86; (C) ProSA Z-score plot indicating a Z-score value of -3.26; and (D) Ramachandran plot analysis demonstrating that 95.6% of residues are located within the most favored regions.

Molecular docking and interaction

Molecular docking of the multi-epitope vaccine construct with TLR4 resulted in a stable protein–protein complex. Interaction analysis of the selected docked model identified ten salt bridges and

35 hydrogen bonds between residues of the vaccine construct and TLR4 [73]. These interactions suggest a strong and well-distributed contact network that may support structural compatibility between the two molecules under computational conditions. The 3D docking conformation and interaction map are illustrated in **Figure 7**.

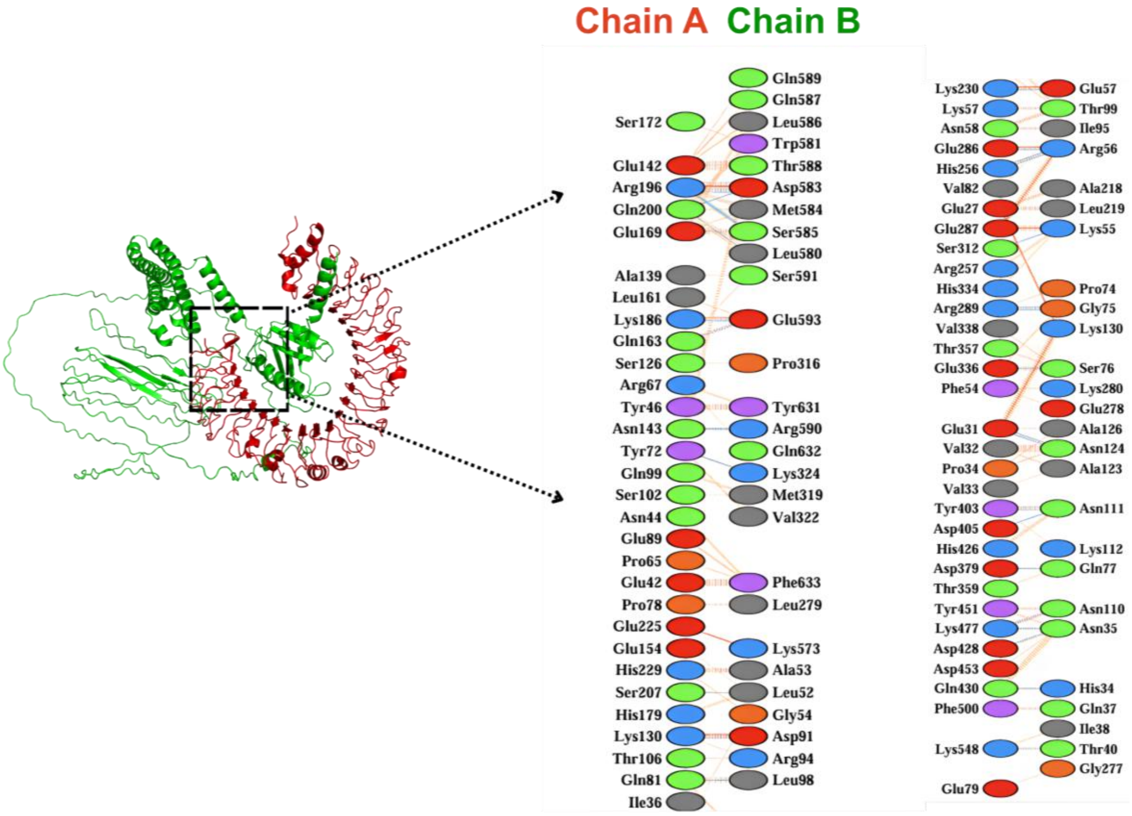

Figure 7. Molecular docking and interaction analysis of the multi-epitope vaccine construct with Toll-like receptor (TLR)-4.

Normal mode analysis (NMA)

NMA of the vaccine-TLR4 complex revealed characteristic flexibility patterns, as presented in **Figure 8**. The deformability profile indicated localized flexible regions (**Figure 8A**), while the low eigenvalue (4.61×10^{-7}) suggested potential for collective motion (**Figure 8B**). Predicted B-factor (**Figure 8C**) and variance profiles (**Figure 8D**) highlighted regions contributing to overall motion. The covariance map demonstrated coordinated and anti-correlated residue movements across the complex (**Figure 8E**) [46,59,67]. The elastic network model further illustrated a well-connected residue interaction framework (**Figure 8F**).

Codon optimization and in silico cloning

The optimized nucleotide sequence exhibited a codon adaptation index of 0.97, GC content of 51%, and a length of 1,893 bp, indicating compatibility with *Escherichia coli* (**Figure 9A**) [74,75]. The sequence was further screened to minimize potential translational constraints, including premature termination signals and undesirable restriction sites. The optimized gene was subsequently inserted in silico into the pET-28a(+) expression vector using *EcoRI* and *BamHI* restriction sites to ensure correct orientation within the multiple cloning site (**Figure 9B**) [67,68]. Simulated agarose gel electrophoresis (1.5%) confirmed the expected fragment sizes of the inserted constructs (**Figure 9C**). The overall in silico cloning strategy is illustrated in **Figure 9**.

Discussion

This study employed a reverse vaccinology framework to design a multi-epitope vaccine candidate against the rabies virus. This vaccine design is tailored specifically to the diverse HLA allele frequencies found across African populations. Through computational epitope screening, the present study identified eleven CTL, nine HTL, and nine linear B-cell epitopes with favorable immunogenicity, non-allergenicity, and antigenicity profiles. Despite the favorable predicted binding affinities of the selected epitopes, it is important to consider that MHC binding does not necessarily guarantee effective antigen presentation in vivo. Antigen processing is a complex, multi-step process involving proteasomal cleavage, peptide transport via the transporter associated with antigen processing (TAP) and loading onto MHC molecules.

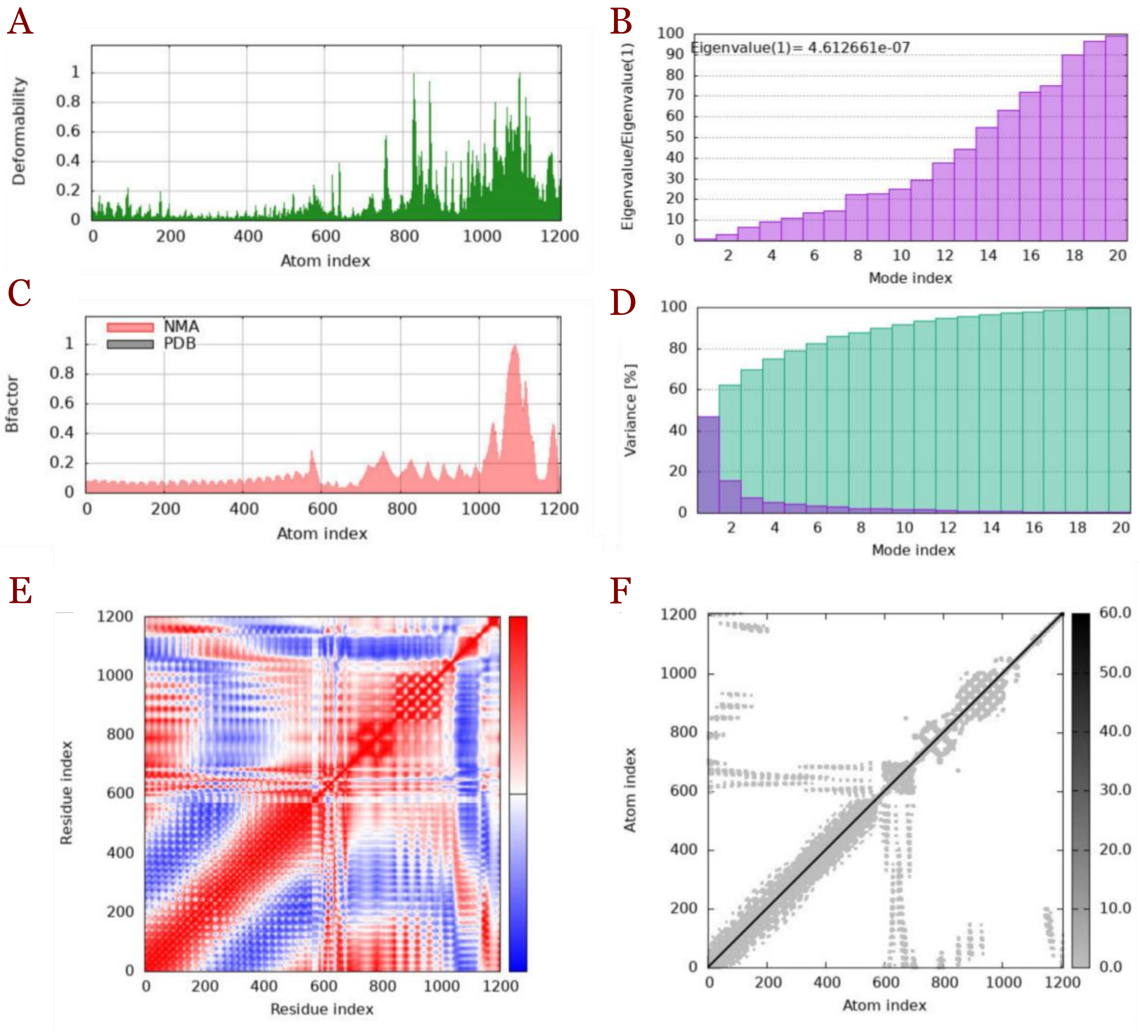

Figure 8. Normal mode analysis (NMA) of the docked vaccine–TLR4 complex generated using iMODS. (A) Deformability profile; (B) eigenvalue plot (4.61×10⁻⁷); (C) B-factor profile; (D) variance associated with normal modes; (E) covariance map showing correlated and anti-correlated residue motions; and (F) elastic network model.

Consequently, some predicted epitopes may not be efficiently generated or presented despite strong binding predictions. In addition, epitope competition and immunodominance hierarchies may influence the actual immune response, as multiple epitopes within a multi-epitope construct may compete for MHC binding or T-cell recognition [76]. This can result in selective presentation of certain dominant epitopes while others remain subdominant or non-immunogenic. The application of multiple sequential filtering criteria may introduce selection bias by prioritizing epitopes that perform well across specific computational metrics, while potentially excluding others that could be biologically relevant [77,78]. This approach reflects a trade-off between increasing confidence in selected candidates and maintaining epitope diversity. Additionally, differences in prediction algorithms and scoring systems may contribute to variability in epitope selection. Therefore, the final epitope set should be interpreted as a subset of high-confidence candidates rather than a comprehensive representation of all potentially immunogenic epitopes.

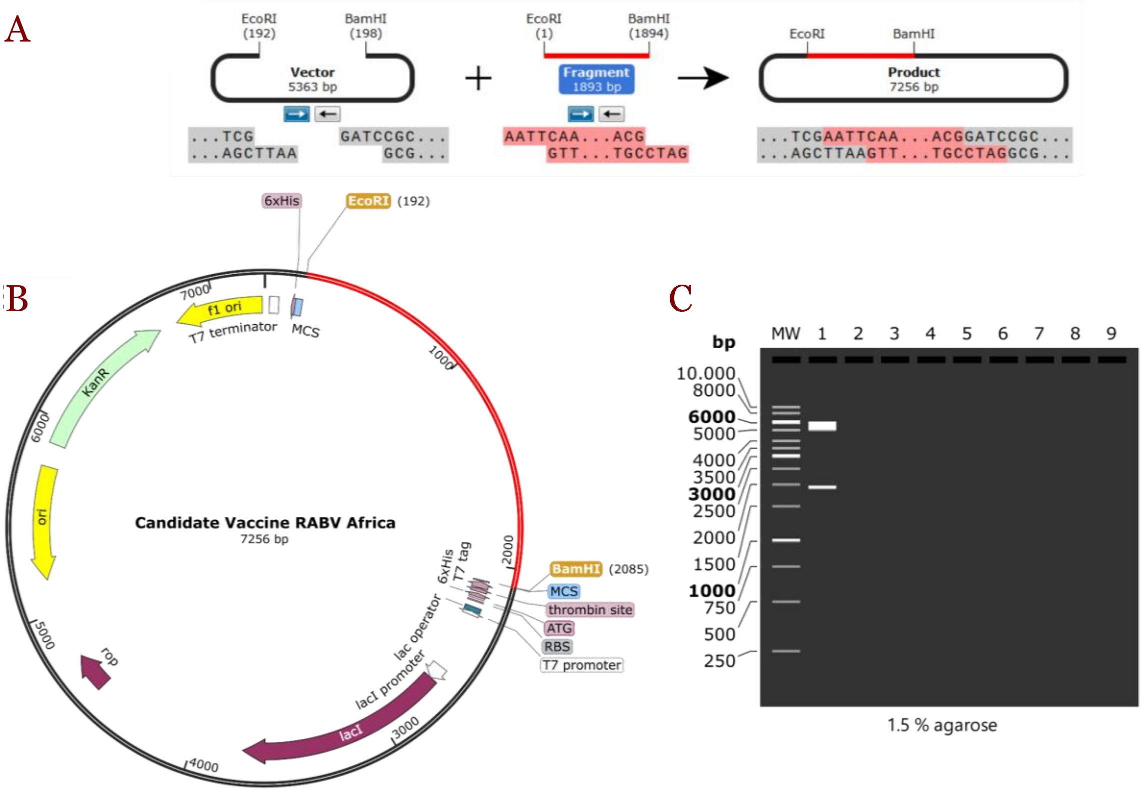

Figure 9. In silico cloning analysis of the multi-epitope vaccine construct. (A) Virtual insertion map generated in SnapGene v8.2; (B) Schematic representation of the insertion of the optimized gene into the pET-28a(+) expression vector using EcoRI and BamHI restriction sites; and (C) Simulated agarose gel electrophoresis.

The final vaccine construct incorporated cholera toxin B subunit as an adjuvant and immunostimulatory linkers, and was evaluated across multiple computational pipelines. The incorporation of multiple functional components, including CTB, PADRE, linkers, and invasin-derived sequences, may introduce potential biological challenges that cannot be fully assessed through computational approaches. These include epitope competition, altered antigen processing, and immunodominance interference, which may affect the overall immune response [43,63]. Therefore, while the construct is theoretically designed to enhance immunogenicity, its biological feasibility and immunological behavior require experimental validation. Furthermore, population coverage analysis revealed 99.95% coverage across five African subregions. This underscores the strength of region-specific HLA optimization, a dimension often overlooked in conventional rabies vaccine designs [74,75]. Notably, the construct displayed an instability index of 29.00 (indicating stability) and a GRAVY score of -0.304. These scores support consistency of hydrophilic behavior with the physicochemical expectations of subunit vaccines [31,71,79]. However, the estimated population coverage should be interpreted with caution, as it is derived from computational modeling and depends on the selected HLA allele dataset and the assumption of independent epitope–HLA interactions. Real-world immune responses may differ due to factors such as epitope competition, antigen processing constraints, and population heterogeneity.

In silico evaluations of structural and immunological behavior further support the theoretical findings of this candidate. The predicted tertiary structure revealed good folding quality with an ERRAT score of 98.86, a Z-score of -3.26, and 95.6% of residues in favored Ramachandran regions [54,72,73]. Docked TLR4 complex predicted 35 hydrogen bonds and ten salt bridges, suggesting a strong and stable potential interaction under computational conditions interface for innate immune recognition [22,52]. The molecular docking analysis with TLR4 was conducted as a theoretical assessment of potential receptor-level interaction. However, it is important to note that binding predictions do not imply immune activation or biological relevance.

Multi-epitope vaccine constructs are not necessarily expected to directly interact with pattern recognition receptors such as TLR4, and such interactions may depend on additional factors, including formulation, delivery system, and adjuvant properties. Moreover, immune simulation suggested patterns of antigen dynamics and antibody responses under modeled conditions over a simulated 1,050 time steps [51]. Such simulations are inherently dependent on predefined parameters and assumptions, and do not fully represent the complexity of real immune systems, including antigen processing, immune regulation, and host variability. Additionally, NMA revealed low eigenvalues and flexible regions, which indicate the complex's ability to accommodate conformational motion while maintaining structural cohesion [63,80]. Finally, successful cloning into the pET-28a(+) vector yielded a codon adaptation index of 0.97 and a GC content of 51%, indicating potential expression in *E. coli*. Experimental validation is required to confirm these findings [67,68].

Notably, the present findings align with prior rabies virus epitope-based vaccine design. Earlier studies utilizing immunoinformatics approaches to design multi-epitope vaccines targeting the rabies glycoprotein have reported vaccine constructs with favorable antigenicity, non-allergenicity, and predicted immunogenicity profiles, similar to those observed in the current study [23,24]. Some studies have further demonstrated the stability of conserved glycoprotein epitopes through protein-protein docking and molecular dynamics simulations [24], while others have reported stable docking performance using peptide-based designs [23]. However, most of these studies relied on globally aggregated datasets and did not explicitly incorporate population-specific HLA allele distributions into the epitope selection process. In contrast, the present study integrates African HLA allele frequency data, which may enhance the theoretical inclusivity of epitope presentation in populations with high immunogenetic diversity. This distinction is particularly relevant given that immunogenetic variability has been associated with differences in vaccine responsiveness across populations [5,7].

Another study further expanded the field by formulating a multi-epitope mRNA vaccine comprising multiple viral proteins, achieving impressive global population coverage (~96%) and strong immune predictions [22]. While their approach emphasizes broad applicability, the population-informed strategy adopted in this study focuses on region-specific immunogenetic representation, which may offer complementary advantages in endemic settings. Nevertheless, it is important to note that these differences are based on computational frameworks, and their impact on real-world vaccine performance remains to be validated experimentally.

This study has several important limitations that should be considered when interpreting the results. All findings are derived from computational predictions and may not fully capture the complexity of in vivo biological systems, including antigen processing, epitope presentation, and immune regulation. In addition, the population coverage analysis is model-dependent and assumes independent epitope–HLA interactions, which may not reflect real-world immune responses. The use of multiple filtering criteria may also introduce selection bias and limit epitope diversity. Furthermore, structural analyses, molecular docking, and immune simulations are based on predefined computational models and cannot reliably predict actual immunogenicity or vaccine efficacy. Therefore, the results of this study should be interpreted as theoretical and hypothesis-generating, and require comprehensive experimental validation.

Conclusion

The final construct, comprising eleven CTL, nine HTL, and nine B-cell epitopes, demonstrated favorable antigenicity, non-allergenicity, and non-toxicity, with predicted population coverage exceeding 99.95% across African subregions. Structural validation metrics, including a Ramachandran favorable residue rate of 95.6%, a Z-score of -3.26, and an ERRAT value of 98.86, indicated excellent stereochemical quality and model reliability. Additional analyses suggested a stable physicochemical profile (instability index: 29.00; GRAVY: -0.304), predicted immune activation, interaction with TLR4, and potential expression in *Escherichia coli* following codon optimization and in silico cloning. These findings provide a theoretical basis for further experimental validation.

Ethics approval

Not required.

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Competing interests

All authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

AI-based language tools, Grammarly and QuillBot, were used solely for language editing, including improving grammar, sentence structure, and readability of the manuscript. These tools were not used for data analysis, interpretation, or generation of scientific content. We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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