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Utilizing Immunoinformatics Methodology to Develop a Monkeypox Virus-Specific Vaccine with Multiple Epitopes

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Abstract

Background:

Monkeypox virus, a DNA virus belonging to the Poxviridae family, is an enveloped virus. It spreads from rodents to primates through infected body fluids, skin lesions, and respiratory droplets. Symptoms of monkeypox virus infection include fever, myalgia, maculopapular rash, and fluid-filled blisters. Distinguishing monkeypox virus from other poxviruses is crucial for accurate diagnosis, which can be achieved through DNA analysis of swab samples. Currently, there is no specific therapy or vaccine available for monkeypox virus, and treatments for other orthopoxvirus infections are used during small outbreaks. In this study, two potential peptides were designed.

Methods:

To design a vaccine, protein sequences were collected, and B- and T-cell epitopes were predicted. Vaccine structures were then predicted and docked with toll-like receptors. The resulting docked complexes were analyzed using iMODS. Additionally, the nucleotide sequences of the vaccine constructs were optimized and expressed in silico.

Results:

The multiepitope vaccine construct (V1) exhibited an antigenicity score of 0.5400, an instability index of 29.33, a z-score of -2.11, and a 42.11% GC content. On the other hand, the COP-A44L vaccine construct (V2) had an antigenicity score of 0.7784, an instability index of 23.33, a zscore of -0.61, and a 48.63% GC content.

Conclusion:

The findings from the in silico analysis demonstrated that both vaccine constructs show promising potential in preventing monkeypox virus. However, multiepitope vaccine construct exhibited better results.

Abbreviations:

MPXV - Monkeypox virus

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IID - Initial Intrusion Duration

CDC - Centers for Disease Control and Prevention

VIG - Vaccinia Immunoglobulins

IEDB - Immune Epitope Database

TLR3 - Toll-like receptor 3

NMA - Normal Mode Analysis

CAI - Codon Adaptation Index

II - Instability Index

Keywords: Multi epitope vaccine constructs; Monkey pox virus; Immunoinformatics; Codon Adaptation Index; Toll-like receptor 3

1. Background

Monkeypox is a zoonotic infection caused by an enveloped monkeypox virus (MPXV) that belongs to the Poxviridae family. It was initially observed in 1959 as a pox-like disease in monkey colonies in Denmark. Subsequent outbreaks have occurred in Central and West African countries, as well as in the United States, with mortality rates ranging from 1% to 10%. Cases have also been reported outside of Africa, including Singapore, South Korea, and most recently, Taiwan Province. The virus primarily spreads from wild animals (rodents and primates) to humans, and human-to-human transmission is common **[1, 2]**. Transmission occurs through contact with contaminated items, infected body fluids, skin lesions, and respiratory droplets. Recent outbreaks have also revealed sexual transmission among bisexual individuals. Following transmission, the virus enters cells and evades antiviral immune responses through viral accessory genes. These genes, also known as host range genes, determine the virus's ability to infect different hosts and hinder various aspects of cellular innate responses. Viral protein B16 suppresses the induction of type I interferon-mediated antiviral signaling, while Tumor Necrosis Factor alpha (TNF-α) and interferon-stimulated genes are not expressed during MPXV infection. In the host, there is an incubation period of 5-21 days, with the first five days referred to as the initial intrusion duration (IID). During IID, patients experience fever, lymph node inflammation, myalgia, severe headache, asthenia, and backache as the main symptoms. After 1-3 days of fever, maculopapular rashes appear, which later develop into pus-filled blisters that burst within ten days. To diagnose monkeypox infection, the analysis of viral DNA extracted from swab samples obtained from vesicle crusts is the most suitable procedure **[3, 4]**. Currently, no specific treatment for MPXV is available according to the Centers for Disease Control and Prevention (CDC). Patients are treated with therapies used for other orthopoxviruses, such as cidofovir, brincidofovir, and tecovirimat. Therefore, the main objective of this research is to design a multiepitope MPXV-specific vaccine using an immunoinformatics approach to aid in the eradication of the viral infection. Two virulent COP genes, A44L and B7R, have been selected for vaccine development. B7R is present in MPXV but absent in variola virus, while A44L

encodes a protein that is 140 amino acids shorter in variola virus compared to MPXV. The protein product of COPA44L, 3-β-hydroxysteroid dehydrogenase, is involved in the conversion of pregnenolone to androstenedione and plays a role in the production of immunosuppressive steroid hormones. Similarly, B7R is a viral protein residing in the endoplasmic reticulum, and its virulence mechanism is unknown. It may impact apoptotic mechanisms or be expressed on the cell surface, possibly influencing immune responses **[5, 6]**.

2. Methodology:

2.1. Retrieval of protein sequences

The poxvirus genome is approximately 200 Kb in size and consists of 200 proteins. It possesses a linear double-stranded DNA genome with covalently closed hairpin ends and 10 Kb inverted terminal repeats at each end. Two specific proteins, COP-A44L and COP-B7R, were selected for analysis. COP-A44L is a full-length protein encoding 140 amino acids, while COP-B7R is a resistance protein found in the monkeypox virus. The complete genome sequence was obtained from the GenBank database at NCBI (https://www.ncbi.nlm.nih.gov/). The protein sequences were also retrieved from GenBank. Physicochemical properties and secondary structure predictions were determined using Expasy (https://www.expasy.org/) and PSIPRED tools (http://bioinf.cs.ucl.ac.uk/psipred/). The number of amino acids and cysteine residues were assessed using the DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA/). The antigenicity score was obtained using the VaxiJen v2.0 online server (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html), while the number of transmembrane helices was determined using the TMHMM v2.0 server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Allergenicity prediction was performed using AllerTOP v. 2.0 (https://ddgpharmfac.net/AllergenFP/). **[22-23].**

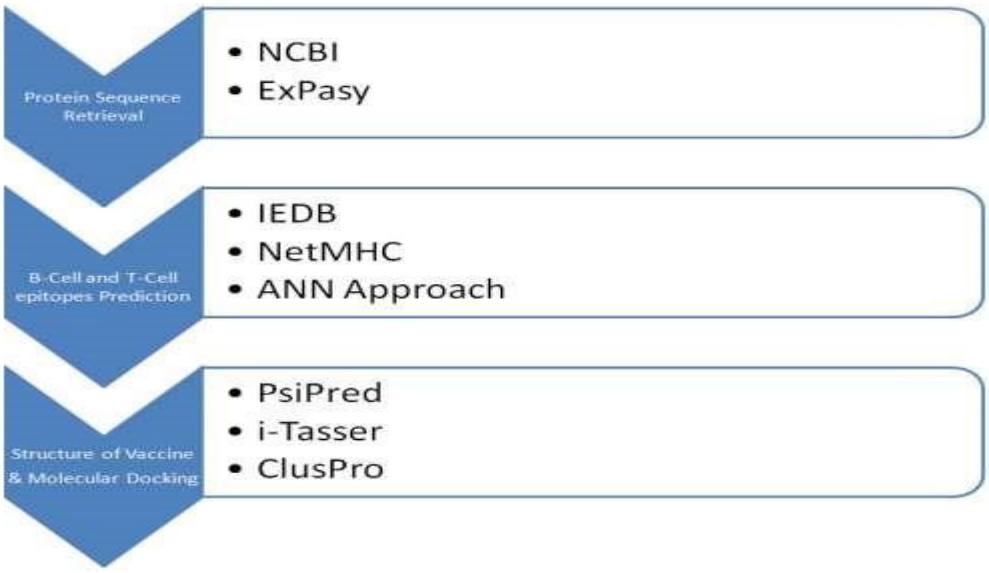

Fig1. Brief flow of methodology deployed in Immunoinformatics scheme for vaccine designing

2.2. Prediction of B- and T-cell epitopes

To predict the B- and T-cell epitopes of COP-A44L and COP-B7R, the immune epitope database (IEDB) analysis resource was utilized. The protein sequences were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/), and the epitopes were predicted separately. Epitopes with an IC50 value ≤ 100, based on their antigenicity and allergenicity, were selected and analyzed for population coverage using the Population Coverage - IEDB Analysis Resource **[24].**

2.3. Prediction of 2D and 3D structures

The 2D structure, which represents the covalent bonds in the molecule, was analyzed using PSIPRED tools (http://bioinf.cs.ucl.ac.uk/psipred/). On the other hand, 3D structures provide the three-dimensional coordinates of a molecule, and SCRATCH Protein Predictor (https://scratch.proteomics.ics.uci.edu/) and I-TASSER (https://zhanggroup.org/I-TASSER/) were employed to determine the 3D structures of COP-A44L and COP-B7R, respectively. The best model was further refined using the Galaxy web server (https://galaxy.seoklab.org/), which assessed the models using the Ramachandran plot, MolProbity, clash score RMSD, and GDTHA. Additionally, the Ramachandran plot for the first model was predicted using RAMPAGE (https://zlab.umassmed.edu/bu/rama/) **[5, 6]**.

2.4. Molecular docking of the designed chimeric protein with Toll-like receptor

The immune response is initiated through the interaction between an antigenic molecule and a specific immune receptor. In this study, Toll-like receptor 3 (TLR3) was selected as the receptor to assess the interaction with the refined protein. The ClusPro server (https://cluspro.bu.edu/) was utilized to identify the native sites within the protein and facilitate protein-protein docking, providing various results. The ClusPro server was employed to perform molecular docking of the multi-epitope vaccine peptide with the TLR3 receptor, aiming to obtain the best docked model. Subsequently, the iMODS server (https://imods.iqfr.csic.es/) was employed to conduct normal mode analysis (NMA) in the internal coordinates of nucleic acid and protein atomic structure **[7]**.

2.5. In silico cloning optimization of the designed vaccine candidate

To enable reverse translation and codon optimization, EMBOSS Backtranseq (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) and NovoPro (https://www.novoprolabs.com/tools/codon-optimization) tools were employed. These tools provided output information such as the codon adaptation index (CAI) and the percentage of GC content, which were used to assess the protein expression level. A CAI score greater than 0.8 is considered favorable, and the GC content should ideally fall within the range of 30-70%. Additionally, SnapGene software was used to perform restriction enzyme cloning, ensuring the expression of the vaccine construct. The optimized gene sequences of the final vaccine constructs were cloned into vectors pET-28c (+) and pET-21a (+). Figure 1 provides a summary of the methodology employed in this study **[25-26].**

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3. Results

3.1. Analysis of protein sequence

The protein sequences of COPA44L and B7R were obtained from the NCBI and utilized for the development of a multi-epitope vaccine against MPXV. The physicochemical properties of the proteins were analyzed using Expasy-ProtParam (https://www.expasy.org/). The analysis revealed that COP-B7R consists of 182 amino acids with a molecular weight of 21,390.08 Da and a theoretical pI of 5.65. The protein was found to have a half-life of 30 h (mammalian reticulocytes, in vitro),> 20 h (yeast, in vivo), and> 10 h (Escherichia coli, in vivo). On the other hand, COP-A44L has a molecular weight of 39,338.36 Da, a sequence length of 346 amino acids, a theoretical pI of 7.06, and a stability index of 34.74 (indicating stability). The estimated halflife for COP-A44L was 30 h,> 20 h, and> 10 h in mammalian reticulocytes (in vitro), yeast (in vivo), and E. coli (in vivo), respectively **[27-28].**

The VaxiJen v2.0 online server (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html) indicated that both protein sequences were potential antigens, with COP-B7R scoring 0.4395 and COP-A44L scoring 0.4016. AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/) predicted that both proteins were non-allergenic. The functional sequences of the proteins were subsequently subjected to linear B- and T-cell epitope prediction **[29-30].**

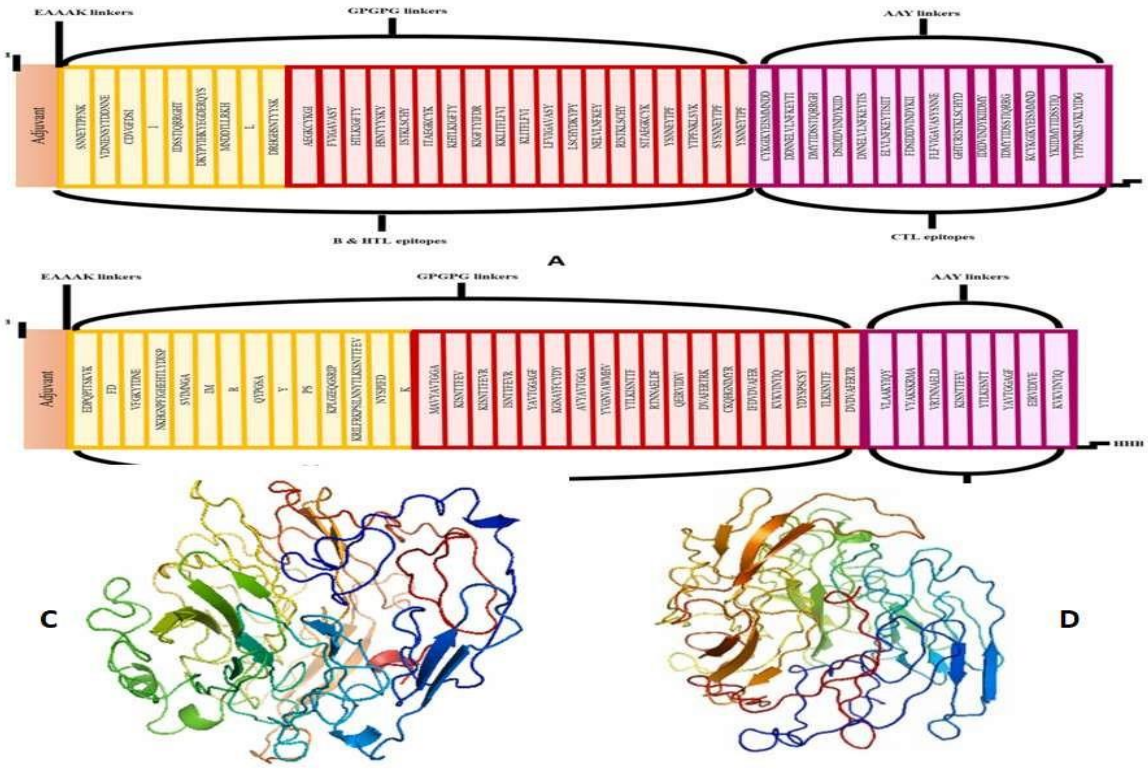

Fig2. Vaccine construct (A) MEV1 or V1 (B) MEV2 or V2 (C & D) Structure of V1 and V2

3.2. Analysis of B- and T-cell epitopes

The Immune Epitope Database (IEDB) Analysis Resource (https://www.iedb.org/) was employed as a repository of computational tools for the analysis and prediction of B- and T-cell epitopes. B-cell epitopes can be categorized as linear or non-linear (discontinuous). Linear B-cell epitopes, derived from protein sequences and composed of sequential residues, are more practical and advanced for prediction compared to discontinuous B-cell epitopes. Discontinuous B-cell epitopes, on the other hand, are derived from various patches of the input protein in a nonsequential manner and require knowledge of the protein's three-dimensional structure. To graft epitopes onto a suitable scaffold for selective antibody production, linear B-cell epitopes are preferred. In this study, BepiPred-2.0, a web server trained on epitope data from the Protein Data Bank (PDB), was employed for the prediction of linear B-cell epitopes from the antigen sequences. BepiPred-2.0 utilizes a random forest algorithm and provides improved prediction accuracy. Epitopes with antigenicity scores>0.5 for COP-B7R and>0.7 for COP-A44L, retrieved from the IEDB (http://tools.iedb.org/bcell/), were selected for vaccine construction **[31-32].**

3.3. Construction of a multi-epitope subunit vaccine

To design a chimera for COPB7R, a total of 9 B-cell epitopes, 19 MHC-I epitopes, and 14 MHCII epitopes were predicted. Similarly, for COPA44L, the vaccine construct consisted of 14 B-cell epitopes, 18 MHC-I epitopes, and 8 MHC-II epitopes, as illustrated in Figure 2. These predicted epitopes were merged together to create a continuous sequence using specific linkers. The B-cell and T-cell epitopes were connected using the GPGPG and AAV linkers. The TLR3 agonist, 50 S ribosomal L7/L12 (Locus RL7_MYCTU) with accession number P9WHE3, was utilized as the adjuvant and added to the amino terminus of the vaccine peptide using the EAAAK linker to induce a specific immune response. Additionally, a 6xHis-tag was appended to the C-terminal for the purpose of protein purification and identification. The resulting peptides had lengths of 494 amino acids for COPB7R and 366 amino acids for COPA44L. Throughout the article, the vaccine construct for COPB7R will be referred to as MEV1, while the vaccine construct for COPA44L will be denoted as V2 **[33-34].**

3.4. Prediction of the antigenicity and allergenicity of the vaccine candidate

The antigenicity and allergenicity of the final sequence of the COP-B7R vaccine construct (MEV1) along with the adjuvant sequence were assessed using VaxiJen v2.0 and AllerTOP v. 2.0. The results indicated an antigenicity score of 0.5400, signifying its antigenic nature, and it was determined to be non-allergenic. The COP-A44L vaccine construct (MEV2) was also predicted to be non-allergenic with an antigenic score of 0.7784. Notably, the antigenic scores obtained for the vaccine constructs were higher compared to previously report in silico vaccine constructs (0.44–0.47 and 0.5311 **[35-36].**

3.5. Physicochemical properties and solubility prediction

The final protein of MEV1 was predicted to have a molecular weight of 57,803.24 Daltons and a theoretical isoelectric point of 6.06. The protein's half-life was estimated to be 1 h (mammalian

reticulocytes, in vitro), 30 min (yeast, in vivo), and> 10 h (Escherichia coli, in vivo). However, solubility prediction using SOLUPROT indicated that MEV1 may not be expressed effectively in E. coli. The protein exhibited an instability index of 29.33, classifying it as stable. Additionally, the aliphatic index was predicted to be 79.90, and the grand average of hydropathicity was 0.416, indicating its hydrophilic nature and ability to interact with water molecules **[37-38].**

In the case of MEV2, its molecular weight was predicted to be 41,377.02 Daltons, and it consisted of 366 residues. The solubility prediction by SOLUPROT indicated a solubility score of 0.522, suggesting that MEV2 could be expressed in E. coli with good solubility. Furthermore, MEV2 had a theoretical isoelectric point of 9.14, an instability index of 23.23, and a hydropathicity of -0.277.

3.6. 2D and 3D structure analysis

3.6.1. Secondary structure prediction

Based on the PSIPRED analysis, MEV1 was found to have three helices in its secondary structure, along with strands and coils. Similarly, MEV2 exhibited six helical conformations, as well as strands and coils, according to the predicted secondary structure. Furthermore, MEMSAT analysis provided insights into the extracellular, transmembrane, and cytoplasmic domains of the vaccine peptide sequences. For MEV2, the analysis revealed a pore lining domain spanning amino acids 173 to 188, with the N-terminal domain designated as the extracellular domain and the C-terminal domain as the intracellular domain **[39].**

3.6.2. Tertiary structure modeling

The I-TASSER tool was utilized to predict the 3D structure of the final proteins in V1. The results presented ten threading templates, with the five best models being 1d2pA, 7w6bA, 3holA, 7w7iA, and 5nxkA. All ten templates exhibited favorable alignment and z-scores ranging from 1.1 to 1.4. Among the five models, the one with the highest C-score was selected for refinement (Fig. 2C), as the C-score varied between -2.53 and -4.76. The chosen model demonstrated an estimated TM-score of 0.42 ± 0.14 and an RMSD of 13.5 ± 4.0 Å.

3.6.3. Tertiary structure refinement

To predict the 3D structure of MEV2, the SCRATCH Protein Predictor was employed. This tool was chosen due to its ability to accurately predict protein sequences with fewer than 400 amino acids in a shorter time frame (Fig. 2C).

For the refinement of the tertiary structure, the Galaxy web server was utilized. This process involved refining Model 1 obtained from both I-TASSER and the SCRATCH Protein Predictor. The GalaxyRefine server generated five models, each assessed based on parameters such as the Ramachandran plot, MolProbity, RMSD, and GDT-HA. Among the models, Model 4 displayed the most favorable characteristics with a GDT-HA score of 0.9160, an RMSD of 0.516, and a MolProbity value of 3.034. Additionally, it had a clash score of 37.5, a poor rotamers score of 2.2, and a Ramachandran plot score of 80.9. This model was selected for further analysis and vaccine construction (Fig. 3). Conversely, Model 1 obtained through GalaxyRefine, which

exhibited a GDT-HA of 0.8854, an RMSD of 0.561, a MolProbity value of 1.854, a clash score of 12.8, a poor rotamers score of 0.6, and a Rama favored score of 96.4, was chosen for additional predictions and analyses of MEV2 (Fig. 3).

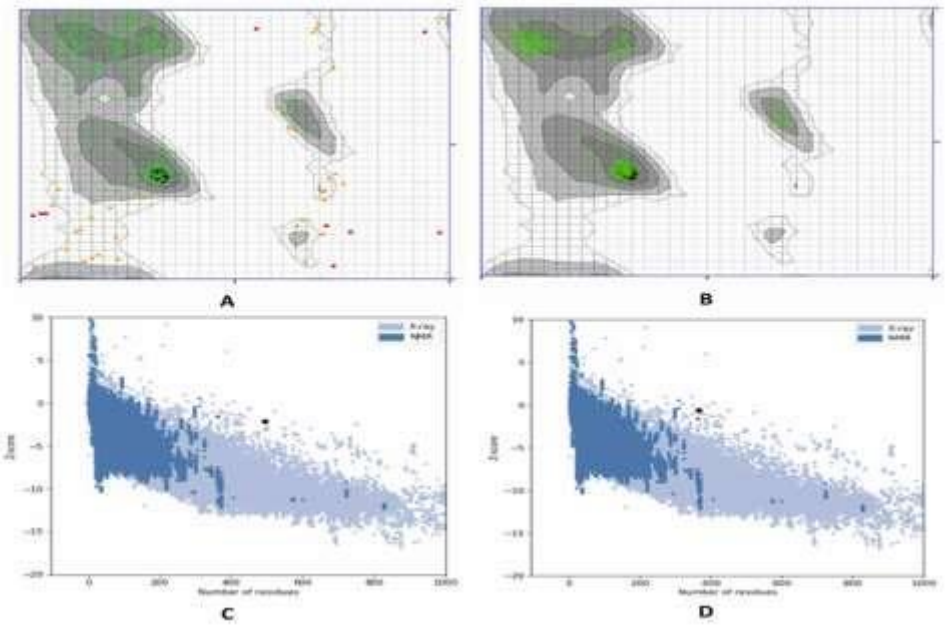

Fig 3. Ramachandran plot and Z-plot analysis for MEV1 (A & C), and MEV2 (B &D).

The Ramachandran plot analysis was performed for MEV1, revealing that 90.6% of the residues fell within the highly preferred region, indicated by green crosses. A small portion of observations (7.3%) were categorized as preferred (brown triangles), and a minor fraction (1.9%) raised questions (red spots) (Fig. 3). In contrast, the Ramachandran plot for MEV2 displayed all residues (328 amino acids) occupying the highly preferred region, denoted by green crosses, with no residues falling into the additional preferred or questionable regions (Fig. 3). To validate the refined models of MEV1 and MEV2, ProSA-web (https://prosa.services.came.sbg.ac.at/prosa.php) was utilized. The z-score obtained for MEV1 was -2.11, while MEV2 yielded a z-score of -0.61 (Fig. 3C and D). A higher z-score signifies a better quality model.

Molecular docking and dynamics simulation of the subunit vaccine with the immune receptor TLR3 were conducted in this study. The ClusPro tool was employed to identify the binding sites and hydrophobic interaction regions on the protein surfaces (Fig. 4). For protein-protein docking analysis and examination of chain deformability and residue deformations, the iMODS server was utilized. The graphs presented in Fig. 4 indicate that MEV1 exhibits higher peaks, indicating greater deformations compared to MEV2, which shows only a few elevated peaks. Additionally, the eigenvalue, which corresponds to the normal mode and represents the rigidity of the model, was predicted. It reflects the energy required to deform the structure, with lower eigenvalues suggesting easier deformation. As depicted in the graphs in Fig. 4, the eigenvalues for MEV1 and MEV2 are 7.088347e-06 and 8.277109e-07, respectively. These values suggest that the

MEV2-TLR3 complex is more prone to deformation. RMSD plot analysis was performed by using NAMD-VMD for 100ns (Fig 4E).

To enhance protein expression, the codon sequence of the final vaccine construct underwent optimization. The EMBOSS Backtranseq tool was initially used to convert the protein residues into a nucleotide sequence, which was then further optimized using NovoPro for maximum expression. The optimized codon sequence for MEV1 had a length of 1482 base pairs, a codon adaptation index (CAI) of 0.84, and an average GC content of 42.11%. These characteristics indicated a favorable potential for expression in the E. coli (strain K12) vector. Finally, the adapted codon sequence was inserted into the pET-21a (+) vector to design a recombinant plasmid, facilitated by SnapGene software.

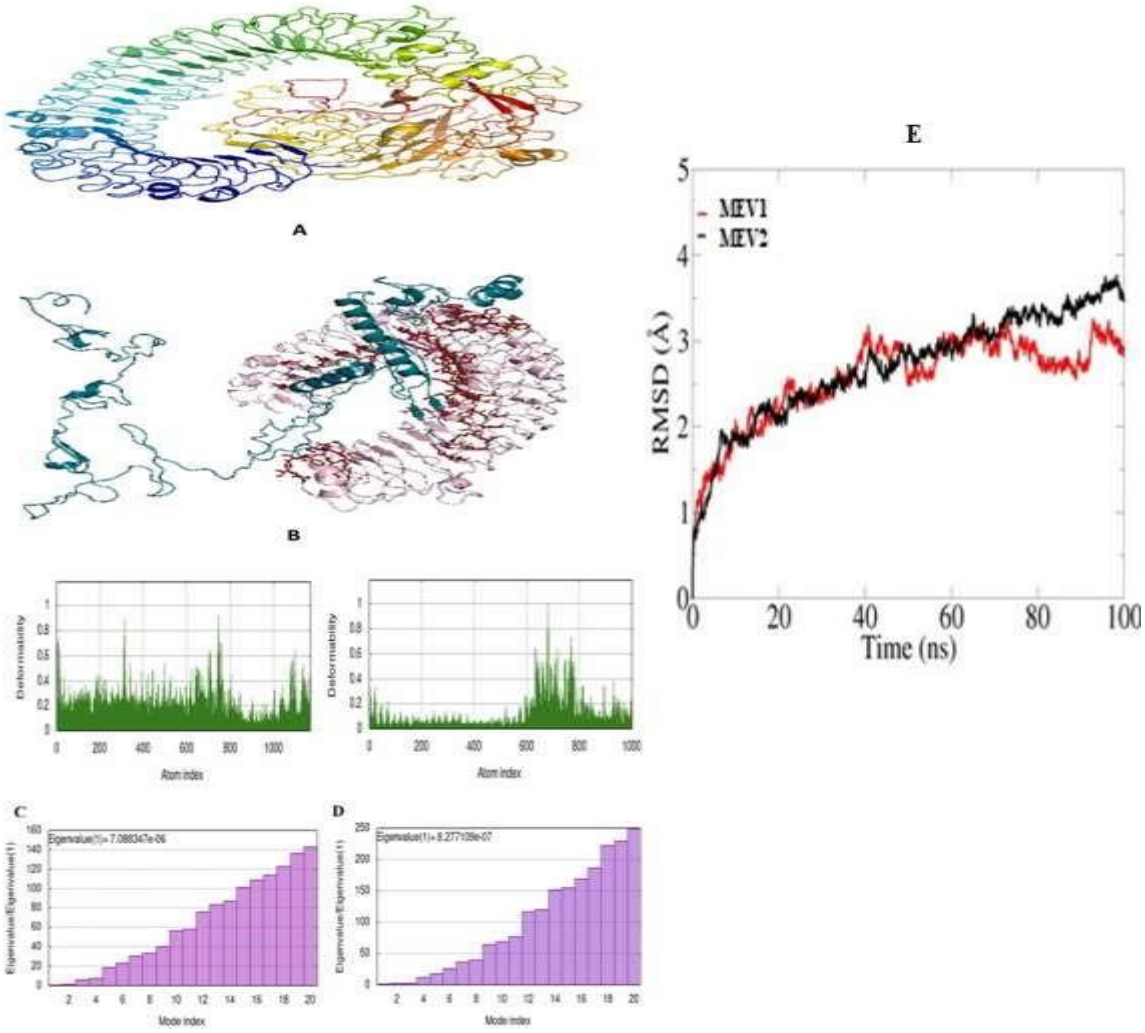

Fig 4. Simulation analysis of Docked complex for MEV1 and MEV2

For the *in silico* restriction enzyme cloning of MEV2, the gene sequence generated by EMBOSS Backtranseq was optimized by NovoPro to enable expression in E. coli (strain K12). Consequently, the codon optimization index (CAI) was improved from 0.65 to 0.80, and the

optimized sequence had a GC content of 48.63% and a length of 1098 base pairs. Analysis of restriction fragments revealed that PpuMI (at 18 bp) and ClaI (at 1040 bp) were the most suitable restriction sites. As a result, the optimized sequence was successfully ligated into the pET-28c (+) plasmid, allowing for expression in E. coli. For MEV1 and MEV2 detailed comparative assessment provided in table 1.

Table 1. Comparison between MEV1 and MEV2 based on properties analysed

Property	**Vaccine MEV1 (COP-B7R)**	**Vaccine MEV2 (COP-A44L)**
Epitopes	9 B-cell, 19 MHC-I, 14 MHC-II	14 B-cell, 18 MHC-I, 8 MHC-II
Molecular Weight	57,803.24 Dalton	41,377.02 Dalton
Solubility	Not soluble in E. coli	Soluble in E. coli
Allergenicity	Non-allergenic	Non-allergenic
Antigenicity	High antigenic score	High antigenic score
Molecular Docking	Stable interaction with TLR3	Favorable interaction with TLR3
Codon Optimization	Length: 1482 base pairs, CAI: 0.84, GC content: 42.11%	Length: 1098 base pairs, CAI: 0.80, GC content: 48.63%

4. Discussion

Monkeypox virus outbreaks have posed significant health threats in Central and West African countries and have recently expanded to other regions outside Africa. To address this ongoing challenge, we developed two novel vaccine constructs, MEV1 (COP-B7R) and MEV2 (COPA44L), aimed at providing specific immunity against the virus. In this discussion, we will analyze and interpret the findings of our study, encompassing the physicochemical properties, allergenicity, antigenicity, molecular dynamics simulation, and cloning of these vaccine constructs **[9-12]**. The design of an effective vaccine relies on various factors, including the selection of epitopes with high population coverage. In our study, both MEV1 and MEV2 were constructed using epitopes predicted to be immunogenic and capable of eliciting a robust immune response. MEV1 consisted of 9 B-cell epitopes, 19 MHC-I epitopes, and 14 MHC-II epitopes, while MEV2 comprised 14 B-cell epitopes, 18 MHC-I epitopes, and 8 MHC-II epitopes. This extensive repertoire of epitopes increases the likelihood of targeting a broad range of individuals and promoting a diverse immune response. The assessment of physicochemical properties is crucial for vaccine development, as it provides insights into factors such as molecular weight, solubility, and stability **[13-16]**. The analysis of MEV1 revealed a molecular weight of 57,803.24 Dalton, whereas MEV2 had a molecular weight of 41,377.02 Dalton. Both constructs exhibited favorable physicochemical properties, with MEV2 demonstrating solubility in E. coli. These characteristics are advantageous for protein expression and purification processes, facilitating the production of the vaccine constructs on a larger scale. Allergenicity and antigenicity are important considerations to ensure the safety and effectiveness of vaccines. Through the use of computational tools, we predicted the allergenicity and antigenicity of our vaccine constructs. The results indicated that both MEV1 and MEV2 were non-allergenic,

reducing the likelihood of adverse allergic reactions. Furthermore, the constructs exhibited high antigenic scores, indicating their potential to stimulate a robust immune response against the target virus. Comparing our vaccine constructs with previously reported ones, our in silico analysis showed higher antigenic scores, suggesting their improved immunogenicity. Molecular docking and dynamics simulations were conducted to investigate the interaction between our vaccine constructs and the immune receptor TLR3. The ClusPro tool enabled the identification of protein binding and hydrophobic interaction sites on the surface of the constructs. The docking analysis provided insights into the potential binding affinity and interaction between the vaccine constructs and TLR3. Additionally, molecular dynamics simulations allowed us to assess the stability and deformability of the vaccine models. These simulations indicated that MEV2 exhibited greater stability and lower deformation energy, suggesting a more favorable interaction with TLR3 compared to MEV1. The optimization of codon sequences is essential for maximizing protein expression **[17-21]**. We employed NovoPro to optimize the codon sequences generated from the EMBOSS Backtranseq tool. The optimized codon sequences for MEV1 and MEV2 exhibited improved codon adaptation indices (CAIs) and GC contents, indicating a higher likelihood of successful expression in the E. coli system. The adapted codon sequences were then inserted into appropriate vectors for recombinant plasmid design.

5. Conclusion

Monkeypox virus outbreaks have been frequently reported in Central and West African countries and have recently spread to countries outside Africa. In this study, we have developed two innovative vaccine constructs to provide targeted immunity against the virus. These vaccine constructs underwent extensive analysis of their physicochemical properties, allergenicity, antigenicity, molecular dynamics simulation, and cloning using restriction enzymes. Both vaccine constructs, namely MEV1 and MEV2, contained epitopes with broad population coverage. They were connected by stable linkers and adjuvants, demonstrating antigenic potential and non-allergenic properties. MEV2, which can be expressed in E. coli, exhibited a higher z-score compared to MEV1. In summary, our bioinformatics approaches revealed that MEV2 yielded superior results compared to MEV1.

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