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Harnessing Immunoinformatics for Precision Vaccines: Designing Epitope-Based Subunit Vaccines against Hepatitis E Virus

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Abstract: Background/Objectives: Hepatitis E virus (HEV) is an RNA virus recognized to be spread mainly by fecal-contaminated water. Its infection is known to be a serious threat to public health globally, mostly in developing countries, in which Africa is one of the regions sternly affected. An African-based vaccine is necessary to actively prevent HEV infection. **Methods:** This study developed an in silico epitope-based subunit vaccine, incorporating CTL, HTL, and BL epitopes with suitable linkers and adjuvants. **Results:** The in silico-designed vaccine construct proved immunogenic, non-allergenic, and non-toxic and displayed appropriate physicochemical properties with high solubility. The 3D structure was modeled and subjected to protein docking with Toll-like receptors 2, 3, 4, 6, 8, and 9, which showed a stable binding efficacy, and the dynamics simulation indicated steady interaction. Furthermore, the immune simulation predicted that the designed vaccine would instigate immune responses when administered to humans. Lastly, using a codon adaptation for the *E. coli* K12 bacterium produced optimum GC content and a high CAI value, which was followed by in silico integration into a pET28 b (+) cloning vector. **Conclusions:** Generally, these results propose that the design of an epitope-based subunit vaccine can function as an outstanding preventive vaccine candidate against HEV, although validation techniques via in vitro and in vivo approaches are required to justify this statement.

Keywords: hepatitis E virus; immunoinformatics; vaccine; capsid protein; immune simulation

1. Introduction

Hepatitis E virus (HEV) has become a significant public health concern [1] and an agent of viral hepatitis in various countries of the world where there are inadequate resources—especially developing countries with few cases of acute hepatitis. HEV was discovered in Afghanistan in 1983 [2], and is a positively stranded, non-enveloped RNA genome of icosahedral symmetry of about 20–30 nm, belonging to the *Hepevirus* genus and *Hepeviridae* family [3]. It encodes open reading frames (ORFs), which are three in number: ORF1 codes for nonstructural proteins, which involves viral genome replication; ORF 2 for the viral capsid protein; and ORF 3 is for multifunctional small proteins [4]. HEV is classified into four genotypes—1, 2, 3, and 4 [5]. Genotypes 1 and 2, which infect only humans, spread through the fecal–oral route [6,7], while genotypes 3 and 4 are mostly found in animals but also in humans, and are easily contracted by feeding on raw or undercooked meat [8]. Major outbreaks have occurred in several developing countries in Africa and Asia because of contaminated food or water intake, which is commonly correlated with genotypes 1 and 2 of HEV [9]. About 20 million people are at risk of infection caused by HEV, among which 3.3 million are cases, and 44,000 die annually across the globe [9]. Among the regions suspected to be greatly affected, Africa is amongst them [10], and its outbreak was first confirmed in Cote d'Ivoire in 1986 [11]. It has infected the human population in 28 of 56 African countries, taking an elevated toll, especially on pregnant women and their fetuses [11].

HEV infection, when accompanied by pregnancy or underlying chronic liver problems, causes considerable mortality [8,10]. In healthy individuals, the disease is self-limiting but can become chronic and lead to severe liver damage in patients with deficient immune systems [12]. In preventing viral diseases, the most effective method is vaccine construction, and scientists have been able to recognize effectual epitopes to use in the development of effective subunit vaccines facilitated by available software algorithms used for genomic studies and immunologic data [13,14]. This subunit vaccine constitutes fragments of immunogenic proteins that can induce an immune response against the target pathogen and imitate the appearance of the natural pathogen [15,16].

In the development of the HEV vaccine, only ORF2 of the genome has proven to be effective because it encodes the viral capsid protein, which is immunogenic by nature and produces neutralizing antibodies [5]. In constructing the HEV vaccine, early efforts have centered on the truncated or short forms of the capsid protein; the reason being that the expression of the whole-capsid protein's immunogenic characteristics was said to be veiled due to insolubility [5,17]. The currently developed vaccine candidates against HEV have been truncated forms of the ORF2 protein. These include the trpE-C2, pE2, HEV 239, 53 kDa, 56 kDa, 62 kDa, rHEV VLP, and T1-ORF2 vaccines [18]. The only assessed vaccines in human clinical trials have been the HEV 239 and 56 kDa vaccines [3]. Among all those vaccine candidates stated, only the HEV 239 vaccine in China qualified to reach a phase III trial, which was licensed by the SFDA China (China's State Food and Drug Administration) in 2011. Currently, there is no available vaccine worldwide to prevent hepatitis E virus infection aside from the one developed and licensed in China [19]. Therefore, an effort was made in this study to construct an effective hepatitis E vaccine with a focus on hepatitis E isolates from African countries, exploiting the diverse tools of bioinformatics and immunoinformatics.

2. Methods

2.1. Retrieval of HEV Protein Sequences

The process of retrieving the sequences of hepatitis E virus (HEV) proteins involved accessing the National Center for Biotechnology Information (NCBI) database Figure 1. These sequences were obtained in protein FASTA format. Specifically, seven capsid protein sequences were retrieved. Among these, one sequence originated from Sudan (AOT06116), one from Nigeria (AF173232), two from Chad (AAW78754, AAW78755), and three from Burkina Faso (QEE82931, QEE82930, and QEE82925).
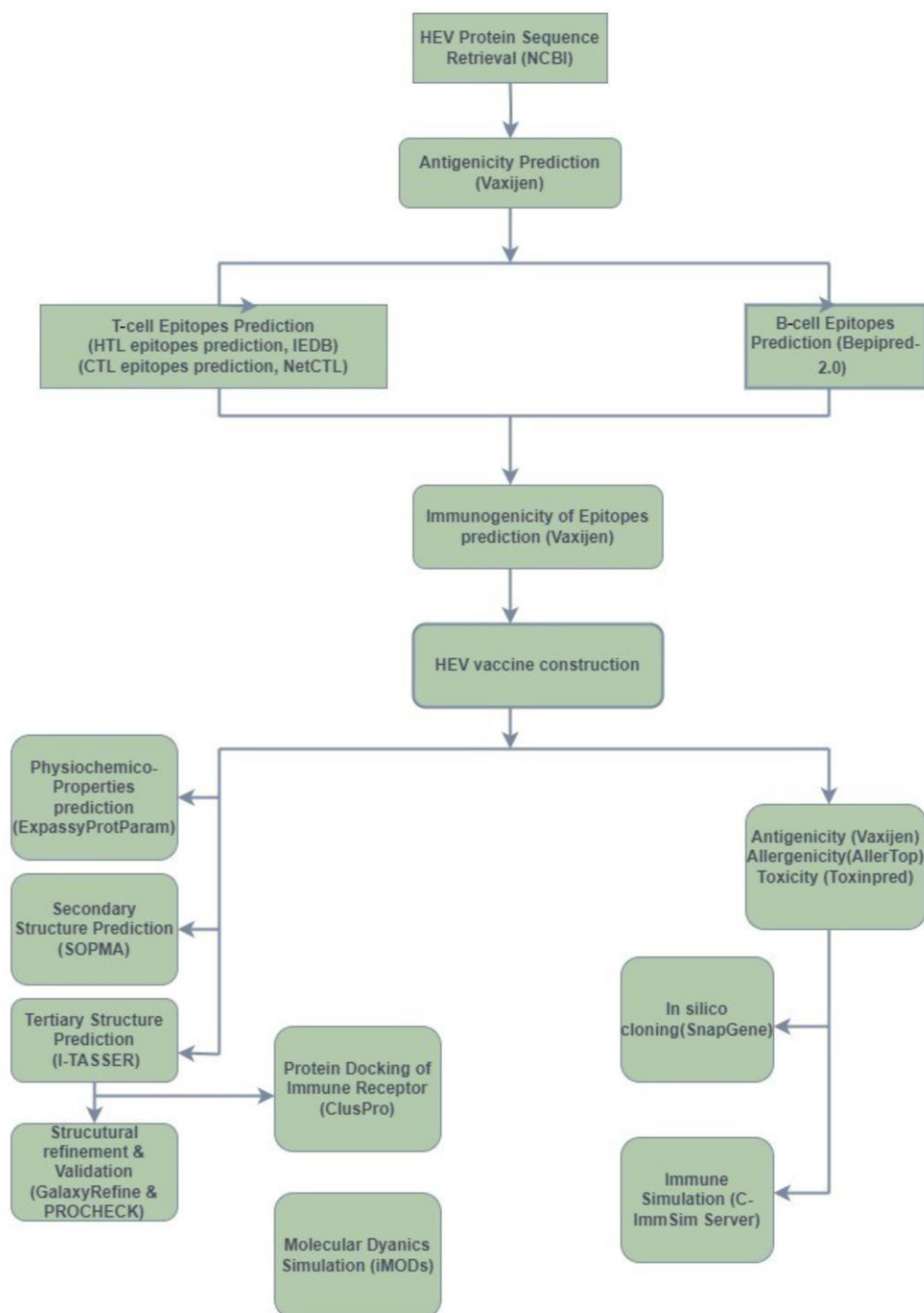
Figure 1. The methodology flowchart of the hepatitis E virus computational vaccine development.

2.2. Antigenicity Testing

The VaxiJen 2.0 server, referenced as [20], is an online computational tool designed for predicting the antigenicity of protein sequences. It utilizes sophisticated algorithms to analyze the physicochemical properties of proteins, thereby predicting their potential to induce an immune response. Widely employed in vaccine development and immunoinformatic research, this tool plays a pivotal role in identifying candidate antigens for vaccine design. Specifically, it serves the purpose of testing the antigenicity of sequences.

2.3. Epitope Predictions

The protein sequences were utilized to predict the epitopes capable of eliciting a response from T-cells and B-cells. This involved analyzing the sequences to identify specific regions or segments that are likely to interact with receptors on T-cells and B-cells, thus triggering an immune response. By predicting these inducing epitopes, we can gain insights into the potential immunogenicity of the protein and its suitability for vaccine development.

2.3.1. T-Cell Epitope Predictions

The Immune Epitope Database (IEDB) [21] was utilized to generate HTL (helper T lymphocyte) epitopes referencing the H2IAd, H2IAb, and H-2-IEd major histocompatibility complex II panel for the production of 15-mer T-cell epitopes at the highest binders, which were selected based on the percentile ranks. The CTL (cytotoxic T lymphocyte) epitope prediction of the retrieved sequences was performed using the NetCTL 1.2 server, which identified 9-mer T-cell epitopes [22]. Parameters such as weight on the proteasomal C-terminal cleavage, weight on the TAP (transporter associated with antigen processing) transport efficiency, and epitope identification were set at 0.15, 0.05, and 0.75, respectively, in the NetCTL-1.2 database.

2.3.2. B-Cell Epitope Predictions

B-cell epitopes were predicted by an online tool BepiPred-2.0, which indicated 14-mer B-cell epitopes for all HEV proteins. The setting of the minimum epitope threshold value was 0.5 [23].

2.4. Peptide Immunogenicity Predictions

VaxiJen v2.0, a web-based bioinformatics software, was used to evaluate the T-cell and B-cell epitopes' antigenicity [24]. The epitopes that were exactly on or surpassed the threshold value (0.4) were selected while non-antigenic epitopes (below the threshold value) were excluded.

2.5. HEV Vaccine Construction

For vaccine construction, HTL epitopes, CTL epitopes, and B-cell epitopes were employed. The adjuvant and first B-cell epitope were combined by the linker EAAAK, between one B-cell and the other GPGPG was used as the linker, likewise, between the B-cell and HTL the linker used was GPGPG, while AAY was used in linking HTLs and CTLs [25,26].

2.6. Different Physicochemical Properties Prediction of Vaccine Constructs

To find out the physicochemical properties of the vaccine, the ExPASy ProtParam tool was utilized [27,28]. This tool predicted different physicochemical features such as the molecular weight, instability index theoretical pI, half-life, aliphatic index, number of amino acids, and GRAVY (grand average of hydropathicity).

2.7. Predictions of the Secondary Structures for Vaccine Constructs

To determine the secondary structures for vaccine constructs, SOPMA was used [29]. The levels of alpha helices, extended strands, beta-turns, and random coils were assessed by SOPMA. The results retrieved show two graphs, one indicating the prediction and the other expressing the score curves.

2.8. Predictions and Refinement of the Tertiary Structures for Vaccine Constructs

The I-TASSER server is an online server that uses I-TASSER-based algorithms for the predictions of protein structure and function [30–32]. This server was used to illustrate the tertiary structure arrangement of the predicted vaccine. The Galaxy Refine server was utilized to polish the structure, showing different models. The refined model was confirmed using the PROCHECK server to produce the Ramachandran plot [33].

2.9. Predictions of Antigenicity, Allergenicity, and Toxicity

The prediction of vaccine antigenicity was also performed on the VaxiJen server [20]. To evaluate whether the vaccine was allergenic, the AllerTOP V2.0 online server was used [34]. Likewise, toxicity was predicted by ToxinPred.

2.10. Protein Docking of the Immune Receptor

To produce an immune reaction, there is a need for an interaction between the immune receptor and antigenic molecule, so the vaccine construct (ligand) and immune receptor (TLR 2: 2z82; TLR 3: 3CIG; TLR 4: 2Z64; TLR 6: 3A79; TLR 8: P58882; TLR 9: 3WPF.) interaction was analyzed by protein–protein docking; the online server used for this was the ClusPro server [35].

2.11. Molecular Dynamics (MD) Simulation

The process of determining the direction and movement of molecules within the vaccine–receptor complex, including the covariance, B-factor, eigenvalues, and deformability, was conducted using the iMODs online server in molecular dynamics (MD) simulation. Deformability is contingent upon the molecule's ability to undergo deformation at its residues. Eigenvalues indicate the rigidity of motion, with lower eigenvalues suggesting structures that are easily deformable. This information was derived from studies by [36], and adopted by many scientific research papers, including [26].

2.12. In Silico Cloning

To improve the expression of genes by the translation machinery of the host, optimization should be performed. Optimization of the codons and reverse translation—using the Java Codon Adaptation Tool—were examined in order to make a fitting expression prediction in vector translation and cloning effectiveness [37]. The organism that was the expression host for the vaccine construct was identified to be *Escherichia coli* (K-12 strain).

2.13. Immune Simulation

The immune response simulation was carried out on the C-ImmSim server [38]. The random seed, simulation volume, and steps were set at 12345, 50 micro–L, and 1000, respectively; likewise, the Host HLA selections for MHC classes I and II were AO101 and DRB1_0101, respectively, while a single injection time step was used in this simulation.

3. Results

3.1. Protein Targets

From the antigenicity prediction output of the target protein set at threshold value—which acts as a predetermined standard for evaluation at a base value of 0.4—the capsid protein identified by the accession number QEE82931.1 demonstrates the highest degree of antigenicity. Subsequently, other proteins, namely QEE8230, QEE82925, AF173232, AOT06116, AAW78754, and AAW78755, follow in descending order of antigenic potency, with the respective values of 0.7528, 0.6825, 0.6250, 0.5672, 0.5317, 0.5214, and 0.5139.

3.2. Predictions of T-Cell Epitopes

Epitopes that were antigenic in the protein sequences were selected. For the seven sequences stated earlier, there were 126 HTL epitopes in total, and for CTLs, 28 epitopes, out of which 88 epitopes and 11 epitopes were found to be antigenic, respectively; these antigenic epitopes were then subjected to advanced study.

3.3. Predictions of B-Cell Epitopes

Out of the seven sequences mentioned previously, a total of 41 B-cell epitopes were subjected to analysis. Among these, 29 epitopes were identified as antigenic and were deemed suitable for more in-depth study.

3.4. HEV Vaccine Sequence Construction

The epitopes that qualified for the final design of T-cell and B-cell epitopes were merged with the AAY and GPGPG linkers, respectively, to form a vaccine construct. Following the addition of linkers and adjuvant, the final design consisted of 232 amino acids and included a total of 14 cell epitopes left after the downstream analyses of antigenicity, allergenicity, and toxicity prediction. Figure 2 below illustrates the HEV vaccine sequence, which consists of an adjuvant (red) at the N-terminal joined with an HEV sequence through an EAAAK linker (sky blue). B-cell lymphocyte (BCL) epitopes are connected by GPGPG linkers (blue), HTL epitopes are linked by GPGPG linkers (blue), and CTL epitopes are connected by AAY linkers (green).
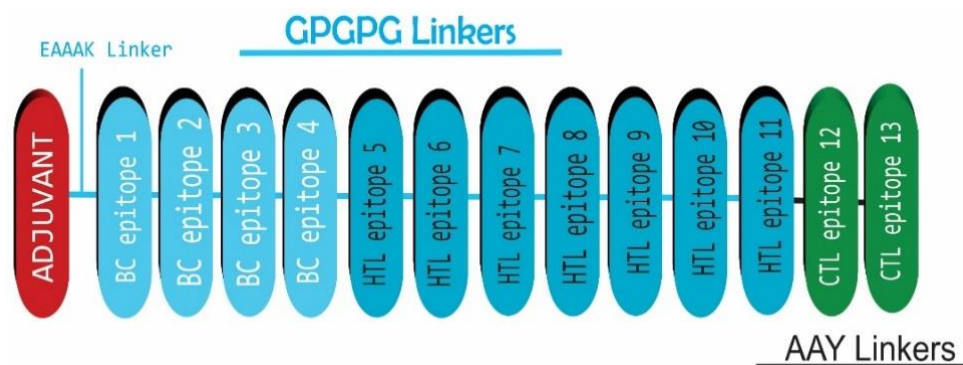

Figure 2. The schematic vaccine construct of HEV.

3.5. Physicochemical Properties of the Vaccine

The physicochemical properties below provide valuable insights into the characteristics of the vaccine construct (Figure 2). A theoretical pI (Isoelectric Point) of 8.70 suggests that the vaccine is likely to carry a net positive charge under typical physiological conditions. The total number of amino acids present in the vaccine construct is 232, with a molecular weight of 22,863.32 g/mol. The aliphatic index reflects the relative volume of aliphatic side chains (valine, isoleucine, and leucine) in a protein sequence. A higher aliphatic index, such as the value of 52.80 provided, suggests greater thermostability, as aliphatic residues contribute to protein stability. The estimated half-life values provided (4.4 h in mammalian reticulocytes, >20 h in yeast, and >10 h in *Escherichia coli*) give insights into the protein's stability and degradation rates in different cellular environments as well as the best compartment to store the vaccine. The instability index value of 22.37 suggests that the protein is relatively stable, as it falls below the threshold value of 40, where II values greater than 40 indicate potential instability. The GRAVY (grand average of hydropathicity) score of -0.23 suggests that the vaccine construct is overall hydrophilic, which may influence its solubility and interactions with other molecules. These properties are crucial for understanding the stability, solubility, and potential immunogenicity of the vaccine construct. They help ensure that the vaccine maintains its structural integrity and efficacy throughout storage, formulation, and administration.

3.6. Predictions of Antigenicity, Allergenicity, and Toxicity

The antigenicity, allergenicity, and toxicity of the vaccine construct show that the nature of the vaccine was antigenic with a score of 0.6831 when the base threshold value was set at 0.4, non-allergenic, and non-toxic.

3.7. Predictions of the Secondary Structures

Among 232 amino acids, 21 amino acids took part in the formation of an α-helix, which represents 9.05% of the structure; 16 in β-strands, which constitute 6.90%; and 141 amino acids produced a random coil, which was 60.78% of the entire vaccine construct (as shown in Figure 3). The formation of secondary structures, such as α-helices and β-strands, can potentially hinder epitope recognition by the host's immune system, especially if the

epitopes are linear; however, the impact on epitope recognition depends on the specific structure–function relationship of the protein and the nature of the epitopes involved.
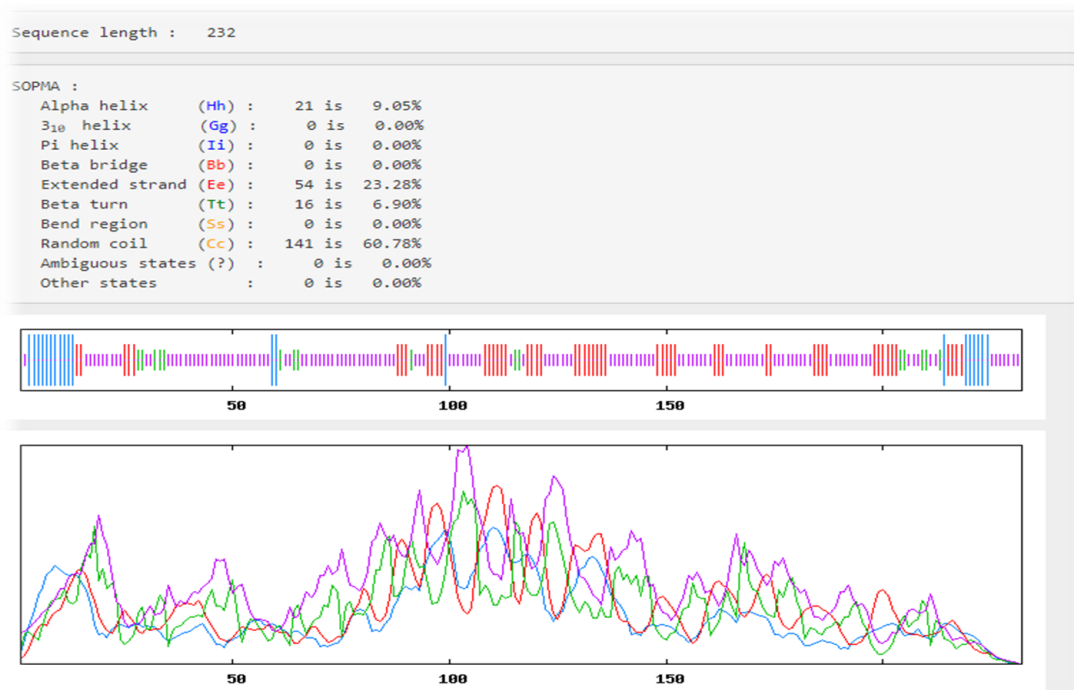

Figure 3. Properties of the secondary structure of the vaccine. From the two graphs displayed, the first graph makes the prediction visible while the second graph includes all the predicted states.

3.8. Three-Dimensional Structure Prediction

In 3D structure prediction, templates of known protein structures are used as references to predict the structure of a target protein with a similar sequence. They provide crucial information about the possible arrangement of amino acids. Out of 10 templates, 7 showed good alignment, with 7bv0, 3jacA, 7bvoA, 4n16A, and 2pffB having the best Z-score values (ranging from 1.21 to 6.08). The C-score values of the top five models (referring to a predicted 3D structure of the target protein) ranged from -4.43 to -2.69, with higher values indicating higher confidence. These models are based on the alignment of the target protein's sequence with known protein structures (templates) and are used to estimate the protein's structural characteristics. The model with a C-score of -2.86, a TM-score of 0.39 ± 0.13, and an estimated RMSD of 12.3 ± 4.3 Å was chosen for further analysis.

3.9. Three-Dimensional Refinement and Validation

The structure refinement process was conducted using the Galaxy Refine server (Figure 4). Out of the five refined models, Model 4 was selected as the best for further study, as indicated in Figure 5a. Model evaluation metrics: The chosen Model 4 was evaluated using various metrics, including GDT-HA (Global Distance Test–High Accuracy), RMSD (Root Mean Square Deviation), Molprobity, clash score, poor rotamers, and Rama favored. These metrics provide information about the overall quality and accuracy of the model; PROCHECK server: The refined Model 4 was further verified using the PROCHECK server, which generated a Ramachandran plot. This plot displayed the distribution of residues in the favored, allowed, and outlier regions of the Ramachandran plot, with 98.0% of residues in the favored region, 2.0% in the allowed region, and 0.0% outliers, as shown in Figure 5b. This indicates that the majority of residues in the model have favorable backbone torsion angles; ProSA-web server: Finally, the structure was validated using the ProSA-web server, which generated a Z-score of -3.17 (Figure 5c). The Z-score assesses the overall quality of the model in comparison to experimental structures, with negative scores indicating higher confidence in the model's accuracy. Overall, the figure demonstrates the thorough

validation process conducted on the refined Model 4 of the vaccine construct, ensuring its structural quality and reliability for further analysis and study.
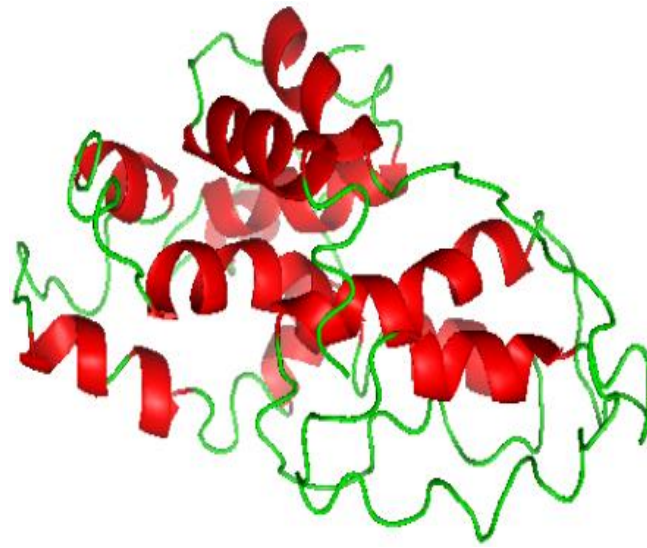

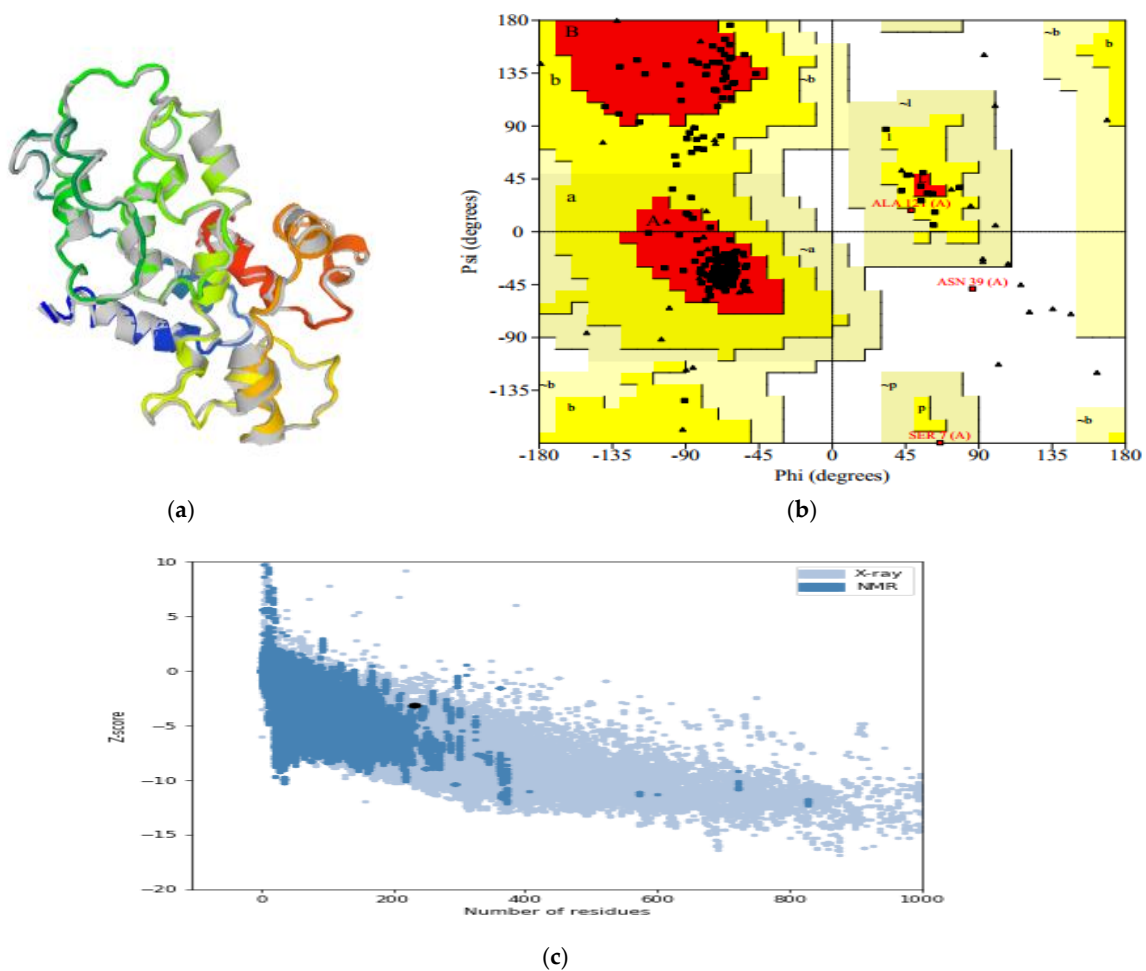**Figure 4.** HEV 3D structure prediction of vaccine construct.

Figure 5. Validation of HEV vaccine construct: (**a**) refined structure of HEV vaccine; (**b**) Ramachandran plot analysis of predicted structure; (**c**) validation of 3D vaccine construct (−3.17).

3.10. Protein–Protein Docking of the Toll-like Receptors (TLRs) and Vaccine Construct

The ClusPro server was used to dock the vaccine construct (ligand) with six different immune receptors (TLRs), which are TLR 2, TLR 3, TLR 4, TLR 6, TLR 8, and TLR 9. A total of 30 models were generated for each immune receptor. Out of the 30 models generated for each of the six TLRs selected, only the lowest-energy-score model was chosen for each of the TLRs (Figure 4). The energy score obtained for TLR 2 was found to be −1172.2, which is of the model 0; the energy scores for TLR3, TLR4, TLR6, TLR8, and TLR 9 are −1259.8, −1262.6, −1276.6, −1182, and −1713.9, respectively (shown in Figure 6a–f).
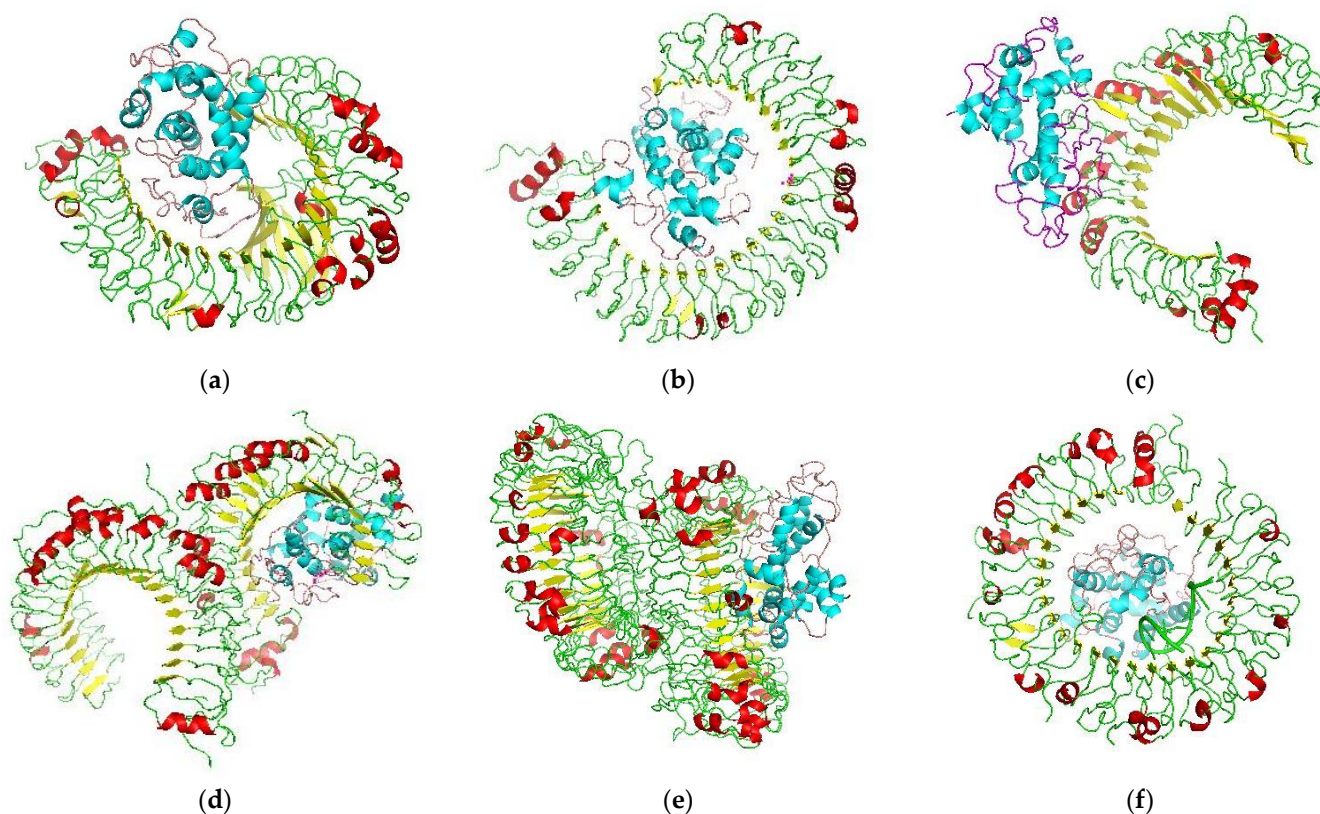

Figure 6. HEV vaccine construct with (**a**) TLR2, (**b**) TLR3, (**c**) TLR4, (**d**) TLR6, (**e**) TLR8, and (**f**) TLR9 docked complexes. Vaccine Construct (Viridian Green).

3.11. Dynamics Simulation

Dynamics simulation was carried out by the iMODS server. The docked files were uploaded to this server in the space for uploading PDB files and the results were shown within a few seconds when keeping all the parameters constant. The results of the dynamics simulation of the vaccine receptor complex of the HEV vaccine were shown in Figures S1a–f–S6a–f in the Supplementary Materials. The eigenvalues provide insights into the dynamic behavior and flexibility of the vaccine–receptor complex, with each value representing a distinct vibrational mode or type of motion within the system. Smaller eigenvalues indicate slower or more localized motions, while larger eigenvalues indicate faster or more global motions. Variances in protein structures denote deviations from average atomic positions, revealing mobility levels; high variances signify flexibility, while low variances indicate rigidity, assessable via methods like PCA or NMA. Deformability measures a molecule's capacity to change shape in response to external factors, with higher values suggesting enhanced flexibility and adaptability to different conformations. The residual index assesses individual residue mobility within a protein, elucidating its relative flexibility; residues with elevated indices exhibit greater mobility, while those with lower values are more constrained. The B-factor, or temperature factor, quantifies thermal atomic

motion, with higher values indicating increased mobility and flexibility, typically depicted visually through color-coded representations on protein structures.

3.12. Codon Adaptation and In Silico Cloning

The Java Codon Adaptation Tool, an online server, was utilized in this study for codon adaptation prediction. The codon sequence optimization had a length of 696 nucleotides. The improved sequence exhibited a Codon Adaptation Index (CAI) value of 0.91, with a GC content of 62.36%. These findings suggest efficient expression of the vaccine construct in *E. coli*, albeit through in silico predictions. Finally, SnapGene software (Version 7.2) was employed to design the recombinant plasmid sequence for the insertion of adapted codon sequences into the pET28b(+) vector, as depicted in Figure 7a,b.
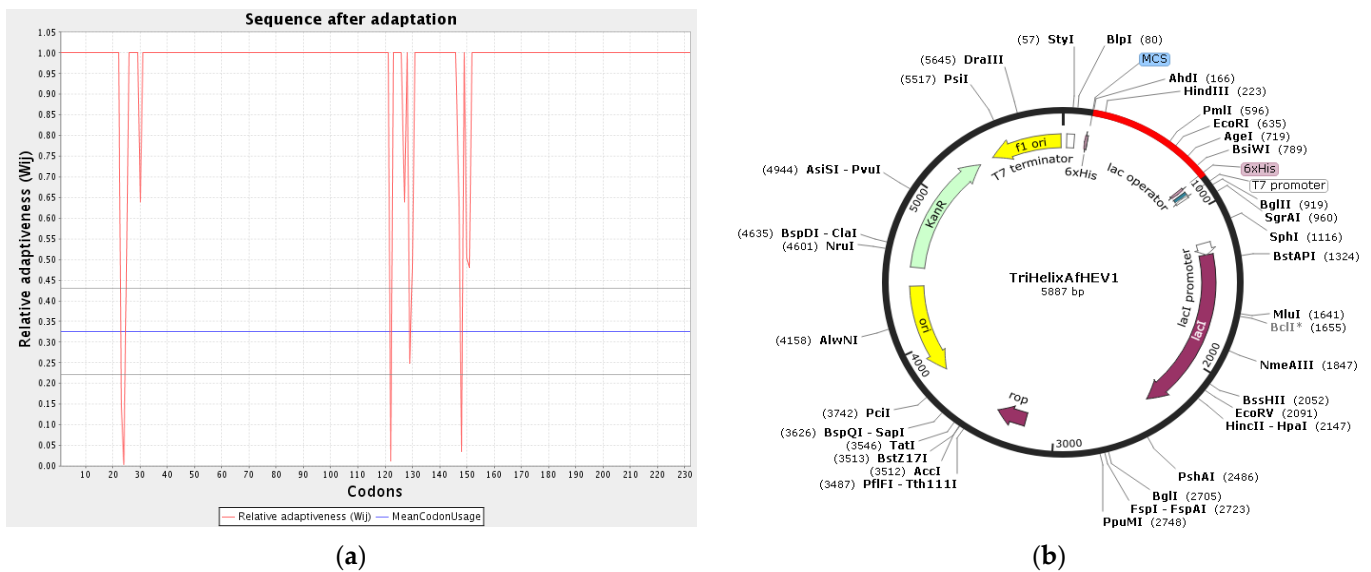

(**a**)   (**b**)

Figure 7. (**a**) The codon adaptation of the HEV vaccine was performed using the JCat server. (**b**) Additionally, the in silico cloning of the adapted vaccine sequence into the pET28b(+) vector is illustrated by black lines, with the region highlighted in red indicating the selected gene encoding the vaccine, flanked by XhoI (158) and XbaI (335) sites.

3.13. Immune Simulation

The initial response to the vaccine was observed within the first fifty days, characterized by high levels of IgM + IgG. Subsequently, there was a rise in IgM, IgG1 + IgG2, and IgG1 levels, coinciding with a rapid decline in antigen concentration, as illustrated in Figure 8a. B-cell activity, particularly B Mem (y2), remained high throughout, indicating the formation of prominent memory cells, while B isotypes showed less memory-cell formation, as depicted in Figure 8b. Similarly, an increased response was observed in the population of Th (helper) and Tc cells, accompanied by memory development, as shown in Figure 8c–i. Additionally, immunization stimulated the production of IFN-y and IL-2.
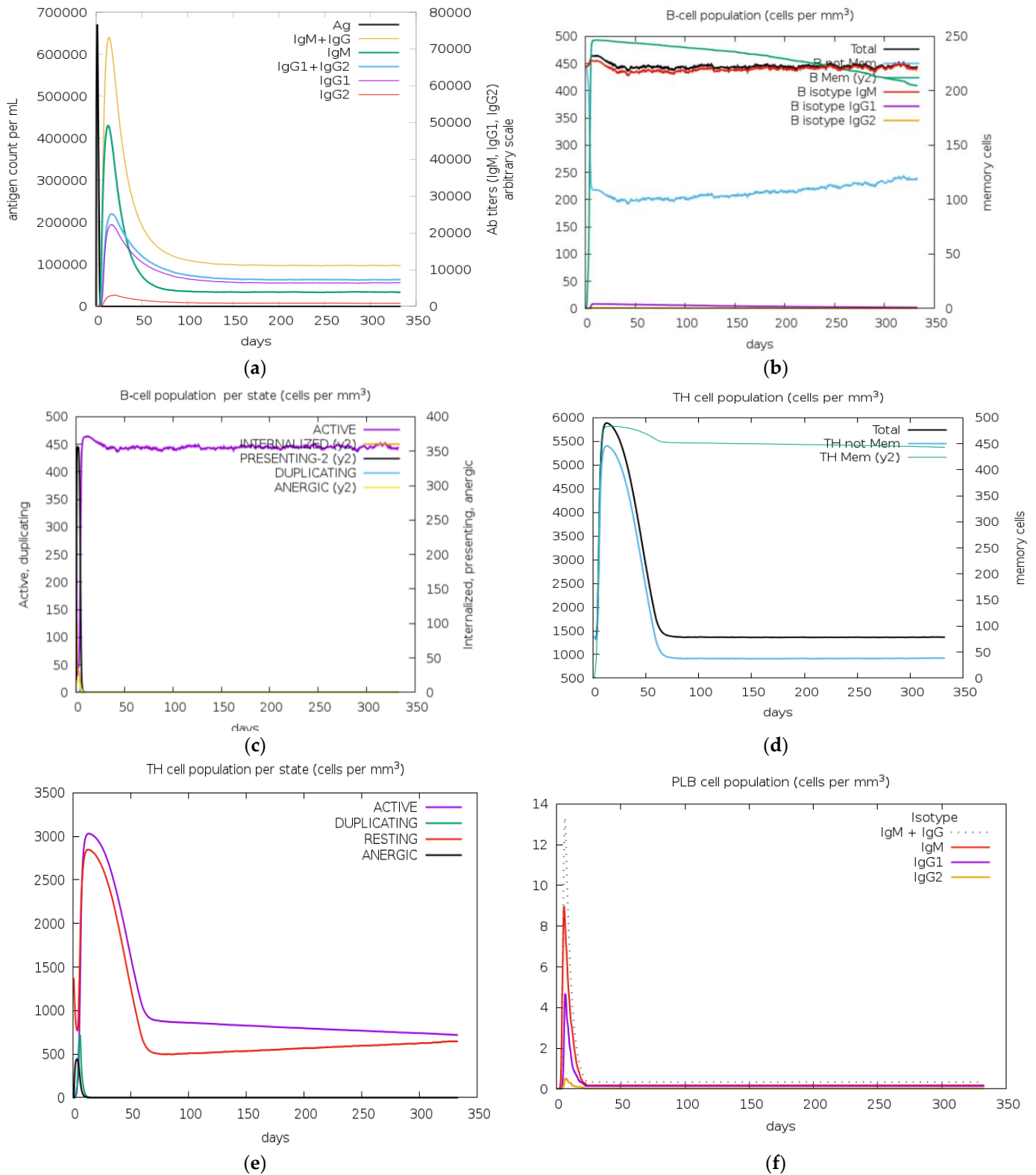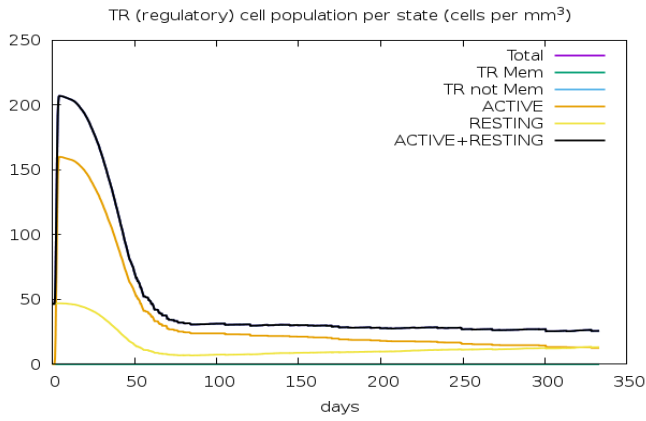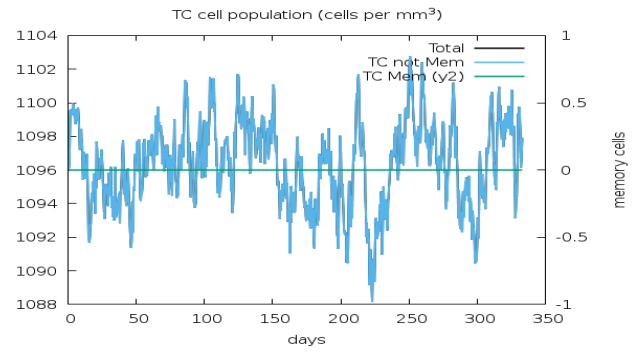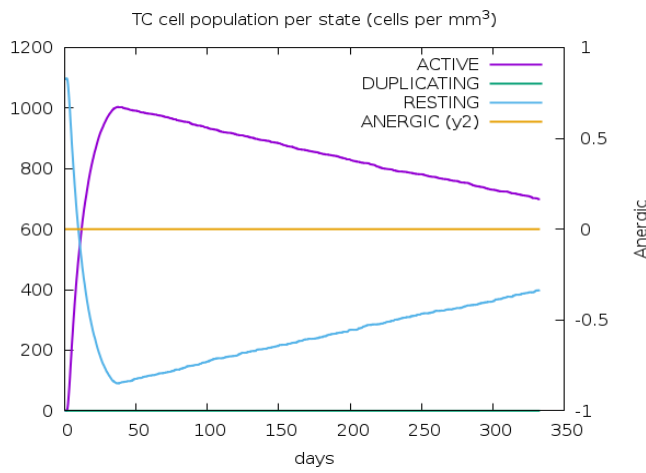
(a)

(b)

(c)

(d)

(e)

(f)

Figure 8. *Cont.*

(**g**)

(**h**)

(**i**)
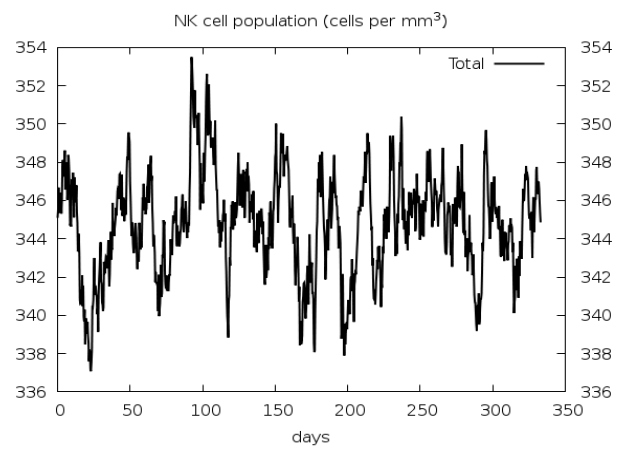
(**j**)

(**k**)

(**l**)

Figure 8. *Cont.*

Figure 8. Immune simulation prediction: (**a**) antigen and immunoglobulins—antibodies are sub-divided per isotype; (**b**) lymphocyte B total count memory cells; (**c**) B lymphocyte population per entity state (i.e., showing counts for active, presenting on class II, internalizing Ag, duplicating, and anergic); (**d**) CD4 T-helper lymphocyte count—total and memory counts are shown on the plot; (**e**) CD4 T-helper lymphocyte count sub-divided per entity state (i.e.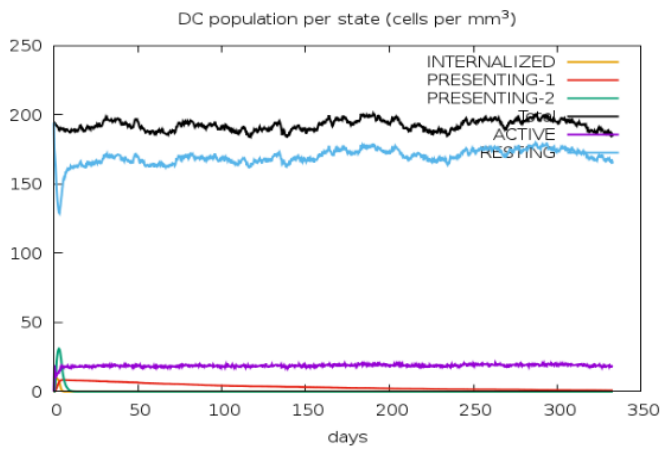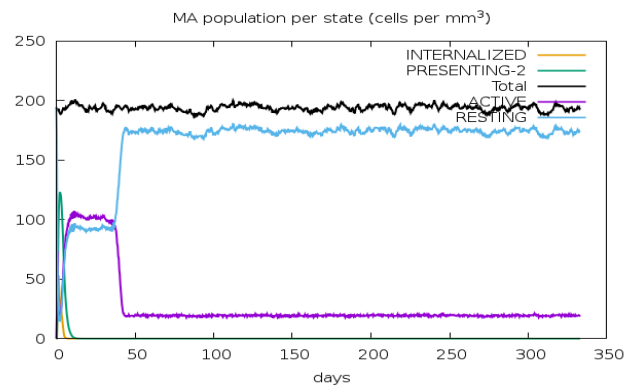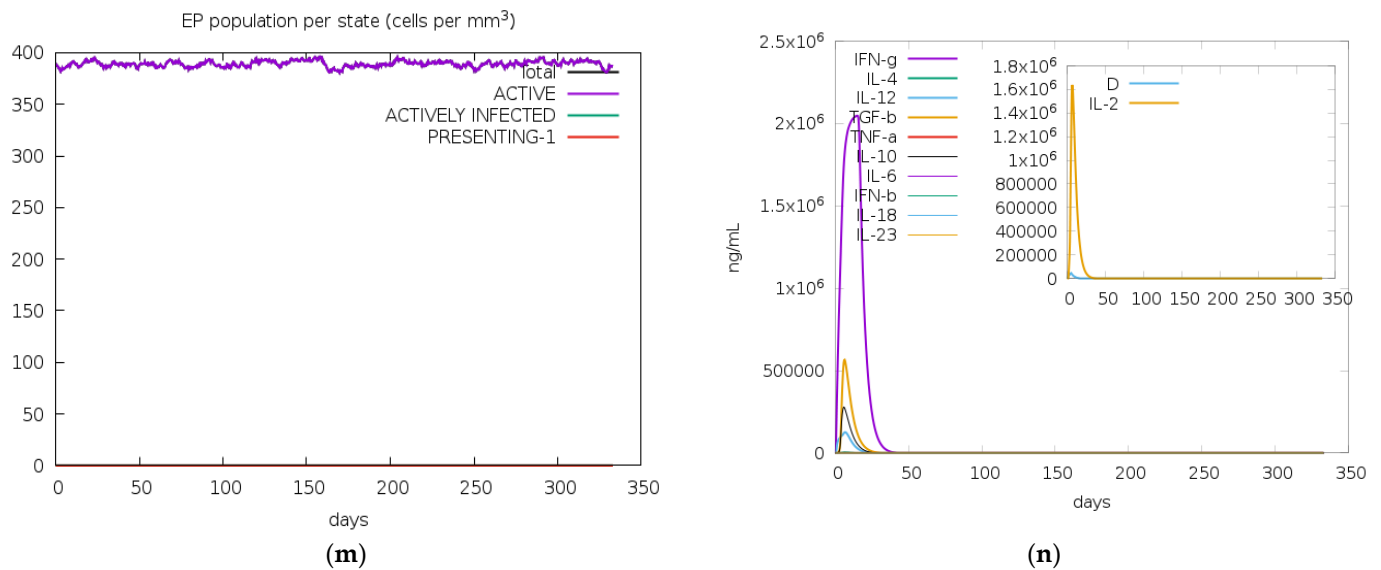, active, resting, anergic, and duplicating); (**f**) plasma B lymphocyte count sub-divided per isotype; (**g**) CD4 T-regulatory lymphocyte count; (**h**) CD8 T-cytotoxic lymphocyte count—total and memory shown; (**i**) CD8 T-cytotoxic lymphocyte count per entity state; (**j**) natural killer cells (total count); (**k**) dendritic cells; (**l**) macrophages—total count, internalized, presenting on MHC class II, active, and resting; (**m**) epithelial cells; (**n**) cytokines (concentration of cytokines and interleukins with D signifying danger).

4. Discussion

The hepatitis E virus (HEV) is a self-limiting virus that can lead to chronic liver infection, increasing disease burden, especially in developing countries like Africa. Developing a preventive method against HEV is important to prevent the occurrence of HEV epidemics, which can be disastrous. Vaccination has been the most desirable means of fighting infectious diseases throughout the globe [10], and researchers have been involved in vaccine development, targeting epitope-based subunit vaccines among others because they are related to enhanced safety profiles and are more achievable [39]. Epitope vaccines, in contrast to traditional vaccines that worsen the state by causing allergic reactions with little advantage, offer increased safety, reduced cost, and a chance for the logical engineering of epitopes for increased effectiveness [40]. Subunit vaccines can concentrate the immune response on conserved epitopes [39]. Antibody epitope estimation, with the use of computational tools, signifies one of the noticeable phases of constructing a vaccine [41].

This study concentrated on the in silico construction of a potential epitope-based subunit vaccine against HEV from ORF 2, which encodes the capsid protein. ORF 2 is specially targeted in vaccine development approaches because it is immunogenic and can stimulate the generation of neutralizing antibodies [5,42,43]. Therefore, immunoinformatic methods were used for this study. Immunoinformatic methods have been used by researchers as a tool to provide advanced models of epitope-driven vaccines against the Ebola virus, Chikungunya, hepatitis C virus, and Dengue virus [44,45]. A total of seven HEV capsid protein sequences from African countries (Sudan, Chad, Nigeria, and Burkina Faso) were retrieved from the NCBI database. These sequences were used to project T-cell and B-cell epitopes, and the derived epitopes were assessed for their antigenic capacity. Hepatitis E (HE) immunity is described to depend on both B-cells and T-cells [45]. Using different immunoinformatic tools, we predicted our CTL, HTL, and B-cell epitopes and evaluated their antigenicity and allergenicity tendencies.

The antigenic competency of a vaccine in instigating immune response is very crucial, likewise, the allergenic potential of the epitopes is necessary in vaccine development; therefore, the epitopes used were antigenic and non-allergenic in nature in that they can induce an antigenic response but are not able to provoke any allergenic reaction in an individual.

For the construction of vaccines, B-cell and T-cell epitope prediction is a necessary step, B-cells are mainly responsible for retaining humoral defense, generating certain cytokines, and presenting antigens to CD4+ T-cells and T-cells involved in cell-mediated immunity [46]. Both cellular and humoral immunities can be stimulated by epitope vaccines, which are beneficial over the monovalent vaccine [47]. This vaccine candidate fits into these criteria of vaccine development. The epitopes chosen were amalgamated using AAY and GPGPG linkers; and the adjuvant was joined to the N-terminal of the vaccine. The essence of this adjuvant in the designed vaccine was to boost immunogenicity and stimulate different mediators and innate immunity, thereby enhancing the intensity, activation, or prolonged existence of the antigen-specific immune responses if utilized in combination with specific vaccine antigens [48]. GPGPG linkers play two roles in the structure, firstly, the role in preventing junctional epitope generation [49]—which is a key concern in constructing epitope-based subunit vaccines—and secondly, it enhances HTL epitopes' immunization and presentation [49,50]. The linker AAY was used to link the CTL epitopes and GPGPG for intra-HTL epitopes. APHAALS and EAAAK linkers were the connectors between the adjuvant from the N-terminal and the remaining components of the vaccine [51,52]. The EAAAK is a functional peptide, which acts as a rigid spacer among proteins [53] to promote a high level of expression and enhanced bioactivity of the merged protein.

The epitope of the subunit vaccine construct was examined for its antigenicity, allergenicity, and toxicity; it was observed to be antigenic, not allergenic, and not toxic—this is in accordance with other vaccine designs of SARS CoV2 by [54,55]. The pI was estimated to be 8.70—this signifies that the final protein is alkaline; the GRAVY score was −0.23—the negative score value signifies that the protein nature is hydrophilic and can relate with water molecules, as suggested by [56,57] in a related study. The protein's stability can be effectively predicted using the instability index, which is calculated based on its amino acid composition. In this study, the obtained value of 22.37 indicates that the protein is relatively stable. This value is notably below the established threshold of 40, beyond which higher instability is suggested. This finding aligns with previous research findings reported by [26,41], further reinforcing its potential for use. Also, the aliphatic index revealed that the protein comprises aliphatic side chains. The whole of these parameters signify that the protein is stable thermally and thus fit to be used in developing countries like Africa.

In vaccine design, the fundamental aspects to know are the secondary and tertiary structures of the aim protein [58]. The analysis of the secondary structure predicted that the final protein involved a 9.05% alpha helix, 23.28% extended strands, and a 60.78% random coil. After the refinement, the vaccine candidate 3D structure was enhanced distinctly, and based on Ramachandran plot forecasts, the properties displayed were apposite [41]. Based on the Ramachandran plot, 85.9% of the refined predicted residues in the model were in favored regions, with an additional 12.2% in allowed regions, 1.3% in generously allowed regions, and 0.6% in disallowed regions, consistent with findings reported by [59]. The ClusPro server was utilized in achieving vaccine-receptor docking [60] and validated by PatchDock [61], which was performed to verify the interaction complex. Toll-like receptors (TLRs) are important receptor proteins in activating the innate immune response [62]. So far, findings have revealed that TLRs found on immune cells are liable to mediate immune responses against RNA viruses, and there are many of these TLRs. Nevertheless, this vaccine was docked against six TLRs, namely TLR2, TLR3, TLR4, TLR6, TLR8, and TLR9, and the vaccine overlapped with mostly all the TLRs. The low binding energy score observed suggests a robust affinity between the molecules [63]. Through the recognition of TLRs, the host's innate immune system spots microorganisms and reacts

to their stimuli. We conducted an in silico prediction of the atomic stability of the vaccine with Toll-like receptors (TLRs) using molecular dynamics simulation techniques. Our findings indicate favorable stability, which is consistent with the results reported by [64]. The B-factor/mobility values of deformability, variance, eigenvalues, co-variance map, and elastic network were provided by this server. The deformability of a protein complex rests on its ability to deform at each of its amino acids. When the eigenvalue—which is allied with the energy that is needed to deform the given structure—is lower, the deformation becomes easier. To determine and measure the protein's flexibility, the iMODS server is easy and has been a fast tool to use [36,65–68]. Protein docking and molecular dynamics simulation gave clues and understanding about the stability, dynamics, and interaction of the vaccine–receptor complex [69]. Immunoreactivity testing through serological testing is one of the first steps in authenticating a designed vaccine [70]. Also, in any vaccine design, its expression in a suitable host is important; therefore, codon adaptation of the designed vaccine was carried out in *E. coli* k 12 to achieve a high expression and the outcome showed that codon optimization resulted in efficacious expression [71].

The immune system simulation creates a possibility of examining how immunogenic generic protein sequences can be and how steady the results are with typical immune responses [72]. After first exposure to the antigen, there was a universal increase in the produced immune responses. Memory B-cell and T-cell development was obvious, with memory B-cells lasting several days. Both Th cells and T cytotoxic cells increased at first and then dropped around day 50 to maintain a range within 50 to 150, and from the cytokine simulation plot, there was an increase noted in amounts of IFN-γ and IL-2 after immunization that were sustained at climaxes for many days.

This study presents a novel approach to combat the hepatitis E virus (HEV) by designing an epitope-based subunit vaccine targeting the capsid protein, utilizing immunoinformatic methods to predict antigenic epitopes. The incorporation of adjuvants and linkers enhances vaccine stability and immunogenicity, while molecular dynamics simulation provides insights into vaccine–receptor interactions. Codon optimization ensures efficient expression in E. coli, and immune system simulations indicate robust immune responses, suggesting the potential effectiveness of the designed vaccine. However, the progression of this vaccine to the pre-clinical and clinical phases is limited by the need to demonstrate its efficacy and safety. Ongoing work aims to address these limitations and further optimize the vaccine before seeking approval.

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