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RESEARCH ARTICLE

Multi-epitope Vaccine Design against Grouper Iridovirus (GIV) Using Immuno-bioinformatics Approach

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Abstract Grouper Iridovirus (GIV) infection induced cell death in grouper spleen cells and caused serious systemic diseases with more than 90% mortality. Therefore, effective strategies are critically needed to prevent economic losses and maintain the sustainability of grouper aquaculture. Using immuno-bioinformatics, this study aimed to create a multi-epitope vaccine (MEV) that would be effective against GIV. The GIV major capsid protein sequences were retrieved from the NCBI proteome database. Out of 284 epitopes, 17 CTL, 12 HTL, and 10 B-cell epitopes were predicted to be antigenic, non-allergenic, and non-toxic. 10 highly antigenic and overlapping epitopes were shortlisted. To generate full-length epitope vaccine candidates, the selected antigenic epitopes were fused with linkers and adjuvants. Four sets of different linker combinations (no linker, GGS, EAAK, GGGS, GPGPG, KK, and AAY) were tested and compared for their antigenicity, allergenicity, and toxicity using several servers. Molecular dynamics simulations with GROMACS were used on the modelled 3D structures to examine their stability. The results of vaccine candidate sequences screening and MD simulation predicted that the structure with GGS linker is relatively stable with a high antigenic index, non-allergenic, and non-toxic. The designed MEV in the present study could be a potential candidate for further vaccine production process against GIV.

Keywords: Iridovirus, immune-bioinformatics, multi-epitope, vaccine, molecular dynamics simulations.

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Introduction

Grouper known as *Epinephelus* spp. is a mariculture fish species that is economically important and widely cultured in Southeast Asian countries including China, Japan, and Taiwan, and occur mostly on corals and rock reefs [1]. According to the Food and Agriculture Organisation (FAO) report for 2022, the global demand for *Epinephelus* species in the production of main aquaculture species has increased by 2.7% [2]. Nevertheless, as the aquaculture industry developed, the possibility of infectious diseases affecting this species has increased [3]. Grouper iridovirus (GIV) has been an emerging concern in Asian countries and shows a broader geographical distribution including China, Japan, South Korea, Taiwan, Singapore, Indonesia, and Malaysia [4], [5], [6], [7], [8], [9], [10]. In Malaysia, epizootic grouper iridovirus isolates from five grouper species have been reported in Sabah including brown-marbled grouper (*Epinephelus fuscoguttatus*), humpback grouper (*Cromileptes altivelis*), giant grouper (*E. lanceolatus*), orange-spotted grouper (*E. coioides*) and hybrid grouper (*E. fuscoguttatus* ♀ x *E. lanceolatus* ♂) [11]. Recently, GIV isolates from two grouper species namely the Tiger grouper hybrid (*Epinephelus sp.*) and the Coral trout (*Plectropomus leopardus*) have been identified in Peninsular Malaysia [5].

GIV has also been recorded from various grouper species in different countries including brown-spotted grouper (*Epinephelus tauvina*) in Singapore [12] tiger grouper (*E. fuscoguttatus*) in Indonesia [13], hybrid grouper (red spotted grouper E. akaara x *E. malabaricus*) in Taiwan [14] yellow grouper (*E. awoara*) [15], giant grouper (*E. lanceolatus*) [16], orange-spotted grouper (*E. coioides*) and hybrid grouper (*E. fuscoguttatus*♀ × *E. lanceolatus*♂) in China [17] Iridoviridae are large icosahedral cytoplasmic DNA viruses with particle sizes varying from 120 to 350 nm. To date, the members of family Iridoviridae are classified into two subfamilies: (1) *Alphairidovirinae* (genera *Ranavirus, Megalocytivirus and Lymphocystivirus*) and (2) *Betairidovirinae* (genera *Iridovirus, Chloriridovirus*, and *Decapodiridovirus*) [18]. Grouper iridovirus belongs to the subfamily *Alphairidovirinae* which infects mainly vertebrates such as bony fish, amphibians, and reptiles. GIV infects a wide range of ages, from fingerlings to spawners and the disease exhibits fatal histopathological signs, including liver and spleen enlargement with haemorrhage [19]. Other symptoms of the infected groupers include abnormal swimming and dark colouration of the body surface [20].

Grouper iridovirus (GIV) poses a severe threat to the aquaculture industry, with devastating economic consequences and potential for widespread impact. Recent studies have quantified its severity, revealing a staggering 93% cumulative mortality rate in giant gourami and grouper populations within just 12 days of infection, as observed in Taiwan [21]. This alarmingly high fatality rate not only decimates grouper stocks but also facilitates the rapid spread of the disease, creating a vicious cycle of infection and population decline [22]. The virus's ability to cross species barriers affects other commercially important fish like dwarf gourami [23], further amplifies its detrimental impact on aquaculture production. With significant economic losses reported across the industry [24], the quantifiable severity of GIV underscores the urgent need for effective management strategies to mitigate its impact on global fish aquaculture.

Reverse vaccinology by immuno-bioinformatics tools has become particularly crucial in human and animal health, and this approach has also recently been applied to marine species. A recent study used reverse vaccinology to screen and identify the potential vaccine against fish pathogen, *Photobacterium damselae subsp. piscicida* [25]. Another software-aided vaccine design was reported to develop a novel peptide vaccine against fish pathogens namely *Edwardsiella tarda* and *Flavobacterium columnare* using a single epitope vaccine [20]. However, this single-epitope vaccine technique is still limited compared to the multi-epitope vaccine design (MEV) where it can recognise immunogenic epitopes with highly targeted immune responses. Therefore, MEV design is an effective strategy to combat most infectious diseases.

The fish immune system is comprised of both innate and adaptive cell-mediated immune mechanisms. The innate immune system fights off initial infections and illnesses first and the main components include cytotoxic T cells, dendritic cells, NK cells, and macrophages [26]. The first line of defence is the physical barriers (skin, gills, and mucous membranes) followed by humoral and cellular defence barriers [27]. The innate immune system plays a crucial role in maintaining homeostasis and preventing the invasion of microbes. It effectively eliminates a wide range of pathogens and aids in activating the adaptive immune response [28]. Recent studies have examined the innate immune mechanisms involved in interactions between pathogens and hosts, and how these mechanisms are conserved across different animal species. These studies have provided new evidence that is reshaping our understanding of innate immunity The adaptive immune system provides a highly specific to a specific antigen and long-lasting response to pathogens [29]. The key components of the adaptive immune system are T cell receptors (TCRs), major histocompatibility complexes (MHC), immunoglobulins (Igs), and recombination-activating genes (RAG). These components first emerged in the first jawed fish, which include cartilaginous and teleost fish [30].

Vaccination stands as a cornerstone in the defence against pathogenic organisms, contributing significantly to the sustainability of aquaculture, as emphasized by [31]. Today's vaccine technology is very precise, focusing on specific pathogenic parts. This is usually done by using subunit or recombinant DNA/RNA particle vaccines [32]. These vaccines centre on the production of antigens, substances that trigger both innate and adaptive immune responses, a fundamental defence mechanism that guards organisms against pathogenic invaders. Notably, the role of certain pathogenic components, like the major capsid protein (MCP), has become more important in the development of vaccines because of how they cause diseases. For example, in viral erythrocytic necrosis (VEN), the virus needs the MCP to get into the host cell. This makes it a great target for lowering the costs of the disease [33]. Furthermore, the major capsid protein of Grouper Iridovirus, as identified by [34], has been recognised not only as a potential biomarker for infected cells but also as a pivotal element in the development of effective vaccines.

The field of immunoinformatics uses prediction tools for many immunobiotechnology and immunomics processes to make vaccines, kits, and biological products that help treat cancer, allergies, and infectious diseases [35]. The use of bioinformatics can help in dangerous substance detection, tissue transplantation ease, and MHC genotyping. Furthermore, bioinformatics enables a better understanding of the functions and interactions of toll-like receptors (TLRs). The first example of such a procedure is reverse vaccinology. Reverse Vaccinology (RV) is a powerful and novel in silico vaccine design approach that overcomes the limitations of current vaccinology methods. It involves a computational-based analysis called subtractive genomics, which is used to prioritise drug targets and vaccine candidates [36]. This approach directly screens genome sequence assemblies, making it a robust method for identifying potential targets and candidates. Reverse vaccinology shortens the time required for vaccine development and evaluation of efficacy against targets [37].

In this study, a MEV against GIV was designed utilising a combination of multiple deep-learning methods in immune-bioinformatics and structural analysis. To optimise the design of a MEV, it is necessary to consider a number of factors, including the selection of included antigenic epitopes, the use of linkers, and the addition of adjuvants.

Materials and Methods

Data Retrieval

The genome of grouper iridovirus (GIV) (GenBank ID: GCA_006465545.1) [38] was used as the basis for this study. From this genomic data, the proteome of GIV was retrieved from the National Biotechnology Information Centre (NCBI). The NCBI provides advanced, significant information through databases such as genomic and genetic data in biotechnology including biomedical, ecological, and agricultural research [39]. Following analysis, six non-redundant protein sequences of major capsid proteins (MCP) were selected as potential antigenic target proteins in FASTA format.

Epitopes Prediction and Validation

Prediction of MHC Class I and MHC Class II Binding Epitope

The prediction of MHC class 1 and MHC class II binding epitopes were performed using the Immune Epitope Database (IEDB) consensus method, the MHCPred server and the RANKEP online servers. The IEDB tool (http://www.iedb. org) was used to predict the MHC class II binding epitope using different prediction methods similar to the MHC class I binding epitope approach [40]. Lengths of 9 and 15 mer were performed using the IEDB recommended method. RANKEP server (http://imed.med.ucm.es/Tools/rankpep.html) was used to identify peptide binders to MHC I and MHC II molecules from protein sequence by position-specific scoring method (PSSM) [41]. MHCPred server (http://www.ddg-pharmfac.net/mhcpred/MHCPred/) predicted binding affinity to MHC II epitopes with an accuracy of about 90 % [41], [42].

Helper T-Lymphocytes (HTL) and Cytotoxic T-Lymphocytes (CTL) Epitopes Mapping

To identify cytotoxic T-lymphocyte (CTL), NetCTL1.2 servers (http://www.cbs.dtu.dk/services/NetCTL/) were used [43]. This server provides an integrative output by combining proteasomal cleavage (C-terminal), Transporter Associated with Antigen Processing (TAP) transport efficiency, and MHC class I affinity. Helper T-lymphocyte (HTL) predicted using NetMHCII pan 4.0 server (https://services.healthtech.dtu.dk/service.php?NetMHCIIpan-4.0) which has an affinity to MHC II [44].

B-Cell Epitope Prediction

Linear B-cell epitopes were predicted by ABCpred and BepiPred. The ABCpred server (http://crdd.osdd.net/raghava/abcpred/) is based on fixed-length patterns of an artificial neural network with a five-fold cross-validation accuracy of 65.93% [45]. An artificial neural network (ANN) network is an information-processing paradigm because of the densely interconnected and parallel structure of the mammalian brain's processing details. BepiPred server (http://www.cbs.dtu.dk/services/BepiPred/) predicted linear B -epitope by applying a combination of a hidden Markov model and propensity scale method [46].

Immunogenicity Prediction and Vaccine Properties Evaluation

Antigenicity Evaluation

Antigenicity evaluation is a vaccine property that shows either epitope or antigen by inducing the immune system with antibodies to develop a mechanism to defence. Based on the antigenicity evolution of the

protein, two servers, VaxiJen and AntigenPro were used. The VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) works based on the alignment-independent prediction of protective antigen with an accuracy of 70% to 89% based on the target organism [47]. ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) server is a sequence-based, alignment-free, and pathogen-independent predictor, which predicts antigenicity based on the obtained result by protein microarray data analysis [48].

Allergenicity Evaluation

The allergenicity evaluation of the designed vaccines was performed using two servers. AllerTOP v.2.0 server (https://www.ddg-pharmfac.net/AllerTOP/index.html) is based on autocross covariance (ACC) transformation of protein sequences into uniform equal-length vectors [49]. The k-nearest (kNN) algorithm is used to sort the proteins into two groups: allergens and non-allergens. It works 88.7% of the time with a training set of 2210 known allergens from different species and 2210 non-allergens from the same species. AlgPred server (http://crdd.osdd.net/raghava/algpred/) classifies between allergens and non-allergens using a hybrid approach with an accuracy of 85% [50].

Toxicity Evaluation

The ToxinPred server (https://webs.iiitd.edu.in/) was used to predict how toxic the epitopes would be. This server uses machine learning to do this based on different properties of the peptides [51].

Construction of Multi-Epitope Vaccine and Structural Analysis

The selected epitopes for the constructed multi-epitope vaccine (MEV) were selected based on being 100% conserved, overlapping, highly immunogenic, non-allergic, non-toxic, and having strong binding affinity with MHC alleles. Clustal Omega serves were used to align and overlap the sequences [52]. The three-dimensional (3D) structure of the vaccine was constructed using I-TASSER [53]. The quality of the model was evaluated using ERRAT and PROCHECK [54], [55], which includes Ramachandran plot analysis to validate the stereochemical quality.

Physicohemical Parameter Evaluation

The ExPasy ProtParam server (http://web.expasy.org/protparam/) was used to figure out the molecular weight (mv), isoelectric point, instability index, aliphatic index, half-life, and gravy score of the target protein [56].

Molecular Interaction of Immunogenic Vaccine Using Molecular Docking

It is essential for the vaccine to interact with the target immune cell receptor and generate a stable immune response. To study the interaction, molecular docking will be performed by High Ambiguity Driven protein−protein DOCKing server (HADDOCK) version 2.4 [57]. The three-dimensional crystal structure of TLR5 (PDB ID: 3J0A, resolution: 26.0 Å) was obtained from the RCSB Protein Data Bank (https://www.rcsb.org/) for molecular docking and simulation analysis with the conjugated vaccine construct. The best structure after refinement from each docked complex will be chosen and their binding affinity will be calculated. HADDOCK server produces a docking score (Z score), which denotes the standard deviations of a given cluster concerning the mean of all the groups generated. The best-docked multi-epitope is one with the minimum Z score. The interacting residues between the vaccine and the receptor will be mapped using PDBsum (https ://www.ebi.ac.uk/thorn ton-srv/databases/pdbsum/Generate.html) [58]. UCSF Chimera and Ligplot software [59] will be used to visualise the 3D and 2D docked complex interactions.

Molecular Dynamic Simulation of Multi-Epitope Vaccine

The designed multi-epitope vaccine and the TLR5-docked complex were subjected to MD simulation using the GROMACS 2021 software package [60]. CHARMM36 force field was chosen for the simulation [61] and the simple point charge (SPC) water model was used for the molecules of water. To preserve the neutrality of the system, sodium and chloride counterions were added. The protein was put into a periodic boundary condition (PBC), and the Particle Mesh Ewald (PME) summation method was used to improve the electrostatic interactions. This method used the Coulomb potential to figure out long-range electrostatics. The system was energy minimized using 5000 steps from the steepest descent algorithm, followed by equilibration for 100 ps of solute-position-restrained MD. We used the Linear Constraints (LINCS) algorithm to set all bond lengths in each system and then did restrained MD with a 2-fs time step on each system. All resulting trajectories were analysed after the MD simulation using GROMACS utilities to assess and evaluate the conformational behaviour of the docked complex. The surfaces of structural conformation in all trajectories were generated using UCSF Chimera [62]. VMD [63] was used to visualise the trajectory of the interaction throughout the simulations. The MD simulations were

conducted in duplicate for a duration of 100 ns (100,000 ps). To assess the stability, flexibility, and hydrogen bonding of the vaccine complex, GROMACS utilities were used to calculate root mean square deviation (RMSD), root mean square fluctuation (RMSF), and hydrogen bonds.

Results and Discussion

Sequence Analysis

The major capsid protein (MCP) of Grouper Iridovirus (GIV) exhibits a high degree of sequence conservation, with 100% homology among five GIV isolates over a 1,392 base pair region and 96-99% similarity with other Ranavirus isolates [64]. This high conservation underscores its crucial structural and functional role in the virus. The MCP is an excellent target for developing epitope vaccines due to several factors: It is the main component of iridoviruses and plays significant roles in virus structure and pathogenesis, making it essential for viral spread [65], [66]. As a highly conserved gene, it ensures broad coverage against multiple strains [34], [67]. The MCP forms the predominant structural component of the virus particle's icosahedral shell, encapsulating the dsDNA complex [68]. This prominence and accessibility on the virus surface make it an ideal target for antibodies. Furthermore, its essential nature means that antibodies targeting MCP are more likely to effectively neutralize the virus. The MCP's potential as a protective antigen further supports its candidacy for vaccine development, as highlighted by recent research [69]. These characteristics, along with minor variations allowing for strain differentiation, make the MCP an optimal choice for epitope-based vaccine development against GIV and related iridoviruses. In this study, data on the proteome of GIV were obtained from the National Biotechnology Information Centre (NCBI) (http://www.ncbi.nlm.gov/), and major capsid proteins were chosen as potential antigenic target proteins. From 126 proteins, 6 non-redundant protein sequences were selected. The selection of six GIV non-redundant protein sequences for epitope prediction was based on a primary criterion of sequence uniqueness. Proteins with 100% sequence identity were considered redundant, and only one representative sequence from such sets was retained. This approach eliminated duplicate sequences, ensuring a diverse set of proteins for epitope analysis.

Epitopes and Immunogenicity Prediction

MHC Class I and MHC Class II Binding Epitope Prediction

To design a comprehensive multi-epitope vaccine against Grouper Iridovirus (GIV), both MHC-I and MHC-II binding epitope predictions were performed using the IEDB and NetMHC server databases. These predictions aimed to identify potential T cell epitopes from GIV proteins that could elicit both cytotoxic T lymphocyte (CTL) and helper T lymphocyte (HTL) responses.

The high-ranked results of the different servers showed several overlaps, indicating segments with a higher probability of antigenicity. The results are presented in Table 1 and Table 2. Epitopes binding to MHC-I and MHC-II and having overlapping properties were chosen for further study. Two key prediction scores were analyzed: IC50 and Rank. The IC50 value represents the concentration of a peptide required to inhibit 50% of the binding of a standard peptide, with lower values indicating stronger binding affinity. Peptides with IC50 < 500 nM are considered strong binders, while those with IC50 < 5000 nM are weak binders. The Rank score represents the percentile rank of the predicted IC50 value compared to a set of random natural peptides, with lower Rank indicating stronger binding.

The MHC-I prediction focused on 9-mer peptides, which are typically presented to $CD8^+$ T cells. Based on IC50 and Rank scores, five CTL epitopes were selected: YTGYHMYSY (amino acids 379-387), ATDIAGGLA (233-241), LANMGVEYY (356-364), FTSVDPYYF (354-362), and YTAASPVYV (311-319). These peptides demonstrated high predicted binding affinities to MHC-I molecules, suggesting their potential to effectively stimulate $CD8^+$ T cell responses against GIV-infected cells. For MHC-II prediction, which represents antigens to $CD4^+$ T cells, we analyzed 15-mer peptides. Three epitopes with high binding affinity scores were selected: TPEIKLLDTNRLGAN (amino acids 86-100), RIGYDNMIGNTSDMT (147-161), and TVEAYVYMTVGLVSN (244-258). These epitopes are predicted to effectively stimulate helper T cell responses, which are crucial for coordinating the overall immune response against GIV.

The identification of both MHC-I and MHC-II binding epitopes is significant for developing a robust multi-epitope vaccine against GIV. MHC-I molecules present antigens to $CD8^+$ T cells, which are essential for direct killing of virus-infected cells. Conversely, MHC-II molecules present antigens to $CD4^+$ T cells, which provide crucial help for both cellular and humoral immune responses [70]. By incorporating both types of epitopes, our proposed vaccine design aims to stimulate a comprehensive immune response

against GIV. The selected CTL and HTL epitopes demonstrate great binding affinity scores for their respective MHC molecules, suggesting they are strong candidates for inclusion in a multi-epitope vaccine construct. This approach could potentially elicit a broad and effective immune response, targeting multiple viral proteins and engaging both arms of the adaptive immune system simultaneously.

Table 1. Predicted MHC I -binding epitopes with strong binding level

Genbank ID	Peptide Sequence	Start (a.a)	End (a.a)	Ic50 (nM)	Rank
QCW63927.1	YTGYHMYSY	379	387	11.96	0.02
	ATDIAGGLA	233	241	212.1	0.25
	LANMGVEYY	356	364	337.65	0.3
AEI85923.1	YTGYHMYSY	379	387	11.96	0.02
	ATDIAGGLA	233	241	212.1	0.25
	LANMGVEYY	356	364	337.65	0.3
ANR02346.1	YTGYHMYSY	379	387	11.96	0.02
	ATDIAGGLA	233	241	212.1	0.25
	LANMGVEYY	356	364	337.65	0.3
AQA28569.1	YTGYHMYSY	379	387	11.96	0.02
	ATDIAGGLA	233	241	212.1	0.25
	LANMGVEYY	356	364	337.65	0.3
AAV91066.1	YTGYHMYSY	379	387	11.96	0.02
	ATDIAGGLA	233	241	212.1	0.25
	LANMGVEYY	356	364	337.65	0.3
	FTSVDPYYF	354	362	338.22	0.3
	YTAASPVYV	311	319	517.15	0.4

Table 2. Predicted MHC II -binding epitopes with strong binding level

GenBank ID	Peptide Sequence	Start (a.a)	End (a.a)	Ic50 (nM)	Rank
QCW63927.1	TPEIKLLETNRLGAN	86	100	5.91	0.18
	RIGYDNMIGNTSDMT	147	161	33.53	4.01
	TVEAYVYMTVGLVSN	244	258	12.37	0.99
AEI85923.1	TPEIKLLETNRLGAN	86	100	5.91	0.18
	RIGYDNMIGNTSDMT	147	161	33.53	4.01
	TVEAYVYMTVGLVSN	244	258	12.37	0.99
ANR02346.1	TPEIKLLDTNRLGAN	86	100	5.91	0.18
	RIGYDNMIGNTSDMT	147	161	33.53	4.01
	TVEAYVYMTVGLVSN	244	258	12.37	0.99
AQA28569.1	TPEIKLLETNRLGAN	86	100	5.91	0.18
	RIGYDNMIGNTSDMT	147	161	33.53	4.01
	TVEAYVYMTVGLVSN	244	258	12.37	0.99
AAV91066.1	TPEIKLLDTNRLGAN	86	100	5.91	0.18
	RIGYDNMIGNTSDMT	147	161	33.53	4.01
	TVEAYVYMTVGLVSN	244	258	12.37	0.99

Helper T-Lymphocytes (HTL) and Cytotoxic T-Lymphocytes (CTL) Epitopes Mapping

The segments that overlapped in the previous investigation were cross-referenced with the results of HTL and CTL epitope mapping. The shared segments were then leveraged for the selection of epitopes in the designed vaccine. Each segment underwent evaluation for allergenicity, antigenicity, and toxicity, with results detailed in Table 3. All data from these analyses were thoroughly compared to pinpoint regions with a variety of epitopes, high antigenicity, and that were non-allergenic and non-toxic - all crucial for integration into the vaccine construct. Epitopes not meeting these criteria were subsequently excluded.

Table 3. Final selected $CD8^+$ and $CD4^+$ T-cell epitopes

GenBank ID	Peptide Sequence	CTL/ HTL	Antigenicity		Allergenicity		Toxicity	
			Vaxigen	Antigen	AllerTop	AlgPred	ToxinPred	Toxicity
QCW63927.1	YTGYHMYSY	CTL	0.6086	ANTIGEN	NON-ALLERGEN	0.31	-0.73	Non-Toxin
	ATDIAGGLA	CTL	0.6061	ANTIGEN	NON-ALLERGEN	0.4	-0.91	Non-Toxin
	LANMGVEYY	CTL	0.7305	ANTIGEN	NON-ALLERGEN	0.26	-0.74	Non-Toxin
	RIGYDNMIGNTSDMT	HTL	0.6197	ANTIGEN	NON-ALLERGEN	0.29	-1.05	Non-Toxin
	TVEAYVYMTVGLVSN	HTL	0.7482	ANTIGEN	NON-ALLERGEN	0.29	-1.57	Non-Toxin
AEI85923.1	YTGYHMYSY	CTL	0.6086	ANTIGEN	NON-ALLERGEN	0.31	-0.73	Non-Toxin
	ATDIAGGLA	CTL	0.6061	ANTIGEN	NON-ALLERGEN	0.4	-0.91	Non-Toxin
	LANMGVEYY	CTL	0.7305	ANTIGEN	NON-ALLERGEN	0.26	-0.74	Non-Toxin
	RIGYDNMIGNTSDMT	HTL	0.6197	ANTIGEN	NON-ALLERGEN	0.29	-1.05	Non-Toxin
	TVEAYVYMTVGLVSN	HTL	0.7482	ANTIGEN	NON-ALLERGEN	0.29	-1.57	Non-Toxin
ANR02346.1	YTGYHMYSY	CTL	0.6086	ANTIGEN	NON-ALLERGEN	0.31	-0.73	Non-Toxin
	ATDIAGGLA	CTL	0.6061	ANTIGEN	NON-ALLERGEN	0.4	-0.91	Non-Toxin
	LANMGVEYY	CTL	0.7305	ANTIGEN	NON-ALLERGEN	0.26	-0.74	Non-Toxin
	TPEIKLLDTNRLGAN	HTL	0.5486	ANTIGEN	NON-ALLERGEN	0.36	-1.15	Non-Toxin
	RIGYDNMIGNTSDMT	HTL	0.6197	ANTIGEN	NON-ALLERGEN	0.29	-1.05	Non-Toxin
	TVEAYVYMTVGLVSN	HTL	0.7482	ANTIGEN	NON-ALLERGEN	0.29	-1.57	Non-Toxin
AQA28569.1	YTGYHMYSY	CTL	0.6086	ANTIGEN	NON-ALLERGEN	0.31	-0.73	Non-Toxin
	ATDIAGGLA	CTL	0.6061	ANTIGEN	NON-ALLERGEN	0.4	-0.91	Non-Toxin
	LANMGVEYY	CTL	0.7305	ANTIGEN	NON-ALLERGEN	0.26	-0.74	Non-Toxin
	RIGYDNMIGNTSDMT	HTL	0.6197	ANTIGEN	NON-ALLERGEN	0.29	-1.05	Non-Toxin
	TVEAYVYMTVGLVSN	HTL	0.7482	ANTIGEN	NON-ALLERGEN	0.29	-1.57	Non-Toxin
AAV91066.1	YTGYHMYSY	CTL	0.6086.	ANTIGEN	NON-ALLERGEN	0.31	-0.73	Non-Toxin
	ATDIAGGLA	CTL	0.6061	ANTIGEN	NON-ALLERGEN	0.4	-0.91	Non-Toxin
	LANMGVEYY	CTL	0.7305	ANTIGEN	NON-ALLERGEN	0.26	-0.74	Non-Toxin
	TPEIKLLDTNRLGAN	HTL	0.5486	ANTIGEN	NON-ALLERGEN	0.36	-1.07	Non-Toxin
	RIGYDNMIGNTSDMT	HTL	0.6197	ANTIGEN	NON-ALLERGEN	0.29	-1.05	Non-Toxin
	TVEAYVYMTVGLVSN	HTL	0.7482	ANTIGEN	NON-ALLERGEN	0.29	-1.57	Non-Toxin
AEI85908.1	FTSVDPYYF	CTL	1.2800	ANTIGEN	NON-ALLERGEN	0.36	-0.75	Non-Toxin
	YTAASPVYV	CTL	0.5953	ANTIGEN	NON-ALLERGEN	0.4	-1.41	Non-Toxin

B-Cell Epitope Prediction (Linear and Conformational B-Cell Epitopes)

The linear B-cell epitopes were identified through a comprehensive analysis using advanced prediction tools including ABCpred and BepiPred. The detailed results of this analysis are presented in Table 4. Out of all the overlapping B-cell segments, only two exhibited both high antigenicity and demonstrated non-allergenic, non-toxic properties. Consequently, these specific epitope sequences were chosen for incorporation into the vaccine construct.

Table 4. Linear B cell epitopes prediction

GenBank ID	Peptide Sequence	Antigenicity		Allergenicity		Toxicity	
		Vaxigen	Antigen	AllerTop	AlgPred	ToxinPred	Toxicity
QCW63927.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin
AEI85923.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin
ANR02346.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin
AQA28569.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin
AAV91066.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin
AEI85908.1	GSTNYGRLTNASITVT	1.2317	ANTIGEN	ALLERGEN	0.01	-1.07	Non-Toxin
	NVHVDMRFSHAVKALF	0.9985	ANTIGEN	NON-ALLERGEN	0.27	-1.27	Non-Toxin
	YVYMTVGLVSNVERCA	0.5517	ANTIGEN	ALLERGEN	0.09	-1.68	Non-Toxin
	NVIPISATDIAGGLAD	1.0242	ANTIGEN	NON-ALLERGEN	0.52	-1.14	Non-Toxin

Construction of Multi-Epitope Vaccine and Structural Analysis

Out of 284 epitopes, 17 CTL, 12 HTL and 10 B-cell epitopes were predicted to be antigenic, non-allergenic, and non-toxic. Ten epitopes that are highly antigenic and overlap were chosen. These include five CTL epitopes, three HTL epitopes, and two B-cell epitopes. These selected epitopes were incorporated into the construct along with two TLR agonist adjuvants. Specifically, the flagellin sections responsible for TLR5 binding and activation [71], were chosen. This encompassed two segments from the N-terminal head and C-terminal tail (Figure 1). In addition, RS09, a short peptide TLR4 agonist [72], [73] , and PADRE, a universal T-helper epitope (Pan HLA-DR reactive epitope) [74] were included. The generation of full-length epitope vaccine candidates involved fusing the selected antigenic epitopes using a combination of linkers and adjuvants (Figure 2).

Four distinct engineered vaccine constructs (V1, V2, V3, and V4) were designed using four sets of different linker combinations (no linker, GGS, EAAK, GGGS, GPGPG, KK, and AAY):

1. V1: The selected antigenic epitopes and adjuvants were fused without a linker.
2. V2: The selected antigenic epitopes and adjuvants were fused using GGS linkers.
3. V3: Adjuvants were joined using EAAAK linkers. CTL, HTL, and B-cell epitopes were joined by GGGS, GPGPG, and KK respectively.
4. V4: Adjuvants were joined using EAAAK linkers. CTL epitopes were joined by AAY linkers, and HTL and B-cell epitopes were joined by GPGPG linkers. AAY linkers were used to connect CTL epitopes, while GPGPG linkers were used to connect HTL and B-cell epitopes.

The Ramachandran plot and ERRAT analyses (Table 5) were used to check the structure quality of the predicted model. All the modelled vaccine structures were evaluated using PROCHECK and contributed to the generation of the Ramachandran plot. For a model to be considered reliable, a minimum of 90% of its residues should reside in the allowed region. All vaccine structures exceeded this threshold, affirming their high reliability. Furthermore, the ERRAT scores for all constructed models exceeded 50%, indicating good quality. All engineered vaccine constructs underwent testing and comparison for their antigenicity, allergenicity, and toxicity using several servers.

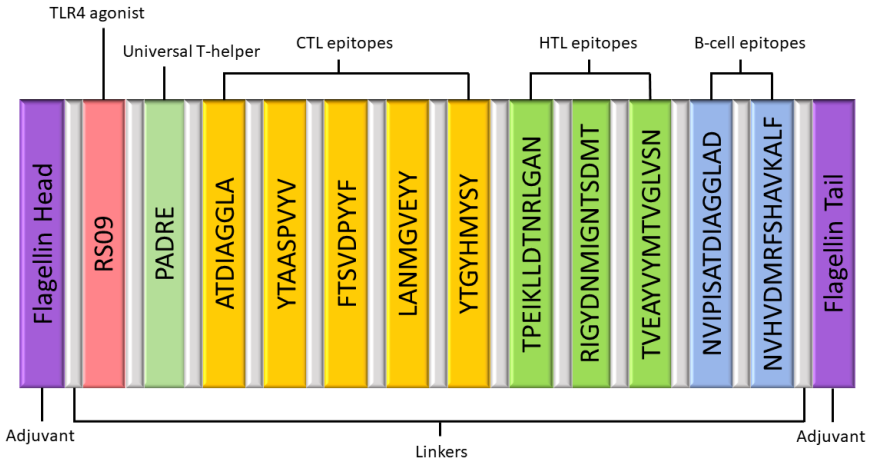

Figure 1. Schematic representation of the final multi-epitope vaccine construct

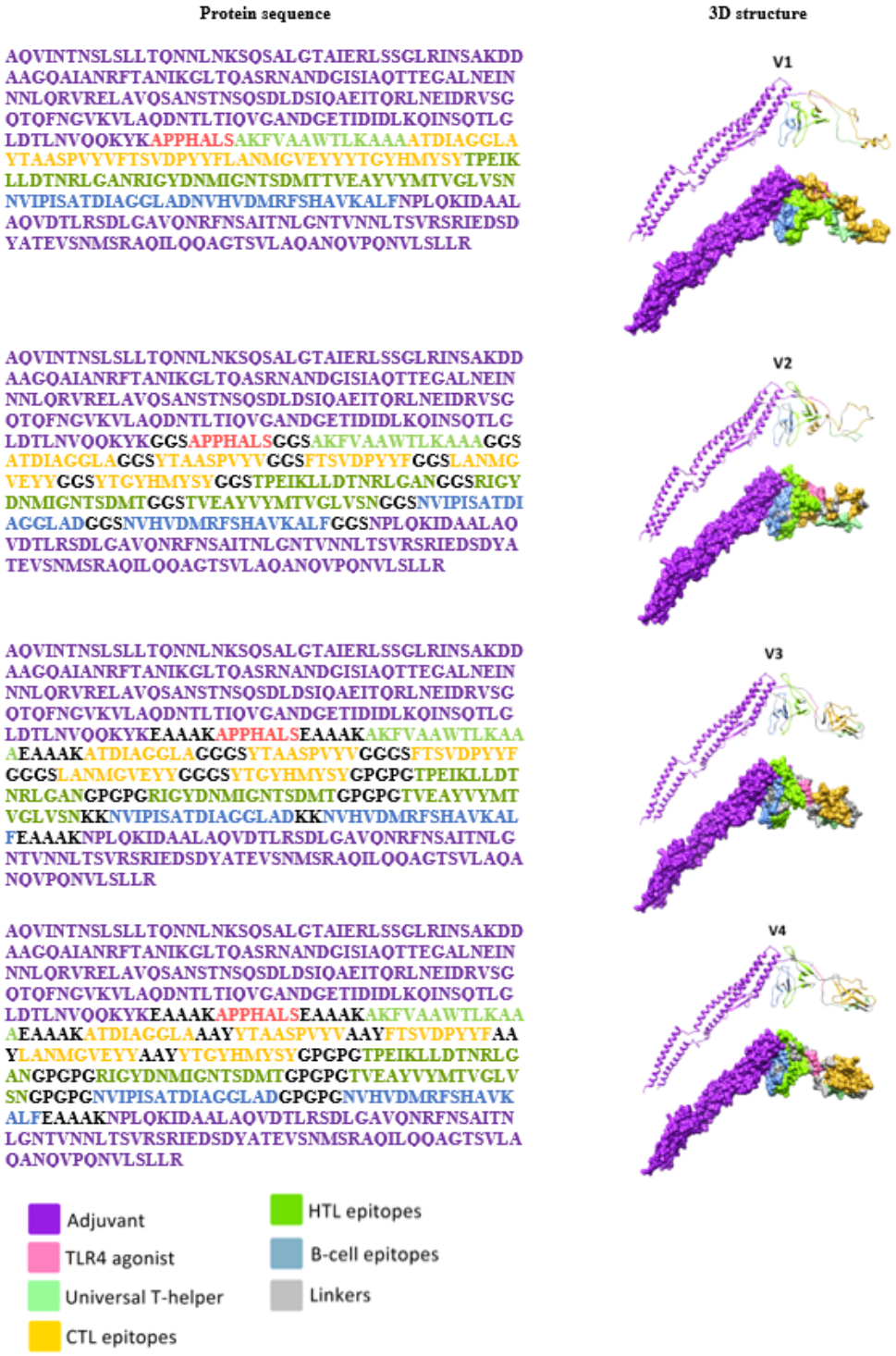

Figure 2. (Left side) Protein sequence of the designed multi-epitope vaccine. (Right side) Predicted 3D structures of the designed multi-epitope vaccine

Table 5. Summary of vaccine validation

Evaluation tools	Evaluation scheme	Vaccine candidates	Score	Normal range of the score
PROCHECK	The number of residues in the allowed regions based on Psi/Phi Ramachandran plot	V1	98.4%	>80%
		V2	99%	
		V3	98%	
		V4	98%	
ERRAT	The overall quality of nonbonded atomic interaction	V1	75.7%	>50%
		V2	82.8%	
		V3	73.8%	
		V4	74.1%	

Immunogenicity Prediction and Vaccine Properties Evaluation

The study looked at four different engineered vaccine constructs (V1, V2, V3, and V4) to see how they affected the immune system, caused allergies, were toxic, and had other physical and chemical properties (Table 6). The prediction of antigenicity was performed using VaxiJen v.2.0 and Antigenpro v.2.0, maintaining a threshold value of 0.4 for Vaxigen v.2.0. Notably, all the developed multi-epitope considered antigens exhibited an antigenicity score greater than 4.0, signifying their substantial immunogenic potential. Predictions of allergenicity using AllerTOP v.2.0 and Algpred consistently yielded non-allergenic profiles for all vaccine constructs, confirming their absence of allergenic properties. Toxicity analysis utilising ToxinPred pinpointed a potential toxin region (Position: 195-204) in V3 and V4, necessitating further investigation and potential modification in this construct. Consequently, V4 and V3 were excluded from further consideration due to the identified potential toxin region. Furthermore, arrays of physicochemical properties were scrutinised, revealing noteworthy characteristics including negative GRAVY scores indicative of hydrophilicity, and stability classifications based on the instability index (II) designating all vaccine constructs as stable. Aliphatic index values (V1: 95.24, V2: 86.97, V3: 86.56, V4: 87.9) further suggested favourable thermostability. Additionally, the molecular weights of the vaccine constructs (V1: 44135.25, V2: 46750.62, V3: 48659.15, V4: 49065.63) aligned with anticipated ranges for effective vaccine candidates. In conclusion, these in-depth analyses demonstrate that the engineered vaccine constructs possess a high immunogenic potential, a low allergenicity level, and other generally favourable physical and chemical properties. This positions them as viable candidates for further refinement and in-depth evaluation in the pursuit of an optimal vaccine candidate.

Table 6. Antigenicity, allergenicity, toxicity, and several physicochemical properties evaluation of the multi-epitope vaccine construct

Linker		V1	V2	V3	V4
Antigenicity Prediction	Vaxigen v.2.0	0.5318	0.5176	0.5691	0.4860
		ANTIGEN	ANTIGEN	ANTIGEN	ANTIGEN
	Antigenpro v.2.0	0.915671	0.937271	0.930226	0.924468
Allergenicity Prediction	AllerTOP v.2.0	NON-ALLERGEN	NON-ALLERGEN	NON-ALLERGEN	NON-ALLERGEN
	Algpred	0.23	0.24	0.24	0.28
		NON-ALLERGEN	NON-ALLERGEN	NON-ALLERGEN	NON-ALLERGEN
Toxicity	ToxinPred	NON-TOXIN	NON-TOXIN	TOXIN-AKAKFVAAWT Position: 195-204	TOXIN-AKAKFVAAWT Position: 195-204
Physicochemical Properties	GRAVY	-0.19	-0.219	-0.263	-0.211
	Molecular Weight (MV)	44135.25	46750.62	48659.15	49065.63
	Instability index (II)	28.93	29.59	28.05	26.25
	Stability based on (II)	stable	stable	stable	stable
	Aliphatic Index	95.24	86.97	86.56	87.9
	Pi	5.18	5.18	5.91	5.25

Molecular Interaction of Immunogenic Vaccine Using Molecular Docking

The HADDOCK 2.4 server was used to perform molecular docking to examine the interaction between V2 and TLR5. 137 structures were clustered into 12 clusters by HADDOCK analysis, accounting for 68% of the water-refined models produced by HADDOCK. The cluster with the lowest HADDOCK score is considered the most reliable cluster. The HADDOCK refinement server was used to further refine a representative model from this top-performing cluster, which resulted in the clustering of 20 structures into one cluster, which accounted for all of the water-refined models produced. A strong binding affinity between the vaccine and the receptor is shown by the HADDOCK score of 140.7 4.4, with a lower number indicating better docking. Notably, the buried surface area (BSA) of 2569.8 +/- 151.9 2 denotes the protein surface's close proximity to and minimal exposure to water. The identification of the complex with the lowest energy and least structural deviation is made possible with the use of the RMSD scores, which are crucial in evaluating the effectiveness of docking experiments. The docked complex's low RMSD score is evidence of its high-calibre model. Figure 3 depicts the docked complex along with multiple strong hydrogen bonds

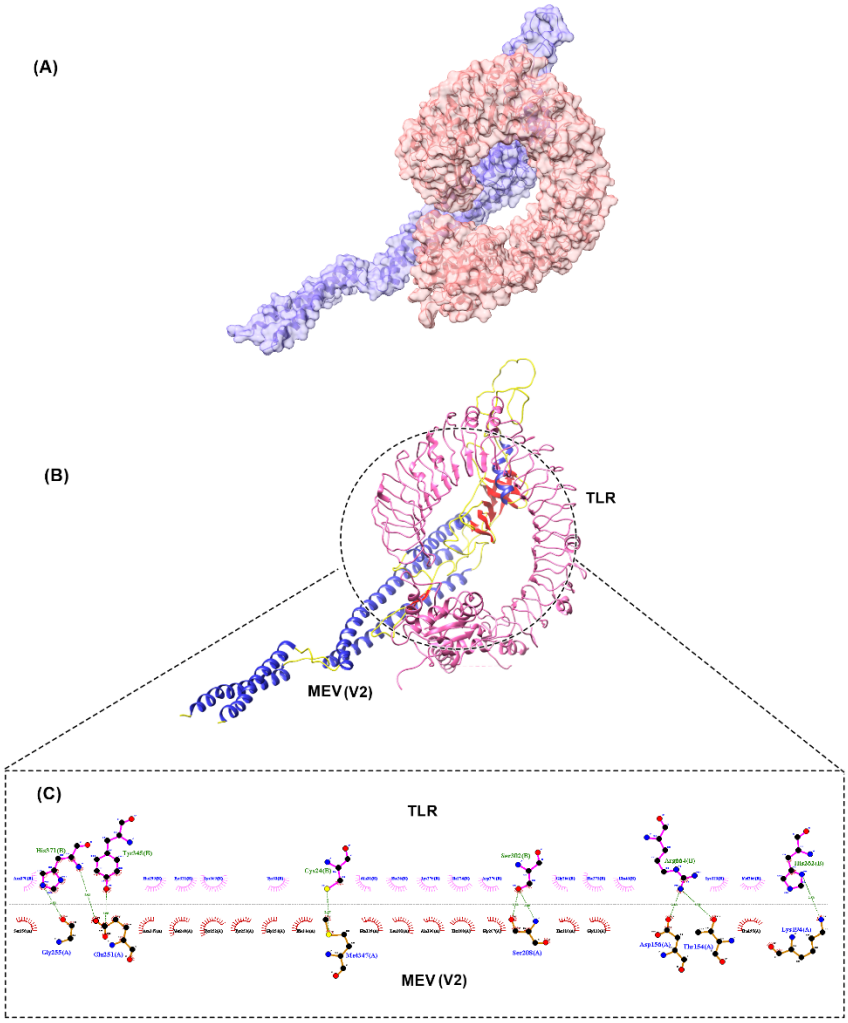

Figure 3. The 3D structure of the designed vaccine (V2) with TLR5: (A) in surface representation and (B) in cartoon representation. (C) LigPlot 2D schematic representation of the interaction profile. Highlighted are the specific binding regions and molecular interactions that facilitate complex formation

The aquaculture industry has been significantly impacted by the emergence of GIV, leading to disease outbreaks that jeopardize grouper production and economic growth [75], [76]. In response, immunoinformatics methods have gained traction for designing multi-epitope vaccines (MEVs), offering a cost-effective and expedited approach to vaccine development [77], [78]. These epitope or peptide-based vaccines present a promising alternative to traditional vaccine methods due to their distinct

advantages. In our study, epitopes were meticulously selected for MEV construction. These epitopes met rigorous criteria, including preservation, overlap, high immunogenicity, and strong MHC allele binding affinity, while also being non-allergenic and non-toxic. The final selection comprised ten epitopes: five cytotoxic T lymphocyte (CTL) epitopes, three helper T lymphocyte (HTL) epitopes, and two B-cell epitopes. However, a challenge with epitope vaccines is their relatively lower protection levels when used alone. To address this, we combined antigenic epitopes with adjuvants and helper peptides in vaccine design. Specifically, we integrated two Toll-like receptor (TLR) agonists, flagellin and RS09, to potentiate the immune response.TLRs, functioning as pattern recognition receptors, are pivotal in recognizing microbial surface antigens, bridging the innate and adaptive immunity [79], [80]. The synergistic effect of combining multiple TLR agonists in vaccines has been validated in prior research [81], [82].

Flagellin's efficacy is enhanced when fused directly to antigens, likely due to synchronized delivery to Antigen-Presenting Cells (APC) and TLR5 activation [83], [84]. Flagellin comprises four domains: D0 and D1, which represent the conserved N and C domains involved in binding and TLR5 signalling and D2 and D3, the middle domains known for their immunodominance and contribution to its antigenicity. To optimize our vaccine's molecular weight and prevent anti-flagellin antibody formation, we modified flagellin by substituting its D2 and D3 domains with selected vaccine components, retaining only its N and C termini (head and tail). This approach aligns with methodologies employed in previous studies [85], [86]. Alongside TLR agonists, we incorporated the helper peptide, PADRE, to further enhance the vaccine's immunogenicity.

Linkers also referred to as 'spacers', play a crucial role in the design of multi-epitope vaccines (MEV) or peptide-based vaccines. They are indispensable for mediating interdomain interactions, ensuring structural stability, and optimising vaccine functionality. Without appropriate linkers, the fusion of epitopes can lead to misfolding in the three-dimensional structure, diminished yield in vaccine production, and impairment of bioactivity. In this study, we developed four distinct engineered vaccine constructs (V1, V2, V3, and V4) utilizing four different sets of linker combinations (no linker, GGS, EAAK, GGGS, GPGPG, KK, and AAY). Subsequently, each of the engineered vaccine constructs was subjected to comprehensive assessment for their antigenicity, allergenicity, and toxicity through multiple server-based analyses.

Of the constructs, V2 emerged as the most promising, exhibiting superior stability, especially when bound to multiple epitopes. While all constructs displayed significant immunogenicity, V3 and V4 were found to contain potential toxin regions, excluding them from further consideration. All the multi-epitope antigens that were evaluated showed significant immunogenicity, above the minimum threshold. Crucially, they demonstrated non-allergenic characteristics, confirming their safety with regards to allergic responses. Nevertheless, the investigation of toxicity revealed the presence of probable toxin regions in V3 and V4, leading to their rejection from further consideration. The physicochemical evaluations demonstrated positive characteristics, highlighting the overall potential of these modified vaccine structures. Proceeding from this preliminary evaluation, molecular dynamics simulations were performed to determine stability. Significantly, V2 consistently exhibited enhanced stability when bound to several epitopes, especially when compared to V1 (supplementary file). The exceptional stability of V2, along with its potent immunogenicity, makes it the most viable option for a multi-epitope vaccine. Further refinement and thorough examination are needed to fully assess its potential, representing tremendous progress in vaccine research.

Molecular docking was utilised to study the interaction between V2 and TLR5. TLR5 is a preferred target for molecular docking studies with vaccine constructs due to its crucial role in immune response activation. In fish, TLR5 exists in membrane-bound (TLR5M) and soluble (TLR5S) forms, both detecting bacterial flagellin but with different roles in the signalling cascade [70]. TLR5 recognition triggers both innate and adaptive immune responses, enhancing antigen presentation and cytokine production. Successful molecular docking of various vaccine constructs, including epitope-based and multi-epitope vaccines, with the TLR5 receptor has been demonstrated in multiple studies [87], [88], [89] . These docking analyses reveal specific binding interactions, such as hydrogen bonds and electrostatic interactions, suggesting the vaccine constructs can effectively bind and activate the TLR5 receptor. These structural insights provide valuable information about potential mechanisms of action and guide vaccine design, making TLR5 a valuable target for developing effective vaccines against both bacterial and viral pathogens in fish.

The docking analysis reveals a complex interaction between the vaccine construct and TLR5, involving nine hydrogen bonds and various amino acid residues. Key TLR5 residues include His371, Tyr345, Cys24, Ser302, Arg664, and His262, which interact with complementary residues on the vaccine

construct. Specifically, the vaccine residues Gly255, Glu251, Met347, Ser208, Asp165, Thr154, and Lys194 form complementary interactions with the TLR5 residues, suggesting a good fit between the vaccine and the receptor. This specific binding profile is crucial for the vaccine's effectiveness, as it ensures proper activation of TLR5 and subsequent immune response initiation.

Targeting this region of TLR5 is a sensible approach for vaccine development against grouper iridovirus (GIV). The multiple interaction points provide specificity and stability, likely leading to sustained receptor activation. This binding can potentially enhance both innate and adaptive immune responses, offering comprehensive protection against the target pathogen. The specificity of the interaction may result in fewer off-target effects, potentially leading to a safer vaccine. Additionally, understanding these specific interactions can guide the design of future vaccines targeting TLR5 or similar receptors. However, while these computational results are promising, further in vivo and in vitro experimental assessments are necessary to fully validate the efficacy and safety of this vaccine candidate against GIV.

Molecular Dynamic Simulation of Multi-Epitope Vaccine

Subsequently, molecular dynamics simulations of V2 were extended to 100 ns to assess its stability with TLR5. The RMSD analysis of the designed multi-epitope vaccine against Grouper Iridovirus reveals distinct stability profiles in its apo form and in complex with TLR5 (Figure 4). In the apo form, the V2 demonstrates moderate flexibility, with RMSD values stabilizing around 1.5-2.0 nm and slight variations between replicates. This flexibility suggests the vaccine can adapt its conformation, which may be beneficial for binding. In contrast, the V2-TLR5 complex exhibits lower and more consistent RMSD values (0.8-1.2 nm after initial equilibration), indicating enhanced structural stability upon binding. This increased stability in the complex form is crucial, as it suggests a persistent and well-defined interaction between the vaccine and its target receptor. The consistency between replicates in the complex form further supports the stabilizing effect of TLR5 binding. These results collectively suggest that the designed vaccine (V2) possesses favourable structural properties - flexibility in its unbound state to facilitate binding, and stability in its bound state to maintain effective interaction with TLR5. Such characteristics support the potential efficacy of the vaccine in engaging the immune system and eliciting a robust response against Grouper Iridovirus.

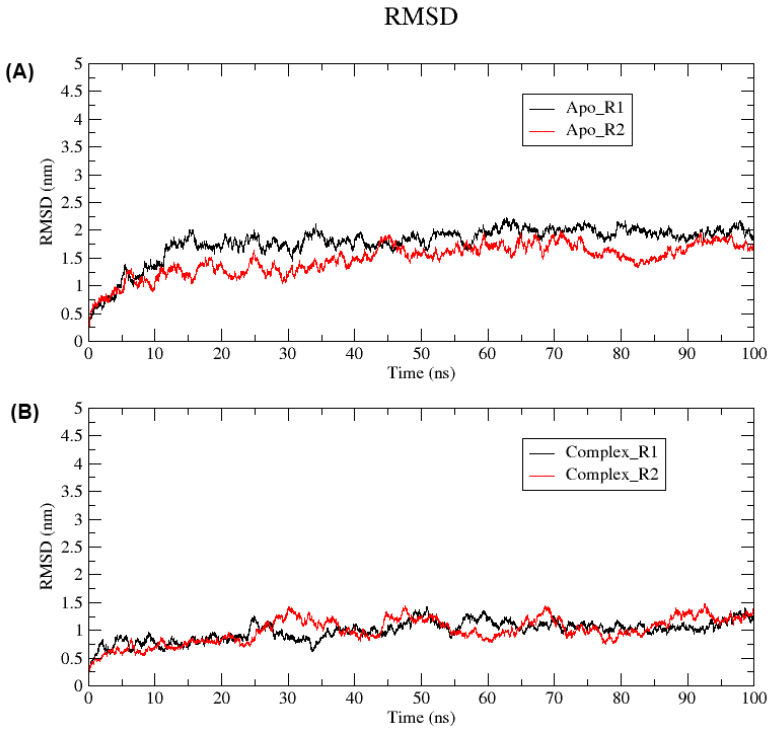

Figure 4. RMSD trajectories of (A) Apo V2 and (B) V2-TLR5 complex during 100 ns molecular dynamics simulation. The MD simulations were conducted in duplicates (referred to as R1 and R2)

The RMSF (Root Mean Square Fluctuation) graphs provide insights into the flexibility of the designed multi-epitope vaccine against Grouper Iridovirus, both in its apo form and in complex with TLR5 (Figure 5). In the apo form, the vaccine exhibits variable flexibility across its residues. Most regions show RMSF values between 0.5-1.5 nm, with some peaks reaching up to 2.0-2.5 nm, particularly around residues 200-250. This suggests areas of high flexibility, which could be important for initial receptor recognition and binding. The two replicates show similar overall patterns, indicating consistency in the flexible regions.The complex form (vaccine with TLR5) demonstrates generally lower RMSF values, mostly below 1.0 nm, with fewer and lower peaks compared to the apo form. This reduced flexibility suggests that binding to TLR5 stabilizes the vaccine structure. Notable exceptions are the N-terminal region (residues 1-50) and C-terminal region (after residue 400), which retain higher flexibility in the complex.

These results indicate that the designed vaccine has adaptable regions in its unbound state, potentially facilitating initial interactions with TLR5. Upon binding, the vaccine structure becomes more rigid, which could promote stable and specific interactions with the receptor. The retained flexibility at the termini in the complex form may allow for fine-tuning of the binding or accommodate interactions with other immune components. The consistency between replicates in both forms supports the reliability of these observations. Overall, this RMSF analysis suggests that the designed vaccine possesses a balance of flexibility and stability that could contribute to its effectiveness in engaging the immune system against Grouper Iridovirus.

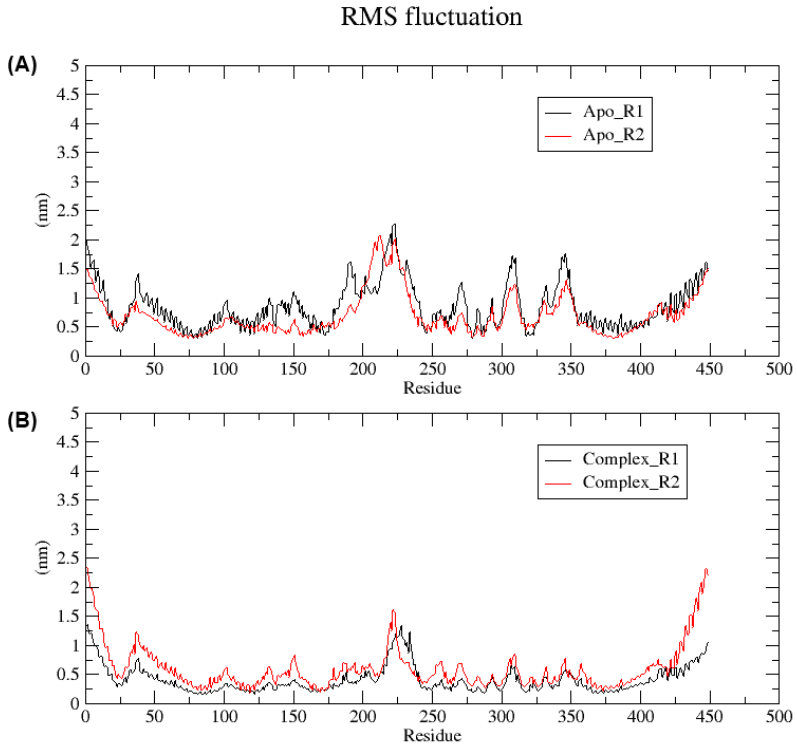

Figure 5. RMSF trajectories of (A) Apo V2 and (B) V2-TLR5 complex during 100 ns molecular dynamics simulation. The MD simulations were conducted in duplicates (referred to as R1 and R2)

The graph illustrates the hydrogen bonding between a designed multi-epitope vaccine and TLR5 over a 100 ns simulation, with two replicates shown in black and red (Figure 6). Both replicates demonstrate a fluctuating but persistent number of hydrogen bonds, typically ranging from 5 to 25. The first replicate (black) shows slightly higher bond numbers on average, with peaks reaching about 25 bonds, while the second replicate (red) generally stays below 20 bonds. These results indicate a stable and dynamic interaction between the vaccine and TLR5 throughout the simulation.

The consistent presence of hydrogen bonds suggests that the designed vaccine maintains a stable association with TLR5, which is crucial for its potential effectiveness. The fluctuations in bond numbers

reflect the dynamic nature of protein interactions in a physiological-like environment. The maintenance of a minimum of around 5 hydrogen bonds in both replicates indicates key interaction points that remain consistently engaged. Overall, this analysis supports the potential efficacy of the designed vaccine against Grouper Iridovirus, suggesting it could effectively engage the immune system through its interaction with TLR5.

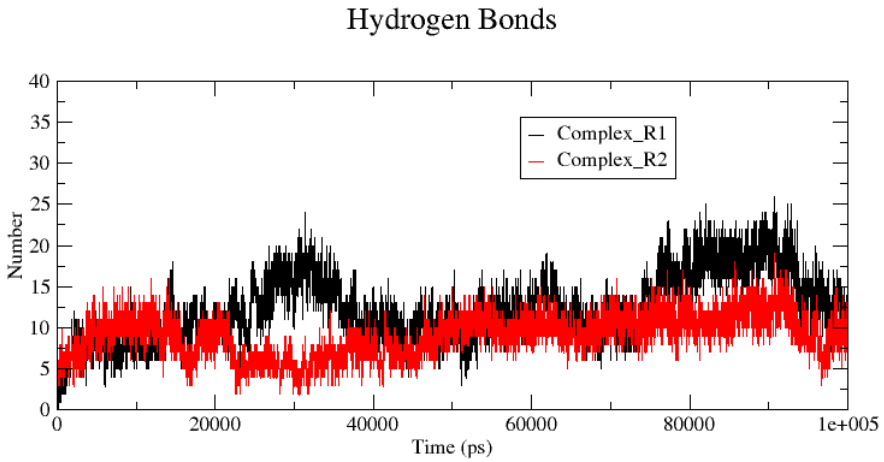

Figure 6. Hydrogen bonds trajectories of V2-TLR5 complex during 100 ns molecular dynamics simulation. The MD simulations were conducted in duplicates (referred to as R1 and R2).

Conclusions

Our findings highlight the potential of the V2 construct as a promising MEV against GIV. While preliminary results are encouraging, further refinement and rigorous testing are essential to validate its efficacy. Subsequent research should focus on in vivo testing of the V2 construct, assessing its immunogenicity, safety, and protective efficacy in relevant animal models. Additionally, exploring alternative adjuvants and optimizing vaccine delivery methods can further enhance its potential.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

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