Antigenicity and Immunogenicity of Three Synthetic Peptides Derived from Predicted B Cell Epitopes of Nervous Necrosis Virus (Nnv) in Asian Seabass (*Lates Calcarifer*)

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Abstract: Nervous necrosis virus (NNV) has spread throughout the world, affecting more than 120 freshwater and marine fish species. While vaccination effectively prevents disease outbreaks, the difficulty of producing sufficient viruses using cell lines continues to be a significant disadvantage for producing inactivated vaccines. This study, therefore, explored the application of synthetic peptides as potential vaccine candidates for the prevention of NNV in Asian seabass (*Lates calcarifer*). Using the epitope prediction tool and molecular docking, three predicted immunogenic B cell epitopes (30-32 aa) derived from NNV coat protein were selected and synthesised, corresponding to amino acid positions 5 to 34 (P1), 133 to 162 (P2) and 181 to 212 (P3). All the predicted peptides interact with Asian sea bass's MHC class II by docking. The antigenicity of these peptides was determined through ELISA and all peptides were able to react with NNV-specific antibodies. Subsequently, the immunogenicity of these synthetic peptides was investigated by immunisation of Asian seabass with individual peptides (30 μg/fish) and a peptide cocktail (P1+P2+P3, 10 μg each/fish) by intraperitoneal injection, followed by a booster dose at day 28 post-primary immunisation. There was a subset of immunised fish that were able to induce upregulation of *CD4* in the head kidney and spleen. Importantly, antibodies derived from fish immunised with synthetic peptides reacted with whole NNV virions. Taken together, these findings indicate that synthetic linear peptides based on predicted B cell epitopes exhibited both antigenic and immunogenic properties, suggesting that they could be potential vaccine candidates for the prevention of NNV in fish.

Keywords: NNV; synthetic peptides; Asian seabass; antigenicity; immunogenicity

1. Introduction

The Nervous Necrosis Virus (NNV) or *Betanodavirus* is the causative agent of a neuropathogenic disease in larvae and juveniles of fish that results in mass mortality of up to 100% [1,2]. This virus has affected more than 120 fish species belonging to freshwater and marine environments [3]. Along with other species, the Asian seabass, commonly known as barramundi, an economically important fish species in Asia and Australia, has been widely affected by NNV. Previously, NNV had been detected in Asian seabass from Australia [4], Taiwan [5], Israel [6], India [7], Malaysia [8], and Iran [9].

NNV is a spherical virus without an envelope with a mean diameter of 25 nm. It is a positive-sense RNA virus having two single strands of RNA (RNA 1 and RNA 2). RNA 1

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has approximately 3,100 nucleotides that code for RNA-dependent RNA polymerase (RdRp), which helps the virus in replication. At the same time, RNA 2 has 1,410 to 1,433 nucleotides that code for capsid protein [3]. This *Nodaviridae* virus affects the central nervous system of fish, causing necrotic vacuolation in the brain, spinal cord, and eye cells [1]. Till now, numerous research has been done to develop vaccines against NNV, which includes traditional inactivated vaccines [10], modern recombinant proteins [11], virus-like particles (VLP) [12] and DNA vaccines [13]. Although these kinds of vaccines could induce neutralising antibodies and provide protective efficacy in fish, their production is costly and time-consuming due to the high expense of cell culture and purification [14]. So, there is a need for a safe and efficacious vaccine that can be produced quickly at an affordable price.

The alternative to the currently available vaccine is the peptide vaccine, which can be synthesised chemically by the solid phase peptide synthesis (SPPS) process, making its production cheap, simple, and rapid on a large scale. Moreover, there is no use of any biological material which eliminates potential contaminants [14,15]. This vaccine consists of a sequence of amino acids (aa) mimicking antigenic epitopes, which upon administration, are recognised by the cells of the immune system. The peptide vaccine aiming to stimulate humoral or antibody response should have a B cell epitope from a pathogen [14]. The entire pathogen has numerous regions, where some are epitopes while others are not, and the success of a peptide-based vaccine depends upon selecting an epitope that is highly antigenic as well as immunogenic [16]. Hence the first step of designing a peptide vaccine is to identify B cell epitopes from the pathogen.

A new field of bioinformatics has emerged, which uses computational tools in vaccine development [17]. The researchers have developed *in silico* tools that have accelerated epitope prediction and antigen selection for vaccine design against emerging infectious diseases [18]. Earlier, these *in silico* tools were used to design a vaccine against human pathogens such as Zika virus [19], COVID-19 [20] and brucellosis [21]. The application of this field in fish vaccines is relatively new [22], with promising achievements of multi-epitope vaccines against streptococcosis and edwardsiellosis in tilapia [23,24]. In this study, epitopes from the coat protein of NNV were identified using *in silico* prediction tools, and the synthetic peptides mimicking these epitopes were evaluated for their antigenicity and immunogenicity in a wet lab.

2. Materials and methods

2.1. Prediction of linear B cell epitope

The amino acid sequence of RGNNV coat protein (accession number UOF76403.1), which originated from diseased fish in Thailand [2], was used in this study. This amino acid sequence was subjected to linear B cell epitope prediction *using Bepipred 2.0 and* Kolaskar and Tongaonkar antigenicity tools available at the Immune Epitope Database (IEDB) server [25]. The threshold value of *Bepipred 2.0, and* Kolaskar and Tongaonkar antigenicity tool was kept at the default value (0.5 and 1, respectively).

2.2 Homology modelling

The 3D structure of predicted epitopes was generated using the homology modelling server SWISS-MODEL [26]. Refinement of selected 3D models was done using the GalaxyRefine server [27]. The model quality was validated using the Ramachandra plot generated by the PROCHECK server [28].

The homology modelling of MHC class II of Asian seabass was performed for molecular docking with predicted epitopes. The amino acid sequence of alpha and beta chains of MHC class II of Asian seabass were retrieved from NCBI with accession numbers XP_018530089.1 and XP_018530088, respectively. At first, the 3D model of the MHC II alpha and the beta chain was generated and refined by the method described above. Using

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the Cluspro server [29], docking the alpha and beta chains of MHC class II was performed to generate the complete 3D structure of MHC class II.

2.3 Molecular docking and visualisation

The docking of 3D models of three peptides with MHC II models of Asian seabass was performed using the Cluspro server. The docked models were refined using the GalaxyWEB server and the quality of the models was checked using the Ramachandra plot. The Prodigy server [30], was used to predict the binding affinity between receptor-ligand, and the PBDsum server [31], was used to check the interaction between peptides and MHC class II. The docked models were further visualised using Pymol software [32].

2.4 Peptide synthesis

The selected epitopes were chemically synthesised as peptides by the ProteoGenix company (France). Acetylation of the N terminal and amidation of the C terminal of the peptides were done to make the peptides more stable and immunogenic. The peptides had >70% purity.

2.5. Inactivated virus preparation and immunisation

The RGNNV strain was cultured in the E-11 cell line as described by Pakingking et al [10] and RT-qPCR revealed that the viral copy number was 1.84×10^9 copies/mL [2]. The viral stock thus obtained was inactivated by adding formalin to reach a final concentration of 0.5%, followed by incubating at 4 °C for 2 h [10]. Complete inactivation of the virus was checked by inoculating the virus to E-11 cells. The inactivated virus was diluted 100 times using 1X phosphate buffer saline (PBS) to reduce formalin residue.

The NNV-specific antibodies used for the antigenicity test were produced in Asian seabass. Fish used in this study were approved by the National Center for Genetic Engineering and Biotechnology IACUC (BT-Animal 14/2563). The healthy fish were obtained from a commercial hatchery in Thailand and were cultured in a recirculatory aquaculture system, which consisted of 200 L culture tanks. The fish were cultured at 10 ppt salinity and fed with commercial feed at 3% body weight daily. Prior to the immunisation experiment, the fish were transferred to the wet lab and distributed into two groups, each containing 20 naïve Asian seabass (21.2 ±1.36 g). The first group was intraperitoneally injected with 100 µL of the inactivated virus (1.84×10^6 copies/fish**)**, while the control group received the same volume of 1X PBS. Blood was collected from fish (n = 5) on day 21 days post-immunisation (dpi) by puncturing the caudal vein with the help of a 1 mL syringe with a $27^1/_2$ G needle. Before immunisation and blood collection, fish were anaesthetised using clove oil at 100 ppm [33]. The collected blood was allowed to coagulate at room temperature for 2 h and centrifuged at 4000 g for 10 min. The isolated serum was then stored at -20° C until use.

2.6. Peptide antigenicity test

The peptide antigenicity test was carried out to assess the ability of selected synthetic peptides to react with NNV-specific antibodies raised in Asian seabass (mentioned above). This was determined by ELISA according to Mai et al. [33], using peptides as coated antigens (Figure 1). At first, 96 well polystyrene plate was washed three times using low salt wash buffer (LSWB, 2 mM Tris; 38 mM NaCl; 0.005% Tween-20; pH 7.3), followed by adding 100 µL of peptides (2 µg), which were allowed to incubate overnight at 4 °C. On the next day, plates were rinsed three times using 100 µL of LSWB, and 250 µL blocking buffer (1X PBS with 1% bovine serum albumin (BSA)) was added and incubated for 2 h at room temperature. After tapping out the blocking buffer, 100 µL of NNV-infected serum or control serum (1:256 diluted in 1X PBS) was added to each well and kept at 4 °C overnight. The following day, the plate was washed five times with high salt wash buffer (HSWB, 2 mM Tris; 50 mM NaCl; 0.02% Tween-20; pH 7.7). After washing, plates

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were incubated with 100 µL of anti-Asian seabass IgM [34], diluted at a 1:50 ratio in 1X PBS with 1% BSA for 1 h at room temperature. The plate was washed five times with HSWB, and goat anti-mouse antibody (Invitrogen, USA) conjugated with HRP was added and allowed to incubate at room temperature for 1 h. The plate was rewashed five times with HSWB, and 100 µL of TMB (Merck, USA) was added to each well for colour development. After 5 to 10 min, the reaction was stopped by adding 50 µL of H_2SO_4 (2 M) stopping reagent to each well. Lastly, optical density (OD) was measured at 450 nm with a microplate reader (Azure Biosystems, USA).

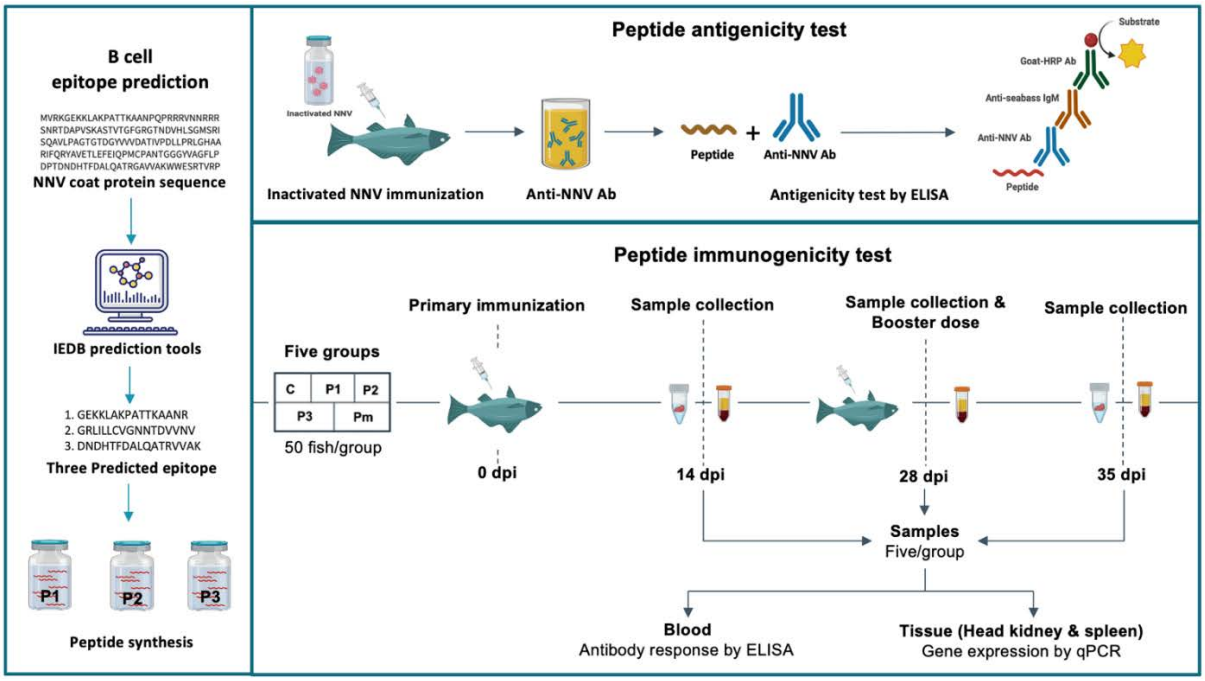

Figure 1. Overall experimental design for peptide immunisation. Control; C, Peptide 1; P1, Peptide 2; P2, Peptide 3; P3 and Peptide mix; Pm (image created in BioRender.com).

2.7. Peptide immunogenicity test

The immunogenicity test was carried out to assess the ability of synthetic peptides to stimulate the immune response in fish. For that, 250 Asian seabass (24.7 ± 3.5 g) were equally distributed into five experimental groups: control (C), peptide 1 (P1), peptide 2 (P2), peptide 3 (P3) and peptide mix (Pm). The immunisation trial was carried out in five 200 L tanks (50 fish/tank). Before vaccination, all fish were anaesthetised with clove oil (100 ppm). The experimental design for peptide immunisation is shown in Figure 1. The fish from the groups P1, P2 and P3 received IP doses of 100 µL of respective peptides (30 µg/fish) mixed with adjuvant (Montanide IMS 1312) at a 1:1 ratio. The Pm group received a mixture of three peptides (10 µg of each peptide/fish) combined with an adjuvant (1:1). Whereas the control group was administered with 100 µL of 1X PBS.

On 28-dpi, booster immunisation was carried out. All vaccinated groups received a dose of 100 µL peptide (10 µg/fish) mixed equally with adjuvant. Five fish from each group were randomly collected for blood and tissue collection on days 14, 28 and 35 dpi. The blood collection and serum isolation were done as the method described above. The tissue sample, including the head kidney and spleen were collected on 14 and 35 dpi, and later stored in 200 µL GENEzol solution (Geneaid, Taiwan province of China) at - 20°C.

2.8. qPCR assay for immune gene expression

The RNA was extracted from 100 mg of the collected tissues (head kidney and spleen) using the GENEzol reagent by following the method recommended by the manufacturer.

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Extracted RNA was treated with DNase I (New England Biolabs, USA) to remove any genomic DNA contamination. The concentration and quality of extracted RNA were determined using nanodrop (Maestrogen, Taiwan province of China). One µg of total RNA was used to synthesise complementary DNA (cDNA) using iScript™ Reverse Transcription Supermix (BioRad, USA).

The mRNA expression level of an immune-related gene (*CD4*) was analysed using quantitative real-time PCR (qPCR). The qPCR was performed using KAPA SYBR® Fast (KAPA Biosystems, Inc.). The 20 µL of reaction mixture consisted of 10 µL of 2× qPCR master mix, 0.4 µL of each forward and reverse primer (10 µM), 7.2 µL of PCR grade water and 2 µL of cDNA. The reaction condition included initial denaturation at 95 °C for 60 s, followed by 40 cycles of denaturation at 95 °C for 15 s and extension at 60 °C for 30 s. *EF-1α* was used as the internal control gene, and obtained C_q values were analysed using the $2^{-\Delta\Delta Ct}$ method [35]. Primers used for qPCR are listed in Table 1.

Table 1. Primers used in this study.

Gene	Accession number	Oligo sequence (5′-3′)	Product size (bp)	Reference
CD4	XM_018672410.1	F: GCAAGTGGACCTGTGTTGTG R: CCCAGGTGATATCAGGAGCA	159	This study
EF-1α	XM_018699074.1	F: GTTGCCTTTGTCCCCATCTC R: CTTCCAGCAGTGTGGTTCCA	130	[36]

2.9. Serum immune response and reactivity to NNV

The reactivity of antibodies generated from the peptide-immunised groups with NNV was determined by ELISA using inactivated NNV (10^8 copies/well) as an antigen. ELISA procedure was followed as the method described above (Section 2.6).

2.10. Statistical analysis

The graph in this study was created using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Statistical analysis of relative fold change in a gene was performed by ANOVA followed by Least Significant Difference (LSD) post hoc test at 95% confidential levels using SPSS 22.0 software (IBM, New York, USA). The difference in OD reading between groups was compared through statistical cut-off value according to Frey et al., [37].

3. Results

3.1. Epitope prediction

In this study, B cell linear epitopes of NNV coat protein of the RGNNV Thai strain were predicted using the IEDB server. The Bepipred 2.0 and Kolaskar and Tongaokar antigenicity tool predicted 13 and 11 epitopes with different amino acid lengths of RGNNV coat protein, respectively. The position of predicted epitopes is shown in Figure 2.

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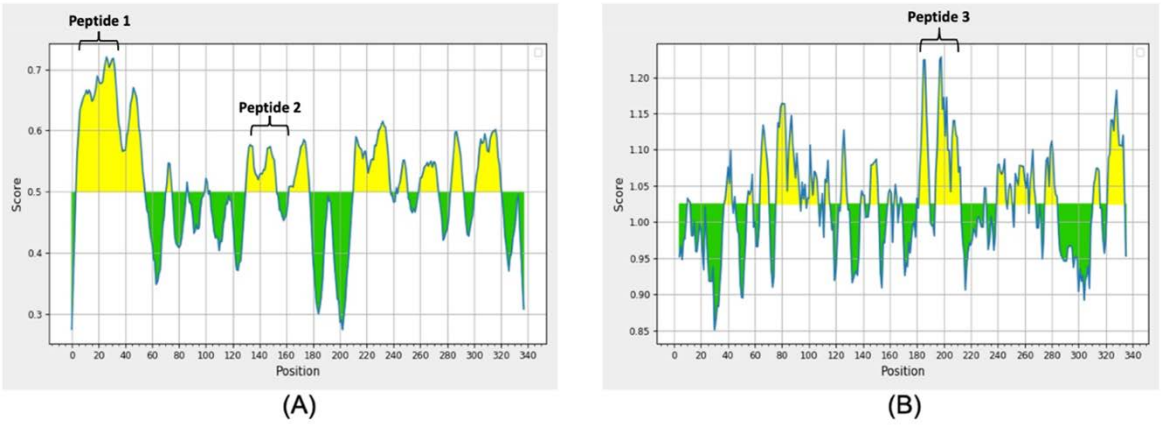

Figure 2. The graphical presentation of the B cell linear epitopes for the coat protein sequence of RGNNV strain predicted using IEDB server. The x and y-axis indicate amino acid positions and scores, respectively, and the yellow peak indicates the linear epitope. (A) B-cell linear epitope prediction by Bepipred 2.0 (threshold: 0.5) and (B) Kolaskar and Tongaonkar antigenicity prediction (threshold: 1.0).

The Bepipred 2.0 prediction showed that epitopes from positions 5 to 55 had the highest average score of 0.644. Other epitopes with an amino acid length of more than 15 residues with scores more than 0.5 (average score) included peptides from positions 131 to 153, 163 to 178, 211 to 238, 260 to 274 and 302 to 321 (Figure 2A). The result from the Kolaskar and Tongaokar antigenicity prediction illustrated that epitope located at position 182-189 had the highest average score (1.135). Another epitope at positions 194 to 212 had the second-highest score of 1.127 (Figure 2B). Among all these predicted epitopes, the amino acid sequences (30-32 aa) corresponding to positions 5-34 (GEKKLAKPATTKAANPQPRRRVNNRRRSNR), 133-162 (DNDHTFDALQATRGAVVAKWWESRTVRPQY) and 181-212 (GRLILLCVGNNTDVVNVSVLCRWSVRLSVPSL) namely Peptides 1 to 3, respectively, were selected and custom synthesised in this study.

3.2. Molecular docking

All the models built using homology modelling were of good quality, as the percentage of residue in the most favoured region of the Ramachandra plot was above 90% (Table S1). Three peptides were able to dock with MHC class II as shown in Figure 3 (A, C and E). The predicted binding affinity of MHC II with peptide 1, peptide 2 and peptide 3 were -13.6, -18.6 and -12.3 (kcal mol^{-1}), respectively. The *in silico* interaction between peptides and the MHC class II model was identified using the PBDsum server. The server identified a total of nine hydrogen bonds between peptide 1 and MHC class II (Figure 3B). Peptide 2 and peptide 3 formed a total of five and ten hydrogen bonds with MHC class II, respectively (Figure 3 D and F). The list of residues forming hydrogen bonds between peptides and MHC II is shown in Table S2-S4.

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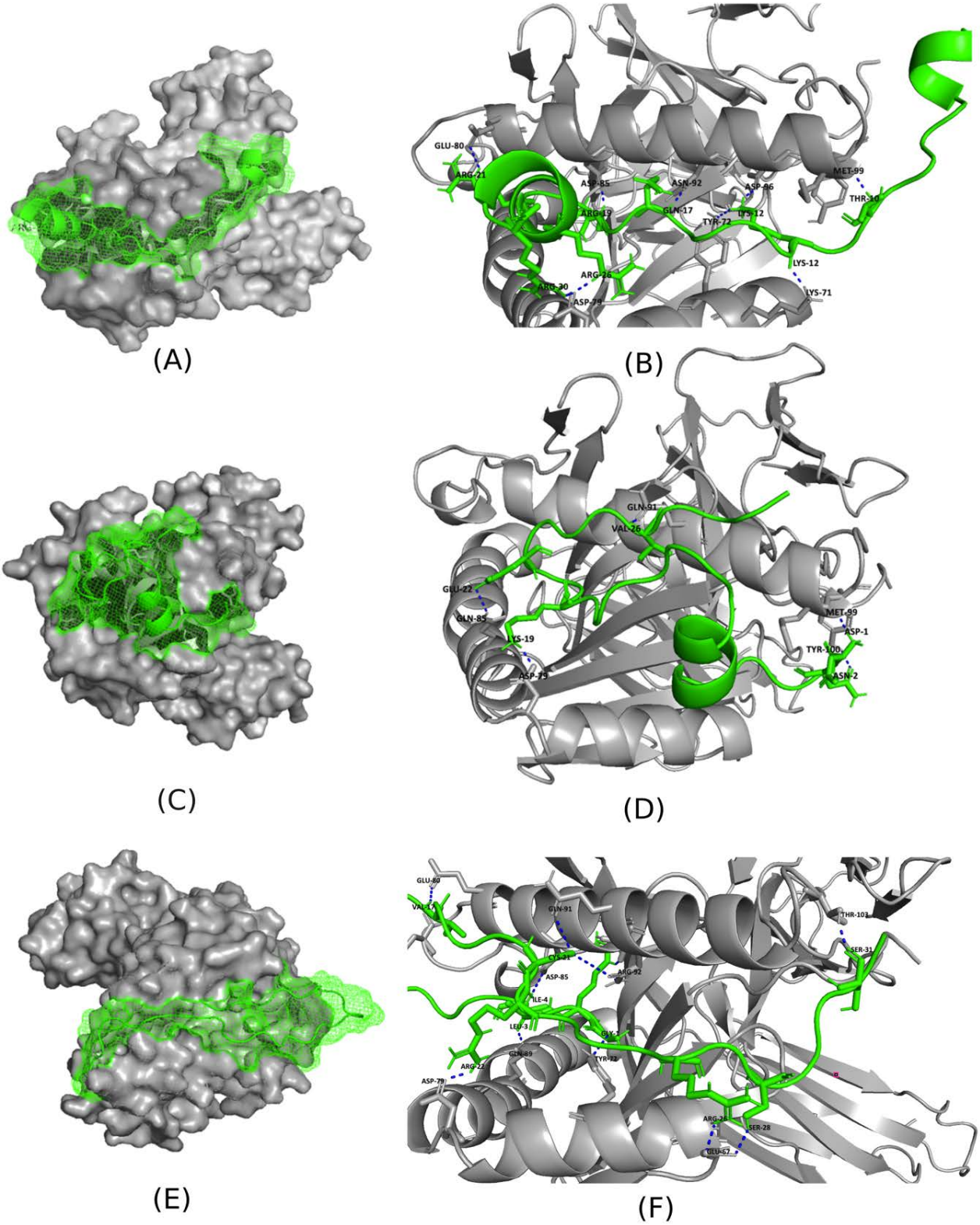

Figure 3. Docked models of MHC class II with Peptide 1 (A, B), Peptide 2 (C, D), Peptide 3 (E, F). Here MHC class II is in grey, while peptides are shown in green. The blue dotted line indicates a hydrogen bond between the residue of two molecules.

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3.3. Peptide antigenicity test

The NNV-specific antibody was raised in Asian seabass by immunisation with the formalin-inactivated virus (FIV). The systemic NNV-specific antibody IgM level on 21 dpi was shown in Figure 4 (A). The average OD_{450} reading of the FIV group (0.315 ± 0.142) was higher than the control group (0.184 ± 0.054) as well as above the cut-off value (0.245) (Table S5).

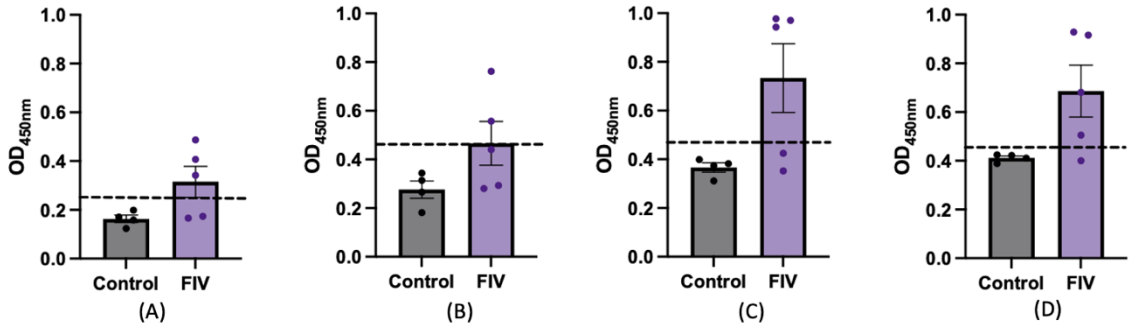

Figure 4. The antigenicity of the selected peptides with NNV specific antibodies was determined by ELISA. The data represented in the figure is as mean ± SE, n = 4 to 5 fish. Serum NNV specific IgM level at 21 dpi derived from the formalin-inactivated virus (FIV) compared to PBS control (A). Antigenicity of Peptide 1 (B), Peptide 2 (C), and Peptide 3 (D) with NNV-specific IgM. All readings were taken at OD_{450}, and fish sera were diluted at 1:256. The statistical cut-off values were used to compare the OD_{450} level. The horizontal dotted line on the graph indicates the cut-off value.

The antigenicity of three peptides was determined by the OD reading value at 450 nm, and a statistical cut-off value was used for comparison (Table S5). All three peptides selected in this study were recognised by the NNV-specific antibodies. Peptides 2 and 3 strongly reacted with NNV-specific antibodies as the mean OD_{450} value of the FIV group (0.733 ± 0.316 and 0.686 ± 0.237, respectively) were above the cut-off value (0.468 and 0.455, respectively) and higher than the control group (0.410 ± 0.104 and 0.436 ± 0.056, respectively) as shown in Figure 4 C-D. Similarly, peptide 1 also reacted with NNV-specific antibodies as the OD value of the FIV (0.466 ± 0.200) group was slightly higher than the cut-off value (0.463) and above the control group (0.325 ± 0.126) (Figure 4B). However, there were approximately 50% of immunised fish whose serum reacted with peptides or viral particles.

3.4. Immune gene expression

In this study, the transcription level of *CD4* immune gene marker in the head kidney and spleen from peptide-immunised fish were compared to the control group. In the head kidney, significant upregulation of the *CD4* compared to control was observed only in the P2 group on 14 dpi, as shown in Figure 5A. The slight upregulation of the *CD4* was found in P1 and P3 group, whereas the Pm group showed downregulation of *CD4* mRNA by 0.85 times compared to the control group. At 35 dpi (one week after a booster dose), all peptide-immunised groups showed an increase in *CD4* mRNA level in comparison to the control, but there was no statistically significant difference ($p > 0.05$).

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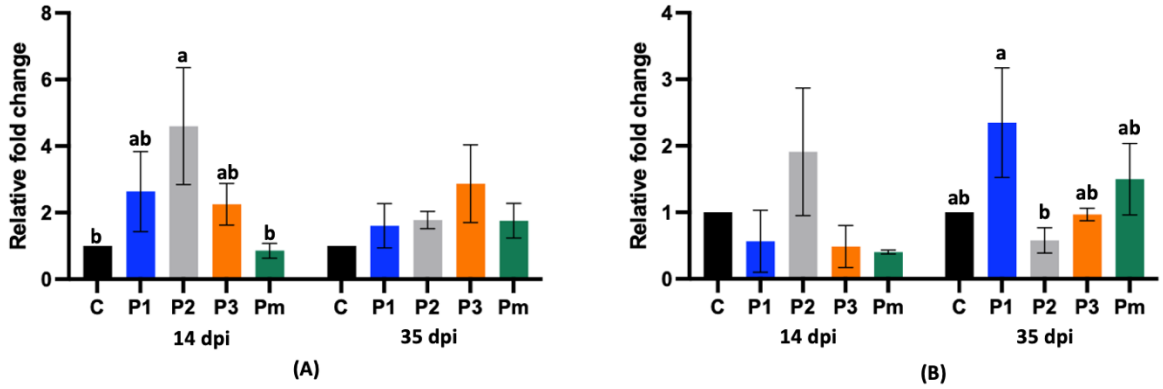

Figure 5. Relative fold change in expression of *CD4* in vaccinated groups compared to control group on 14 and 35 dpi. The data in the figure is represented as mean ± SE, n=5. Expression of the *CD4* in the head kidney (A) and spleen (B). Significant difference ($p > 0.05$) is shown in small alphabets.

In the spleen, non-significant upregulation of the *CD4* was observed in the P2 group, whereas in the other three groups (P1, P3 and Pm), downregulation of the same gene compared to the control group was observed on 14 dpi. However, there was no significant difference among the groups ($p > 0.05$). On 35 dpi, there was an increase in the transcription level of the *CD4* mRNA in P1 and Pm compared to the control group. However, P1 group was significantly upregulated compared to P2 group only ($p < 0.05$) (Figure 5B).

3.5. Serum antibody response and reactivity to NNV

The serum-specific immune response and reactivity of antipeptide antibodies from the serum of synthetic peptide-immunised fish were analysed through ELISA. The IgM antibody responses on 14, 28 and 35 dpi were indicated by OD at 450 nm, and the statistical cut-off value was used to compare the groups (Table S6). The result obtained by ELISA showed that the antibodies generated by all four immunised groups of Asian seabass reacted with NNV.

On 14 dpi, the mean OD reading of all peptide immunised groups were above the cut-off value (0.222) and higher than the control group (0.159 ± 0.02), as shown in Figure 6A. On the same day, the P2 group had the highest OD reading of 0.396 ± 0.179. On 28 dpi, the mean OD readings of P1, P2, P3 and Pm were 0.300 ± 0.101, 0.192 ± 005, 0.214 ± 0.04, and 0.375 ± 0.297, respectively. However, only the Pm group had an OD value higher than the cut-off value (Figure 6B).

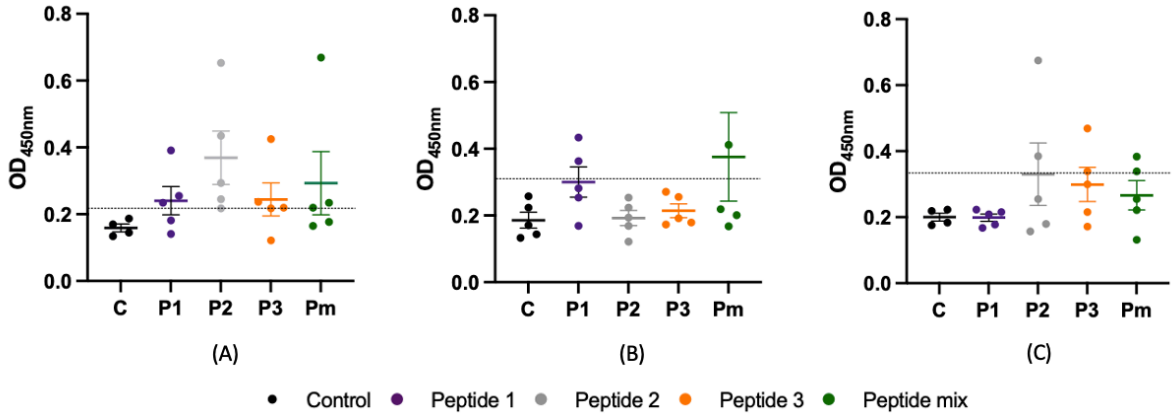

Figure 6. The level of NNV-specific antibodies (IgM) from sera of peptides immunised fish was determined by ELISA on 14 dpi (A), 28 dpi (B) and 35 dpi (C), n=5. All readings were taken at OD_{450}, and fish sera were diluted at 1:256. The statistical cut-off values were used to compare OD_{450} level. The horizontal dotted line on the graph indicates the cut-off value. Control; C, Peptide 1; P1, Peptide 2; P2, Peptide 3; P3 and Peptide mix; Pm.

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One week after a booster dose (35 dpi), none of the peptide-immunised groups had OD reading above the cut-off value (0.366). However, OD readings of P2 (0.330 ± 0.212), P3 (0.299 ± 0.115), and Pm (0.266 ± 0.099) were still higher than control (0.220 ± 0.049), while the P1 (0.198 ± 0.024) group had the lowest OD reading among all groups, including the control, as shown in Figure 6C. Wide variation in antibody levels among individuals from all groups was observed at each time point.

4. Discussion

Vaccination plays an important role in preventing disease outbreaks caused by infectious agents. Previous studies have been conducted to find potential vaccine candidates for NNV. However, the expense of manufacturing and the difficulty of scaling up those vaccines is one of the major challenges to creating an affordable vaccine for farmers [14]. In this study, three synthetic peptides derived from the predicted B cell epitopes of NNV coat protein were tested as alternatives to traditional vaccines that used whole viral particles. This could be a promising approach for the development and mass production of vaccines against NNV without a complicated and expensive cell culture system [15,16].

Selecting the correct epitope is crucial for the success of the synthetic peptide vaccine. Using advanced computational tools and algorithms, we identified three potential epitope regions from NNV coat protein, some of which have already been reported as B cell epitopes. The relatively short epitopes (12 aa) identified earlier were located within the amino acid regions 5 to 55, 131 to 178 and 181 to 212 [38]. In this study, three long peptides (30 to 32 aa) were chosen, as binding of the epitope to the B cell receptor needs a conformational structure similar to a native pathogen, which small peptides may not achieve [15, 39].

In theory, antigen-presenting cells (APCs) would present phagocytosed antigens containing B cell epitope(s) to T helper (T_H) cells through MHC class II receptor. This process activates the humoral immune response (B cell response) to produce specific antibodies [39]. All three peptides designed in this study were able to interact with MHC class II with negative binding affinity *in silico* prediction. Previous studies suggested that at least four hydrogen bonds between two molecules are required to form a stable interaction [40]. Here, all three peptides had more than four predicted hydrogen bond interactions with MHC class II, suggesting that the selected peptides can potentially form a stable interaction with MHC class II and possibly activate T_H cells.

The synthetic peptide epitopes must be antigenic in order to be used for immunisation. In this study, it was observed that all three synthetic peptides were able to react with the NNV-specific antibodies raised from whole viral particles. Three of the five sera samples from inactivated NNV-immunised fish contained detectable levels of specific IgM. These three samples reacted strongly to synthetic peptides, indicating that the peptides were antigenic. An earlier study reported that 12 aa length peptides corresponding to coat protein region 1-32 aa, 91-162 aa, and 181-212 aa reacted with a serum of European seabass (*Dicentrarchus labrax*) naturally infected with NNV. However, the immunogenicity of these peptides was not elucidated [38]. Therefore, in this study, immunogenicity of the peptide was determined by *in vivo* immunisation in Asian seabass with the assessment of specific antibody production and the expression of *CD4*, a co-receptor on T cells. Followed by immunisation of fish with peptides, upregulation of *CD4* gene was observed. This $CD4^+$ receptor is involved in activating T_H cells, and its activation produces cytokines stimulating plasma cells to differentiate into B cells producing specific antibodies [41]. The findings in the present study reveal that the synthetic B cell epitope peptides can stimulate specific humoral response pathways (B cell response) in Asian seabass.

In this study, all peptides were able to elicit an IgM-specific antibody, but not all immunised fish exhibited a positive response. Low immunogenicity of peptides, the unoptimised dose, the absence of an appropriate adjuvant, and variation in individual host's immunity might explain the differences in *CD4* expression and antibody response. Moreover, the serum-specific immune response of all four immunised groups was found to be

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different, with peptide 2 stimulating higher antibodies level than other groups. This is possibly due to different T_H cell epitopes present within the B cell epitope. The peptide with a strong T_H cell epitope can stimulate a better immunological response compared to a weak T_H cell epitope [42].

In this immunisation trial, one fish group received a cocktail of three peptides. However, antibody levels generated by the peptide mix group were relatively low compared to individual peptide groups. It is possible that the low immunisation dose (10 µg/peptide, which is three times lower than the dose of individual peptide groups) contributed to this outcome. Further optimisation of immunised doses is, therefore, necessary.

Monomeric peptides are considered to have poorer immunogenicity than whole virus particles in the inactivated vaccine [43]. Hence booster dose was given in order to induce a significant immunological response. Except for P1, OD readings increased one week after the booster dose (35 dpi) in all immunised groups, indicating that the booster dose effectively induces an antibody response. However, most OD readings of fish in immunised groups were still below the threshold. Since *CD4* upregulation continued at this time point, this suggests that the humoral immune response was still active and that it might take a longer time (not investigated in this study) to see higher levels of antibodies, particularly in the P1 and Pm groups. Nonetheless, not all immunised fish exhibited a positive response. This might have resulted from an unoptimised low dose (10 µg, which was three times lower than the dose of primary vaccination).

For a vaccine to be effective, it must generate antibodies capable of recognising and neutralising the known native pathogen. Previously, studies on a synthetic peptide vaccine against the foot and mouth disease virus (FMDV) showed that it protected guinea pigs and produced neutralising antibodies [42, 44]. The present study revealed that the antibodies generated by all four epitope-based synthetic peptide groups could recognise the native NNV (RGNNV) protein from whole viral particles. Further study should address whether these antibodies are neutralising antibodies that help to protect the fish from NNV infection. Moreover, to make these peptides effective vaccine candidates against NNV, further optimisation of the antigen dosage, adjuvant, and booster scheme is necessary.

In conclusion, this study reports the immunogenicity and antigenicity of long synthetic peptides (30-32 aa) based on predicted B cell epitopes of NNV in Asian seabass. The three synthetic peptides derived from NNV coat protein were able to interact with MHC class II by molecular docking and stimulate specific humoral immune response pathways involving *CD4* lymphocytes and antibody production when immunised in Asian seabass. We demonstrated that 30-32 aa-long synthetic peptide epitopes that have been proven to be antigenic and immunogenic are potential candidates for a chimeric multi-epitope vaccine against NNV in fish and warrant further study.

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References

1. Zorriehzahra, M. J. Viral nervous necrosis disease. *Emerging and Reemerging Viral Pathogens* **2019**, 673–703. 10.1016/B978-0-12-819400-3.00030-2
2. Jungi, S. V.; Sangsuriya, P.; Taengphu, S.; Phiwsaiya, K.; Sonthi, M.; Nuangsaeng, B.; Salin, K. R.; Senapin, S.; and Dong, H. T. Detection of nervous necrosis virus RGNNV genotype in pearl gentian grouper (*Epinephelus lanceolatus* ♂ ×*E. fuscoguttatus* ♀) fry imported to Thailand. *Aquaculture* **2022**, *561*, 738659. https://doi.org/10.1016/j.aquaculture.2022.738659
3. Bandín, I.; Souto, S. Betanodavirus and VER disease: A 30-year research review. *Pathogens* **2020**, *9*, 106. https://doi.org/10.3390/pathogens9020106.
4. Glazebrook, J.S.; Heasman, M.P.; and de Beer S.W. Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* Bloch. *Journal of Fish Diseases* **1990**, *13*, 245-249. https://doi.org/10.1111/j.1365-2761.1990.tb00780.x
5. Chi, S.C.; Shieh, J.R.; Lin, S.J. Genetic and antigenic analysis of betanodaviruses isolated from aquatic organisms in Taiwan. *Disease of Aquatic Organisms* **2003**, *55*, 221–228. https://doi.org/10.3354/dao055221
6. Ucko, M.; Colorni, A.; and Diamant, A. Nodavirus infections in Israeli mariculture. *Journal of Fish Diseases* **2004**, *27*, 459-469. https://doi.org/10.1111/j.1365-2761.2004.00565.x
7. Parameswaran, V.; Kumar, S. R.; Ahmed, V. I.; and Hameed, A. S. A fish nodavirus associated with mass mortality in hatchery-reared Asian Sea bass, *Lates calcarifer*. *Aquaculture* **2008**, *275*, 366–369. https://doi.org/10.1016/j.aquaculture.2008.01.023
8. Ransangan, J.; & Manin, B. O. Mass mortality of hatchery-produced larvae of Asian seabass, *Lates calcarifer* (Bloch), associated with viral nervous necrosis in Sabah, Malaysia. *Veterinary microbiology* **2010**, *145*, 153–157. https://doi.org/10.1016/j.vetmic.2010.03.016
9. Ziarati, M.; Zorriehzahra, M.J.; Kafilzadeh, F.; and Kargar, M. Molecular monitoring and phylogenetic analysis of *Betanodavirus* in Groupers (*Epinephelus* spp.) and Asian Sea bass (*Lates calcarifer*) of Iranian northern waters of the Persian Gulf. *Current Microbioloogy* **2020**, *77*, 3919–3926. https://doi.org/10.1007/s00284-020-02222-w
10. Pakingking, R.; Jr, Seron, R.; dela Peña, L.; Mori, K.; Yamashita, H.; and Nakai, T. Immune responses of Asian sea bass, *Lates calcarifer* (Bloch), against an inactivated betanodavirus vaccine. *Journal of fish diseases* **2009**, *32*, 457–463. https://doi.org/10.1111/j.1365-2761.2009.01040.x
11. Kim, J. O.; Kim, J. O.; Kim, W. S.; & Oh, M. J. Development of a recombinant protein vaccine based on cell-free protein synthesis for sevenband grouper *Epinephelus septemfasciatus* against viral nervous necrosis. *Journal of microbiology and biotechnology* **2015**, *25*, 1761–1767. https://doi.org/10.4014/jmb.1507.07004
12. Barsøe, S.; Skovgaard, K.; Sepúlveda, D.; Stratmann, A.; Vendramin, N. ; & Lorenzen, N. Nervous necrosis virus-like particle (VLP) vaccine stimulates European sea bass innate and adaptive immune responses and induces long-term protection against disease. *Pathogens* **2021**, 10, 1477. https://doi.org/ 10.3390/pathogens10111477
13. Vimal, S.; Farook, M.A.; Madan, N.; Abdul Majeed, S.; Nambi, K.S.N.; Taju, G.; Sundar raj, N.; Venu, S.; Subburaj, R.; Thirunavukkarasu, A.R.; and Sahul Hameed, A.S. Development, distribution and expression of a DNA vaccine against nodavirus in Asian Seabass, *Lates calcarifier* (Bloch, 1790). *Aquaculture Research* **2016**, *47*, 1209-1220. https://doi.org/10.1111/are.12578
14. Moisa, A. A.; & Kolesanova, E. F. Synthetic peptide vaccines. *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry* **2010**, *4*, 321–332. https://doi.org/10.1134/s1990750810040025
15. Skwarczynski, M.; & Toth, I. Peptide-based synthetic vaccines. *Chemical science* **2016**, *7*, 842–854. https://doi.org/10.1039/c5sc03892h
16. Li, W.; Joshi, M. D.; Singhania, S.; Ramsey, K. H.; & Murthy, A. K. Peptide vaccine: progress and challenges. *Vaccines* **2014**, *2*, 515–536. https://doi.org/10.3390/vaccines2030515
17. De, R. K.; & Tomar, N. *Immunoinformatics (Methods in Molecular Biology, 1184)* (Softcover reprint of the original 2nd ed. 2014). *Springer* **2016**.
18. De Groot, A. S.; Moise, L.; Terry, F.; Gutierrez, A. H.; Hindocha, P.; Richard, G.; Hoft, D. F.; Ross, T. M.; Noe, A. R.; Takahashi, Y.; Kotraiah, V.; Silk, S. E.; Nielsen, C. M.; Minassian, A. M.; Ashfield, R.; Ardito, M.; Draper, S. J.; & Martin, W. D. Better epitope discovery, precision immune engineering, and accelerated vaccine design using immunoinformatics tools. *Frontiers in immunology* **2020**, *11*, 442. https://doi.org/10.3389/fimmu.2020.00442
19. Kumar Pandey, R.; Ojha, R., Mishra, A.; & Kumar Prajapati, V. Designing B- and T-cell multi-epitope based subunit vaccine using immunoinformatics approach to control Zika virus infection. *Journal of cellular biochemistry* **2018**, *119*, 7631–7642. https://doi.org/10.1002/jcb.27110
20. Abdelmageed, M. I.; Abdelmoneim, A. H.; Mustafa, M. I.; Elfadol, N. M.; Murshed, N. S.; Shantier, S. W.; & Makhawi, A. M. (2020). Design of a multiepitope-based peptide vaccine against the E protein of human COVID-19: An immunoinformatics approach. *BioMed research international*, **2020**, 2683286. https://doi.org/10.1155/2020/2683286
21. Saadi, M.; Karkhah, A.; & Nouri, H. R. Development of a multi-epitope peptide vaccine inducing robust T cell responses against brucellosis using immunoinformatics based approaches. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases* **2017**, *51*, 227–234. https://doi.org/10.1016/j.meegid.2017.04.009
22. Momtaz, F.; Foysal, J.; Rahman, M.; & Fotedar, R. Design of epitope based vaccine against shrimp white spot syndrome virus (WSSV) by targeting the envelope proteins: an immunoinformatic approach. *Turkish Journal of Fisheries and Aquatic Sciences* **2019**, *19*, 149-159. http://doi.org/10.4194/1303-2712-v19_2_07

23. Pumchan, A.; Krobthong, S.; Roytrakul, S.; Sawatdichaikul, O.; Kondo, H.; Hirono, I.; Areechon, N.; & Unajak, S. Novel chimeric multiepitope vaccine for streptococcosis disease in Nile tilapia (*Oreochromis niloticus*). *Scientific reports* **2020**, *10*, 603. https://doi.org/10.1038/s41598-019-57283-0
24. Machimbirike, V. I.; Pornputtapong, N.; Senapin, S.; Wangkahart, E.; Srisapoome, P.; Khunrae, P.; & Rattanarojpong, T. A multi-epitope chimeric protein elicited a strong antibody response and partial protection against *Edwardsiella ictaluri* in Nile tilapia. *Journal of fish diseases* **2022**, *45*(1), 1–18. https://doi.org/10.1111/jfd.13525
25. Vita, R.; Mahajan, S.; Overton, J. A.; Dhanda, S. K.; Martini, S.; Cantrell, J. R.; Wheeler, D. K.; Sette, A.; & Peters, B. The Immune epitope database (IEDB): 2018 update. *Nucleic acids research* **2019**, *47*, 339-343. https://doi.org/10.1093/nar/gky1006
26. Waterhouse, A.; Bertoni, M.; Bienert, S.; Studer, G.; Tauriello, G.; Gumienny, R.; Heer, F. T.; De Beer, T. A.; Rempfer, C.; Bordoli, L.; Lepore, R.; & Schwede, T. SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Research* **2018**, *46*, 296–303. https://doi.org/10.1093/nar/gky427
27. Shin, W. H.; Kim, J. K.; Kim, D. S.; & Seok, C. GalaxyDock2: protein-ligand docking using beta-complex and global optimisation. *Journal of computational chemistry* **2013**, *34*, 2647–2656. https://doi.org/10.1002/jcc.23438
28. Laskowski, R.A.; MacArthur, M.W.; Moss, D.S. and Thornton, J.M. PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Cryst.***1993**, *26*, 283-291. https://doi.org/10.1107/S0021889892009944
29. Porter, K. A.; Xia, B., Beglov, D.; Bohnuud, T.; Alam, N., Schueler-Furman, O., & Kozakov, D. ClusPro PeptiDock: efficient global docking of peptide recognition motifs using FFT. *Bioinformatics (Oxford, England)* **2017**, *33*, 3299–3301. https://doi.org/10.1093/bioinformatics/btx216
30. Xue, L. C. ; Rodrigues, J. P. ; Kastritis, P. L. ; Bonvin, A. M. ; & Vangone, A. PRODIGY: a web server for predicting the binding affinity of protein-protein complexes. *Bioinformatics (Oxford, England)* **2016**, *32*, 3676–3678. https://doi.org/10.1093/bioinformatics/btw514
31. Laskowski, R. A.; Jabłońska, J.; Pravda, L.; Vařeková, R. S.; & Thornton, J. M. PDBsum: Structural summaries of PDB entries. *Protein science : a publication of the Protein Society* **2018**, *27*, 129–134. https://doi.org/10.1002/pro.3289
32. Schrödinger, L.; & DeLano, W. PyMOL. **2020**. Retrieved from http://www.pymol.org/pymol
33. Mai, T. T.; Kayansamruaj, P.; Taengphu, S.; Senapin, S.; Costa, J. Z.; Del-Pozo, J.; Thompson, K. D.; Rodkhum, C.; & Dong, H. T. Efficacy of heat-killed and formalin-killed vaccines against Tilapia tilapinevirus in juvenile Nile tilapia (*Oreochromis niloticus*). *Journal of fish diseases* **2021**, *44*, 2097–2109. https://doi.org/10.1111/jfd.13523
34. Soonthonsrima, T.; Wangman, P.; Pengsuk, C.; Sithigorngul, P.; Longyant, S. Production of a monoclonal antibody specific to immunoglobulin of Asian sea bass *Lates calcarifer*. *Burapha Science Journal*, **2019**, 24, 1237-1249.
35. Livak, K. J.; & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)* **2001**, *25*, 402–408. https://doi.org/10.1006/meth.2001.1262
36. Paria, A.; Dong, J.; Babu, P.; Makesh, M.; Chaudhari, A.; Thirunavukkarasu, A. R.; Purushothaman, C. S.; & Rajendran, K. V. Evaluation of candidate reference genes for quantitative expression studies in Asian seabass (*Lates calcarifer*) during ontogenesis and in tissues of healthy and infected fishes. *Indian journal of experimental biology* **2016**, *54*, 597–605.
37. Frey,A.; Di Canzio, J.; & Zurakowski, D. A statistically defined endpoint titer determination method for immunoassays. *Journal of immunological methods* **1998**, *221*, 35–41. https://doi.org/10.1016/s0022-1759(98)00170-7
38. Costa, J. Z.; Adams, A.; Bron, J. E.; Thompson, K. D.; Starkey, W. G.; & Richards, R. H. Identification of B-cell epitopes on the betanodavirus capsid protein. *Journal of fish diseases* **2007**, *30*, 419–426. https://doi.org/10.1111/j.1365-2761.2007.00824.x
39. Zhang, R.; & Ulery, B. D. Synthetic Vaccine Characterization and Design. *Journal of Bionanoscience* **2018**, *12*(1), 1–11. https://doi.org/10.1166/jbns.2018.1498
40. Mahendran, R. ; Jeyabaskar, S. ; Sitharaman, G. ; Michael, R. D. ; & Paul, A. V. Computer-aided vaccine designing approach against fish pathogens *Edwardsiella tarda* and *Flavobacterium columnare* using bioinformatics softwares. *Drug design, development and therapy* **2016**, *10*, 1703–1714. https://doi.org/10.2147/DDDT.S95691
41. Roberts, R. J. *Fish pathology*. Blackwell Publishing Ltd. **2012**
42. Zhang, Z.; Pan, L.; Ding, Y.; Zhou, P.; Lv, J.; Chen, H.; Fang, Y.; Liu, X.; Chang, H., Zhang, J., Shao, J., Lin, T., Zhao, F., Zhang, Y., & Wang, Y. Efficacy of synthetic peptide candidate vaccines against serotype-A foot-and-mouth disease virus in cattle. *Applied microbiology and biotechnology* **2015**, *99*, 1389–1398. https://doi.org/10.1007/s00253-014-6129-1
43. Malonis, R. J.; Lai, J. R.; & Vergnolle, O. Peptide-based vaccines: Current progress and future challenges. *Chemical reviews* **2020**, *120*, 3210–3229. https://doi.org/10.1021/acs.chemrev.9b00472
44. Wang, C. Y.; Chang, T. Y.; Walfield, A. M.; Ye, J.; Shen, M.; Chen, S. P.; Li, M. C.; Lin, Y. L.; Jong, M. H.; Yang, P. C.; Chyr, N.; Kramer, E.; & Brown, F. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. *Vaccine* **2002**, *20*, 2603–2610. https://doi.org/10.1016/s0264-410x(02)00148-2