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Novel subunit vaccine with linear array epitope protect giant grouper against nervous necrosis virus infection

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ABSTRACT

Viral nervous necrosis caused by nervous necrosis virus (NNV) is one of the most severe diseases resulting in high fish mortality rates and high economic losses in the giant grouper industry. Various NNV vaccines have been evaluated, such as inactivated viruses, virus-like particles (VLPs), recombinant coat proteins, synthetic peptides of coat proteins, and DNA vaccines. However, a cheaper manufacturing process and effective protection of NNV vaccines for commercial application are yet to be established. Hence, the present study developed a novel subunit vaccine composed of a carrier protein, receptor-binding domain of *Pseudomonas* exotoxin A, and tandem-repeated NNV coat protein epitopes by using the structural basis of epitope prediction and the linear array epitope (LAE) technique. On the basis of the crystal structure of the NNV coat protein, the epitope was predicted from the putative target cell receptor-binding region to elicit neutralizing immune responses. The safety of the LAE vaccine was evaluated, and all vaccinated fish survived without any physiological changes. The coat protein-specific antibody titers in the vaccinated fish increased after vaccine administration and exerted NNV-neutralizing effects. The efficacy tests revealed that the relative percent survival (RPS) of LAE antigen formulated with adjuvant was above 72% and LAE vaccine was effective for preventing NNV infection in giant grouper. This study is the first to develop an NNV vaccine by using epitope repeats, which provided effective protection to giant grouper against virus infection. The LAE construct can be used as a vaccine design platform against various pathogenic diseases.

1. Introduction

The giant grouper (*Epinephelus lanceolatus*) is an economic marine fish species because of the high value placed on the flesh quality of such fish species [1,2]. However, over the past two decades, severe production losses in giant grouper cultures have been caused by large-scale mass mortality due to disease outbreaks [3]. One of the most severe diseases is viral nervous necrosis caused by fish nodavirus, which has been reported to infect many marine fish species worldwide [4–6]. Nervous necrosis virus (NNV), belonging to the genus *Betanodavirus* and family nodavirus, typically infects giant grouper larvae, fry or juveniles; diseased fish commonly exhibit spiral swimming, loss of appetite, and brain vacuolization, typically resulting in 80%–100% death [7].

NNV is a nonenveloped virus consisting of two genome segments, RNA1 and RNA2. RNA1 encodes the viral part of the RNA-dependent RNA polymerase and protein B2, whereas RNA2 encodes the coat protein [8,9]. The coat protein is the only outer structural protein of the virus, which is necessary for viral particle assembly, host specificity, virus entry, and diagnostic recognition [10,11]. A recent study determined the crystal structure of virus-like particles (VLPs) of grouper NNV (GNNV) and revealed that the coat protein is divided into four distinct domains: N-terminal arm, shell domain (S-domain), linker region, and protrusion domain (P-domain); furthermore, the P-domain located on the virus surface may have significant roles in the initial attachment with host cells and cellular uptake [12]. Several studies have suggested that the structural analysis of the virus surface protein can provide a structural basis of the virus-host cell binding affinity, and the epitope on the receptor-binding region can act as a vaccine for the

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induction of host immune responses for virus neutralization [13,14].

Currently, intensive fish vaccine preparations against NNV infection have been evaluated, including vaccines realized using inactivated viruses [15–18], recombinant betanodavirus coat proteins [19–22], VLPs expressed in various expression systems [23–25], synthetic peptides of coat proteins [26], and coat protein-encoding DNA constructs [27]. These vaccines provide high to low levels of protection against virus infection in fish [26,27]. Because inactivated virus vaccines have a risk of infection and virus replication resulting from incomplete inactivation [28,29], subunit vaccines comprising portions of the immunogenic viral proteins are potentially safer options. VLP vaccines consist of NNV coat proteins self-assembled into a similar structure of virus particles [25]. VLP vaccines mimic inactivated virus vaccines because they are structurally similar to infectious viruses; therefore, VLP vaccines are as effective as inactivated vaccines against NNV infection [23–25]. Compared with VLP vaccines, vaccines containing recombinant NNV coat proteins expressed in prokaryotic cells or synthetic peptides of the coat protein are easily produced. However, these subunit vaccines typically elicit weaker immune responses and have less protective efficacy in fish [19–22].

Such peptide-induced poorer immune responses can be overcome by using a polymerase chain reaction (PCR)-based method, referred to as the linear array epitope (LAE) technique, to prepare an immunogen containing multiple linear epitope repeats [30,31]. Studies have demonstrated that constructs containing multiple copies of the designed antigen epitope fused to a carrier protein, the receptor-binding domain (domain Ia) of *Pseudomonas* exotoxin (PE) A yielded high titers of antigen-specific antibodies and resulted in physiological changes [30,31]. *Pseudomonas* exotoxin A contains four functional domains, and the domain Ia is responsible for recognition of receptors on the cells to induce immune responses [32,33]. Because the fusion protein comprised a carrier protein and multiple antigen epitope copies, it elicited strong antibody activities [30,31]. Therefore, this fusion protein platform can be applied in novel vaccine development for preventing pathogenic infections.

The present study predicted the epitope of the GNNV coat protein based on the crystal structure of GNNV and used the LAE technique to yield a multiple tandem-repeated coat protein epitope, which was obtained through the conjugation of the epitope repeats and domain Ia of PE. The *in vivo* safety and potency were compared between this novel NNV LAE vaccine and VLP vaccines. The results indicated that the epitope prediction of the pathogen protein structure and fusion protein containing the carrier protein and tandem-repeated epitopes can be used as a vaccine design platform against infectious diseases.

2. Material and methods

2.1. Virus isolation and titer quantification

The grouper fin cell line (GF-1, BCRC 960094) was obtained from the Bioresource Collection and Research Center (BCRC) in Taiwan. GNNV was obtained from naturally infected giant grouper larvae (*E. lanceolatus*) collected in 2008 from Jiading farm no. 11 [34]. NNV detection was performed through quantitative PCR (qPCR) by using cDNA samples from total RNA isolated from grouper tissues [34]. The virus was isolated from the tissues and propagated in GF-1 cells at 28 °C in Leibovitz's L-15 (L15) medium (Invitrogen, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Invitrogen, USA) for several days until the occurrence of a cytopathic effect (CPE) in 90% of the cells. Virus isolation and purification were performed as described previously [34]. In brief, the culture supernatants were passed through a 0.22-μm filter, and free viruses in the clarified supernatant were frozen at −80 °C. Virus quantity was represented by 50% tissue culture infective doses ($TCID_{50}$), which were calculated from the correlation of the cell number with the CPE and the serial NNV dilution by using the Reed–Muench method [35,36].

Table 1
Primers and plasmids used in this study.

Name	Sequence (5′→3′)/Characterization	Purpose/ Source
Primers		
NS-oligoA	GACATTGCCCCTGATGGAGCAGTCTTCCAG	TR-PCR
NS-oligoB	ATCTGGGGCAATGTCCTGGAAGACTGCTCC	TR-PCR
NS-5S	GATCCCGCGGCGACATTGCCCCTGATGGA	Adaptor-PCR
NS-3E	GATCGAATTCTAATCAGGGGCAATGTCCTG	Adaptor-PCR
NNV-qF	GACGCGCTTCAAGCAACTC	qPCR
NNV-qR	CGAACACTCCAGCGACACAGCA	qPCR
Plasmids		
pSUMO-NNVCP	Expression of recombinant GNNV coat protein for assembly to VLPs	Chen *et al.* [12]
pPEIa-His_6	Recombinant PEIa protein	Hsu *et al.* [30,31]
pPEIa-S5E	Recombinant fusion protein containing PEIa and GNNV coat protein epitope repeats	This study

2.2. Construction of GNNV coat protein epitope repeats by using LAE

According to the crystal structure of the GNNV coat protein and the linear epitope prediction results of the FBCPred method (http://ailab.ist.psu.edu/bcpred/predict.html) [12,37], a peptide containing 10 amino acids (residues 249–258) was selected as the epitope. The epitope was tandem-repeated for plasmid construction in two steps, tandem repeat (TR)-PCR and adaptor (AD)-PCR. Two primers, NS-oligoA and NS-oligoB (see Table 1), were used as templates, and *Pfu* DNA polymerase (Promega, USA) was used in TR-PCR. The PCR programs involved 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and polymerization at 72 °C for 30 s, followed by the final polymerization step at 72 °C for 10 min. The TR-PCR product was then used as a template for AD-PCR. NS-5S and NS-3E primers and Taq polymerase (Bioman, Taiwan) were used in this reaction under the following PCR conditions: 5 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and polymerization at 72 °C for 30 s, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and polymerization at 72 °C for 30 s. Finally, the reaction was terminated with a polymerization step at 72 °C for 10 min. The AD-PCR product was cloned into a pGEM-T easy vector (Promega, USA) for sequencing, and the desired fragment of five epitope repeats was then cloned into the *Bam*HI and *Xho*I sites of the pPEIa-His_6 vector for expression [30,31]. The resulting plasmid was named as pPEIa-S5E.

2.3. Preparation of subunit vaccines

The construction and purification of the recombinant GNNV coat protein, which then assembled into VLPs, were performed as described previously [12]. VLP integrity was analyzed by subjecting negatively stained samples to electron microscopy (Supplementary Fig. 1) [12], and the purified VLPs were stored at −80 °C until further use. The plasmids, pPEIa-His_6 and pPEIa-S5E, were used to transform *Escherichia coli* BL21(DE3) (Yeastern Biotech, Taiwan), and the clones were cultured at 37 °C in Luria–Bertani medium containing 100 μg/mL of ampicillin (Sigma-Aldrich, USA). At a cell density (OD_{600}) of 0.3–0.4, a final concentration of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich, USA) was added. After 3 h of induction, the cells were harvested through centrifugation (8000 × *g*, 4 °C, 20 min) and disrupted through sonication in lysis buffer. Recombinant proteins were purified in HisTrap HP columns (GE Healthcare, UK) and then stored at −80 °C until further use. The purified VLPs, recombinant PEIa protein, and recombinant fusion protein containing PEIa and GNNV coat protein epitope repeats, referred to as NNV-VLP, PEIa, and NNVCP-S5E, respectively, were quantified using the Pierce BCA Protein Assay Kit