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Short communication

Immunity to nervous necrosis virus infections of orange-spotted grouper (*Epinephelus coioides*) by vaccination with virus-like particles

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ABSTRACT

Nervous necrosis virus (NNV) is a kind of the betanodaviruses, which can cause viral nervous necrosis (VNN) and massive mortality in larval and juvenile stages of orange-spotted grouper (*Epinephelus coioides*). Due to the lack of viral genomes, virus-like particles (VLPs) are considered as one of the most promising candidates in vaccine study to control this disease. In this study, a type of VLPs, which was engineered on the basis of orange-spotted grouper nervous necrosis virus (OGNNV), was produced from prokaryotes. They possessed the similar structure and size to the native NNV. In addition, synthetic oligodeoxynucleotide (ODN) containing CpG motif was added in vaccines, and the expression patterns of several genes were analyzed after injecting with VLP and VLP with adjuvant (VA) to assess the regulation effect of vaccine for inducing immune responses. RT-PCR assays showed that six related genes in healthy tissues were ubiquitously expressed in all nine tested tissues. The vaccine alone was able to enhance the expression of genes, including MHCIa, MyD88, TLR3, TLR9 and TLR22 after vaccination, indicating that the vaccine was able to induce immune response in grouper. In liver, spleen and kidney, the gene expressions of VA group were all significantly higher than that of VLP group at 72 h post-stimulation, showing that the fish of VA challenge group obtained the longer-lasting protective immunity and resistance to pathogen challenge than that of VLP group. The data indicated that the efficacy of vaccine could be further enhanced by CpG ODN after vaccination and provided the reference for the development of future viral vaccine in grouper.

1. Introduction

Betanodaviruses are icosahedral and non-enveloped virus particles, belonging to the family *Nodaviridae* (genus *Betanodavirus*). Its genome contains two single-stranded, positive-sense RNA molecules, called RNA1 and RNA2 [1]. RNA1 is the large genomic segment, encoding RNA-dependent RNA polymerase, while RNA2 belongs to the small genomic segment and mainly encodes the coat protein (CP) [2,3]. Nervous necrosis virus (NNV) is a nodavirus, which can cause viral nervous necrosis (VNN) in 30 or more species of marine fish worldwide [4].

Infection with NNV causes massive mortality in larval and juvenile stages of fish (80–100%), resulting in significant economic losses in aquaculture industry [5]. The experiments indicated that brain and eye were the main organs for NNV replication [6]. NNV attack the central nervous system of the host, causing deadly viral encephalopathy and retinopathy (VER) in fish [7]. The infected fish showed the symptoms of neurological disorders, including focal vacuolization of the central nervous system (CNS) and retina, spiral swimming, uncoordinated darting, together with abnormalities of swimming bladder control [7].

The orange-spotted grouper (*Epinephelus coioides*) serves as an important commercial mariculture fish species, due to its excellent food quality, abundant nutrients and rapid growth, and has been widely cultured in South China and Southeast Asian countries. With the serious issue of environment pollution, the grouper farming industry present even greater challenges than ever. Infectious pathogens are a serious problem in aquaculture, and VNN is a

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particularly important disease of *E. coioides* that is caused by different strains of NNV [8].

Vaccination is one of the most effective methods of combating threatening viral diseases in fish. A number of vaccines are currently commercially available for use in the aquaculture industry, but currently-available traditional vaccines are ineffective against certain NNV serotypes [9–11]. In addition, the producing vaccines based on inactivated viruses must rely on large scale cultivation of piscine cells to allow the viruses to replicate, and the cost is usually too high to make this strategy economically viable [12]. Therefore, there is a considerable current interest to develop the novel drugs for preventing and treating NNV infection.

Virus-like particles (VLPs) are protein complexes consisting of recombinant virus capsid proteins, whose shapes are similar to native virions. In specific conditions, they can assemble spontaneously from single or multiple structure proteins to induce protective antibody responses in fish [13–16]. Due to the lack of viral genomes, VLPs are not infectious and safer than attenuated vaccines. Therefore, VLPs are considered as one of the most promising candidates in vaccine study. VLPs can be produced from eukaryotes or prokaryotes, and the prokaryotic system is easier for gene manipulation, produces higher yields and is cheaper [17]. *Escherichia coli* is the simplest prokaryotic host system, which is the most widely utilized for producing recombinant proteins, has also been used to express certain viral VLPs [18].

Traditionally, fish vaccines can be administered via immersion, orally and injection. The ideal immunization procedure for aquaculture should be easier and faster, but intramuscular injection seems to be the most effective way for administering vaccines presently. In addition, synthetic oligodeoxynucleotide (ODN) containing CpG motif has been widely added in piscine vaccines for inducing both innate and adaptive immune responses in fish body [19–21]. Because of the potent adjuvant effect and extremely low damage to tissues at injection, CpG ODN has been regarded as a gold standard for vaccine adjuvant [22,23].

In the present study, orange-spotted grouper NNV (OGNNV) VLPs were produced as vaccine in *E. coli* for immunizing orange-spotted grouper by intramuscular (IM) injection. Although vaccines are expected to elicit long-lived, lymphocyte-mediated immune protection in fish, little is known about the molecular mechanisms of vaccine-induced immune protection in teleost fish. Moreover, teleost immune systems play a crucial role in protecting organism from virus infection. Thus, the expression profiles of several innate and adaptive immunity genes were detected to evaluate vaccine efficiency.

2. Materials and methods

2.1. NNV virus purification

Orange-spotted grouper with the classical VNN symptom, which were close to death, were collected from fish farms of Zhangzhou (Fujian, China). The method for virus purification was modified from that of Poulos et al. [24]. The infectious fish were homogenized in PBS buffer in the proportion of one to five at 0 °C, and the suspension was clarified by low-speed centrifugation. The penicillin streptomycin solution was joined into the supernatant fluid after clarification. The supernatant was stored at 4 °C over night, and then was filtered using 45 nm membrane filter. Collected filtrate was ultracentrifuged for 3.5 h at 205,000 × g (Beckman Optima LE-80K). The resulting pellet was resuspended in PBS buffer (pH 7.5) and then was fractionated on a 20–50% (w/w) sucrose gradient at 200,000 × g for 3 h. Various gradient fractions were collected, diluted with PBS and centrifuged for 3.5 h at 205,000 × g to remove the sucrose. The pellets were resuspended with appropriate PBS, and the viral structure and shape were verified through negative staining and electron microscopy (JEOL JEM-1230).

2.2. Vector construction and preparation of OGNNV VLPs

The open reading frame of CP gene (GenBank accession number: EF558369) from cDNA of viral RNA2 was synthesized and subcloned into a prokaryotic expression vector pET30a (Novagen) to generate the capsid-expressing plasmid, pET-CP. The plasmid without His-tag was then transformed into DE3 *E. coli* and used for VLPs production. *E. coli* producing OGNNV VLPs were cultured in containing 50 μg ml^{-1} kanamycin LB-broth medium at 37 °C. After the cell density reached 0.3–0.5 (OD600), the culture was cooled down to 30 °C and 0.9 mM IPTG was added in medium for inducing cell growth, and then the cells were continued to be cultured for 16 h. The cells were harvested by centrifugation at 8000 × g for 15 min under 4 °C, and then the cells were disrupted by the ultrasonic wave and the cell lysate was collected using centrifugation. Finally, VLPs of OGNNV were purified by high-performance liquid chromatography. In addition, SDS-PAGE, Western-Blot and electron microscopy were utilized to examine the VLPs.

2.3. Fish

Healthy orange-spotted grouper (meanweight 10 g) were obtained from fish farms of Xiamen (Fujian, China). All fishes were maintained in aerated flow-through seawater tanks at room temperature with a natural photoperiod. After one week of acclimatizing, three fishes were randomly selected as control samples and used to verify the virus-free of grouper by polymerase chain reaction (PCR). And then vaccination experiments were performed.

2.4. Vaccination challenge and sampling

Fish were randomly divided into three groups, each consisting of 35 fishes. VLPs were injected with the dosages of 2 μg per gram of fish body weight [15]. The negative control group received intramuscularly 50 μl of PBS. CpG DNA adjuvant was dissolved in PBS to prepare a solution with 0.14 μg/μl density (Table 1). One VLP injection group was given IM injection of 20 μg of purified VLPs that prepared in 50 μl PBS with CpG DNA adjuvant. Another VLP injection group of fishes were injected with the same dosages of VLPs without adjuvant. The control and infected fish samples were killed at 0 h, 4 h, 8 h, 12 h, 16 h, 24 h, 36 h, 48 h, and 72 h after injection, respectively. Three individuals of each group were killed in every time point and nine tissue samples from the heart, kidney, eye, gill, intestine, brain, spleen, liver and blood were collected and then kept at −80 °C until use.

2.5. RNA isolation and cDNA synthesis

Samples were placed into 1 ml of Trizol reagent (Qiagen) and homogenized, respectively. Total RNA was extracted from the different tissues using Trizol reagent according to the manufacturer's instructions. cDNA synthesis was performed using a QuantScript RT Kit (TIANGEN) in accordance with the manufacturer's protocol, and cDNAs were then stored at −20 °C for later use.

2.6. Primer design and real-time quantitative PCR (qRT-PCR)

CP primer was designed according to the sequences of NNV coat protein gene in orange-spotted grouper for virus-free testing (Table 1). Other primers were designed to implement the qRT-PCR analysis (Table 1). Expressions levels of six selected genes were