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Immune gene expressions in grouper larvae (*Epinephelus coioides*) induced by bath and oral vaccinations with inactivated betanodavirus

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ABSTRACT

Nervous necrosis virus (NNV) has caused mass mortality in many mariculture fish species. Bath vaccination of inactivated NNV and oral immunization of recombinant NNV coat protein are reported to protect grouper larvae against NNV infection. However, the information of immune gene expression in grouper larvae (*Epinephelus coioides*) after bath and oral immunizations is still limited. In this study, grouper larvae were respectively bath- and orally immunized with binary ethylenimine (BEI)-inactivated NNV, and the expression levels of immune genes were analyzed. Significant gene expressions of IL-1β, Mx, MHC-I, MHC-II, CD8α, IgM and IgT were observed in bath- and orally immunized fish 1–4 weeks post immunization (wpi). Particularly, the up-regulation of IL-1β and Mx gene expression lasted for 4 weeks. The IgT gene expression in gill was only induced by bath immunization, while that in gut was only stimulated by oral immunization. Both immunizations elicited MHC-I and CD8α gene expression relative to cellular immunity. Furthermore, NNV RNA genome, which was detected in inactivated NNV, could induce Mx gene expression in grouper brain (GB) cells, indicating that NNV RNA genome could be recognized by pathogen-recognition receptors (PRRs). In summary, bath and oral vaccinations with BEI-inactivated NNV triggered the gene expression of not only humoral immunity but also cellular immunity.

1. Introduction

Nervous necrosis virus (NNV), the causative agent of viral nervous necrosis disease, is one of the most important viral diseases in aquaculture and has caused mass mortality in more than 34 fish species at larval and juvenile stages [1]. The nervous system is the target for NNV replication, and the pathological characteristic is vacuolation of the brain and retina. NNV is a non-enveloped virus and belongs to Betanodavirus of *Nodaviridae*. The NNV genome consists of two single-stranded positive-sense RNA. RNA1 and RNA2 respectively encodes RNA-dependent RNA polymerase and capsid protein [2]. Based on partial sequences of RNA2, four genotypes are identified, including barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), and redspotted grouper nervous necrosis virus (RGNNV) [3]. Betanodaviruses of RGNNV genotype are commonly isolated from warm-water fish [3,4], and all Taiwan NNV isolates belong to the RGNNV genotype [5].

Groupers (*Epinephelus* spp.) are economically important fish species in the aquaculture industry in Southeast Asia and are very sensitive to NNV infection. In Taiwan, the highest mortality (80–100%) caused by NNV occurs at larval stage [6,7]. Horizontal and vertical transmission of NNV has been recorded in many fish species [8–14]. Several types of NNV vaccines are developed and applied by intramuscular or intraperitoneal injection, including recombinant capsid protein [15–18], synthetic peptides [19], virus-like particle [20,21], and inactivated virion [22]. In order to immunize grouper larvae to achieve earlier protection, bath and oral immunizations of NNV vaccine have been applied. Oral immunization with subunit NNV vaccine was reported to provide protection of grouper larvae against NNV infection [23,24]. Furthermore, our previous study proved that bath immunization with inactivated NNV effectively protected grouper larvae from NNV challenges [25]. In addition, fish mucosa is the first tissue invaded by the pathogens. For example, grouper fin has been reported to be a primary tissue for NNV infection [26]. Therefore, activation of mucosal immunity by bath and oral vaccinations will be helpful to antagonize pathogen attack.

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In bath and oral immunizations, the fish mucosa, including skin, gut and gill, will first contact with the antigens. However, the information about the induction of immune gene expressions in those mucosal tissues after both immunizations is very limited, and the relationship between vaccine-induced protection and immune gene expressions is unclear. Therefore, this study aimed at examination of the immune gene expressions in mucosal tissues after bath and oral vaccinations with inactivated NNV, including the genes of pro-inflammatory cytokines (IL-1β and TNF-α), type I interferon (IFN) inducible antiviral protein Mx, MHC molecules, T_C cell marker CD8α, and immunoglobulins (IgM and IgT). Through the analysis of immune gene expressions, it will help realize what kinds of immune responses are induced by bath and oral vaccinations and are possibly enrolled in the protection of groupers larvae against NNV infection.

2. Materials and methods

2.1. Fish and cell line

A total number of 600 orange-spotted grouper (*Epinephelus coioides*) larvae with age of 40 days post hatchery (dph) and average body weight of 0.18 g (~2.3 cm) were obtained from a private grouper hatchery farm in southern Taiwan. The fish were kept to adapt for 3 days in a 400-L tank supplied with ozone-treated and re-circulated seawater. Ten larvae were randomly sampled for NNV detection by RT-PCR and nest PCR described by Chi et al. (2003) [5]. The F1/R3 primer set was used to amplify NNV RNA2 T2 fragment by RT-PCR, and F2/R3 primer set was selected to amplify NNV RNA2 T4 fragment by nested PCR [3]. The primer sequences are listed in Table 1. All the tested larvae were concluded to be NNV-free.

Grouper brain (GB) cell line (unpublished cell line) was derived from the brain tissue of orange-spotted grouper (*Epinephelus coioides*) and has been subcultured for more than 70 times, using Leibovitz's L-15 medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 28 °C. GB cells are sensitive to NNV infection.

2.2. Preparation of inactivated NNV vaccine

The NNV used for vaccine preparation was HGNNV strain. HGNNV was isolated from the NNV-infected humpback grouper (*Cromileptes altivelis*) and was identified as RGNNV genotype [5]. HGNNV was propagated in GF-1 cell line [27] with multiplicity of infection (MOI) being 1. Until complete cytopathic effect (CPE) was observed, the whole culture fluid was collected and centrifuged at 2000 × *g* for 10 min at 4 °C, and the culture supernatant was titrated in GF-1 cells.

The inactivated NNV vaccine was prepared according to the method described in our previous report [25]. Viral supernatant with titer of $10^{10.5}$ $TCID_{50}$ ml^{-1} was inactivated by incubating with 4 mM binary ethylenimine (BEI) at 25 °C for 72 h and then dialyzed with PBS. The safety of the inactivated vaccine was confirmed via three passages in GF-1 cells without appearance of CPE. The BEI-inactivated NNV vaccine was further nano-encapsulated by Alarvita Biolife Company (Taipei, Taiwan) using patented materials and method (Patent: TW 90133170, 91136029; U.S. 10/316926; E.P. 02022037.2; C.N. 2130595; J 2002-381638). The average diameter of the nanoencapsulated particle was about 80 nm.

The NNV RNA genome in the inactivated NNV vaccine was detected by RT-PCR described in Section 2.1.

2.3. Bath and oral immunizations

Grouper larvae were divided into three groups (200 fish per group) for bath and oral immunizations and non-immunization, and each group was reared in a 60 L-aquarium at 24–27 °C. The titer ($TCID_{50}$) of the virus before inactivated is used to represent the dose of inactivated NNV vaccine. For bath immunization, larvae were immersed in 13 L seawater containing encapsulated BEI-inactivated NNV vaccine at a concentration of 5×10^5 $TCID_{50}$ ml^{-1} for 60 min. For oral immunization, artemia eggs (Ocean star international INC, USA.) were hatched in ozone-treated 2.2% seawater at 28 °C. Two days post hatching, approximately 60,000 artemia were mixed with encapsulated BEI-inactivated NNV vaccine at a dose of 10^8 $TCID_{50}$ in 10 ml seawater for 1 h. The artemia were washed by seawater to remove the unattached vaccine and then were fed to the fish twice daily for three days.

To detect the early immune response induced by bath and oral immunizations, five fish of each group were sampled at 1, 3 and 5 days post immunization (dpi). The size of larvae at this stage was so small that the whole fish body was homogenized for RNA extraction and analysis of immune gene expression by real-time PCR. At 1, 2 and 4 weeks post immunization (wpi), three fish of each group were sampled, and the viscera (the mixture of brain, liver, spleen and kidney), skin, gut (the whole intestine), and gill of the fish were removed for examination of immune gene expression.

2.4. Reverse transcription and real-time PCR

The procedures of RNA extraction, reverse transcription (RT), real-time PCR for analysis of immune gene expression were the same as that described by Wu et al. (2010) [28]. Total RNA of all samples were extracted by acid guanidinium thiocyanate-phenol-chloroform method [29] and were transcribed into cDNA by M-MLV reverse transcriptase (Promega) and oligo dT_{20}. The cDNA of 0.25 μl was added into each PCR mixture with a final volume of 10 μl containing 0.5 μM forward and reverse primers in 1 × iQ SYBR Green Super-Mix (Bio-Rad). The amplification was carried out in CFX384 Real-time PCR Detection System (Bio-Rad) with an initial denaturing step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and fluorescence detection at 76 °C for 20 s. All samples were analyzed in triplicate. The primer sequences used for detection of immune gene expression are listed in Table 1. The expression level of each immune gene was normalized with internal control (β-actin). Relative gene

Table 1
Primer sequences.

Primer name	Primer sequence
NNV F1	5′-GGATTTGGACGTGCGACCAA-3′
NNV F2	5′-CGTGTCAGTGCTGTGTCGCT-3′
NNV R3	5′-CGAGTCAACACGGGTGAAGA-3′
MHC-I F	5′-CGACCTCACTCAGCATTGTCCT-3′
MHC-I R	5′-GTAGAAACCTGTAGCGTGGCG-3′
MHC-II F	5′-GGACATCAGACCCTGGACCAA-3′
MHC-II R	5′-ACACCGAGCAGACCGACAGT-3′
CD8α F	5′-CACTAACAAGCCAGGGGAAA-3′
CD8α R	5′-GGTGGCGATGAGGAGTAGAA-3′
IgM F	5′-ACCGTGACCCTGACTTGCTATG-3′
IgM R	5′-CCCGATGGACCTGACAATAGC-3′
IgT F	5′-TGTGTCAAAGTCTGCCTGGGATTCA-3′
IgT R	5′-CTTAGGAGGTGGAGGAGGCTTTTG-3′
TNF-α F	5′-ACGCAATCAGGCCAAAGAG-3′
TNF-α R	5′-AAGCCGCCCTGAGCAAAC-3′
IL-1β F	5′-AGCGACATGGTGCGGTTTCTC-3′
IL-1β R	5′-CTCTGTAGCGGCTGGTGGACTC-3′
Mx F	5′-TGAGGAGAAGGTGCGTCC-3′
Mx R	5′-GCGCCTCCAACACGGAGCTC-3′
β-actin F	5′-GGCCGCGACCTCACAGACTACCTC-3′
β-actin R	5′-CCTCTGGGCAACGGAACCTCTCAT-3′