한국어병학회지 제34권 제2호 (2021)
J. Fish Pathol., 34(2) : 177~184

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2021.34.2.177

Use of G gene-deleted single-cycle viral hemorrhagic septicemia virus (VHSV) for delivery of nervous necrosis virus (NNV)-like particles

Jeong In Yang[1], Min Sun Kim[2††] and Ki Hong Kim[1†]

[1]Department of Aquatic Life Medicine, Pukyong National University, Busan 48513, South Korea
[2]Department of Biological Sciences, Kongju National University, Gongju 32588, South Korea

Vaccines based on single-cycle viruses that are replication-incompetent due to knockout of replication-related structural gene(s) are more immunogenic than inactivated or subunit vaccines and can be used as delivery vehicles for foreign antigens without concerns on the reverting to virulent forms. The aim of this study was to develop a delivery vehicle for nervous necrosis virus (NNV)-like particles (VLPs) using G gene deleted single-cycle VHSV (rVHSV-ΔG). Recombinant single-cycle VHSVs carrying NNV capsid protein gene between N and P gene of rVHSV-ΔG genome (rVHSV-ΔG-NNV_{Cap}) were rescued by reverse genetic technology. The successful expression of NNV capsid protein in cells infected with rVHSV-ΔG-NNV_{Cap} was demonstrated by Western blot analysis, and the production of NNV VLPs in infected cells was confirmed using an electron microscopy. The results suggest that single-cycle VHSVs can be used as a safe delivery vehicle for NNV VLPs, and can be extended to other pathogens for the development of prophylactic vaccines.

Key words: Single-cycle VHSV, NNV Capsid-expressing VHSV, NNV VLPs, Delivery vehicle

Introduction

Viral nervous necrosis (VNN) caused by nervous necrosis virus (NNV) infection has been an important cause of massive mortalities in various economically important marine fish worldwide (Munday *et al.* 2002). NNV belongs to the genus *Betanodavirus* of the family *Nodaviridae*, and has a bipartite positive sense RNA genome, RNA1 encoding the RNA-dependent RNA polymerase, RNA2 mainly encoding the capsid protein, and a subgenomic RNA3 encoding B2 protein. NNVs can be divided into four genotypes based on the sequence of the capsid protein (Nishizawa *et al.* 1995; Doan *et al.* 2017). In Korea, the red-spotted grouper nervous necrosis virus (RGNNV) type has been recorded from various marine fish species causing mass mortalities in cultured fish such as sevenband grouper (*Hyporthodus septemfasciatus*), rock bream (*Oplegnathus fasciatus*), red drum (*Sciaenops ocellatus*) and olive flounder (*Paralichthys olivaceus*) (Sohn *et al.* 1998; Oh *et al.* 2005; Cha *et al*. 2007; Gomez *et al.* 2008), and the barfin flounder virus (BFNNV) type was recently found from various domestic and imported shellfish (Kim *et al.* 2018).

The induction of protective immunity against NNV by the immunization of fish with inactivated viruses, virus-like particles (VLPs), or recombinant capsid protein has been reported (Kim *et al.* 2015a;

†Corresponding author: Ki Hong Kim
Tel: +82-51-629-5943; Fax: +82-51-629-5938
E-mail: khkim@pknu.ac.kr
††Corresponding author: Min Sun Kim
Tel: +82-41-850-8503; Fax: +82-41-850-0957
E-mail: mskim0825@kongju.ac.kr

Lin *et al.* 2016; Nunez-Ortiz *et al.* 2016). Generally, inactivated or subunit vaccines need a large amount of antigen and boost immunizations, and often have to be accompany with adjuvants to increase immunogenicity, which can lead to the increase of side effects and vaccine price. Recently, there have been several reports on the use of bacteria or yeast for the expression and delivery of NNV capsid protein to fish hosts (Chen *et al.* 2011; Kim *et al.* 2014; Cho *et al.* 2017).

Recombinant viruses made through reverse genetics can be used as a delivery vehicle for foreign antigens. However, the recombinant live viruses have been limited in their practical use due to the concerns on the reverting to virulent types. Previously, we generated single-cycle viral hemorrhagic septicemia virus (VHSV) that lacking the glycoprotein (G) gene in the genome (rVHSV-ΔG) (Kim *et al.* 2015b; Kim *et al.* 2016a). As the G gene-deleted rhabdoviruses are replication-incompetent, we can use them as safe vaccines or delivery vehicles for foreign molecules without concerns on the reverting to virulent forms. The genome of rhabdoviruses is so flexible that foreign genes can be inserted between viral genes, through which various recombinant rhabdoviruses can be generated and can be used as delivery vehicles for various proteins (Kim *et al.* 2016b). In this study, we produced recombinant VHSV (rVHSV-ΔG-NNV_{Cap}) that is carrying the NNV capsid protein gene between the nucleoprotein (N) and phosphoprotein (P) gene of the rVHSV-ΔG genome, and evaluated the potential of rVHSV-ΔG-NNV_{Cap} as a safe delivery vehicle for NNV VLPs.

Materials and methods

Cells and viruses

Epithelioma papulosum cyprini (EPC) and E11 cells were grown at 25°C in Leibovitz medium (L-15, Sigma) supplemented with penicillin (100 U/ml. Gibco), streptomycin (100 μg/ml, Gibco) and 10% fetal bovine serum (FBS, Gibco). The wild-type VHSV (VHSV KJ2008) (Kim *et al.* 2011a) were propagated in monolayer of EPC cells at 15°C in the presence of 2% FBS. A RGNNV isolate recovered from olive flounder was propagated in E11 cells at 25°C with a medium containing 2% FBS. EPC cells expressing VHSV G protein were used for the propagation of G gene-deleted single-cycle VHSV (rVHSV-ΔG) that was generated from our previous study (Kim *et al.* 2015b).

Construction of single-cycle VHSV vector containing NNV capsid protein gene (pVHSV-ΔG-NNV_{Cap})

To get a clone of NNV capsid protein gene, complementary DNA (cDNA) was synthesized using Hyper Script First strand synthesis kit (GeneAll) from E11 cells infected with RGNNV. The NNV capsid protein gene ORF amplified with a primer pair that had *Nde*I (forward) and *Sal*I (reverse) sites (Table 1) was cloned in pGEM T-easy vector (pNNV_{Cap}; Promega). To insert NNV capsid protein ORF between N and P genes of VHSV, previously constructed pVHSV-A-RFP (pVHSV containing a red fluorescent protein (RFP) gene between N and P genes) (Kim *et al.* 2013) and the pNNV_{Cap} were digested with *Nde*I and *Sal*I, then, the RFP gene ORF was replaced with the NNV capsid protein gene ORF, resulting in pVHSV-A-NNV_{Cap}. To delete G gene in the vector pVHSV-A-NNV_{Cap}, previously constructed pVHSV-ΔG (pVHSV lacking the G gene) and pVHSV-A-NNV_{Cap} were digested with *Nae*I and *Age*I, then, the N-P-M fragment in the pVHSV-ΔG was replaced with the N-NNV_{Cap}-P-M fragment of the pVHSV-A-NNV_{Cap}, and named as pVHSV-ΔG-NNV_{Cap}.

Recombinant VHSV production

EPC cells expressing T7 RNA polymerase were grown to about 80% confluence and transfected with a mixture of pVHSV-ΔG-NNV_{Cap} (2 μg) and helper plasmids, pCMV-N (500 ng), pCMV-P (300 ng),

Table 1. Summary of primers used in this study

Name of primer		Sequence (5' to 3')
For construction of pVHSV-ΔG-NNV_{Cap}		
NNV_{Cap} ORF	F	CATATGGTACGCAAAGGTGAGAAG
	R	GTCGACTTAGTTTTCCGAGTCAACCCTGG
For verification of production of rVHSV-ΔG-NNV_{Cap}		
N - NNV_{Cap}	F	CCCCACCACCCAGTCAAGGGC
	R	TTAGTTTTCCGAGTCAACCCTGG
NNV_{Cap} - P	F	ATGGTACGCAAAGGTGAGAAG
	R	GGGACAGCTTGATACGTCTTAG
P - M	F	GATCAGCTCTCTTTTCGTCC
	R	CTACCGGGGTCGGACAGAGGG
M - NV	F	CCAGGTCGATAAGATCTGCATGG
	R	GGTCCTTAGATCCTCTGAGACT
G - NV	F	CCTCCAACTGCAGAAAAAACTCC
	R	GGTCCTTAGATCCTCTGAGACT
G - L	F	CCTCCAACTGCAGAAAAAACTCC
	R	ATCGTCCTCAAGGCCAAATC
For construction of His-tagged NNV capsid protein (pET-NNV_{Cap})		
NNV_{Cap} ORF	F	CATATGGTACGCAAAGGTGAGAAG
	R	AAGCTTGTTTTCCGAGTCAACCCTGG

The underlined characters represent restriction enzyme sites

pCMV-L (200 ng), and also pCMV-G (250 ng), using FuGENE HD (Promega) according to manufacturer's instructions. Transfected cells were incubated for 12 h at 28°C and shifted to 15°C. Cultures displaying total cytopathic effect (CPE) were harvested and centrifuged at 4,000 g for 10 min. The resulting supernatant was inoculated to EPC cells expressing G protein of VHSV, and incubated at 15°C in L-15 supplemented with antibiotics and 2% FBS. At 7-10 days post-inoculation, the supernatant was harvested, filtered and stored at -80°C.

Verification of rVHSV-ΔG-NNV_{Cap} and titer analysis

To confirm the production of rVHSV-ΔG-NNV_{Cap} by reverse transcriptase PCR (RT-PCR), total RNA was extracted from 1 ml of the virus supernatant using Trizol reagent (Invitrogen), and synthesized first-strand cDNA using HyperScript First strand synthesis Kit with random primer (GeneAll) according to the manufacturer's instruction. The insertion of NNV capsid protein gene between N and P genes and the deletion of VHSV G gene in the genome were analyzed by RT-PCR using primer sets in Table 1. The PCR products were visualized on a 1% agarose gel containing Nucleic acid stain (Korea Labtec). The titer of rVHSV-ΔG-NNV_{Cap} was determined by plaque assay (Burke and Mulcahy 1980). Briefly, EPC cells monolayer expressing G protein of VHSV was inoculated with the above rVHSV-ΔG-NNV_{Cap} serially diluted from 10^{-2} to 10^{-4}. After 1 h of incubation at 15°C, the cells were overlaid with plaquing medium (0.7% agarose in L-15 containing 2% FBS and antibiotics). After 10 days of incubation, the cells were fixed by 10% formalin and stained with 3% crystal violet for 30 min at RT. After rinsing of the cells with distilled water, the plaque-forming units (PFU) were counted.

Analysis of the ability of rVHSV-ΔG-NNV_{Cap} to produce infective viral particles

To analyze whether rVHSV-ΔG-NNV_{Cap} infection can generate infective viral particles without trans-supply of G protein, supernatant of rVHSV-ΔG-NNV_{Cap} that passed through 3 times in EPC cells expressing G protein was inoculated into normal EPC cells that did not express G protein. After 2 h of the inoculation, cells were washed with PBS for 3 times, and incubated at 15°C in the culture medium containing 2% FBS and antibiotics. After 7 days, the supernatant was isolated and conducted plaque assay using normal EPC cells.

Expression and purification of NNV capsid protein

To produce His-tagged NNV capsid protein, first, NNV capsid protein gene ORF containing a *Nde*I (forward) and a *Hind*III (reverse) restriction enzyme site was amplified by PCR with primer pair in Table 1. The resulting fragment was cloned in pGEM T-easy vector and subcloned into the *Nde*I and *Hind*III sites of the pET28a vector (Novagen), resulting in pET28a-NNV_{Cap}. The recombinant His-tagged NNV capsid protein was expressed in *E. coli* BL21 (DE3) by inducing with IPTG of 1 mM at 20°C for overnight. The His-tagged NNV capsid protein was purified by chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the manufacturer's protocols (Novagen). Protein purity was monitored by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue, and the protein concentration was determined using the BCA protein assay (Sigma).

Production of antiserum against NNV capsid protein

To produce polyclonal antibody against NNV capsid protein, a rabbit was immunized 3 times with His-tagged NNV capsid protein (330 μg/ml). The rabbit experiment was conducted by AbFrontier, Korea. At 9 weeks after initial immunization, whole serum was collected, and stored at -80°C until analysis.

Western blot

EPC cells expressing VHSV G protein were seeded in 6-well plate (1×10^6 cells/well) at 28°C, and shifted to 15°C, and cells monolayer was inoculated with rVHSV-ΔG-NNV_{Cap} at a MOI of 0.1. At 1d, 3d, 5d, 7d, 10d post-infection, cells were harvested by centrifugation with 4,000 g for 10 min, then, were solubilized in SDS-PAGE loading buffer (GeneAll), boiled for 5 min and loaded on a 10% SDS-PAGE gel. Non-infected EPC cells and RGNNV stock solution was used as a negative and a positive control, respectively. Proteins were then transferred from gel to nitrocellulose membrane and incubated with diluted rabbit anti-NNV_{Cap} antiserum (1:1000) for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (1:2000, Santa Cruz Biotechnology) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

Purification of NNV VLPs and transmission electron microscopy

EPC cells were grown to about 80% confluence and inoculated with rVHSV-ΔG-NNV_{Cap} at a MOI of 0.01. After 7 days post-infection, cells were freeze-thawed 2 times were centrifuged (4,000 g, 5 min), then the collected supernatant was filtered using a 0.2 μm syringe filter. To purify NNV VLPs, the supernatant was ultracentrifuged at 32,000 rpm for 1 h (Beckman) against a 25 % sucrose cushion, and pelleted NNV VLPs were suspended in 1ml of 50 mM Tris-HCl (pH 7). The collected NNV VLPs were put on a Formvar carbon grid, then 4% uranyl-acetate was placed on the grid for negative staining. After 40 seconds, the staining solution was

removed, and the NNV VLPs were observed by transmission electron microscopy (TEM).

Results

Generation of rVHSV-ΔG-NNV$_{Cap}$

The procedure to construct a vector pVHSV-ΔG-NNV$_{Cap}$ is shown in Fig. 1a. Evident CPE was observed from T7 RNA polymerase-expressing EPC cells that were transfected with pVHSV-ΔG-NNV$_{Cap}$ and helper plasmids. The insertion of NNV capsid protein ORF between N and P gene and the deletion of G gene in the genome of rVHSV-ΔG-NNV$_{Cap}$ were verified by RT-PCR (Fig. 1b), and were also confirmed by full genome sequencing (data not shown). In plaque assay, the virus titer was 7.4×10^4 PFU/ml in EPC cells that were expressing VHSV G protein.

Analysis of rVHSV-ΔG-NNV$_{Cap}$ ability to produce infective viral particles

The rVHSV-ΔG-NNV$_{Cap}$ could induce CPE and form plaques only when inoculated to EPC cells that were expressing G protein. No plaque formation was observed in EPC cells lacking G protein supply cassette (Fig. 2)

Analysis of NNV capsid protein expression by rVHSV-ΔG-NNV$_{Cap}$

To examine the production of NNV capsid protein in the cytoplasm of cells infected with rVHSV-ΔG-NNV$_{Cap}$, cells harvested at 1, 3, 5, 7, and 10 d post-infection were examined by Western blot analysis using rabbit antiserum against the NNV capsid protein. As a result, about 37 kDa band that was corresponding to the MW of NNV capsid protein was detected in all loaded samples except non-infected cells lysate (Fig. 3).

Transmission electron microscopic analysis of NNV VLPs

The NNV VLPs from EPC cells inoculated with rVHSV-ΔG-NNV$_{Cap}$ were observed as regular shaped and variable sized (mean diameter of about 30 to 50

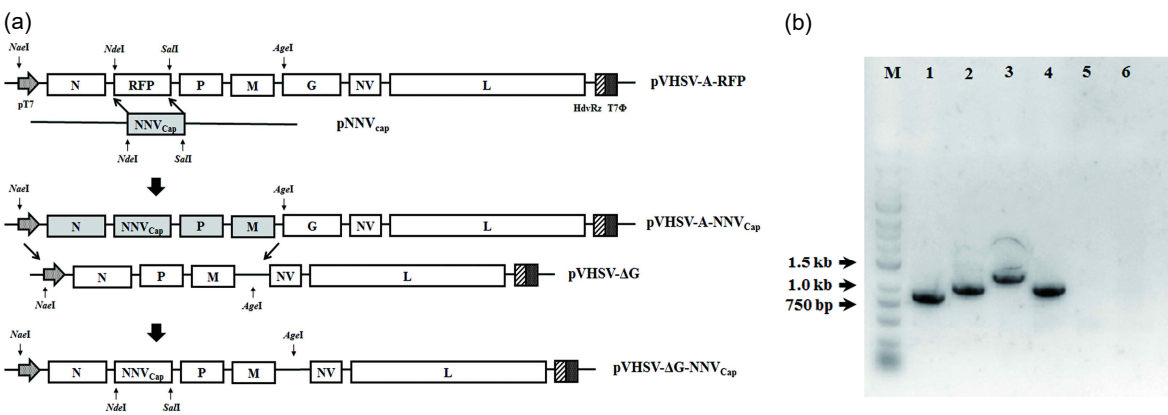

Fig. 1. (a) Construction of single-cycle VHSV vector containing NNV capsid protein gene (pVHSV-ΔG-NNV$_{Cap}$). The plasmid, pVHSV-A-NNV$_{Cap}$, was firstly generated by replacement of the RFP gene in the pVHSV-A-RFP with the NNV capsid protein gene using NdeI and SalI restriction enzyme sites. And then, to delete the G gene, the fragment from pT7 to M gene harboring NNV capsid protein gene was inserted into the pVHSV-ΔG using NaeI and AgeI restriction enzyme sites. (b) Verification of both NNV capsid protein gene insertion and G gene deletion in the genome of rVHSV-ΔG-NNV$_{Cap}$ by RT-PCR. The regions across N - NNV$_{Cap}$ genes (Lane 1), NNV$_{Cap}$ - P genes (Lane 2), P - M genes (Lane 3), and M - NV genes without G gene (Lane 4) were exactly amplified in the rVHSV-ΔG-NNV$_{Cap}$. The G - NV genes (Lane 5) and G - L genes (Lane 6) were not amplified at all.

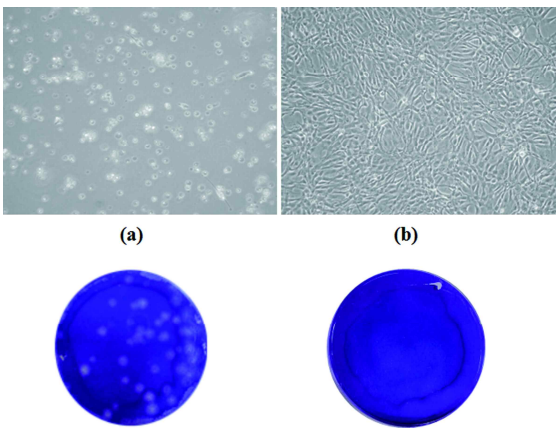

Fig. 2. The induction of CPE and the formation of plaque in EPC cells by inoculation of rVHSV-ΔG-NNV$_{Cap}$. (a) The evident CPE and many plaques were presented in G protein expressing EPC cells, (b) No CPE and plaque was observed without trans-supply of G protein.

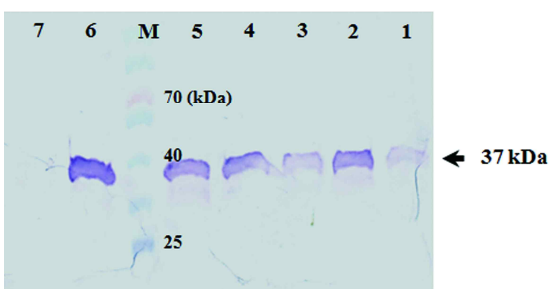

Fig. 3. Analysis of NNV capsid protein expression in the cytoplasm of cells infected with rVHSV-ΔG-NNV$_{Cap}$ by Western blot analysis using rabbit antiserum against the NNV capsid protein. M: prestained protein ladder (Thermo), Lane 1-5: cells lysates infected with rVHSV-ΔG-NNV$_{Cap}$ at 1, 3, 5, 7, and 10 d post-inoculation, Lane 6: cells lysates infected with wild-type RGNNV as a positive control, Lane 7: no-infected cells lysates as a negative control.

nm) particles by an electron microscopy (Fig. 4).

Discussion

The technologies of reverse genetics have allowed us to produce safety-guaranteed and functionality-enhanced recombinant viruses. In fish viruses, VHSV has been a main target for the production of recombinant viruses, and reverse genetically produced recombinant VHSVs have been used in immunoprophylaxes (Kim *et al.* 2011b; Kim *et al.* 2015b). Recently, Nzonza *et al.* (2014) and Rouxel *et al.* (2016) reported the availability of recombinant VHSVs that are expressing mammalian viral antigens such as West Nile virus's E glycoprotein or influenza virus's hemagglutinin for the development of prophylactic vaccines. However, studies on the use of recombinant VHSVs as a delivery vehicle for other fish pathogen's antigens are rare.

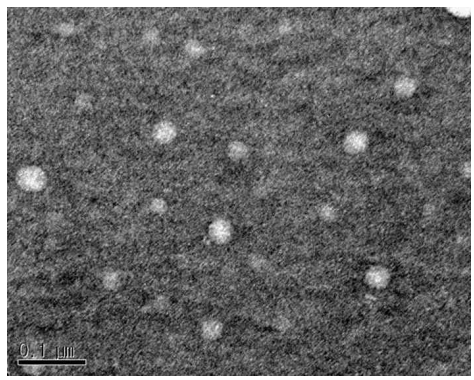

Fig. 4. NNV VLPs observed by a transmission electron microscope (TEM). Bar scale, 0.1 μm.

Replication-competent attenuated viruses can be pathogenic to immunocompromised individuals and have a possibility to regain virulence. Therefore, although the protective efficacy may be lowered, replication-incompetent viral replicon particle can be an alternative to guarantee safety. In the present study, we evaluated the potential of G gene deleted single-cycle rVHSV-ΔG to be used as a delivery vehicle for NNV VLPs by inserting the NNV capsid gene between N and P gene of VHSV genome. The results showed that rVHSV-ΔG-NNV$_{Cap}$ successfully produced NNV capsid protein in the infected cells, indicating that rVHSV-ΔG replicon particles can be used as a delivery vehicle for NNV capsid protein to fish. Moreover, as rVHSV-ΔG-NNV$_{Cap}$ cannot replicate unless the G protein is trans-supplied, the safety could be largely enhanced. Although rVHSV-Δ

G-NNV$_{Cap}$ cannot produce viral particles in the infected cells, they still have an ability to transcript their genes using N, P, and L proteins, which might confer them the enhanced ability to induce protective immunity.

In this study, we showed the possible use of single-cycle VHSV as a safe delivery vehicle for NNV VLPs. To utilize the present rVHSV-ΔG-NNV$_{Cap}$ for practical vaccine purposes, further researches on the in vivo immunization should be conducted.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science and ICT) (No. NRF-2020R1A2C4001511).

References

Burke, J.A. and Mulcahy, D.: Plaquing procedure for infectious hematopoietic necrosis virus. Appl. Environ. Microbiol., 39: 872-876, 1980.

Cha, S.J., Do, J.W., Lee, N.S., An, E.J., Kim, Y.C., Kim, J.W. and Park, J.W.: Phylogenetic analysis of betanodaviruses isolated from cultured fish in Korea. Dis. Aquat. Org., 77: 181-189, 2007.

Chen, Y.-M., Shih, C.-H., Liu, H.-C., Wu, C.-L., Lin, C.-C., Wang, H.-C., Chen, T.-Y., Yang, H.-L. and Lin, J.H.-Y.: An oral nervous necrosis virus vaccine using Vibrio anguillarum as an expression host provides early protection. Aquaculture 321: 26-33, 2011.

Cho, S.Y., Kim, H.J., Lan, N.T., Han, H.J., Lee, D.C., Hwang, J.Y., Kwon, M.G., Kang, B.K., Han, S.Y., Moon, H., Kang, H.A. and Kim, H.J.: Oral vaccination through voluntary consumption of the convict grouper Epinephelus septemfasciatus with yeast producing the capsid protein of red-spotted grouper nervous necrosis virus. Vet. Microbiol., 204: 159-164, 2017.

Doan, Q.K., Vandeputte, M., Chatain, B., Morin, T. and Allal, F.: Viral encephalopathy and retinopathy in aquaculture: a review. J. Fish Dis., 40: 717-742, 2017.

Gomez, D.K., Baeck, G.W., Kim, J.H., Choresca, C.H., Jr. and Park, S.C.: Molecular detection of betanodavirus in wild marine fish populations in Korea. J. Vet. Diagn. Invest., 20: 38-44, 2008.

Kim, H.J., Lee, J.Y., Kang, H.A., Lee, Y., Park, E.J. and Kim, H.J.: Oral immunization with whole yeast producing viral capsid antigen provokes a stronger humoral immune response than purified viral capsid antigen. Lett. Appl. Microbiol., 58: 285-291,2014.

Kim, J.O., Kim, J.O., Kim, W.S. and Oh, M.J.: Development of a recombinant protein vaccine based on cell-free protein synthesis for sevenband grouper *Epinephelus septemfasciatus* against viral nervous necrosis. J. Microbiol. Biotechnol., 25: 1761-1767, 2015a.

Kim, M.S., Choi, S.H. and Kim, K.H.: Effect of G gene-deleted recombinant viral hemorrhagic septicemia virus (rVHSV-DeltaG) on the replication of wild type VHSV in a fish cell line and in olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol., 54: 598-601,2016a.

Kim, M.S., Kim, D.S. and Kim, K.H.: Generation and characterization of NV gene-knockout recombinant viral hemorrhagic septicemia virus (VHSV) genotype IVa. Dis. Aquat. Org., 97: 25-35, 2011a.

Kim, M.S., Kim, D.S. and Kim, K.H.: Oral immunization of olive flounder (*Paralichthys olivaceus*) with recombinant live viral hemorrhagic septicemia virus (VHSV) induces protection against VHSV infection. Fish Shellfish Immunol., 31: 212-216, 2011b.

Kim, M.S., Lee, S.J., Kim, D.S. and Kim, K.H.: Generation of recombinant viral hemorrhagic septicemia virus (rVHSV) expressing two foreign proteins and effect of lengthened viral genome on viral growth and in vivo virulence. Mol. Biotechnol., 58: 280-286, 2016b.

Kim, M.S., Park, J.S. and Kim, K.H.: Optimal place of a foreign gene in the genome of viral haemorrhagic septicaemia virus (VHSV) for development of VHSV-based viral-vectored vaccines. J. Appl. Microbiol., 114: 1866-1873, 2013.

Kim, M.S., Park, J.S. and Kim, K.H.: Generation of G gene-deleted viral hemorrhagic septicemia virus (VHSV) and evaluation of its vaccine potential in olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol., 45: 666-671,2015b.

Kim, Y.C., Kwon, W.J., Kim, M.S., Kim, K.I., Min, J.G. and Jeong, H.D.: High prevalence of betanodavirus barfin flounder nervous necrosis virus as well as red-spotted grouper nervous necrosis virus genotype in shellfish. J. Fish Dis., 41: 233-246, 2018.

Lin, K., Zhu, Z., Ge, H., Zheng, L., Huang, Z. and Wu, S.: Immunity to nervous necrosis virus infections of

orange-spotted grouper (Epinephelus coioides) by vaccination with virus-like particles. Fish Shellfish Immunol., 56: 136-143, 2016.

Munday, B.L., Kwang, J. and Moody, N.: Betanodavirus infections of teleost fish: a review. J. Fish Dis., 25: 127-142, 2002.

Nishizawa, T., Mori, K., Furuhashi, M., Nakai, T., Furusawa, I. and Muroga, K.: Comparison of the coat protein genes of five fish nodaviruses, the causative agents of viral nervous necrosis in marine fish. J. Gen. Virol., 76(Pt 7): 1563-1569, 1995.

Nunez-Ortiz, N., Pascoli, F., Picchietti, S., Buonocore, F., Bernini, C., Toson, M., Scapigliati, G. and Toffan, A.: A formalin-inactivated immunogen against viral encephalopathy and retinopathy (VER) disease in European sea bass (*Dicentrarchus labrax*): immunological and protection effects. Vet. Res., 47: 89, 2016.

Nzonza, A., Lecollinet, S., Chat, S., Lowenski, S., Merour, E., Biacchesi, S. and Bremont, M.: A recombinant novirhabdovirus presenting at the surface the E Glycoprotein from West Nile Virus (WNV) is immunogenic and provides partial protection against lethal WNV challenge in BALB/c mice. PloS One 9: e91766, 2014.

Oh, M.-J., Jung, S.-J. and Kitamura, S.-I.: Comparison of the Coat Protein Gene of Nervous Necrosis Virus (NNV) Detected from Marine Fishes in Korea. J. World Aquac. Soc., 36: 223-227, 2005.

Rouxel, R.N., Merour, E., Biacchesi, S. and Bremont, M.: Complete Protection against Influenza Virus H1N1 Strain A/PR/8/34 Challenge in Mice Immunized with Non-Adjuvanted Novirhabdovirus Vaccines. PloS One 11: e0164245, 2016.

Sohn, S.-G., Park, M.-A., Oh, M.-J., and Chun, S.-K.: A fish nodavirus isolated from cultured sevenband grouper, *Epinephelus septemfasciatus*. J. Fish Pathol., 11: 97-104, 1998.

Manuscript Received : Oct 27, 2021
Revised : Nov 08, 2021
Accepted : Nov 08, 2021