Efficacy of the Inactivated Nervous Necrosis Virus Vaccine Against Viral Nervous Necrosis in Pond-Reared Orange-Spotted Grouper *Epinephelus coioides*

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Abstract

The field efficacy of the formalin-inactivated nervous necrosis virus (NNV) against viral nervous necrosis (VNN) in orange-spotted grouper (*Epinephelus coioides*) reared in floating net cages in earthen pond was investigated. Seroneutralization assay conducted on the sera of vaccinated fish exhibited the occurrence of neutralizing antibody titers from Day 30 (mean titer 1:1792±701) to Day 150 (1:704±351) with the highest titer observed at Day 60 (1:6656±3435) post-vaccination. Because mortality attributed to VNN was not encountered during the pond experiment, intramuscular challenges of vaccinated and unvaccinated (L-15 injected) fish with NNV ($10^{6.5}$ $TCID_{50}$/fish) were conducted in indoor tanks at Day 30 (Mean body weight [MBW]: vaccinated [21±3.4 g]; unvaccinated [20.6±1 g) and Day 120 (MBW: vaccinated [178±27 g]; unvaccinated [176±19 g) post-vaccination, respectively, to demonstrate the *in vivo* efficacy of the inactivated vaccine. Nil and 25 % mortality rate were obtained in vaccinated and control fish, respectively, challenged with NNV at Day 30 post-vaccination. On the contrary, nil mortality were obtained in both groups challenged with NNV at Day 120 post-vaccination. Although nil mortality was obtained in NNV-challenged unvaccinated fish, 30 % of the fish manifested dark coloration of the skin and abnormal swimming behavior that commenced and disappeared at Day 3 and Day 7 post-NNV challenge, respectively, suggesting an age/weight-dependent resistance to the disease. Our current data illustrate that single vaccination with inactivated vaccine could mount the production of protective antibodies and concomitant conferment of protection against VNN in groupers especially during the early phase of grow-out culture in earthen ponds where they are highly susceptible to the disease.

Key words: Inactivated vaccine, viral nervous necrosis, VNN, Epinephelus coioides

Introduction

Viral nervous necrosis (VNN) is a destructive disease of both farmed and wild fish, with more than 120 species belonging to 30 families from 11 different orders being susceptible to the disease (Bandín and Souto, 2020). Betanodaviruses (family *Nodaviridae*), the causal agents of VNN, are small, non-enveloped, spherical (25–30 nm) viruses with a genome composed of two single-stranded RNA segments: RNA1 (3.1 kb) and RNA 2 (1.4 kb) which encode the viral replicase (110 kDa) and the coat

protein (42 kDa), respectively (Comps *et al*., 1994; Mori *et al*., 1992; Thiery *et al*., 2012). A third RNA segment, RNA3 (0.4 kb), is sub-genomically transcribed from RNA1 in the infected cells and correspondingly encode a protein with potent RNA silencing-suppression activity (Iwamoto *et al*., 2005; Sommerset and Nerland, 2004). Four genotypes have been designated based on the coat protein gene sequences including striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), redspotted grouper nervous necrosis virus (RGNNV), and barfin flounder nervous necrosis virus (BFNNV) (Nishizawa *et al*., 1997). Among these, RGNNV and genetically related viruses have by far been implicated in mass mortalities of hatchery-reared high-value marine fish species including groupers (*Epinephelus* spp.), sea bass (*Lates calcarifer*), and pompano (*Trachinotus blochii*) in the Philippines (Maeno *et al*., 2004, 2002; Pakingking *et al*., 2011).

Control of VNN via elimination of virus-carrying broodfish by RT-PCR (Nishizawa *et al*., 1994) and disinfection of eggs by ozonation (Mori *et al*., 1998) has been carried out but despite the rigid screening of broodstocks for nervous necrosis virus (NNV) by RT-PCR and cell culture isolation, sporadic outbreaks of VNN still occur. Because of the ubiquity of NNV in the marine environment, unpredictable outbreaks of VNN in floating net cages in the open sea is also inevitable (Nakai *et al*., 2009). In addition, broodstocks obtained from the wild, though apparently healthy, most often than not carry some residual infectious NNV in their system that may proliferate rapidly once these fish are confronted with abiotic disease-causing agents such as abrupt temperature changes and abnormal water physicochemical parameters (Gomez *et al*., 2004; Mushiake *et al*., 1994, 1992).

In our previous bioassay experiments in tanks, we successfully demonstrated the efficacy of the formalin-inactivated Philippine strain of NNV in sea bass, groupers, and pompano juveniles against experimental challenges, i.e. via injection and immersion, with the homologous NNV (Pakingking *et al*., 2011, 2010, 2009). We also noted that a potent anamnestic response would arise when vaccinated groupers that survived NNV challenge were re-exposed to infectious NNV as indicated by substantial increases, i.e. up to six-folds or higher, in NNV-neutralizing antibody titers in NNV re-challenged fish (Pakingking *et al*., 2010). Although our tank studies have clearly demonstrated the efficacy of the formalin-inactivated Philippine strain of NNV vaccine against experimental NNV challenge, however, its field efficacy has not yet been thoroughly investigated. Thus, in this context, the field efficacy of the formalin-inactivated Philippine strain of NNV vaccine was tested in orange-spotted grouper, a highly susceptible fish species to VNN, reared in floating net cages in earthen pond in Dumangas Brackish Water Station (DBS) of the Aquaculture Department, Southeast Asian Fisheries Development Center (SEAFDEC/AQD). Specifically, we examined the immunogenicity and protective immunity of the inactivated-NNV vaccine in the context of its ability to induce the production of NNV-neutralizing antibodies and conferment of protection in fish against artificial NNV challenge.

Materials and methods

Fish

A total of 210 healthy orange-spotted grouper juveniles (*E. coioides*) with mean body weight (MBW) of 8 g, obtained from a private hatchery in Roxas City, Capiz, Philippines, were stocked in 1000-L tank supplied with sand-filtered and flow-through seawater at 28 ˚C. Fish were allowed to acclimate for 2 weeks before the start of the experiment. They were fed SEAFDEC/AQD formulated diet (3 %

body weight) once a day throughout the experiment. Prior to the conduct of the vaccination experiment, brain samples of 10 randomly chosen fish were aseptically dissected and screened for NNV by nested-step RT-PCR (Nishizawa *et al*., 1994). All samples examined were negative for NNV.

Virus strain

The OSGBF1E strain (Genotype: red spotted grouper nervous necrosis virus; RGNNV) of NNV was used as vaccine immunogen in the preparation of the vaccine (Pakingking *et al*., 2009). NNV was propagated in E-11 cells (Iwamoto *et al*., 2000) using L-15 medium supplemented with 2 % fetal bovine serum (FBS).

Preparation of the vaccine

The formalin-inactivated vaccine was prepared following the published method of Pakingking *et al*. (2009, 2010, 2011). Briefly, the OSGBF1E strain at a titer of $10^{9.2}$ $TCID_{50}$ ml^{-1} was inactivated with 0.5 % formalin, followed by incubation at 4 ˚C for 10 days. The vaccine was ascertained to be completely inactivated, i.e. free of any residual infective virus, by showing no CPE in E-11 cells after three serial passages.

Vaccination

Vaccination of groupers was carried out by first anesthetizing fish in holding aquaria (500 L) with MS222. Two hundred grouper juveniles (MBW: 8.3±1.2 g) were randomly divided into two groups. The first group composed of 100 fish were intraperitoneally (IP) injected with 100 µl of the vaccine (pre-inactivation titer: $10^{9.2}$ $TCID_{50}$ ml^{-1}) and stocked in 2×3×1.0 m floating net cage in the pond. Correspondingly, the same number of individuals were also injected with an equal volume of L-15 medium (control group). At different time points post-vaccination, i.e. Days 30, 60, 90, 120, and 150, blood samples were collected from the caudal veins of 5 fish randomly collected from each of the cages. Quantification of the neutralizing antibody titers in the sera of fish were done following the method described in the succeeding section. Both vaccinated and unvaccinated fish were monitored periodically for any signs of VNN.

Neutralizing antibody assay

Neutralizing antibody titers in the sera of both vaccinated and unvaccinated fish were quantified using the method of Pakingking *et al*. (2011, 2010, 2009, 2009). At each sampling time, fish (n=5) were randomly collected from each of the vaccinated and unvaccinated group, anesthetized, followed by the collection of blood from the caudal vein of fish. After allowing the blood to clot at 4 ˚C overnight, the serum was obtained by centrifugation at 1500 × g for 15 min. The serum was then divided into several aliquots and stored at -20˚C until used. To quantify the NNV-neutralizing antibody titer in the sera of fish, a seroneutralization assay was conducted following the method of Pakingking *et al*. (2011, 2010, 2009). Briefly, fish sera were diluted with 39 volumes of Hanks' balanced salt solution supplemented with penicillin (100 IU ml^{-1}) and streptomycin (100 µg ml^{-1}) (HBSS-PS). They were then diluted twofold with HBSS-PS and mixed with an equal volume (50 µl) of the viral suspension (50 µl, 10^2 $TCID_{50}$). Immediately after incubating the mixture at 25 ˚C for 60 min, aliquots of each mixture were inoculated into four wells of the 96-well plate seeded with E-11 cells at approximately 80 % confluency. Cytopathic effect (CPE) was observed daily for ten days and the NNV-neutralizing antibody titer was calculated according to Reed and Muench (1938).

Virus challenge

At day 30 and 120 post-vaccination or L-15 injection, twenty and ten fish respectively

from each vaccinated and unvaccinated group were randomly collected and brought to the Infection Building, Tigbauan Main Station, SEAFDEC/AQD. These fish were intramuscularly challenged with NNV at an inoculum dose of $10^{6.5}$ $TCID_{50}$/ fish and periodically observed for 14 days post-NNV challenge. Brains and kidneys of dead and surviving fish were aseptically collected and subjected to virus titrations. Prior to dissection, blood samples were taken from the caudal vein of surviving fish for NNV-neutralizing antibody detection.

Virus titrations

NNV titers in the brains of dead and surviving fish were quantified following a protocol adapted from the previous study of (Pakingking *et al*., 2011, 2010, 2009).

NNV detection in fish by RT-PCR amplification

The brains of vaccinated and unvaccinated fish at the termination of the experiment were examined by nested-step RT-PCR (Nishizawa *et al*., 1994).

Statistical analysis

Statistical analysis was carried out using Fisher's exact probability test for fish mortalities and Mann Whitney's U-test for neutralizing antibody titers (Pakingking *et al*., 2010).

Results and discussion

The field efficacy of the inactivated Philippine strain of NNV vaccine in orange-spotted grouper juveniles, a highly susceptible fish species to VNN, via intraperitoneal (IP) injection was investigated in the current study. We employed the IP injection because this mode of vaccine administration has been proven efficient in upregulating the immune system of fish to produce potent NNV-neutralizing antibodies as documented in our previous reports (Pakingking *et al*., 2018, 2011, 2010, 2009). Accordingly, we were able to establish and compare the kinetics of NNV-neutralizing antibody productions in vaccinated groupers reared in floating net cages in earthen pond at different time points post-vaccination and with our previous data generated on tank trials, respectively (Pakingking *et al*., 2011, 2010, 2009).

The result of seroneutralization assay conducted prior to the IP injection of groupers with either L-15 (control) or inactivated NNV vaccine showed the absence of NNV-neutralizing antibodies (<1:80) in the sera of fish. However, at Day 30 post-vaccination, potent NNV-neutralizing antibodies (mean titer: 1:1792±701) were detected in the sera of fish (n=5) that peaked at Day 60 (1:6656±3435) and thereafter started to gradually decline but still detectable at Day 150 (1:704±351) post-vaccination (**Figure 2**). On the contrary, NNV-neutralizing antibodies were not detected (<1:80) in groupers examined at scheduled intervals post-L15 injection. By far, the mean NNV-neutralizing antibody titers in the sera of orange spotted groupers quantified at different time points post-vaccination in the current study strongly corroborate with the results of our previous tank trials in brown marbled groupers (Pakingking *et al*., 2010). These results clearly indicate that the inactivated Philippine strain of NNV is highly immunogenic to groupers as evidenced by high levels of NNV-neutralizing antibodies in the sera of vaccinated fish. Notably, no significant differences were noted in the mean body weights between the vaccinated and unvaccinated fish examined at different time points post-vaccination or L-15 injection (**Figure 1**), clearly suggesting that the vaccine has no inadvertent effect on the growth of fish.

Because natural occurrence of VNN in net-caged groupers in earthen pond in DBS was not encountered during the course of the pond experiment despite several cases of VNN in grouper juveniles encountered in the same area in the past, the field efficacy of the inactivated vaccine in grouper could not be clearly elucidated. Thus, to circumvent this problem, representative samples from both vaccinated and unvaccinated fish were randomly collected from the pond, brought to TMS of SEAFDEC/AQD and were subsequently challenged via intramuscular injection with the homologous virus at a dose of $10^{6.5}$ TCID50/ fish at Day 30 post-vaccination. As a result, 25 % and nil mortality were obtained in unvaccinated and vaccinated fish, respectively (**Figure 3**). The survival rate obtained in pond-reared vaccinated fish challenged with the homologous NNV was evidently in consonance with the data that we obtained in our previous tank trials in brown marbled grouper (Pakingking *et al*., 2010). However, the mortality rate (25 %) obtained in Day 30 post-L-15 injected fish in the current study is lower compared with the result, i.e. 70 %, of our previous NNV challenge in brown marbled grouper similarly conducted at Day 30 post-L-15 injection. The difference observed in our previous and current study could be attributed to various factors including the body weight, age, species, and rearing conditions of the fish, and infectivity of the NNV used in the challenge experiments among others. For instance, in our previous report, the mean body weight (MBW) of tank-reared brown marbled groupers intramuscularly challenged with NNV at Day 30 post-L15 injection that resulted in 70 % mortality was only 12±2 g whereas in the current study, the MBW of orange-spotted groupers challenged with NNV that resulted in 25 % mortality was significantly higher, i.e. 21±1 g. Moreover, this hypothesis pertinent to the weight-dependent susceptibility of groupers to NNV is further backed up by nil mortality obtained in groupers with a mean body weight of 176±19 g that were challenged with NNV at Day 120 post-L-15 injection (de la Peña *et al*., 2017). It is worth noting that approximately 50 % of the Day 120 L15-injected groupers challenged with NNV at $10^{6.5}$ $TCID_{50}$/fish commenced manifesting lethargy at Day 3 followed by abnormal swimming behavior at Day 4 post-NNV injection (**Table 1**). The abnormal swimming behavior however lasted for only about 7 days after NNV challenge. Surprisingly, these fish gradually resumed normal swimming behavior and apparent recovery at the termination of the experiment. High NNV titers (>10^9 $TCID_{50}$/g) were detected in the brains of dead unvaccinated fish (**Table 1**). On the contrary, NNV was not detected in the brains of surviving vaccinated fish challenged with NNV (**Table 1**).

In summary, single administration of the monovalent formalin-inactivated NNV vaccine can effectively upregulate the production of NNV-neutralizing antibodies and concomitant conferment of protection against VNN in groupers especially during the early phase of grow-out culture in earthen ponds when these fish species are highly susceptible to the disease. Additionally, our current data also indicate the potential use of this inactivated vaccine against NNV infection in other grouper species and other warm-water marine fish species such as sea bass and pompano, particularly during the early phase of grow-out culture in earthen ponds or floating net-cages in the open sea, since mortalities of these fish species have been found to be caused by NNV strains belonging to a single genotype, i.e. RGNNV type.

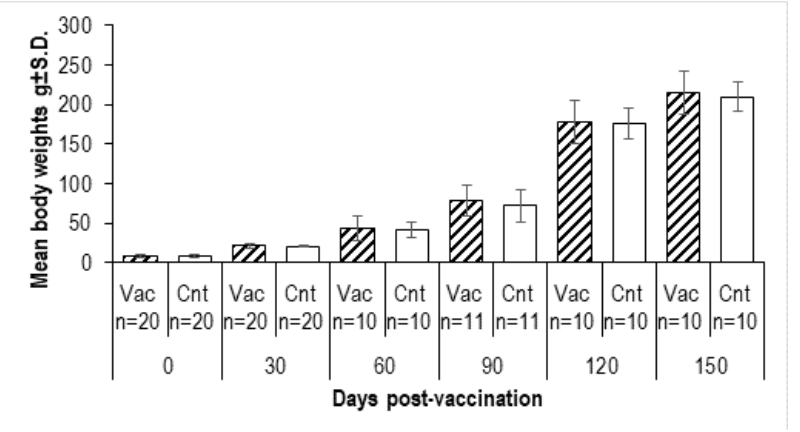

Figure1. Body weights (Mean ± SD) of vaccinated and unvaccinated orange-spotted groupers (*Epinephelus coioides*) examined at different time points post-vaccination

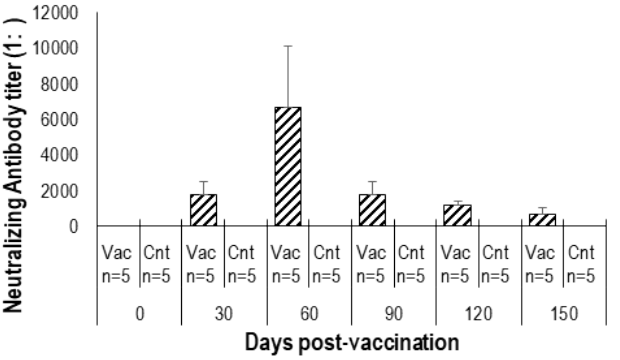

Figure 2. Neutralizing antibody titer (Mean ± SD) in the sera of vaccinated (Vac) and unvaccinated (Cnt) orange-spotted groupers (*Epinephelus coioides*) examined at different time points post-vaccination. The lowest detection limit is 1:80

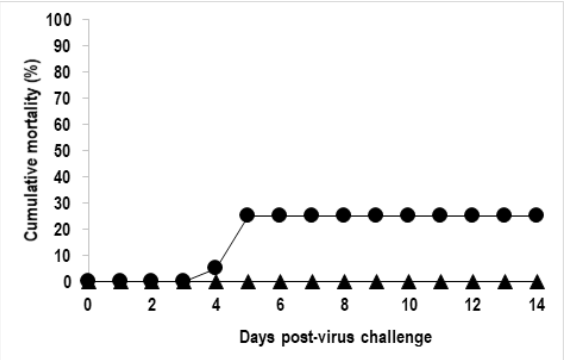

Figure 3. Cumulative mortalities of unvaccinated (●) and vaccinated (▲) orange-spotted grouper (*Epinephelus coioides*) juveniles intramuscularly injected with nervous necrosis virus at an inoculum dose of $10^{6.5}$ $TCID_{50}$ $fish^{-1}$.

Table 1. Cumulative mortality, serum antibody titers and nervous necrosis virus (NNV) titers in orange-spotted groupers intramuscularly challenged with NNV (106.5 TCID50 fish-1) at Days 30 and 120 post-vaccination or L-15 injection (control).

Days post vaccination	No. of fish that died of NNV infection/No. of fish challenged with NNV (%) [Mean body weight (g)± SD]		Antibody titer before the NNV challenge (No. of fish examined)		Antibody titer 1 month after the NNV challenge (No. of fish examined)		No. of fish that exhibited abnormal swimming behavior & dark skin coloration*/ No. of fish challenged with NNV (%)		No. of surviving fish that recovered from abnormal swimming behavior & dark skin coloration**/ No. of fish that survived NNV challenge (%)		NNV Titer $Log_{10}TCID_{50}\ g^{-1}$ in the brains of surviving fish examined at 1 month post NNV challenge (No. of positive / total no. of fish examined)	
	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control
Day 30	0/20 (0) [21±3.4]	5a /20 (25%) [21±1]	1792±701 (5)	<80 (5)	8192±2804 (5)	576±143 (5)	0/20 (0)	13/20 (65)	NA (0)	8/15 (53)	—[b] (0/5)	4.42 ± 0.02^{b} (5/5)
Day 120	0/20 (0) [178±27]	0/20 (0) [176±19]	1152±286 (5)	<80 (5)	14336±5609 (5)	704±351 (5)	0/20 (0)	6/20 (30)	NA (0)	6/20 (30)	—[b] (0/5)	4.35 ± 0.42^{b} (5/5)

* abnormal swimming behavior & dark coloration commenced from Day 3 to Day 4 post-NNV challenge; ** abnormal swimming behavior & dark coloration started to disappear from Day 8 post-NNV challenge; *** [a] Virus titer in the brains of dead fish (n=5) $10^{11.32\pm0.1}\ TCID_{50}g^{-1}$; [b] $<10^{2}\ Log_{10}TCID_{50}\ g^{-1}$; NA, not applicable

Acknowledgment

This study was funded by Government of Japan Trust Fund VI through the Regional Fish Disease Project (study code: FH01-F2015-T) and in part by SEAFDEC/ AQD. We express our heartfelt gratitude to Dr. Takuro Shibuno and Dr. Chihaya Nakayasu, former GOJ-TF managers, Dr. Koh Ichiro Mori, current GOJ-TF manager, and the Marine Fish Hatchery staff especially Mr. A. Gamuza.

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