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Efficacies of inactivated vaccines against betanodavirus in grouper larvae (*Epinephelus coioides*) by bath immunization

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Summary Betanodavirus is the pathogen of viral nervous necrosis (VNN) disease that has caused mass mortality among many species of marine fish at larval stage. In this study, the efficacy of inactivated betanodavirus was evaluated by bath-immunization and bath-challenge of orange-spotted grouper (*Epinephelus coioides*) at early larval stage. Two kinds of chemicals were used for inactivation of the virus, and the relative percent survival (RPS) values of 0.4 mM binary ethylenimine (BEI)-inactivated vaccine was revealed to be 79–95, higher than that of 0.1–0.2% formalin-inactivated vaccines (39–43). Three lengths of bath immunization time were tested, and 20 min immersion of BEI-inactivated betanodavirus at a concentration of 10^6 $TICD_{50}$/ml was sufficient to induce high protection (RPS > 75). Protection of the BEI-inactivated vaccine was evaluated at different time post immunization, and the peak of protection was observed 30 days post vaccination, and retained for at least 3 months. The efficacies of formalin-inactivated vaccines with or without encapsulation were compared, and the result revealed that the efficacy of formalin-inactivated vaccine could be significantly improved by nano-encapsulation (RPS = 85). All these data strongly suggested that bath immunization with nano-encapsulated formalin-inactivated or BEI-inactivated betanodavirus vaccines is an effective strategy to protect grouper larvae against VNN.

Introduction

Viral nervous necrosis (VNN) is one of the most important viral diseases in aquaculture. It affects more than 34 species of culture fish including grouper [1,2]. The causative agent of VNN is a non-enveloped virus with a size of 25–30 nm, classified as betanodavirus in the family Nodaviridae. The genome of betanodvirus consists of two single-stranded

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sense RNAs. The RNA1 encodes RNA-dependent RNA polymerase and RNA2 encodes coat protein [3]. In addition, a subgenomic RNA transcribed from the 3′ end of RNA1, named RNA3 [4], encodes a protein B2 with unknown function. Four genotypes are identified based on partial sequences of RNA2: barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) and red spotted grouper nervous necrosis virus (RGNNV) [5]. Fish nodaviruses of RGNNV genotype are commonly isolated from warm-water fish [5,6], and all Taiwan NNV isolates belong to the RGNNV genotype [2].

Vertical and horizontal transmissions of NNV have been demonstrated in many fish species [7—14], and different control strategies have been reported, including the screening of broodstock [8,9], ozone sterilization of water and eggs [15,16] and vaccination [17—19]. Several types of NNV vaccines have developed, including recombinant protein [17,18,20], synthetic peptide [21], inactivated virion [22], DNA vaccine [23] and virus-like particles (VLP) [24,25], but the efficacies of those vaccines were all evaluated on juvenile or young fish by intramuscular (IM) or intraperitoneal (IP) immunization and challenge. The highest mortality occurred at the larval stage; however the size of most fish species at the larval stage is too small to be immunized by injection.

Grouper is an economically important fish species in the aquaculture industry in Taiwan. The annual market of grouper larvae is worth more than 300 millions of US dollars. Since 1994, VNN disease has been continuously reported in cultured groupers (*Epinephelus* spp.), and the highest mortalities (80—100%) always occur among 1-month-old larvae with total body lengths (TBL) of 2.0 cm to 2-month-old larvae (TBL = 4.0 cm) [2,26,27].

The aim of this study was to induce protection of grouper larvae against VNN disease by bath immunization of inactivated-betanodavirus vaccine. The efficacies of the vaccines prepared by different methods were compared, and the optimal conditions for bath immunization were established.

Materials and methods

Fish and virus

A total number of 3000 orange-spotted grouper larvae (*Epinephelus coioides*) 40 days post hatchery (dph) with body weight (BW) of 0.2 g and TBL of 2.4 cm were obtained from a private grouper hatchery farm of southern Taiwan. Before immunization, the fish were allowed to adapt for 1 week in a 400 l-tank supplied with ozone-treated and recirculated seawater, and fed with commercial dried pellets (Uni-President Enterprises Corp., Taiwan) six times a day. Ten fish were collected at random for NNV screening using RT-PCR and nested PCR. All samples were found to be negative.

A betanodavirus strain HGNNV isolated from hump-back grouper (*Cromileptes altivelis*) [2], was used for preparation of inactivated vaccine. The HGNNV was inoculated into GF-1 cells [28] with multiplicity of infection (MOI) 1, and the cells were then incubated at 28 °C in Leibovitz's L-15 medium supplemented with 1% fetal bovine serum (FBS). Once complete cytopathic effect (CPE) was observed, the culture fluid was centrifuged at 2000 × *g* for 10 min, and the supernatant was titrated. The titer of the supernatant of HGNNV-infected GF-1 cells can reach as high as 10^{11} $TCID_{50}$/ml.

Inactivation of virus

Either formalin or binary ethylenimine (BEI) were used to inactivate HGNNV separately. Formalin was added into virus supernatant to a final concentration of 0.1% or 0.2%, and separately incubated at 4, 24 and 30 °C for 1—10 days (Table 1 for details). For the second means of inactivation, freshly prepared 0.1 M BEI was mixed with virus supernatant to a final concentration of 4 mM, and incubated at 25 °C for 72 h. After inactivation, the virus supernatants were dialyzed with phosphate buffer (PBS) at 4 °C overnight. The safety of each inactivated vaccine was examined by cell culture assay [29]. The vaccines were confirmed to be completely inactivated by showing no viral titer in GF-1 cells after three serial passages, and were used for the following immunization tests.

Encapsulation of inactivated vaccine

Part of the 0.2% formalin-inactivated NNV vaccine was further nano-encapsulated by Alarvita Biolife Company (Taipei, Taiwan) using patented substances 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 nano-encapsulated particle was about 80 nm.

Vaccination and challenge test

As described in Fig. 1, five independent trials were performed. The total fish we obtained from the hatchery farm for this study were 3000. The first stock had 1000 fish and was used for trial 1 and some pretests. The second stock had 2000 fish for all the experiments in trial 2—5. Although the fish prepared for this study were from two stocks, they all belong to the same species of grouper with the same age and size, and came from the same hatchery farm.

Within each trial, fish were divided into several vaccine groups and a control group (n = 100 per group). Each group was kept in a separate 60 l-aquarium prior to bath immunization and reared at 24—27 °C. The fish in control group were mock-immunized by PBS. The vaccines used in this study were all prepared by inactivating the culture supernatant of virus-infected cells instead of purified viral particles. The viral supernatant was titrated before inactivation, and the titer of the virus ($TCID_{50}$/ml) was used herein to represent the concentration of the inactivated vaccine.

In trial 1, the efficacies of formalin- and BEI-inactivated HGNNV vaccines were compared under the same dose (10^7 $TCID_{50}$/ml), and the viruses were separately inactivated by two concentrations of formalin (0.1% and 0.2%) and 4 mM BEI. The purpose of trail 2 is to evaluate the concentration effect of BEI-inactivated HGNNV vaccines on immunization, so the fish were bath-immunized with HGNNV vaccine at concentrations of 10^5, 10^6 and 10^7 $TCID_{50}$/ml. In trail 3, three lengths of immersion time (20, 60 and 120 min) for one dose of BEI-inactivated vaccine

Table 1 Safety test of formalin-inactivated HGNNV after different treatments

Formalin concentration (%)	Reaction temperature (°C)	Days for treatment	Viral titer post inactivation ($\log TCID_{50}$/ml)	CPE under each passage number		
				1	2	3
0.1	4	7	6.5	+	+	+
		10	5.5	+	+	+
	24	2	4.0	+	+	+
		3	3.0	+	+	+
		5	<1.0	—	—	+
		7	<1.0	—	—	—
	30	1	2.7	+	+	+
		2	<1.0	—	—	+
		3	<1.0	—	—	+
0.2	4	7	6.5	+	+	+
		10	6.0	+	+	+
	24	2	2.7	+	+	+
		3	<1.0	—	+	+
		5	<1.0	—	—	+
		7	<1.0	—	—	—
	30	1	<1.0	—	+	+
		2	<1.0	—	—	+
		3	<1.0	—	—	—

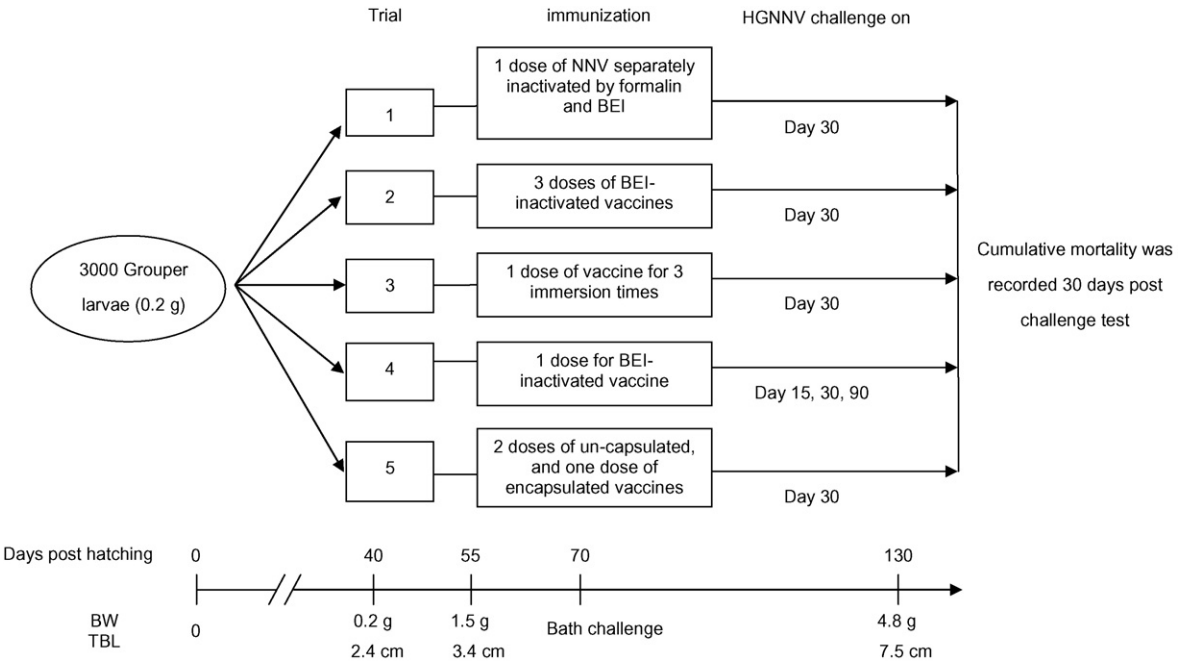

Figure 1 Experimental design for the bath immunization study. Each trial includes several vaccine groups and one control group ($n = 100$ per group). Fish in each control group for trials 1—5 were mock immunized with PBS, and challenged at the respective time points. Trial 1, comparing the efficacies of the same dose of NNV vaccines inactivated by two chemicals including formalin (0.1% and 0.2%) and BEI (4 mM); trial 2, comparing the concentration effect of BEI-inactivated NNV vaccines; trial 3, testing the effect of immersion time of BEI-inactivated vaccine; trial 4, checking the retention of protection induced by BEI-inactivated vaccine after immunization; trial 5, comparing the efficacies of formalin-inactivated vaccines with or without encapsulation.

(10^7 $TCID_{50}$/ml) were tested. In trial 4, the retention of protection induced by HGNNV vaccine was evaluated, so the fish were bath-immunized with one dose of BEI-inactivated HGNNV (10^7 $TCID_{50}$/ml) vaccine and then bath-challenged on days 15, 30 and 90 post immunization. The purpose of trial 5 is to compare the efficacies of non-capsulated and encapsulated formalin-inactivated vaccines; therefore, two doses of vaccine (10^6 and 10^7 $TCID_{50}$/ml) without encapsulation, and one dose of vaccine (10^5 $TCID_{50}$/ml) with encapsulation were used for bath immunization. The immersion time of vaccines for the fish in trials 1, 2, 4 and 5 was 120 min.

Bath challenge tests were done 30 days post immunization in trials 1, 2, 3 and 5, but on days 15, 30 and 90 post immunization in trial 4. Each trial had its own control group (PBS-mock immunized fish), and the control fish were challenged accompanied with vaccine groups at the respective time points. The HGNNV dose for bath challenge test was 1.6×10^6 $TCID_{50}$/ml. In trial 4, the size of fish on day 90 post immunization became larger (BW = 4.6 g, BL = 7.5 cm), and thus an intramuscular challenge test with dose of 1.0×10^7 $TCID_{50}$/fish was done in addition to bath challenge test. The cumulated mortality was recorded 1-month post challenge.

Detection of NNV by RT-PCR and semi-nested PCR

Viral RNA was extracted from the brain and retina of the tested fish, and was amplified by RT-PCR and semi-nested PCR by methods described by Chi et al. [2]. The primers for RT-PCR include forward primer F1 (5′-GGATTTGGA CGTGCGACCAA-3′) and reverse primer R3 (5′-CGAGTCAACACGGGTGAAGA-3′), and primers for semi-nested PCR are F2 (5′-CGTGTCAGTGCTGTGTCGCT-3′) and R3. Primer sequences of F1, F2 and R3 were based on those described by Nishizawa et al. [30] with minor modification. The target product for primer set (F1, R3) is T2 (870 bp), and for (F2, R3) is T4 (421 bp).

Statistical analysis

Differences in mortality were tested for statistical significance by the Chi-square contingency table test with the Yates' correction [31]. The cumulative mortality after challenge was recorded over 30 days, and the relative percent survival (RPS; [32]) was calculated by the formula: RPS = [1 − (% cumulative mortality of vaccinated group/% of cumulative mortality of control group)] × 100.

Results

Safety of the inactivated vaccines

The safety of the inactivated HGNNV vaccines under different preparations was tested by three serial passages in GF-1 cells. No viral titers were observed in GF-1 cells inoculated by the inactivated vaccines that were treated with 0.1% or 0.2% formalin at 24 °C for 7 days, 0.2% formalin at 30 °C for 3 days and 4 mM BEI at 25 °C for 3 days, respectively (Table 1). Moreover, 10 fish in each group were examined by RT-PCR and nested PCR 30 days post immunization, and no NNV was found at all.

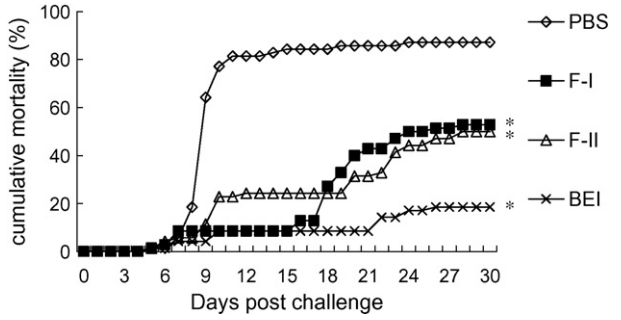

Figure 2 Cumulative mortality of grouper larvae after bath challenge with NNV. Grouper larvae were bath-immunized with 0.1% formalin-inactivated (F-I), 0.2% formalin-inactivated (F-II) and 4 mM BEI-inactivated NNV vaccine with viral concentration of 10^7 $TCID_{50}$/ml, and then bath-challenged with NNV (1.6×10^6 $TCID_{50}$/ml) 30 days post immunization. In negative control group, PBS was used instead of viral vaccine. *$p < 0.001$ (Chi-square).

Efficacy trail: chemicals for inactivation of viral antigen

To compare the efficacy of formalin-inactivated and BEI-inactivated vaccines, the final concentrations of the vaccines during bath immunization were adjusted to 10^7 $TCID_{50}$/ml. There were no abnormalities in any of the vaccinated or control fish before challenge. The fish in each group were then bath-challenged 30 days post immunization. The cumulative mortality of the challenged fish was 87% for the control group, 53% for 0.1% formalin-inactivated vaccine group, 50% for 0.2% formalin-inactivated vaccine group and 19% for BEI-inactivated vaccine group (Fig. 2). The RPS value of the fish immunized by BEI-inactivated vaccine was 79, i.e. much higher than that of the fish immunized by formalin-inactivated vaccine (RPS = 39). In addition, NNV was found in all the dead fish of each immunized group and negative control group after challenge test by RT-PCR examination.

Effective trial: dosage of vaccination

To determine the effective dosage of BEI-inactivated HGNNV vaccine, grouper larvae were bath-immunized with different dosages of BEI-inactivated HGNNV. The cumulative mortality of the fish in each group after HGNNV challenge is shown in Fig. 3. The RPS values of the fish bath-immunized with HGNNV vaccine with final concentration of 10^5, 10^6 and 10^7 $TCID_{50}$/ml were 7.5, 87.5 and 95, respectively. Therefore, the minimal effective dosage of BEI-inactivated HGNNV vaccine for bath immunization was 10^6 $TCID_{50}$/ml.

Efficacy trial: immersion time for bath immunization

Grouper larvae were bath-immunized with BEI-inactivated vaccine with a final concentration of 10^7 $TCID_{50}$/ml for 20, 60

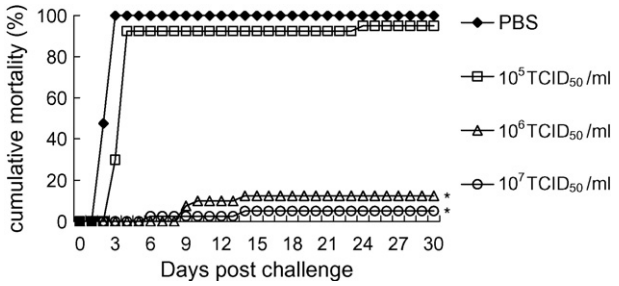

Figure 3 Cumulative mortality of grouper larvae after bath challenge of NNV. Grouper larvae were separately bath-immunized with BEI-inactivated vaccines with viral concentrations of 10^5, 10^6 and 10^7 $TCID_{50}$/ml, and then bath-challenged with NNV (1.6×10^6 $TCID_{50}$/ml) 30 days post immunization. *$p < 0.001$ (Chi-square).

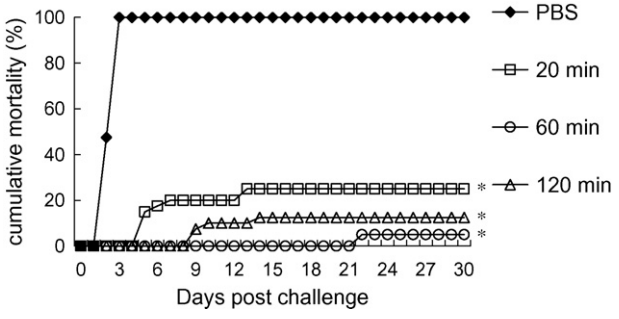

Figure 4 Cumulative mortality of grouper larvae after bath challenge of NNV. Grouper larvae were bath-immunized with BEI-inactivated NNV vaccine through different immersion time (20, 60 and 120 min) and then bath-challenged with NNV (1.6×10^6 $TCID_{50}$/ml) 30 days post vaccination. *$p < 0.001$ (Chi-square).

and 120 min, separately. The cumulative mortality of fish in each group after challenge is shown in Fig. 4. The cumulated mortalities of all the immunized groups were significantly lower than that of the control group. The RPS values of 20, 60 and 120 min were 75, 95 and 88, respectively.

Efficacy trial: retention of protection induced by BEI-inactivated vaccine

Grouper larvae after bath-immunization by BEI-inactivated vaccine with a final concentration of 10^7 $TCID_{50}$/ml were challenged with NNV with titer of 1.6×10^6 $TCID_{50}$/ml on the 15th, 30th and 90th day post vaccination. Table 2 indicates that the cumulative mortality and RPS value of each group. The RPS value of the 15-day group was 30, much lower than that of the 30-day group (RPS = 87), indicating that 15 days were not enough for the larvae to develop efficient immunity against NNV infection. The RPS value of the 90-day group was 82, as high as that of the 30-day group, indicating that efficient protection induced by immunization could last for at least 3 months. If the fish of the 90-day group were challenged by IM injection, the RPS value decreased to 8.

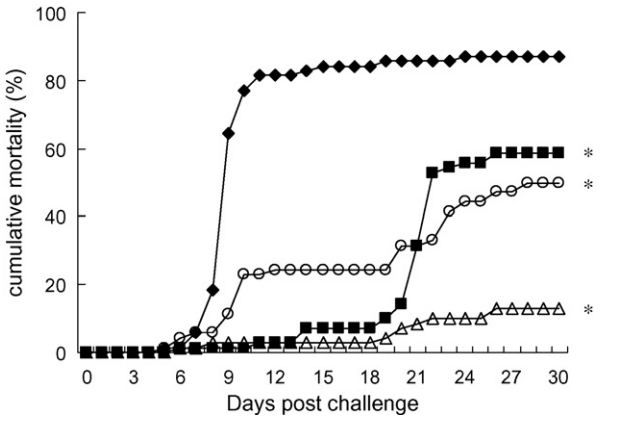

Figure 5 Cumulative mortality of grouper larvae after bath challenge with NNV. Grouper larvae were bath-immunized with 0.2% formalin-inactivated NNV vaccines with or without capsulation, and then bath challenged with NNV (1.6×10^6 $TCID_{50}$/ml) 30 days post immunization. (♦) PBS; (■) uncapsulated vaccine with viral concentration of 10^7 $TCID_{50}$/ml; (◯) uncapsulated vaccine with viral concentration of 10^6 $TCID_{50}$/ml; (△) encapsulated vaccine with viral concentration of 10^5 $TCID_{50}$/ml. *$p < 0.001$ (Chi-square).

Efficacy trial: nano-encapsulation of the inactivated vaccine

Part of the 0.2% formalin-inactivated NNV was further nano-encapsulated. The original concentration of NNV in the prepared nano-capsulated vaccine was 10^8 $TCID_{50}$/ml, and was then 1000-fold diluted into the final concentration of 10^5 $TCID_{50}$/ml during bath immunization. The cumulative mortality of negative control fish was 85%, and that of the fish immunized with uncapsulated vaccine with an immersion concentration of 10^6 and 10^7 $TCID_{50}$/ml were 58.6% and 50%, respectively (Fig. 5). However, the cumulative mortality of the fish immunized by nano-encapsulated vaccine

Table 2 Cumulative mortality of grouper larvae challenged with HGNNV (1.6×10^6 $TCID_{50}$/ml for bath challenge and 1.0×10^7 $TCID_{50}$/fish for IM challenge) 15, 30 and 90 days post immunization (dpi) by BEI-inactivated HGNNV vaccine

Challenge mode	Treatment	Cumulative mortality (RPS)		
		15 dpi	30 dpi	90 dpi
Bath	Vaccine	43% (30)	13% (87)	10% (82)
	PBS	61%	100%	55%
IM injection	Vaccine	ND	ND	60% (8)
	PBS	ND	ND	65%

with immersion concentration of 10^5 $TCID_{50}$/ml was as low as 12.9% (RPS = 83).

Discussion

Although the control of VNN could be based on selecting virus-free breeders and disinfection of fertilized eggs by ozone [16,33], the safe dosage of ozone for the eggs will be different from species to species, and the procedures are still difficult to apply in practice. Nevertheless, vaccination is widely accepted to be a major improvement to lead effective control for fish viral diseases.

To our knowledge, this is the first report demonstrating the use of inactivated vaccine applied on grouper larvae 40 dph (TBL = 2.4 cm, BW = 0.2 g) against NNV infection through bath immunization. Although grouper larvae at this stage are small, the immune organs (thymus, head kidney) are already developed [34]. In addition, the expression of recombination activating genes (rag-1 and rag-2) which are markers for the differentiation of lymphocytes [35,36], were detected in the grouper (*Epinephelus malabaricus*) since 21 days post hatchery (personal communication with Dr. Lin H.T.). Hence, it will not induce immune tolerance in grouper larvae 40-day old. However, the specific antibodies and neutralization antibodies were not monitored in the immunized larvae due to their small size. Besides, the relationship between antibody efficacy *in vitro* (ELISA and neutralization) and *in vivo* (reduction in mortality) is not always correlated [21]. Therefore, the efficacies of the inactivated HGNNV vaccines were directly evaluated by challenge test.

Most available fish vaccines against viral diseases are traditional inactivated vaccines [19]. Instead of the viruses purified by complex procedures including ultracentrifugation, the culture supernatants of NNV-infected GF-1 cells were directly used for vaccine preparation. The GF-1 cell line was derived from grouper fin, and is highly permissive to NNV isolates of RGNNV genotype [2,28]. The titer of NNV amplified in this cell line can reach as high as 10^{11} $TCID_{50}$/ml. Moreover, the doubling time of GF-1 cells is about 24 h cultured in L-15 medium (5% FBS) at 28 °C, and complete CPE appeared in the NNV-infected cells (MOI = 1) cultured in L-15 (1% FBS) within 4 days. The short doubling time, the low requirement of FBS and the high efficiency of NNV proliferation of GF-1 cells make the inactivated NNV vaccine less expensive.

Fish nodaviruses were reported to be less susceptible to low concentrations of formalin (0.16% and 0.025%) [15,37], but were inactivated by 0.5% or higher concentrations of formalin [22]. Here, we found that NNV could be completely inactivated by 0.1% or 0.2% formalin after 7-day incubation at 24 °C or by 0.2% formalin after 3-day incubation at 30 °C. The inactivation time at 30 °C was shorter than that at 24 °C since the chemical reaction is temperature dependent and sensitive. The efficacies of these two formalin-inactivated vaccines were similar, indicating that increasing the temperature was not harmful for NNV antigenicity and could save time during vaccine preparation.

Although most of the viral vaccines with inactivated antigen were prepared using formalin, in recent years an aziridine compound, binary ethylenimine was recommended as a superior inactivation agent to formalin [38]. Since BEI does not react with proteins, the vaccines of many RNA viruses and DNA viruses prepared with BEI were reported to be antigenically superior to the vaccines inactivated by formalin [39]. In this study, antigenicity superiority was also found in BEI-inactivated NNV vaccines (RPS = 79—95), whereas the RPS values of formalin-inactivated NNV vaccines were 39—50.

A new technique for preparing solid lipid nanoparticles (SLN) was applied for nano-encapsulation of NNV vaccine. The inactivated NNV vaccine was mixed with melted hydrogenated plant oil and other elements, cooled, and sprayed to form nano-encapsulated particles. Due to the stability and consistency of hydrogenated plant oil (solid oil) which characteristically excludes water and oxygen, it is possible to encapsulate and conserve a vaccine. During bath immunization, the encapsulated vaccine was able to enter the fish through its body surface or via the digestive tract. The encapsulated vaccine is more stable in the water, and also more resistant to stomach acid; therefore, it will not be wasted during bath immunization. The RPS value of nano-encapsulated formalin-inactivated vaccine with viral concentration of 10^5 $TCID_{50}$/ml in bath immunization was 87, while the RPS values of uncapsulated formalin-inactivated NNV vaccine with viral concentrations of 10^7 and 10^6 $TCID_{50}$/ml in bath immunization were 50 and 41.5, respectively. Compared with uncapsulated vaccine, nano-encapsulated vaccine significantly increased the protection of immunized fish and reduced nearly 100-fold concentration of viral antigens ($p < 0.001$).

In order to examine the influence of immersion time, grouper larvae were bath-immunized through 20, 60 and 120 min. It was found that the 20 min bath immunization of BEI-inactivated NNV vaccine was enough to elicit high protection of grouper larvae against NNV challenge (RPS = 75). There is no significant difference of efficacies among the three immersion times. In Taiwan, when the larvae in the hatchery farms grow to the body size of 2.0—2.4 cm, they are generally sold to other farms for juvenile-stage culture. Most grouper farms are located in southern Taiwan, and the transportation time varies from 20 to 120 min. The larvae are moved from the large tanks or pounds in the hatchery farms to the small tanks on the truck. Therefore, it is proper to bath-immunize the larvae during transportation due to the economical volume of vaccines being needed and the improved convenience to farmers.

In previous reported papers [17,20—22,24], betanodavirus vaccines were all tested at the juvenile or adult stages. However, the mortality induced by VNN disease is always higher in larvae than in juveniles or adult fish. Therefore, the bath immunization of NNV vaccine at the larval stage will greatly reduce the loss of fish at the late larval and early juvenile stages. This study demonstrated that the peak of protection induced by BEI-inactivated NNV vaccine was observed 1-month post vaccination (RPS = 87), and remained high even 3-month post vaccination (RPS = 82). Three months were long enough for grouper larvae growing into juveniles with TBL of 10—12 cm, and the fish are large enough for a boost of vaccine by IM or IP injection. In a few species of grouper, fish at the juvenile stage are more resistant to NNV infection, and the mortality induced by natural infection becomes negligible.

It is interesting to note that the fish on the 3rd month post bath immunization showed only 10% cumulative mortality by bath challenge, but 60% cumulative mortality by intramuscular challenge. The 50% difference of mortalities might be caused by the different viral dosage in each model (1.6×10^7 $TCID_{50}$/fish for IM challenge and 1.6×10^6 $TCID_{50}$/ml for bath challenge), but the mortality of the negative control in IM challenge was only 10% higher than that of bath challenge. Betanodaviruses basically affect fish at early developmental stage, and in naturally NNV-affected or bath-challenged young larvae, NNV has been detected in the epithelial cells of the skin concurrently with the nerve cells of central nerve system (CNS), indicating that epithelial cells of young larvae are also susceptible to NNV infection, and virus may gain access to CNS via sensory and/or motor nerves link to the epithelium of skin [39,40]. By IM challenge model, NNV may directly pass through peripheral nervous system in lateral muscular tissue and transport to CNS without getting through the skin, or may invade the CNS via blood circulation from the injection site [41]. Teleost fish have been shown to have an effective immune response in cutaneous mucus [42]. Moreover, bath immunization has been demonstrated to elicit significant antibody response in cutaneous mucus but low serum antibodies levels in some species of fish [42]. Therefore, the different mortalities between bath challenge and IM challenge is possible due to different immune responses in cutaneous mucus and in serum. It is thus suggested that cutaneous immune protection induced by bath immunization is important for larvae against betanodavirus infection. How bath immunization of inactivated NNV vaccine induces specific and/or non-specific immunity in the cutaneous mucus on the skin of grouper, and how the immune response protect the fish away from NNV natural infection will be clarified in the future.

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