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Immunization regimen in Asian sea bass (*Lates calcarifer*) broodfish: A practical strategy to control vertical transmission of nervous necrosis virus during seed production

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ABSTRACT

Outbreaks of viral nervous necrosis (VNN) in Asian sea bass (*Lates calcarifer*) at the larval stages via vertical transmission of nervous necrosis virus (NNV) from asymptomatic broodfish remain as a major deterrent during seed production. A five-year study was conducted to produce NNV-specific-free sea bass broodfish reared in land-based tanks through an annual immunization regimen with the formalin-inactivated NNV. We primarily immunized (intraperitoneal injection) sea bass juveniles (5 g) and monitored the neutralizing antibody (Nab) titers in the sera of these fish at scheduled intervals post-immunization. Nab titers in the sera of immunized fish peaked at Month 2 (titer: 1:4480 ± 1185) but thereafter gradually declined and significantly dropped (1:260 ± 83) at Month 12 post-primary immunization. Booster immunization of these fish at Month 12 post-immunization led to abrupt increases in Nab titers in booster immunized (B-Im) fish at Month 1 (1:12800 ± 6704) but thereafter declined and dropped at Month 12 (1:480 ± 165) post-booster immunization. The annual booster injections with the inactivated vaccine or L-15 (Unimmunized [U-Im]) were consecutively conducted for 4 years until the fish became sexually mature. Mature fish from both groups were successively induced to spawn twice (1-month interval) via intramuscular injection with luteinizing hormone-releasing hormone analogue (LHRH-a; 100 μg/kg BW). NNV was not detected by RT-PCR in oocytes and milts, and spawned eggs of B-Im fish. In contrast, oocytes and milts, and spawned eggs of U-Im fish were NNV positive. Spawned eggs of B-Im broodfish exhibited Nab titers ranging from 1:192 ± 34 to 1:240 while such was not detected (<1:40) in eggs of U-Im fish. Taken together, current data clearly demonstrate that annual immunization regimen with inactivated NNV vaccine is a pragmatic approach for sustaining immunocompetent sea bass broodfish reared in land-based tanks and circumvent the risk of vertical transmission of NNV from asymptomatic broodfish to their offspring under stress of repetitive spawning.

1. Introduction

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is a devastating disease that affects both farmed and wild fish, with more than 120 species belonging to 30 families from 11 different orders being susceptible to this disease [1]. Betanodaviruses (family *Nodaviridae*), the causative agents of VNN, are small, non-enveloped, spherical (25–30 nm) viruses with a genome consisting of two single-stranded RNA segments: RNA1 (3.1 kb) which encodes the viral replicase (110 kDa) and RNA 2 (1.4 kb) which encodes the coat protein (42 kDa) [2–4]. A third RNA segment, RNA3 (0.4 kb) is sub-genomically transcribed from RNA1 in the infected cells [5] and encodes a protein with potent RNA silencing-suppression activity [6]. Four genotypes, i.e. 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) have been designated based on the coat protein gene sequences [7]. Among these, RGNNV and genetically related viruses have by far been implicated in mass mortalities of hatchery-reared high-value marine fish species including sea bass (*Lates calcarifer*), groupers (*Epinephelus* spp.), and pompano (*Trachinotus blochii*) in the Philippines [8–10].

At the hatchery of the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD), the primary outbreak of VNN in 2001 resulted in mass mortalities of 14-day

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old sea bass larvae [8]. The vertical route of nervous necrosis virus (NNV) infection was speculated as the plausible cause since the sea bass broodfish used in the induced spawning at that time were sourced from the wild and maybe carriers of NNV because the virus is ubiquitous in the wild. This, coupled with stress from repetitive induced spawning could have facilitated the proliferation of NNV from these asymptomatic fish and transfer to the developing spermatozoa or oocytes [11]. Control strategies aimed at preventing the spread of NNV in hatcheries through elimination of NNV-carrying broodfish and chemical disinfection of eggs and rearing water by iodine or ozone were accordingly instituted over the past years [12,13]. However, despite tedious screening of sea bass broodstocks' intraovarian oocytes or milt by RT-PCR prior to induced spawning and chemical disinfection of spawned eggs by iodine, sporadic outbreaks of VNN in our hatchery still inadvertently occurred. The inefficiency of chemical disinfection of eggs could be explained by the recent report that NNV does not only attach to the surface of the eggs but rather penetrates the egg membrane and multiplies in the developing embryo [14]. It is also worth noting that the immune system of sea bass at the early larval stages is not mature enough to accept oral or bath immunization and therefore underscores the need to boost the immunity of sea bass broodfish against VNN so that vertical transmission of the virus could be intercepted by maternal antibodies.

We previously demonstrated the potentiality of the formalin-inactivated Philippine strain of NNV in aborting the replication of the homologous virus in the brains and kidneys of vaccinated sea bass juveniles (*L. calcarifer*) [15]. We also verified the efficacy of the same vaccine in grouper (*E. fuscoguttatus*) juveniles. Importantly we demonstrated that an effective anamnestic response would arise when immunized groupers that survived NNV challenge were re-exposed to the homologous NNV as indicated by remarkable increases in NNV-neutralizing antibody titers of up to six-folds or higher in NNV re-challenged fish [16]. These data served as springboard in conceptualizing our novel and pragmatic strategy of producing immunocompetent (NNV-specific-free) sea bass broodfish that could be sustainably maintained in land-based tanks through an annual immunization regimen with an inactivated Philippine strain of NNV as an attempt to feasibly control the vertical transmission of NNV during seed production. The presence of NNV-neutralizing antibodies in the sera and infectious NNV in the milts and oocytes prior to induced spawning, and as well as in spawned eggs from both booster-immunized and unimmunized sea bass broodfish, respectively, were monitored to evaluate the efficacy of the immunization regimen.

2. Materials and methods

2.1. Experimental fish

A total of 100 healthy sea bass juveniles with mean body weight (MBW) of 5 g were obtained from the hatchery of SEAFDEC/AQD. Fish were stocked in 1000-L tank supplied with sand-filtered and flow-through seawater at 28 °C and allowed to acclimate for 2 weeks prior to the start of the experiment. They were fed SEAFDEC/AQD formulated diet once a day throughout the experiment. Prior to the experiment, 10 fish were randomly collected from the holding tank and examined for NNV by reverse transcriptase polymerase chain reaction (RT-PCR) [17]. NNV was not detected in any of the brain sample examined.

2.2. Virus and vaccine preparation

The Philippine strain of NNV designated as OSGBF1E (RGNNV type) originally isolated from orange-spotted grouper (*E. coioides*) [15] was used in the vaccine preparation and NNV-neutralizing antibody assays. E-11 cells, originally cloned from SSN-1 cell line, were used for the propagation of the virus [18]. These cells were grown and maintained at 25 °C in Leibovitz L-15 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) in 25 cm^2 culture flasks (Falcon). Monolayer cultures of E-11 cells were washed twice with Hanks' balanced salt solution (HBSS) and then inoculated with OSGBF1E at a multiplicity of infection (MOI) of 0.01. The cells were eventually incubated at 25 °C in L-15 medium containing 2% FBS. When cytopathic effect (CPE) was extensive, usually after 3 days of incubation, the culture supernatant was centrifuged at 2000*g* for 10 min at 4 °C and stored at −80 °C until used. The infective titer ($TCID_{50}$ ml^{-1}) of the virus was determined by end point dilution [19] in 96-well plates seeded with E-11 cells.

The inactivated vaccine was prepared following our published protocol [10,15,16]. Briefly, formalin was added to the virus stock with a titer of $10^{10.5}$ $TCID_{50}$ ml^{-1} at a final concentration of 0.5%, followed by incubation at 4 °C for 10 days. Complete inactivation of the vaccine was ascertained, i.e. free of any residual infective virus, by cell culture assay using E-11 cells.

2.3. Primary and booster immunizations of fish

A total of 40 sea bass juveniles with MBW of 5 g were first anesthetized in a holding aquarium using 2-phenoxyethanol (SIGMA) before they were initially injected intraperitoneally (IP) with 100 μl of the inactivated NNV with a pre-inactivation titer of $10^{10.5}$ $TCID_{50}$ ml^{-1}. The same number of fish (n = 40) were likewise anesthetized and IP injected with L-15 medium to serve as control. Right after injection, the fish were immediately returned to the aquaria and allowed to recover. Because the MBW of immunized fish significantly increased to 0.53 kg after I year of rearing, the volume of the inactivated vaccine IP-injected to fish during the 1st booster-immunization was increased to 0.5 ml per fish. In addition, the volumes of the vaccine IP-injected to sea bass during the 2nd (MBW: 1.8 kg), 3rd (2.9 kg), and 4th (3.4 kg) annual booster immunizations, were also adjusted depending on the body weights of the fish, such that those with body weights ranging from 1 to 2 kg were IP-injected with 1 ml of the vaccine per fish while those with body weights of ≥3 kg received 1.5 ml of the inactivated vaccine per fish, respectively. Annual booster injections with L-15 (Control) were similarly conducted on sea bass with MBWs of 0.4, 1.5, 2.5, and 3.5 kg, respectively.

2.4. Assay of NNV-neutralizing antibodies in primary and booster immunized fish

Before the primary immunization or L-15 injection of fish, blood samples were taken from the caudal vein of 10 fish and the serum samples were individually subjected to seroneutralization assay [10,15,16]. All serum samples examined had no detectable NNV-neutralizing antibodies (<1:40). The collection of blood samples (n = 5–8) in both immunized and L-15 injected fish (control) for seroneutralization assay were subsequently conducted at various time points post-primary/ booster immunizations or L-15 injections (Fig. 1). At each sampling time, fish randomly collected from each of the immunized and unimmunized group were anesthetized with 2-phenoxyethanol and blood was collected from the caudal vein of the fish. Right after the blood extraction, the fish were immediately returned to the aquaria and allowed to recover. After allowing the blood to clot at 4 °C overnight, the serum was collected by centrifugation at 1500*g* for 15 min, divided into several aliquots, and stored at −80 °C until used [16]. Briefly, the fish sera were diluted with 39 volumes of HBSS, then diluted twofold with HBSS and eventually mixed with an equal volume (50 μl) of the viral suspension (50 μl, 10^2 $TCID_{50}$). After incubating the mixture

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