Short communication

An Oral Vaccination Method with the Aid of Capsaicin against Viral Nervous Necrosis (VNN)

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ABSTRACT—To develop an oral vaccination method for viral nervous necrosis (VNN) caused by piscine nodavirus (betanodavirus), sevenband grouper *Epinephelus septemfasciatus* was immunized with inactivated redspotted grouper nervous necrosis virus (RGNNV) vaccine. Seven-day consecutive oral administration of the RGNNV vaccine via feed failed to induce protection in fish against the virus challenge at 21 days post-immunization. However, anal intubation of the vaccine conferred protective immunity on fish, although virus neutralizing antibodies could not be detected. Similar protective immunity was also confirmed by oral administration of vaccine via feed supplemented with capsaicin, suggesting that capsaicin modulated the intestinal mucosal barrier to induce immune response.

Key words: viral nervous necrosis, betanodavirus, oral vaccination, capsaicin, *Epinephelus septemfasciatus*

Fish farming has always involved a risk of microbial infections, especially those caused by pathogenic viruses, for which chemotherapeutics are generally ineffective. Viral nervous necrosis (VNN), otherwise known as viral encephalopathy and retinopathy (VER), caused

by piscine nodavirus (the Betanodavirus, Nodaviridae), has caused major constraints on aquaculture of fish all over the world since the first description of the disease in 1990 (Yoshikoshi and Inoue, 1990; Glazebrook *et al.*, 1990), as reviewed in a number of papers (e.g., Munday *et al.*, 2002; Costa and Thompson, 2016). Currently, the Betanodavirus consists of four genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), and redspotted grouper nervous necrosis virus (RGNNV) (Thiéry *et al.*, 2012). Of these genotypic variants, the RGNNV is the most prevalent among a variety of farmed warm-water fish species in Asian, Oceanian, and the Mediterranean countries (OIE, 2016).

Prophylactic methods applicable to reduce losses due to the disease are urgently needed, and an inactivated RGNNV vaccine by injection recently became commercially available for sevenband grouper *Epinephelus septemfasciatus* in Japan (Brudeseth *et al.*, 2013; OIE, 2016). However, this injective vaccine cannot be applied to younger fish at the early juvenile stage, which are more susceptible to betanodaviruses than older fish. Although oral administration of vaccine via feed is the most ideal delivery method for immunization of fish in aquaculture practice, it is in general low or inconsistent in inducing protective immunity against microbial infections, compared with parenteral methods (Ellis, 1988).

For the establishment of the oral immunization method using particulate vaccine against systemic infection, a large quantity of innate particles (antigens) must be absorbed from intestine for induction of systemic immunity (Neutra and Kozlowski, 2006). A problem is that such absorption of antigens is difficult under the presence of feed which disturbs direct contact with epithelial cells. For induction of systemic immunity, therefore, it is essential to enhance antigen uptake through the intestinal mucosal barrier. In this regard, substances such as toxins, enzymes, or chemicals that modulate passage of vaccine particles through the intestinal epithelial layer have been used in terrestrial animals (Vajdy *et al.*, 2004), and it is also known that capsaicin (a natural substance exerting intestinal inflammatory reaction) causes the reversible opening of cell tight junctions (Shiobara *et al.*, 2013; Kaiser *et al.*, 2015).

Our previous study, in which goldfish *Carassius auratus* as a model fish was anally intubated with the inactivated RGNNV vaccine, proved that capsaicin was effective to aid the transepithelial passage of the virus antigens (Gaafar *et al.*, 2015). In the present study, we examined the effect of capsaicin on protective immunity of sevenband grouper following oral immunization with the inactivated RGNNV vaccine via feed.

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Materials and Methods

Fish

Sevenband grouper, which were produced and reared at facility of the Fishery Research Center of the Ehime Research Institute of Agriculture of Japan were used in this study. Prior to the experiments, fish (brain, $n = 20$) randomly sampled from stocks were examined for betanodavirus detection by reverse transcription polymerase chain reaction (RT-PCR) according to a previously described method (Nishioka *et al.*, 2016). No specific PCR products were detected in any samples examined.

Virus and vaccine preparation

A betanodavirus SGEhi00 strain (RGNNV genotype) was propagated in E-11 cells cultured at 25°C in Leibovitz's L-15 medium supplemented with 5% fetal bovine serum, titrated, and stored at -80°C until use, according to the method of Iwamoto *et al.* (2000). For vaccine preparation, the virus (10^{10} TCID₅₀/mL) was inactivated by 0.5% formalin-treatment at 4°C for 10 days, as described previously (Yamashita *et al.*, 2005).

Immunization and virus challenge

Two trials were separately performed using fish with mean body weight of 10 g (Exp. 1) and 45 g (Exp. 2). In Exp. 1, an experimental group of fish was fed with commercial feed (pellets) soaked in vaccine solution (10^{10} TCID₅₀/mL) once a day for seven consecutive days by a natural feeding manner. The vaccine dose fed to fish was approximately 10^8 TCID₅₀/fish/day. Another experimental group of fish was anesthetized with Eugenol (FA-100, Tanabe seiyaku) and given 0.1 mL of the vaccine solution by anal intubation twice at a one-week interval. In Exp. 2, commercial feed or vaccine-incorporated feed was soaked in capsaicin (Wako chemicals) previously dissolved in a small amount of ethanol and fed to fish at a dose of approximately 0.5 mg of capsaicin/fish/day for seven consecutive days. The dose of capsaicin was determined based on the result in the previous study (Gaafar *et al.*, 2015). Thereafter, commercial feed was administered to fish once a day until and during virus challenge. No apparent difference was observed in feeding behavior of fish between vaccine or capsaicin group and control group.

Twenty-one days after the first immunization, fish were challenged with SGEhi00 strain by intramuscular (IM) injection at doses of $10^{4.0}$ and $10^{3.0}$ TCID₅₀/fish (Exp. 1) or drainage method (Exp. 2). In the drainage method (Nishi *et al.*, 2016), fish (recipient) were exposed to drainage from an aquarium of fish (donor, $n = 20$) infected with the virus by IM injection ($10^{5.5}$ TCID₅₀/fish), and dead fish were removed from the tank on the day. During immunization and challenge periods, fish were kept in 45-L plastic tanks with a flow-through system at a

density of 20–25 individuals/tank. The water temperature of tanks was adjusted at 25°C ± 1°C. Dead fish were subjected to the RT-PCR test for RGNNV.

Neutralizing antibody assay

Blood was collected from the caudal veins of fish at 21 days post-immunization (dpi), and the serum samples were stored at -20°C until use. Virus-neutralizing antibody titers were determined using E-11 cells and SGEhi00 strain, as described previously (Yamashita *et al.*, 2005). The detection limit of the antibody titer was 1:80. This assay was done only for fish in Exp. 1.

Histopathological and immunohistological examinations

Sevenband grouper (each $n = 5$) with an average body weight of 45 g were anesthetized with Eugenol, then anally intubated with 0.1 mL of the vaccine solution (10^{10} TCID₅₀/mL), capsaicin solution (5 mg/mL), vaccine-capsaicin mixture, or phosphate-buffered saline (PBS). Fish were kept in 45-L plastic tanks with aeration at 25°C ± 1°C for 3 h, and the removed intestine samples were fixed with Davidson's fixative for 24 h and then transferred to 70% ethanol until use. The 4-μm-thick tissue sections were subjected to microscopic observation after staining with haematoxylin and eosin (H&E) or immunohistochemistry (IHC) and indirect immunofluorescent antibody technique (IFAT) using a rabbit anti-RGNNV serum according to the method previously described (Gaafar *et al.*, 2015).

Statistical analysis

Statistical analysis was carried out using Fisher's exact probability test for fish mortality in the challenge experiments.

Results and Discussion

All brain samples of fish died after virus challenge were positive for RGNNV in RT-PCR tests. In Exp. 1, the fish group administered the vaccine-incorporated feed did not show protection against any IM injection challenges, while the fish group that received anal intubation of the vaccine showed a significantly low mortality ($p < 0.01$) against the IM injection challenge at low dose (Table 1), but neutralizing antibodies were not detected in both immunized and control groups (titer < 1:80). In Exp. 2, all fish of the donor group previously injected with virus died between 4 and 6 dpi (Table 2). When fish were challenged by the drainage method, mortality in the recipient groups was observed between 11 and 19 dpi, except for the fish group administered vaccine-capsaicin-incorporated feed, which showed no mortality ($p < 0.001$) (Table 2), suggesting that oral administration of the inactivated RGNNV vaccine via feed induced protective immunity in fish, with the aid of capsaicin.

The drainage challenge method used in Exp. 2 is

Table 1. Betanodavirus challenge of sevenband grouper vaccinated with inactivated RGNNV (Exp. 1)

Fish group	IM-injection dose (TCID ₅₀ /fish)	Mortality (no. of dead fish/fish examined)	Mean (range) lethal time of fish after virus challenge
Vaccinated by oral route	10 ^{4.0}	87% (20/23)	9.2 (4–15) days
	10 ^{3.0}	60% (15/25)	9.3 (6–16)
Vaccinated by anal route	10 ^{4.0}	79% (19/24)	10.9 (5–18)
	10 ^{3.0}	20% (5/25)*	11.8 (8–16)
Control	10 ^{4.0}	88% (22/25)	8.3 (4–17)
	10 ^{3.0}	56% (14/25)	11.3 (5–19)

Fish (10 g in mean body weight) fed inactivated RGNNV vaccine with feed for consecutive seven days or received twice the vaccine by anal intubation. At 21 day post-immunization, fish were challenged with RGNNV by intramuscular (IM) injection and observed for 20 days. * $p < 0.01$

Table 2. Betanodavirus challenge of sevenband grouper vaccinated with inactivated RGNNV (Exp. 2)

Fish group	Mortality (no. of dead fish/fish examined)	Mean (range) lethal time of fish after virus challenge
Donor (injection of virus)	100% (20/20)	5.0 (4–6) days
Recipient		
Vaccine-feed	17.5% (7/40)	12.9 (11–18)
Capsaicin-feed	17.5% (7/40)	13.1 (12–19)
Vaccine-capsaicin-feed	0% (0/40)*	–
Control (feed)	25.0% (10/40)	13.2 (11–18)

Fish (45 g in mean body weight) fed inactivated RGNNV vaccine-feed with/without capsaicin for consecutive seven days. At 21 day post-immunization, fish (recipient) were exposed to drainage from an tank of fish (donor) previously infected with RGNNV and observed for 25 days. * $p < 0.001$

less stressful to fish and mimics natural infection, compared with the injection, bath, or oral gavage challenge methods, although it inevitably resulted in relatively lower mortality as in the previous study (Nishi *et al.*, 2016). However, the use of one drainage as an infection source is superior in ensuring the uniformity of infection pressure among tanks as compared to a similar challenge method by cohabitation with diseased fish in the same tank. Therefore, this drainage method is preferable for the evaluation of the protective effect of oral vaccine that is difficult to be verified under more stressful challenge conditions.

Histopathological examination revealed moderate congestion, increased mucous cell secretions and frequent villi destruction in the intestinal epithelial layers of fish that received anal intubation of capsaicin or vaccine-capsaicin mixture, but not in fish that received vaccine or PBS (Fig. 1). The pathological changes observed in sevenband grouper are consistent with those of goldfish in the previous study (Gaafar *et al.*, 2015). This suggests that capsaicin gave a modulating effect on the normal intestinal mucosal barrier of fish that, in turn, enhances uptake of vaccine particles (RGNNV antigens) by intestinal mucosal phagocytes, as revealed by immunohistological methods in the previous goldfish study (Gaafar *et al.*, 2015). However, we could not clearly

Fig. 1. Histopathological changes in the intestine of sevenband grouper received anal intubation of (A) PBS (control), (B) RGNNV vaccine, (C) capsaicin, and (D) RGNNV vaccine + capsaicin. Letters: (c) congestion, (m) mucous secretion, (d) villi destruction, (g) goblet cells. bar = 50 μ m, H&E stain.

detect RGNNV antigen signals in the present grouper intestine by IHC or IFAT (data not shown), and thus it remains unclear whether or not the capsaicin effect can enhance antigen uptake in grouper. More detailed examinations at a cellular or molecular level might be necessary to solve it.

There have been many studies on vaccine candidates for VNN [inactivated, recombinant protein, virus-like particle (VLP), synthetic peptide or DNA vaccine], and vaccination methods (parenteral injection, bath or oral), as reviewed in Costa and Thompson (2016). In general, parenteral injections of vaccine resulted in high protection of fish by provoking production of anti-betanodavirus neutralizing antibodies. Our previous study (Yamashita *et al.*, 2009) revealed a correlation between neutralizing antibody titer and protective immunity (i.e., fish carrying antibody at 1:200 or higher titers have enough protection against injection challenge of RGNNV). On the other hand, published data on oral immunization of VNN and neutralizing antibody production are very limited. Wi *et al.* (2015) reported that a

single oral gavage of RGNNV VLPs (50 µg/fish) without feed to sevenband grouper resulted in a slight protective effect against challenge by intraperitoneal injection of RGNNV and production of neutralizing antibodies at low titer levels (mean titer 1:75). However, we failed in detecting neutralizing antibody in the present anal intubation of inactivated RGNNV, in which a high level of protection was observed in the immunized fish (Table 1). This discrepancy may be partly due to a technical issue that the detection limit of titer in our study was 1:80. As we did not perform neutralizing antibody test in fish orally immunized with capsaicin-vaccine via feed in the present study, it remains unknown whether neutralizing antibody-mediated immunity plays a pivotal role in oral vaccination against VNN.

Our oral vaccine strategy against VNN is to use it for non-injectable juvenile fish sizes before use of injective vaccine. For application of the present oral vaccination method with capsaicin to aquaculture, pepper may be used as a capsaicin source, but the inactivated virus vaccine has a problem in its commercial mass production. Further studies using more easily mass producible vaccines such as recombinant proteins or VLPs are required for development of practical oral vaccine against VNN.

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