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Protection of Japanese flounder *Paralichthys olivaceus* from viral hemorrhagic septicemia (VHS) by Poly(I:C) immunization

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ABSTRACT: In immunization of fish with polyinosinic-polycytidylic acid (poly[I:C], a synthetic double-stranded RNA), injection of Poly(I:C) followed by challenge with a live virus induces a transient, non-specific antiviral state by interferon activity. When exposed to a virus while in this antiviral state, the fish acquire a specific and protective immunity against the corresponding viral disease and survive. In the present study, the effiacy of Poly(I:C) immunization was investigated in japanese flounder *Paralichthys olivaceus* using viral hemorrhagic septicemia virus (VHSV) as a model; the minimum dose of Poly(I:C) required for inducing protection and the duration of the antiviral state were determined, and a potentially curative effect of Poly(I:C) administration was assessed. The antiviral state was induced by administration of Poly(I:C) doses ranging from 12.5 to 200 µg fish^{-1}. Minimum dose to induce the antiviral state (relative percentage survival, RPS: 90%) was 12.5 µg fish^{-1}. No curative effect of Poly(I:C) was observed in fish pre-infected with VHSV. Fish injected with 200 µg Poly(I:C) fish^{-1} were highly protected (RPS: 100%) from an artificial challenge with VHSV, and specific antibodies against VHSV were detected. The corresponding high level of antiviral state against VHSV was attained 1 d post Poly(I:C) injection, lasted for 6 d and susequently decreased. Moreover, the surviving fish were highly protected from re-challenge with VHSV (RPS: 100%). Thus, it was considered that an immunity against viral hemorrhagic septicemia was induced in the Japanese flounder by injecting live VHSV following Poly(I:C) administration.

KEY WORDS: Poly(I:C) · Immunization · Viral hemorrhagic septicemia virus · VHSV · Japanese flounder · Vaccine · Antiviral state

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV), a member of the genus *Novirhabdovirus* (Trdo et al. 2005), is the etiological agent of viral hemorrhagic septicemia (VHS), which is among the most serious viral diseases affecting farmed rainbow trout *Oncorhynchus mykiss* in continental Europe (Wolf 1988, Smail 1999). VHSV has been isolated from anadromous salmon in North America (Winton et al. 1989) and also from various marine fish species worldwide (Jensen et al. 1979, Vestergård-Jørgensen & Olesen 1987, Schlotfeldt et al. 1991, Meyers et al. 1992, 1994, 1999, Ross et al. 1994, Meyers & Winton 1995, Mortensen et al. 1999, Takano et al. 2000). VHSV has been isolated from a broad range of free-living marine fish species around the world (Dixon et al. 1997, Smail 1999, Winton & Einer-Jensen 2002).

In Japan, VHSV was first isolated from the free-living Japanese flounder *Paralichthys olivaceus* in 1999 (Takano et al. 2000, 2001). VHS occurred in cultured Japanese flounder in the Seto Inland Sea of Japan (Isshiki et al. 2001). Since then, VHS has been among the most serious diseases affecting farmed Japanese flounder in Far East Asia. In seed production facilities for Japanese flounder, stable production of VHSV-free fish has been accomplished by preventing VHSV transmission (Yoshimizu 2009). However, VHS

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still occurs at grow-out and adult stages in sea cages of open aquaculture systems, a possible source of infection for wild fish populations. For example, VHSV has been detected in apparently healthy wild Japanese flounder around the sea cages (Watanabe et al. 2002). Therefore, development of a vaccine against the disease is essential for pen-culture of Japanese flounder, and different kinds of experimental VHS vaccines have been developed, such as killed or attenuated vaccines, recombinant vaccines with VHSV glycoprotein (Lorenzen & Olesen 1997), and DNA vaccines encoding the surface glycoprotein (Heppell et al. 1998, Lorenzen et al. 1998, 2002). However, none of these vaccines is currently commercially available.

A novel immunization method using polyinosinic-polycytidylic acid (Poly[I:C]), a synthetic double-stranded RNA, was devised recently and its efficacy was experimentally confirmed for infectious hematopoietic necrosis in rainbow trout and viral nervous necrosis (VNN) in sevenband grouper (Kim et al. 2009, Nishizawa et al. 2009). In Poly(I:C) immunization, fish are immunized with live pathogenic virus followed by Poly(I:C) administration, wherein Poly(I:C) induces a transient, non-specific antiviral state by interferon activity that enables the fish to survive the initial immunization with live virus that would otherwise be lethal. Moreover, in the antiviral state, the fish infected with the live virus are able to mount a specific protective immune response against the injected pathogenic virus. This immunization scheme has several advantages: (1) the injected Poly(I:C) does not remain in fish tissues because Poly(I:C) is unstable like other RNAs, indicating that there may be no problem using the immunized fish as food source and also that it is harmless to the fish-rearing environment; (2) neither attenuation nor inactivation of the virus is required, and high levels of immunity can be expected; (3) recombinant DNA technology is not necessary, suggesting relatively low costs for vaccine development, and it would be more acceptable to consumers who are skeptical of food products produced with recombinant DNA technology (Nishizawa et al. 2009). Therefore, it is conceivable that Poly(I:C) immunization will be applicable to a wide range of fish species as well as other viruses.

In the present study, the efficacy of Poly(I:C) immunization was investigated for VHSV infection in the Japanese flounder *Paralichthys olivaceus*. The minimum dose of Poly(I:C) necessary for inducing an antiviral state as well as the duration and any curative effect of the antiviral state were assessed.

MATERIALS AND METHODS

Fish and virus. Japanese flounder weighing 31.7 ± 2.9 g reared at the Nagasaki Prefectural Institute of Fisheries (NaPIF) were used in this study. The fish were maintained using UV-sterilized seawater at 16 ± 1°C in a separate facility at NaPIF. Some of these fish were sampled for virus isolation using fathead minnow (FHM) cells to confirm a VHSV-free status prior to the experiments.

VHSV (Obama25) isolated from free-living Japanese flounder in 1999 (Takano et al. 2000, 2001, Nishizawa et al. 2002) was cultured in FHM cells maintained at 20°C in minimum essential medium (MEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 150 IU ml^{-1} penicillin G, and 100 µg ml^{-1} streptomycin. Following the development of complete cytopathic effect (CPE), the cell culture supernatant was centrifuged (12 000 × *g*, 10 min, 4°C), subdivided into small aliquots, and stored at –80°C until use. Titration of viral infectivity was performed using 96-well microplates seeded with FHM cells. After 14 d of culturing, the appearance of CPE was evaluated to determine the 50% tissue culture infectious dose ($TCID_{50}$).

Poly(I:C) administration and primary challenge with VHSV. Poly(I:C) was dissolved in diethylpyrocarbonate (DEPC)-treated water (Sigma) at predetermined concentrations before use in each experiment. In total, 126 Japanese flounders were reared in 3 aquaria (n = 42 each) with 200 l of flowing UV-sterilized seawater at 16 ± 1°C (14 cycles d^{-1}). Poly(I:C) was administered intramuscularly into the fish in 2 aquaria at a dose of 200 µg 100 $µl^{-1}$ $fish^{-1}$, while 100 µl of DEPC-treated water (control) $fish^{-1}$ was injected into fish in the third aquarium. To prevent the leakage of inoculum from the injection site, a thin needle was inserted slightly into the fish muscle at an angle, and then the direction of insertion was changed, decreasing the angle formed between the needle and fish body surface to insert the needle more deeply. After 2 d, the fish in the first 2 aquaria were challenged intramuscularly with VHSV at a dose of $10^{4.3}$ $TCID_{50}$ 100 $µl^{-1}$ $fish^{-1}$ (Poly[I:C]-VHSV and control-VHSV groups), while fish administered Poly(I:C) in the remaining aquarium were mock-challenged with 100 µl MEM $fish^{-1}$ (Poly[I:C]-mock groups). The challenged fish were reared for an additional 28 d with sufficient feeding once daily, and mortality was monitored daily. Relative percent survival (RPS) values were calculated according to the method of Amend (1981). Twenty-five days following the VHSV challenge, sera were collected from the survivors in each group and analyzed for detection of antibodies against VHSV using an ELISA system as described below.

Secondary challenge of fish surviving the primary challenge with VHSV. Four weeks after the primary challenge with VHSV, the surviving fish in the Poly(I:C)-VHSV group were divided into 2 aquaria with 21 fish in each, and challenged intramuscularly

with VHSV at a dose of $10^{4.3}$ $TCID_{50}$ 100 μl^{-1} $fish^{-1}$ or MEM (Poly[I:C]-VHSV-VHSV and Poly[I:C]-VHSV-mock groups). As a positive control, 21 naïve fish were reared in another aquarium and challenged with the same dose of VHSV (naive-VHSV group). Fish in each group were reared for an additional 20 d, and mortality was monitored. Twenty days following the secondary challenge, sera were collected from the survivors and analyzed for detection of antibodies against VHSV using ELISA.

Minimum dose of Poly(I:C) for inducing the antiviral state. Sixty Japanese flounders were maintained in UV-sterilized seawater at 16 ± 1°C (360 exchanges d^{-1}) in an individual rearing system with 1 l cubic tanks, which was a modification of the system by Kokawa et al. (2008). Groups of 10 fish were administered 2-fold diluted Poly(I:C) in 5 doses ranging from 200 to 12.5 µg 100 μl^{-1} $fish^{-1}$ or 100 µl $fish^{-1}$ of DEPC-treated water. The fish were challenged intramuscularly with VHSV at a dose of $10^{4.3}$ $TCID_{50}$ 100 μl^{-1} $fish^{-1}$ 2 d after Poly(I:C) administration, and mortality was monitored for 28 d.

Curative effect and duration of antiviral state by Poly(I:C) injection. Seventy Japanese flounders were allocated to 7 aquaria (40 l $aquarium^{-1}$), each containing 10 fish. All fish were administered Poly(I:C) at a dose of 200 µg 100 μl^{-1} $fish^{-1}$ and challenged intramuscularly with VHSV at a dose of $10^{4.3}$ $TCID_{50}$ 100 μl^{-1} $fish^{-1}$. To evaluate any curative effect of Poly(I:C), fish in 1 of the 7 aquaria were challenged with VHSV 2 d before Poly(I:C) administration. In the other aquaria, fish were challenged with VHSV 0, 1, 2, 4, 7, and 14 d after Poly(I:C) administration to evaluate the duration of the antiviral state in fish induced by Poly(I:C) administration. The fish were reared for additional 21 d, and mortality was monitored.

Antibody detection using ELISA. Detection of antibodies against VHSV was performed using an ELISA system according to a modification of the methods of Kim et al. (2007) and Nishizawa et al. (2009). Briefly, culture fluids of VHSV with $10^{8.3}$ $TCID_{50}$ ml^{-1} were diluted 10-fold with distilled water, 50 µl of the fluid were loaded into ELISA plate wells, and the viral antigen was fixed by drying overnight at 37°C. Sera were diluted 1:40 with phosphate-buffered saline (PBS) containing 5% skim milk and incubated at 25°C for 1 h, then loaded in duplicate ELISA plate wells and incubated at 25°C for 1 h, followed by 3 rinses with PBS containing 0.05% Tween 20 (T-PBS). Fish immunoglobuline M (IgM) captured by the VHSV antigen was detected using rabbit (secondary) antiserum against Japanese flounder IgM, horseradish peroxidase-conjugated swine serum against rabbit IgG (Dako), and substrate (100 mM Na_2HPO_4, 50 mM citric acid, 1.0 mg ml^{-1} *o*-phenylenediamine, 0.03% H_2O_2). After color development, the reaction was stopped with 2 N H_2SO_4 and absorbance at an optical density of 492 nm (OD_{492}) was read using a microplate reader (MTP-300, Corona). The ELISA values among challenged groups were statistically analyzed using a Tukey-Kramer multiple comparisons test at a significance level of 1%.

RESULTS AND DISCUSSION

The survival rate in Japanese flounder after VHSV challenge following Poly(I:C) administration is shown in Fig. 1A. No mortality was observed in the fish challenged with VHSV (Poly[I:C]-VHSV group) or in the mock-challenged fish (Poly[I:C]-mock group) following Poly(I:C) administration. In fish without Poly(I:C) administration typical signs of VHS and mortality were observed starting on Day 4 after the challenge, and all fish died within 9 d (control-VHSV group). These results demonstrate that fish administered Poly(I:C) in advance were highly protected from VHS. Survival rates of the fish given a secondary challenge with

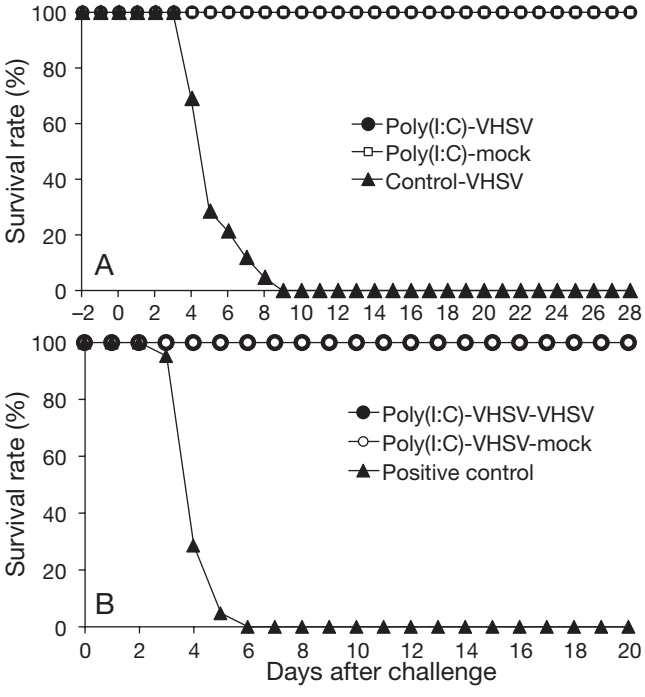

Fig. 1. *Paralichthys olivaceus.* Survival rate of fish challenged with viral hemorrhagic septicemia virus (VHSV) following Poly(I:C) administration. (A) Fish were intramuscularly challenged with VHSV ($10^{4.3}$ 50% tissue culture infectious dose [$TCID_{50}$] $fish^{-1}$) or minimum essential medium (MEM; mock challenge) 2 d after intramuscular administration of 200 µg Poly(I:C) $fish^{-1}$ or diethylpyrocarbonate (DEPC) water (Control). (B) Survivors from the primary VHSV challenge following Poly(I:C) administration were re-challenged with VHSV ($10^{4.3}$ $TCID_{50}$ $fish^{-1}$) or MEM (mock challenge); naïve fish challenged equally with VHSV served as positive control

VHSV as well as that of the positive control group (naïve-VHSV group) are shown in Fig. 1B. All fish of the Poly(I:C)-VHSV group survived the secondary challenge with VHSV (Poly[I:C]-VHSV-VHSV group), while fish in the positive control group (naïve-VHSV group) showed 0% survival within 6 d after VHSV challenge. No mortality was seen in the fish with secondary mock challenge (Poly[I:C]-VHSV-mock group). It is considered that the protection of fish against VHS in the Poly(I:C)-VHSV group could be due to non-specific immunity because the antiviral state induced by Poly(I:C) administration confers a non-specific immunity against homologous and heterologous viral infection (Eaton 1990).

Fish sera collected from the survivors in the Poly(I:C)-VHSV and Poly(I:C)-mock groups were assayed for antibody detection ELISA (Fig. 2A). ELISA absorbance values (OD_{492}) of sera from the survivors of the Poly(I:C)-VHSV group ranged from 0.02 to 1.15 (average: 0.13), but those from mock-challenged survivors of the Poly(I:C)-mock group were less than 0.09 (average: 0.03). According to the criterion for antibody detection ELISA by Kim et al. (2008, 2009), fish sera with ELISA values less than 0.1 are considered negative for specific antibodies. Based on this criterion, all sera from the survivors in the Poly(I:C)-mock group were negative, while those from 14 out of 42 survivors in the Poly(I:C)-VHSV group were positive for antibodies against VHSV (0% and 33%, respectively). The results for the Poly(I:C)-mock group were as expected, but the low incidence of positives in the Poly(I:C)-VHSV group was entirely unexpected.

The ELISA values of fish sera obtained 20 d after the secondary challenge with VHSV (corresponding to 48 d after the primary challenge) are shown in Fig. 2B. ELISA values of sera from the Poly(I:C)-VHSV-VHSV group ranged from 0.11 to 1.29 (average: 0.36), demonstrating that all sera were positive for antibodies against VHSV (ELISA value: ≥0.1). Interestingly, the ELISA values of sera from the Poly(I:C)-VHSV-mock group ranged from 0.10 to 1.12 (average: 0.33), indicating that they were positive for VHSV-specific antibodies. This indicated that all fish from the Poly(I:C)-VHSV group with negative sera after primary challenge eventually increased specific antibody titers so that their sera became positive between Days 28 and 48 without secondary challenge. The same tendency was observed in our previous experiments on Poly(I:C) immunization with rainbow trout and sevenband grouper, i.e. ELISA values representing specific antibody titers were clearly lower 21 d after immunization than 35 to 49 d after immunization (Kim et al. 2009, Nishizawa et al. 2009). The present results demonstrated that immunity against VHS was established in the fish injected with VHSV followed by Poly(I:C)

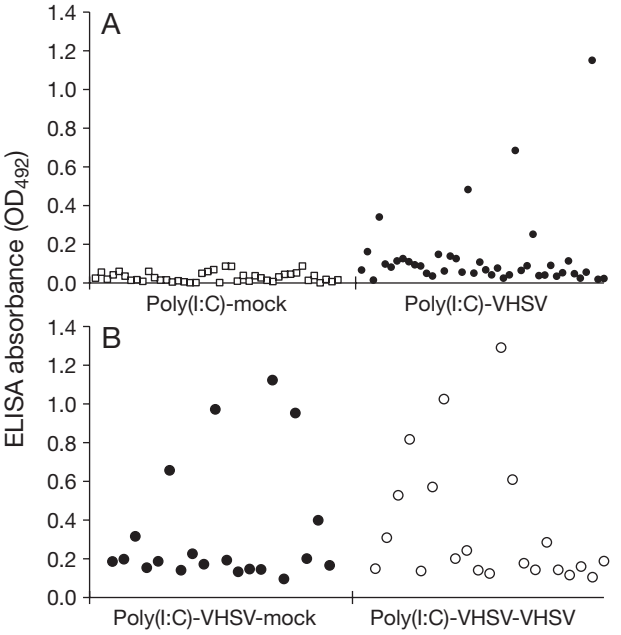

Fig. 2. *Paralichthys olivaceus.* Detection of specific antibodies against VHSV from sera of the survivors in the experiment shown in Fig. 1. (A) Fish sera obtained from survivors (n = 42) 25 d after the primary VHSV challenge. (B) Sera obtained from survivors (n = 21) 48 d after the primary VHSV challenge (corresponding to 20 d after the secondary VHSV challenge). OD_{492}: optical density at 492 nm

administration, suggesting that Poly(I:C) immunization with live VHSV is protective against VHS in Japanese flounder similar to previous results with sevenband grouper and rainbow trout Poly(I:C)-immunized with live fish nodavirus and infectious hematopoietic necrosis virus (IHNV), respectively (Nishizawa et al. 2009, Kim et al. 2009). Establishment of immunity determined by antibody detection ELISA was confirmed more than 6 to 7 wk after virus injection; however, immunity seems to be guaranteed even if specific antibodies in immunized fish sera are below the detectable level. This delay could be due to a definite period required for specific antibodies to reach a detectable level for ELISA after immunization. In addition, cell-mediated specific immunity could be established at sufficient levels in these fish by immunization with Poly(I:C) and live virus.

After Poly(I:C) administration at different doses, Japanese flounders were challenged with VHSV to determine the minimum dose of Poly(I:C) required for inducing an antiviral state (Fig. 3A). The fish immunized with Poly(I:C) at doses of 25 µg $fish^{-1}$ or more showed a 100% survival rate following VHSV challenge, while those immunized with Poly(I:C) at a dose of 12.5 µg $fish^{-1}$ showed a 90% survival rate. All fish without Poly(I:C) administration died within 9 d of VHSV challenge (data not shown). It was thus consid-

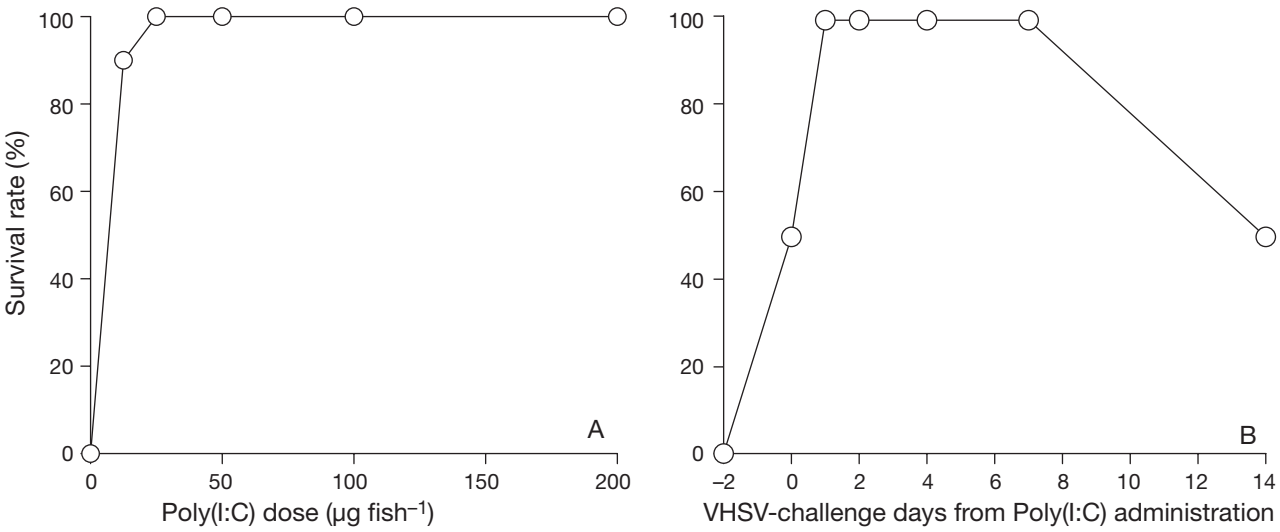

Fig. 3. *Paralichthys olivaceus.* Minimum Poly(I:C) dose for the induction of an antiviral state and the duration of the induced antiviral state. (A) Final survival rate (28 d post challenge) of fish challenged intramuscularly with VHSV ($10^{4.3}$ 50% tissue culture infectious dose [$TCID_{50}$] $fish^{-1}$) 2 d after administration of different doses of Poly(I:C). (B) Final survival rate (21 d post treatment) of fish challenged intramuscularly with VHSV at $10^{4.3}$ $TCID_{50}$ $fish^{-1}$ either 2 d before administration of 200 µg Poly(I:C) $fish^{-1}$ to evaluate the curative effect of Poly(I:C), or 0, 1, 2, 4, 7, or 14 d after Poly(I:C) administration to evaluate the duration of the antiviral state

ered that the required dose of Poly(I:C) for inducing the antiviral state in more than 90% of Japanese flounder was at least 12.5 µg $fish^{-1}$. This dose in Japanese flounder was less than one quarter of that in sevenband grouper (Nishizawa et al. 2009), suggesting that the required dose of Poly(I:C) could be different depending on the fish species.

To investigate the curative effect and duration of the antiviral state, Japanese flounders were administered Poly(I:C) at 200 µg $fish^{-1}$ before and after VHSV challenge (Fig. 3B). The fish challenged with VHSV 2 d before Poly(I:C) administration showed 0% survival, demonstrating no curative effect of Poly(I:C) in the fish pre-infected with VHSV. In the present experiments, typical disease signs were observed after 2 to 3 d of VHSV challenge (data not shown), and, without Poly(I:C) immunization, all challenged fish died within 6 to 9 d after VHSV challenge (Fig. 1), suggesting that the inoculated VHSV might propagate within 2 d up to a level that will kill the fish. The same tendency was also observed in our previous studies on sevenband grouper with red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 2009). Thus, no curative effect of Poly(I:C) could be observed, at least in an acute stage of viral infection.

The fish challenged with VHSV on the same day or 14 d after Poly(I:C) administration showed 50% survival rate, while no mortality was observed in the fish challenged after 1, 2, 4, or 7 d of Poly(I:C) administration (Fig. 3B). It was thus considered that the antiviral state appeared 1 d after Poly(I:C) administration and lasted for at least 6 d, but subsequently decreased. These results are comparable to some extent with those of previous experiments in salmonids and other fish species in which the fish Mx gene response following Poly(I:C) administration peaked 1 d after administration and subsequently disappeared within 15 d (Lockhart et al. 2004, Purcell et al. 2004, Plant et al. 2005, Saint-Jean & Pérez-Prieto 2007, Fernandez-Trujillo et al. 2008). In our previous study on sevenband grouper, a high survival rate (95%) was observed in the fish receiving both Poly(I:C) administration and RGNNV challenge at the same time (Nishizawa et al. 2009); whereas the results of the present study recommend vaccination with live VHSV in Japanese flounder to be carried out 1 d after Poly(I:C) administration (Fig. 3B).

In conclusion, it was confirmed that Poly(I:C) immunization against VHS is efficacious in Japanese flounder. The fact that the required dose of Poly(I:C) for Japanese flounder was less than one quarter of that for sevenband grouper underlines that there are some differences in the required dose of Poly(I:C) and the duration of the antiviral state among host fish species. Furthermore, the response of Japanese flounder to Poly(I:C) administration was slightly slower than that of sevenband grouper. These results suggest that the dose of Poly(I:C) and the appropriate time-point of virus injection should be investigated prior to starting Poly(I:C) immunization procedure for other fish species and viruses. In addition, the fish rearing temperature may also be an important factor influencing fish

response to Poly(I:C), and should be addressed in future experiments. Finally, difficulties were encountered during the grow-out and adult stages in sea cages of open aquaculture systems due to horizontal transmission of fish pathogenic viruses in Japan, although a stable seed production in separate facilities was accomplished by preventing viral transmission (Mushiake et al. 1994, Watanabe et al. 2000, Yoshimizu 2003, 2009), suggesting that the environment of the culture system was contaminated with fish pathogenic viruses. Moreover, a new problem of mixed infection with unknown pathogenic viruses was also revealed in VNN-affected fish in Japan (Kokawa et al. 2008). Under these circumstances, Poly(I:C) immunization could be useful for cultured fish species in such sea cages. In the present study, virus isolation from the fish surviving VHSV challenges was not conducted because viral infectivities in the Poly(I:C)-administered survivors of IHNV or fish nodavirus infection were all under the detection limit in the previous studies (Kim et al. 2009, Nishizawa et al. 2009). It is not guaranteed that the inoculated live virus is eliminated completely from the Poly(I:C)-immunized fish, meaning that the possibility of the vaccinated fish becoming sub-clinical carriers may remain. Therefore, movement of the Poly(I:C)-immunized fish should be restricted between fish farms using different rearing-water sources until the risk of vaccinated fish becoming sub-clinical carriers is solved.

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