

# Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or Poly(I:C)

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**ABSTRACT:** It was recently reported that prophylaxis against infectious hematopoietic necrosis virus (IHN) in fish was induced by pre-exposure to the infectious pancreatic necrosis virus (IPNV). Here the establishment of IHN immunity in rainbow trout *Oncorhynchus mykiss* was investigated by IHN challenge following non-lethal pre-infection with IPNV. Also, synthetic double-stranded RNA polyinosinic polycytidylic acid, Poly(I:C), an inducer for interferon (IFN), was evaluated as a substitute for IPNV induction of the non-specific antiviral state and subsequent IHN-specific immunity in fish. Rainbow trout pre-infected with IPNV were protected from IHN challenge 7 d later (relative percentage survival, RPS: 68.8%), and IHN-specific antibodies were detected in sera from the survivors. Moreover, these surviving fish showed 91.6% RPS when re-challenged with IHN 28 d after the primary IHN challenge. Thus, fish appear to acquire IHN-specific immunity through the IHN challenge following pre-injection with IPNV. Fish pre-injected with Poly(I:C) were also highly protected from IHN challenge 2 d later (RPS: 95.2%), and IHN-specific antibodies were also detected amongst survivors. The survivors showed a 100% survival rate following re-challenge with IHN both 21 and 49 d after the primary IHN challenge. Thus, IHN immunity in rainbow trout is induced by challenge with live IHN following pre-injection with either IPNV or Poly(I:C). The use of Poly(I:C) to induce an anti-viral state protecting rainbow trout from an otherwise lethal vaccination dose of IHN may have application to a wider range of fish species and fish pathogenic viruses.

**KEY WORDS:** Immunization · Infectious hematopoietic necrosis virus · IHN · Poly(I:C) · Interferon · Antiviral state · Dual infection · Vaccine

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## INTRODUCTION

Infectious hematopoietic necrosis (IHN) is one of the most serious diseases of salmonids. Because outbreaks of IHN can result in losses of over 80%, depending on the species and size of fish, virus strain and environmental conditions (Wolf 1988). The causative agent, IHN virus (IHN), is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Kurath et al. 1985, Tordo et al. 2005). IHN is currently enzootic throughout the Pacific Northwest

of North America, but was spread by movement of IHN-contaminated fish or fish eggs to Asian and European countries in the 1960s to 1970s (Kimura & Yoshimizu 1991, Winton 1991, Nishizawa et al. 2006a). The imported IHN has evolved rapidly in the rainbow trout *Oncorhynchus mykiss* farm environment in Japan (Nishizawa et al. 2006a), and some of the Japanese isolates of IHN have spread to Korea (Kim et al. 2007b). In recent years, serious losses due to IHN have been observed in adult and market size rainbow trout, although IHN tends to

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be more pathogenic in juveniles. The evolutionary divergence of IHNV influences its pathogenicity, but this has not been elucidated scientifically (Nishizawa et al. 2006a).

In seed production facilities for salmonids, stable production of specific-pathogen-free (SPF) fish has been accomplished by measures for the prevention of IHNV transmission, such as disinfection of eggs with iodine, and sterilization of rearing water and facilities. Furthermore, maintenance of SPF-spawners monitored by antibody detection (ELISA) is also important and helpful to prevent vertical transmission of IHNV (Yoshimizu 1996, 2003). However, it is still difficult to prevent horizontal transmission of IHNV through water flow in rainbow trout farms. Therefore, development of an IHNV vaccine is essential, and several kinds of efficacious IHNV vaccines have been developed, e.g. killed or attenuated vaccines (Winton 1997, Biering et al. 2005), recombinant vaccines with IHNV G protein (Xu et al. 1991, Cain et al. 1999) and DNA vaccines with IHNV G gene (Anderson et al. 1996, Kim et al. 2000, Lorenzen et al. 2002).

Protection of salmonids from IHNV, viral hemorrhagic septicemia virus (VHSV) and/or infectious salmon anemia virus (ISAV) can be induced by pre-exposure of fish to infectious pancreatic necrosis virus (IPNV), aquareovirus or picornavirus (de Kinkelin et al. 1992, Hedrick et al. 1994, LaPatra et al. 1995, Johansen & Sommer 2001, Byrne et al. 2008). Furthermore, in marine fishes, primary infection of Japanese flounder and sevenband grouper *Epinephelus septemfasciatus* by aquabirnavirus confers protection from other pathogenic viruses, such as VHSV and fish nodavirus (Pakingking et al. 2003, 2005). The protection from the secondary infection of virus was due to an interferon (IFN) activity induced in the fish by the primary infection (Pakingking et al. 2004, Das et al. 2007, McBeath et al. 2007). Polyinosinic polycytidylic acid (Poly(I:C)), a synthetic double-stranded RNA, is commonly used as an IFN inducer in many different animals. IFN is induced in rainbow trout by Poly(I:C) injection, causing the injected fish to develop an antiviral state (Eaton 1990, Trobridge et al. 1997, Purcell et al. 2004).

In the present study, establishment of IHNV immunity in rainbow trout *Oncorhynchus mykiss* that survived IHNV infection following pre-infection with non-lethal IPNV was investigated by re-challenge with IHNV. Moreover, Poly(I:C) was evaluated for use as a substitute for non-lethal IPNV for the induction of an antiviral state in fish. It was demonstrated that injection with IPNV or Poly(I:C) allows the fish to survive the subsequent IHNV infection, which then stimulates specific immunity against IHNV.

## MATERIALS AND METHODS

**Viruses.** IHNV RtNag96 and IPNV RtNag00 were used for experimental infection of rainbow trout, while IHNV RtNag96 and VHSV Obama25 were used as viral antigens for ELISA antibody detection. IHNV RtNag96 was isolated from IHN-affected rainbow trout in Nagano Prefecture, Japan in 1996, and classified into the genogroup JRt (Nishizawa et al. 2006a). IPNV RtNag00 was obtained from apparently healthy rainbow trout in Nagano Prefecture in 2000, and classified into the Genogroup I of the genus *Aquabirnavirus* (Nishizawa et al. 2005). IPNV RtNag00 is not, or only seldom lethal to rainbow trout. No mortality was observed in rainbow trout injected with this isolate at a  $10^{6.3}$  50% tissue culture infectious dose (TCID<sub>50</sub>) fish<sup>-1</sup> using a standard challenge system (data not shown), although the mechanism is not clear. VHSV Obama25 was isolated from free-living Japanese flounder *Paralichthys olivaceus* caught in the Obama coastal area of the Wakasa Bay, Japan, in 1999 (Takano et al. 2000, 2001), and classified into the Genogroup IV of VHSV (Nishizawa et al. 2002, 2006b).

The viruses were propagated in epithelioma papulosum cyprini (EPC) cells, which were maintained at 15°C with Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum, 100 IU ml<sup>-1</sup> penicillin G and 100 µg ml<sup>-1</sup> streptomycin sulfate (MEM<sub>10</sub>). Titration of viral infectivity was performed using 96-well microplates, seeded with EPC cells. After culture at 15°C for 14 d, cytopathic effect (CPE) was evaluated to determine the TCID<sub>50</sub>.

**IHNV challenge for fish pre-infected with IPNV.** SPF rainbow trout juveniles of  $1.3 \pm 0.2$  g body weight were kindly contributed by Mr. K. Kohara in Nagano Prefectural Fisheries Experimental Station, in Japan. The SPF nature of the fish was cleared not only for IPNV and IHNV but also for other rainbow trout viruses by culture-isolation test using RTG-2, EPC and CHSE-214 cell lines. The fish were reared in 10 l tanks at 10 to 11°C with 24 exchanges per day, and fed a commercial dry-pellet diet at approximately 1 to 2% of body weight d<sup>-1</sup> during the following experiments. The experimental design scheme of IPNV-injection and IHNV challenges is shown in Fig. 1. After 2 wk for acclimation, IPNV at a dose of  $10^{6.3}$  TCID<sub>50</sub> 50 µl<sup>-1</sup> fish<sup>-1</sup> or the same volume of MEM<sub>10</sub> (mock infection) was intraperitoneally injected into 300 and 70 fish, respectively. Seven days after IPNV injection, 200 of the IPNV-injected fish were intraperitoneally challenged with IHNV at a dose of  $10^{5.0}$  TCID<sub>50</sub> 50 µl<sup>-1</sup> fish<sup>-1</sup> (IPNV-IHNV group), and the remaining 100 fish were mock challenged with MEM<sub>10</sub> (IPNV-Mock group). Fifty out of the 70 fish injected with MEM<sub>10</sub> (mock infection) were divided into 2 groups, and were

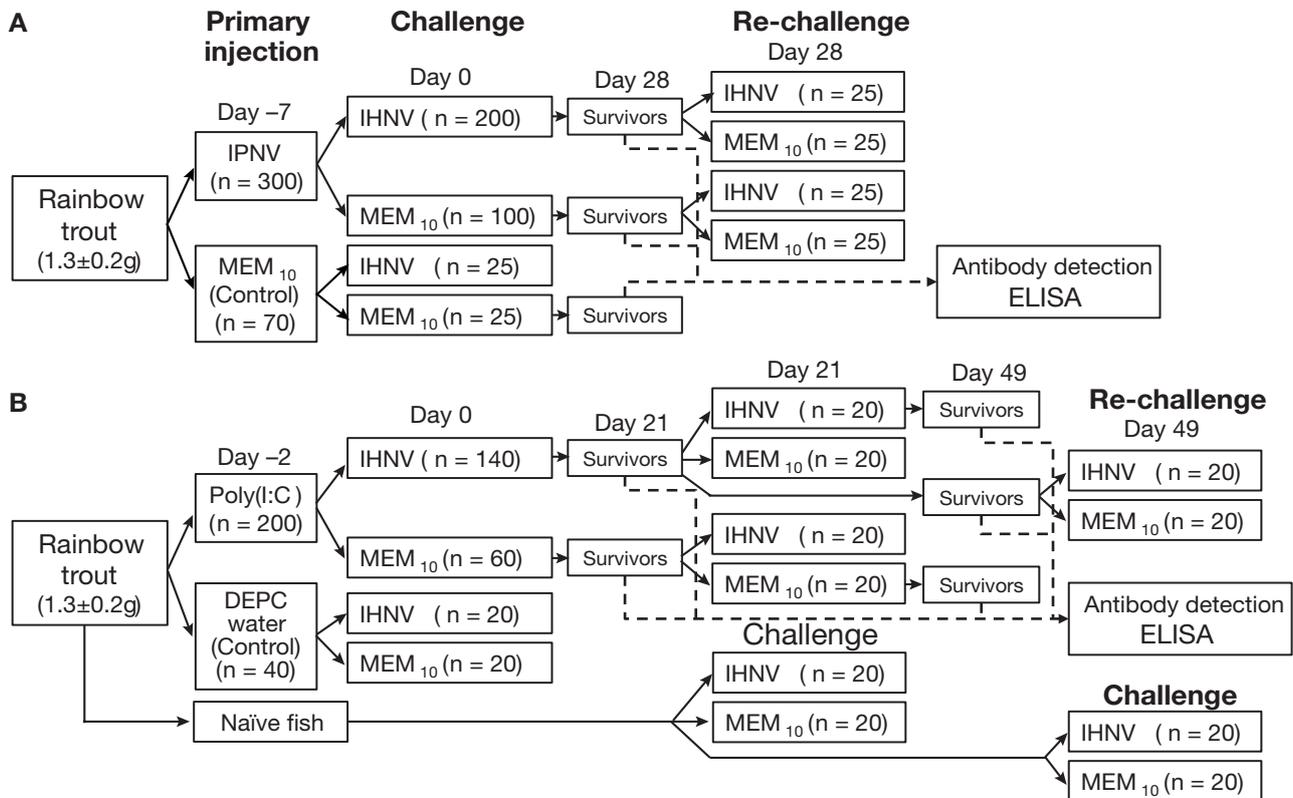


Fig. 1. Experimental design scheme of infectious pancreatic necrosis virus (IPNV)-infection, polyinosinic polycytidylic acid (Poly(I:C))-injection, infectious hematopoietic necrosis virus (IHNV) challenges and collection of fish sera for antibody detection via ELISA. (A) Scheme for IPNV protection; (B) scheme for Poly (I:C) protection. MEM<sub>10</sub>: supplemented minimum essential medium; DEPC: diethyl pyrocarbonate. Dashed lines: collection of fish sera from survivors for the antibody ELISA

intraperitoneally challenged with IHNV at a dose of  $10^{5.0}$  TCID<sub>50</sub>  $50 \mu\text{l}^{-1}$  fish<sup>-1</sup> (Mock-IHNV group) or MEM<sub>10</sub> (Mock-Mock group). Fish mortality was monitored daily for an additional 28 d, and the relative percentage survival (RPS) was calculated at the end of the experiment according to the method of Amend (1981).

At the end of the experiment, sera were collected from 10 or 20 surviving fish out of each group, and subjected to IHNV-specific antibody detection ELISA, as described below (Fig. 1). Kidney tissues, obtained from 10 dead fish of the Mock-IHNV group and 5 survivors of each of the other groups, were homogenized with 9 volumes of Hanks' balanced salt solution (HBSS), and titrated in cell culture to determine the viral load in the tissue.

**Re-challenge experiment with surviving fish from the primary IHNV challenge.** On Day 28 after the primary IHNV challenge, 50 of the surviving fish from the IPNV-IHNV group were divided into 2 groups, and were intraperitoneally re-challenged with IHNV at a dose of  $10^{5.0}$  TCID<sub>50</sub>  $50 \mu\text{l}^{-1}$  fish<sup>-1</sup> (IPNV-IHNV-IHNV group) or MEM<sub>10</sub> (IPNV-IHNV-Mock) (Fig. 1). In the same manner, 50 survivors of the IPNV-Mock group were challenged with IHNV (IPNV-Mock-IHNV

group) or MEM<sub>10</sub> (IPNV-Mock-Mock group). Mortality was monitored daily for an additional 28 d, and the RPS was calculated.

**IHNV challenge for fish pre-injected with Poly(I:C).** Poly(I:C) (Sigma) was dissolved in diethyl pyrocarbonate (DEPC)-treated water (Nippon Gene) at  $1 \text{ mg ml}^{-1}$  before using for each experiment. As shown in Fig. 1, a total of 200 fish were intraperitoneally injected with Poly(I:C) at  $50 \mu\text{g } 50 \mu\text{l}^{-1}$  fish<sup>-1</sup>, while the control group (40 fish) were injected with the same volume of DEPC water. Two days after Poly(I:C)-injection, 140 fish with Poly(I:C)-injection were challenged with IHNV at a dose of  $10^{5.0}$  TCID<sub>50</sub>  $50 \mu\text{l}^{-1}$  fish<sup>-1</sup> (Poly(I:C)-IHNV group), and the remaining 60 fish with Poly(I:C)-injection were mock challenged with MEM<sub>10</sub> (Poly(I:C)-Mock group). At the same time, fish of the control group without Poly(I:C)-injection were challenged with either IHNV or MEM<sub>10</sub> in the same manner (Mock-IHNV and Mock-Mock groups, respectively). Mortality was monitored daily for an additional 21 d. At the end of the experiment, sera and kidneys were collected from a few survivors of each group for IHNV-specific antibody detection ELISA and titration of viral infectivity, respectively.

**Re-challenge experiment with Poly(I:C)-injected fish surviving IHNV challenge.** At 21 d after the primary IHNV challenge, 20 survivors in each of the Poly(I:C)-IHNV and Poly(I:C)-Mock groups were intraperitoneally re-challenged with IHNV at a dose of  $10^{5.0}$  TCID<sub>50</sub>  $50 \mu\text{l}^{-1}$  fish<sup>-1</sup> (Poly(I:C)-IHNV-IHNV and Poly(I:C)-Mock-IHNV groups, respectively), and mock challenged with MEM<sub>10</sub> (Poly(I:C)-IHNV-Mock and Poly(I:C)-Mock-Mock groups, respectively). In the same manner, 49 d after the primary IHNV challenge, 20 survivors in the Poly(I:C)-IHNV group were re-challenged with IHNV, and mock challenged with MEM<sub>10</sub> respectively. As additional positive and negative controls, 20 naïve fish, which had not been handled previously, were also challenged with IHNV or MEM<sub>10</sub> (Naïve-IHNV and Naïve-Mock groups, respectively). Mortality was monitored daily for a further 21 d after the re-challenge with IHNV. At the end of the experiment, sera and kidneys were collected from 10 survivors of each group for IHNV-specific antibody detection ELISA and for titration of viral infectivity, respectively.

**ELISA for detection of IHNV-specific antibodies from fish sera.** Ca. 5 to 10  $\mu\text{l}$  of blood collected from caudal venous puncture, from survivors, was clotted at 4°C for 16 h, and centrifuged at  $12000 \times g$  for 10 min at 4°C. The obtained fish sera were stored at -20°C until use. Detection of IHNV-specific antibodies by ELISA was performed according to the method of Kim et al. (2007a, 2008). After clarification by centrifugation ( $19000 \times g$ , 30 min, 4°C), supernatants of IHNV (target antigens) and VHSV (control antigens) containing a dose of approximately  $10^{8.0}$  TCID<sub>50</sub>  $\text{ml}^{-1}$  were diluted 10-fold with phosphate buffered saline (PBS), placed into ELISA plate wells at  $50 \mu\text{l}$  well<sup>-1</sup> and incubated at 4°C overnight. ELISA wells were washed 3 times with PBS containing 0.05% Tween 20 (T-PBS), and blocked with 5% skim milk in PBS at 25°C for 1 h. The ELISA wells were washed 3 times with T-PBS, and then incubated with  $50 \mu\text{l}$  of fish sera (primary) diluted 1:40 in 5% skim milk at 25°C for 1 h. Plates were then incubated at 25°C for 30 min with rabbit (secondary) antiserum specific for rainbow trout IgM and anti-rabbit IgG swine Ig (tertiary) conjugated with horseradish peroxidase (Dako). Before use, both rabbit and swine sera were diluted 1:1000 in 5% skim milk. After washing 3 times with T-PBS,  $50 \mu\text{l}$  of substrate solution ( $1 \text{ mg ml}^{-1}$  *o*-phenylenediamine, 0.03% H<sub>2</sub>O<sub>2</sub>, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM citric acid) was added to each well and incubated at 25°C for 30 min. The reaction was stopped with 2N H<sub>2</sub>SO<sub>4</sub>, and then the optical density at 492 nm (OD<sub>492</sub>) was read using a microplate reader (MTP-300, Corona), and the OD values for IHNV-specific antibodies were corrected according to Kim et al.

(2008). The ELISA values among challenged groups were statistically analyzed using the Steel-Dwass test with a significance level of 5%.

## RESULTS

### Protection of rainbow trout pre-infected with IPNV against IHNV infection

Rainbow trout, pre-infected with IPNV or mock-infected, were challenged 7 d later with IHNV (Fig. 2A). Infectivity titers of IPNV in kidney of the fish pre-infected with IPNV were between  $10^{3.3}$  and  $10^{4.8}$  TCID<sub>50</sub>  $\text{g}^{-1}$  tissue 3 d after the IPNV injection, but decreased below detection limit ( $<10^{2.8}$ ) to  $10^{3.8}$  TCID<sub>50</sub>  $\text{g}^{-1}$  tissue at the time of IHNV challenge, i.e. at 7 d after the IPNV injection (data not shown). The fish pre-infected with IPNV (IPNV-IHNV group) began to die 5 d after the IHNV challenge, but the survival rate was 68.8% at the end of the experiment. The fish without pre-infection with IPNV (Mock-IHNV group) began to

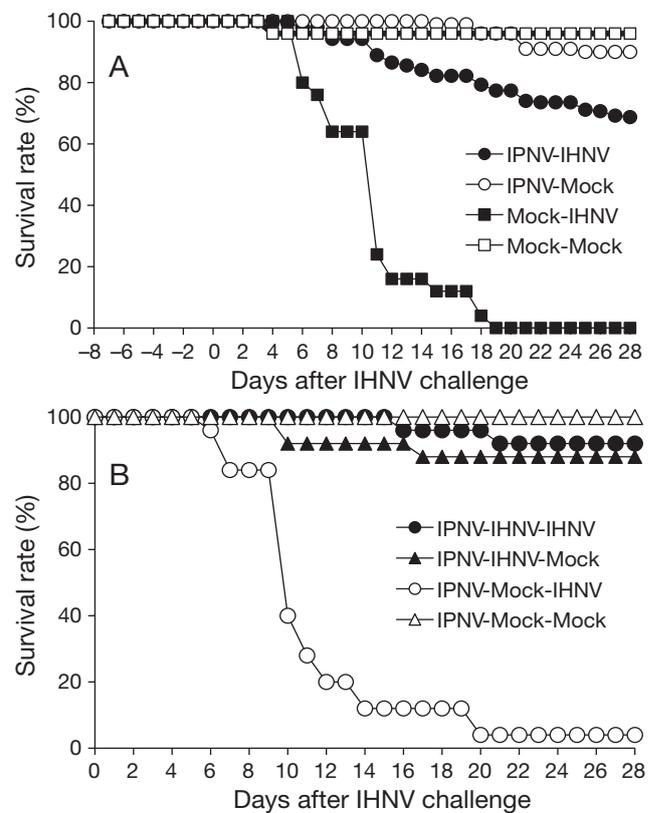


Fig. 2. *Oncorhynchus mykiss*. Survival rates following challenge with infectious hematopoietic necrosis virus (IHNV) in rainbow trout with and without pre-infection with non-lethal infectious pancreatic necrosis virus (IPNV). (A) Fish were challenged with IHNV 7 d after pre-infection with IPNV. (B) Surviving fish from IHNV and mock challenges following pre-infection with IPNV were re-challenged with IHNV

die 4 d after the IHNV challenge, and all died within 19 d (0% survival rate). The RPS value of the IPNV-IHNV group versus Mock-IHNV group was 68.8%. The survival rates of fish in the IPNV-Mock and Mock-Mock groups were 90.0 and 96.0%, respectively. At the end of the experiment, neither IPNV nor IHNV was detected among survivors of IPNV-IHNV, IPNV-Mock and Mock-Mock groups, while IHNV at doses of  $10^{5.1}$  to  $10^{6.1}$  TCID<sub>50</sub> g<sup>-1</sup> tissue was detected in kidneys of dead fish in the Mock-IHNV group. These results demonstrate that fish pre-infected with IPNV were highly protected from IHNV-infection in comparison with non-pre-infected fish.

The survivors of the IPNV-IHNV and IPNV-Mock groups were re-challenged with IHNV 28 d after the primary IHNV challenge (Fig. 2B). Fish in the IPNV-Mock-IHNV group began to die 6 d after the re-challenge with IHNV, and the final survival rate was 4%. In contrast, final survival rate of the other 3 groups, IPNV-IHNV-IHNV, IPNV-IHNV-Mock and IPNV-Mock-Mock, were 92.0, 88.0 and 100%, respectively. Thus, the RPS value of the IPNV-IHNV-IHNV group versus the IPNV-Mock-IHNV group was 91.6%. These results demonstrate that protection from IHNV challenge was observed in fish, which survived dual infection with IHNV and IPNV, but not in those which survived single infection with IPNV. Fish surviving from the dual infection remained alive throughout the experiment.

#### Detection of IHNV-specific antibodies in fish that survived IPNV and IHNV infection

Before the secondary IHNV challenge (28 d after the primary IHNV challenge), sera were collected

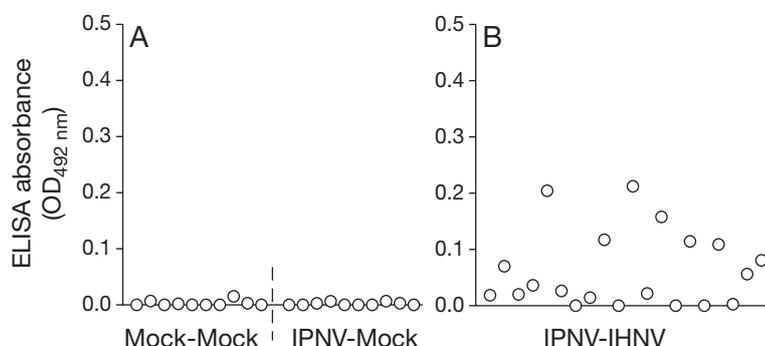


Fig. 3. *Oncorhynchus mykiss*. Detection of infectious hematopoietic necrosis virus (IHNV)-specific antibodies in sera of the surviving fish from infectious pancreatic necrosis virus (IPNV) and IHNV infection. Sera were collected 28 d after IHNV challenge. (A) Mock challenge; (B) IHNV challenge. Mock-Mock: sera from fish with neither IPNV-infection nor IHNV-infection; IPNV-Mock: sera from fish with mock challenge following pre-infection with IPNV; IPNV-IHNV: sera from the fish with IHNV challenge following pre-infection with IPNV. OD<sub>492</sub>: optical density at 492 nm

from some of the surviving fish in the Mock-Mock, IPNV-Mock and IPNV-IHNV groups for detection of IHNV-specific antibodies (Fig. 3). ELISA absorbance (OD<sub>492</sub>) of sera collected from the fish in the IPNV-IHNV group ranged from 0 to 0.21 (Fig. 3B), while absorbance values from fish in the Mock-Mock and IPNV-Mock groups were all less than 0.02 (Fig. 3A), i.e. a significant difference was observed in the ELISA values for IHNV antigens between sera of the IPNV-IHNV and those of the IPNV-Mock group or Mock-Mock group ( $p < 0.05$ ). These results demonstrate that antibodies against IHNV antigens were produced in a large proportion of the surviving fish of IPNV-IHNV group during the primary IHNV challenge.

#### Protection of rainbow trout injected with Poly(I:C) against IHNV infection

Rainbow trout injected with or without Poly(I:C) were challenged 2 d later with IHNV, and mortalities were monitored (Fig. 4). Fish pre-injected with Poly(I:C) (Poly(I:C)-IHNV group) showed 95.7% survival rate, which was clearly higher than that of fish pre-injected with DEPC water (Control group). RPS of the Poly(I:C)-IHNV group versus the Control-IHNV group was 95.2%. No infectivity of IHNV was detected in kidneys of the survivors in the Poly(I:C)-IHNV group, although doses of  $10^{7.1}$  to  $10^{7.6}$  TCID<sub>50</sub> g<sup>-1</sup> tissue of IHNV were detected in dead fish collected 6 to 18 d after the challenge in the Control-IHNV group (data not shown). No mortality was observed in fish of the Poly(I:C)-Mock and Control-Mock groups (Fig. 4A), and no IHNV was detected (data not shown).

Mortalities in surviving fish of the Poly(I:C)-IHNV and Poly(I:C)-Mock groups after re-challenge with IHNV 21 d after the primary IHNV challenge were shown in Fig. 4B. No mortality was observed in the Poly(I:C)-IHNV-IHNV group after re-challenge with IHNV (final survival rate: 100%). Seven days after IHNV challenge in the Poly(I:C)-Mock-IHNV (re-challenge) and Naïve-IHNV (initial challenge) groups, fish began to die, and the final survival rate of each group was 10%. RPS of the Poly(I:C)-IHNV-IHNV group versus Poly(I:C)-Mock-IHNV or Naïve-IHNV groups were both 100%. Only one fish of the Poly(I:C)-IHNV-Mock group died 9 d after the re-challenge with IHNV (final survival rate: 95%), while no mortality was observed in the Poly(I:C)-Mock-Mock and Naïve-Mock groups.

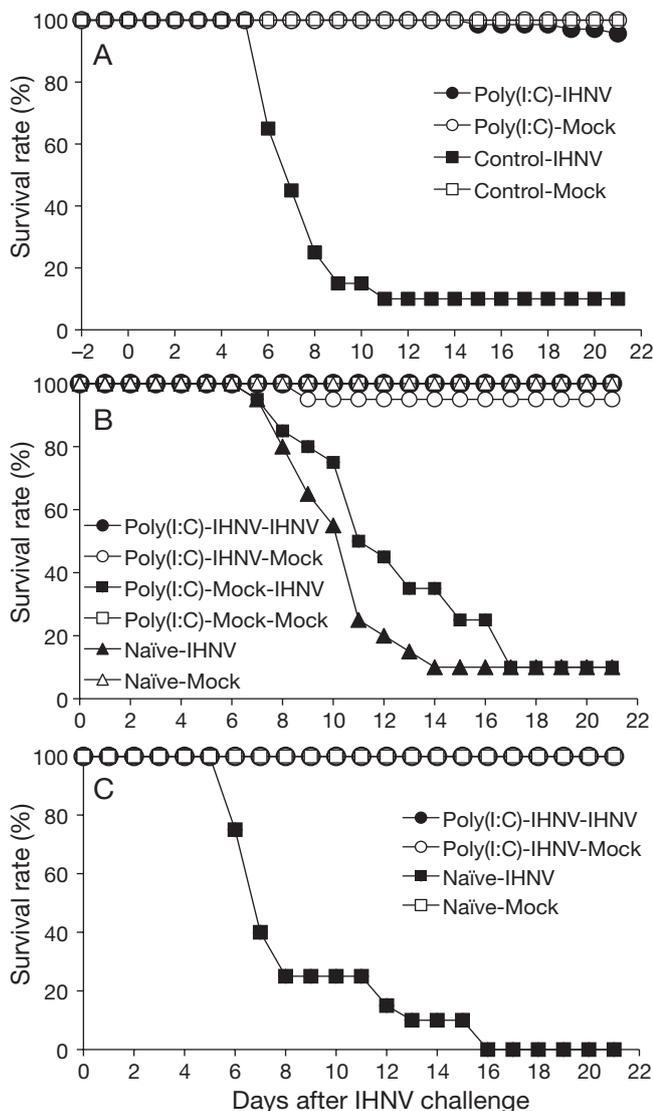


Fig. 4. *Oncorhynchus mykiss*. Survival rates following challenge with infectious hematopoietic necrosis virus (IHN) in rainbow trout with and without polyinosinic polycytidylic (Poly(I:C)) pre-injection. (A) Fish were challenged with IHN 2 d after pre-injection with Poly(I:C). (B) Surviving fish from IHN and mock challenges following pre-injection with Poly(I:C) were re-challenged with IHN 21 d after the primary IHN challenge. (C) Further challenge with IHN 49 d after the primary IHN challenge

In a separate experiment, survivors of the Poly(I:C)-IHN and Poly(I:C)-Mock groups, as well as the fish in the Naïve-IHN group, were also challenged with IHN 49 d after the primary IHN challenge (Fig. 4C). All fish in the Naïve-IHN group died within 16 d after the challenge with IHN, while no mortality was observed in Poly(I:C)-IHN-IHN, Poly(I:C)-IHN-Mock and Naïve-Mock groups. RPS of the Poly(I:C)-IHN-IHN group versus Naïve-IHN group was 100%.

#### Detection of IHNV-specific antibodies in fish that survived IHN challenge following Poly(I:C)-injection

ELISA absorbance values of sera obtained from Poly(I:C)-injected survivors of the IHN challenges are shown in Fig. 5. Firstly, sera were collected from survivors in the Poly(I:C)-Mock and Poly(I:C)-IHN groups 21 d after the primary IHN challenge, i.e. before the secondary IHN challenge. Sera from the Poly(I:C)-Mock group showed ELISA values of  $<0.02$ , and those from the Poly(I:C)-IHN group showed ELISA values ranging from 0 to 0.03 (Fig. 5A). The ELISA values of sera from the Poly(I:C)-IHN group seemed to be slightly higher than those from the Poly(I:C)-Mock group although no statistical difference ( $p < 0.05$ ) was observed between them, suggesting that the ELISA values from the Poly(I:C)-IHN group were not sufficiently positive for IHNV-specific antibodies.

Secondly, sera were collected from survivors in the Poly(I:C)-Mock-Mock, Poly(I:C)-IHN-Mock and Poly(I:C)-IHN-IHN groups 49 d after the primary IHN challenge, i.e. 28 d after the secondary IHN challenge (Fig. 5B). ELISA values of sera from Poly(I:C)-Mock-Mock group were all less than 0.01, and those from Poly(I:C)-IHN-Mock and Poly(I:C)-IHN-IHN groups ranged from 0.0 to 0.80 (average: 0.15) and from 0.06 to 0.58 (average: 0.24), respectively (Fig. 5B). Statistical differences ( $p < 0.05$ ) were observed in the ELISA values between the Poly(I:C)-Mock-Mock and Poly(I:C)-IHN-Mock or Poly(I:C)-IHN-IHN groups, but not between the Poly(I:C)-IHN-Mock and Poly(I:C)-IHN-IHN groups. These results demonstrate that IHNV-specific antibodies were produced in those fish with Poly(I:C)-injection by IHN challenge between 21 and 49 d after the challenge. From the present data, it was unfortunately difficult to confirm that the level of IHNV-specific antibodies was enhanced by the secondary IHN challenge.

#### DISCUSSION

Rainbow trout pre-infected with IPNV were highly protected from IHN infection, as the survival rate in the Mock-IHN group was zero, but 68.8% in the IPNV-IHN group (Fig. 2A). It was reported recently that protection from IHN infection induced by pre-exposure to IPNV (de Kinkelin et al. 1992, Byrne et al. 2008) was due to induction of IFN activity by the pre-infection with IPNV (McBeath et al. 2007, Byrne et al. 2008). Thus, the protection from the IHN challenge observed in the IPNV-IHN group could likewise be due to induction of IFN activity by pre-infection with IPNV, although expression of *Mx* gene, an

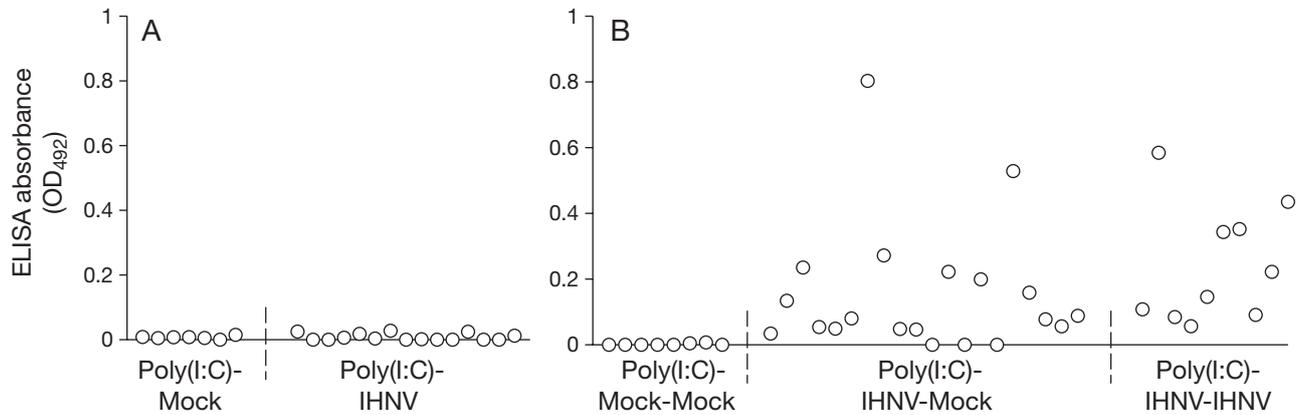


Fig. 5. *Oncorhynchus mykiss*. Detection of infectious hematopoietic necrosis virus (IHNV)-specific antibodies from sera of the surviving fish from IHNV challenge following pre-injection with polyinosinic polycytidylic acid (Poly(I:C)). Sera were collected (A) 21 d, (B) 49 d after the primary IHNV challenge. Poly(I:C)-Mock: fish with Poly(I:C)-injection and mock challenge; Poly(I:C)-IHNV: with Poly(I:C)-injection and IHNV challenge; Poly(I:C)-IHNV-IHNV: with Poly(I:C)-injection and 2 subsequent IHNV challenges. OD<sub>492</sub>: optical density at 492 nm

indicator for IFN induction, was not confirmed in the present study.

By antibody detection ELISA, it was confirmed that IHNV-specific antibodies were produced in the surviving fish from the IHNV challenge following IPNV pre-infection (IPNV-IHNV group) (Fig. 3B). Moreover, it was demonstrated that those survivors were highly protected from re-challenge with IHNV (IPNV-IHNV-IHNV group, Fig. 2B), suggesting that fish immunity against IHNV was established in the survivors of the IPNV-IHNV group. These phenomena could be explained as follows: through induction of IFN by primary infection with non-lethal IPNV, fish developed an antiviral state, which allowed the fish to survive the secondary infection with IHNV and thus to acquire IHNV-specific immunity. Thus, we speculate that immunization against a pathogenic virus can be established by intentional infection with the live pathogenic virus during the phase of IFN induction by pre-infection with a non-lethal virus.

Poly(I:C), a synthetic double-stranded RNA, is well known as an inducer of IFNs in many different fish models (Eaton 1990, Jensen & Robertsen 2002, Jensen et al. 2002, Plant et al. 2005, Fernandez-Trujillo et al. 2008). In the present study, Poly(I:C) was evaluated as a substitute for a non-lethal IPNV to induce the antiviral state in rainbow trout (Fig. 4A). The fish pre-injected with Poly(I:C) were highly protected from IHNV challenge, as the survival rate of the control-IHNV group was only 10%, while the survival rate of the Poly(I:C)-IHNV group was 95.7% (RPS: 95.2%, Fig. 4A). Moreover, no infectious IHNV was detected among the survivors of the Poly(I:C)-IHNV group. Thus, it can be confirmed that an antiviral state was induced in the fish of Poly(I:C)-IHNV group by pre-injection with Poly(I:C) 2 d prior to IHNV challenge, so Poly(I:C) could be useful as a substitute for non-lethal

viruses. However, the antiviral state induced by Poly(I:C)-injection completely disappeared within a short period, as the surviving fish of the Poly(I:C)-Mock group showed only 10% survival rate (RPS: 0%) after the IHNV challenge 21 d later after the Poly(I:C)-injection (Fig. 4B). These results agreed with previous data where Mx gene responses against Poly(I:C) peaked 1 d after the Poly(I:C)-injection in rainbow trout and Atlantic salmon, and subsequently disappeared within 15 d (Lockhart et al. 2004, Purcell et al. 2004). Similarly, the antiviral state against fish nodavirus induced by Poly(I:C)-injection lasted for a few days after injection in sevenband grouper *Epinephelus septemfasciatus* and subsequently decreased (Nishizawa et al. 2009, this issue).

The surviving fish of the Poly(I:C)-IHNV group showed 100% survival rate (RPS: 100%) against the re-challenge with IHNV 21 d after the primary challenge (Fig. 4B). However, the sera collected from those survivors had no detectable IHNV-specific antibody, and no clear difference was observed in ELISA values of sera between Poly(I:C)-IHNV and Poly(I:C)-Mock groups (Fig. 5A). Sera collected from the Poly(I:C)-IHNV-Mock group 49 d after the primary IHNV challenge were positive for IHNV-specific antibodies, because ELISA values of these sera were clearly higher than those from the Poly(I:C)-Mock-Mock group (negative control), but the same level as those from the surviving fish with re-challenge of IHNV (Poly(I:C)-IHNV-IHNV group) (Fig. 5B). It was noted that antibody levels in fish sera of the Poly(I:C)-IHNV-Mock group were the same as those of the Poly(I:C)-IHNV group, and thus it was considered that the IHNV-specific antibody level in sera from the Poly(I:C)-IHNV group increased 21 d after the primary IHNV challenge. Therefore, it is suggested that the

surviving fish of the Poly(I:C)-IHNV group could have already acquired IHNV-immunity at 21 d after the primary challenge even though IHNV-specific antibodies were still under the detection limit of the present ELISA system. Moreover, it can be considered that the IHNV-immunity acquired in fish could last a long time, because the surviving fish in the Poly(I:C)-IHNV groups showed no mortality (RPS: 100%) after the re-challenge with IHNV 49 d after the primary challenge (Fig. 4C). As mentioned above, duration of the antiviral state induced in fish by Poly(I:C)-injection lasted only a few days after the Poly(I:C)-injection, so IHNV should be injected into fish within the antiviral state for establishment of IHNV-immunity in fish.

In conclusion, the present study demonstrated that IHNV-specific immunity was established in rainbow trout by intentional infection with IHNV during the phase of antiviral state by preceding infection with non-lethal IPNV. Moreover, Poly(I:C) could be used as a substitute for non-lethal IPNV. Although several efficacious vaccines for fish viruses have been developed, such as killed, attenuated, subunit, recombinant and DNA vaccines (Xu et al. 1991, Anderson et al. 1996, Winton 1997, Cain et al. 1999, Kim et al. 2000, Lorenzen et al. 2002, Biering et al. 2005), the present method with IHNV challenge following pre-injection with non-lethal IPNV or Poly(I:C) is a novel immunization method for inducing protection against IHNV. It uses pre-infection with a non-lethal dose of IPNV, or injection with Poly(I:C), to stimulate a transient, non-specific anti-viral state that allows the fish to survive an IHNV infection that would otherwise be lethal. In our preliminary experiments, usefulness of the present method was also confirmed for protection of sevenband grouper against fish nodavirus, a causative agent of viral nervous necrosis (Nishizawa et al. 2009), and in Japanese flounder against viral hemorrhagic septicemia virus (VHSV) (I. Takami, T. Nishizawa & M. Yoshimizu unpubl.). Thus, we expect that the present method can be applied to various other viruses in other fish species, including non-culturable and non-identified viruses. However, more detailed experiments are required before application of the present method to open aquaculture, since some risks remain e.g. re-induction of the live virus used for immunization under stress conditions such as spawning maturation of the fish, and the spread of the virus used for immunization beyond the target.

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