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Development of a stringent ELISA protocol to evaluate anti-viral hemorrhagic septicemia virus-specific antibodies in olive flounder *Paralichthys olivaceus* with improved specificity

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Olive flounder were vaccinated with polyinosinic:polycytidylic acid [Poly (I:C)] to prevent viral hemorrhagic septicemia (VHS). Vaccine efficacy was verified by detection of anti-VHS virus (VHSV) antibodies using enzyme-linked immunosorbent assay (ELISA). In the study, ELISA absorbance values of the negative control group [Poly (I:C)-MEM$_{10}$] were saturated when an ELISA protocol, that includes pretreatment of the fish sera with 5% skim milk, was used. However, the saturated OD values in the negative control did not correlate with a specific immune response against VHSV, because the group showed low survival rate (only 10%) following the VHSV challenge. Also, OD values of Poly (I:C)-VHSV group were high, and the group showed high survival rate (97.5%) against VHSV challenge test. It was suggested that the high OD values were possibly due to the presence of anti-fetal bovine serum (FBS) cross-reactivity. To compensate this, we subtracted the absorbance of infectious hematopoietic necrosis (IHNV)-Ag plates from those of the VHSV-Ag plates. However, the average value for the Poly (I:C)-VHSV group (0.167) was lower than expected even though high survival rate. We used an advanced ELISA system to pre-treat fish sera with 5% skim milk and two novirhabdoviruses as capture antigens as well as 50% FBS. The corrected absorbance values for pre-treated fish sera from the negative control Poly (I:C)-MEM$_{10}$ and experimental Poly (I:C)-VHSV groups averaged 0.033 and 0.579, respectively. The specific VHSV antibody response of the vaccinated group was assessed using fish sera pretreated with skim milk and FBS and by calculating the corrected absorbance values from ELISA with two novirhabdovirus capture antigens.

Keywords: VHSV, ELISA, specificity, olive flounder

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Introduction

Fish inoculated with polyinosinic-polycytidylic acid [Poly (I:C)] and a live pathogenic virus produce a specific immune response against the virus (Kim *et al*., 2009; Nishizawa *et al*., 2009; Takami *et al*., 2010; Nishizawa *et al*., 2011a, 2011b; Oh *et al*., 2012, 2013). Previous *in vivo* studies have shown that Poly (I:C) immunization protects rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus (IHNV) (Kim *et al*., 2009), seven-band grouper (*Epinephelus septemfasciatus*) against nervous necrosis virus (Nishizawa *et al*., 2009, 2011a, 2011b; Oh *et al*., 2013), and Japanese flounder (*Paralichthys olivaceus*) against viral hemorrhagic septicemia virus (Takami *et al*., 2010). The specificity of the immune response was tested by challenging fish with live pathogenic virus, followed by detection of antiviral antibodies in the sera of vaccinated fish by enzyme-linked immunosorbent assay (ELISA) (Kim *et al*., 2009; Nishizawa *et al*., 2009; Takami *et al*., 2010). ELISA is convenient for routine, high-throughput screening, provides high sensitivity, enables rapid detection, is economical, and allows serum sampling that does not harm the fish (Kim *et al*., 2008). However, fish antibody detection by ELISA has poor reproducibility due to various factors, including high background signal caused by interactions between fish antibodies and non-specific antigens (Olesen *et al*., 1991; Höglund and Pilström, 1995; Knopf *et al*., 2000; Kibenge *et al*., 2002; Guo and Woo, 2004; Kim *et al*., 2007, 2008).

In this study, we investigated whether the high background signal associated with antibody detection in fish sera occurs due to the binding of fish serum to cell culture debris and components of fetal bovine serum (FBS). These antibodies may be present in the sera, as Poly (I:C) immunization includes injection of live pathogenic virus together with a cell culture solution that contains non-specific binding agents such as FBS. We developed an ELISA protocol to detect viral hemorrhagic septicemia virus (VHSV)-specific antibodies in fish with a reduced signal from FBS-reacting antibodies.

Materials and Methods

Viruses

VHSV KJ2008 was used to infect olive flounder, and VHSV KJ2008 or IHNV RtWanju09 was used as the viral antigen for ELISA antibody detection. VHSV KJ2008 was isolated in 2008 from a moribund olive flounder that was infected during a natural VHS outbreak on a commercial farm in

Korea, and was kindly provided by Prof. K. H. Kim (Department of Aquatic Medicine, Pukyong National University). The virus was identified as genotype IVa by sequencing the viral N and G genes (Kim and Kim, 2011). IHNV RtWanju09 was isolated in 2009 from the eggs of IHNV-infected rainbow trout from Wanju Prefecture, Korea, and classified into the JRt genogroup (Kim, 2010).

The viruses were propagated in epithelioma papulosum cyprini (EPC) cells maintained at 15°C in supplemented Eagle's minimum essential medium (MEM_{10}), which consisted of Eagle's minimum essential medium (Gibco, Invitrogen Co.) supplemented with 10% FBS, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate. The viruses were harvested after observation of cytopathic effects (CPEs), and were collected by centrifugation at 12,000 × *g* for 10 min at 4°C. The CPE culture supernatants were divided into aliquots and stored at -80°C. Viral infectivity was determined in 96-well microplates seeded with EPC cells. After propagating the EPC cells at 15°C for 14 d, the virus titer was quantified by assessing the 50% tissue culture infective dose ($TCID_{50}$).

Fish

Olive flounder (average body weight, 42.23 g each) were kindly provided by M. C. Choi of the Incheon Fisheries Research Institute, Korea. The fish were tested for VHSV, aquabirnavirus, and other olive-flounder virus infections by performing culture isolation tests with the EPC, RTG-2, and BF-2 cell lines. The fish were reared in 200 L flowing, UV-sterilized seawater at 17 ± 1°C and fed a commercial dry pellet diet (1–2% body weight/day).

Poly (I:C) and VHSV immunization

Poly (I:C) was dissolved in diethyl pyrocarbonate (DEPC)-treated water at a concentration of 1 mg/ml immediately before use and intraperitoneally injected at a concentration of 100 μg·100 μl^{-1}·$fish^{-1}$. The control group (20 fish) received the same volume of DEPC water. Two days after injection, VHSV was intraperitoneally injected at a dose of 10^6 $TCID_{50}$·100 μl^{-1}·$fish^{-1}$ (or the same volume of MEM_{10}) into 120 VHSV-inoculated fish [Poly (I:C)-VHSV group] or 120 MEM_{10}-injected fish [Poly (I:C)-MEM_{10} group]. The 20 fish injected with DEPC water [DEPC-VHSV group] were challenged with the same dose of VHSV. The unhandled fish group (negative controls) included 100 fish (Naïve group). Fish mortality was monitored daily for an additional 28 d.

Antibody detection

Procedures for fish sera pre-treatment and antibody detection by ELISA were performed as described previously (Kim *et al.*, 2007, 2008; Kwon *et al.*, 2010). On day 28 post-VHSV inoculation, sera were collected from surviving fish in the Naïve, Poly (I:C)-MEM_{10}, and Poly (I:C)-VHSV groups for VHSV-specific antibody detection. Two ELISA plates (Corning) were coated with viral antigens by adding 50 μl VHSV ($10^{8.05}$ $TCID_{50}$ ml^{-1}) or IHNV ($10^{8.05}$ $TCID_{50}$ ml^{-1}) per well and then incubating the plates at 37°C overnight. The antigens were diluted 10-fold in distilled water. Next, the plates were washed three times with phosphate-buffered saline (PBS; pH 7.5) containing 0.05% Tween 20 (T-PBS), blocked with 5% skim milk in PBS at 25°C for 1 h, and washed three times with T-PBS. Fish sera were diluted 40-fold with PBS containing 5% skim milk and incubated at 25°C for 1 h. Some samples from the Poly (I:C)-MEM_{10} group were diluted with 5% skim milk supplemented with 0%, 5%, 10%, 25%, 50%, and 75% FBS (Kwon *et al.*, 2010). The pre-treated fish sera were loaded (50 μl/well) in duplicate wells of VHSV antigen (Ag)- and IHNV-Ag plates and incubated at 25°C for 1 h. The plates were then washed three times with T-PBS and incubated at 25°C for 30 min with rabbit serum against olive flounder IgM (1:1,000 with PBS containing 5% skim milk), followed by incubation at 25°C for 30 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1,000, Santa Cruz) that was diluted 1,000-fold. After washing the plates three times with T-PBS, 50 μl of substrate solution (1 mg/ml *O*-phenylenediamine, 0.03% H_2O_2, 100 mM Na_2HPO_4, and 50 mM citric acid) was added to each well. Following incubation for 30 min at 25°C, the reaction was stopped with 2 N H_2SO_4 and absorbance was measured at 490 nm (Abs_{490}) on a microplate reader (Emax Precision Microplate Reader, Molecular Devices). The VHSV-specific antibody titers were calculated by subtracting the absorbance of the IHNV-Ag plates from those of the VHSV-Ag plates.

VHSV challenge test

On day 28 after VHSV immunization, 20 survivors from each group [Poly (I:C)-VHSV, Poly (I:C)-MEM_{10}, and Naïve group] were intraperitoneally injected with VHSV at a dose of 10^6 $TCID_{50}$ 100 μl^{-1} $fish^{-1}$. Fish mortality was monitored daily for an additional 28 d.

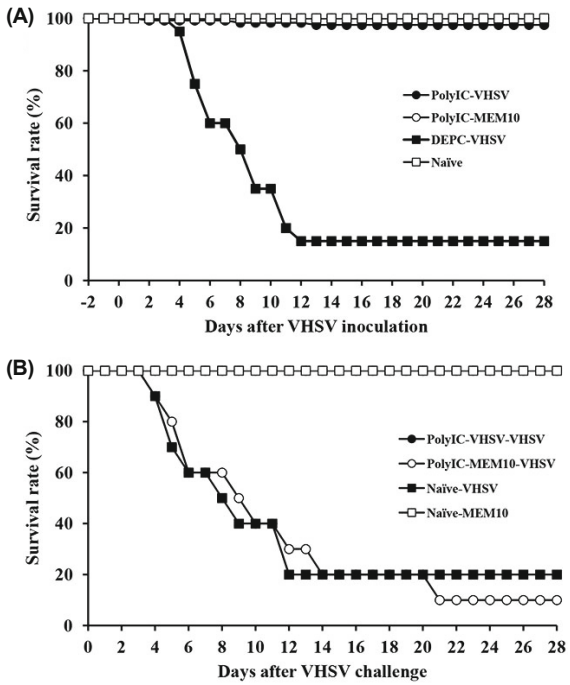

Fig. 1. Olive flounder survival following challenge with viral hemorrhagic septicemia virus (VHSV). (A) Fish were challenged with VHSV on day 2 after pre-injection with Poly (I:C). (B) Twenty-eight days following inoculation, Naïve and Poly (I:C)-injected fish were challenged with VHSV, or subjected to mock challenge.

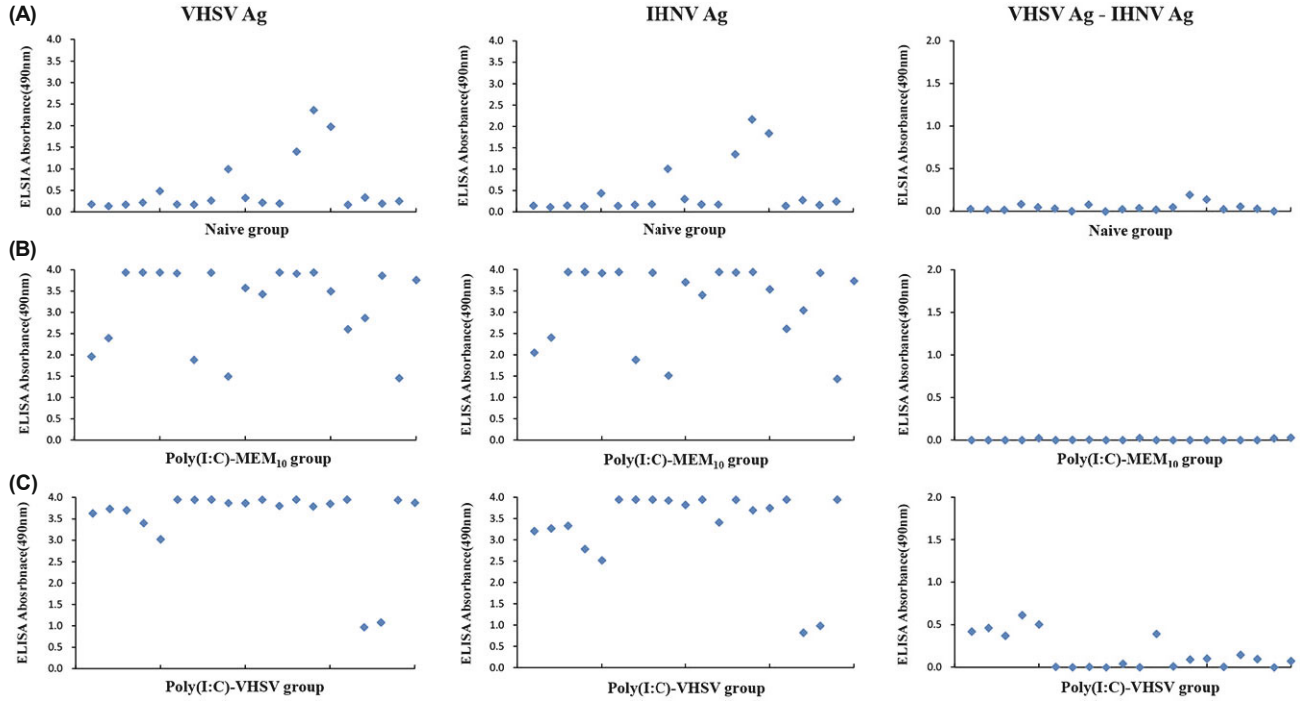

Fig. 2. A comparison of the results obtained by VHSV-Ag ELISA and IHNV-Ag ELISA of fish sera pre-treated with 5% skim milk. ELISA titers of fish sera in the Naïve group (A), Poly (I:C)-MEM$_{10}$ group (B), and Poly (I:C)-VHSV group (C). The anti-VHSV-specific antibody titers (right column) were calculated by subtracting the values of sera from the IHNV-Ag ELISA from those obtained using the VHSV-Ag ELISA.

Results and Discussion

Recently, Takami *et al.* (2010) reported induction of specific immunity against VHSV in Japanese flounder injected with live VHSV following Poly (I:C) administration. In the present study, the Poly (I:C)-VHSV group was injected with Poly (I:C), and then inoculated twice with VHSV (1 inoculation per day); this group exhibited a 2.5% mortality rate in the 28 d following inoculation (Fig. 1A). As expected, there were no mortalities in the Poly (I:C)-MEM$_{10}$ and Naïve control groups (Fig. 1A). In contrast, the DEPC-VHSV group suffered 85% mortality, demonstrating that the VHSV KJ2008 strain is highly pathogenic (Fig. 1A). On day 28 after inoculation, 20 fish from each group were challenged with VHSV and mortality was monitored for 28 d (Fig. 1B). Survival rates at day 28 were as follows: Naïve-MEM$_{10}$, 100%; Naïve-VHSV, 15%; Poly (I:C)-MEM$_{10}$-VHSV, 10%; and Poly (I:C)-VHSV-VHSV, 100% (Fig. 1B), confirming the development of specific immunity against VHSV in the surviving Poly (I:C)-immunized fish. These findings are consistent with the previously published results reported by Takami *et al.* (2010).

To suppress non-specific absorption of antibodies to ELISA plates, Kim *et al.* (2007) reported the efficacy of pre-treatment of fish sera with a 5% skim milk solution on ELISA plate wells. Recently, Choi *et al.* (2014) reported that nervous necrosis virus antigen is attached to ELISA plate at the 320-fold dilution ratio. In addition, IHNV-specific antibody levels in IHN-surviving fish were determined by subtracting the VHSV-Ag plate absorbance values from IHNV-Ag plate absorbance values for all samples (Kim *et al.*, 2008). We used ELISA to detect anti-VHSV-specific antibodies in the sera of olive flounder injected with live VHSV following Poly (I:C) administration, as described previously (Kim *et al.*, 2007, 2008). Absorbance from Naïve samples on the VHSV-Ag plate ranged from 0.135 to 2.361 (mean, 0.539), whereas on the IHNV-Ag plate, these values ranged from 0.115 to 2.167 (mean, 0.492; Fig. 2A). Subtraction of the IHNV-Ag values from the VHSV-Ag values ranged from 0 to 0.194

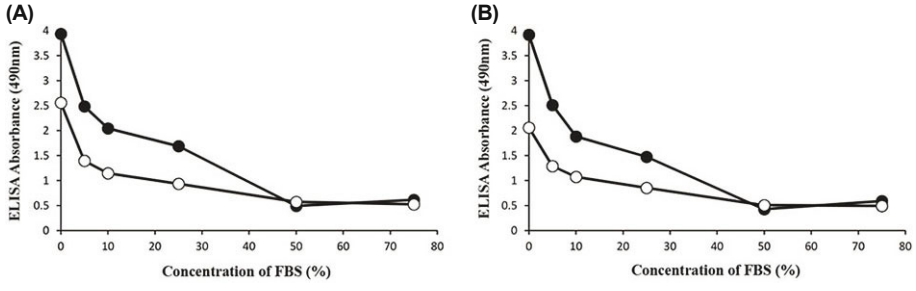

Fig. 3. Background signal reduction for fish sera from the Poly (I:C)-MEM$_{10}$ group after treatment with 5% skim milk and various concentrations of FBS. VHSV (A) and IHNV (B) culture supernatants were used as antigens. Filled circles, fish #6; empty circles, fish #7.

(mean, 0.033; Fig. 2A), confirming that non-specific values were dramatically reduced by pre-treatment with a 5% skim milk solution and subtracting the IHNV-Ag values from the VHSV-Ag values in the Naïve group.

The absorbance values for sera from the Poly (I:C)-MEM_{10} mock-infection group on the VHSV-Ag plate ranged from 1.456 to 3.940 (mean, 3.213); the range on the IHNV-Ag plate ranged from 1.434 to 3.940 (mean, 3.235; Fig. 2B). Among the 20 fish samples assessed on the VHSV-Ag or IHNV-Ag plates, 11 (55%) yielded values ranging from 3.5 to 4.0 (saturation of the ELISA signal). This suggests that the high values in the Poly (I:C)-MEM_{10} group were not associated with a specific immune response against VHSV, since we also observed elevated values in the IHNV-negative control group, and low survival rates (10%) following the VHSV challenge. In addition, the mean subtracted values for the Poly (I:C)-MEM_{10} and Poly (I:C)-VHSV groups were similar to those of fish with and without VHSV inoculation. Of 20 sera samples, 16 (80%) on the VHSV-Ag plate and 11 (55%) on the IHNV-Ag plate yielded values ranging from 3.5 to 4.0 (Fig. 2C). Although Poly (I:C)-VHSV sera yielded low signal specific to VHSV-Ag (mean, 0.167; Fig. 2C), these values were greater than for the control groups. The low signal may indeed result from specific immunity against VHSV, as the group exhibited 100% survival against the VHSV re-challenge. Thus, the extremely high absorbance values from the Poly (I:C)-MEM_{10} and Poly (I:C)-VHSV groups may be due to cross-reactivity from other antibodies that are highly reactive in both the VHSV-Ag and IHNV-Ag assays.

Kwon *et al.* (2010) reported that FBS proteins in viral culture fluid interfere with detection of specific antibodies against red sea bream iridovirus (RSIV) in yellowtail (*Seriola quinqueradiata*) immunized with a formalin-inactivated RSIV vaccine; cross-reactive antibodies were blocked by pre-treatment with 50% FBS. To evaluate whether FBS-antibody interactions had lead to the extremely high background we observed in our ELISA experiments, we evaluated the FBS-reactivity of sera from fish in the Poly (I:C)-MEM_{10} group. Sera from two fish (#6 and #7) in the Poly (I:C)-MEM_{10} group were treated with various concentrations of FBS, and the absorbance values decreased in a dose-dependent manner (Fig. 3). The VHSV-Ag plates for sera from fish #6 treated with FBS (0%, 5%, 10%, 25%, 50%, and 75%) yielded absorbance values of 3.934, 2.483, 2.042, 1.687, 0.572, and 0.612, respectively. Absorbance values for sera from fish #7 treated with FBS (0%, 5%, 10%, 25%, 50%, and 75%) were 2.557, 1.395, 1.145, 0.935, 0.572, and 0.522, respectively (Fig. 3A). On the IHNV-Ag plate, the values were 3.916, 2.509, 1.88, 1.473, 0.506, and 0.589, respectively. Absorbance values for fish sera #7 treated with FBS (0%, 5%, 10%, 25%, 50%, and 75%) were 2.058, 1.287, 1.072, 0.852, 0.506, and 0.490, respectively (Fig. 3B). Absorbance values decreased with increasing FBS concentration, demonstrating the production of anti-FBS antibody in olive flounder, accounting for the non-specific immune response against VHSV-Ag and IHNV-Ag in this ELISA system.

Fish sera treated with 5% skim milk and 50% FBS were assessed by ELISA with two novirhabdoviruses as capture antigens (Fig. 4). Mean values for the Poly (I:C)-MEM_{10} group in the VHSV-Ag assay were 4.86-fold lower, decreasing from 3.213 to 0.661; in the IHNV-Ag assay, values were 4.98-fold lower, with the mean decreasing from 3.235 to 0.650. Subtracting the mean IHNV-Ag value from the VHSV-Ag va-

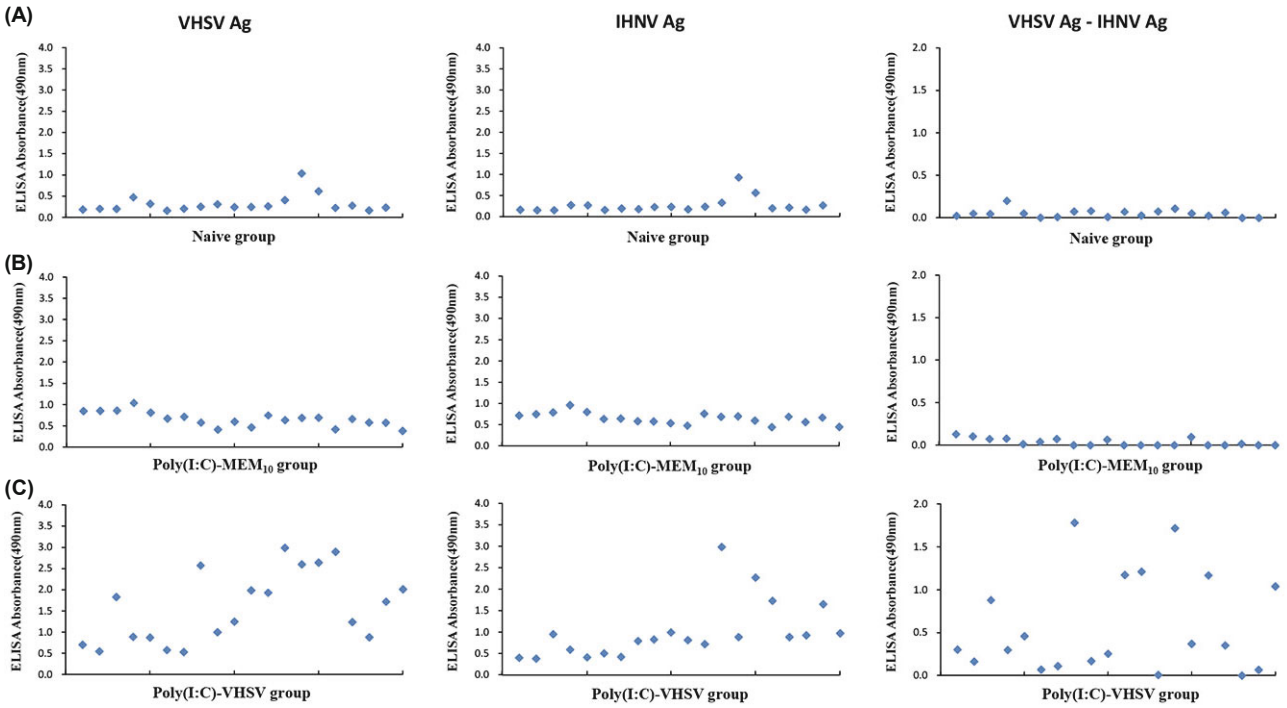

Fig. 4. A comparison of the results obtained by VHSV-Ag ELISA and IHNV-Ag ELISA of fish sera pre-treated with 5% skim milk and 50% FBS. ELISA titers of fish sera from Naïve (A), Poly (I:C)-MEM_{10} (B), and Poly (I:C)-VHSV groups (C). The final anti-VHSV titers were obtained by subtracting the antibody titer value of the IHNV-Ag assay from that of VHSV-Ag assay for each sample.

lue yielded a difference of 0.033, indicating that anti-VHSV antibodies were not produced (Fig. 4B). Average values for VHSV-Ag reactivity from the Poly (I:C)-VHSV group was 2.22-fold lower, decreasing from 3.512 to 1.582; on the IHNV-Ag plate, the decrease was 3.31 fold, from 3.323 to 1.005. However, the average subtracted value of the IHNV-Ag from the VHSV-Ag increased 3.47-fold, from 0.167 to 0.579 (Fig. 4C). Thus, the specificity of our assay to determine the antibody response against VHSV in the Poly (I:C)-VHSV group could be greatly enhanced with pretreatment of the sera with 5% skim milk and 50% FBS, and subsequently subtracting the signal from two novirhabdoviruses from the specific VHSV signal.

In this study, ELISA was used to detect anti-VHSV specific antibodies in the sera of olive flounder injected with live VHSV following Poly (I:C) administration. The addition of the Poly (I:C)-MEM_{10} group was a novel negative control for a live virus vaccine system. We suggest that for accurate detection by ELISA of specific antibodies against live or formalin-inactivated vaccines in cell culture medium containing FBS, it is necessary to select an appropriate negative control group and eliminate the signal from antibodies against FBS.

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References

Choi, B., Gye, H.J., Oh, M.J., and Nishizawa, T. 2014. Cell culture medium inhibits antigen binding used in an ELISA for detection of antibodies against nervous necrosis virus. *J. Aquat. Anim. Health* **26**, 168–172.

Guo, F.C. and Woo, P.T. 2004. Detection and quantification of *Spironucleus barkhanus* in experimentally infected Atlantic salmon *Salmo salar*. *Dis. Aquat. Organ.* **61**, 175–178.

Höglund, J. and Pilström, L. 1995. Mechanical isolation and characterization of antigens from adult *Anguillicola crassus*. *Fish Shellfish Immunol.* **5**, 51–60.

Kibenge, M.T., Opazo, B., Rojas, A.H., and Kibenge, F.S. 2002. Serological evidence of infectious salmon anaemia virus (ISAV) infection in farmed fishes, using an indirect enzyme-linked immunosorbent assay (ELISA). *Dis. Aquat. Organ.* **51**, 1–11.

Kim, H.J. 2010. Phylogenetic analysis of infectious hematopoietic necrosis virus (IHNV) isolated from cultured rainbow trout *Oncorhynchus mykiss* in Korea. *J. Fish Pathol.* **23**, 1–8.

Kim, M.S. and Kim, K.H. 2011. Inhibition of viral hemorrhagic septicemia virus replication using a short hairpin RNA targeting the G gene. *Arch. Virol.* **156**, 457–464.

Kim, W.S., Mochizaki, M., Nishizawa, T., and Yoshimizu, M. 2008. Detection of specific antibodies against infectious hematopoietic necrosis virus from rainbow trout sera by ELISA using two Novirhabdoviruses. *Fish Pathol.* **43**, 112–116.

Kim, W.S., Nishizawa, T., and Yoshimizu, M. 2007. Non-specific adsorption of fish immunoglobulin M (IgM) to blocking reagents on ELISA plate wells. *Dis. Aquat. Organ.* **78**, 55–59.

Kim, H.J., Oseko, N., Nishizawa, T., and Yoshimizu, M. 2009. Protection of rainbow trout from infectious hematopoietic necrosis (IHN) by injection of infectious pancreatic necrosis virus (IPNV) or Poly(I:C). *Dis. Aquat. Organ.* **83**, 105–113.

Knopf, K., Naser, K., van der Heijden, M.H., and Taraschewski, H. 2000. Evaluation of an ELISA and immunoblotting for studying the humoral immune response in *Anguillicola crassus* infected European eel *Anguilla anguilla*. *Dis. Aquat. Organ.* **43**, 39–48.

Kwon, S.R., Nishizawa, T., Takami, I., and Yoshimizu, M. 2010. Antibody detection against red sea bream iridovirus (RSIV) in Yellowtail *Seriola quinqueradiata* using ELISA. *Fish Pathol.* **45**, 73–76.

Nishizawa, T., Takami, I., Kokawa, Y., and Yoshimizu, M. 2009. Fish immunization using a synthetic double-stranded RNA Poly(I:C), an interferon inducer, offers protection against RGNNV, a fish nodavirus. *Dis. Aquat. Organ.* **83**, 115–122.

Nishizawa, T., Takami, I., Yang, M., and Oh, M.J. 2011a. Live vaccine of viral hemorrhagic septicemia virus (VHSV) for Japanese flounder at fish rearing temperature of 21°C instead of Poly (I:C) administration. *Vaccine* **29**, 8397–8404.

Nishizawa, T., Takami, I., Yoshimizu, M., and Oh, M.J. 2011b. Required dose of fish nervous necrosis virus (VNN) for poly (I:C) immunization of sevenband grouper *Epinephelus septemfasciatus*. *Aquaculture* **311**, 100–104.

Oh, M.J., Kim, W.S., Seo, H.G., Gye, H.J., and Nishizawa, T. 2013. Change in infectivity titre of nervous necrosis virus (NNV) in brain tissue of sevenband grouper, *Epinephalus fasciatus* Thunberg, with Poly(I:C) administration. *J. Fish Dis.* **36**, 159–162.

Oh, M.J., Takami, I., Nishizawa, T., Kim, W.S., Kim, C.S., Kim, S.R., and Park, M.A. 2012. Field tests of Poly(I:C) immunization with nervous necrosis virus (NNV) in sevenband grouper *Epinephelus septemfasciatus* (Thunberg). *J. Fish Dis.* **35**, 187–191.

Olesen, N.J., Lorenzen, N., and Jørgensen, P.E.V. 1991. Detection of rainbow trout antibody to Egtved virus by enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), and plaque neutralization tests (50% PNT). *Dis. Aquat. Organ.* **10**, 31–38.

Takami, I., Kwon, S.R., Nishizawa, T., and Yoshimizu, M. 2010. Protection of Japanese flounder *Paralichthys olivaceus* from viral hemorrhagic septicemia (VHS) by Poly(I:C) immunization. *Dis. Aquat. Organ.* **89**, 109–115.