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Detection of Specific Antibodies against Infectious Hematopoietic Necrosis Virus from Rainbow Trout Sera by ELISA using Two Novirhabdoviruses

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ABSTRACT—An enzyme-linked immunosorbent assay (ELISA) with viral culture fluids as capture antigens was performed to detect specific antibodies against infectious hematopoietic necrosis virus (IHN) from rainbow trout *Oncorhynchus mykiss* sera. When using IHN antigen (IHN-Ag), ELISA OD values for IHN-survived (IHN-Surv) rainbow trout sera were relatively higher than those for specific pathogen free (SPF) fish sera. However, some of the SPF sera were diagnosed as positive due to high OD values (> 0.2). To clarify reasons for these high OD values, each of five IHN-Surv and SPF sera was subjected to ELISA with viral hemorrhagic septicemia virus (VHSV) and hiramé rhabdovirus (HIRRV) antigens (VHSV-Ag and HIRRV-Ag). Some of the IHN-Surv and SPF sera showed high OD values in both VHSV-Ag and HIRRV-Ag plates even though there was no possibility that those sera contained antibodies against VHSV or HIRRV antigen. These results suggest that some of the sera contained antibodies against impurities in viral culture fluids such as FBS and cell debris, and caused the false-positive reactions. The corrected OD values, subtracted the OD values in VHSV-Ag plates from those in IHN-Ag plates, were all < 0.2, in SPF sera (n = 148) while those in IHN-Surv sera (n = 238) were randomly distributed from 0 to 0.7. It was considered that the corrected OD values may represent true values recognized by IHN-specific antibodies.

Key words: antibody detection, ELISA, *Oncorhynchus mykiss*, IHN, background

Infectious hematopoietic necrosis (IHN) is one of the most serious virus diseases for salmonid fish, because outbreaks of IHN result in losses more than 70% depending on the species and size of the fish, the virus strain and environmental conditions (Wolf, 1988). IHN virus (IHN) is a member of the genus *Novirhabdovirus* in the family *Rhabdoviridae*, and consists of a linear single-strand, negative-sense RNA genome with approximately 11 k nucleotides and five structural proteins; nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L). Moreover, a non-virion protein (NV) encoded between G and L genes is an important common character among novirhabdoviruses (Kurath *et al.*, 1985; Tordo *et al.*, 2005). IHN is originally enzootic in sockeye salmon *Oncorhynchus nerka* in the Pacific Northwest of North America, but it was spread to Asian and European

countries by transportation of IHN-contaminated fish eggs (Winton, 1991; Bootland and Leong, 1999). The introduced IHN was spread throughout wide areas of Japan in the 1970s, and then adapted and evolved in rainbow trout *O. mykiss* farm environments (Nishizawa *et al.*, 2006). Moreover, parts of the evolved IHN isolates in Japan were transported to Korea (Nishizawa *et al.*, 2006; Kim *et al.*, 2007a). In general, pathogenicity of IHN has a bias toward alevin to fry. However, in recent years significant mortalities by IHN have occurred in juvenile and marketable grown-up fish of rainbow trout. Under the circumstance on the epidemiology of IHN in Japan, it was interested in how the evolutionary divergence influences viral pathogenicity (Nishizawa *et al.*, 2006).

Various methods for direct detection of IHN were established, such as a neutralization test, immunofluorescent antibody techniques (IFAT), enzyme-linked immunosorbent assay (ELISA) and RT-PCR, etc. Especially, ELISA is convenient for routine screening of

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numerous samples satisfying high sensitivity, rapidity, low cost and sampling of sera without killing fish. Specific antibody detection ELISA will be useful for indirect detection of IHN to elucidate infection history of fish. A lot of studies using antibody detection ELISA have been reported in fish (Jorgensen *et al.*, 1991; Olesen *et al.*, 1991; Yoshimizu *et al.*, 1992; Ristow *et al.*, 1993; LaPatra, 1996; Nishida *et al.*, 1998; Watanabe *et al.*, 1998; Kibenge *et al.*, 2002). However, it has not been internationally accepted due to low reproducibility, which came from high background of non-specific reaction of fish antibodies (Olesen *et al.*, 1991; Höglund and Pilström, 1994; 1995; Knopf *et al.*, 2000; Kibenge *et al.*, 2002; Kim *et al.*, 2007b). In our previous study, it was reported that fish antibodies adsorbed non-specifically to ELISA blocking reagents, and moreover the non-specific adsorption could be suppressed by pre-treatment of fish sera with skim milk solution (Kim *et al.*, 2007b).

In the present study, according to procedures by Kim *et al.* (2007b), sera of IHN-survived and specific pathogen free (SPF) rainbow trout were subjected to antibody detection ELISA with IHN-culture fluid as IHN antigens. It was revealed that some of those sera reacted with impurities in IHN-culture fluids, moreover that IHN-specific antibodies were detectable by subtraction of ELISA values due to such impurities in viral culture fluids using an ELISA system with serologically heterologous novirhabdoviruses, viral hemorrhagic septicemia virus (VHSV) or hiram rhabdovirus (HIRRV).

Materials and Methods

Viruses for ELISA antigens

IHN ChAb7601 from chum salmon *O. keta* (Yoshimizu *et al.*, 1988), and VHSV Obama25 (Nishizawa *et al.*, 2002) and HIRRV H8401-H (Kimura *et al.*, 1986; Nishizawa *et al.*, 1991) from Japanese flounder *Paralichthys olivaceus* were used in the present study. The viruses were propagated in epithelioma papulosum cyprini (EPC) cells maintained at 15°C with Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin G and 100 mg/mL streptomycin sulfate. Viral culture supernatants were clarified by centrifugation at $19,000 \times g$ for 30 min to eliminate cell debris. The infectivity titers of IHN, VHSV and HIRRV were $10^{8.3}$, $10^{8.1}$ and $10^{7.8}$ TCID₅₀/mL, respectively. The clarified viral solutions were stored at -80°C until use for ELISA antigens.

Rainbow trout sera

Blood samples of IHN-survived rainbow trout (10–2,020 g of body weight) were obtained from 238 individuals reared at four farms (named A, B, C and D) in Shizuoka Prefecture, Japan in 2006 and 2007 (IHN-Surv

sera), and those of SPF rainbow trout (2–330 g of body weight) were from 148 individuals at other four farms (E, F, G and H) in Shizuoka and Yamagata Prefectures, Japan (SPF sera). Fish sera were collected by centrifugation of clotted blood samples at $2,000 \times g$ for 10 min at 4°C and stored at -20°C until use.

Antibody detection ELISA

ELISA plates (Greiner) were coated with 50 µL of the viral antigens (IHN, VHSV or HIRRV), diluted 1:10 with Dulbecco's phosphate buffered saline (PBS), and then incubated at 4°C overnight. After addition of 50 µL neutrally buffered formalin (0.74%), the plates were incubated at 4°C for additional overnight to inactivate the virus for preventing an efflux of the pathogen. Plates were washed 3 times with PBS containing 0.05% Tween 20 (T-PBS), blocked with 5% skim milk in PBS at 25°C for 1 h, and washed again three times with T-PBS. Procedures for pre-treatment of fish sera and antibody detection ELISA were carried out according to the method described by Kim *et al.* (2007b). Briefly, ELISA plates were incubated with 50 µL 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 against 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 three times with T-PBS, 50 µL of substrate solution (1 mg/mL *o*-phenylenediamine, 0.03% H₂O₂, 100 mM Na₂HPO₄, 50 mM citric acid) was added to each well. After 30 min of incubation at 25°C, the reaction was stopped with 2 N H₂SO₄, and then the absorbance at 492 nm (OD₄₉₂) was read using a microplate reader (MTP-300, Corona), and results expressed as OD values.

Results and Discussion

ELISA absorbance values of IHN-survived and SPF rainbow trout sera were compared using the ELISA plate with IHN antigen (Fig. 1). ELISA values for the IHN-survived fish sera (IHN-Surv sera) ranged from 0.26 to 1.03 (0.55 ± 0.17), and those of SPF fish sera (SPF sera) ranged from 0.04 to 0.64 (0.18 ± 0.15). Although the OD values for IHN-Surv sera were generally higher than those of SPF sera, ten out of 33 SPF sera (30%) showed over 0.2 of OD values, which seemed to be positive as IHN-specific antibodies. The high OD values observed in some of the SPF sera were not considered due to non-specific adsorption of fish IgMs, because all the fish sera were pre-treated with 5% skim milk solution to prevent it as described by Kim *et al.* (2007b). Therefore, we considered that fish IgMs in those SPF sera could react with some antigens derived from IHN culture fluids on the ELISA plate wells.

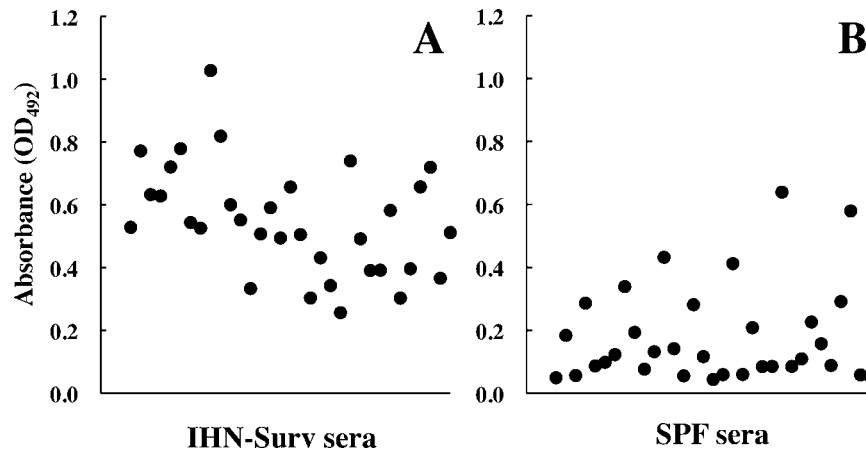


Fig. 1. Comparison of ELISA absorbance values (OD₄₉₂) for IHN-survived and SPF rainbow trout sera using IHN culture fluid as an antigen. A: IHN-survived fish sera, B: SPF fish sera.

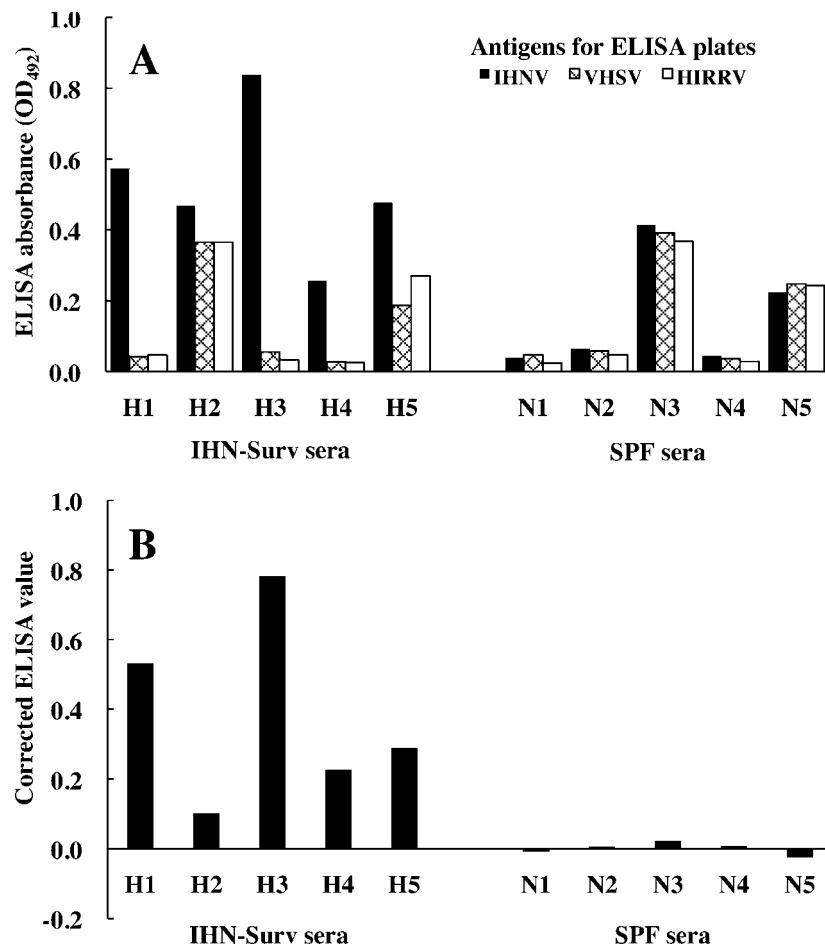


Fig. 2. Comparison of ELISA absorbance values (OD₄₉₂) for IHN-survived and SPF rainbow trout sera using different antigens, IHN, VHS and HIR culture fluids as capture antigens (A). The ELISA values for specific reaction of fish antibodies against IHN were calculated by subtracting the ELISA values with VHS antigen from those with IHN antigen (B).

To explain our presumption about the reactions between fish IgMs and IHN culture fluids, five IHN-Surv and five SPF sera were selected from the previous experiment, and were subjected to ELISA with IHN,

VHS and HIR culture fluids (Fig. 2A). The IHN-Surv sera showed high ELISA values ranging from 0.26 to 0.84 in the ELISA plate with IHN-Ag plate). Of the five IHN-Surv sera, three sera (H1, H3

and H4) showed < 0.1 of OD values in VHSV or HIRRV antigen (VHSV-Ag or HIRRV-Ag plate, respectively), which were distinctly lower than those in the IHNV-Ag plate. With the remaining two IHN-Surv sera (H2 and H5), the OD values in VHSV-Ag or HIRRV-Ag plate ranged from 0.19 to 0.37, which were slightly lower than those in IHNV-Ag plates (Fig. 2A). On the other hand, the SPF sera showed OD values from 0.04 to 0.41 in IHNV-Ag plates, and the OD values for the sera N3 and N5 were relatively higher than those for other three sera, N1, N2 and N4. These data imply that the N3 and N5 sera are positive as IHNV-specific antibodies even though they are SPF sera. Interestingly, both the SPF sera, N3 and N5, also showed high OD values in the VHSV-Ag or HIRRV-Ag plate, the values of which were almost the same as those in the IHNV-Ag plate (Fig. 2A).

In the present results, two interesting features were observed as follows. The first one was that no significant difference was observed in the OD values between the VHSV-Ag and HIRRV-Ag plates regardless of IHNV infection history of each serum. The second feature was that the IHN-Surv sera showed significantly higher OD values in IHNV-Ag plates than those in VHSV-Ag and HIRRV-Ag plates. However, the SPF sera showed almost the same OD values among in IHNV-Ag, VHSV-Ag and HIRRV-Ag plates. All of the present fish sera had no possibility containing antibodies against VHSV and HIRRV antigens, because VHSV or HIRRV infection have never been recorded at any rainbow trout farms in Japan. Moreover, cross-reaction of IHNV-specific antibodies with VHSV antigen was negligible level in ELISA, 50% plaque neutralization test and IFAT, although some common antigens were detected among IHNV, VHSV and HIRRV in Western blot analysis with rabbit sera against IHNV and HIRRV (Jorgensen *et al.*, 1991; Nishizawa *et al.*, 1991). Therefore, it is considered that the OD values for fish sera observed in the VHSV-Ag or HIRRV-Ag plate might be caused by specific reaction of fish antibodies with impurities commonly existing among the IHNV, VHSV and HIRRV culture fluids, for example

antigens derived from cultured cells or cell debris. It was confirmed that those SPF sera with high OD values in ELISA with IHNV-Ag, VHSV-Ag and HIRRV-Ag plates was due to reaction with FBS and/or cell debris in culture fluids (data not shown). The present results suggest that corrected OD values, obtained by subtracting the OD values in VHSV-Ag or HIRRV-Ag plates from those in IHNV-Ag plates, are close to true OD values at least due to IHNV-specific antibodies reacted with IHNV antigens. In fact, the corrected OD values for IHN-Surv sera were ranged from 0.1 to 0.78, while those of SPF sera were less than 0.02 (Fig. 2B), meaning that all SPF sera were negative for IHNV-specific antibodies, which were completely consisted with fish histories of IHNV epizootics. It was therefore concluded that high ELISA values for SPF sera observed in IHNV-Ag plates were false positive reactions due to impurities in virus culture fluids, such as FBS and cell debris.

To evaluate the IHNV-specific antibody detection ELISA system using IHNV-Ag and VHSV-Ag plates, 238 IHN-Surv sera from the farms A to D in Shizuoka Prefecture and 148 SPF sera from the farms E to H in Shizuoka and Yamagata Prefectures were subjected (Fig. 3). The corrected OD values for each serum (Fig. 3) were calculated by subtracting the OD values in the VHSV-Ag plate from those in the IHNV-Ag plate. The corrected OD values for IHN-Surv sera were randomly distributed from 0 to 0.7 regardless of the farms. Although only three out of 148 SPF sera showed OD values between 0.1 and 0.2, the other SPF sera showed all less than 0.1. That is, the corrected OD values were clearly different between IHN-Surv and SPF sera, and almost all of the SPF sera showed < 0.1 of OD values. These results also support that the corrected OD values are infinitely near to the true values by IHNV-specific antibodies in rainbow trout sera.

In the present study, it was obvious that rainbow trout sera contained antibodies reacting to impurities derived from the viral culture fluids although such antibodies reacting with those impurities were quantitatively

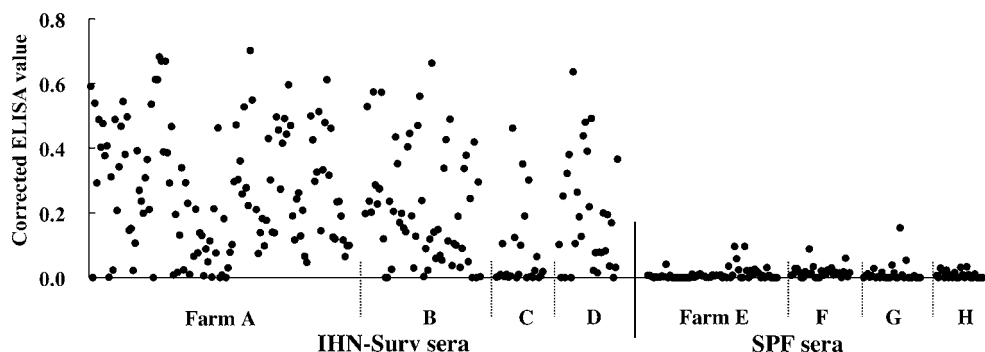


Fig. 3. Detection of IHNV-specific antibodies from IHN-survived and SPF rainbow trout by antibody-detection ELISA using two viral antigens. The IHN-survived sera were obtained from fish reared in the farms A to D, while SPF sera were from the farms E to H. The ELISA values were calculated by subtracting the ELISA values with VHSV antigen from those with IHNV antigen.

different among the individual sera. Kibenge *et al.* (2002) previously reported similar phenomena that antibodies in sera of Atlantic salmon *Salmo salar* and coho salmon *O. kisutch* reacted with cellular antigens in antibody detection ELISA. We speculate that such antibodies cause false positive reactions in fish antibody detection ELISA. Therefore, culture fluids of serologically different viruses should be useful as ELISA capture antigens for the detection of specific antibodies in fish sera. Highly purified IHN virus antigens are difficult to prepare because IHN virus has an envelope derived from host cell membranes. The devised method with two viral culture fluids made it possible to detect specific antibodies in fish sera. Moreover, one of highlighted advantages in the present method is of using virus culture fluids, which is easier than purification of viral antigens.

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