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Evaluation of enzyme-linked immunosorbent assay (ELISA) for detection of olive flounder antibodies to viral hemorrhagic septicemia virus (VHSV, genotype IVa) using two *Novirhabdovirus* antigens

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An enzyme-linked immunosorbent assay (ELISA) with two *Novirhabdovirus* antigens (viral hemorrhagic septicemia virus, VHSV and infectious hematopoietic necrosis virus, IHNV) was used to detect specific antibodies against VHSV from olive flounder (*Paralichthys olivaceus*) sera. In ELISA plates with VHSV culture supernatants (VHSV-Ag plate), optical density (OD) values for sera from olive flounder with VHS history (VHS sera) ranged from 0.64±0.36, and those of sera from fish without VHS history (non-VHS sera) ranged from 0.26±0.26. In IHNV-Ag plate, the OD values (0.43±0.28) for VHS sera were quite low compared to those in VHSV-Ag plates, while the OD values for non-VHS sera were almost similar. When the OD values for each serum were calculated by subtracting the OD values in the IHNV-Ag plate from those in the VHSV-Ag plate, the corrected OD values were significantly different between VHS sera and non-VHS sera. The results were completely in line with fish histories of VHS epizootics. It was considered that the corrected OD values may represent the true values recognized by VHSV-specific antibodies.

Key words: Antibody detection·ELISA·*Paralichthys olivaceus*·VHSV·two antigens

Introduction

Viral hemorrhagic septicemia virus (VHSV) is the etiological agent of viral hemorrhagic septicemia (VHS). VHS is one of the most serious viral diseases affecting farmed rainbow trout (*Oncorhynchus mykiss*) in European countries and olive flounder (*Paralichthys olivaceus*) in Far East Asia (Isshiki *et al*., 2001; Kim *et al*., 2009; Smail, 1999; Skall *et al*., 2005; Wolf, 1988). The Far East Asian VHSV isolates (genotype IVa) from olive flounder are phylogenetically distinguishable from North American and European isolates, with a low virulence to rainbow trout fry (Kim *et al*., 2011a; Kim *et al*., 2011b; Nishizawa *et al*., 2002; Skall *et al*., 2005).

VHSV is a member of the genus *Novirhabdovirus* belonging to family *Rhabdoviridae*, and consists of a negative single-stranded RNA genome with approximately 11,000 nucleotides and five structural proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase (L) (Trdo *et al*., 2005). It is genetically and serologically distinguishable from other fish novirhabdoviruses, such as the infectious hematopoietic virus (IHNV) and hirame rhabdovirus (HIRRV) (Isshiki *et al*., 2001; Kimura *et al*., 1986; Trdo *et al*., 2005).

VHSV is generally isolated from fish tissues in cell culture followed by confirmation of the isolated virus

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by reverse-transcriptase polymerase chain reaction (RT-PCR) (WOAH, 2021). During and immediately following VHS outbreaks, VHSV is readily isolated using this technique, although detection of the virus from non-clinical fish is a challenge. Antibody detection assays, such as neutralization tests and enzyme-linked immunosorbent assay (ELISA) are used to elucidate the history of infection in live and clinically healthy fish (Kim *et al*., 2008; LaPatra, 1996; Watanabe *et al*., 1998). ELISA is a convenient and rapid technique for routine screening of numerous samples with cost effectiveness. However, antibody-detection ELISA is not a recognized method endorsed by the WOAH for international diagnostic testing because it is associated with low reproducibility partly due to high background optical density (OD) caused by non-specific reactions between fish antibodies and antigens and non-specific adsorption of fish immunoglobulin M (IgM) (Kibenge *et al*., 2002; Kim *et al*., 2007a; Knopf *et al*., 2000; Olesen *et al*., 1991). In our previous study, the non-specific adsorption of fish IgM was suppressed by pre-treatment of fish sera with skim milk solution (Kim *et al*., 2007a).

In addition, IHNV-specific antibodies were detected in rainbow trout sera by subtraction of ELISA values using an ELISA system with two serologically heterologous *Novirhabdovirus* antigens, IHNV and VHSV or HIRRV (Kim *et al*., 2008). VHSV (genotype IVb)-specific antibodies were detected in muskellunge sera by competitive ELISA, which the fish sera were heat-treated and pre-incubated with nonfat milk, and purified virus was used (Millard *et al*., 2014). Thus, the specific antibodies in fish can be detected by partly modifying ELISA. In the present study, we evaluated an ELISA system with two *Novirhabdovirus* antigens (VHSV and IHNV) to detect VHSV (genotype IVa)-specific antibodies in olive flounder.

Materials and Methods

Viruses for ELISA antigens

VHSV (isolate: FYeosu05, genotype IVa) from olive flounder and IHNV (isolate: RtUi02) from rainbow trout were used in this study (Kim *et al*., 2007b; Kim *et al*., 2009). The viruses were propagated with epithelioma papilosum cyprini (EPC) cells maintained at 15°C in Eagle's minimum essential medium (EMEM) 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 4,000 × g for 30 min to eliminate cell debris. The clarified viral solutions were stored at -80°C until use for ELISA. The infectivity titers of VHSV and IHNV were $10^{8.3}$ and $10^{7.8}$ tissue culture infective dose $(TCID)_{50}$/ml, respectively.

Olive flounder sera

Blood samples (VHS sera) of olive flounder (body weight: 26.1-435 g) with VHS history were obtained from 109 individuals reared at six farms (labeled A, B, C, D, E and F) in Jeju and Wando from 2015 to 2017, and those (non-VHS sera) of olive flounder (1.35-310 g) without VHS history were sampled from 115 individuals at seven other farms (g, h, i, j, k, l, and m) in Hampyeong, Muan, Wando and Yeosu (Table 1). Fishes of VHS occurred farm were observed mass mortality and clinical signs of the disease. Blood samples were taken from caudal vein and fish sera were collected by centrifugation of clotted blood samples at 2,000 × g for 10 min at 4ºC and stored at -80ºC until use.

Antibody detection: ELISA

ELISA was conducted using two *Novirhabdovirus* antigens (VHSV and IHNV) according to the methods described by previous studies (Kim *et al*., 2007a; Kim *et al*., 2008). ELISA plates (Greiner, Germany) were coated with 50 µl of each virus culture supernatant (VHSV and IHNV), diluted 1:320 with distilled water, and incubated at 37ºC overnight. ELISA plates with VHSV and IHNV antigens (VHSV-Ag plate and IHNV-Ag plate) were washed 3 times with phosphate

Table 1. Information on olive flounder used for antibody detection ELISA

Farm	Place	Period of VHS outbreak	Cumulative mortality rate (%)	Sampling		Fish	
				Date	Water temp. (ºC)	Number	Weight (g)
A	Jeju	Mar-Jun, 2015	60	Jun, 2015	20	26	4.9±0.3
B	Wando	Dec, 2015 - Jan, 2016	30	Feb, 2016	12	20	90.5±25.5
C		Mar - Apr, 2016	66	May, 2016	17.8	18	37.3±6.7
D		Mar - Apr, 2016	95	May, 2016	18.5	20	39.0±6.1
E		Dec, 2016 - Apr, 2017	50	Jun, 2017	17	13	314.2±38.5
F		Jan - Mar, 2017	25	Jun, 2017	19.5	12	317.9±55.4
g	Hampyeong	-	0	Mar, 2015	18	32	3.6±0.8
h	Muan	-	0	Jun, 2015	16	30	4.9±0.6
i	Wando	-	0	Sep, 2016	24.5	12	192.0±39.5
j		-	0	Sep, 2016	25	11	180.0±22.7
k	Yeosu	-	0	Dec, 2016	8.5	10	103.5±23.2
l		-	0	Dec, 2016	8.5	10	130.0±21.0
m		-	0	Feb, 2017	5	10	85.8±26.3

buffered saline containing 0.05% Tween 20 (T-PBS), blocked with 5% skim milk in PBS at 25ºC for 1 h, and washed again three times with T-PBS. During blocking of ELISA plate, fish sera were diluted 1:40 in 5% skim milk and reacted at 25ºC for 1h to prevent the non-specific adsorption of fish IgM. ELISA plates were incubated with 50 µl of fish sera (primary) for 1 h, and then incubated at 25ºC for 1h with monoclonal antibody (secondary) against olive flounder IgM (Aquatic Diagnostics Ltd, UK) and anti-mouse IgG swine Ig (tertiary) conjugated with horseradish peroxidase (Younginfrontier, Korea). Before use, both secondary and tertiary antibodies were diluted 1:1,000 in 5% skim milk. After washing 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, 50 mM citric acid) was added to each well. After 30 min of incubation at 25ºC, the reaction was stopped with 2 N H_2SO_4, and the absorbance at 490 nm (OD_{490}) was read using a microplate reader (Molecular Devices, USA), and results expressed as OD values. Fish serum samples were analyzed in duplicate. Each serum obtained from VHS-surviving olive flounder and VHSV-free fish was used in ELISA as positive and negative controls.

Results and Discussion

One hundred nine VHS sera (Farms A to F) and 115 non-VHS sera (Farms g to m) were subjected to ELISA with two *Novirhabdovirus* antigens, VHSV and IHNV to detect VHSV specific antibodies in olive flounder (Table 1 and Fig. 1). In VHSV-Ag plates, OD values for the VHS sera ranged from 0.08 to 2.96 (mean OD value: 0.64 ± 0.36), and those of non-VHS sera ranged from 0.04 to 3.32 (0.26 ± 0.26) (Fig. 1A). The OD values for VHS sera were generally higher than those of non-VHS sera except for sera from i farm. In IHNV-Ag plates, OD values for the VHS sera ranged from 0.09 to 2.61 (0.43 ± 0.28), and those of non-VHS sera ranged from 0.04 to 3.38 (0.28 ± 0.26) (Fig. 1B). The OD values for VHS sera were different from those observed in VHSV-Ag plates, while the OD values for non-VHS sera were almost

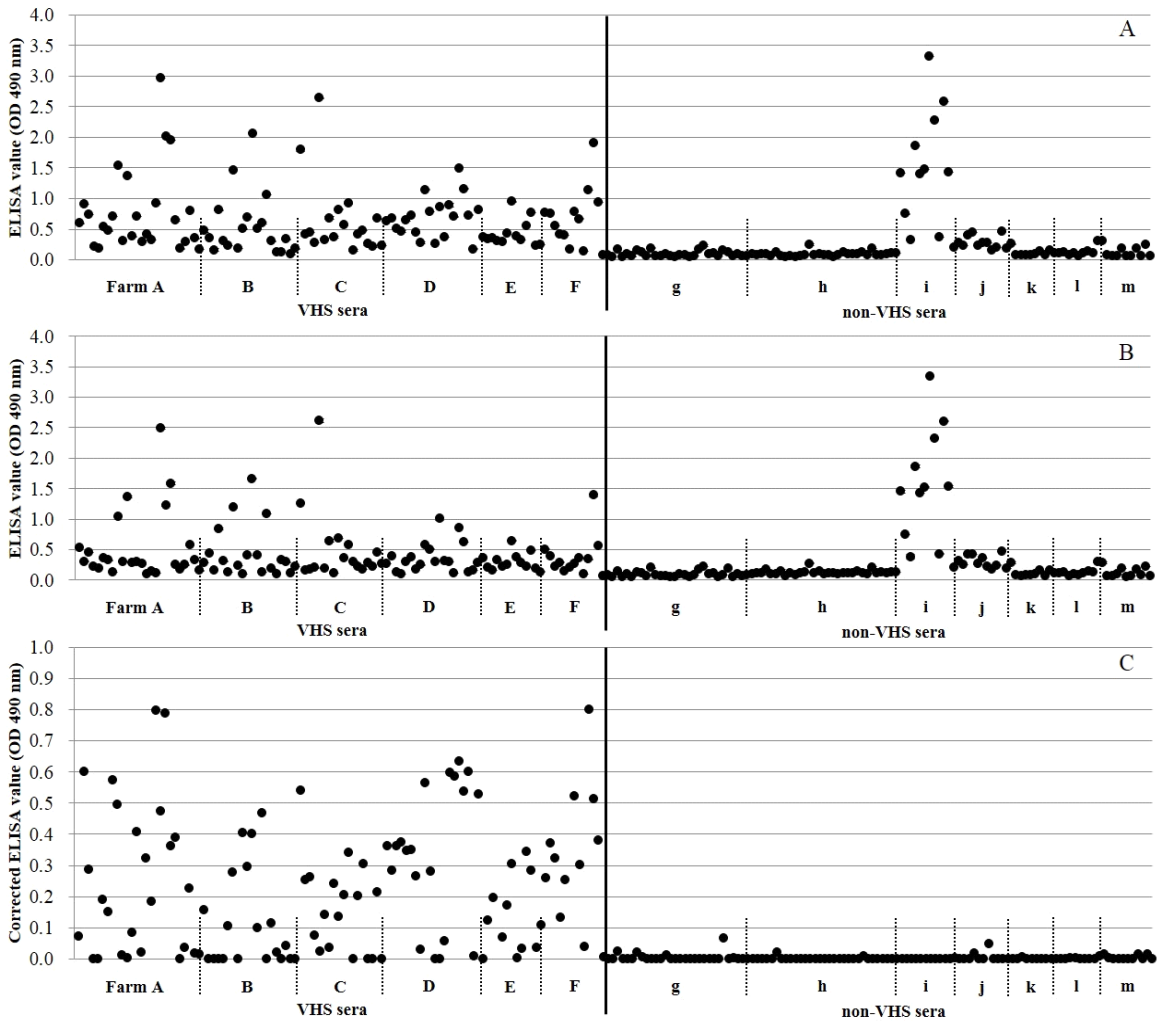

Fig. 1. Comparison of absorbance values (OD_{490}) in olive flounder sera with VHS history (VHS sera) and non-VHS history (non-VHS sera) in ELISA with VHSV and IHNV culture fluids (A: VHSV antigen, B: IHNV antigen). The ELISA values for specific reaction of fish antibodies against VHSV were calculated by subtracting the ELISA values with IHNV antigen from those with VHSV antigen (C). The VHS sera were obtained from olive flounder in farms A to F, while non-VHS sera were obtained from the farms g to m.

similar. When the OD values for each serum were calculated by subtracting the OD values in the IHNV-Ag plate from those in the VHSV-Ag plate, the OD values for VHS sera were distributed from 0 to 0.79 regardless of the farms, while all the 115 non-VHS sera showed less than 0.06 (Fig. 1C), suggesting that the corrected OD values varied clearly between VHS and non-VHS sera. These results were completely consistent with histories of VHS epizootics, indicating that the corrected OD values were close to true OD values at least due to VHSV-specific antibodies reacting VHSV. The 115 fish sera without VHS history all showed OD value less than 0.06. These results supposed that fish sera of OD value less than 0.06 had no VHSV-specific antibodies. However, further studies are needed to establish a cut-off line of OD

value through a large number of sera without VHSV infection history.

The high OD values were observed in a few olive flounder sera in IHNV-Ag plates (Fig. 1B), even though IHNV was never isolated or detected from any marine fishes in Korea. A few non-VHS sera also showed high OD values in VHSV-Ag and IHNV-Ag plates (Fig. 1A and 1B). Therefore, we considered that a few olive flounder IgMs react with specific antigens derived from virus culture supernatants on the ELISA plate. To evaluate our hypothesis of the reaction between fish IgMs and viral culture fluids, each of the 15 VHS sera and non-VHS sera were selected from the previous experiment, and subjected to ELISA with viral (VHSV and IHNV) culture supernatants and 10% FBS because culture fluids contained 10% FBS (Fig. 2). The VHS sera showed high OD values ranging from 0.32 to 1.34 in VHSV-Ag plate, while the OD values in IHNV-Ag and FBS-Ag plates ranged from 0.09 to 0.71, which were distinctly lower than in VHSV-Ag plate. The OD values of six VHS sera (V1 to V6) in IHNV-Ag plate were slightly higher than in FBS-Ag plate, while the OD values of eight sera (V8 to V15) were almost similar to both IHNV-Ag and FBS-Ag plates. The VHS sera had no possible antibodies against IHNV, because IHNV infection was never recorded at any olive flounder farms. Therefore, the similar OD values in IHNV-Ag and FBS-Ag plates may be attributed to the reaction of fish IgMs with FBS. In addition, the slightly higher OD values in IHNV-Ag plate than in FBS-Ag plate are likely caused by reaction of fish IgMs with cell debris existing in IHNV culture fluids. On the other hand, the non-VHS sera showed OD values ranging from 0.15 to 1.77 in VHSV-Ag plate, which were almost similar to those in IHNV-Ag and FBS-Ag plates. These results showed that the high OD values for non-VHS sera in VHSV and IHNV-Ag plates were mostly due to the reaction with FBS in the VHSV and IHNV culture fluids. Kim *et al*. (2008) was reported a similar reaction between rainbow trout IgMs and FBS or cell debris in antibody detection using ELISA. Thus, it was obvious that at least a few rainbow trout and olive flounder sera contained antibodies reacting to FBS and cell debris, although such antibodies varied quantitatively among the individual sera.

Conventional VHSV purification methods based on sucrose or cesium gradient ultracentrifugation are tedious, difficult, and require access to special instrumentation. These methods also often result in low virus recovery. Moreover, VHSV has an envelope derived from host cell membranes. Therefore, the present ELISA system with two serologically heterologous *Novirhabdovirus* culture supernatants is useful for the detection of VHSV-specific antibodies in olive flounder without difficulty of antigen preparation.

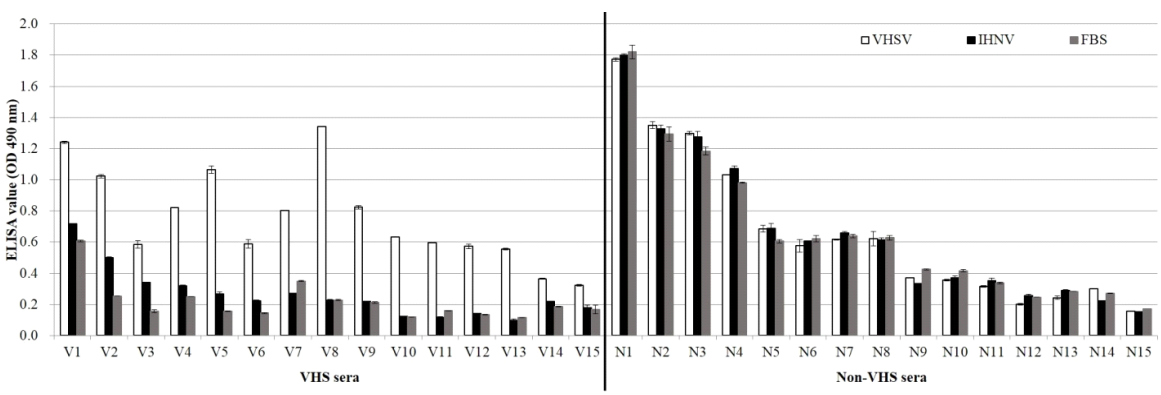

Fig. 2. Comparison of absorbance values (OD_{490}) for olive flounder sera with VHS history (VHS sera) and non-VHS history (non-VHS sera) in ELISA conducted with viral (VHSV and IHNV) culture supernatants and 10% FBS.

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