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Survey of Viral Hemorrhagic Septicemia Virus (VHSV) in Olive Flounder, *Paralichthys olivaceus* Hatchery in Korea

(Tinjauan Virus Berdarah Septisemia Viral (VHSV) di Tempat Penetasan *Olive Flounder, Paralichthys olivaceus* di Korea)

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ABSTRACT

*Viral hemorrhagic septicemia (VHS) generally occurs after juvenile olive flounders (*Paralichthys olivaceus*) are moved from the hatchery to on-growing system in Korea during spring. However, it remains unclear whether fish are infected by VHS virus (VHSV) in the hatchery or the on-growing system. In the present study, a survey was conducted to investigate VHSV infection in 39 olive flounder hatcheries from 2014 to 2017. Fish were tested for the presence of VHSV by inoculating sample to fathead minnow (FHM) and chinook salmon embryo (CHSE-214) cells to observe cytopathic effect, reverse transcription-polymerase chain reaction (RT-PCR), and antibody detection enzyme-linked immunosorbent assay (ELISA). VHSV was not detected in any of the 2,430 fish (from 461 pooled and 156 unpooled samples), although 34 (20.3%) of 167 samples was found to be positive for marine birnavirus (MABV) by cell culture and RT-PCR. Antibody detection ELISA results showed that all 212 fish sera have optical density (OD) values below of 0.1, suggesting that these fish had no VHSV-specific antibodies. Moreover, VHSV was not detected in any of 40 pooled samples (172 fish) collected after shifting rearing water temperature from 17-21°C to 10-15°C. In conclusion, the 39 olive flounder hatcheries surveyed in Korea was not infected by VHSV.*

Keywords: Hatchery; olive flounder; survey; viral hemorrhagic septicemia virus

ABSTRAK

Virus septisemia berdarah (VHS) biasanya berlaku selepas juvenil olive flounders *(*Paralichthys olivaceus*) berpindah dari tempat penetasan ke sistem perkembangan di Korea semasa musim bunga. Walau bagaimanapun, ia masih tidak jelas sama ada ikan telah dijangkiti oleh virus VHS (VHSV) di tempat penetasan atau dalam sistem perkembangan. Dalam kajian ini, suatu tinjauan telah dijalankan untuk mengkaji jangkitan VHSV pada 39 tempat penetasan* olive flounder *dari 2014 untuk 2017. Ikan telah diuji untuk kehadiran VHSV melalui inokulasi sampel* fathead minnow *(FHM) dan sel embrio* chinook salmon *(CHSE-214) untuk memerhati kesan sitopati, tindak balas rantai transkripsi-polimerase berbalik (RT-PCR) dan pengesanan antibodi enzim berkaitan cerakin imunosorben (ELISA). VHSV tidak dikesan dalam mana-mana 2,430 sampel ikan (dari 461 sampel tergembleng dan 156 tak tergembleng), walaupun 34 (20.3%) 167 adalah positif untuk birnavirus laut (MABV) melalui kultur sel dan RT-PCR. Keputusan pengesanan antibodi ELISA menunjukkan bahawa kesemua 212 sera ikan mempunyai nilai ketumpatan optik (OD) di bawah 0.1 yang menyarankan bahawa ikan ini tidak mempunyai antibodi khusus VHSV. Selain itu, VHSV juga tidak dikesan dalam mana-mana 40 sampel tergembleng (172 ikan) yang diambil selepas perubahan suhu air ternakan daripada 17-21°C kepada 10-15°C. Kesimpulannya, 39 tempat penetasan* olive flounders *yang dikaji di Korea tidak dijangkiti dengan VHSV.*

Kata kunci: Olive flounder*; tempat penetasan; tinjauan; virus septisemia berdarah viral*

INTRODUCTION

Viral hemorrhagic septicemia virus (VHSV) is the etiological agent of viral hemorrhagic septicemia (VHS), 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; Skall et al. 2005; Smail 1999; Wolf 1988). VHSV isolates from Far East Asia belong to genotype IVa and they are phylogenetically different from North American and European isolates of VHSV (Kim et al. 2011; Nishizawa et al. 2002).

VHSV had never been detected in marine or salmonid fishes in Far East Asia until 1999. However in 1999, VHSV was first isolated from wild Japanese flounder in Japan (Watanabe et al. 2002). Since then, VHSV infections with severe mortalities in olive flounder farms have frequently occurred in both Korea and Japan (Isshiki et al. 2001; Kim et al. 2009). VHSV has also been detected from several marine fish species in coasts of Korea and Japan (Kim & Park 2004a; Kim et al. 2011; Takano et al. 2000; Watanabe et al. 2002).

Olive flounders are cultured in land-based facilities in Korea. Generally, the development of olive flounder eggs

to juvenile fish stages are kept at water temperature (WT) about 18ºC in hatcheries from winter to spring. After that, juvenile fish are transferred to on-growing system during spring (March-May, WT: about 10-17ºC). VHS mainly occurs after juvenile fish are moved from hatchery to on-growing system. However, it remains unclear whether fish are infected with VHSV in the hatchery or in the on-growing system. Therefore, the objective of the present study was to determine if VHSV infection happened before the juvenile fish were transferred to the grow-out facilities by conducting a survey in a total of 39 olive flounder hatcheries in Korea from years 2014 to 2017.

Materials and Methods

Experimental fish and fish sera

A total of 2,430 juvenile olive flounders were used for VHSV detection (Table 1). Fish were randomly collected from 39 olive flounder hatcheries at Hampyeong, Yeonggwang, Muan, Gochang, Taean, Hwaseong, Buan, Incheon, Heanam and Seosan during 2014-2017 (Figure 1). Most of the fishes were wrapped in oxygen filled double plastic bags and transported to the laboratory in a cooler with sufficient ice gels. In the laboratory, they were immediately subjected to VHSV examination. Blood samples were obtained from 212 fish. Clotted blood samples were centrifuged at 2,000 × g for 10 min at 4ºC to collect sera. Sera samples were stored at -80ºC until required for further experiments.

Detection of VHSV

Most of the tissue sampled from experimental fishes were pooled as 5 fishes per sample according to the weight of the tissues except four samples that were pooled from 4 or less number of fishes (Table 1). Kidney, spleen and heart samples were homogenized in 10 volumes of Hanks' balanced salt solution (Gibco, USA) and centrifuged at 3,000 × g for 30 min. The supernatant was filtered through a 450 nm membrane filter. Then 100 µL of homogenate was inoculated into fathead minnow (FHM) and chinook salmon embryo (CHSE-214) cells in 24-well tissue culture plates (Nunc, Denmark). Inoculated cells were then incubated at 15°C for 14 days and examined daily for cytopathic effect (CPE). All cells were maintained in Dulbeco's minimum essential medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 IU penicillin/mL and 100 µg streptomycin/mL, Gibco, USA). If CPE was observed, cell culture supernatant was subjected to reverse transcription-polymerase chain reaction (RT-PCR) for the detection of VHSV and marine birnavirus (MABV, a tentative species in genus *Aquabirnavirus*). RNA was extracted using TRIZOL reagent (Gibco, USA). Reverse transcription (RT) was performed using ReverTra Ace® qPCR RT Kit (Toyobo, Japan) according to the manufacturer's instruction. PCR was performed using the following three primer sets: VN forward (5'-ATGGAAGGAGGAATTCGTGAAGCG-3') and VN reverse (5'-GCGGTGAAGTGCTGCAGTTCCC-3') primers; VGsense (5'-CCAGCTCAACTCAGGTGTCC-3') and VGanti (5'-GTCACYGTGCATGCCATTGT-3')

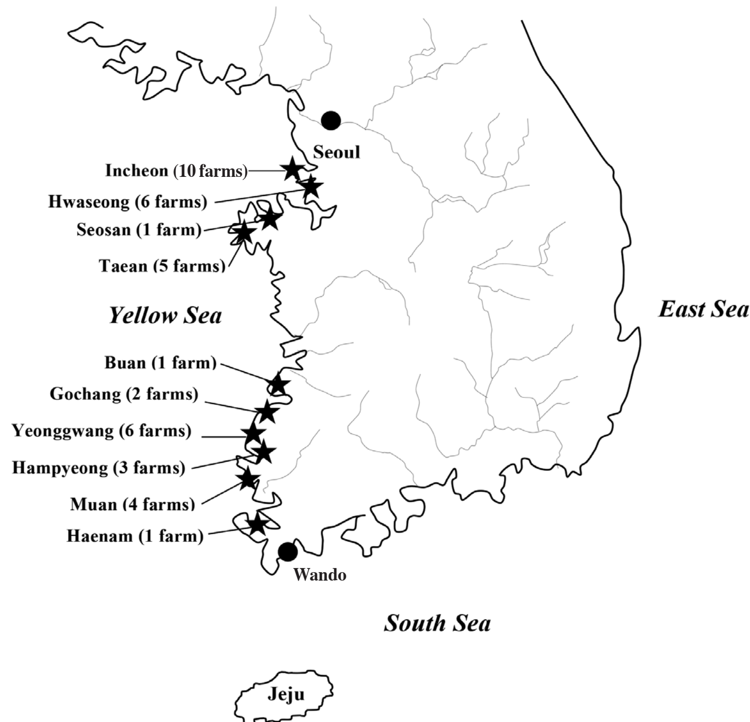

FIGURE 1. Sampling locations of olive flounder hatcheries in South Korea between years 2014 and 2017 (★)

primers for VHSV detection and P1 (5'-AGAGATCACTGACTTCACAAGTGAC-3') and P2 (5'-TGTGCACCACAGGAAAGATGACTC-3') for MABV detection (Nishizawa et al. 2005, 2002; OIE 2017; Suzuki et al. 1997). PCR amplification was performed in a MyGenie32 Thermal Block (Bioneer, Korea) with 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min for N gene of VHSV detection, 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72 min for G gene of VHSV detection, and 30 cycles of 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min for MABV detection. Amplified PCR products were subjected to electrophoresis using 1.5% agarose gels containing ethidium bromide. They were visualized under UV light. In addition, kidney, spleen, and heart tissues collected from 1,933 fish (from 384 pooled and 36 unpooled samples) were directly examined for the presence of VHSV using PCR (Table 1).

DETECTION OF VHSV-SPFECIFIC ANTIBODY

To detect VHSV-specific antibodies in olive flounders, enzyme-linked immunosorbent assay (ELISA) was conducted using two Novirhabdovirus antigens (VHSV and infectious hematopoietic virus, IHNV) by modification of the procedures described by Kim et al. (2008). VHSV (isolate: FYeosu05) from olive flounder and IHNV (isolate: RtUi02) from rainbow trout were used for preparation of ELISA antigens (Kim et al. 2009, 2007). The viruses were propagated with epithelioma papilosum cyprini (EPC) cells maintained at 15ºC 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 19,000 × g for 30 min to eliminate cell debris. The clarified viral solutions were stored at -80ºC 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.

ELISA plates (Greiner, Germany) were coated with 50 µL of VHSV culture medium (VHSV-Ag plate) and IHNV culture medium (IHNV-Ag plate) diluted with sterile distilled water at 1:320 followed by incubation at 37ºC overnight. Plates were washed three times with sterile phosphate 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. ELISA plates were incubated with 50 µL of fish sera (primary antibody), monoclonal antibody against olive flounder immunoglobulin M (IgM, secondary antibody, Aquatic Diagnostics Ltd, UK) and anti-mouse IgG swine Ig conjugated with horseradish peroxidase (tertiary antibody, Younginfrontier, Korea) each for 1 h and washed three times with T-PBS after each step. Fish sera were pre-treated with 5% skim milk (1:40) for 1 h to prevent the non-specific adsorption of fish IgM. Both secondary and tertiary antibodies were diluted in 5% skim milk at 1:1,000. 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, and 50 mM citric acid) was added to each well. After incubating at 25ºC for 30 min, the reaction was stopped with 2 N H_2SO_4. The absorbance value at 490 nm was measured with a microplate reader (Molecular Devices, USA). The results are expressed as optical density (OD) values calculated by subtracting the OD values of IHNV-Ag plate from those of VHSV-Ag plate. Each one serum from VHS-survived olive flounder and VHS-free fish was used in ELISA as positive and negative controls. ELISA was conducted in duplicates.

DETECTION OF VHSV AFTER SHIFTING THE REARING WT

A total of 172 fish were randomly collected from four hatcheries (rearing WT: 17-21ºC) at Yeonggwang and Hampyeong counties during 2014-2015 (Table 2). They were transported to the laboratory in live condition. After that, fish were reared at about 5ºC WT lower than that in the hatchery. Fourteen to 32 fish were randomly sampled at 7 and 14 days after shifting the rearing WT. VHSV examination was conducted according to method described.

RESULTS AND DISCUSSION

Results of VHSV detection from 2,430 fish in 39 hatcheries are shown in Table 1. No clinical sign was observed in any fish tested. Virus was isolated from 5 (2.99%) of 167 samples inoculated to FHM cells and 34 (20.3%) of 167 samples inoculated to CHSE-214 cells. In PCR analysis, no VHSV was detected from 39 virus-culture media of FHM and CHSE-214 cells or 1,933 fishes. However, MABV was detected from all (100%) virus-culture media. Therefore, VHSV was not isolated or detected in any of the 2,400 fish tested (from 461 pooled and 126 unpooled samples), even though 20.3% (34/167 samples) of them was MABV-positive. Based on antibody detection ELISA, 212 fish sera showed OD values below of 0.1. In our previously study, an ELISA with VHSV and IHNV antigens were conducted to detect specific antibodies against VHSV from olive flounder sera (109 with VHS history and 115 without VHS history). The OD values of 109 fish sera with VHS history were distributed from 0 to 0.79, while 115 sera without VHS history were all showed OD value less than 0.1. The OD values of fish sera with and without VHS history were clearly different and fish sera without VHS history showed less than OD 0.1, which was predicted negative for VHSV-specific antibodies. These results suggested that the 212 fish tested in this study had no VHSV-specific antibodies.

In the culture field, VHS generally occurs after juvenile fish are moved from the hatchery (WT: 16-22ºC) to the on-growing system (WT: 11-16ºC). Therefore, fish are exposed to WT about 5ºC lower than that in the hatchery. Thus, VHSV examination was conducted using 172 fish (40 pooled samples) after shifting the rearing WT from 17-21ºC to 10-15ºC (Table 2). No clinical sign was observed from fish tested. Virus was isolated from 4 (10%) of 40 pooled samples inoculated to FHM cells and 27 (67.5%)

TABLE 1. Detection of VHSV in olive flounder samples collected from 39 hatcheries in South Korea conducted between years 2014-2017

Place	Farm	Sampling date	Water temp. (℃)	Fish		Virus isolation		PCR		ELISA (OD 0.1≤)
				Tested sample (fish No.)	Weight (g)	FHM	CHSE-214	VHSV	MABV	
Hampyeong	A	Nov, 2014	17	15 (15)	3.9	NT	NT	NT	NT	0% (0/15)¦
	B	Nov, 2014	17	15 (15)	5.5	NT	NT	NT	NT	0% (0/15)¦
	C	Mar, 2015	18	8* (32)	3.7	0% (0/8)†	100% (8/8)†	0% (0/8)‡	100% (8/8)‡	0% (0/32)¦
Yeonggwang	A	Sep, 2014	19	5* (25)	1.2	20% (1/5)†	40% (2/5)†	0% (0/3)‡	100% (3/3)‡	NT
		May, 2015	19	40 (40)	8.8	0% (0/40)†	0% (0/40)†	NT	NT	NT
		Dec, 2015	17.5	4* (20)	2	NT	NT	0% (0/4)‡	NT	NT
		Jun, 2017	NT	4* (20)	3.32	NT	NT	0% (0/4)‡	NT	NT
		Aug, 2017	NT	4* (20)	0.63	NT	NT	0% (0/4)‡	NT	NT
		Sep, 2017	NT	6 (6)	7.91	NT	NT	0% (0/6)‡§	NT	NT
		Oct, 2017	NT	4 (4)	36.7	NT	NT	0% (0/4)‡§	NT	NT
		Dec, 2017	NT	4* (12)	2.35	NT	NT	0% (0/4)‡§	NT	NT
	B	Sep, 2014	21	4* (20)	3.6	100% (4/4)†	100% (4/4)†	0% (0/8)‡	100% (8/8)‡	0% (0/20)¦
	C	Feb, 2015	17	4* (20)	3.4	0% (0/4)†	0% (0/4)†	NT	NT	0% (0/20)¦
	D	Sep, 2015	20	6* (30)	2	NT	NT	0% (0/6)‡§	NT	0% (0/30)¦
	E	Apr, 2016	18	4* (20)	2.29	NT	NT	0% (0/4)‡§	NT	NT
	F	Apr, 2016	18	6* (30)	2.29	0% (0/6)†	0% (0/6)†	NT	NT	NT
Muan	A	Feb, 2015	15	5* (30)	0.8	0% (0/5)†	0% (0/5)†	NT	NT	NT
	B	May, 2015	19	4* (20)	1.9	0% (0/4)†	0% (0/4)†	NT	NT	NT
	C	Jun, 2015	16	30 (30)	4.9	0% (0/30)†	36.7% (11/30)†	0% (0/11)‡	100% (11/11)‡	0% (0/30)¦
	D	Oct, 2017	NT	4* (20)	3.86	NT	NT	0% (0/4)‡§	NT	NT

NT = not tested, * Pooled sample no., † Virus isolation rate % (isolation no./ total no.), ‡ PCR for targeting N gene of VHSV, § PCR for targeting G gene of VHSV, ¦ Anti-VHSV antibody detection rate (detection no./ total no.)

TABLE 1. *Continued*

Place	Farm	Sampling date	Water temp. (°C)	Fish		Virus isolation		PCR		ELISA (OD 0.1≤)
				Tested sample (fish No.)	Weight (g)	FHM	CHSE-214	VHSV	MABV	
Gochang	A	Jan, 2015	16.5	12* (60)	2.5	0% (0/12)†	0% (0/12)†	NT	NT	NT
		Jan, 2015	16.5	4* (20)	2.47	NT	NT	0% (0/4)‡	NT	NT
	B	Apr, 2015	22	10* (50)	1.8	0% (0/10)†	0% (0/10)†	NT	NT	NT
Taean	A	Mar, 2014	22	10 (10)	1~1.5	NT	NT	0% (0/10)‡	0% (0/10)‡	NT
		Oct, 2014	18	4* (20)	7.4~8.1	NT	NT	0% (0/4)‡	0% (0/4)‡	NT
		Sep, 2015	22	10 (10)	0.8~1.0	NT	NT	0% (0/10)‡	0% (0/10)‡	NT
	B	Nov, 2015	21	5* (25)	2.1~4.8	NT	NT	0% (0/5)‡	0% (0/5)‡	NT
	C	Apr, 2015	17	10* (50)	2	0% (0/10)†	30% (3/10)†	0% (0/3)‡	100% (3/3)‡	0% (0/10)¦
	D	Apr, 2016	16.8	10 (10)	2.51	0% (0/10)†	0% (0/10)†	NT	NT	0% (0/10)¦
	E	Nov, 2016	18	3* (10)	2.7	0% (0/3)†	0% (0/3)†	NT	NT	NT
Hwaseong	A	May, 2014	18	30* (150)	5.9~7.3	NT	NT	0% (0/30)‡	NT	NT
		Apr, 2015	19	10* (50)	5.4~7.0	NT	NT	0% (0/10)‡	NT	NT
	B	May, 2014	17	10* (50)	6.2~7.3	NT	NT	0% (0/10)‡	NT	NT
		May, 2015	18	10* (50)	5.8~7.4	NT	NT	0% (0/10)‡	NT	NT
	C	May, 2014	17	10* (50)	7.2~8.6	NT	NT	0% (0/10)‡	NT	NT
		Jun, 2014	18	10* (50)	8.8~12.7	NT	NT	0% (0/10)‡	NT	NT
		Jun, 2015	18	10* (50)	6.0~8.7	NT	NT	0% (0/10)‡	NT	NT
	D	Feb, 2016	15	4* (20)	2.45	NT	NT	0% (0/4)‡	NT	0% (0/20)¦
		Mar, 2016	14	4* (20)	5.5	NT	NT	0% (0/4)‡	NT	NT
Buan	A	May, 2016	18	6* (30)	1.19	0% (0/6)†	0% (0/6)†	NT	NT	NT
		May, 2016	18	4* (20)	1.19	NT	NT	0% (0/4)‡§	NT	NT

NT = not tested, * Pooled sample no., † Virus isolation rate % (isolation no./ total no.), ‡ PCR for targeting N gene of VHSV, § PCR for targeting G gene of VHSV, ¦ Anti-VHSV antibody detection rate (detection no./ total no.)

TABLE 1. *Continued*

Place	Farm	Sampling date	Water temp. (ºC)	Fish		Virus isolation		PCR		ELISA (OD 0.1≤)
				Tested sample (fish No.)	Weight (g)	FHM	CHSE-214	VHSV	MABV	
Incheon	A	May, 2014	17	30* (150)	6.2~10.4	NT	NT	0% (0/30)‡	NT	NT
		Jul, 2014	20	10* (50)	7.7~11.0	NT	NT	0% (0/10)‡	NT	NT
	B	May, 2014	17	30* (150)	4.8~7.9	NT	NT	0% (0/30)‡	NT	NT
		Oct, 2014	19	10* (50)	6.9~9.8	NT	NT	0% (0/10)‡	NT	NT
	C	May, 2014	18	30* (150)	6.5~9.6	NT	NT	0% (0/30)‡	NT	NT
	D	May, 2014	17	30* (150)	6.5~9.7	NT	NT	0% (0/30)‡	NT	NT
	E	May, 2014	17	20* (100)	5.5~9.5	NT	NT	0% (0/20)‡	NT	NT
	F	May, 2014	16	20* (100)	5.9~9.5	NT	NT	0% (0/20)‡	NT	NT
	G	May, 2014	16	10* (50)	6.2~8.9	NT	NT	0% (0/10)‡	NT	NT
	H	May, 2015	17	10* (50)	6.2~10.4	NT	NT	0% (0/10)‡	NT	NT
		Jul, 2016	18	10* (50)	5.0~8.7	NT	NT	0% (0/10)‡	NT	NT
	I	May, 2015	18	10* (50)	4.9~8.3	NT	NT	0% (0/10)‡	NT	NT
	J	Jun, 2016	21	10 (10)	4.75	0% (0/10)†	60% (6/10)†	0% (0/6)‡	100% (6/6)‡	0% (0/10)¦
Haenam	A	Apr, 2017	NT	5* (10)	1.38	NT	NT	0% (0/5)‡§	0% (0/5)‡	NT
Seosan	A	Aug, 2017	NT	6 (6)	35.65	NT	NT	0% (0/6)‡§	NT	NT
Total	39			617 (2,430)		2.99% (5/167)	20.3% (34/167)	0% (0/459)	53.4% (39/73)	0% (0/212)

NT = not tested, * Pooled sample no., † Virus isolation rate % (isolation no./ total no.), ‡ PCR for targeting N gene of VHSV, § PCR for targeting G gene of VHSV, ¦ Anti-VHSV antibody detection rate (detection no./ total no.)

TABLE 2. Detection of VHSV from olive flounder samples collected between years 2014-2015 after shifting water temperature from 17-21℃ to 10-15℃

Place	Farm	Sampling date	Water temp. (℃) (examination day)	Fish Tested sample (fish No.)	Virus isolation†		PCR‡	
					FHM	CHSE-214	VHSV	MABV
Yeonggwang	A	Sep, 2014	19 → 15 (7)	4* (20)	50% (2/4)	100% (4/4)	0% (0/6)	100% (6/6)
			19 → 15 (14)	4* (20)	50% (2/4)	75% (3/4)	0% (0/5)	100% (5/5)
Yeonggwang	B	Sep, 2014	21 → 15 (7)	3* (14)	0% (0/3)	66.6% (2/3)	0% (0/2)	100% (2/2)
			21 → 15 (14)	3* (14)	0% (0/3)	100% (3/3)	0% (0/3)	100% (3/3)
Yeonggwang	C	Feb, 2015	17 → 10 (7)	5* (20)	0% (0/5)	0% (0/5)	NT	NT
			17 → 10 (14)	5* (20)	0% (0/5)	20% (1/5)	0% (0/1)	100% (1/1)
Hampyeong	C	Mar, 2015	18 → 14 (7)	8* (32)	0% (0/8)	87.5% (7/8)	0% (0/7)	100% (7/7)
			18 → 14 (14)	8* (32)	0% (0/8)	87.5% (7/8)	0% (0/7)	100% (7/7)
Total	4			40 (172)	10% (4/40)	67.6% (27/40)	0% (0/31)	100% (31/31)

NT = not tested, * Pooled sample no., † Virus isolation rate % (isolation no./ total no.), ‡ Virus detection rate % (detection no./ total no.)

of 40 pooled samples inoculated to CHSE-214 cells. In PCR analysis, VHSV was not detected in any of 31 virus-culture media collected from FHM and CHSE-214 cell culture after inoculation with fish sample. However, MABV was detected from all (100%) 31 virus-culture media after inoculation with fish sample. These results confirmed that all 40 pooled samples (172 fish) collected after WT shifting contained no VHSV.

Most olive flounder hatcheries are located on the western coast of Korea, while most on-growing systems are located in Wando and Jeju on the south coast of Korea (accountings for more than 85% of total olive flounder production in Korea), where fish are kept 5-28ºC WT. In the present study, a survey was conducted to investigate VHSV infection in 39 olive flounder hatcheries. The results of the present study showed that olive flounders collected in 39 hatcheries had neither VHSV nor VHSV-specific antibody. VHSV was not also detected in olive flounder samples collected after WT shifting either. Moreover, VHSV has not been reported in hatcheries in Korea at the present time. These results suggested that juvenile olive flounders are most likely not being infected by VHSV in the hatcheries located on the western coast of Korea. The following possibility may explain why fish are not infected by VHSV in the hatcheries. First, while VHS mainly occurred at lower 17ºC in Korea (Kim et al. 2009; Kim et al. 2003; Kim & Park 2004b), the development of olive flounder eggs to juvenile fish stages are generally kept at above 18ºC in hatcheries. Second, the olive flounder hatcheries are located on the western coast of Korea which is far apart from Wando or Jeju area, the main place for VHS outbreaks. Unfortunately, we could not collect fish in hatcheries at Wando and Jeju and 416 pooled samples were examined for the presence of VHSV using PCR with VHSV VN primer set listed in the OIE manual, which the sensitivity of the Korean VHSV isolates (genotype IVa) is not a high (Kim 2015). We inferred that the absence of VHSV in olive flounder of hatchery is either due to hatchery rearing temperature or geographical location of the hatchery which is isolated from usual VHS outbreak areas. Presumably VHSV infection occurs after the seeds being transferred to grow-out facilities from the hatchery. Therefore, further studies are needed to confirm if fish are not infected with VHSV in hatcheries or fish are infected with VHSV in few hatcheries through follow-up survey.

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