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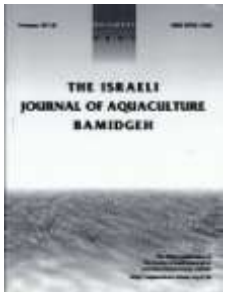

Isolation and Infectious Temperature Optimization of Genetically Similar VHSV Isolates in Farmed Olive Flounder, *Paralichthys olivaceus*

Yeoung-Hwan Jang[1], Subramanian Dharaneedharan[2], Bong-Jo Kang[1], Moon-Soo Heo[2]*

[1] *Jeju Special Self-Governing Province Ocean and Fisheries Research Institute, Pyoseon-myeon, Segwipo-si, Jeju 697-914, South Korea*

[2] *Marine Applied Microbes and Aquatic Organism Disease Control Lab, Department of Aquatic Biomedical Sciences, School of Marine Biomedical Sciences & Marine and Environmental Research Institute, Jeju National University, Jeju 690-756, South Korea*

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Key words: viral hemorrhagic septicemia virus, VHS, N-gene, infection temperature, flounder fish

Abstract

Viral hemorrhagic septicemia virus (VHSV) was isolated from farmed olive flounder (*Paralichthys olivaceus*). The viral N-gene was amplified by reverse transcriptase PCR, cloned, and sequenced for phylogenetic analysis to identify the genotype (I-IV). Virus isolates were cultured on *Epithelioma papulosum cyprini* cell line and, after completion of the cytopathic effects, the supernatant was collected and used to challenge virus-free flounder. The infected founder were reared in 16°C, 21°C, or 25°C, and compared to an unchallenged control. Virus titration was measured in the head kidneys, spleens, livers, brains, muscles, and gills of challenged fish using real-time quantification of the VHSV G-gene. Phylogenetic analysis confirmed that the isolates were VHSV Genogroup IV. The VHSV-challenged fish in the 16°C group showed 100% mortality with significantly increased expression of viral G-gene mRNA in the spleen, compared to fish reared in other temperatures and the control fish, suggesting that fish reared in 16°C are more susceptible to VHSV infection.

* Corresponding author. E-mail: msheo@cheju.ac.Kr

Introduction

Viral hemorrhagic septicemia (VHS) is a deadly infectious fish disease caused by the viral hemorrhagic septicemia virus (VHSV), a negative-sense single-stranded RNA virus belonging to the family *Rhabdoviridae* and genus *Novirhabdovirus* (Walker et al., 2000). The VHSV genome comprises six nonvirion (NV) proteins and five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and RNA polymerase (L), in the order 3'-N-P-M-G-NV-L-6', with about 11,200 nucleotides.

VHSV was first isolated in 1965 and VHS was first reported as a serious economic problem in rainbow trout farms in Europe (Wolf, 1988; Smail, 1999), particularly France and Denmark (Castric and de Kinkelin, 1980; Horlyck et al., 1984), and Germany and Scotland (Schlotfeldt et al., 1991; Ross et al., 1994). VHSV was also isolated from cultured olive flounders (*Paralichthys olivaceus*) in the Seto Inland Sea of Japan in 1996 where clinical signs included darkening of skin, an expanded abdomen due to ascites, hemorrhaging in the head and fin bases, a congested liver, splenomegaly, and a swollen kidney (Isshiki et al., 2001). The virus was identified and isolated from juvenile flounder in aquaculture farms in Korea in 2001 (Kim et al., 2003). In 2005, mortality rose from 40% to 60%, particularly in the spring and winter seasons, due to VHSV infection in juvenile and market-sized olive flounder (Kim et al., 2009).

The VHSV Genogroup IV is restricted to North American and Asian marine and freshwater species (Snow et al., 2004). The present study reports on the identification and characterization of VHSV Genogroup IV as the presumed cause of mortality during the cold season from 2008 to 2011 in olive flounder (*Paralichthys olivaceus*) in Jeju island, South Korea, and on viral titration of organs of challenged fish raised in temperatures ranging 16-25°C.

Materials and Methods

In-vitro virus culture. Epithelioma papulosum cyprini (EPC) cells were cultured in minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (MEM-10) and 1% antibiotic-antimycotic (Gibco), incubated at 20°C, and subcultured every 7-10 days. Kidneys of diseased olive flounders were collected from commercial farms on Jeju Island (Table 1), excised, and subjected to virus isolation tests in aseptic conditions. Briefly, whole kidneys were homogenized in 10 ml Hanks' balanced salt solution (HBSS, Cellgro) and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through a sterilized 0.2-µm membrane and inoculated over an EPC cell monolayer in 25 cm^2 flasks. When the cytopathic effect (CPE) was nearly complete, five days after infection, the cells were scraped off the culture flasks, centrifuged at 1000 × *g* for 20 min, and stored at -80°C. The virus-containing supernatant was tittered according to Reed and Muench (1938).

Table 1. Fish farms in South Korea from which diseased olive flounder (*Paralichthys olivaceus*) were collected.

Farm	*Location*
Pyoseon	33°19'35.38"N, 126°49'53.90"E
Namwon	33°17'18.70"N, 126°42'14.89"E
Aewol	33°27'43.01"N, 126°19'46.29"E
Daejeong	33°13'36.53"N, 126°15'8.01"E
Handong	33°31'9.57"N, 126°48'39.97"E
Seongsan	33°22'51.83"N, 126°52'36.32"E

RNA extraction. Viral RNA was extracted from the supernatant of the CPE cells using an RNeasy Plus Mini Kit (Qiagen, Germany), following the manufacturer's instructions. RNA samples were eluted in 50 µl diethylpyrocarbonate-water, the RNA concentration was determined by spectrophotometer at 260 nm, and the solution was adjusted to 0.1 µg/µl for each sample. Aliquots were stored at -80°C until use. cDNA from the total viral RNA was synthesized by mixing 2.5 ng/µl random primers (Invitrogen, USA) and 9 µl extracted total RNA in nuclease-free water. The mixture was incubated at 95°C for 5 min, held on ice for at least 1 min, and then a reverse transcription mixture containing 200 U/µl Superscript III RT (Invitrogen, USA), 0.5 mM dNTPs, and 0.05 M DTT in 1× first strand buffer was added. The mixture was incubated at 25°C for 10 min, followed by 50°C for 50 min, and the reaction was stopped by heating at 85°C for 5 min. The synthesized cDNA was stored at 4°C until use.

Primers. Primers for internal control were designed from the β-actin gene nucleotide sequence from the NCBI Genbank database (accession no. HQ386788.1) using Primer Express 3 software from Applied Biosystems, USA (Table 2). For the VHSV G-gene, previously reported primers 1018F and 1018R were used to amplify the target region of about 80 bp (Cutrin et al., 2009). Two primer pairs were used for SYBR Green assay. The presence of the VHSV N-gene was screened using a conventional PCR method with the previously reported primer pair for the N-gene (Snow et al., 2004).

Table 2. PCR primer and amplicon size.

Primer	*Nucleotide sequence (5′ → 3′)*	*Amplicon size (bp)*
1018F	CTCATTTCTCCTCTCAAAGTTTCG	80
1018R	CCGTCTGTGTTGTTGTCTACC	
β-actin F	AGGCGCAGAGCCTTGATG	191
β-actin R	GTCAAGCGCCAAAAATAACTGA	
VN Forward	ATGGAAGGAGGAATTCGTGAAGCG	505
VN Reverse	GCGGTGAAGTGCTGCAGTTCCC	

Viral screening PCR. cDNA obtained from infected fish was used to confirm viral infection by conventional PCR. Amplification was performed in 25 µl volumes containing 2 µl cDNA, 15 pmol of each primer, 10 mM of each dNTP Mix, 5 U/µl Diastar^{TM} Taq DNA polymerase (Solgent, Korea), 10 × Taq reaction buffer (25 mM $MgCl_2$ mixed), and 5 × Band Doctor^{TM}. Amplification was conducted on a Mycycler thermocycler (Bio-Rad, USA) and the temperature profile consisted of initial denaturation at 94°C for 4 min, 30 denaturing cycles of 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. The amplified segment was cloned into a pCR2.1-TOPO Vector (Invitrogen, USA) under conditions recommended by the supplier, and positive clones were subcultured. Plasmid DNA was purified with a QIAprep Spin Miniprep kit (Qiagen, Germany) and sequenced on an ABI 3730XL DNA analyser (Applied Biosystems, USA).

Phylogenetic analysis. Multiple sequence alignment was performed to assess similarities using ClustalW program in MEGA (ver. 4.1). A phylogenetic tree was constructed based on deduced amino acid sequences using the neighbor-joining algorithm, and the reliability of the branching was compared with partial N-gene sequences from the NCBI Genbank, using 1,000 bootstrap samplings of the data.

Real-time PCR analysis of genes. Real-time PCR was carried out in an Mx 3000P (Stratagene, USA) using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent technologies, USA) with 2 µl diluted cDNA as the template (in triplicate). Two gene specific primer pairs (1018 F/R and β-actin F/R) were used to amplify 80 and 191 bp, respectively. The β-actin gene was used as an endogenous control for the real-time gene expression analysis. PCR reaction included an initial 10 min Taq activation step at 95°C and 40 cycles of 95°C for 10 s, 57°C for 5 s, and 72°C for 30 s. Results were analyzed using MX Pro, a program designed to analyze real-time quantitative data using a modified delta-Ct method.

Experimental infection. PCR-certified VHSV-free olive flounder (18-22 g) were obtained from a local commercial farm and acclimated to 16°C, 21°C, or 25°C for two weeks in three 50-l tanks. The fishes were divided into six treatments of 30 fish/group, with triplicates of each treatment. Three groups were intraperitoneally injected with 100 µl viral suspension (10^5 $TCID_{50}$/fish) and raised at 16±0.5°C, 21±0.5°C, or 25±0.5°C. Three control groups received 100 µl phosphate buffered saline and were likewise raised in 16±0.5°C, 21±0.5°C, or 25±0.5°C. When all fish in the 16°C treatment died (fish in the other treatments were still alive), head kidneys, spleens, livers, brains, muscles, and gills were aseptically dissected and total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Germany). Cumulative mortality and VHSV titration in the vital organs were recorded.

Statistical analysis. Data were subjected to Student's *t* test. Differences were considered statistically significant when $p<0.05$.

Results

Nucleotide sequencing and phylogenetic analysis. The plasmid sequence with the target gene was BLAST searched. Over 60 VHSV N-gene sequences were used to construct the

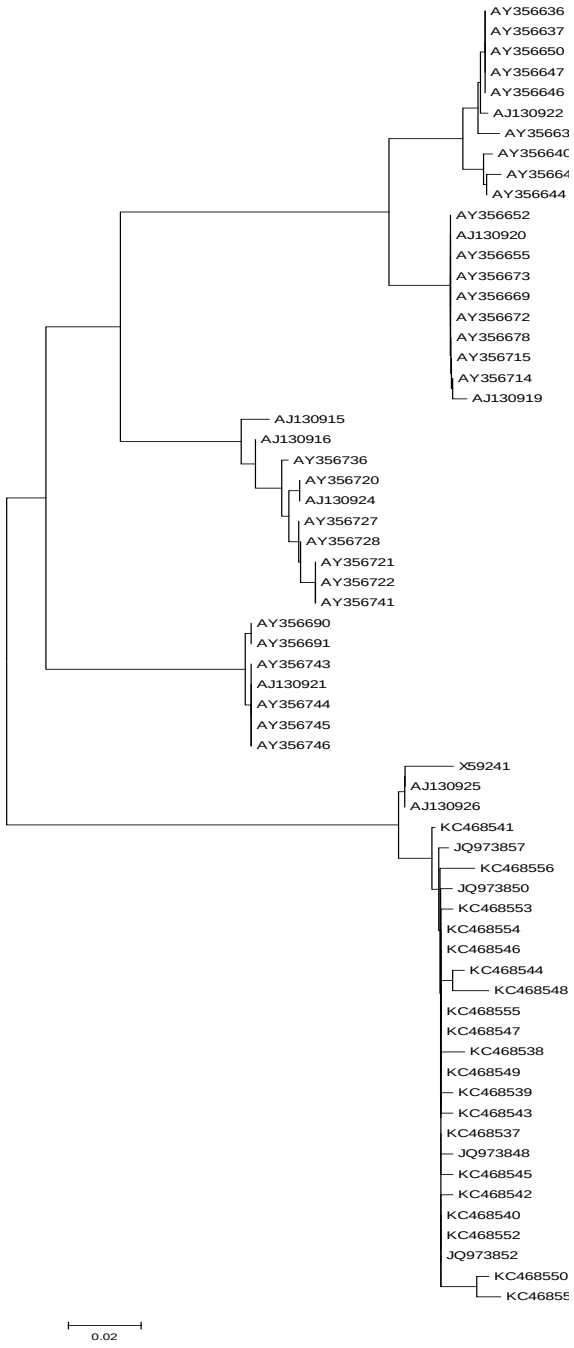

Fig. 1. Phylogenetic classification of major genotypes using the neighbor-joining method for VHSV isolates.

phylogenetic tree (Fig. 1). When compared with VHSV N-gene sequences from the NCBI Genbank, all 21 of our VHSV isolates were classified as VHSV Genotype IV (Table 3).

Cumulative mortality. Clinical signs of hemorrhagic fins, ascites, hemorrhages in the muscle, and swollen internal organs were seen from the fifth day after injection in infected fish reared in 16°C; mortality was 100% twenty-five days after infection. In fish raised in 21°C, mortality was 3%, while in fish raised in 25°C and in control fish, there was no mortality.

Real-time RT-PCR analysis of gene expression after infection. Relative expression of the VHSV G-gene in fish raised in 16°C was significantly higher than in fish raised in other temperatures, particularly in the spleen ($6.27\pm5.3 \times 10^8$). Viral gene expression was very low in the spleen ($14.1\pm1.9 \times 10^6$) and kidney ($2.47\pm2.8 \times 10^7$) two days after injection in fish kept at 21°C while fish kept at 25°C showed no viral gene expression in any of the samples (Fig. 2).

Discussion

Sequence analysis confirmed the occurrence of VHSV in Jeju waters and isolates were of the Genogroup IV, identified as the prevailing VHSV in the Pacific waters of North America, Japan, and Korea (Snow et al., 2004; Einer-Jensen et al., 2005).

Phylogenetic analysis also confirmed that the virus isolates from the Jeju flounder farms belong to the VHSV Genogroup IV, consistent with earlier phylogenetic analyses of the four genotypes of VHSV (Einer-Jensen et al., 2004; Snow et al., 2004). Genotypes I, II, and III are considered European types, although Genotype III comprises isolates from both the European and the North American sides of the North Atlantic (Lopez-Vazquez et al., 2006). On the other hand, Genogroup IV includes isolates from the Pacific Ocean (North American, Japanese, Korean isolates). Genetically similar VHSV isolates from flounder farms in the southern part of Korea revealed greater pathogenicity than the Jeju isolates (Cho et al., 2012). Other fish viruses from flounder farms in Korea include infectious hematopoietic necrosis virus (IHNV; Park et al., 1993; Kim et al., 2007), infectious hematopoietic necrosis virus (HIRRV; Kim et al., 2005), marine birna virus (Sohn et al., 1995), beta nodavirus (Cha et al., 2007), and iridovirus (Do et al., 2005).

The present study indicates that the optimum temperatures for culturing VHSV *in vitro* and *in vivo* are 20°C and 16°C, respectively. Fish raised in 21°C or 25°C showed no clinical signs and cumulative mortality was 3% and 0%, respectively, suggesting that

Table 3. Viral hemorrhagic septicemia virus isolates used for phylogenetic analysis.

Genotype	*Geographic origin*	*Isolate name*	*Year of isolation*	*Host species*	*GenBank no.*
Ia	Denmark	DK-6143		*Oncorhynchus mykiss*	AY356645
Ia	Denmark	DK-6047	1991	*Oncorhynchus mykiss*	AY356644
Ia	Denmark	DK-3955	1987	*Oncorhynchus mykiss*	AY356640
Ia	Denmark	DK-7217	1994	*Oncorhynchus mykiss*	AY356646
Ia	Denmark	DK-7300	1994	*Oncorhynchus mykiss*	AY356647
Ia	Denmark	DK-7483	1995	*Oncorhynchus mykiss*	AY356650
Ia	Denmark	DK-9795159	1997	*Oncorhynchus mykiss*	AY356636
Ia	Germany	DE-7321 (5927)	1991	*Scophthalmus maximus*	AJ130922
Ia	Denmark	DK-9795265	1997	*Oncorhynchus mykiss*	AY356637
Ia	Denmark	DK-9695265	1996	*Oncorhynchus mykiss*	AY356634
Ib	Baltic Sea	DK-7e97	2001	*Clupea harengus*	AY356714
Ib	Baltic Sea	DK-1p40	1996	*Rhinonemus cimbrius*	AJ130919
Ib	Kattegat	DK-5p251	1998	*Clupea harengus*	AY356678
Ib	Skagerrak	DK-5p11	1998	*Pleuronectes platessa*	AY356672
Ib	Kattegat	DK-5e59	1998	*Limanda limanda*	AY356669
Ib	Kattegat	DK-5p26	1998	*Limanda limanda*	AY356673
Ib	Baltic Sea	DK-1p85	1996	*Clupea harengus*	AY356655
Ib	Baltic Sea	DK-1p50	1996	*Sprattus sprattus*	AJ130920
Ib	Baltic Sea	DK-1p8	1996	*Clupea harengus*	AY356652
Ib	Baltic Sea	DK-7p37	2001	*Pomatoschistus minutes*	AY356715
II	Baltic Sea	DK-5p551	1998	*Clupea harengus*	AY356690
II	Baltic Sea	DK-5p557	1998	*Clupea harengus*	AY356691
II	Baltic Sea	DK-1p49	1996	*Clupea harengus*	AY356743
II	Baltic Sea	DK-1p53	1996	*Clupea harengus*	AJ130921
II	Baltic Sea	DK-1p52	1996	*Sprattus sprattus*	AY356744
II	Baltic Sea	DK-1p54	1996	*Gadus morhua*	AY356745
II	Baltic Sea	DK-1p55	1996	*Sprattus sprattus*	AY356746
III	Gigha	UK-860/94	1994	*Scophthalmus maximus*	AJ130915
III	SW Ireland	IR-F 13.02.97	1997	*Scophthalmus maximus*	AJ130916
III	North Sea	UK-H17/1/95	1995	*Melanogrammus aeglefinus*	AY356720
III	North Sea	UK-H17/2/95	1995	*Melanogrammus aeglefinus*	AJ130924
III	North Sea	DK-4p51	1997	*Argentina sphyraena*	AY356736
III	North Sea	UK-MLA98/6PT16	1998	*Trisopterus esmarkii*	AY356722
III	North Atlantic Sea	UK-MLA98/4PT6	1998	*Trisopterus esmarkii*	AY356741
III	North Sea	UK-MLA98/6PT13	1998	*Trisopterus esmarkii*	AY356721
III	North Sea	UK-H17/5/93	1993	*Gadus morhua*	AY356727
III	North Sea	UK-H19/1/93	1993	*Gadus morhua*	AY356728
IV	Alaska, USA	US-pws-ak90	1996	*Gadus macrocephalus*	AJ130926
IV	Washington, USA	US-eby-wa93	1993	*Clupea pallasii*	AJ130925
IV	Washington, USA	US-mak-wa88	1988	*Oncoryhychus kisutch*	X59241
IV	South Korea	GCVP-05	2010	*Paralichthys olivaceus*	JQ973850
IV	South Korea	GCVP-24	2008	*Paralichthys olivaceus*	JQ973857
IV	South Korea	GCVP-10	2010	*Paralichthys olivaceus*	JQ973852
IV	South Korea	GCVP-01	2010	*Paralichthys olivaceus*	JQ973848
Present study					
IVa	Jeju, South Korea	JE1	2008	*Paralichthys olivaceus*	KC468537
IVa	Jeju, South Korea	JE2	2008	*Paralichthys olivaceus*	KC468538
IVa	Jeju, South Korea	JE3	2009	*Paralichthys olivaceus*	KC468539
IVa	Jeju, South Korea	JE4	2009	*Paralichthys olivaceus*	KC468540
IVa	Jeju, South Korea	JE5	2009	*Paralichthys olivaceus*	KC468541
IVa	Jeju, South Korea	JE6	2009	*Paralichthys olivaceus*	KC468542
IVa	Jeju, South Korea	JE7	2009	*Paralichthys olivaceus*	KC468543
IVa	Jeju, South Korea	JE8	2009	*Paralichthys olivaceus*	KC468544
IVa	Jeju, South Korea	JE9	2010	*Paralichthys olivaceus*	KC468545
IVa	Jeju, South Korea	JE10	2010	*Paralichthys olivaceus*	KC468546
IVa	Jeju, South Korea	JE11	2010	*Paralichthys olivaceus*	KC468547
IVa	Jeju, South Korea	JE12	2010	*Paralichthys olivaceus*	KC468548
IVa	Jeju, South Korea	JE13	2010	*Paralichthys olivaceus*	KC468549
IVa	Jeju, South Korea	JE14	2010	*Paralichthys olivaceus*	KC468550
IVa	Jeju, South Korea	JE15	2011	*Paralichthys olivaceus*	KC468551
IVa	Jeju, South Korea	JE17	2011	*Paralichthys olivaceus*	KC468552
IVa	Jeju, South Korea	JE18	2011	*Paralichthys olivaceus*	KC468553
IVa	Jeju, South Korea	JE19	2011	*Paralichthys olivaceus*	KC468554
IVa	Jeju, South Korea	JE20	2011	*Paralichthys olivaceus*	KC468555
IVa	Jeju, South Korea	JE21	2011	*Paralichthys olivaceus*	KC468556

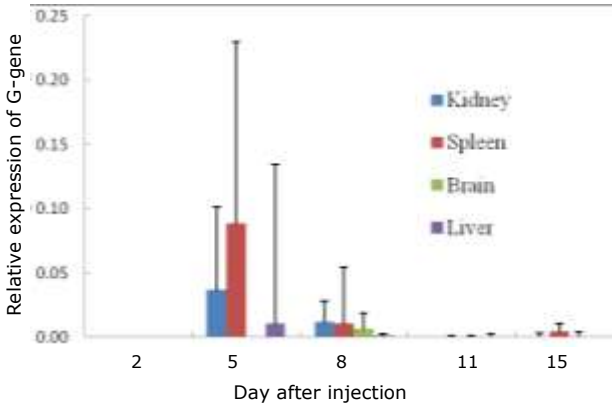

Fig. 2. Relative mRNA expression of VHSV G-gene (× 10^9 increase from the control β-actin gene expression level) in kidney, spleen, brain, and liver of infected *Paralichthys olivaceus* raised in 16°C.

flounder are more prone to VHSV infection in cold water than in warm, as reported for Pacific sardine infected by Genogroup IV (Arkush et al., 2006). Fish raised in 21°C had no mortality after the second day after injection and there was no mortality at all in fish raised in 25°C, suggesting that viral multiplication in the fish was inhibited by the expression of immune gene interferon (IFN), a well-known antiviral protein that interferes with viral replication in host cells, as reported in experimentally infected rainbow trout (de Kinkelin and Dorson, 1973).

VHSV G-gene expression in the infected flounder held at 16°C showed increased mortality associated with increased virus titration particularly in the spleen. This result agrees with earlier observations of VHSV Genogroup IV infected flounder reared at 20°C and viral titers in different organs (Iida et al., 2003).

Hence, the present study shows the optimum isolation and culture temperature for VHSV from Jeju waters. *In vitro* and *in vivo* experimental results show increased propagation in 16-20°C. Comparative sequence analysis confirmed the isolates are close to VHSH Genogroup IVa. Further studies are required to test other physico-chemical and biological factors responsible for viral culture and their pathogenic effect in various stages of host fish. Such studies are needed to validate the biology and pathogenicity of VHSV to prevent infection in commercially important aquatic organisms.

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