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Genetic positioning of Korean viral hemorrhagic septicemia virus (VHSV) from cultured and wild marine fishes

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Viral haemorrhagic septicaemia virus (VHSV) is an epidemic virus in olive flounder *Paralichthys olivaceus* farms in Korea, since the virus have first isolated in 2001. In the present study, partial glycoprotein (G) gene nucleotide sequences of seven Korean VHSV from cultured olive flounder and wild marine fishes in coastal areas of Korea were analyzed to evaluate their genetic relatedness to worldwide isolates. Phylogenetically, all Korean VHSV formed only one minor cluster including Japanese isolates, in genotype IVa, while the North America isolates formed a different minor cluster in genotype IVa. These results suggest that Korean VHSV could be an indigenous virus in Korean and Japanese coastal areas, but have not been introduced from North America.

Key words : Viral hemorrhagic septicemia virus, Glycoprotein gene, Phylogeny, Korea

Viral haemorrhagic septicaemia (VHS) is an infectious disease causing extensive losses in rainbow trout *Oncorhynchus mykiss* farms in European countries (Wolf, 1988; Smail, 1999), and it is listed as notifiable disease by the Aquatic Animal Health Code of the World Organization for Animal Health (OIE). VHS virus (VHSV), the causative agent of VHS, was first isolated from freshwater-cultured rainbow trout in Denmark in 1963 (Jensen, 1963). Over the succeeding four decades, VHSV has been isolated as the predominant pathogen of not only rainbow trout but other fish species including marine fishes in European countries (Wolf, 1988; Mortensen *et al*., 1999; Skall *et al*., 2005). At the end of the 1980s, VHSV was also isolated from anadromous salmon and marine fish on the west coast of North America, marking the first detection of VHSV on that continent (Winton *et al*., 1989; Meyers and Winton, 1995; Meyers *et al*., 1999). Moreover, it has also been found in olive flounder *Paralichthys olivaceus* and other marine fish in Far Eastern Asia (Takano *et al*., 2000; Isshiki *et al*., 2001; Watanabe *et al*., 2002; Kim *et al*., 2009). Thus, VHSV is ubiquitous in a variety of freshwater and marine fishes throughout the northern hemisphere, including North America, Asia and Europe.

As a member of the genus *Novirhabdovirus* in the

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family *Rhabdoviridae*, VHSV has a negative- and single-stranded RNA genome with approximately 11.1 kb containing six genes, nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV) and polymerase (L) genes, in the order 3'-N-P-M-G-NV-L-5' (Trdo *et al*., 2005). Based on phylogenetic analyses with nucleotide sequences of N and G genes, worldwide isolates of VHSV were classified into four genotypes. Genotype I includes a wide range of isolates originating in freshwater and marine fish species in continental Europe, genotype II is composed of marine isolates recovered from the Baltic Sea, genotype III comprises isolates distributed in the North Sea coastal area of the United Kingdom and Ireland, and genotype IV is comprised of isolates from Japan, and the Pacific coast and Great Lakes regions of North America (Benmansour *et al*., 1997; Stone *et al*., 1997; Snow *et al*., 1999; Nishizawa *et al*., 2002; Einer-Jensen *et al*., 2004; Snow *et al*., 2004; Skall *et al*., 2005; Elsayed *et al*., 2006; Nishizawa *et al*., 2006; Gagné *et al*., 2007). It is considered that genetic differences of VHSV are more related to geographic areas than to host fish species.

VHSV was not detected in Korea before 2000. In 2001, VHSV infection occurred in juveniles of farmed olive flounder at Pohang, in eastern Korea (Kim *et al*., 2003). Thereafter, more widespread VHS outbreaks occurred in Korean olive flounder farms (Kim *et al*., 2009), with VHS becoming an endemic problem in the Korean aquaculture industry. Furthermore, VHSV has been detected from wild marine fishes in Korean coastal areas (Kim and Park, 2004; Lee *et al*., 2007). Our hypothesis asserts that the original source of VHSV in Korea may be introduced from outside of Korea by the movement of VHSV-contaminated fish in nature or imported fish from many countries as a feed source in aquaculture industry, because the disease was not reported prior to 2001. However, there is no evidence to support this hypothesis. Thus, in the present study, we analyzed partial nucleotide G gene sequences of seven Korean VHSV from cultured olive flounder and wild marine fishes in coastal areas of Korea, and compared the sequences with those of existing Korean, Japanese and worldwide isolates for evaluation of the origin of Korean VHSV.

Materials and Methods

VHSV for nucleotide sequence analysis

Two isolates of VHSV from cultured olive flounder and five VHSV from apparently healthy wild marine fishes were subjected to nucleotide sequence analysis of partial G gene (Fig. 1). The FWando08 and FYG08 were isolated from moribund juveniles of olive flounder involved in VHS epidemic outbreaks at Wando and Yeonggwang in 2008. BF05-1 and BF05-2 were detected from wild buffer fish *Pampus argenteus*, while KF05 was from wild Korean flounder *Glyptocephalus stelleri*; the fish were captured at a coastal area of the western part of Korea in 2005. LH03 and YBS05 were detected from wild largehead hairtail *Trichiums lepturus* and yellowback seabream *Dentex tumifrons* captured at a coastal area of the southern part of Korea in 2003 and 2005, respectively. FWando08 and FYG08 isolates were propagated in fathead minnow (FHM) cells, which were maintained at 20°C with Eagle's MEM supplemented with

10% fetal bovine serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate. The isolates used for nucleotide sequence analysis had undergone two passages in cell culture. In addition, each 0.2 g tissues (spleen and kidney) from wild marine fishes were homogenized in a 4-fold volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged at 2500 g for 10 min at 4℃. The supernatants were used for reverse transcription (RT)-polymerase chain reaction (PCR) of VHSV.

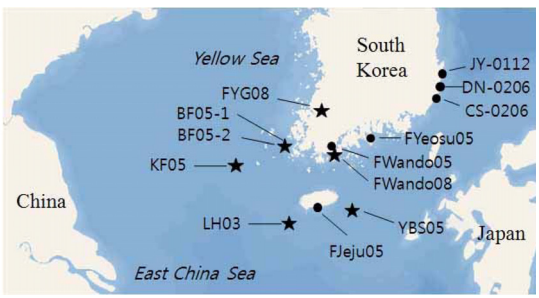

Fig. 1. Locations where VHSV have been detected in cultured olive flounder or wild marine fish from or near coastal areas of Korea. Stars indicate locations of Korean VHSV detected in this study, and circles indicate locations of Korean VHSV reported previously (Kim *et al*., 2003; Kim *et al*., 2009).

RT-PCR

Viral RNA was extracted from the tissue homogenates and viral-culture fluid using TRIZOL reagent (Gibco, Gaithersburg, MD, USA) according to the manufacturer's instructions for RT-PCR amplification with two PCR primer sets. PCR primers VG1 (5'-ATGGAATGGAAC ACTTTTTTC-3')-VD3 (5'-TGTGATCATGGGTCCT GGTG-3') (one-step PCR) and VD5 (5'-TCCCGCTAT CAGTCACCAG-3')-VD3 (nested PCR) were used for amplification of a 697 and 444 base-region of the VHSV G gene, respectively (Miller *et al*., 1998). Briefly, total RNA was isolated using TRIZOL reagent, precipitated with isopropanol, and resuspended with 20 μl of diethyl pyrocarbonate (DEPC)-DW (Bioneer, Daejon, Korea). Viral genomic RNA was denatured at 95°C for 5 min, and then incubated at 42°C for 30 min in 10 μl of RT buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 100 U M-MLV reverse transcriptase (Bioneer, Daejon, Korea), 2.5 μM forward primer, 1 mM dNTP and 5 mM $MgCl_2$ for reverse transcription. The synthesized cDNA was amplified in 50 μl of PCR buffer containing 0.5 μM each PCR primer, 1.25 U Ex-Taq DNA polymerase (Takara, Shiga, Japan), 0.2 mM dNTP and 2 mM $MgCl_2$ using a thermal cycler (MyGenie 96 thermal block, Bioneer, Daejon, Korea) with 30 cycles (95°C for 1 min, 54°C for 1min, and 72°C for 1min). When PCR using primers VG1 and VD3 was negative at first amplification, a nested-PCR was performed using the same conditions as above.

Nucleotide sequence analysis

PCR products were analyzed by 1.5% agarose gel electrophoresis. PCR products were purified with QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) for nucleotide sequence analysis using an ABI PRISM 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The resulting sequences were assembled with Genetyx Win Ver. 5.1 software and construction of multiple alignments was done to infer geneticrelationships with neighbor-joining criteria using Clustal X software (Thompson *et al*., 1997). The final phylogenetic tree was drawn with the MEGA4 program (Tamura *et al*., 2007). The determined nucleotide sequences were registered in GenBank as accession numbers GU265809 - GU265815. The seven Korean VHSV in the present study were compared by

other 41 of worldwide isolates and other 6 Korean isolates reported in olive flounder (Table 1).

Table 1. Data for the 54 VHSV used in this study for phylogenetic analysis

Genotype	Isolate name	Origin	Year	Host species	GenBank no.
Ia	FR-07-71	France	1971	Rainbow trout	AJ233396
Ia	DK-7380	Denmark	1994	Rainbow trout	AY546594
Ib	DK-M.rhabdo	Baltic Sea	1979	Atlantic cod	Z93414
Ib	DK-6p403	Skagerrak	1999	Atlantic herring	AY546584
Ib	KRRV9601	Japan	1996	Olive flounder	DQ401190
Ib	DK-5e59	Kattegat	1998	Dab	AY546583
Ib	UK-MLA98/6 HE1	North Sea	1998	Atlantic herring	AY546584
Ib	UK-96-43	English Channel	1996	Atlantic herring	AF143862
Ic	DK-2835	Denmark	1982	Rainbow trout	AY546585
Ic	DK-5131	Denmark	1988	Rainbow trout	AF345858
Id	FI-ka-422	Gulf of Bothnia	2000	Rainbow trout	AY546615
Id	NO-A16368G	Norway	1968	Rainbow trout	AY546621
Ie	GE-1.2	Georgia	1981	Rainbow trout	AY546619
Ie	TR-Bs13/15H	Black Sea	2005	Turbot	AB231161
II	DK-1p52	Baltic Sea	1996	Sprat	AY546576
II	DK-1p53	Baltic Sea	1996	Atlantic herring	AY546577
III	DK-4p101	North Sea	1997	Whiting	AY546581
III	DK-4p168	Skagerrak	1997	Atlantic herring	AY546582
III	UK-H17/5/93	North Sea	1993	Atlantic cod	AY546630
III	UK860/94	Gigha	1994	Turbot	AY546628
IVa	US-Makah	USA, Washinton	1988	Coho salmon	U28747
IVa	Obama25	Japan	1999	Olive flounder	DQ401191
IVa	JF00Oit1*	Japan	2000	Olive flounder	Not reported
IVa	KRRV9822*	Japan	1998	Olive flounder	Not reported
IVa	Fukuya00*	Japan	2000	Olive flounder	Not reported
IVa	Cbr-BC93	Canada, British Columbia	1993	Pacific herring	U88051
IVa	Clw-WA91	USA, Washinton	1991	Coho salmon	U88050
IVa	Eby-WA93	USA, Washinton	1993	Pacific herring	U88055
IVa	Pws-AK90	USA, Alaska	1990	Pacific cod	U88052
IVa	Pws-AK93	USA, Alaska	1993	Pacific herring	U88053
IVa	BC99-292	Canada, Pacific	1999	Atlantic salmon	DQ401188
IVa	BC98-250	Canada, Pacific	1998	Atlantic salmon	DQ401187
IVa	BC99-001	Canada, Pacific	1999	Pacific sardine	DQ401195
IVa	BC99-010	Canada, Pacific	1999	Pacific herring	DQ401194
IVa	BC93-372	Canada, Pacific	1993	Pacific herring	DQ401186
IVa	ME03	USA, Maine	2003	Atlantic herring	DQ401192
IVa	FJeju05	Korea	2005	Olive flounder	FJ811902
IVa	FYeosu05	Korea	2005	Olive flounder	FJ811901
IVa	FWando05	Korea	2005	Olive flounder	FJ811900
IVa	JY-0112	Korea	2001	Olive flounder	AY167587
IVa	DN-0206†	Korea	2002	Olive flounder	Not reported
IVa	CS-0206†	Korea	2002	Olive flounder	Not reported
IVa	FWando08	Korea	2008	Olive flounder	In this study
IVa	FYG08	Korea	2008	Olive flounder	In this study
IVa	BF05-1	Korea	2005	Buffer fish	In this study
IVa	BF05-2	Korea	2005	Buffer fish	In this study
IVa	KF05	Korea	2005	Korean flounder	In this study
IVa	LH03	Korea	2003	Largehead hairtail	In this study
IVa	YBS05	Korea	2005	Yellowback seabream	In this study
IVb	MI03GL	USA, Michigan	2003	Muskellunge	DQ401193
IVb	CA-NB00-01	Canada, Atlantic	2000	Mummichog	EF079896
IVb	CA-NB02-01	Canada, Atlantic	2002	Striped bass	EF079897
IVb	CA-NB04-01	Canada, Atlantic	2004	Striped bass	EF079898
IVb	CA-NS04-01	Canada, Atlantic	2004	Brown trout	EF079899

* Same nucleotide sequences as Obama25

† Same nucleotide sequences as JY-0112

Results and Discussion

PCR products of approximately 690 bp were obtained by RT-PCR with the primers VG1 and VD3 (one-step PCR) for the G gene from two Korean VHSV isolates (FWando08 and FYG08) from cultured olive flounder, while approximately 440 bp of PCR products were amplified with the primers sets (VG1-VD3 and VD5-VD3; nested PCR) from five Korean VHSV (LH03, BF05-1, BF05-2, KF05 and YBS05) from wild marine fish (data not shown). The PCR products were sequenced and the determined nucleotide sequences of the partial G gene (nt 273-677) without the PCR primer sequences were compared with those of 47 worldwide VHSV isolates. The nucleotide sequences showed more than 99.5% identity among Korean VHSV, and more than 78.3% identity among 54 worldwide VHSV (data not shown). A radial tree of phylogeny based on the determined G gene nucleotide sequences revealed the four major clusters for genotypes I-IV, as described in previous studies (Fig. 2) (Benmansour *et al*., 1997; Stone *et al*., 1997; Nishizawa *et al*., 2002; Einer-Jensen *et al*., 2004; Elsayed *et al*., 2006; Gagné *et al*., 2007). Moreover, two minor clusters for classes IVa and IVb were observed for genotype IV. Genotype IVa represented isolates in Japan and in the Pacific coast areas of North America, and IVb included isolates originating from the eastern Atlantic coast of Canada and the Great Lakes of the North America. All eight Korean isolates (JY-0112, DN-0206, CS-0206, FJeju05, FYeosu05, FWando05, FWando08 and FYG08) from cultured olive flounder were classified into genotype IVa in the present tree (Fig. 2) and were closely related to isolates from the Pacific coast areas of North America and Japan. Also, all five Korean VHSV (BF05-1, BF05-2, KF05, LH03 and YBS05) from wild marine fish grouped in genotype IVa. Thus, VHSV belonging to genotype IVa may be responsible for the epizootic episodes of VHS in olive flounder farms in Korea.

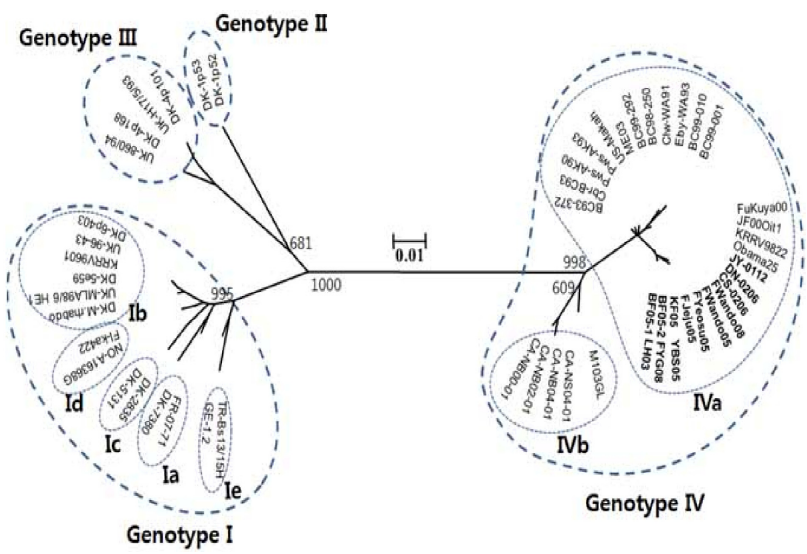

Fig. 2a

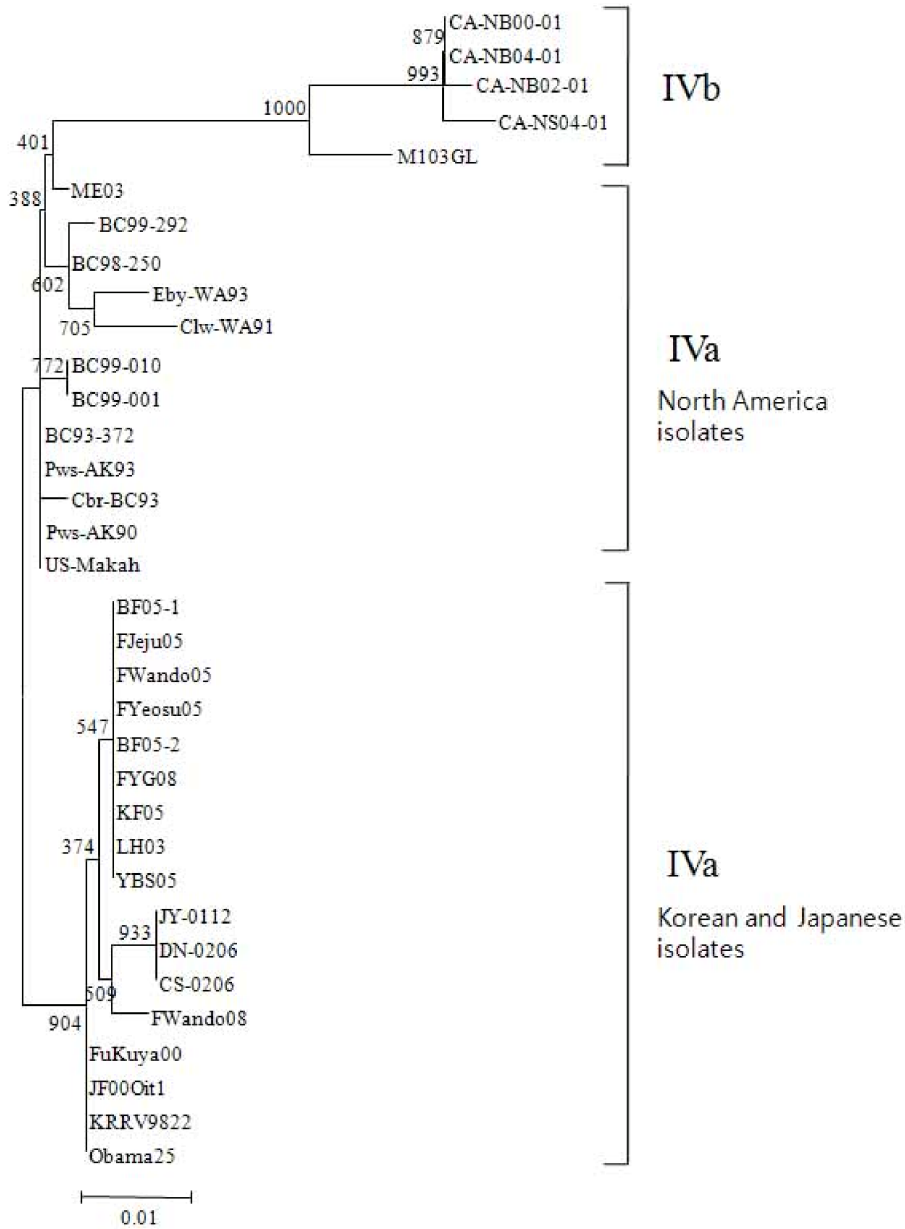

Fig. 2b

Fig. 2. Molecular phylogenetic tree showing the genetic relationships among 54 VHSV based on the nucleotide sequence of the partial G gene (nt 273-677) (a) and a phylogram using isolates belong to genotype IV (b). Bootstrap values at 1000 times of construction are shown at major nodes in the tree. Distance marker refers to the expected number of substitutions per site.

A recent study of VHSV in Japan suggests at least two different origins for VHSV in Japan (Nishizawa *et al*., 2002). In one route, Japanese isolates could be an indigenous virus in Japanese coastal areas, but not

have been introduced from North America because the Japanese isolates fall into one minor cluster in genotype IVa, which does not contain any American isolates. Alternatively, Japanese isolate may have originated outside of Japan because the isolate (KRRV9601) falls into the traditional European genotype Ib. However, the situation for Korean VHSV seemed different from that of Japanese isolates, because Korean VHSV was not included in the genotypes I, II and III. In addition, all Korean VHSV including the oldest Korean isolate (JY-0112) formed only one minor cluster including Japanese isolates, in genotype IVa, while the North America isolates formed a different minor cluster in genotype IVa (Fig. 2b). These results suggest that the Korean VHSV could be an indigenous virus in Korean and Japanese coastal areas, indicating that the origin of Korean VHSV may be from coastal water sharing Korea and Japan.

VHSV has been isolated from several wild marine fish species (Stone *et al*., 1997; Skall *et al*., 2005). Feeding of VHSV-infected marine fish to domestic stock may be a route of infection (Stone *et al*., 1997; Skall *et al*., 2005). In Korea, fresh minced marine fish containing of herring, chub mackerel *Scomber japonicus*, horse mackerel *Trachurus japonicas*, etc are commonly used in the flounder-farming industry as feed. Additionally, VHSV has been isolated or detected from several species of wild marine fish including chub mackerel in coastal area of Korea (Kim and Park 2004; present study). The previous and present data highlight the potential threat to the flounder farming industry from marine fish reservoirs of VHSV, and in particular the use of untreated trash fish as feed.

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