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Phylogenetic Analysis of Viral Hemorrhagic Septicemia Virus (VHSV) Isolated from Olive Flounders (*Paralichthys olivaceus*)

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Abstract: Viral Hemorrhagic Septicemia Virus (VHSV) is the most important viral pathogens that infects the olive flounders reared in Korean aquaculture. Thirty VHSV isolates were identified in farmed olive flounders from 2007 to 2011 by using the Epithelioma Papulosum Cyprini (EPC) cell line. The genetic diversity of the VHSV was evaluated by analyzing the nucleotide sequences of the partial Glycoprotein (*G*) and Nucleocapsid protein (*N*) genes of the 30 isolates. Researchers found that all the isolates were closely related to the Japanese and North American genotype IVa which is clearly distinct from the 3 European genotypes. The isolates formed a unique subgroup which was genetically separated from the Japanese and North American isolates. The nucleotide sequences of the 30 isolates exhibited very close identity with each other (>98.6% identity). This study shows that the VHSV genotype IVa strains are widely distributed throughout olive flounder farms in Korea.

Key words: VHSV, nucleotide, glycoprotein, pathogens, Korea

INTRODUCTION

Viral Hemorrhagic Septicemia (VHS) is known as one of the most serious viral diseases in farmed rainbow trout *Oncorhynchus mykiss* in European countries (Einer-Jensen *et al*., 2005a). Since, the first isolation of VHSV from the wild Atlantic cod (*Gadus morhua*) (Jensen *et al*., 1979) VHSV has been isolated from a variety of species of freshwater and marine fishes (Kim *et al*., 2011). VHSV belong to the genus Novirhabdovirus with in the family Rhabdoviridae. It has a single molecule of linear, negative-sense, single-strand RNA (~11.1 kb) with 6 genes in the order 3'-N-P-M-G-NV-L-5' which encode 5 structural proteins: Nucleoprotein (N), Phosphoprotein (P), Matrix protein (M), Glycoprotein (G), RNA polymerase (L) and a Nonstructural protein (NV) (Einer-Jensen *et al*., 2005b).

VHSV has been classified into 4 major genotypes (Genotype I-IV) on the basis of the *N* and *G* genes (Jensen *et al*., 1979; Kim *et al*., 2011). Each of these genotypes has a specific geographic and host range based on the associations. Genotype I for example, consists of 5 sub-lineages and includes a wide range of viruses isolated from fish caught in European freshwaters and the Baltic Sea. Interestingly, VHSV genotype IV has been isolated only once from farmed Japanese flounder in Japan, the origin of which has never been determined (Nishizawa *et al*., 2002). Genotype II includes isolates originating from a narrow region in the Baltic Sea while and Genotype III includes isolates from the North Sea and coastal waters of the United Kingdom. The fourth genotype of VHSV isolates genotype IV has been recovered from fishes in North America, Japan and Korea (Snow *et al*., 2004). Recently, a new sub-lineage of the North American genotype (genotype IVb) was found in muskellunge *Esox masquinongy* in the great lakes of the United States (Elsayed *et al*., 2006).

VHS is also known as one of the most important viral diseases of farmed olive flounder (Kim *et al*., 2009), a major species used in the aquaculture industry in Korea. VHSV was isolated from farmed olive flounders in 2001 and 2002. Korean isolates have been found to be closely related to the Japanese and North American genotypes based on the partial nucleotide sequences of the *G* and *N* genes (Kim *et al*., 2003) in both farmed and wild marine fishes from the coastal areas of Korea (Kim *et al*., 2011). Since, the primary genetic grouping of Korean VHSV was established using only 5 isolates recovered from olive founder in 2001 and 2002 (Kim *et al*., 2003). There is currently insufficient data available regarding the

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prevalence and phylogenetic analysis of VHSV distribution in the farmed olive flounder in Korea. Researchers investigated the prevalence of VHSV from 2007 to 2011 in Korea and reported the results of a phylogenetic analysis of the isolates on the basis of the partial sequences of their *G* and *N* genes.

MATERIALS AND METHODS

Samples: Olive flounders (length range: 5.5-23.5 cm) from 55 aqua farms located on Haenam, Wando and Jeju islands in South Korea were submitted to the Research Unit of Green Cross Veterinary Products and Ocean and Fisheries Research Institute, Jeju Special Self-Governing Province between 2007 and 2011. The submitted specimens showed the typical clinical signs of viral hemorrhagic septicemia including darkened coloration, clear ascitic fluid in the abdomen and hernia.

Virus isolation: Virus isolation was performed as described by Isshik *et al*. (2001) with some modifications. Briefly, the spleen, kidney and the heart tissues were homogenized in 9 volumes of Hank's Balanced Salt Solution (HBSS, SIGMA). Tissue homogenates were centrifuged at 8000 rpm for 20 min and the supernatants were filtered through a 0.20 μm syringe filter (Sartorius). The filtered supernatant was inoculated onto an Epithelioma Papulosum Cyprini (EPC) cell line. Cells were cultured in Eagle's Minimum Essential Medium (MEM, SIGMA) supplemented with 10% (v/v) Fetal Bovine Serum (FBS, GIBCO) and penicillin-streptomycin solution (at final concentrations of 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, Cellgro). Inoculated cells were incubated at 20°C and examined for the appearance of Cytopathic Effect (CPE) for 7 days after inoculation. The medium from the cell cultures showing CPE was stored in aliquots at -80°C for future use as stock virus and viral isolates were identified using RT-PCR. The data on the VHSV isolates recovered is shown in Table 1.

RNA extraction and RT-PCR amplification: Viral RNA was extracted from cell culture supernatants using TRIzol LS (Invitrogen) according to the manufacturer's instructions then eluted in 30 μL of DEPC-water. Reverse transcription was performed as previously described (Lee *et al*., 2010). Amplification of the partial viral Glycoprotein (*G*) gene and the Nucleocapsid protein (*N*) gene sequences was performed as described previously (Elsayed *et al*., 2006; Nishizawa *et al*., 2002) with 2 different primer sets. Briefly, the first primer set, VGsense (5'-CCA GCT CAA CTC AGG TGT CC-3') and VGanti (5'-GTC ACY GTG CAT GCC ATT GT-3') was used for amplification of 587 bp (nt 175-761) encoding the *G* gene fragment. The second primer set consisted of 5'-N+(5'-ATG ATG AGT TAT GTT ACA RGG-3') and 5'-N-(5'-TTG TCC ACC GAG TAC TTG GT-3'), targeting a 587 bp (nt 13-599) region of the *N* gene. In the RT-PCR reaction, annealing temperatures of each primer set for the *G* and *N* genes were 64 and 55°C, respectively. PCR amplification was performed using a Maxim PCR PreMix kit (INTRON) according to the manufacturer's instructions. PCR products were loaded on a 1.5% agarose gel and visualized by ethidium bromide staining.

Sequence analysis: PCR products were sequenced using a commercial service (SolGent Co., Ltd.) with the sequence of each product being determined twice. Sequence analysis was performed as previously described (Lee *et al*., 2010). Briefly, the phylogenetic trees were generated using the MEGA 4.0 program and ClustalX 3.81 Alignment Algorithm Software. The neighbor-joining tree was drawn using the Kimura-two Parameter Model to estimate the distance and percent frequencies of the groupings were determined after 1,000 bootstrap evaluations. Pairwise sequence alignments were performed using the Bioedit v7.0.9.0 Program to determine nucleotide sequence similarities. The reference viruses used in this study to compare the *G* and *N* genes are listed in Table 1.

Table 1: Sources of viral hemorrhagic septicemia virus strains in this study

					GeneBank Accession No.	
Genotypes	Strains	Host fish	Locality	Years	*G* gene	*N* gene
I	DK-Hededam	Rainbow trout	Denmark	1972	Z93412	Z93412
Ia	DK-6137	Rainbow trout	Denmark	1991	AY546593	DQ159190
Ia	AU-8/95	Rainbow trout	Austria	1995	AY546570	DQ159192
Ia	FR-0771	Rainbow trout	France	1971	AJ233396	AJ233396
Ia	FR-2375	Brown trout	France	1975	AY546617	DQ159191
Ib	DK-M.rhabdo	Atlantic cod	Baltic Sea	1979	Z93414	AY356632
Ib	DK-1p40	Rockling	Baltic Sea	1996	AY546575	AJ130919
Ib	JP-96KRRV9601	Olive flounder	Japan	1996	DQ401190	-
Ib	UK-96-43	Atlantic herring	English Channel	1997	-	AF143862
Ic	DK-2835	Rainbow trout	Denmark	1982	AY546585	-

Table 1: Continue

Genotypes	Strains	Host fish	Locality	Years	GeneBank Accession No.	
					G gene	*N* gene
Ic	DK-5131	Rainbow trout	Denmark	1988	AF345858	-
Id	NO-A16368G	Rainbow trout	Norway	1968	AY546621	-
Id	FI-ka422	Rainbow trout	Gulf of Bothnia	2000	AY546615	-
Ie	GE-1.2	Rainbow trout	Georgia	1981	AY546619	DQ159189
II	DK-1p52	Sprat	Baltic Sea	1996	AY546576	AY356744
	DK-1p53	Herring	Baltic Sea	1996	AY546577	AJ130921
	DK-1p55	Sprat	Baltic Sea	1996	AY546578	AY356746
III	FR-L59X	Eel	France	1987	AY546618	-
	UK-860/94	Turbot	Gigha	1994	AY546628	AJ130915
	UK-H17/2/95	Haddock	North Sea	1995	AY546629	AJ130924
	DK-4p101	Whiting	North Sea	1997	AY546581	AJ130918
	DK-4p168	Atlantic herring	Skagerrak	1997	-	AY356724
	UK-MLA98/6PT10	Norway pout	North Sea	1998	-	AY356723
	UK-MLA98/6 PT11	Norway pout	North Sea	1998	AY546632	-
IVa	US-Pws-AK90	Pacific cod	Alaska, USA	1990	U88052	-
	NA-7	Pacific cod	Alaska, USA	1991	Z93425	-
	NA-8	Coho salmon	Washington, USA	1991	Z93426	-
	US-PhrgElbay93	Pacific herring	Washington, USA	1993	-	AJ130925
	US-PcodAK93	Cod	Alaska, USA	1993	-	AJ130926
	US-Makah	Coho salmon	Washington, USA	1998	U28747	X59241
	BC99-010	Pacific herring	Pacific, Canada	1999	DQ401194	-
	BC99-001	Pacific sardine	Pacific, Canada	1999	DQ401195	-
	ME03	Atlantic herring	Marine, USA	2003	DQ401192	-
	JP-KRRV9822	Japanese flounder	Japan	1998	AB179621	AB179621
	JP99Obama25	Japanese flounder	Japan	1999	DQ401191	-
	JP-JF00Ehi1	Japanese flounder	Japan	2000	AB490792	AB490792
	JY-0112	Olive flounder	Korea	2001	AY167587	-
	FWando05	Olive flounder	Korea	2005	FJ811900	-
	FYeosu05	Olive flounder	Korea	2005	FJ811901	-
	FJeju05	Olive flounder	Korea	2005	FJ811902	-
	FWando08	Olive flounder	Korea	2008	GU265811	-
	FYG08	Olive flounder	Korea	2008	GU265812	-
	GCVP-23	Olive flounder	Jeju island, Korea	2007	-	JQ973856
	GCVP-13	Olive flounder	Jeju island, Korea	2008	-	-
	GCVP-14	Olive flounder	Jeju island, Korea	2008	-	JQ973853
	GCVP-15	Olive flounder	Jeju island, Korea	2008	-	-
	GCVP-24	Olive flounder	Jeju island, Korea	2008	-	JQ973857
	GCVP-25	Olive flounder	Jeju island, Korea	2008	-	-
	GCVP-16	Olive flounder	Jeju island, Korea	2009	-	JQ973854
	GCVP-17	Olive flounder	Jeju island, Korea	2009	-	JQ973855
	GCVP-18	Olive flounder	Jeju island, Korea	2009	JQ952780	-
	CGVP-01	Olive flounder	Jeju island, Korea	2010	JQ952775	JQ973848
	GCVP-02	Olive flounder	Jeju island, Korea	2010	-	JQ973849
	GCVP-03	Olive flounder	Jeju island, Korea	2010	JQ952776	-
	GCVP-04	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-05	Olive flounder	Jeju island, Korea	2010	-	JQ973850
	GCVP-06	Olive flounder	Jeju island, Korea	2010	-	JQ973851
	GCVP-07	Olive flounder	Jeju island, Korea	2010	JQ952777	-
	GCVP-08	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-09	Olive flounder	Jeju island, Korea	2010	JQ952778	-
	GCVP-10	Olive flounder	Jeju island, Korea	2010	-	JQ973852
	GCVP-11	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-12	Olive flounder	Jeju island, Korea	2010	JQ952779	-
	GCVP-19	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-20	Olive flounder	Jeju island, Korea	2010	JQ952781	-
	GCVP-21	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-22	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-26	Olive flounder	Jeju island, Korea	2010	-	-
	GCVP-27	Olive flounder	Jeju island, Korea	2011	JQ952782	JQ973858
	GCVP-28	Olive flounder	Wando, Korea	2011	-	-
	GCVP-29	Olive flounder	Haenam, Korea	2011	-	-
	GCVP-30	Olive flounder	Wando, Korea	2011	-	-
IVb	MI03GL	Muskellunge	Michigan, USA	2003	DQ401193	DQ427105

RESULTS

Nucleotide sequence analysis: Researchers identified the partial nucleotide sequences of both the *G* and *N* genes in 30 VHSV isolates. The partial *G* and *N* gene sequences were submitted to GeneBank as JQ952775-JQ952782 and JQ973848-JQ973858, respectively (Table 1). The determined nucleotide sequences of the partial G (nt 361-720) and *N* genes (nt 111-421) of the VHSV isolates were compared with those of other known North American, Japanese and European VHSV isolates in GeneBank. The nucleotide sequences of the partial *G* genes from the isolates were 99.1-99.7, 95.5-99.1 and 80.5-84.7% homologous to those of previously identified Japanese, North American and European isolates, respectively. While the identity of the *G* genes among the Korean isolates was 98.8-100% similar, all isolates showed 98.6-100% homology with previously identified isolates (JY-0112, FWando08, FYG08, FJeju05, FYeosu05 and FWando05). Nucleotide sequences of the partial *N* genes exhibited similar results. The sequence homology with the Japanese, North American and European isolates showed 97.7-98.3, 93.2-98.7 and 80-85.2% similarity, respectively and 98.6-100% identity among the Korean isolates.

Phylogenetic analysis: A neighbor-joining phylogenetic tree based on the sequences of the partial *G* and *N* genes is shown in Fig. 1. The VHSV genotypes were divided into 4 major clusters which agrees with previously published result (Einer-Jensen *et al*., 2005b). Korean VHSV isolates were closely related to the Japanese and North American genotype IVa which is clearly distinct from the 3 European genotypes. Interestingly, the Korean isolates formed a unique subgroup separate from the Japanese and North American isolates.

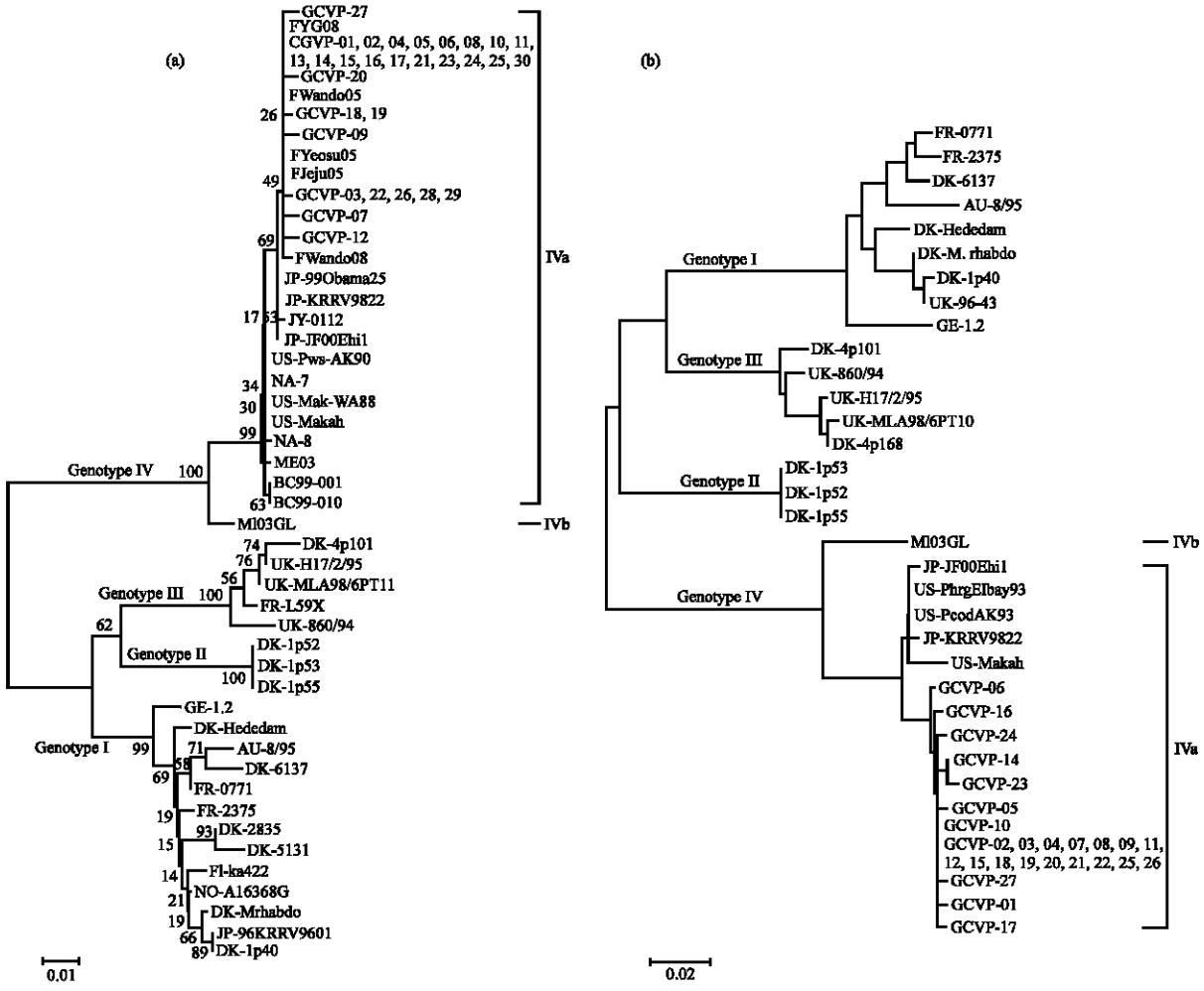

Fig. 1: Neighbor-joining phylogenetic tree based on the nucleotide sequences of the: a) *G* and b) *N* genes of the VHSV isolates in Korea

DISCUSSION

Since, the first report of VHSV isolated from olive flounder farms in the Eastern coastal regions of Korea (Kim *et al*., 2011), several studies have focused on VHSV isolated from farmed olive flounders and wild marine fish in the coastal areas of Korea (Kim and Park, 2004; Kim *et al*., 2011; Lee *et al*., 2007). To the best of the knowledge, this is the first study to assess the genetic characteristics of a large number of VHSV isolates isolated from olive flounders in Korea. To confirm the genetic characteristics of the VHSV isolates, researchers collected samples from Je-ju and Wan-do islands which are the major aquafarming regions for olive flounders in Korea.

Nucleotide sequence analysis of the *G* and *N* genes has been demonstrated to be useful for phylogenetic analysis of VHSV (Einer-Jensen *et al*., 2005b). Many previous studies based on *G* or *N* gene sequences have confirmed the existence of 4 major genotypes of VHSV isolates worldwide (Einer-Jensen *et al*., 2005b; Kim *et al*., 2011; Snow *et al*., 2004). The phylogenetic tree obtained in the present study agreed with these studies and the 30 VHSV isolates were classified into the genotype IVa along with the previous Korean isolates (Kim *et al*., 2003; Kim *et al*., 2011). Genotype IVa isolates have been primarily recovered in North America, Japan and Korea (Kim *et al*., 2011). The Korean VHSV isolates have been shown to be more closely related to the Japanese isolates than to the North American isolates the genetic similarities could be attributed to the fact that both Japan and Korea share a common sea from which the isolates were obtained. Stone *et al*. (1997) suggested that all marine fish species are potential carriers of VHSV. Genotype IVa VHSV has previously been detected in several species of wild marine fish in the coastal areas of Korea and Japan (Kim and Park, 2004; Lee *et al*., 2007; Nishizawa *et al*., 2002). It is possible that these marine reservoirs of the virus played a role in the emergence of VHSV between Korea and Japan. Nishizawa *et al*. (2002) suggested that the Japanese VHSV isolates are the native virus in Japanese coastal areas because the Japanese isolates form a minor cluster distinct from the North American isolates in genotype IVa.

In the present phylogenetic tree based on the partial nucleotide sequences of *G* and *N* genes, Korean VHSV isolates were shown to form a minor subgroup separate from the North American and Japanese isolates. Therefore, the Korean VHSV is also an indigenous virus of the coastal areas of Korea and Japan as suggested by Kim and Park (2004). However, the phylogenetic tree based on the *G* gene, JY-0112was grouped along with the Japanese isolates (JP-99Obama25, JP-KRRV9822 and JP-JF00Ehil) and therefore they are more closely related to the Japanese isolates (99.4%) than to the other Korean isolates (98.6-99.1%, Fig. 1). Since, JY-0112 was first isolated from olive flounders in Korea in 2001 this indicates that Korean VHSV evolved independently in the coastal areas of Korea. The VHSV isolates used in this study exhibited very close identity to each other. Although, RNA viruses are known to be highly adaptable and exhibit a high mutation rate (Snow *et al*., 2004), the nucleotide sequences of the Korean VHSV isolates were highly homologous inspite of the amount of elapsed time (2001-2011).

CONCLUSION

The aim of the present study was to analyze the prevalence and genetic characteristics of VHSV isolated from farmed olive flounders in Korea from between 2007 and 2011. The VHSV genotype IVa strains are widely distributed throughout the olive flounder farms in Korea and that the nucleotide sequences of these VHSV isolates have been conserved.

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