The Journal of Microbiology, June 2008, p. 265-273 Vol. 46, No. 3
DOI 10.1007/s12275-008-0004-7
Copyright ⓒ 2008, The Microbiological Society of Korea

Distribution of Marine Birnavirus in Cultured Olive Flounder *Paralichthys olivaceus* in Korea

Sung-Ju Jung[1*], Seok-Ryel Kim[1], Il-Yong Joung[1], Shin-Ichi Kitamura[1#], Hee-Taek Ceong[2], and Myung-Joo Oh[1]

[1]*Department of Aqualife Medicine, Chonnam National University, Yeosu 550-749, Republic of Korea*
[2]*Department of Mobile Software, Chonnam National University, Yeosu 550-749, Republic of Korea*
[#]*Present address: Center for Marine Environmental Studies (CEMES), Ehime University, Matsuyama 790-8577, Japan*

(Received December 28, 2007 / Accepted April 11, 2008)

Surveys of marine birnavirus (MABV) were undertaken in cultured olive flounder *Paralichthys olivaceus* from the south and west coastal areas and Jeju in Korea during the period January 1999 to April 2007. MABV was detected in all seasons from the fry, juveniles and adult fish from the areas examined. Evident cytopathic effects of the virus including rounding and cell lysis were observed in chinook salmon embryo (CHSE-214) and rainbow trout gonad (RTG-2) cells, but not in fathead minnow (FHM) and epithelial papilloma of carp (EPC) cells. Nucleotide sequences of the VP2/NS junction region of the Korean isolates showed 97.8%~100% similarity, and they belonged to the same genogroup. Cross neutralization tests with serotype-specific rabbit antisera against MABV strains exhibited a close antigenic relationships between strains, and were distinct from infectious pancreatic necrosis virus (IPNV) strains. Coinfection of MABV with bacteria (*Streptococcus iniae*, *Vibrio* spp.) and viruses (nervous necrosis virus, lymphocystis disease virus, viral hemorrhagic septicemia virus) was observed.

***Keywords*:** prevalence, marine birnavirus, aquatic birnavirus, olive flounder, genogroup, serogroup

Aquatic birnaviruses are the largest and most diverse group of viruses in the family Birnaviridae and include a variety of viruses from many species of fish and marine shellfish worldwide (Dobos *et al*., 1979; Reno, 1998; Jung *et al*., 1999). The viruses have been isolated from salmonids (Wolf, 1988), Japanese eels *Anguilla japonica* (Lee *et al*., 1999) and marine fish and shellfish species (Sorimachi and Hara, 1985; Kusuda *et al*., 1989; Nakajima *et al*., 1993; Suzuki *et al*., 1997a, 1997b; Suzuki *et al*., 1998). Many of the aquatic birnaviruses from a variety of marine fish and shellfish species in Asia are closely related to members of the infectious pancreatic necrosis virus (IPNV) Ab serotype, whereas most of the VR-299 serotype viruses from Asia have been isolated from salmonids (Lipipun *et al*., 1989; Chou *et al*., 1993). Birnaviruses having a marine origin can be distinguished from IPNV by serotyping and genogrouping according to sequences of the VP2/NS junction regions (Kusuda *et al*., 1993; Hosono *et al*., 1994; Hosono *et al*., 1996). Suzuki *et al*. (1997b) proposed the name "marine birnavirus" (MABV) for those birnaviruses of marine fish and shellfish origin, including yellowtail ascites virus (YTAV). MABVs have been isolated from many cultured fish species such as yellowtail *Seriola quinqueradiata*, amberjack *Seriola dumerili*, makogarei *Limanda yokohamae*, and rockfish *Sebastes schlegeli* (Joh *et al*., 2000b; Isshiki *et al*., 2004).

In Korea, IPNVs that are serologically similar to the VR-299 and Ab strain have been isolated from cultured chum salmon *Oncorhynchus keta* and goldfish *Carassius auratus*, respectively (Hah *et al.,* 1984; Hedrick *et al*., 1985). In addition, MABV from olive flounder *Paralichthys olivaceus*, rockfish, sea bass *Lateolabrax japonicus*, blue mussel *Mytilus galloprovincialia,* and sea squirts *Halocynthia roretzi* has been described (Oh *et al*., 1999b; Joh *et al*., 2000b; Oh *et al*., 2006a; Azumi *et al*., 2007; Kitamura *et al*., 2007). In this study, we summarize our laboratory diagnosis results of MABV detection in cultured olive flounder between January 1999 and April 2007 and the results of serological and sequence comparisons among isolated stains.

Materials and Methods

Fish

Samples were delivered by fish farmers or by express mail to the laboratory for diagnosis when abnormal mortality occurred in olive flounder farms. When a serious mortality was reported, a laboratory member went to the farm to collect diseased fish. Diagnoses were carried out during the period January 1999 to April 2007.

Microscopic observation

Skin, gills, fins, and brain were observed in wet preparations at 40, 100, and 400 magnifications using microscope to confirm parasites and lymphocystis cells.

Bacteria isolation

Bacteria was isolated from the kidney and spleen using agar plates of Brain Heart Infusion (BHI) (Difco, USA), Salmonella Shigella (SS) (Difco), and Thiosulfate Citrate Bile

* To whom correspondence should be addressed.
(Tel) 82-61-659-3175; (Fax) 82-61-659-3175
(E-mail) sungju@chonnam.ac.kr

Sucrose (TCBS) (Difco) if necessary.

Virus isolation and cell lines

The kidney and spleen pool was homogenized in Hank's solution. For fry, all visceral organs were homogenized. The homogenate was centrifuged 2,000×g for 10 min and the filtered supernatant was inoculated onto chinook salmon embryo (CHSE-214) cells. The CHSE-214 cells were cultured in Eagle's Minimum Essential Medium (EMEM) (Gibco BRL, USA), supplemented with 10% fetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 μg/ml) at 15°C. Cell susceptibility assays were conducted using three fish cell lines: rainbow trout gonad (RTG-2), epithelial papilloma of carp (EPC) and fathead minnow (FHM) cells.

Polymerase chain reaction (PCR) and sequence analysis

The virus genome was extracted by the method of Oh *et al*. (1999a). Reverse transcription-PCR and nested-PCR were performed as described by Suzuki *et al*. (1997a, 1997b). Briefly, viral genomic RNA extracted using Trizol regent (Gibco BRL) and chloroform was used as the template for RT-PCR. PCR primers P1-P2 (P1; 5'-AGAGATCACTGAC TTCACAAGTGAC-3', P2; 5'-TGTGCACCACAGGAAAGAT GACTC-3') and P3-P4 (P3; 5'-CAACACTCTTCCCCATG-3', P4; 5'-AGAACCTCC CAGTGTCT) were used for RT-PCR and nested PCR, respectively. PCR amplification was performed using an AccuPower™ PCR Premix Kit (Bioneer) according to the manufacturer's instructions. Cycle sequencing reactions of amplified fragments were carried out using the manufacturer's recommended conditions. Sequences were analyzed by Genetyx-Mac version 8.0. The sequences were compared with previously reported data for the MABV isolates. Alignments were analyzed with the MEGA program version 4.0 (Tamura *et al.*, 2007) to produce neighbor-joining (NJ) trees using the Kimura two-parameter model. Confidence in the NJ trees was determined by analyzing 1,000 bootstrap replicates using the MEGA program. The nucleotide sequences used in this paper are available from the GenBank databases and corresponding countries, host species and accession numbers are provided in Table 1.

Other viruses infecting olive flounder such as hiramerhabdovirus (HIRRV), viral hemorrhagic septicemia virus (VHSV), and nervous necrosis virus (NNV) were examined using PCR as our previous reports (Oh and Choi, 1998; Oh

Table 1. Aquatic birnavirus strains employed in this study for comparison

Strain/Isolate	Geographic origin	Host species	GenBank accession no.	Genogroup
TN3-A89	Canada	-[a]	L13991	I
Tomakomai	Japan	Rainbow trout	AB179708	
VR299	USA	Trout	AF343572	
11	USA	Trout	AY026347	
93	USA	Trout	AY026346	
AM-98	Japan	Amago	AY283780	
Buhl	USA	Trout	AF343573	
Ab	Denmark	Trout	AF342729	II
578	Spain	Turbot	AJ489228	
Sole virus	Spain	Sole	EF493156	III
S-IPNV-SH2-98	Norway	-	DQ536091	
Sp	Denmark	Trout	AJ829474	
Sp116	Norway	Atlantic salmon	AY354520	
ASV	Canada	Atlantic salmon	AY026490	IV
Canada1	Canada	Trout	L13979	
Canada2	Canada	Trout	AF342733	V
He	USA	Pike	AF342730	VI
Hecht	Germany	Pike	L40583	
Obama10	Japan	Olive flounder	AB179705	VII
Obama29	Japan	Olive flounder	AB179706	
AKK02SS	Japan	Snowy sculpin	AB179701	
Y-6	Japan	Yellowtail	AY283781	
Izu18	Japan	Olive flounder	AB179704	
SY	Japan	Yellowtail	D61385	
DS	Korea	Olive flounder	AY064395	

[a] Not reported

Table 2. MABV detection from diseased olive flounder from 1999 to 2007

Date		Area	Average fish size (cm)	Isolation	PCR	Strain[a]	Other pathogens
1999	1.25	Yeonggwang	9.3	−	+		Nervous necrosis virus
	4.14	Jindo	3.2	+	+	NC1	
	5. 1	Goheung	2.4	+	+	NC2	
	10.25	Yeonggwang	6.0	−	+		
2000	3.18	Goheung	1.8	+	+	FK	
	3.19	Goheung	3.1	+	+	FG1	
	4.17	Yeonggwang	1.7	+[b]	ND		
	4.25	Hampyeong	2.8	+	+	FH1	
	4.25	Hampyeong	1.7	+	+	FH2	
	10. 8	Yeosu	2.1	−	+		Herpesvirus
2001	1.10	Muan	3.0	+	+	FM1	
	1.16	Goseong	12.0	+	+	FJ	Scuticociliates
	3. 8	Muan	2.7	+	+	FM2	
	3.22	Muan	2.9	+	+		
	4.12	Haenam	13.2	+[b]	ND		
	4.19	Namhae	11.4	+	+	FSA1	
	4.19	Namhae	12.5	+	+	FSA2	
	4.19	Wando	14.7	+	+		
	7. 4	Wando	4.3	+	+		
	7.28	Wando	17.0	+		FWA	
	11. 8	Yeonggwang	9.8	+	+	FY1	
	11.23	Tongyeong	5.0	+	+	FC	
	1.12	Jindo	ND	+	+		
	3. 8	Yeonggwang	Eggs	−	+		
	3.15	Yeonggwang	1.2	+	+	FY2	
	4.22	Hampyeong	2.2	ND	+		
	5.20	Wando	7.0	−	+		
	6. 5	Wando	45.3	+	+		*Streptococcus iniae*
	7.25	Jindo	ND	+	+		
	8.16	Jindo	24.6	+	+		
2003	4.22	Muan	6.5	+	+		
	8. 1	Wando	16.7	+	+		
	8.19	Wando	12.0	+	+		
	8.21	Geoje	24.4	+	+		
2004	8. 6	Seogwipo	17.5	ND	+		*Vibrio* spp.
	10. 2	Tongyoung	2.7	+	+		
2006	4.18	Wando	17.1	+	+		VHSV[c]
	4.18	Wando	15.6	+	+		VHSV, *Vibrio* spp.
	6.22	Seogwipo	30.9	+	+		*Streptococcus iniae*
	7. 5	Namhae	17.5	ND	+		
	8. 7	Yeosu	18.0	ND	+		LCDV[d]
	8.25	Namhae	25.7	ND	+		LCDV
2007	4.10	Wando	20.8	−	+		

[a] Strain names are given in case sequence data are shown.
[b] Cytopathic effect in CHSE-214 cell line, but not in FHM and EPC cell line.
[c] Viral hemorrhagic septicemia virus
[d] Lymphocystis disease virus
ND, not determined

et al., 2002; Oh *et al*., 2005; Lee *et al*., 2007).

Preparation of antisera

Sera were prepared in rabbits by inoculation of NC1 and NC2 virus from olive flounder that had been purified by centrifugation on 30% sucrose and 40% CsCl. The partially purified viruses were mixed with complete Freund's adjuvant and injected into the backs of New Zealand white rabbits. Three weeks later, 1 ml of virus mixed with incomplete Freund's adjuvant was inoculated by subcutaneous injection. Serum was collected from blood taken four days after the final injection in the marginal ear vein and the antisera were inactivated by heating for 30 min at 56°C.

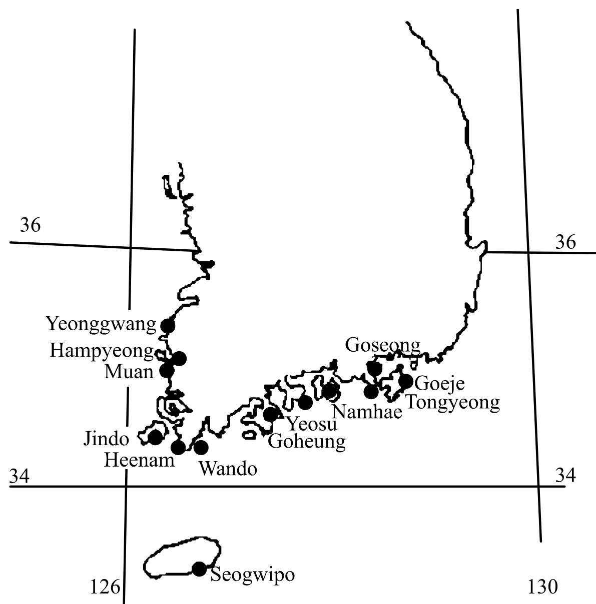

Fig. 1. Map of olive flounder *Paralichthys olivaceus* farms where marine birnavirus (MABV) was detected (●).

Cross-neutralization assay

The relationships of the six Korean isolates to the reference strains of IPNV (Ab, Sp, and VR-299) and MABV (Y-6) were examined. The neutralization test was carried out according to the method of Okamoto *et al*. (1983). In brief, viruses were diluted in EMEM to obtain a titer of approximately 100~1,000 $TCID_{50}$/50 μl and serial twofold dilutions of the rabbit antisera were made. Viruses and antisera dilutions were mixed (1:1) and incubated for 1 h at room temperature. The amount of remaining virus infectivity was determined by $TCID_{50}$ analysis. The antigenic relatedness (1/r) between the viruses were calculated according to Archetti and Horsfall (1950), from the formula $r=r1\times r2$ where r1 and r2 are the titer ratios (heterologous titers ÷ homologous titers) for antisera prepared for the two viruses being compared.

Results

Clinical signs

Most of dead and moribund juveniles and fry exhibited a dark body color, and a distended abdomen due to accumulation of ascitic fluid in the abdominal cavity. Gills were pale, and the stomach and intestine were invariably empty

```
NC1   GCGGCACCAC  TCATCGGAGC  CGCCGACCAA  TTCATCGGGG  ACCTGACCAA   50
FK    **********  *******G**  **********  **********  **********
FJ    **********  **********  **********  **********  **********
FWA   **********  **********  T*********  **********  **********
Y-6   **********  **********  **********  ********A*  **********

NC1   GACCAACGCA  GCCGGAGGCC  GCTACCTAAC  ACATGCAGCA  GGAGGACGCT   100
FK    **********  **********  **********  **********  **********
FJ    **********  **********  **********  **********  **********
FWA   **********  **********  **********  **********  **********
Y-6   **********  **********  **********  **********  **********

NC1   ACACTGACGT  AATGGACTCC  TGGGCCAGCG  GCAC                     134
FK    **********  **********  **********  ****
FJ    **********  **********  ****G*****  ****
FWA   **********  **********  **********  **T*
Y-6   **********  **********  **********  ****
```

Fig. 2. Nucleotide sequences of the nested PCR products (134 bp, i.e. 168 excluding primer length) for the 15 Korean strains. NC1 is representative for the NC2, FG1, FH1, FH2, FM2, FY1, FY2, FC, FSA1, and FSA2. FJ and FM strains exhibit same sequences. Sequences for the MABV Y-6, isolated from yellowtail *Seriola quinqueradiata* in Japan, are shown for comparison. (*) indicates nucleotide identical to that of strain NC1.

and filled with mucus exudates. Adult fish showed symptoms of ascitic fluid in the abdominal cavity, congestion in the liver and no food in the intestine with hemorrhages. Some fish exhibited hemorrhages on the body surface and white nodules in the kidney and spleen. Clump of lymphocystis cells due to lymphocystis disease virus (LCDV) was seen in dorsal and caudal fins by gross observations in some large fish.

Microscopic observation

Scuticociliate (parasitic ciliate) was observed from the ulcer lesions in the skeletal muscles (Table 2). Typical lymphocystis cells with enlarged size, thick transparent membrane was confirmed by microscopy. In addition, massive epithelial cell growth in fins on microscopic observation was confirmed due to herpesvirus using electron microscope (data not shown).

Virus isolation

A cytopathic effect (CPE) was observed 3~5 days after inoculation of tissue homogenates onto CHSE-214 cells. The CPE initiated from rounding and subsequently the granulation of cytoplasm, pyknosis, and final cell lysis were observed. The cell lines most susceptible to MABV were CHSE-214 and RTG-2, but the FHM and EPC cell lines did not produce CPE. When CPE was only observed in CHSE-214 cells but not in FHM cells, the sample was tentatively regarded as MABV positive and processed for PCR. MABV was isolated from the south and west coastal areas and Jeju in all seasons from fry, juveniles, and adult fish (Fig. 1 and Table 2).

Virus detection by PCR

Marine birnavirus was detected as 170 bp PCR product by nested-PCR in olive flounder cultured in the Yeonggwang,

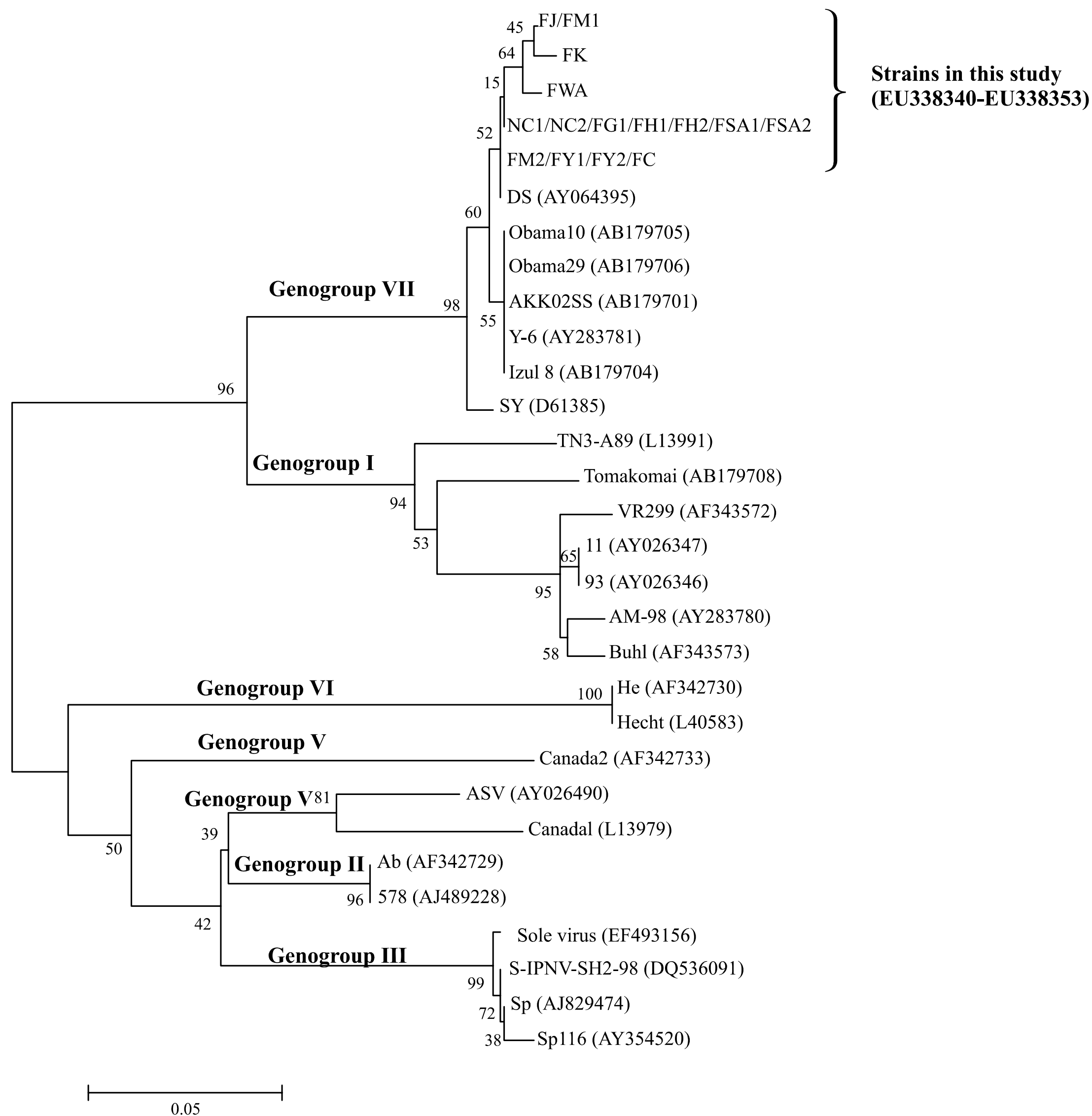

Fig. 3. Molecular phylogenetic tree based on nucleotide sequences of the VP2/NS junction region among MABV and IPNV. Corresponding GenBank accession numbers of each strain are shown in a round bracket.

Table 3. Antigenic relationship based on 1/r between birnaviruses

Virus	Virus					
	NC1	NC2	Y-6	Ab	Sp	VR-299
MABV NC1	1	0.78	0.75	3.05	17.54	8.41
MABV NC2		1	0.76	-	-	-
MABV Y-6			1	9.04	47.73	13.69
IPNV Ab				1	43.87	21.27
IPNV Sp					1	15.41
IPNV VR-299						1

* Data are given as 1/r, with r=r1×r2, and r1 and r2 are the titer ratios, calculated as the ratio between the heterologous and the homologous titers. Higher 1/r value means less antigenic relations.

Hampyeong, Muan, Jindo, Wando, Goheung, Yeosu, Namhae, Tongyeong, Goseong, Geoje, and Seogwipo areas during all seasons of the year (Fig. 1 and Table 2). Nervous necrosis virus (NNV) and viral hemorrhagic septicemia virus (VHSV) were detected from the MABV detected fish at Yeonggwang (1999) and Wando (2006) sample, respectively (Table 2).

Sequences of the VP2/NS junction region

Figure 2 shows the 134 base-long nucleotide sequences of the Korean isolates and the MABV Y-6 strain for the reference stain isolated from yellowtail in Japan. The determined nucleotide sequences were registered with GenBank under accession no. EU338340-EU338353. Korean isolates showed a high homology of 97.8%~100% among the 15 strains. Eleven strains of NC1, NC2, FG1, FH1, FH2, FM2, FY1, FY2, FC, FSA1, and FSA2 exhibited same sequences. In addition, Korean isolates exhibited 97.8%~99.3% similarity with Y-6. Genomic mutations were found in the nucleotide at the 18th, 21th, and 125th positions between Korean strains. Amino acid mutation was observed at the 125th position G of FJ and FM1 strain, resulting in an amino acid change of alanine to glycine. All isolates from olive flounder in this study and MABV Y-6 were included in the same genogroup, Genogroup VII (Fig. 3).

Virus neutralization

The antiserum against the flounder isolates totally neutralized the reference MABV Y-6 originally isolated from yellowtail in Japan, but the neutralization titer was low with anti-IPNV. Cross neutralization ratios of 1/r for NC1 and NC2 with reference strains are 0.78 as given in Table 3. The 1/r value between NC1 and MABV Y-6 was 0.75 and that between NC2 and MABV Y-6 was 0.76, indicating a close antigenic relationship between these viruses. 1/r value of MABV NC1 and Y-6 with IPNV stains was between 3.05 and 47.73.

Discussion

A decade has passed since MABV was first reported in marine fish from Korea (Sohn *et al*., 1995; Oh *et al*., 1999a, 1999b). It has caused high mortality in the early stages of olive flounder and is regarded as an important disease of olive flounder. Therefore, regulations are currently in place to reduce the dissemination of diseases by restriction of the movement of MABV carrier fish between national boundaries. However, this study showed that MABV has widely infected olive flounder in the south and west coasts and Jeju where most of the olive flounder culture farms are located. When an area is already contaminated, restriction of movement of carrier fish may not a solution for disease control. This study shows most of the coastal areas are contaminated by MABV and indicates the need for strategies other than restricting the movement of carrier fish, to reduce economic damages.

With the aid of the advanced aquaculture technology, seed production of olive flounder is totally under control. Olive flounder culture is totally based on hatchery-produced larvae, and is mostly practiced in a flow-through system in land-based facilities. However, many hatcheries do not keep brood stock and buy fertilized eggs from a hatchery holding brood stock (mostly from the Jeju region). When the brood stock is contaminated, the disease can easily spread all over the culture farms through eggs. In the case of IPNV, fish that survive an IPNV epizootic become an active carrier of the virus, and these fish are capable of transmitting the virus to their offspring in either the spermatozoa or eggs (Ahne and Neyele, 1985; Dorson and Torchy, 1985). Oh *et al*. (2000) also detected MABV in eggs, ovarian fluid and seminal fluid with virus titers of $10^{2.3}$~$10^{4.3}$ $TCID_{50}$/g. Effective diseases control of brood stocks for seed production is one of the key issues in olive flounder aquaculture to prevent the spread of the disease.

The junction region between VP2 and NS in the genome segment A of 15 Korean isolates and one reference strain from Japan was highly homologous. They exhibited nucleotide differences at three bases. Among the differences, 39th base G from olive flounder was comparable with A from the genus *Seriola* and mollusks (Suzuki and Nojima, 1999). Isshiki *et al.* (2004) also reported six strains of MABV from olive flounder exhibiting G at the 39th base. The mutation seems to be specific to olive flounder. Although the nucleotide mutation did not cause amino acid changes, it is an interesting phenomenon shown in MABV isolated from olive flounder. The VP2 and NS junction region is known to be variable and thus is useful for genogrouping of aquabirnaviruses (Heppell *et al*., 1992, 1993; Hosono *et al*., 1996; Nishizawa *et al*., 2005). Heppell *et al*. (1992) reported that the VP2/NS junction region is suitable for the analysis of genomic variation between IPNV strains. This genomic part

of the MABV was analyzed by Hosono *et al*. (1996), who concluded that MABV is in a distinct genogroup with IPNV. The nucleotide sequences of the VP2/NS junction of the 15 Korean isolates in this study are highly homologous to each other and are included in genogroup VII, the same as the other MABV strains in Korea and Japan. From these results, it can be concluded that serologically and genetically similar types of MABV occur in olive flounder in coastal areas of Korea and Japan. Similarly, 2,919 bp nucleotide sequence of genomic segment A from two isolates from Korea (NC1 strain in this study and UR1 from sea squirts) and 10 isolates from Japan were clustered into one genogroup, which was distinct from the other six IPNV genogroups (Zhang and Suzuki, 2004).

In the present study, NC1 and NC2 isolates of MABV from Korea could be included in the same serogroup distinct from IPNV. In addition, MABV isolates from brood stock (E-2-E, S-2-S, S-3-E, and W-1-S) in our previous study (Oh *et al*., 2000) were also included in the same serogroup with NC1 and NC2. The cross-neutralization titers among NC1, NC2, and the reference strain of Y-6, which was isolated from yellowtail in Japan, were extremely similar to each other. In addition, anti-NC1 serum successfully neutralized NC1 (homologous strain), FM1, FSA1, and FWA with neutralization titer (reciprocal of the 50% neutralization endpoint) of 19808, 14202, 15012, and 15758, respectively. The results indicate that these isolates are serologically homologous. Novoa and Figueras (1996) reported that six marine birnaviruses isolated from turbot *Scophthalmus maximus* in a restricted geographic area off the coast of Galicia (northwestern Spain) revealed a high heterogeneity of nucleotide sequences and polypeptide electrophoretic patterns and their reactions with the monoclonal antibodies. In addition, the turbot isolates were not exactly the same as the reference strains of IPNV Ab, Sp, and VR-299. Cutrin *et al*. (2000) also described the diversity of birnavirus isolates from fish, shellfish, and other reservoirs in Galicia. They compared 231 strains, and demonstrated that most of the Galician isolates were of the European Ab and Sp serotype. In contrast, our results demonstrated that MABV isolates from olive flounder could be included in a serotype. In addition, MABV Y-6 originally isolated from yellowtail in Japan was also homologous with Korean strains. MABV Y-6 was also homologous with other MABV isolates from several marine fish in Japan (Kusuda *et al*., 1993). Therefore, birnavirus from various marine fish in coastal areas of Korea and Japan are serologically homologous, in contrast to other studies in Europe (Novoa and Figueras, 1996; Cutrin *et al*., 2000). Nishizawa *et al.* (2005) also suggested that aquabirnavirus isolated from marine fish in Japan were indigenous to Japan, based on a phylogenetic analysis.

It is considered that fry and juvenile fish under 10 g in body weight are most susceptible to MABV (Nakajima *et al*., 1998), and the pathogenicity of MABV is higher in smaller fish (Sorimachi and Egusa, 1986; Kusuda *et al*., 1989). In our study, for fry and juvenile fish less than 1 g with clinical signs of ascites in the abdominal cavity and pale gills, their mortality was considered to be related to MABV infection. The peak of mortality was observed in 24- to 30-day-old fish that were metamorphosing and started to settle on the bottom of the culture tanks. Isshiki *et al*. (2001) reported MABV can infect various fish species without clinical signs with titers of under 10^6 PFU/g in the infected fish. Pakingking *et al.* (2004) also reported that MABV-infected olive flounder weighing 18 g did not show any abnormality, even when extremely high concentrations of MABV were detected ($10^{8.3}$ $TCID_{50}$/g). It is very similar to IPNV, which is only lethal to young animals, although the virus can be readily isolated in high concentrations from the viscera of infected fish during the lifespan of the host. Accordingly, extensive care of young stages of olive flounder is especially important. For hatchery and brood stock, using underground seawater is an option to reduce infection risks from serious disease.

In this study, MABV was isolated from fish over 10 g with coinfection of other pathogens as cases shown in Table 2. In our previous study, MABV alone did not cause mortality in 68~99 g fish, similar to other studies. However, when fish with MABV were coinfected with bacteria such as *Vibrio harveyi* and *Edwardsiella tarda*, the mortality was higher than only for bacterial infection (Oh *et al*., 2006b). Therefore, although MABV alone is not regarded as a serious pathogen of fish over 10 g, careful attention is needed to understand the effect of coinfection in fish. Information in this study on MABV distribution, genogroup, serogroup, and coinfection cases can be useful to build further disease control strategy.

Acknowledgements

This research was supported by the MIC (Ministry of Information and Communication), Korea, under the ITRC (Information Technology Research Center) support program supervised by the IITA (Institute of Information Technology Advancement) (IITA-2007-C1090-0701-0001)

References

Ahne, W. and R.D. Neyele. 1985. Studies on the transmission of infectious pancreatic necrosis virus via eyed eggs and sexual products of salmonid fish, p. 262-270. *In* A. Ellis (ed.), Fish and shellfish pathology. Academic Press, London, UK.

Archetti, I. and F.L. Horsfall. 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization *in vivo* with heterologous immune serum. *J. Exp. Med*. 92, 441-462.

Azumi, K., S. Nakamura, S.I. Kitamura, S.J. Jung, K. Kanehira, H. Iwata, S. Tanabe, and S. Suzuki. 2007. Accumulation of organotin compounds and marine birnavirus detection in Korean ascidians. *Fisheries Sci.* 73, 263-269.

Chou, H.Y., C.F. Lo, M.C. Tung, C.H. Wang, H. Fukuda, and T. Sano. 1993. The general characteristics of a birnavirus isolated from cultured loach (*Misgurnus anguillicaudatus*) in Taiwan. *Fish Pathol.* 28, 1-7.

Cutrin, J.M., J.G. Olveira, J.L. Barja, and C.P. Dopazo. 2000. Diversity of infectious pancreatic necrosis virus strains isolated from fish, shellfish, and other reservoirs in Northwestern Spain. *Appl. Environ. Microbiol*. 66, 839-843.

Dobos, P., B.J. Hill, R. Hallet, D.T.C. Kells, and H. Becht. 1979. Teninges biophysical and biochemical characterization of five animal viruses with bisegmented double-stranded RNA Genomes. *J. Virol.* 32, 593-605.

Dorson, M. and C. Torchy. 1985. Experimental transmission of infectious pancreatic necrosis via the sexual products, p. 251-261. *In* A. Ellis (ed.), Fish and shellfish pathology. Academic Press, London, UK.

Hah, Y.C., S.W. Hong, M.H. Kim, J.L. Fryer, and J.R. Winton. 1984. Isolation of infectious pancreatic necrosis virus from goldfish (*Carassius auratus*) and chum salmon (*Oncorhynchus keta*) in Korea. *Korean J. Microbiol.* 22, 85-90.

Hedrick, R.P., W.D. Eaton, J.L. Fryer, Y.C. Hah, J.W. Park, and S.W. Hong. 1985. Biochemical and serological properties of birnavirus isolated from fish in Korea. *Fish Pathol.* 20, 463-468.

Heppell, J., L. Berthiaume, F. Corbin, E. Tarrab, J. Lecomte, and M. Arella. 1993. Comparison of amino acid sequences deduced from cDNA fragment from infectious pancreatic necrosis virus (IPNV) strains of different serotypes. *Virology* 195, 840-844.

Heppell, J., L. Berthiaume, E. Tarrab, J. Lecomte, and M. Arella. 1992. Evidence of genomic variations between infectious pancreatic necrosis virus strains determined by restriction fragment profile. *J. Gen. Virol.* 72, 2863-2870.

Hosono, N., S. Suzuki, and R. Kusuda. 1994. Evidence for relatedness of Japanese isolates of birnaviruses from marine fish to IPNV. *J. Fish Dis.* 17, 433-437.

Hosono, N., S. Suzuki, and R. Kusuda. 1996. Genogrouping of birnavirus isolated from marine fish: a comparison of VP2/NS junction regions on genome segment A. *J. Fish Dis.* 19, 295-302.

Isshiki, T., T. Nagano, K. Kanehira, and S. Suzuki. 2004. Distribution of marine birnavirus in cultured marine fish species from Kagawa prefecture, Japan. *J. Fish Dis*. 27, 89-98.

Isshiki, T., T. Nagano, and S. Suzuki. 2001. Infectivity of aquabirnavirus strains to various marine fish species. *Dis. Aquat. Org.* 46, 109-114.

Joh, S.J., J.H. Kim, and G.J. Heo. 2000a. Genetic analysis of the VP2/NS junction region on segment A of marine birnavirus isolated from cultured flounder *Paralichthys olivaceus*. *J. Microbiol.* 38, 44-47.

Joh, S.J., D.W. Kim, J.H. Kim, and G.J. Heo. 2000b. Detection of marine birnavirus (MBV) from rockfish *Sebastes schlegeli* using reverse transcription and nested PCR. *J. Microbiol.* 38, 260-264.

Jung, S.J., S.I. Kitamura, K. Kawai, and S. Suzuki. 1999. Isolation of different types of birnavirus from ayu *Plecoglossus altivelis* and amago salmon *Oncorhynchus rhodurus* cultured in the same geographic area. *Dis. Aquat. Org.* 38, 87-91.

Kitamura, S.I., J.Y. Ko, W.L. Lee, S.R. Kim, J.Y. Song, D.K. Kim, S.J. Jung, and M.J. Oh. 2007. Seasonal prevalence of lymphocystis disease virus and aquabirnavirus in Japanese flounder *Paralichthys olivaceous* and blue mussel *Mytilus galloprovincialis*. *Aquaculture* 266, 26-31.

Kusuda, R., K. Kado, Y. Takeuchi, and K. Kawai. 1989. Characteristics of two virus strains isolated from young Japanese flounder *Paralichthys olivaceous*. *Suisan Zoushoku* 37, 115-120.

Kusuda, R., Y. Nishi, N. Hosono, and S. Suzuki. 1993. Serological comparison of birnavirus isolated from several species of marine fish in south west Japan. *Fish Pathol*. 28, 91-92.

Lee, N.S., Y. Nomura, and T. Miyazaki. 1999. Gill lamellar pillar cell necrosis, a new birnavirus disease in Japanese eels. *Dis. Aquat. Org.* 37, 13-21.

Lee, W.L., H.M. Yun, S.R. Kim, S.J. Jung, and M.J. Oh. 2007. Detection of viral hemorrhagic septicemia virus (VHSV) from marine fish in the south western coastal area and east China sea. *J. Fish Pathol*. 20, 201-209.

Lipipun, V., P. Caswell-Reno, Y.L. Hsu, J.L. Wu, M.C. Tung, P.W. Reno, W. Wattanavjarn, and B.L. Nicholson. 1989. Antigenic analysis of Asian aquatic birnavirus isolates using monoclonal antibodies. *Fish Pathol*. 24, 155-160.

Nakajima, K., K. Inoue, and M. Sorimachi. 1998. Viral diseases in cultured marine fish in Japan. *Fish Pathol.* 33, 181-188.

Nakajima, K., Y. Maeno, M. Arimoto, K. Inoue, and M. Sorimachi. 1993. Viral deformity of yellowtail fingerlings. *Fish Pathol.* 28, 125-129 (In Japanese with English summary).

Nishizawa, T., S. Kinoshita, and M. Yoshimizu. 2005. An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. *J. Gen. Virol.* 86, 1973-1978.

Novoa, B. and A. Figueras. 1996. Heterogeneity of marine birnaviruses isolated from turbot (*Scophthalmus maximus*). *Fish Pathol.* 31, 145-150.

Oh, M.J. and T.J. Choi. 1998. A new rhabdovirus (HRV-like) isolated in Korea from cultured Japanese flounder *Paralichthys olivaceus*. *J. Fish Pathol.* 11, 129-136.

Oh, M.J., S.J. Jung, and H.R. Kim. 1999a. Biological and serological characteristics of birnavirus isolated from cultured olive flounder in 1999. *J. Fish Pathol*. 12, 56-62.

Oh, M.J., S.J. Jung, and Y.J. Kim. 1999b. Detection of birnavirus from cultured marine fish using polymerase chain reaction. *J. Fish Pathol*. 12, 49-55.

Oh, M.J., S.J. Jung, Y.J. Kim, H.R. Kim, T.S. Jung, and I.K. Yeo. 2000. The screening of marine birnavirus (MABV) infected in brood stocks of flounder, *Paralichthys olivaceous. J. Fish Pathol*. 13, 53-59.

Oh, M.J., S.J. Jung, S.R. Kim, K.V. Rajendran, Y.J. Kim, T.J. Choi, H.R. Kim, and J.D. Kim. 2002. A fish nodavirus associated with mass mortality in hatchery-reared red drum, *Sciaenops ocellatus*. *Aquaculture* 211, 1-7.

Oh, M.J., S.J. Jung, and S.I. Kitamura. 2005. Comparison of the coat protein gene of nervous necrosis virus (VNN) detected from marine fishes in Korea. *J. World Aquac. Soc*. 36, 223-227.

Oh, M.J., S.J. Jung, S.I. Kitamura, H.R. Kim, and S.Y. Kang. 2006a. Viral diseases of olive flounder in Korean hatchery. *J. Ocean Univ. China.* 5, 45-48.

Oh, M.J., W.S. Kim, S.I. Kitamura, H.K. Lee, B.W. Son, T.S. Jung, and S.J. Jung. 2006b. Change of pathogenicity in olive flounder *Paralichthys olivaceous* by co-infection of *Vibrio harveyi*, *Edwardsiella tarda* and marine birnavirus. *Aquaculture* 257, 156-160.

Okamoto, N., T. Sano, R.P. Hedrick, and J.L. Fryer. 1983. Antigenic relationship of selected strains of infectious pancreatic necrosis virus and European eel virus. *J. Fish Dis.* 6, 19-25.

Pakingking, R., Jr., Y. Okinaka, K.I. Mori, M. Arimoto, K. Muroga, and T. Nakai. 2004. *In vivo* and *in vitro* analysis of the resistance against viral haemorrhagic septicaemia virus in Japanese flounder (*Paralichthys olivaceous*) precedingly infected with aquabirnavirus. *Fish Shellfish Immunol*. 17, 1-11.

Reno, P.W. 1998. Infectious pancreatic necrosis and associated aquatic birnaviuses, p. 1-55. *In* P.T.K. Woo and D.W. Bruno (eds.), Fish Diseases and Disorders. CABI Publishing, New York, NY, USA.

Sohn, S.G., M.A. Park, J.W. Do, J.Y. Choi, and J.W. Park. 1995. Birnavirus isolated from cultured flounder in Korea. *Fish Pathol.* 30, 279-280.

Sorimachi, M. and S. Egusa. 1986. Experimental infection of yellowtail ascites virus (YAV) to yellowtail. *Fish Pathol.* 21, 133-134.

Sorimachi, M. and T. Hara. 1985. Characteristics and pathogenicity of a virus isolated from yellowtail fingerlings showing ascites. *Fish Pathol.* 19, 231-238.

Suzuki, S., N. Hosono, and R. Kusuda. 1997a. Detection of aquatic birnavirus gene from marine fish using a combination of reverse transcription-and nested PCR. *J. Mar. Biotechnol.* 5, 205-209.

Suzuki, S., M. Kamakura, and R. Kusuda. 1998. Isolation of birnavirus from Japanese pearl oyster *Pinctada fucata*. *Fisheries Sci.* 64, 342-343.

Suzuki, S., T. Nakata., M. Kamakura, M. Yoshimoto, Y. Furukawa, Y. Yamashita, and R. Kusuda. 1997b. Isolation of birnavirus from Agemaki (Jack Knife Clam) *Sinonovacura constricta* and survey of the virus using PCR technique. *Fisheries Sci.* 63,

563-566.

Suzuki, S. and M. Nojima. 1999. Detection of a marine birnavirus in wild molluscan shellfish species from Japan. *Fish Pathol.* 34, 121-125.

Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. 24, 1596-1599.

Wolf, K. 1988. Infectious Pancreatic Necrosis, p. 115-157. *In* Fish viruses and fish viral diseases. Cornell University Press, New York, NY, USA.

Zhang, C.X. and S. Suzuki. 2004. Aquabirnaviruses isolated from marine organisms form a distinct genogroup from other aquabirnaviruses. *J. Fish Dis.* 27, 633-643.