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NOTE

# Genetic Analysis of the VP2/NS Junction Region on Segment A of Marine Birnavirus Isolated from Cultured Flounder *Paralichthys olivaceous*

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**The cDNA of VP2/NS junction region on segment A of the marine birnavirus (MBV) isolated from flounder (DS strain) was amplified using the reverse transcription (RT)-polymerase chain reaction (PCR). Its cDNA nucleotide and deduced amino acid sequences were analyzed, and compared with the reference strains of MBV and infectious pancreatic necrosis virus (IPNV). Analyses of the nucleotide and deduced amino acid sequences revealed that the DS strain is very similar to the reference strains of MBV, distant from the IPNV.**

*Key words:* RT-PCR, marine birnavirus, flounder, sequencing

Marine birnavirus (MBV) was first isolated from the yellowtail (*Seriola quiqueradiata*) in Japan (16). This contagious virus is called the yellowtail ascites virus (YAV) and causes a high mortality rate in the yellowtail fry. MBV belongs to the birnaviridae and is similar to the infectious pancreatic necrosis virus (IPNV) in several biological and pathological characteristics (3, 4, 16). After initial isolation of YAV, other MBVs have been isolated from several marine fish with ascites or deformity (9, 10, 11, 12, 17).

As the cultured marine fish became more intensified, economic losses due to viral diseases have become a serious problem in Korea. Recently, MBVs isolated from flounder (*Paralichthys olivaceus*) and rockfish (*Sebastes schlegeli*) have been reported to cause lethal diseases (14, 15).

Cloning and nucleotide sequences of IPNV have been performed in a few strains (1, 2, 5). cDNA of the VP2/NS junction region of IPNV strains was amplified by polymerase chain reaction (PCR) (6). It was found that the junction region is suitable for studying genomic variations of IPNV and for genogrouping (6, 7).

This finding suggests that this region might enable classification of MBV at the genetic level. Examination of this region would also be useful for clarifying variations of NS proteins. Therefore, Hosono *et al.* (8) reported that isolates of MBV formed a birnavirus genogroup which was distinct from other IPNV genogroups.

In this study, the nucleotide sequence of reverse transcription (RT)-PCR products from the VP2/NS junction region on segment A of MBV isolated from flounder (DS strain) in Korea and its deduced amino acid sequence were compared with those of MBV and IPNV strains. It was found that the DS strain is similar to the reference strains of MBV but distant from IPNV strains.

The chinook salmon embryo cell line (CHSE-214) was supplied by Dr. Park and grown at 18°C in Eagle's minimum essential medium (GibcoBRL, U.S.A.) supplemented with 10% fetal bovine serum (FBS, GibcoBRL, U.S.A.). The medium containing 2% FBS was used for the maintenance.

DS strain used in this study was supplied by Dr. Sohn (15) and has been maintained in our laboratory. GC-1 strain was first isolated in our laboratory (14) and other strains of MBV and four strains of IPNV were supplied by Dr. Park and used as the reference strains (Table 1). Confluent cells were infected by virus and incubated at 18°C. When cytopathic effects were extended to 70~90% of the layer, the cells were scraped off and centrifuged at 2,000×g. The pelleted cells were resuspended in TNE buffer (0.01 M Tris HCl, pH 7.8, 0.1 M NaCl, and 0.05 M EDTA). After centrifugation at 2,000×g for 20 min, the supernatant was combined with the culture supernatant and this was used as the crude virus. Extraction of the

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**Table 1.** Sources of virus strains used in this study

| Virus | Strain | Host | Origin | Year |
|---|---|---|---|---|
| MBV | DS | Flounder | Korea | 1995 |
| | GC-1 | Rockfish | Korea | 1998 |
| | H-1 | Flounder | Japan | 1996 |
| | Y-6 | Yellowtail | Japan | 1996 |
| IPNV | DRT | Rainbow trout | Korea | 1989 |
| | SP | Trout | Denmark | 1989 |
| | Ab | Trout | Denmark | 1989 |
| | VR-299 | Rainbow trout | U.S.A | 1963 |

viral RNA was performed by treatment with a solution containing 10 mM Tris-HCl, pH 7.8, 0.5% SDS, 0.05 M EDTA and 0.1 mg $ml^{-1}$ proteinase K (Sigma) at 55°C for 2 h. This was extracted then phenol plus chloroform , and RNA was precipitated with sodium acetate and ethanol. The RNA was dried and resuspended in TE buffer (10mM Tris-HCl, pH 7.5, 1 mM EDTA) (13). Viral RNAs from the DS strain and reference strains were separated by electrophoresis on a 1.5% agarose gel.

Two primers used for amplification of cDNA have been described previously (6). The forward primer (5'-AGAGATCACTGACTTCACAAGTGAC-3'; primer 1) corresponds to 1403-1427, and the reverse primer (5'-TGTGCACCACAGGAAAGATGACTC-3'; primer 2) corresponds to 1746-1761 of the IPNV Jasper strain segment A, respectively. A mixture of viral dsRNA and primers 1 and 2 was heated at 100°C for 5 min and cooled on ice. The mixture was then added to a RT buffer [50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each of deoxyribonucleotide and AMV Reverse Transcriptase XL (Takara)]. RT was performed in a final volume of 50 μl at 37°C for 1 h. RNA-DNA hybrid was denatured by heating at 100°C for 5 min. PCR amplification by *Ex Taq* polymerase (Takara) involved 35 cycles: 1 min at 95°C for denaturation, 2 min at 50°C to allow annealing and 3 min at 72°C for extension. The amplified DNA was visualized by ethidium bromide following 2% agarose gel electrophoresis. The RT-PCR products were purified by using a QIAquick PCR purification kit (Qiagen, U.S.A.). The purified product was ligated into pGEM-T Easy Vector (Promega, U.S.A.) by using T4 DNA ligase and then incubated at 16°C for 24 h.

The ligated plasmid was transformed into competent *E. coli* DH5α cells. The recombinant plasmid DNA was isolated by using LB/ampicillin/IPTG/X-Gal plates and white colonies which contained recombinant plasmid DNA were selected. After the white colonies were grown

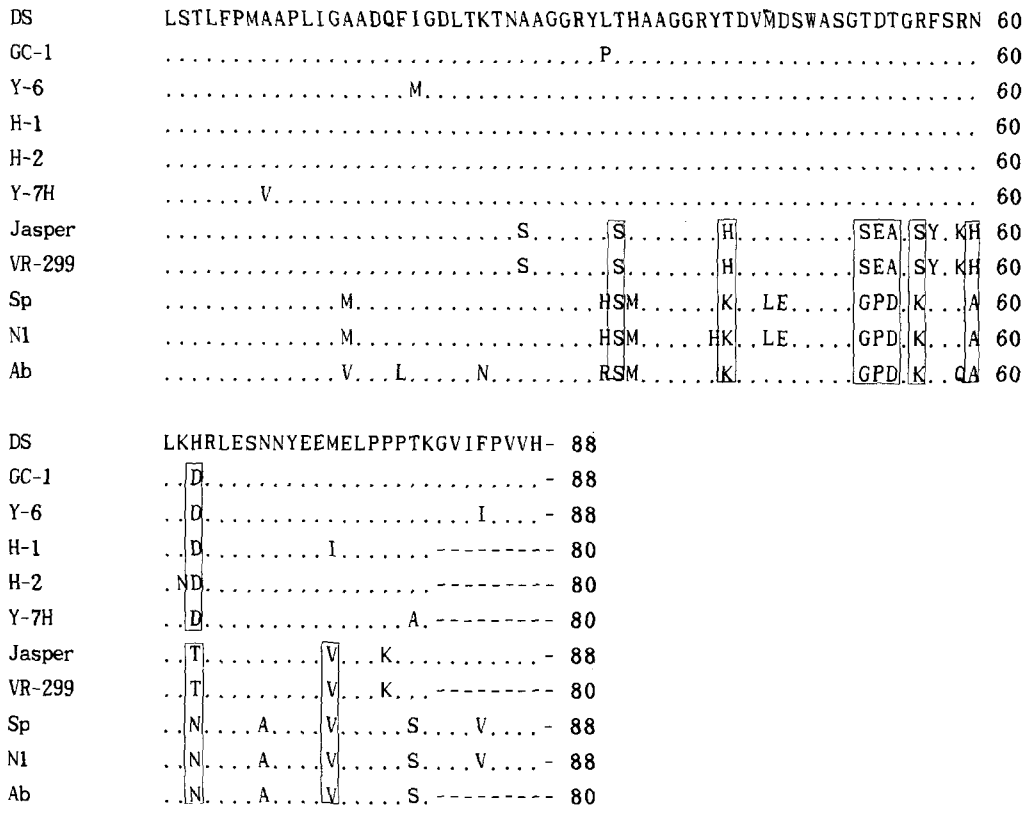


**Fig. 1.** Amino acid sequence deduced from cDNA fragments obtained from DS strain and their corresponding portion on the sequences of other MBV and IPNV strains with the following accession number: D61384 (Y-6), D61386 (H-1), D61387 (H-2), D61388 (Y-7H), L40584 (VR-299), M18049 (Jasper), L40580 (Ab), D00701 (N1) and L13988 (Sp). Homology to DS strain is indicated by dots. Hyphens represent gaps introduced for optimal alignment.

**Table 2.** Comparison of amino acid homology of DS strain with MBV and IPNV strains

| Strain | | MBV | | | | | IPNV | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | DS | Y-6 | H-1 | H-2 | Y-7H | Jasper | VR-299 | Sp | N1 | Ab |
| MBV | DS | 100 | | | | | | | | | |
| | Y-6 | 96 | 100 | | | | | | | | |
| | H-1 | 97 | 97 | 100 | | | | | | | |
| | H-2 | 97 | 97 | 97 | 100 | | | | | | |
| | Y-7H | 95 | 95 | 95 | 95 | 100 | | | | | |
| IPNV | Jasper | 85 | 82 | 83 | 82 | 80 | 100 | | | | |
| | VR-299 | 83 | 82 | 83 | 82 | 80 | 100 | 100 | | | |
| | Sp | 80 | 79 | 80 | 78 | 77 | 78 | 77 | 100 | | |
| | N1 | 79 | 78 | 78 | 77 | 76 | 77 | 76 | 98 | 100 | |
| | Ab | 78 | 77 | 78 | 77 | 76 | 77 | 77 | 91 | 90 | 100 |

in LB broth, plasmid extraction was performed by using the alkaline lysis method (13). The cloned inserts were digested with *Sac*II and *EcoR*I, visualized by staining with ethidium bromide after 1.5% agarose gel electrophoresis. The cloned plasmid was purified by using a QIAEX agarose gel extraction kit (Qiagen, U.S.A.). The VP2/NS junction region on segment A of the DS strain was sequenced by using the Ampli Taq cycle sequencing kit (ABI, Foster, CA) provided by the Korean Research Institute of Bioscience and Biotechnology (KRIBB). The nucleotide and amino acid sequence were analyzed using BLAST, ORF finder (NCBI, U.S.A.) and ClustalW program (EMBL, U.K). A dendrogram was drawn to show the relationship of the DS strain with reference MBV and IPNV on the basis of amino acid sequence homology.

The extracted RNAs of the DS strain and reference strains were analyzed by 1.5% agarose gel electrophoresis. The extracted RNA of the DS strain and all the reference strains appeared to consist of two segments of RNA (data were not shown). RNA of the DS strain was very similar in size to RNAs of the reference strains of MBV and IPNV.

The amplified 359-bp cDNA fragments from the DS strain was compared with all the reference strains of MBV and IPNV. The amplified DNA product of the DS strain was the same as those of all the reference strains (data were not shown).

The cDNA sequence of the VP2/NS junction region on segment A of DS strain was compared with the reference strains of MBV and IPNV. The nucleotide sequences of the reference strains of MBV and IPNV were obtained from DDBJ, EMBL and GenBank nucleotide sequence databases.

The deduced amino acid sequence from the nucleotide sequence of the DS strain was compared with the reference strains of MBV and IPNV (Fig. 1). The amino acid sequence of the DS strain was very similar to the reference strains of MBV but one major variation was observed at the 63rd position. This difference is thought to be caused by the geographical variation and host selectivity of the virus. When compared with the reference strains of IPNV, the sequence homology was low and 9 major variations (at 34, 42, 52, 53, 54, 56, 60, 63, 73) were observed. Similar results were reported by Hosono *et al.* (8) and this difference might be caused by the geographical variation and host selectivity of viruses which are based on historical events in the evolution of genes. The specific amino acids of the DS strain were: T at 34, 42, 52 and 54, D at 53, R at 56, N at 60, H at 63 and M at 73. The amino acid sequence homology of DS strain with MBV strains was 95~97%, 83~85% with IPNV genogroup and 78~80% with IPNV genogroup. These findings are summarized in Table 2. It showed that although the DS strain is very similar to the reference strains of MBV and different from the IPNV, its homology is the lowest among the reference strains.

A dendrogram based on the amino acid homology is shown in Fig. 2. This shows that the DS strain is similar to MBV reference strains and different from IPNV. From these results, it is suggested that the DS strain is included in the genogroup of MBV strains and distinguished from the genogroup of IPNV.

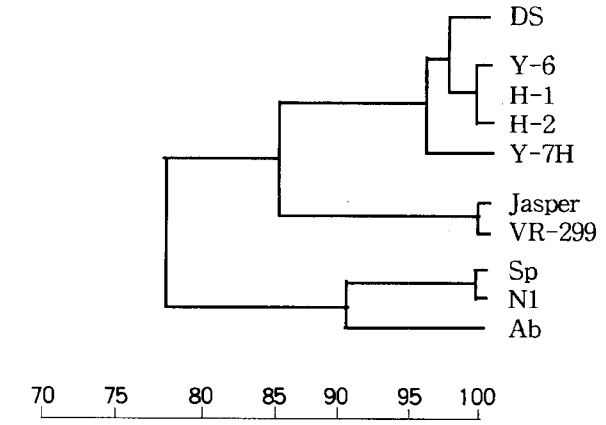


**Fig. 2.** Dendrogram representing the relationships between DS strain and 9 published reference strains as determined by amino acid sequence homology.

For development of the an efficient vaccine for the control of the viral disease caused by MBV, we need further understanding of the molecular basis for the genetic differences and the geographical relationship between MBV.

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