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A Fish Nodavirus Isolated from Cultured Sevenband Grouper, *Epinephelus septemfasciatus*

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Since 1989, mass mortality has repeatly occurred in cage-cultured sevenband grouper, *Epinephelus septemfasciatus* along the southern coast of Korea in the summer season and usually reached over 80% within a few months. Diseased fish showed the clinical signs of anorexia, dark coloration, loss of eqilibrium, spinal swimming behaviour, vertebral deformity and inflation of swim bladder. Histopathologically, necrosis and/or vacuolation of the nerve cells in the brain and retina were observed. We previously reported that the causative agent was filtrable. The causative agent was not culturable in various fish cells; RTG-2, CHSE-214, BF-2, EPC and FHM. However, electron microscopic observation revealed unenveloped icosahedral viral particles with about 30 nm in diameter in the cytoplasm of nerve cells of the brain. The characteristics of the virus was tested by an artificial infection with the filtrate of the homogenate of diseased fish. The pathogenicity of the virus was retained after treatment with ether or heat (50°C, 30 min) but partly lost by pH 3 or 11 treatment. These results suggest that the causative agent are similar to the fish nodavirus. In order to compare the causative agent with a fish nodavirus, Striped Jack Nervous Necrosis Virus (SJNNV), a polymerase chain reaction (PCR) was performed with primers specific to SJNNV. As a result, about 430 bp PCR products were detected from the brain and the eye of both naturally and artificially infected sevenband grouper. All these results represent that the mass mortality in the cultured sevenband grouper is caused by the infection of a nodavirus similar to SJNNV and this is the first report of a fish nodavirus from the cultured sevenband grouper in Korea.

Key words: Sevenband grouper, VNN, PCR, Fish nodavirus

Nodavirus is a nonenveloped spherical virus with 25-34 nm in diameter and has bipartite positive-sense RNA genomes. Fish nodavirus has been reported to cause viral nervous necrosis in various fish such as redspotted grouper (Mori *et al.*, 1991), striped jack (Arimoto *et al.*, 1993), kelp grouper and tiger puffer (Nakai *et al.*, 1994), Japanese flounder (Nguyen *et al.*, 1994), grouper (Boonyaratpalin *et al.*, 1996), sevenband grouper (Fukuda *et al.*, 1996) and sea bass (Frerichs *et al.*, 1996). Nishizawa *et al.* (1997) compared 25 isolates of fish nodavirus and classified them into four groups based on the nucleotide sequences of the coat protein gene.

Marine fish culture in Korea has rapidly grown with the development of the artificial seed production since 1980s. On the other hand, mass mortalities have occurred frequently among cultured fish due to environmental pollution, overstocking and pathogens and the loss increased every year. Among the pathogens, virus was one of the major causative agents of diseases in cultured marine fishes of Korea.

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Net-cage culture of sevenband grouper, *Epinephelus septemfasciatus*, relying on fingerlings collected from the wild has expanded along the southern coast of Korea. Unfortunately, a new epizootic disease has repeatedly occurred among this fish in the summer season (July to October) since 1989 and the mortality reached over 80%. Previously, we examined the causative agent of the disease but could not detect any pathogenic parasites and bacteria except a few *Trichodina* sp. However, the filtered homogenate of organs from diseased grouper caused the same symptom to the healthy grouper by intramuscular injection, suggesting that the causative agent might be virus (Sohn *et al.*, 1991). In this paper, we examined the characteristics of the casuative agent in detail and report that it is a fish nodavirus similar to SJNNV.

Materials and Methods

Fish

Diseased sevenband grouper, *E. septemfasciatus* (29 to 250 g in body weight) were sampled from cage-culture farms at southern coast of Korea in summer seasion from 1990 to 1992 and stored at -85°C until use.

Histological examination

Tissues of gill, brain, eyes and other internal organs from moribund fish were`fixed in Bouin's fixative and processed for standard histological preparations. Paraffin sections (4 µm in thickness) were stained with haematoxylin-eosin and observed by light microscopy.

Virus preparations

Pooled organs (about 50 g) of 5 diseased fish were homogenized with sea sand and 9-volumes of Dulbecco's modified eagle's medium (DMEM) at low temperature, and centrifuged at 5000×g for 15 min. The supernatant was recentrifuged at 12000×g for 2 min and filtered with a 0.45 µm membrane filter. The filtrate was used as a virus inoculum in cell culture and artificial infection.

Cells and virus isolation

Four fish cell lines, RTG-2, CHSE-214, BF-2 and EPC were grown in Eagle's modified minimum essential medium (EMEM) at 20°C. To isolate a causative virus from diseased fish, the filtrate was inoculated to four fish cell lines and inocubated at 20°C and/or 25°C for 3 weeks. A blind passage was carried out twice if cytopatic effect (CPE) was not formed.

Virus characteristics

Due to the failure of isolation of virus by cell culture, characteristics of the causative virus were tested by the pathogenicity of artificial infection according to the menthod described by Iida *et al.* (1989). To test the resistance to ether, 1 ml of ethyl ether was added to 4 ml of the filtrate and allowed to stand at 4°C for 18 hr, and then the ether was evaporated from the filtrate by shaking at room temperature. In pH stability, 45 ml of DMEM was added to 5 ml filtrate and standed for 3 hr after adjusting to pH 3, 7 and 11. Thermostability was tested by incubating the filtrate for 30 min at 50°C and 4°C.

Each 1 ml of the filtrates treated as mentioned above was intramuscularly injected into 10 healthy fish per experimental group and fish were transferred to 250 l tanks with moderate aeration at 22-24°C for 30 days, and then mortality was monitored. DMEM and mock-treated filtrate were also injected as controls.

Electron microscopy

Tissues of brain, eyes, kidney, spleen and liver from moribund fish were fixed in 2.5% glutaraldehyde sol. (pH 7.2) and postfixed in 1% osmium tetroxide sol. (pH 7.2) and embedded in Epon 812. Ultrathin sections were stained with 1% uranyl acetate and 1% lead citrate, and examined with a Hitach-7200 electon microscopy at 80 kV accelerating voltage.

Polymerase chain reaction

PCR was conducted in order to confirm the presence of the causative virus in the various organs of the diseased fish, brain, eyes, kidney, spleen and liver. Each organ of infected fish was homogenized with RNA zol (Biotex) followed by chloroform extraction and centrifuged at 12000×g for 15 min. The total nucleic acids were precipitated by addition of isopropanol and RNA Tack Resin (Biotex), washed with 75% ethanol twice and dried in a speedvaccum (Heto). The pellet was dissolved in distilled water. For the reverse transcription and PCR amplification, a primer set (Table 1) designed for T4 region (about 430 bp) in the open reading frame of SJNNV coat protein gene by Nishizawa *et al.* (1994) was used. For reverse transcription, total nucleic acids from tissues of diseased fish was pre-heated at 90 °C for 5 min and incubated at 42°C for 30 min in 20 μl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl) containing 2.5 U of M-MLV reverse transcriptase (USB), 1 U of ribo-nuclease inhibitor (Toyobo), 0.5 μM of reverse primer, 1 mM each of 4 deoxynucleotide triphosphates (dNTP), and 5 mM of $MgCl_2$. Following cDNA synthesis, the mixture was incubated at 99°C for 10 min to incubate the reverse transcriptase and then dilluted 5-fold with PCR buffer containing 0.1 μM of forward primer (F_2), 2.5 U of Tth Version 2.0 DNA polymerase and 2 mM of $MgCl_2$. The mixture was incubated in an automatic thermal cycler (Perkin Elmer) programmed for 1 cycle at 95°C for 5 min and 35 cycles at 55°C for 40 sec and at 72°C for 40 sec, and finally held at 72°C for 5 min. Amplified product was electrophoresed in 1.5% agarose gel and stained with ethidium bromide.

Table 1. Sequence of the oligonucleotide primers

Primer	Sequence	Location on cloned cDNA
F_2	5'-CGTGTCAGTCATGTGTCGCT-3'	592-611
F_1	5'-CGAGTCAACACGGGTGAAGA-3'	998-1017

Results

Clinical signs

Diseased fish exhibited the symptoms of anorexia, dark coloration, loss of equilibrium, spinal swimming behaviour, vertebral deformity and inflation of swimbladder. The exophthalmus were observed in some fish.

Histopathological changes

Remarkable histopathological changes were observed mainly in the brain and retina of the diseased fish. Moribund fish showed vacuolar degeneration of the retina in eye (Fig. 1) and severe necrosis and/or vacuolation in the brain (Fig. 2).

Virus morphology

Previous study (Sohn *et al.*, 1991) suggested that the causative agent was filtrable and would be a virus. The morphology of the virus was observed under electron microscope. Virus particles were found in the cytoplasm of the nerve cells from the brain of infected fish. Virions were unenveloped, icosahedral and were about 30 nm in diameter (Fig. 3).

Virus isolation

In order to isolate the causative virus, the filtrate of the organs of the diseased fish was inoculated

Fig. 1. Vacuolar necrosis of the retina in the eye of diseased sevenband grouper.

Fig. 3. Electron micrograph showing virus particles (about 30 nn in diameter) in the cytoplasm of nerve cells from the brain of diseased sevenband grouper. Inset shows higher magnification of particles. Bar is 200 nm.

into various fish cells, RTG-2, CHSE-214, EPC, FHM and BF-2. All the cells used in this experiments showed no cytopathic effect (CPE) until 3 weeks postinfection. There was no CPE even after a blind passage (Table 2).

Virus characteristics

In order to identify the physicochemical characteristics of the causative virus, the filtrate of homogenate of diseased fish was treated with ether, high temperature (50°C), acidic (pH 3) and basic (pH 11) conditions and the pathogenicity of the filtrates was determined by cumulative mortality. Mock-treated filtrate as a control, showed cumulative mortality of 90% during the experimental periods of 30 days. Ether- and heat-treated filtrates showed the same cumulative mortalities of 80%. However, acid- and base-treated filtrates showed cumulative mortalities of 30% and 60%, respectively (Table 3). These results represent that the causative virus is ether- and heat- stable but labile to extreme pH, particularly to high acidity.

Virus detection by PCR

The clinical syndroms, electron microscopic morphology and physicochemical characteristics of the causative virus were very similar to those of fish nodavirus. For the further study, a RT-PCR was conducted using a primer set designed from nucleotide sequences of a fish nodavirus, SJNNV (Nishizawa *et al.*, 1994). The predicted product of the PCR with this set was about 430 bp in SJNNV. As shown in the Fig. 4, no product was detected from the normal fish. However, PCR products of about 430 bp were obtained from both naturally and artificially infected fish. In order to identify the main target

Table 2. Susceptibility of fish cell lines to the causative virus

Cell lines	Incubation Temp. (°C)	No. of blind passage	CPE
RTG-2	20	2	-*
CHSE-214	20	2	-
	25	2	-
EPC	20	2	-
	25	2	-
FHM	20	2	-
	25	2	-
BF-2	20	2	-
	25	2	-

* No cytopathic effect.

Table 3. Effect of physicochemical treatments on the pathogenicity of the causative virus

Treatment	Dosage of inoculum (ml/fish)	Body weight (g)	No. of fish tested	No. of fish died (30 days)	Mortality (%)
Ether	0.1	53±5	10	8	80
DMEM (control)	0.1	52±8	10	0	90
pH 3	0.1	49±3	10	3	30
pH 11	0.1	50±5	10	6	60
pH 7 (control)	0.1	48±7	10	8	80
50°C	0.1	51±7	10	8	80
4°C (control)	0.1	55±4	10	8	80

Fig. 4. Detection of a virus gene from the brain of sevenband grouper by PCR amplification. Amplified products in agarose gel were stained with ethidium bromide. Lanes: (M) DNA ladder marker, (1) normal sevenband grouper, (2) naturally infected sevenband grouper, (3) artificially infected sevenband grouper.

tissue of this virus, various organs were prepared separately from the diseased fish and were used for RT-PCR. The PCR products were only detected in the brain and eye tissues of diseased fish (Fig. 5). These results represent that the causative virus of the disease in the sevenband grouper is a fish nodavirus similar to SJNNV and the major target of this virus is brain and eye tissues.

Discussion

Mass mortalities of sevenband grouper cultured in net cages at southern coast of Korea have been occurred in the summer season (July to October) since 1989 and usually reached over 80% within a

Fig. 5. Detection of a virus gene in organs artificially infected sevenband grouper by PCR amplification. Lane: (M) DNA ladder (1) brain, (2) eye, (3) kidney, (4) spleen, (5) liver.

few months. Sohn *et al.* (1991) suggested that the virus would be responsible for mass mortality of sevenband grouper because healthy fish inoculated intramuscularly with a filtrated homogenate of pooled organs from diseased fish showed the same symptoms as naturally infected fish did. This study was conducted to identify the causative virus responsible for the mass mortality of sevenbanded grouper.

Diseased sevenband grouper exhibited clinical symptoms similar to viral nervous necrosis (VNN) which was characterized by abnomal swimming, dark coloration and vacuolation of the central nervous tissues. Even though isolation of the virus in fish cell cultures was not successful, the morphology of the virus could be observed from the the cytoplasm of brain nerve cells in the the diseased fish. The virions were

nonenveloped spherical-shaped and were about 30 nm in diameter. The physicochemical characteristics of the virus was also determined by artificial infection. The virus was ether-resistant and heat-stable but partially labile to extreme pH condition, particularly to high acidity. All these results suggest that the disease is viral nervous necrosis (VNN) and causative virus is a fish nodavirus.

Until now, VNNs caused by fish nodavirus have been reported in redspotted grouper (Mori *et al.*, 1991), striped jack (Arimoto *et al.*, 1993), kelp grouper and tiger puffer (Nakai *et al.*, 1994), Japanese flounder (Nguyen *et al.*, 1994), grouper (Boonyaratpalin *et al.*, 1996), sevenband grouper (Fukuda *et al.*, 1996) and sea bass (Frerichs *et al.*, 1996). Nishizawa *et al.* (1995) found that the nucleotide sequences of coat proteins of fish nodaviruses were so conserved that a PCR primer designed from SJNNV could be used to amplify the coat protein genes of other four fish nodaviruses. They found that the sequence similarities among the SJNNV and four other fish nodavirus were greater than 75.8% in nucleotide level and greater than 80.9% in amino acid level.

In order to identify the causative virus in Korea, we conducted gene amplification of the causative virus using the PCR primers designed for SJNNV by Nishizawa *et al.* (1995). As a result, we obtained a PCR product of about 430 bp from the diseased fish. The size of this PCR product was similar to that of SJNNV. This result represents that the causative virus from sevenband grouper in Korea is a nodavirus similar to SJNNV. Recently, based on the nucleotide sequences of the coat protein gene, the fish nodavirus was classified into four groups, tiger puffer nervous necrosis virus, striped jack nervous necrosis virus (SJNNV), berfin flounder nervous necrosis virus and red-spotted grouper nervous necrosis virus (Nishizawa *et al.*, 1997). Now, it is not certain whether the causative virus of the sevenband grouper belongs to the group of SJNNV. Further study on the nucleotide sequencing of the PCR product may confirm this.

Mass mortality of the sevenband grouper caused by VNN in Korea has occurred in adult as well as in fry in summer season. As the water temperature became below 20°C, the mortality caused by VNN subsided and at the water temperature of below 15 °C, artificially injected virus did not show any pathogenicity (Data not shown). These represent that a high rearing water temperature rather than fish size seems to be a predisposing factor to manifest VNN disease in sevenband grouper.

In this study we investigated the causative virus of mass mortality of sevenband grouper and represent that the causative virus is a fish nodavirus similar to SJNNV.

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양식 능성어로 부터 Fish Nodavirus 분리

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1989년부터 남해안 일원 해상 가두리 양식장에서 사육중이던 능성어가 고수온기에 대량폐사하여 폐사율이 80%이었다. 발병한 병어는 채색흑화, 안구돌출, 평행감각 상실, 회전운동과 몸통이 휘어지는 증상을 나타냈고, 조직학적으로는 뇌조직 및 안구망막의 신경세포가 공포 또는 괴사되었다. 병어로 부터 어류주화세포를 이용한 세포배양법으로 원인 바이러스는 분리되지 않았지만, 뇌 신경세포의 세포질에서 크기가 약 30 nm이며 외막(envelop)이 없는 다면체 모양의 바이러스 입자가 전자현미경적으로 관찰되었다. 능성어로 부터 분리된 바이러스는 에테르나 열(50℃, 30분)처리에 의해 병원성이 실활되지 않았으나, 강산(pH 3)이나 강알카리(pH 11)에는 부분적으로 병원성이 실활되었다. 그리고 fish nodavirus 인 Striped Jack Nervous Necrosis Virus(SJNNV)의 primer를 이용하여 인위감염 및 자연감염된 능성어를 대상으로 PCR을 한 결과 감염된 능성어의 뇌 및 안구조직에서 약 430 bp 정도의 PCR 증폭산물이 검출되므로서 능성어를 폐사시키는 원인 바이러스는 SJNNV와 아주 유사한 nodavirus 였다.

Key words: Sevenband grouper, VNN, PCR, Fish nodavirus