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Characterization of Betanodaviruses in the Philippines

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Abstract

Viral nervous necrosis caused by betanodaviruses is one of the most devastating diseases in cultured marine finfish. In the Philippines, mass mortalities occurred in sea bass, *Lates calcarifer* larvae and grouper, *Epinephelus coioides* broodstock. The virus was isolated using SSN-1 fish cell line and confirmed by PCR. Cytopathic effect started to develop in the cell line 2 days post infection (p.i) with tissue filtrates until the cells completely disintegrated and detached from the flask at 5 days p.i. and the viral protein was detected by immunofluorescence. Sequence analysis revealed that VNN isolated from the brain of grouper broodstock and sea bass larvae were 98.6 % similar. Sequence analysis between the Philippine isolates and red-spotted grouper nervous necrosis virus (RGNNV) genotype is 96.9% similar as compared to 72.0% and 64.0% similar with the barfin flounder nervous necrosis virus (BFNNV) and tiger puffer nervous necrosis virus (TPNNV) genotypes, respectively. These results confirm that the Philippine isolates belong to RGNNV genotype.

Introduction

Viral nervous necrosis (VNN) also known as viral encephalopathy and retinopathy (VER) is one of the most devastating diseases in cultured marine finfish. The disease affects larvae and juveniles resulting in high mortalities and in rare cases, also adults (Munday & Nakai, 1997). The first outbreak was reported in hatchery-reared larvae and juveniles of Japanese parrotfish in 1990 (Yoshikoshi & Inoue, 1990). Since then, the virus was reported in 19 species of marine fish worldwide (Munday & Nakai, 1997). In the Philippines, Maeno et al. (2002) documented the first record of VNN infection in hatchery-reared orange-spotted grouper.

The disease is characterized by extensive necrosis of nervous tissues (spinal cord, brain) and retina (Yoshikoshi & Inoue, 1990; Arimoto

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et al., 1993). The causative virus is a non-enveloped, round-shaped, about 25-34 nm in diameter with a single-stranded, positive sense RNAs without poly (A) sequences at the 3' terminus (Mori et al., 1992; Comps et al., 1994). Fish nodaviruses have been classified into four genotypes based on their coat protein gene sequences, namely: tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). A serological relationship among genotypic variants of betanodaviruses was evaluated and it was found the sero-grouping was consistent with the genotypes: serotype A for SJNNV, serotype B for TPNNV and serotype C for RGNNV and BFNNV genotypes (Mori et al., 2003). However, in 2004, Johansen et al. characterized the nodavirus obtained from farmed turbot, *Scophthalmus maximus* and the sequence alignment showed that the turbot nodavirus (TNV) was different from previously described fish nodaviruses, suggesting a fifth genotype.

In Taiwan, Chi et al. (2001) characterized NNV isolated from grouper larvae, *Epinephelus* sp. and the result of the T2 sequence alignment with four fish nodavirus genotypes indicated that NNV isolates from Taiwan shared 99% similarity with RGNNV isolate from Japan. In 2003, a similar study was done by Chi et al. using different species of cultured marine finfish. The nucleotide and deduced amino acid sequences from all isolates were strongly homologous (>97%) with RGNNV genotype (Chi et al., 2003). In this study, we report the molecular characterization of betanodaviruses isolated from grouper broodstock and sea bass larvae in the Philippines using sequence analysis of the T4 region and comparison with the homologous gene sequences from other betanodaviruses.

Materials and methods

Fish samples

The virus was obtained from diseased sea bass, *Lates calcalifer* larvae and orange-spotted grouper, *Epinephelus coioides* broodstock reared at Southeast Asian Fisheries Development Center, Aquaculture Department (SEAFDEC AQD), Iloilo, Philippines. Moribund sea bass larvae, retinal and brain tissues from diseased grouper were aseptically dissected and stored at -80°C until use. The virus was confirmed by reverse-transcriptase-polymerase chain reaction (RT-PCR), histopathology and cell culture using SSN-1 cell line (Frerichs et al., 1991).

Viral isolation

Isolation was done following Iwamoto et al. (1999). Briefly, tissues were individually homogenized in Hanks' balanced salt solution (HBSS) and centrifuged at 1,200 *g* for 10 min at 4°C. The supernatant was filtered using 0.22 μm filter and stored at -80°C until use.

SSN-1 cells were cultured in 25-cm^2 tissue culture flask using Leibovitz L-15 medium supplemented with 10% FBS and 1% antibiotic. The cells were incubated at 25°C until the monolayer is approximately 70% confluent. The monolayer was washed twice with HBSS and the filtrate was inoculated into the cell line. Cells were incubated at 25°C until the appearance of cytophatic effect (CPE).

Immunofluorescent staining

Rabbit anti-SJNNV serum needed for indirect fluorescent antibody test (IFAT) was produced following the protocol of Mori et al (2003). Briefly, the purified virus was emulsified with Freund's complete adjuvant (FCA) and injected into rabbits. Subsequent injections without FCA were given to the rabbits until neutralization titers against homologous virus reached 10,000 or higher. The produced antiserum was sterilized using 0.45mm membrane filters and stored at -80°C until use.

For IFAT test, 100 µl filtrate was inoculated in a 24-well plate containing 70% confluent SSN-1 cells and incubated until the cells exhibit CPE. Cells were washed, air dried, fixed with methanol and washed with phosphate buffered saline (PBS). Cells were added with 100 µl rabbit anti-SJNNV serum, incubated at 37°C for 30 min, washed, reacted with fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulin (Dako, Denmark), incubated and washed again. Mounting medium (1 part PBS in 9 parts non-fluorescent glycerin) was added and observed under a fluorescence microscope.

Extraction of RNA

RNA was extracted using TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. Briefly, 50-100 mg tissue were homogenized in 1 mL TRIzol and centrifuged at 10,000 *g*. The supernatant was added with chloroform, mixed and centrifuged at 10,000 *g* for 15 min. RNA was precipitated with iso-propanol, washed with 75% ethanol, dissolved in diethyl-pyrocarbonate-treated distilled water (DEPC-DW) and stored in -80°C until use.

RT-PCR

Viral detection was carried out using primer sets designed for T4 region (430 bp) following the protocol of Nishizawa et al. (1994). Briefly, 1 µL RNA was incubated at 42°C for 30 min in 20 µL of reverse-transcription mastermix. Following cDNA synthesis, the mixture was incubated at 99°C for 10 min then added with PCR mastermix containing forward primer and ExTaq DNA Polymerase (Takara, Japan) and amplified using iCycler (Bio-Rad, USA). Viral detection by a nested step was carried out using the primer set RGNNV-NFRG and RGNNV-NRRG with the expected amplified product of 258 bp. After addition of 1 mL of the RT-PCR product to the PCR mixture, amplification was done using the same thermal cycler. Amplified DNA was analyzed by agarose gel electrophoresis using 2% agarose in Tris-Borate-EDTA (TBE) buffer, stained with ethidium bromide and viewed using a gel documentation system.

Nucleotide sequence analysis

The 430 bp 1-step PCR product was excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen, Germany). The purified PCR product was sent to a commercial sequencing company in Japan for DNA sequencing.

Results

Development of CPE and immunofluorescent staining

CPE developed as areas of cytoplasmic vacuolation in SSN-1 cell line, which started to appear 2 days after inoculation with the tissue filtrates. CPE further progressed to 50-75% at 3-4 days p.i. until the cells completely disintegrated and detached from the flask at

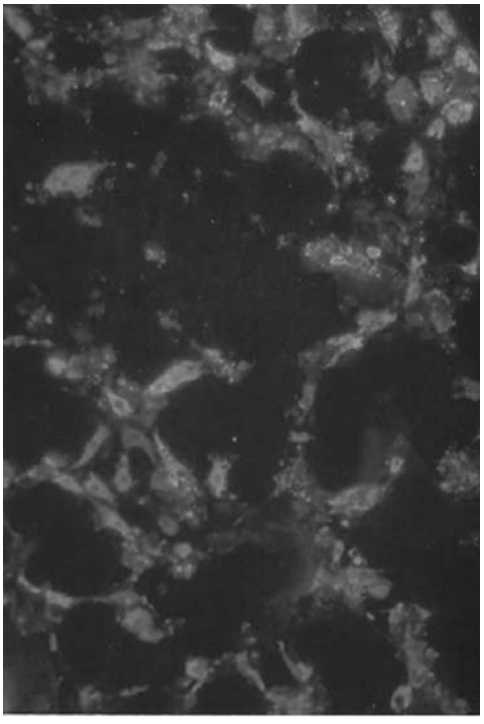

Figure 1. Immunofluorescent stain of VNN-infected SSN-1 cells at 5 days p.i.

Figure 2. Agarose gel electrophoresis of PCR products. Lanes: (1) DNA marker; (2) RGNNV positive control; (3) SJNNV positive control; (4) TPNNV positive control; (5) BFNNV positive control; (6) sea bass larvae; (7-10) grouper broodstock brain and retinal (11) negative control.

5 days p.i. The virus isolated from sea bass larvae and grouper broodstock were reactive to homologous antiserum. Positive fluorescence was observed up to 1000-fold dilution of the serum at five days p.i. (Figure 1). Uninoculated SSN-1 cells were used as negative control.

One-step and nested PCR

Figure 2 shows the agarose gel electrophoresis of PCR products of sea bass and grouper isolates. In order to determine the genotype, PCR analyses were carried out using RG-specific primer pairs. RNAs from known RG, SJ, TP and BF genotypes were used as positive controls. As a result, only RNA from the known RG genotype produced the expected amplicon size whereas SJ, TP and BF genotypes did not. Interestingly, RNA samples from the diseased grouper and sea bass produced the desired 430 bp product, suggesting that it is of RGNNV genotype.

Nucleotide sequence and comparative analyses

In order to determine the sequence similarity of the isolates, nucleotide sequence analysis was carried out (Figure 3) using the Genetyx program version 6.1.0 (Software Development). A molecular phylogenetic tree of the nucleotide sequences of isolates was built by the maximum likelihood criteria with the Dnaml program of PHYLIP 3.573c (Felsenstein, 1993). Results revealed that VNN isolated from grouper broodstock brain (GBBPhi101) and sea bass larvae (SBBLPhi101) were 98.6 % similar (Figure 4). The sequence analysis between grouper and sea bass isolates from the Philippines and RGNNV genotype (RG40ka, Nishizawa) are 96.9% similar.

Discussion

VNN caused infection in marine fish. Infected fish die within several days after the appearance of abnormal swimming behavior

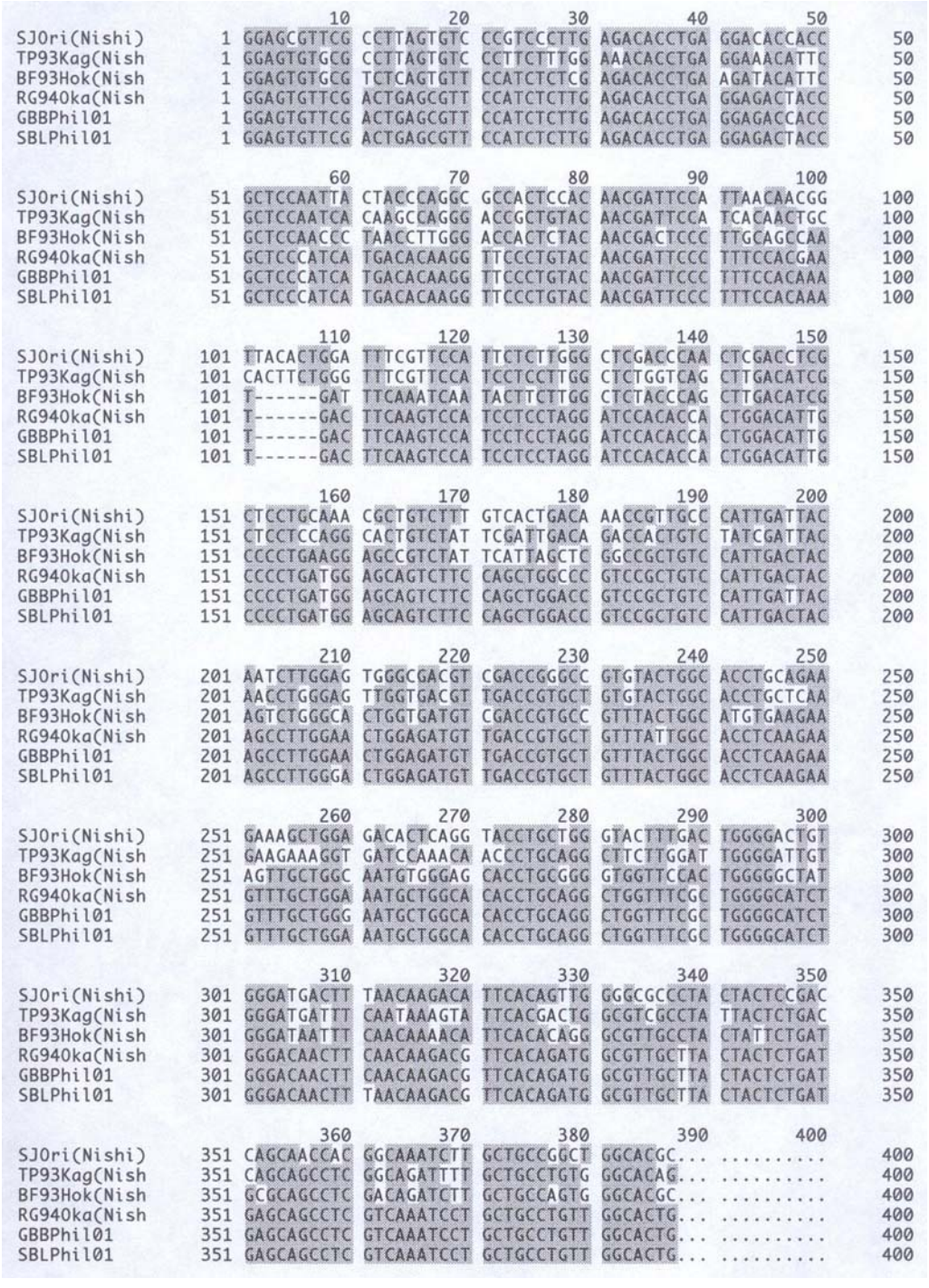

Figure 3. Multiple alignment of nucleotide sequence (T4 region) of isolates from the Philippines (GBBPhil01 and SBLPhil01) compared with four betanodavirus genotypes.

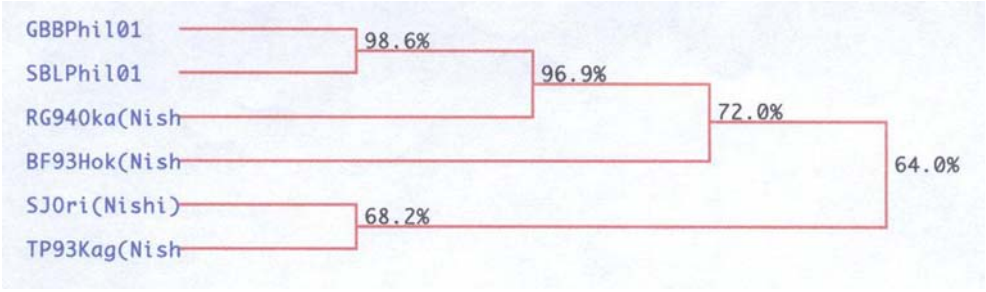

Figure 4. Molecular phylogenetic analysis of T4 region of nucleotide sequence of coat protein gene of VNN isolated from grouper broodstock (GBBPhil101) and sea bass larvae (SBLPhil101) compared with four betanodavirus genotypes RGNNV (RG940ka, Nish); BFNNV (BFHok, Nish); SJNNV (SJ0ri, Nishi) and TPNNV (TP93Kag, Nish).

and loss of appetite. The causative agent was isolated and confirmed by histopathology, PCR and cell culture. The viral agent was characterized using sequence analysis and compared with the homologous gene sequences of the four betanodavirus genotypes TPNNV, SJNNV, BFNNV and RGNNV.

CPE developed initially in SSN-1 cell line infected with the viral isolates 2 days p.i. After 5 days p.i., cells were completely disintegrated and detached from the flask. This suggests the presence of nervous necrosis virus (NNV) since SSN-1 cell line is susceptible to NNV. The cells also displayed fluorescence in IFAT after viral infection. Positive fluorescence was observed up to 1000-fold dilution of the serum at 5 days p.i.

PCR analysis was done to partially determine the genotype of the isolates. Results revealed that NNV isolated from both sea bass and grouper produced the expected PCR product suggesting that they belong to RGNNV genotype.

In order to determine the sequence similarity of the isolates and to further confirm the genotype, nucleotide sequence analysis was carried out. Results revealed that the two isolates were 98.6% similar. Conversely, the sequence analysis between Philippine isolates and a known RGNNV isolate is 96.9% similar as compared to 72.0% and 64.0% similar with the known BFNNV and TPNNV isolates, respectively.

These results conform to earlier reports that RGNNV genotype has been isolated from a wide range of warm water fish species, including grouper and sea bass (Dayanadol et al., 1995, Chi et al., 1997, le Breton et al., 1997). Chi et al., (2001) reported that the host range of RGNNV genotype includes 11 species of fish. Nishizawa et al., (1997) also reported that it was observed in at least 8 countries across Europe and Asia. Warm-water temperature in the Philippines might explain the presence of RGNNV genotype. The optimal growth temperatures of the 4 genotypes in the E-11 cell line are 15°C to 20°C for BFNNV, 20°C for TPNNV, 20°C to 25°C for SJNNV and 25°C to 30°C for RGNNV (Iwamoto et al., 2000). In contrast, BFNNV genotype has only been isolated from cold-water species (Bloch et al., 1991, Watanabe et

al., 1999), while SJNNV and TPNNV genotypes have only been limited to striped jack (Mori et al., 1992) and tiger puffer (Nakai et al., 1994), respectively. The low-host specificity of RGNNV increases the spread of NNV through its inter-specific transmission among fish reared in the same aquatic area (Chi et al., 2003).

This study confirmed the presence of RGNNV genotype in sea bass and grouper in the Philippines.

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