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BRIEF REPORT

Genetic analysis and pathogenicity of betanodavirus isolated from wild redspotted grouper *Epinephelus akaara* with clinical signs

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Abstract Diseased wild redspotted grouper *Epinephelus akaara* were collected from Seto Inland Sea, Ehime Prefecture, in August 2002. Fish showed erratic swimming behavior and inflation of the swim bladder. The fish brains were positive for nodavirus in both RT-PCR and nested PCR. The sequence of the nested PCR product (177 nt) was closely related to that of a known betanodavirus, redspotted grouper nervous necrosis virus. When juvenile sevenband grouper *E. septemfasciatus* were challenged intravitreously with virus, abnormal swimming behavior and high mortality were observed. This is the first report on viral nervous necrosis in a wild population of redspotted grouper with clinical signs.

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Betanodaviruses infection, also known as viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER), is considered one of the most serious viral diseases that occur particularly during the seedling and culture process in a variety of marine fish in Japan [14] and in other parts of the world [15]. VNN was first reported in hatchery-reared Japanese parrotfish *Oplegnathus fasciatus* [19] and barramundi *Lates calcarifer* [6], and later in other marine fishes [4, 12, 15] such as redspotted grouper *Epinephelus akaara* [11]. The transmission of VNN maybe vertical [3] or horizontal [2, 7]. The characteristics of VNN are necrosis and vacuolation of central nervous tissues and retina of the affected larvae and juvenile fishes showing high mortality [4]. Nodavirus is a member of the genus *Betanodavirus* of the family *Nodaviridae*, and it is non-enveloped, 25 to 30 nm in diameter, spherical in shape, and contains two segments of positive-sense single-stranded RNA. The RNA 1 (3.1 kb) encodes the viral replicase, and RNA 2 (1.4 kb) encodes the coat protein [1]. Genomic classification of betanodaviruses has revealed four major types: striped jack nervous necrosis virus (SJNNV)-type, tiger puffer nervous necrosis virus (TPNNV)-type, barfin flounder nervous necrosis virus (BFNNV)-type and redspotted grouper nervous necrosis virus (RGNNV)-type [16]. Recently, a fifth group was suggested as turbot nodavirus (TNV)-type from turbot *Scophthalmus maximus* [10]. In the present study, moribund wild juvenile redspotted grouper *E. akaara* with clinical signs of VNN were collected and studied. A partial nucleotide sequence of the RNA2 coat protein gene was analyzed to elucidate the genetic relatedness of this wild redspotted grouper isolate to other known betanodavirus isolates.

Ten moribund wild juvenile redspotted grouper *E. akaara* (average body weight 85 g) that showed erratic swimming behavior and inflation of the swim bladder were obtained

from Ehime Prefectural Chuyo Fisheries Experimental Station. These wild fish were captured near the aquaculture area in Seto Inland Sea, Japan, in August 2002. The brains were aseptically collected and stored at −80°C until use for virus isolation. Ten brain samples were used for virus isolation using an E-11 cell line [8] previously cloned from the SSN-1 cell line [5]. The cells were maintained at 25°C with Leibovitz L-15 medium (Invitrogen, USA) supplemented with 5% (V/V) fetal bovine serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin. Briefly, brain tissue homogenate with nine volumes of Hank's balanced salt solution (HBSS; Nissui, Japan) was membrane-filtered (0.45 μm; Millipore) and inoculated onto cells seeded in 24-well tissue culture plates [8]. The ten isolated samples inoculated in E-11 cells were incubated at 25°C for 10 days, and the supernatant of the cells of all samples showing cytopathic effect (CPE) at three passages were harvested and stocked at −80°C until used for pathogenicity testing. Titration of virus infectivity in a serial 10-fold dilution of the supernatant was performed with E-11 cells seeded in a 96-well tissue culture plate. The virus infectivity titers ($TCID_{50}$/g) were read and determined by Reed and Muench method [17]. Total RNA was extracted from the five representative brain samples using an RNA extraction kit (ISOGEN; Nippon Gene, Japan) according to the manufacturer's instructions. In brief, the tissues were homogenized with ISOGEN and shaken with chloroform, then centrifuged at 12,000 × *g* for 15 min at 4°C. RNA in the aqueous phase was precipitated with isopropanol and then dissolved in diethylpyrocarbonate-treated water (DEPC; Invitrogen, USA). RT-PCR and nested PCR amplification were conducted using five oligonucleotide primers designed from sevenband grouper nervous necrosis virus (SGNNV) RNA2, as described previously [7]. After reverse transcription using Reverse Transcriptase M-MLV (TaKaRa, Japan) at 45°C for 60 min, PCR was conducted using Ex *Taq* polymerase (TaKaRa, Japan) with 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 20 s, and extension at 72°C for 60 s. Nested PCR was conducted using the same protocol described above. The amplified products were analyzed by 2% agarose gel electrophoresis and visualized under UV irradiation after being stained with ethidium bromide. The RNA from uninfected (negative control) and infected [11] (positive control) redspotted grouper larvae were used for RT-PCR and nested PCR. A positive nested PCR product from one representative isolate RG02Ehi was recovered from an agarose gel and was purified using the QIAquick Gel Extraction kit (Qiagen, Germany) as described in the manufacturer's instructions. The purified amplification product was subjected to nucleotide sequence analysis according to the method described previously [13]. As the nested PCR product included variable and homologous regions commonly observed in betanodaviruses [9], for practical reasons, the variable region of the SGNNV RNA2 sequence (177 nt from nt no. 666–842) [9] was selected, and extra sequences were trimmed and then realigned using the multiple alignment algorithms in the MegAlign package Windows Version 3.12e (DNASTAR, USA) along with the known betanodavirus sequences obtained from the GenBank database to give a phylogenetic tree. The tree was constructed based on neighbor-joining method (multiple alignment data using the Jotun Hein Method in the MegAlign package). The GenBank accession numbers of the known betanodavirus sequences used in this study were: redspotted grouper, RG91tok (D38636); striped jack, SJOri (D30814); barfin flounder *Verasper moseri*, BF93hok (D38635); tiger puffer *Takifugu rubripes*, TP93Kag (D38637); guppy *Poicelia reticulata*, Gup (AF499774); white grouper *E. aeneus*, EA040799-IL (AY284963); sevenband grouper *E. septemfasciatus*, SGWak97 (AY324870); dragon grouper *E. lanceolatus*, DGNNV (AY721615); orange-spotted grouper *E. coioides*, ECNNV (EF492143); greasy grouper *E. tauvina*, ETNNV1 (AF281657); European seabass *Dicentrarchus labrax*, DL040899-IL (AY284969); white star snapper *Lutjanus stellatus*, WSSNNV (AY835642); greasy grouper *E. tauvina*, ETNNV2 (NC_004136); greasy grouper *E. tauvina*, GGNNV (AF318942); gray mullet *Mugil cephalus*, MC061198-IL (AY284962) and barramundi *L. calcarifer*, LC220800 (AY284973). One representative isolate of RG02Ehi was used for experimental infections with healthy juvenile sevenband grouper *E. septemfasciatus* (average body weight 63 g), which were reared at Kamiura Station, National Research Institute of Aquaculture, Fisheries Research Agency. Prior to the infection experiment, the brains of some healthy sevenband grouper were randomly examined for nodavirus by PCR assays [7], and all were negative for nodavirus (data not shown). For the experimental challenge of juvenile sevenband grouper, a total of 30 fish were stocked in three 70-l aquaria at ten fish per aquarium. The aquaria were maintained at 23–25°C for 14 days, supplied with ozonated seawater. Fish were fed once a day with commercially prepared pellets. The RG02Ehi (experimental treatment) and SGWak97 (positive control) isolates $10^6 TCID_{50}$/fish/0.03 ml/fish or HBSS (negative control) was injected into each fish intravitreously. During experimental infections, dead fish were removed daily and kept at −80°C; surviving fish were collected at 14 days after challenge. Mortality of fish was observed for 2 weeks, and recovery of nodavirus from the brain of all fish in the challenge and control groups was conducted to fulfill Koch's postulates.

All of the brains of the five representative fish samples were positive for nodavirus in both RT-PCR and nested PCR. A partial coat protein gene (RNA2) sequence corresponding to nucleotides (nt) 666–842 of one representative isolate, RG02Ehi, was amplified by nested PCR for nucleotide

sequence analysis. A comparison of the sequences of RG02Ehi and RG91tok (D38636) showed nearly 100% sequence identity, with only 2 nt differences. These 2 nt differences, at positions 736 and 775, did not affect the encoded amino acid residues. It also showed 96.6–100% sequence identity against other known isolates: AF499774, AY284963, AY324870, AY721615, EF492143, AF281657, AY284969, AY835642, NC_004136, AF318942, AY284962 and AY284973. On the other hand, RG02Ehi showed sequence identities of 71.2% with BF93hok (D38635), 62.7% in TP93Kag (D38637) and 67.8% in SJOri (D30814). Phylogenetic analysis confirmed that the wild redspotted grouper isolate RG02Ehi belongs to the RGNNV genotype (Fig. 1). In this study, we reported the isolation of betanodavirus from the brain of wild juvenile redspotted grouper swimming near the aquaculture area in Seto Inland Sea. We determined the viral strain classification using PCR-based amplification techniques with SGNNV RNA2-specific primer sets [7], nucleotide sequencing, phylogenetic analysis, virus isolation and pathogenicity testing. The isolated virus is closely related to a known betanodavirus, redspotted grouper nervous necrosis virus (RGNNV) [18]. It was also confirmed that the virus isolate was genetically distinguishable from other three major genotypes: SJNNV, BFNNV and TPNNV, which further elucidates the differences between these betanodavirus strains. So far, this is the first record of VNN in the wild marine fish population in Japan where the fish exhibited clinical signs. Although some research facilities and rearing ponds (Hiroshima and Tokushima Prefectural Fisheries Experiment Stations) near the Seto Inland Sea had already experienced and reported several outbreaks of VNN from August to September 1990, these outbreaks had only affected the cultured larvae and juvenile stages of redspotted grouper [11]. It is believed that the RGNNV genotype is the main cause of disease in these wild populations of redspotted grouper, since this fish has been reported to be one of its susceptible hosts [11]. We also think that the virus isolate could be an indigenous type of betanodavirus distributed in the Seto Inland Sea based on the results of the present study and the past reported cases of VNN in that area.

Sevenband grouper injected with RG02Ehi started to show abnormal swimming behavior, loss of body balance and poor feeding on the fourth day. Ten percent mortality occurred on the ninth day post-injection (p.i.) and reached 60% by 2 weeks p.i. The ranges of virus titers from the dead fish and survivors were $10^{7.3}$ $TCID_{50}$/g to $10^{8.6}$ $TCID_{50}$/g and $10^{5.2}$ $TCID_{50}$/g to $10^{6.6}$ $TCID_{50}$/g, respectively. The levels of pathogenicity were also similar to those of sevenband grouper injected with virus isolate SGWak97 (positive control). These fish started to show abnormal swimming and poor feeding behavior on the fifth day p.i. Forty percent mortality occurred on the 9th day and reached 80% on the 14th day p.i., with virus titers ranging from $10^{6.4}$ $TCID_{50}$/g to $10^{8.6}$ $TCID_{50}$/g. Twenty percent of the fish survived, with virus titers ranging from $10^{5.7}$ $TCID_{50}$/g to $10^{5.9}$ $TCID_{50}$/g. Moreover, neither fish abnormality nor virus titers ($<10^{2.05}$ $TCID_{50}$/g) was observed in the HBSS group (negative control). It should be noted that Tanaka et al. [18] reported the pathogenecity of nodavirus isolated from diseased sevenband grouper (RGNNV type) injected to healthy cultured redspotted grouper induced similar behavioral abnormalities starting at 2 days p.i. with 20%

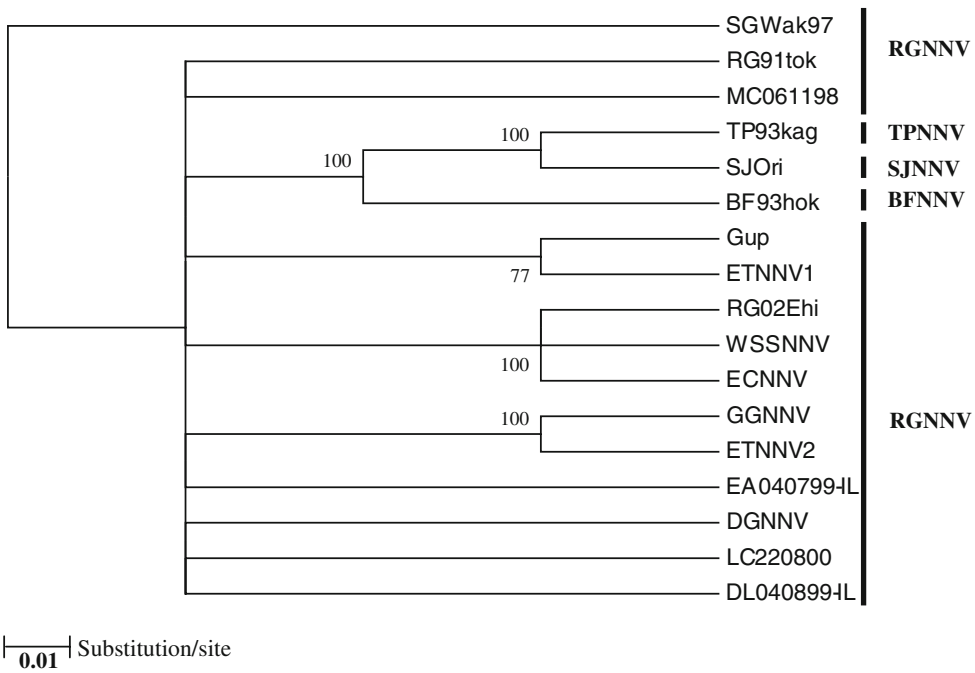

Fig. 1 Phylogenetic tree based on partial nucleotide sequences (nt 666–842) of the RNA2 coat protein gene of RG02Ehi and other known betanodavirus isolates. This tree was constructed by the neighbor-joining method in the MegAlign package. *Numbers* at branch nodes indicate percentage bootstrap support for the node with 1,000 replications

mortality. Previous studies also reported 30% mortality in healthy cultured redspotted grouper by experimental infection with isolated virus from the same species of grouper [11]. Furthermore, similar clinical signs (erratic and corkscrew swimming behavior as well as inflation of the swim bladder) displayed by the affected fish was observed to be a characteristic of VNN disease among cultured redspotted grouper [4, 11] affected by the RGNNV genotype.

It has been discovered that betanodavirus isolates from groupers that belong to the same RGNNV genotype proliferate well at higher temperature in cultured cells compared with the other three genotypes (SJNNV, TPNNV, BFNNV), indicating that this genotypic variant originates from warm-water fish [8]. Another finding in groupers is that the VNN infection usually occurs in cultured or hatchery-reared stages, but in the present study, it occurs in the wild population. We speculate that these wild redspotted grouper populations have possibly been infected horizontally with RGNNV-type nodavirus originating from subclinically infected or diseased cultured marine fish that were released to the open sea by sea farming. Another possibility is that RGNNV-type nodavirus could be transmitted from other subclinically infected or diseased wild marine fish that were there swimming around the mariculture area. Moreover, RGNNV-type nodavirus could also be transmitted via environmental water and other biological organisms that were infected in the environment. Therefore, the present result indicates that these wild redspotted grouper juveniles were severely affected by betanodavirus (RGNNV-type) infection and showed clinical signs of VNN. Furthermore, these affected wild redspotted grouper may be a persistent potential source of RGNNV-type nodavirus to the other susceptible healthy cultured marine fish species near the mariculture areas.

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