Short communication

Isolation of Nervous Necrosis Virus from Hybrid Grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) Cultured in Guangxi, China

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ABSTRACT—Outbreaks of suspected viral nervous necrosis emerged in cultured groupers in Guangxi, China in August 2018. Histopathology showed severe vacuolation in the brain tissue of the diseased juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂). A nervous necrosis virus (NNV) was isolated from pooled sample of the brain and eyes using a grouper cell line EAGB. Cytopathic effect with multiple cytoplasmic vacuolation cells developed in EAGB cells. The coat protein gene sequence of the isolate (GNNV-Gx) showed that it belonged to RGNNV genotype and was most closely related to that of a tiger grouper NNV registered in GenBank. The infectivity of the isolate decreased remarkably at pH 3 and pH 10 and was susceptible to 60°C.

Key words: VNN, grouper NNV, hybrid grouper

The agent of viral nervous necrosis (VNN), nervous necrosis virus (NNV) is a member of nodaviridae, which could be classified into four genotypes: tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV), striped jack nervous necrosis virus (SJNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.*, 1997). NNV can infect more than 69 fish species, including grouper species (Chi *et al.*, 2001; Liu *et al.*, 2015). As one of the most devastating disease in mariculture, VNN caused mortality in excess of 90% and even up to 100% in the severe outbreaks (Harikrishnan *et al.*, 2011; Wang, 2013). Groupers *Epinephelus* spp. are economically important marine fish species cultured in China and Southeast Asian countries (Li *et al.*, 2015). Reportedly, the viruses detected from groupers are usually RGNNV genotype (Harikrishnan *et al.*, 2011; Ma *et al.*, 2015). As an important marine fish culture center in China, the grouper aquaculture in Guangxi Province has grown rapidly in recent years, and the main farmed species is a hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) (Li *et al.*, 2018). Thus, outbreaks of suspected VNN in cultured groupers in Guangxi usually emerged at water temperature 20°C–28°C from April to October every year. The sustainable development of grouper aquaculture in Guangxi is potentially threatened by the pathogen. However, few researches on VNN including virus isolation and characterization in grouper aquaculture in Guangxi has been conducted. In this study, we investigated a mortality case of the juvenile hybrid grouper in August 2018.

Materials and Methods

The suspected NNV outbreaks occurred in the grouper farm in Beihai city, Guangxi province, and the mortality rate was over 90%. The hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂) with suspected VNN, whose body lengths of 3–10 cm, were collected at water temperatures 26°C–28°C in August 2018. The diseased hybrid groupers showed some typical disease signs of VNN, such as whirling, losing the balance and inappetence, and then a large number of deaths occurred in one or two days. The pooled samples of brains and eyes were collected from two diseased fish (body length 5–8 cm; body weight 6–9 g) and subjected to routine histopathology and virus isolation. EAGB cell line derived from the brain of grouper (Huang *et al.*, 2011) was used for virus isolation. The tissue homogenate was centrifuged at 5,000 ×g for 30 min at 4°C, then the supernatants was passed through a 0.45-μm pore size filter. The filtration was inoculated to EAGB cells seeded in 24-well plate (Shengyou Biotechnology, Hangzhou, China) using Leibovitz's L15 medium (L15, Gibco), and the plate was incubated at 28°C. Cytopathic effect (CPE) was observed under a laser

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Table 1. The primers used in this study

Primer	Sequences
CP-F	5'-AGCCGGGACAGGAACAGAC-3'
CP-R	5'-CCAACAGGCAGCAGGATTT-3'
qCP-F	5'-CAACTGACAACGATCACACCTTC-3'
qCP-R	5'-CAATCGAAGACTCCAGCGACA-3'
β -actin F	5'-TACGAGCTGCCTGACGGACA-3'
β -actin R	5'-GGCTGTGATCTCCTTCTGCA-3'

scanning confocal microscope (LSCM; Nikon).

In order to identify the virus isolated in EAGB cells, RT-PCR for NNV was carried out (Huang *et al.*, 2011; Li *et al.*, 2016). Total RNA of the virus culture was extracted by Viral DNA/RNA kit (Takara), and reversely transcribed into cDNA by ReverTra Ace qPCR RT Kit (Toyobo). The pair of primers (CP-F, CP-R) (Zhou *et al.*, 2017) applied for detecting coat protein (CP) gene of NNV was shown in Table 1. The thermal cycling profile of PCR involved 35 amplification cycles (92°C for 1 min, 55°C for 45 s, 72°C for 1 min) followed by a final extension at 72°C for 10 min. The PCR products were detected by agarose gel electrophoresis. Then the expected PCR products were purified by gel extraction and sequenced. Genetical analysis of the obtained sequence was performed using molecular evolutionary genetics analysis software (MEGA 7) and BLASTN (NCBI database).

The effects of temperature and pH on viral infectivity of the isolate were further analyzed. The isolate was incubated in L15 at different pH (pH 3, 5, 7, 7.5 and 10) and different temperatures (4°C, 25°C, 40°C, 50°C and 60°C) for 1 h. EAGB cells (2×10^5 cells/well) were seeded in 24-well plates for 18 h. Then each treated virus solution was inoculated on the EAGB cells in 24-well plate at a multiplicity of infection of 0.5, and the cells were incubated at 28°C for 48 h. qRT-PCR was applied to analyze the virus replication in EAGB cells. At 48 h after inoculation, the cells and culture supernatant were collected for total RNA extraction. The extracted total RNA was reversely transcribed into cDNA by ReverTra Ace qPCR RT kit (Toyobo), and then RNA copy number was quantified by qPCR for CP gene using a primer pair (qCP-F, qCP-R) (Zhou *et al.*, 2017) shown in Table 1. The β -actin gene was used as an internal control. The average value of three independent experiments was calculated. Intergroup differences were compared using a one-way analysis of variance by SPSS statistical software (IBM). The results of comparisons with $P < 0.01$ were considered to represent statistically significant differences.

Results and Discussion

Histopathology on the diseased hybrid grouper revealed severe vacuolation in the brain tissue (Fig. 1). A virus was isolated from the brain and eyes of the fish

Fig. 1. Histopathological section of the brain of diseased juvenile hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂). Note severe vacuolation in the brain (arrows). H&E. Bar = 200 μ m

Fig. 2. Virus isolation in EAGB cells. Compared to control EAGB cells (A), EAGB cells inoculated with the brain and eye tissue homogenates of diseased hybrid grouper showed cytopathic effect with multiple cytoplasmic vacuolations after 48 h at 28°C (B).

in EAGB cells. CPE of the cells was multiple cytoplasmic vacuolations typical for NNV (Fig. 2). To identify the isolate, RT-PCR was applied using a primer pair for NNV CP gene. A specific band was amplified by the RT-PCR with the expected size of 1,017 bp (Fig. 3A). The nucleotide sequences of the amplicon (Accession number: MK107836) were obtained, and MEGA 7 and BLASTN analyses showed that the isolate was most closely related to a RGNNV detected from tiger grouper (HQ859940.1) (Fig. 3B). Therefore, we tentatively named the isolate a grouper NNV, Guangxi (GNNV-Gx).

Fig. 3. Identification of the virus isolate GNNV-Gx from diseased hybrid grouper. (A) A specific PCR band (1,017 bp) of nervous necrosis virus coat protein gene was amplified. (B) Phylogenetic analysis based on coat protein gene.

Fig. 4. The effects of pH and temperature on infectivity of the virus isolate GNNV-Gx. The virus was exposed to various pH (A) and heat (B). Virus quantity in the EAGB cells inoculated with each treated virus was measured by RT-qPCR. Bars with different letters are significantly different ($P < 0.01$) by the one-way analysis of variance.

qRT-PCR was applied to analyze the effects of temperature and pH on infectivity of GNNV-Gx isolate. The results showed that the isolate was sensitive to exposure to pH 3 and pH 10 (Fig. 4A). The virus was resistant to 4°C–50°C, but sensitive to 60°C (Fig. 4B). As reported previously, SJNNV could be resistant to pH 3 and be inactivated by high alkalinity of pH 12, and also inactivated by heat treatment at 60°C (Arimoto *et al.*, 1996). A grouper NNV isolated from moribund grouper larvae of *Epinephelus* sp. was sensitive to pH 3 and pH 10–12, and the infectivity was totally blocked by heat treatment at 60°C (Chi *et al.*, 2001). An NNV isolated from juvenile sea bass was resistant to pH 2–9 (Frerichs *et al.*, 1996). Although this study shows GNNV-Gx isolate is sensitive to pH 3, sensitivity of NNV strains to pH previously reported differ from each other (Chi *et al.*, 2001).

Thus this study demonstrated that VNN was implicated in the mortalities in hybrid grouper cultured in

Guangxi Province, China.

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