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Short communication

Experimental evidence of horizontal transmission of *Betanodavirus* in hatchery-produced Asian seabass, *Lates calcarifer* and brown-marbled grouper, *Epinephelus fuscoguttatus* fingerling

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ABSTRACT

In the present study we report the experimental evidence of horizontal transmission of betanodavirus in hatchery-produced Asian seabass (*Lates calcarifer*) and brown-marbled grouper (*Epinephelus fuscogutattus*) fingerling. The experiment was conducted by incubating fish fingerlings in aerated sterile natural seawater inoculated with tissue homogenate of betanodavirus infected fish fingerling at 28°C for 30 min and after which they are transferred into the aquarium. Dead fish were collected daily for 10 days and subjected to RT-PCR and histological examinations. The cDNA of coat protein gene of betanodavirus from positive fish specimens were subjected to RFLP-PCR and DNA sequencing analyses. The result showed high fish mortality in treatment than in the control experiment. The RT-PCR and histological analyses showed all fish specimens in treatment groups except for D2 and E2 were successfully infected with *Betanodavirus*. In contrast, all fish specimens in control groups remained uninfected. The result of the present study indicate that mixing of fish fingerlings obtained from different sources and the use of surplus fish eggs for feeding supplement can potentially promote the horizontal transmission of *Betanodavirus* in hatchery. This can threaten the sustainability of aquaculture industry in Malaysia.

1. Introduction

Asian seabass, *Lates calcarifer* and brown-marbled grouper, *Epinephelus fuscoguttatus* are two of the most extensively cultured marine fish species in Malaysia. This is driven by high demand and market price of these fish especially during festive seasons (Othman, 2008). The aquaculture of these species is carried out in net cages installed in the coastal waters (Othman, 2008). Most of the seeds are produced in hatcheries (Ransangan and Manin, 2010). However, seeds are also imported from neighboring countries. Due to space limitations in most hatcheries, seeds of the same fish species but obtained from different sources are mixed in the same nursery tank. In addition, surplus eggs are sometimes used as feed supplement during the larval rearing. These practices could have been the reasons for many outbreaks of viral nervous necrosis (VNN) in Malaysia (Ransangan et al., 2011; Ransangan and Manin, 2010).

Viral nervous necrosis (VNN) is caused by a RNA virus called the *Betanodavirus* under the family Nodaviridae. The virus can be transmitted both in horizontal and vertical fashions (Boonyaratpalin et al., 1996; Castric et al., 2001; Oh et al., 2002; Watanabe et al., 2000). The horizontal transmission of the virus is known to occur from infected fish, contaminated trash fish and contaminated water supply (Chérif et al., 2009; Gomez et al., 2008, 2010; Watanabe et al., 2000). The cannibalistic nature of Asian seabass and brown-marbled grouper fingerlings may also facilitate the horizontal transmission of virus in the culture tank. This study was conducted to experimentally verify the horizontal transmission of *Betanodavirus* in Asian seabass and brown-marbled grouper seeds through culture water contaminated with tissue homogenate of *Betanodavirus*-infected fish fingerlings.

2. Methods and materials

2.1. Fish specimens

Asian seabass (10dph, 26dph and 40dph) and brown-marbled grouper (10dph) fingerlings were obtained from a hatchery in Tuaran, Sabah. The seeds were maintained in the aquarium (capacity 100 L) with re-circulated sea water and aerated throughout the experiment. The fish specimens were fed twice a day with artemia or/and commercial pellet. A day before the experiment, three pools (20 individuals) of Asian seabass (10dph and 26dph), brown-marbled grouper (10dph) and three individuals of Asian seabass (40dph) fingerlings were first randomly checked for *Betanodavirus* infection using RT-PCR and histopathological methods. Subsequently, only fish specimens from negative batches were then used in the subsequent experiments.

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2.2. Preparation of tissue homogenates

The tissue homogenates were prepared from *Betanodavirus*-infected fish fingerlings (TGNNV0708, SBNNV1108, TGNNV0809 and SBNNV0909). First, 2.0 g of infected fish fingerlings were weighed and homogenized in 20 ml sterile phosphate buffered saline (PBS, pH7.4) solution. Each homogenate was checked for the presence of *Betanodavirus* by RT-PCR as described previously (Ransangan et al., 2011). Only the positive tissue homogenates were used for challenge experiments. Meanwhile, 500 μl of viral stock culture (GPNNV) was used as positive control (Ransangan et al., 2011).

2.3. Exposure of fish specimens to Betanodavirus

Fish fingerlings were divided into fourteen groups as described in Table 1. The control groups were labeled as groups A1, B1, C1, D1, E1, F1, and G1. Meanwhile, the treatment groups were labeled as groups A2, B2, C2, D2, E2, F2 and G2. The fish specimens in control and treatment groups were transferred into 500 ml beakers filled with sterile natural seawater (28 ppt). Then, 500 μl PBS, 500 μl GPNNV stock culture, 20 ml tissue homogenates of each non-infected and infected fish fingerling were added into respective beakers as described in Table 1. The fish seeds were exposed for 30 min with aeration. Subsequently, the fish specimens were transferred into 7.0 L tanks. Number of fish specimens was the same for control and treatment groups. The fish specimens were fed twice a day with artemia or/and commercial pellet. The daily water exchange was set at 50%. The observation was conducted for 10 days with fish mortality recorded daily. The dead and surviving fish specimens were subjected to RT-PCR and histological analyses.

2.4. RT-PCR analysis

Representative fish specimens from each control and treatment group (A to G) were subjected to total RNA extraction using commercial TRIzol® reagent (Invitrogen™) according to the manufacturer's instructions. The extracted RNA was dissolved in 200 μl diethyl pyrocarbonate (DEPC) treated water and stored at –20 °C until use. Subsequently, the RNA was reverse transcribed to cDNA and amplified following the method described by Ransangan et al. (2011). The PCR fragments were analyzed on 1.5% agarose gel electrophoresis.

2.5. Histopathological analysis

The moribund and surviving fish specimens from control and treatment groups were subjected to histological examination according to the method described by Ransangan and Manin (2010). However, no histological sectioning was carried out for fish specimens in control group B1 and treatment group B2 due to the freshness issue of the specimens.

2.6. RFLP-PCR analysis

The RFLP-PCR analysis was done using six different restriction enzymes (AflIII, AlwNI, BbsI, BsaXI, BseRI and BsiEI). PCR fragments of the RNA1 (RdRp) and RNA2 (Cp) of *Betanodavirus* were amplified according to the method described by Ransangan et al. (2011) and digested using the six restriction enzymes (New England Biolabs, NEB) according to the manufacturer's instructions. RFLP-PCR fragments were separated on 1.5% agarose gel electrophoresis and photographed using the Image Analyzer (Alpha Innotech Corporation).

2.7. DNA sequencing of coat protein (Cp) gene

The Cp gene of *Betanodavirus* in infected fish specimens were amplified according to the method described by Ransangan et al. (2011). The PCR product (1363 bp) was cloned in pGEM®-T Easy vector (Promega) and transformed into *Escherichia coli* strain JM109 (Promega) according to the manufacturer's instructions. Subsequently, plasmids were extracted and purified by using the PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) according to the manufacturer's instructions. The purified plasmids containing the correct DNA insert were sent to AITBIOTECH, Singapore for sequencing. The sequencing was done using M13 (−20) forward (5′ – GTAAAACGACGGCCAGT – 3′) and M13 (−24) reverse (5′– GGAAACAGCTATGACCATG – 3′) primers. The DNA sequences were assembled using SeqMan (DNASTAR, v7.1) and compared with the DNA sequences of the viral source.

3. Results

3.1. The clinical signs and cumulative mortality

During the 10 days of observation, no clear clinical signs of VNN were observed in the fish specimens both in control and treatment groups. However, some of the fish in the treatment groups lost appetite and preferred to rest at the bottom of the tank. The treatment groups, except for D2 and E2 recorded higher mortality than the control groups. The first mortality occurred at day 1 in group B2, day 3 in A2, day 5 in G2, day 6 in F2 and day 7 in C2, respectively. After 10 days of observation, 100% mortality was recorded in group B2, 60.0% in G2, 56.7% in F2, 53.3% in A2 and 50.0% in C2, respectively. Control groups recorded first mortality at day 8 in B1, day 9 in G1 and day 10 in A1, respectively. The control groups A1 and G1 recorded 6.7% mortality while group B1 recorded 23.3% mortality. No mortality was recorded in the other control groups. The percentage cumulative mortality recorded in each group is shown Fig. 1.

Table 1
Fish species and viral source used in the study.

Control group	Description	Treatment group	Description	Number of specimens in each group
A1	Asian seabass (10dph) inoculated with 500 μl sterile PBS	A2	Asian seabass (10dph) inoculated with 500 μl GPNNV	30
B1	Brown-marbled grouper (10dph) inoculated with 20 ml tissue homogenate of virus-free tissue from Asian seabass	B2	Brown-marbled grouper (10dph) inoculated with 20 ml tissue homogenate of virus-infected Asian seabass (SBNNV1108)	30
C1	Asian seabass (40dph) inoculated with 500 μl sterile PBS	C2	Asian seabass (40dph) inoculated with 500 μl GPNNV	30
D1	Asian seabass (40dph) inoculated with 20 ml tissue homogenate of virus-free brown-marbled grouper	D2	Asian seabass (40dph) inoculated with 20 ml tissue homogenate of virus-infected brown-marbled grouper (TGNNV0809)	30
E1	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-free brown-marbled grouper	E2	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-infected brown-marbled grouper (TGNNV0708)	30
F1	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-free Asian seabass	F2	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-infected Asian seabass (SBNNV1108)	30
G1	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-free Asian seabass	G2	Asian seabass (26dph) inoculated with 20 ml tissue homogenate of virus-infected Asian seabass (SBNNV0909)	30

Note: dph = day-post hatching.