Susceptibility of Different Weight Ranges of 
*Epinephelus coioides* to Piscine Nodavirus

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Abstract

The susceptibility of different weight ranges of orange-spotted grouper (*Epinephelus coioides*) to piscine nodavirus was studied by experimental infection. Size ranges of 1-2 g, 6-8 g, 10-13 g, 30-60 g, and 90-120 g were intramuscularly injected with 50 µL 10^6 TCID<sub>50</sub>/mL of the cell culture supernatant. Clinical signs were observed and mortality occurred from 4 days post infection (dpi) for weight range 1-2 g to 7 dpi for weight range 30-60 g. Fish with weight ranges 1-2 g and 6-8 g showed highest susceptibility with mortality rate of 80% while fish with weight range 90-120 g were no longer susceptible. Mortality rates for weight ranges 10-13 g and 30-60 g were 50% and 20%, respectively. No mortality was observed in the 90-120 g weight range and the control group. These data show that the susceptibility of orange-spotted grouper to VNN is weight-dependent.

Introduction

Rapid development of marine fish aquaculture has been significantly hindered due to pathogenic diseases. Among the fish pathogens, viruses rank high in causing heavy devastation to the aquaculture industry and among these one of the most threatening is the viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) caused by a piscine nodavirus classified to the genus *Betanodavirus*, a member of the family *Nodaviridae* (Ball et al., 2000). This disease has affected hatchery-reared larvae and juveniles of Japanese parrotfish (*Oplegnathus fasciatus*) in Japan between 1986 to 1987 (Yoshikoshi and Inoue, 1990). Since then, the virus has been reported in more than 50 species of marine fish distributed worldwide (Sano et al., 2011).

Clinical signs differ for every species but generally include mortalities of up to 100%, abnormal coloration, lethargy, anorexia and abrupt swimming behaviour in a corkscrew fashion. The disease mostly affects larvae and juveniles, although significant mortalities are also observed in older fish up to broodstock. Vacuolations on the brain, spinal cord and retina can be observed microscopically (Costa and Thompson, 2016).

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In the Philippines, the disease was first reported in broodstock and larvae of hatchery-reared grouper, *Epinephelus coioides*, in 2001 (Maeno et al., 2002). This was followed in the same year by a report on VNN causing mass mortalities of hatchery-reared Asian sea bass, *Lates calcarifer*, larvae (de la Peña et al., 2008).

The susceptibility of fish species to VNN has been documented as age-dependent. Although there are variations, in general, the earlier the signs of disease occur, the greater is the mortality (Munday et al., 2002). In this study, the susceptibility of different weight ranges of orange-spotted grouper (*E. coioides*) to VNN was investigated to determine the most susceptible weight range. The result will serve as a basis to determine the most favourable weight for stocking in the ponds or cages for grow-out culture.

**Materials and methods**

**Virus preparation**

VNN-infected tissues from the brain and retina of *E. coioides* were obtained from the study of Maeno et al. (2007). Based on the sequence analysis of the virus by de la Peña et al. (2008) using the same source of infected tissues, it was determined that the virus belonged to the red-spotted grouper nervous necrosis virus (RGNNV) genotype. The virus was then inoculated in the E-11 cell line for tissue passage. The cells, propagated in 24-well tissue culture plates, were maintained with Leibovitz’s L-15 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA) and antibiotics, 100 units/mL each of penicillin and streptomycin (Invitrogen, USA) then incubated at 25°C. Cell cultures were observed daily for cytopathic effects (CPE) after 3-7 days under an inverted microscope. The cells were then harvested and the supernatant was checked for the presence of VNN using reverse transcription polymerase chain reaction (RT-PCR) following the method described below. The supernatant, volume of 50 µL, was then inoculated in grouper juveniles with average body weight (ABW) of 8 g for its first tissue passage, and the fish were observed daily for mortalities. Brain and eye tissues of moribund juveniles were homogenised using 1 mL of Hanks’ Balanced Salt Solution (HBSS) (Invitrogen, USA) and centrifuged at 1,500 x g for 10 min. Supernatant was filtered using 0.45 µm disposable filter (Millipore, USA). A portion was submitted for the detection of VNN using RT-PCR. Another aliquot of virus was re-inoculated onto fresh E-11 cell cultures for its second tissue passage. The same method was repeated until the third tissue passage to increase its virulence.

**Fish**

Fish were obtained from SEAFDEC/AQD nursery and were acclimatised in 200 L tanks for 1 week at the Infection Building. The fish were maintained in well-aerated, flow-through, UV-sterilised seawater with salinity of 32 ppt and at room temperature (29-31°C). Fish were fed twice daily with a commercial pelleted feed.

**Experimental infection**

Ten VNN-free hatchery-reared orange-spotted grouper for each weight range: 1-2 g, 6-8 g, 10-13 g, 30-60 g and 90-120 g, were intramuscularly injected with 50 µL 10⁶ TCID₅₀/mL of the cell culture supernatant. A control group with 10 fish for each weight range was injected with 50 µL of HBSS. Experimental fish were not subjected to anesthesia - instead a soft cotton cloth was used to wrap around the whole body of the fish to immobilise them prior to injection. The
fish were kept under observation for 14 days post challenge. The survival was closely monitored and moribund fish were collected. Moribund and freshly dead fish were dissected aseptically and submitted for RT-PCR, histology and viral cell culture examinations. The fish were not fed for the whole duration of the experiment. The seawater supplied to the fish was UV-sterilised and well-aerated at room temperature (29-31°C) with salinity of 32 ppt. Eighty percent of the rearing seawater was replaced daily.

RNA extraction

For RT-PCR, total RNA was extracted from brain tissue sample weighing 50-100 mg using 1 mL TRIzol reagent (Invitrogen, USA), homogenized and prepared for phase separation, followed by addition of 200 µL chloroform, then centrifuged for 15 min at 12,000 x g at 4°C. RNA was precipitated out of the aqueous supernatant with 500 µL isopropyl alcohol at room temperature for 10 min then centrifuged for 8 min at 12,000 x g at 4°C. RNA was then washed with 75% ethanol, centrifuged for 5 min at 7,500 x g at 4°C, briefly air-dried and dissolved in diethylpyrocarbonate-treated distilled water (DEPC-DW) then incubated at 55-60°C for 10-15 min. The RNA was then used for RT-PCR or stored at -80°C until use.

RT-PCR

RNA samples were submitted for one-step PCR using the primers designed by Nishizawa et al. (1994) and nested PCR using the RGNNV genotype-specific primers by Nishioka et al. (2010). One µL of RNA was subjected to reverse transcription at 42°C for 30 min followed by 99°C for 10 min. Afterwards, cDNA produced in the reverse transcription was subjected to one-step PCR in a 50 µL reaction mixture composed of 5X PCR Buffer, 25 mM MgCl$_2$, 0.6 U Taq DNA Polymerase (Invitrogen, USA) and 1.0 µM forward primer with sequence F2 – 5’ CGT GTC AGT CAT GTG TCG CT – 3’. Amplification parameters were an initial denaturation of 1 cycle at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 40 sec, annealing at 55°C for 40 sec, and extension at 72°C for 40 sec, then final extension at 72°C for 5 min. PCR products were kept at 4°C until ready for analysis using gel electrophoresis. Briefly, in the nested PCR step, 1 µL of the one-step PCR product was used as template for PCR amplification using primer pairs RGNNV-NFRG and RGNNV-NRRG with sequences 5’ – ACC TGA GGA GAC TAC CGC TC – 3’ and 5’ –CAG CGA AAC CAG CCT GCA GG – 3’ for forward and reverse nested-primers, respectively. Amplification profile for the nested PCR was an initial denaturation at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec, and extension at 72°C for 30 sec, then final extension at 72°C for 5 min. PCR products for nested step PCR were also kept at 4°C until ready for gel electrophoresis. The products were separated in 2.0% agarose 1000 (Invitrogen, USA) gel in TBE buffer, stained with ethidium bromide and visualised using a Gel Documentation System (Syngene, GeneGenius, UK). The outer and inner primer pairs amplified products of 430 and 280 bp, respectively.

Virus isolation

Virus isolation trials were done by inoculation of the filtered homogenate of brain tissue into E-11 cell line according to standard procedures, and incubated at 25°C for 14 days.

Histological examination

Eye tissue samples from moribund fish were
fixed in 10% buffered formalin and processed using standard histological techniques for paraffin-embedding, sectioned at 5-7 μm prior to staining with haematoxylin and eosin (H & E). Two fish per weight range were processed.

**Results**

**Fish mortality**

Fish with weight ranges 1-2 g and 6-8 g started to exhibit mortality at 4 dpi and reached 80% at 6 dpi (Table 1). For fish with weight range 10-13 g, onset of mortality was 5 dpi where 50% was reached at 8 dpi. Finally, for fish with weight range 30-60 g, 20% mortality was observed only after 7 dpi (Figure 1). The fish displayed anorexia, pale-grey skin pigmentation, loss of equilibrium and corkscrew-like swimming behaviour prior to death. No mortalities were observed in fish with weight range 90-120 g and in all control groups (Figure 1).

**RT-PCR**

By RT-PCR amplification with primers designed by Nishizawa et al. (1994), 430 bp amplicon (Figure 2) were detected for the one-step PCR cycling while the nested-step produced 280 bp amplicon.

**Table 1. Summary of nested-step PCR, viral cell culture isolation and histopathological results of different grouper weight ranges infected with tissue homogenates of VNN-infected grouper.**

<table>
<thead>
<tr>
<th>Weight range (g)</th>
<th>Mortality (%)</th>
<th>Nested PCR (%)</th>
<th>Cell culture (%)</th>
<th>Histopathology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>80 (8/10)</td>
<td>100 (8/8)</td>
<td>50 (4/8)</td>
<td>100 (2/2)</td>
</tr>
<tr>
<td>6-8</td>
<td>80 (8/10)</td>
<td>100 (8/8)</td>
<td>75 (6/8)</td>
<td>100 (2/2)</td>
</tr>
<tr>
<td>10-13</td>
<td>50 (5/10)</td>
<td>100 (5/5)</td>
<td>80 (4/5)</td>
<td>100 (2/2)</td>
</tr>
<tr>
<td>30-60</td>
<td>20 (2/10)</td>
<td>100 (2/2)</td>
<td>100 (2/2)</td>
<td>100 (2/2)</td>
</tr>
<tr>
<td>90-120</td>
<td>0 (0/10)</td>
<td>0 (0/2)</td>
<td>0 (0/2)</td>
<td>0 (0/2)</td>
</tr>
</tbody>
</table>

Results are given as number of fish positive for the virus/number of fish examined.

![Figure 1. Mortality rates of different grouper weight ranges after intramuscular injection challenge.](image-url)
Virus isolation
CPE were detected in 50-100% of the E-11 cell cultures inoculated with the filtrate of brain tissue samples from moribund fish for all weight ranges except 90-120 g (Table 1). Characteristic cytoplasmic vacuole formations were observed as early as 3 dpi for weight range 1-2 g and as late as 8 dpi for 10-13 g.

Histological examination
The results of the histological observations are summarised in Table 1. Representative samples were submitted for histology and all samples exhibited necrosis with vacuolation in the retina. All samples exhibited typical histopathological changes concurrent with those observed in VNN-infected tissues.

Discussion
In Asia, VNN is considered as one of the most dreaded diseases seriously affecting a long list of host range (Curtis et al., 2001). In the Philippines, mass mortalities have been observed (de la Peña et al., 2008; Maeno et al., 2007) and continue to devastate high value cultured marine fish such as grouper and sea bass. Methods to minimise, if not eliminate, the increased economic losses caused by the disease are continuously being explored. These methods include chemical treatment of eggs, PCR screening of the broodstock populations, vaccination using formalin-killed virus and implementation of biosecurity programs in the culture system (de la Peña, 2010; Pakingking et al., 2010).

In this study, grouper with weight ranges 1-2 g and 6-8 g were the most susceptible to VNN having a mortality rate of 80% with the onset as early as 4 dpi. Weight range 10-13 g only had 50% mortality, while the 30-60 g weight range exhibited 20%. It was observed that fish greater than 90 g were no longer susceptible to the virus since no mortality was observed during the infection challenge. However, the survivors from the experimental infection were not tested for the presence of VNN. Even if no mortality was observed or the fish survived the infection challenge, a possibility of subclinical infection...
exists in the survivors. In the study of Maeno et al. (2007), the virus was re-isolated from more than 50% of the survivors of the experimental infection done on orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish. It is therefore important to test for the presence of the virus to prevent transmission of the disease. In the study of Nguyen et al. (1997), betanodavirus was detected in multiple organs including gonads and intestines of subclinical striped jack \((Pseudocaranx dentex)\) broodstock but not in their central nervous system. This suggests the possibility of vertical transmission of the disease through the gametes (eggs or sperm) or faeces.

Based on the results of the nested PCR, the virus was confirmed as an RGNNV genotype. This conforms to the results of the study of de la Peña et al. (2008) wherein the same primers were also used. Infection of E-11 cell cultures with tissue filtrate from infected fish tissue showed extensive CPE parallel to the study of Maeno et al. (2002). The clinical signs and histopathological changes are also consistent with those described by Munday and Nakai (1997). This paper clearly describes that the susceptibility of orange-spotted grouper is weight-dependent. The results of this study suggest that the favourable size for stocking of grouper in the ponds or net cages should be 90 g or greater since this weight range exhibited no mortality upon exposure to the VNN.

**Acknowledgements**

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