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Susceptibility of Fish Species Cultured in Mangrove Brackish Area to Piscine Nodavirus

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Abstract

Susceptibility of orange-spotted grouper *Epinephelus coioides*, Asian sea bass *Lates calcarifer*, mangrove red snapper *Lutjanus argentimaculatus*, milkfish *Chanos chanos*, and rabbitfish *Siganus guttatus* to piscine nodavirus from orange-spotted grouper was studied by experimental infection. The fish were intraperitoneally injected with 0.05 mL of the filtrate homogenate of infected organs from diseased grouper at $10^{6.8}$, $10^{5.8}$ or $10^{4.8}$ $TCID_{50}$/fish, while the control group received 0.05 mL of Hanks' balanced salt solution. Clinical signs such as lethargy, anorexia and darkened pigmentation were observed in the orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish injected with high and medium doses of the homogenate. Although no or little mortality occurred in the experimentally-infected fish 10 days post-inoculation, viral nervous necrosis specific lesions such as severe necrosis and vacuolation in the brain and retina were produced in these four fish species. The virus was reisolated in SSN-1 cells inoculated with the filtrated tissue homogenate of survivors in all doses for all four fish species. However, in the experimentally infected rabbitfish no histological lesion was observed, and no virus was reisolated. These results indicate that grouper, sea bass, mangrove red snapper, and milkfish are susceptible to the piscine nodavirus isolated from diseased grouper.

Discipline: Aquaculture
Additional key words: host range, VNN

Introduction

In Southeast Asia, aquaculture production has grown rapidly for the last decade, and has contributed significantly to worldwide food supply and provided the means to generate increased revenue for the countries. There is an increasing demand for high value fish species such as orange-spotted grouper *Epinephelus coioides*, mangrove red snapper *Lutjanus argentimaculatus*, Asian sea bass *Lates calcarifer*, and rabbitfish *Siganus guttatus* as they command a higher market price[12]. The cage culture of these fish has expanded in the Philippines, Thailand and Malaysia. The spawning and seed production techniques are still in the experimental stages, thus, larvae used for the aquaculture mostly rely on the capture from the wild. Milkfish *Chanos chanos* has been the most important food fish cultured in the Philippines, Indonesia and Taiwan with the advantage of a low trophic level species. The techniques for breeding and seed production have been developed, but still continue to be refined. The intensification of aquaculture in these countries has led to the occurrence of various infectious diseases, and disease is one of the suppressing factors for sustainable production of these species, especially during the larval and juvenile stages.

Viral nervous necrosis (VNN) (alternative term: viral encephalopathy and retinopathy, VER), which is the

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disease listed by the Office International des Epizooties (OIE)[17], has been a major problem in the production of marine fish worldwide during the last decade[6,8,15,20]. The identification of the causative virus as a member of the family *Nodaviridae* was achieved by investigating the nucleic acids and structural proteins of purified virus from striped jack *Pseudocaranx dentex* larvae. The nodaviruses are small, non-enveloped icosahedral viruses with a genome consisting of 2 single stranded RNAs[13]. Piscine nodaviruses (betanodaviruses) have been shown to infect more than 30 marine fish species, especially at the larval and juvenile stages, and the infection usually results in high mortality[14]. Piscine nodaviruses can be classified into 4 genotypes based on the nucleotide sequences of the coat protein gene[16]: SJNNV (striped jack nervous necrosis virus), RGNNV (redspotted grouper nervous necrosis virus), TPNNV (tiger puffer nervous necrosis virus), and BFNNV (barfin flounder nervous necrosis virus).

Piscine nodavirus infection has been associated with high mortalities in cultured grouper species in Taiwan[4], Singapore[5], Thailand[7], China[9], and Indonesia[21]. Recently we documented outbreaks of VNN among hatchery-reared larvae of orange-spotted grouper and Asian sea bass in the Philippines[10,11]. By phylogenetic analysis, the isolates from the orange-spotted grouper and Asian sea bass belonged to RGNNV genotype (unpublished data). Piscine nodavirus infection has the potential threat to cause severe damage to other aquaculture species in this region. Hence there is an urgent need to determine the host range of this virus. The objective of the present study is to examine the susceptibility of orange-spotted grouper, Asian sea bass, mangrove red snapper, milkfish, and rabbitfish to the piscine nodavirus obtained from VNN-infected orange-spotted grouper by an experimental inoculation.

Materials and methods

1. Viral inoculum

VNN-infected orange-spotted grouper larvae showing abnormal swimming behavior, were collected during natural epizootics in 2001 and were stored at –80ºC until use.

The head portions of the diseased fish were thawed and homogenized at a dilution of 1:10 w/v in Hanks' balanced salt solution (HBSS). After centrifuging at 1,500 rpm for 10 min at 4ºC, the resulting supernatant was passed through a membrane filter (0.45 µm), further diluted with HBSS when required, and used as viral inoculum.

Virus titration was carried out using SSN-1 cells. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated based on the appearance of cytopathic effects (CPE) after a 10-day incubation period at 25ºC.

2. Fish

Healthy hatchery-reared larvae of orange-spotted grouper (body weight: 1.0–2.0 g), Asian sea bass (body weight: 0.6–0.9 g), mangrove red snapper (body weight: 1.5–3.6 g), milkfish (body weight: 6.9–12.5 g), and rabbitfish (body weight: 3.2–4.9 g) were intraperitoneally injected with the tissue homogenate at concentrations of $10^{6.8}$, $10^{5.8}$ or $10^{4.8}$ $TCID_{50}$/fish. For histological examination fish were inoculated with the tissue homogenate at concentration of $10^{6.8}$ $TCID_{50}$/fish. The control group with 10 fish for each species received 50 µL of HBSS. Ten fish for each dose were maintained in glass aquaria with 30 L aerated seawater at room temperature (25.5–28.7ºC) and observed daily for 10 days. Fish were fed once daily with a commercial diet. All aquaria were cleaned to remove waste material and half of the water volume was changed daily.

3. Histological examination

At 5 and 10 days post-inoculation (p.i.), 5 fish were randomly taken for histological examination. For light microscopy, the brain and eye of fish were fixed in 10% buffered formalin solution. The samples were processed using standard histological technique, embedded in paraffin wax, and 5–7 µm sections were stained with haematoxylin and eosin (H & E).

4. Virus isolation

For reisolation of the virus, surviving fish in the three infection doses in each species at day 10 p.i. were stored at –80ºC until assay. The head portions of the fish were processed and inoculated onto SSN-1 cells at 25ºC for 10 days.

Results

1. Fish

The orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish inoculated with $10^{6.8}$ or $10^{5.8}$ $TCID_{50}$/fish of the tissue homogenate exhibited dark pigmentation from 3 to 4 days p.i. Some of the orange-spotted grouper, mangrove red snapper and milkfish inoculated with $10^{6.8}$ or $10^{5.8}$ $TCID_{50}$/fish showed lethargy and abnormal swimming behavior including loss of equilibrium around the water surface or tank bottom. No abnormalities were observed in these fish inoculated with $10^{4.8}$ $TCID_{50}$/fish and in all dose groups of rabbitfish as well as in control groups.

In orange-spotted grouper, one fish died in both the $10^{6.8}$ and $10^{5.8}$ $TCID_{50}$/fish inoculation groups at 5 days p.i. In mangrove red snapper, two and one fish died in the $10^{6.8}$ and $10^{5.8}$ $TCID_{50}$/fish inoculation groups at 7 days p.i., respectively. In milkfish, one fish died in the $10^{6.8}$ $TCID_{50}$/fish inoculation group at 6 and 8 days p.i. No mortality was observed in sea bass and rabbitfish during the experimental period.

2. Histological examination

The results of the histopathological observations are summarized in Table 1. VNN-specific histological lesions such as necrosis with vacuolation in the brain and retina of orange-spotted grouper (Fig. 1), Asian sea bass (Fig. 2),

Table 1. Histopathological changes in the grouper, sea bass, snapper, milkfish, and rabbitfish experimentally infected with tissue homogenates of VNN-infected grouper

Fish species	Sampling Date (days post-inoculation)	Brain	Retina
Orange-spotted grouper			
	5	5/5	5/5
	10	5/5	2/5
Control	10	0/10	0/10
Asian sea bass			
	5	5/5	5/5
	10	5/5	5/5
Control	10	0/10	0/10
Mangrove red snapper			
	5	5/5	3/5
	10	5/5	4/5
Control	10	0/10	0/10
Milkfish			
	5	5/5	5/5
	10	5/5	5/5
Control	10	0/10	0/10
Rabbitfish			
	5	0/5	0/5
	10	0/5	0/5
Control	10	0/10	0/10

Results are given as number of fish with necrosis and vacuolation in the brain and retina/ number of fish examined.

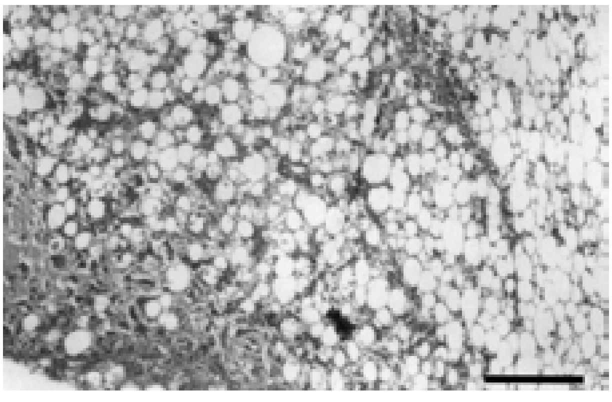

Fig. 1. Light micrograph showing vacuolation in the brain of experimentally infected orange-spotted grouper on day 10 post-inoculation
H&E stain. Scale bar = 100 µm.

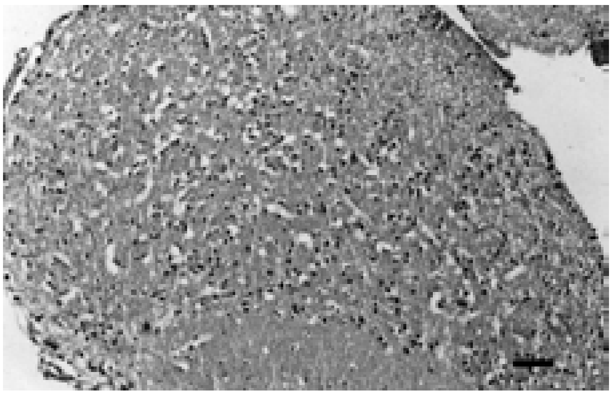

Fig. 2. Light micrograph showing vacuolation in the brain of experimentally infected Asian sea bass on day 5 post-inoculation
H&E stain. Scale bar = 100 µm.

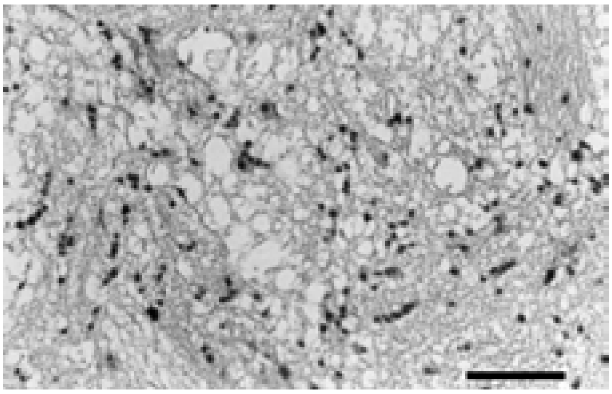

Fig. 3. Light micrograph showing vacuolation in the brain of experimentally infected mangrove red snapper on day 10 post-inoculation
H&E stain. Scale bar = 50 µm.

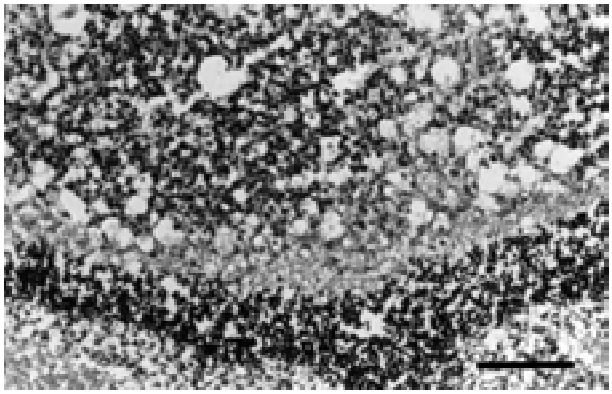

Fig. 4. Light micrograph showing vacuolation in the retina of experimentally infected milkfish on day 5 post-inoculation
H&E stain. Scale bar = 50 µm.

Table 2. Detection of piscine nodavirus from the survivors in experimental infection

	$10^{6.8}$ $TCID_{50}$/fish	$10^{5.8}$ $TCID_{50}$/fish	$10^{4.8}$ $TCID_{50}$/fish	Control
Orange-spotted grouper	5/9	6/9	6/10	0/10
Asian sea bass	6/10	4/10	5/10	0/10
Mangrove red snapper	6/8	4/9	7/10	0/10
Milkfish	6/8	6/10	6/10	0/10
Rabbitfish	0/10	0/10	0/10	0/10

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

mangrove red snapper (Fig. 3), and milkfish (Fig. 4) were observed at 5 and 10 days p.i., although no histopathological change was found in those of rabbitfish as well as in control groups.

3. Virus isolation

The results of the detection of piscine nodavirus from the survivors of all five species in SSN-1 cell cultures are shown in Table 2. In orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish, the virus was reisolated from all dose groups of each species. However, no virus was reisolated from rabbitfish as well as from the control groups.

Discussion

In a previous study, histopathological lesions such as marked necrosis with vacuolation in the brain and retina were observed in orange-spotted grouper naturally infected with piscine nodavirus[10]. In the present study, histopathological features in the brain and retina similar to those of naturally infected orange-spotted grouper were reproduced in experimentally infected orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish. Reisolation of the virus in SSN-1 cells inoculated with samples from survivors of all experimental groups of these four fish species was obtained with the development of extensive CPE. However, in rabbitfish no histopathological lesion was observed in the brain and retina, and the virus was not reisolated. These results indicate that orange-spotted grouper, Asian sea bass, mangrove red snapper, and milkfish were susceptible to a piscine nodavirus from diseased orange-spotted grouper.

Natural infection of VNN is acute, and the disease outbreak can be severe when the occurrence of virus is coupled with stress factors such as high stocking density and high water temperature in the culture system[2,19]. Combination of these factors could cause high mortality during the rearing period. However, a lower mortality rate was observed in the present study. In the case of natural infection in orange-spotted grouper, mass mortality occurred in 34-day-old larvae less than 0.20 g in body weight[10], while fish used in the present work were bigger (>1 g). The differences in mortality rate might be explained by the stage of development in the immune system of grouper. Another possible explanation for the low mortality is the route of infection. In the experimental transmission of piscine nodavirus to juvenile sea bream *Sparus aurata* and sea bass *Dicentrachus labrax*, high mortality occurred in fish challenged by intramuscular injection. However, no or low mortality was observed in fish challenged by intraperitoneal injection[1,18]. This might be caused by more efficient non-specific immune defense mechanisms triggered in the peritoneal cavity[3]. Further studies should be carried out to investigate the pathogenicity of the piscine nodavirus *in vivo*, the factors influencing its virulence, and the fish immune response to the virus.

Although there is no outbreak of VNN in mangrove red snapper and milkfish at the SEAFDEC AQD hatchery, it is possible that these species could be in a carrier state of infection or reservoir of the virus. Thus, it is urgently needed to examine the pathogenicity of the virus to these species in detail.

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