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Short Communication

Prevalence, Risk Factors and Transmission of Nervous Necrosis Virus in A Hatchery Producing Hybrid Grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*) Fry

Atirah Khairiah Ahmad[1], Mohammad Noor Azmai Amal[1,2*], Mohd Zamri Saad[1,3], Munira Murni[4], Azila Abdullah[4], Sufian Mustafa[5] and Nik Haiha Nik Yusof[5]

[1]Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[3]Department of Veterinary Laboratory Diagnosis, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4]National Fish Health Research Division, Fisheries Research Institute, 11900 Batu Maung, Penang, Malaysia
[5]Marine Fish Aquaculture Research Division, Fisheries Research Institute, Tanjung Demong, 22200 Besut, Terengganu, Malaysia

ABSTRACT

This study investigates the prevalence, risk factors, and transmission of nervous necrosis virus (NNV) in a hatchery producing hybrid grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*) fry. The eggs and sperm of giant grouper (GG) and tiger groupers (TG) that were collected for breeding purposes within the 12-month study period were sampled to detect NNV. At the same time, three breeding attempts of different NNV status of broodstocks, which were NNV-positive GG × NNV-positive TG, NNV-positive GG × NNV-negative TG and NNV-negative GG × NNV-negative TG were conducted. The produced hybrid grouper (HG) fry was then sampled at 5, 10, 20, 30, 40, 60, 90, and 120 days post-hatched to detect the presence of NNV. The fresh fish, live feed, and commercial fish pellet that were used to feed the broodstocks or HG fry throughout the study period were also sampled for NNV detection. The water's physico-chemical parameters during each sampling were determined. The results revealed that the broodstocks had a low

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E-mail addresses:
atirahahmad92@gmail.com (Atirah Khairiah Ahmad)
mnamal@upm.edu.my (Mohammad Noor Azmai Amal)
mzamri@upm.edu.my (Mohd Zamri Saad)
munira_murni24@yahoo.com (Munira Murni)
azadullah@gmail.com (Azila Abdullah)
sufnor96@yahoo.com (Sufian Mustafa)
nhaiha@hotmail.com (Nik Haiha Nik Yusof)
* Corresponding author

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prevalence to NNV. However, when at least one of the broodstocks was NNV-positive, all batches of the fry were NNV-positive at high prevalence. There were consistent associations of ammonia and iron with the presence of NNV in both broodstocks and fry. Phylogenetic tree indicates the possible horizontal and vertical transmissions of NNV in the hatchery culture system. Understandings the epidemiology of NNV in a real hatchery condition can provide significant information for control and prevention of the disease.

Keywords: Hatchery, prevalence, risk factors, transmission, viral nervous necrosis

INTRODUCTION

The marine aquaculture industry is rapidly expanding in Malaysia. As a major commodity for protein supply, the total production from marine aquaculture (excluding seaweeds) was ~98,050 metric tonnes (MT), with an estimated wholesale value of USD500 million in 2016 alone (Annual Fisheries Statistic [AFS], 2016). The productions of groupers (*Epinephelus* spp.), snappers (*Lutjanus* spp.) and Asian seabass (*Lates calcarifer*) were 6,167 MT, 16,020 MT and 15,026 MT, with estimated wholesale values of USD 55 million, USD 97 million and USD 63 million, respectively, dominating the marine aquaculture production in Malaysia (AFS, 2016). It is expected that the production of these highly valuable marine fishes will increase in the future due to the intensification and commercialization of the industry (AFS, 2016).

The hybrid grouper (♂ giant grouper *E. lanceolatus* × ♀ tiger grouper *E. fuscoguttatus*) was first introduced in Malaysia by Ch'ng and Senoo (2008). Now, it is a favourite among marine fish farmers in Malaysia for their fast growth rate compared to the commonly cultured grouper species (Sufian & Nik-Haiha, 2015). Furthermore, it possesses better resistant towards disease, temperature, and salinity, making it an important target species in aquaculture (Senoo, 2010).

Betanodavirus, the nervous necrosis virus (NNV), causes a disease known as viral nervous necrosis (VNN). It is currently classified into four major genotypes based on the T4 variable region of RNA2: the striped jack nervous necrosis virus (SJNNV), the tiger puffer nervous necrosis virus (TPNNV), the barfin flounder nervous necrosis virus (BFNNV), and the red grouper nervous necrosis virus (RGNNV) (Nishizawa et al., 1997). NNV infects more than 70 species of marine and freshwater fish globally (Doan et al., 2017). The disease is characterised by extensive neuropathy and retinopathy of the brain and eye of affected fish larvae and fry. High mortality rate can reach 100% is usually observed in larvae and fry, but larger fish could also be infected (Kua et al., 2013). Mortality usually occurs following abnormal swimming behaviours, especially at juvenile stages (Nakai & Mori, 2016). In Malaysia, NNV has been detected in marine cultured Asian seabass, humpback grouper (*Cromileptis altivelis*), brown marbled grouper (*E. fuscoguttatus*), golden pompano (*Trichinotus blochii*), and

cobia (*Rachycentron canadum*) (Abdullah et al., 2017; Kua et al., 2013; Manin & Ransangan, 2011; Rangsangan et al., 2011; Ransangan & Manin, 2010). This study intends to determine the prevalence, risk factors, and transmission of NNV in a hatchery producing hybrid grouper (HG) fry. It was designed without any intervention of the hatchery management and practices to allow us to understand the epidemiology of NNV in real hatchery conditions.

MATERIALS AND METHODS

Sampling Site

The study was conducted in a marine fish hatchery located in Besut, Terengganu, Malaysia. The broodstocks of the male giant grouper (GG) (*E. lanceolatus*) and female tiger grouper (TG) (*E. fuscoguttatus*) that were used in this study were reared in cylindrical concrete and rectangular fiber tanks. The broodstocks were fed daily with commercial fish pellet and fresh fish.

Samples Collection from Broodstock and Fresh Fish

Sampling was conducted between March 2016 and April 2017. During the study period, breeding was conducted at monthly intervals, as previously described (Ch'ng & Senoo, 2008; Sufian & Nik-Haiha, 2015). Briefly, the eggs and sperm were stripped from matured TG and GG broodstocks (Glamuzina et al., 1998). In each striping and hormone injection process, the broodstocks were anesthetized using MS-222 (Sigma-Aldrich, Kuala Lumpur, Malaysia) at a rate of 0.1 ml/L of water. Strippings were carried out as soon after the ovulation process occurred, within 6 to 12 hours following the injection of human chorionic gonadotropin hormone (Pregnyl®, Baxter Oncology, Halle, Germany), at a dose of 1000 IU/kg for TG. The total length and body weight of individual broodstocks were recorded before the hormone injection. Prior to the start of breeding, the eggs and sperm of TG and GG broodstocks were sampled to detect the presence of NNV. Two to three broodstocks of GG and TG were used monthly for breeding purpose (Table 1). At the same

Table 1
The status of NNV in grouper's broodstocks throughout the study period

Month and Year	GG♂		TG♀		GG♂	TG♀	GG♂	TG♀
	Length (cm)	Weight (kg)	Length (cm)	Weight (kg)	VNN Status	VNN Status	% Positive	% Positive
March 2016	155.1	80.58	64.0	5.88	-	-		
	160.3	90.64	52.0	3.09	-	-	0	0
	167.0	92.78	65.3	7.33	-	-		
April 2016	150.2	75.95	47.5	2.50	-	-		
			48.8	2.13		-	50	33.3
	146.2	85.84	46.9	2.07	+	+		

Table 1 *(continue)*

Month and Year	GG♂		TG♀		GG♂	TG♀	GG♂	TG♀
	Length (cm)	Weight (kg)	Length (cm)	Weight (kg)	VNN Status	VNN Status	% Positive	% Positive
July 2016	145.6	70.35	51.5	3.46	-	-	0	0
	150.8	80.78	52.5	3.50	-	-		
August 2016	151.0	80.68	51.5	3.80	-	-	0	0
	145.0	70.88	68.9	6.05	-	-		
September 2016	134.6	80.74	54.0	4.60	-	-	0	0
	167.8	93.32	52.4	3.10	-	-		
October 2016	150.3	80.02	55.4	6.24	-	-	0	0
	146.6	85.12	47.9	4.18	-	-		
November 2016	150.8	80.31	53.5	2.89	-	-		
			67.5	5.66		-	0	0
	140.5	80.2	55.5	4.77	-	-		
December 2016	150.1	80.03	73.5	5.54	-	-		
	140.7	65.51	54.5	3.81	-	-	0	0
	155.7	80.41	75.6	5.17	-	-		
January 2017	150.6	80.32	53.5	5.00	-	-		
	150.8	75.45	52.5	3.47	-	-	0	0
	140.6	66.77	50.0	5.11	-	-		
February 2017	140.8	78.00	47.0	4.70	-	-		
			66.5	5.50		-	0	0
	141.8	78.25	62.8	5.10	-	-		
March 2017	155.4	80.52	60.8	7.00	-	-		
	150.6	80.23	56.9	6.50	-	-	0	0
	155.5	81.89	50.3	4.90	-	-		
			54.1	6.40		-		
April 2017	141.4	66.54	60.9	5.60	-	-		
			48.4	4.20		-	0	0
	146.4	86.36	52.9	3.30	-	-		

GG♂: Male giant grouper; TG♀: Female tiger grouper

day, 10 individual fresh fish that were used to feed the broodstocks were collected randomly, before the eyes and brains were pooled for detection of NNV.

Samples Collection from Produced Fry, Live Feed and Commercial Pellet

In NNV's vertical transmission study, three breeding attempts of different NNV status of broodstocks, which were 1) NNV-positive GG × NNV-positive TG (GG+ × TG+); 2) NNV-positive GG × NNV-negative TG (GG+ × TG-); and 3) NNV-negative GG × NNV-negative TG (GG- × TG-) were conducted as detailed above. Broodstocks of two GG and three TG with different status of NNV as determined earlier were used for these experiments. Following fertilization,

the obtained HG fry was reared and cultured in separated tanks until 120 days post-hatch (dph).

The management and rearing procedures for the newly hatched HG fry were conducted as previously described (Ch'ng & Senoo, 2008; Sufian & Nik-Haiha, 2015). After fertilization, each batch of the fry was placed into separate tanks. At 5, 10 and 20 dph, ~1000 whole body fry in each batch were sampled to detect the presence of NNV. The samples were divided into five different replicate tubes (200 fry/replicate). However, on days 30, 40, 60, 90 and 120 post-hatching, only the eyes and brains were collected from 150 juveniles. The samples were pooled and divided into five different replicates (50 juveniles/replicate). Twenty individuals at each sampling times were collected for total length and body weight measurements.

Moreover, on days 5, 10, 20 and 30 post-hatching, the rotifer that was used as live feed for the fry were also sampled at a rate of ~1000 rotifer/tube in five replicates for the detection of NNV. The commercial fish pellet that fed to the fry from 40 to 120 dph were also detected for NNV.

Water Quality Determination

Water temperature, pH, dissolved oxygen, salinity, conductivity and dissolved particles were measured *in situ* using an YSI 556 MPS probe (YSI Incorporation, NY, USA). The water sample was collected in sterilized polyethylene sampling bottles in replicates from two points within the hatchery, which were tanks containing the broodstocks and the newly produced HG fry. The levels of iron, ammonia, nitrate, nitrite, and phosphate were measured using a DR2800 spectrophotometer (Hach Company, Loveland, USA). The water qualities were determined every time prior to sampling of the broodstocks egg, sperm, and post-hatch HG fry.

Detection of NNV

In order to detect the presence of NNV, the total RNA of the eggs and sperm, fresh fish, live feed, and the HG fry were extracted using Viral RNA Mini Kit (Invitrogen, California, USA), according to the manufacturer's instructions. RT-PCR was performed using MyTaq™ One Step RT-PCR kit and MyTaq™ kit (Bioline, London, UK). The RT-PCR was carried out according to previously outlined methods (Nishizawa et al., 1994; World Organisation for Animal Health [OIE], 2017). The primers used were forward primer F2 (5' - CGTGTCAGTCATGTGTCGCT-3') and reverse primer R3 (5'-CGAGTCAACACGGGTGAAGA-3'). The PCR amplifications were performed using an Eppendorf Mastercycler Pro Thermal Cycler (Eppendorf, Hamburg, Germany) with reverse transcription at 45°C for 20 minutes, polymerase activation at 95°C for 1 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 30 seconds. After that, the first PCR product was later subjected for nested PCR using primer set of RGNNV-NFRG (5' - ACCTGAGGAGACTACCGCTC

- 3') and RGNNV-NRRG (5' - CAGCGAAACCAGCCTGCAGG - 3') as described by Nishioka et al. (2010). The amplification of cDNA was performed for one cycle at 95°C for 1 minute, 35 cycles of denaturation at 95°C for 15 seconds, annealing at 58°C for 15 seconds, and extension at 72°C for 10 seconds. Then, the PCR product was electrophoresed with 1.5% agarose gel.

Sequencing and Data Analysis

A total of nine purified PCR products of NNV were used for sequencing (First Base, Kuala Lumpur, Malaysia). The nucleotide sequences of the NNV were compared with the known sequences in the GenBank database using Nucleotide Basic Local Alignment Search Tool (BLAST) program. Phylogenetic tree for NNV was generated by Neighborjoining of the MEGA 6.06 software (Tamura et al., 2013).

Pearson's correlation coefficient (*r*) (Statistix 9, Analytical Software, Tallahassee, FL, USA) was used to determine the possible correlation between the mean individual of water quality parameters in each sampling time with the presence of NNV. A p value at <0.05 indicates statistical significance.

RESULTS

Prevalence of NNV in Broodstocks

The prevalence of NNV among broodstocks between March 2016 and April 2017 is shown in Table 1. Low rate of NNV was detected among the broodstocks in the hatchery. The NNV was detected among broodstocks only in April 2016 in 50% of the males and 33.3% of the females. However, the NNV-positive broodstocks showed no symptom or clinical sign indicating of NNV infection. The nested PCR confirmed the detection with amplification of the 280 bp band (Figure 1).

Associations between Water Quality and NNV

The associations between the water quality with the detection of NNV among broodstocks and HG fry are presented

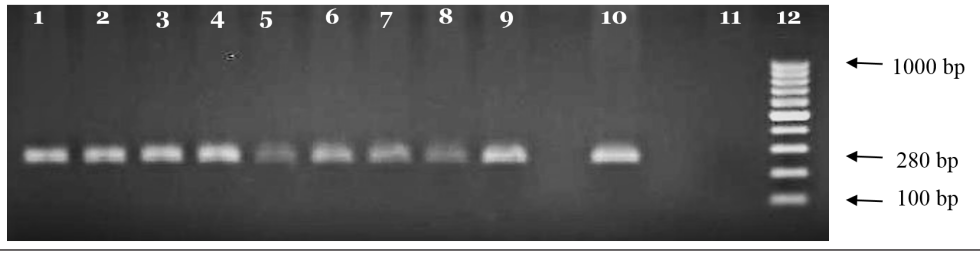

Figure 1. Detection of NNV in GG and TG broodstocks, and produced HG fry at different day post-hatch. Lane 1: GG ♂ broodstock; Lane 2: TG ♀ broodstock; Lane 3: Juvenile 40 dph (GG+ × TG-); Lane 4: Juvenile 40 dph (GG+ × TG+); Lane 5: Juvenile 60 dph (GG+ × TG-); Lane 6: Juvenile 90 dph (GG+ × TG-); Lane 7: Juvenile 90 dph (GG+ × TG+); Lane 8: Juvenile 120 dph (GG+ × TG-); Lane 9: Juvenile 120 dph (GG+ × TG+); Lane 10: Positive control of NNV (Abdullah et al., 2017); Lane 11: Negative control of NNV; Lane 12: 100 bp molecular weight marker (Fermentas). GG: Giant grouper; TG: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; dph: days post-hatch

in Table 2 (Full water quality data in Appendices 1 to 5). Iron and ammonia consistently showed significant ($p<0.05$) and positive correlations with the detection of NNV in broodstocks of GG, TG, and GG+ × TG+ fry, while other parameters such as conductivity, dissolved oxygen, dissolved particles, nitrite, salinity and pH also showed significant positive/negative correlations with the detection of NNV, but without consistency.

Transmission of NNV to Produced HG's Fry

High rate of NNV detection between 60% and 100% were observed among HG fry produced from parents that at least one positive to NNV (Table 3). However, the NNV-positive HG fry showed no symptom or clinical sign indicating of NNV infection. No detection of NNV was also observed in fry that were produced by parent that both tested negative to NNV (Figure 1). Throughout the study period, NNV was not detected in the fresh fish, live feed, and commercial fish pellets.

Phylogenetic Tree Analysis

Analysis of the phylogenetic tree revealed that the detected NNV strains in this study were closely related to each other but distinctively grouped from other strains of NNV, including those isolated from east Malaysia (HQ859945 and HQ859922), other virus strains such as *Iridovirus* (DM015883.1), and the lymphocytes disease virus (KJ408273). The nucleotide sequences for NNV strains in this study, including from the broodstocks and the HG fry (40 dph to 120 dph) were deposited into the GenBank database with accession number from MG581289 to MG581297 (Figure 2).

Table 2
Relationships between the water qualities with the detection of NNV in grouper's broodstocks and produced HG fry from different NNV status of broodstocks

Water Quality	***r* value**				
	GG♂	**TG♀**	**GG+ × TG-**	**GG- × TG-**	**GG+ × TG+**
Conductivity (µS/cm)	0.7847	-0.9840*	0.8491	NA	-0.8646
Dissolve oxygen (mg/L)	0.7594	0.9548*	-0.9648	NA	-0.9795
Dissolve particles (mg/L)	0.8437*	-0.8854	0.6201	NA	0.4329
Iron (mg/L)	0.8783*	0.9649*	0.8660	NA	1.0000*
Ammonia (mg/L)	0.8731*	0.9573*	0.8885	NA	1.0000*
Nitrate (mg/L)	0.2740	0.9428	-0.0822	NA	-0.5000
Nitrite (mg/L)	0.8648*	0.9661*	0.0000	NA	0.8660
Salinity (ppt)	-0.9769*	-0.8193	0.8833	NA	0.9377
Temperature (°C)	-0.4995	-0.8430	0.7587	NA	0.3346
pH (1-14)	-0.9559*	-0.6572	0.9784	NA	0.0251

GG♂: Giant grouper; TG♀: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; *: indicate statistically significant at $p < 0.05$

Table 3
Rate of detection of NNV in broodstocks and HG fry at different days post-hatch and the feed

Days Post-hatch	GG+ × TG+			GG+ × TG-			GG- × TG-			Fresh Fish	Live Feed (rotifer)	Pellet
	Length (cm)	Weight (g)	NNV Status (%)	Length (cm)	Weight (g)	NNV Status (%)	Length (cm)	Weight (g)	NNV Status (%)			
GG broodstock	140.0	70000	+	140.0	70000	+	150.0	80000	-	-	ND	ND
TG broodstock	46.0	2070	+	48.0	2130	-	73.0	5540	-	-	ND	ND
5	2.81 ± 0.51	0.15 ± 0.05	+ (100)	2.71 ± 1.08	0.11 ± 0.05	+ (100)	2.81 ± 0.32	0.18 ± 0.05	-	ND	-	ND
10	2.90 ± 1.23	0.39 ± 0.09	+ (100)	2.92 ± 1.19	0.35 ± 0.15	+ (60)	3.00 ± 0.55	0.47 ± 0.03	-	ND	-	ND
20	3.91 ± 1.09	1.63 ± 0.27	+ (80)	4.11 ± 1.34	1.57 ± 0.76	+ (100)	4.31 ± 0.53	1.69 ± 0.11	-	ND	-	ND
30	5.83 ± 1.33	3.74 ± 1.25	+ (100)	5.60 ± 1.31	3.69 ± 1.22	+ (80)	6.08 ± 0.67	4.07 ± 1.23	-	ND	-	ND
40	7.72 ± 1.04	16.23 ± 1.36	+ (80)	7.22 ± 1.57	11.54 ± 2.48	+ (80)	7.81 ± 0.89	17.02 ± 2.54	-	ND	ND	-
60	8.91 ± 1.22	61.11 ± 2.33	+ (80)	8.32 ± 1.88	56.41 ± 2.65	+ (80)	9.05 ± 0.12	69.85 ± 2.66	-	ND	ND	-
90	11.11 ± 1.34	132.11 ± 3.34	+ (100)	10.41 ± 1.69	120.00 ± 4.67	+ (100)	11.50 ± 0.40	151.43 ± 4.58	-	ND	ND	-
120	12.60 ± 1.25	250.00 ± 2.47	+ (80)	11.91 ± 2.64	220.00 ± 3.87	+ (100)	12.90 ± 0.54	289.00 ± 4.66	-	ND	ND	-

GG: Giant grouper; TG: Tiger grouper; +: positive to NNV; -: negative to NNV; ×: crossbreed; ND: not determined. The detection rate of NNV in fresh fish, live feed and commercial fish pellet were similar for all experiments

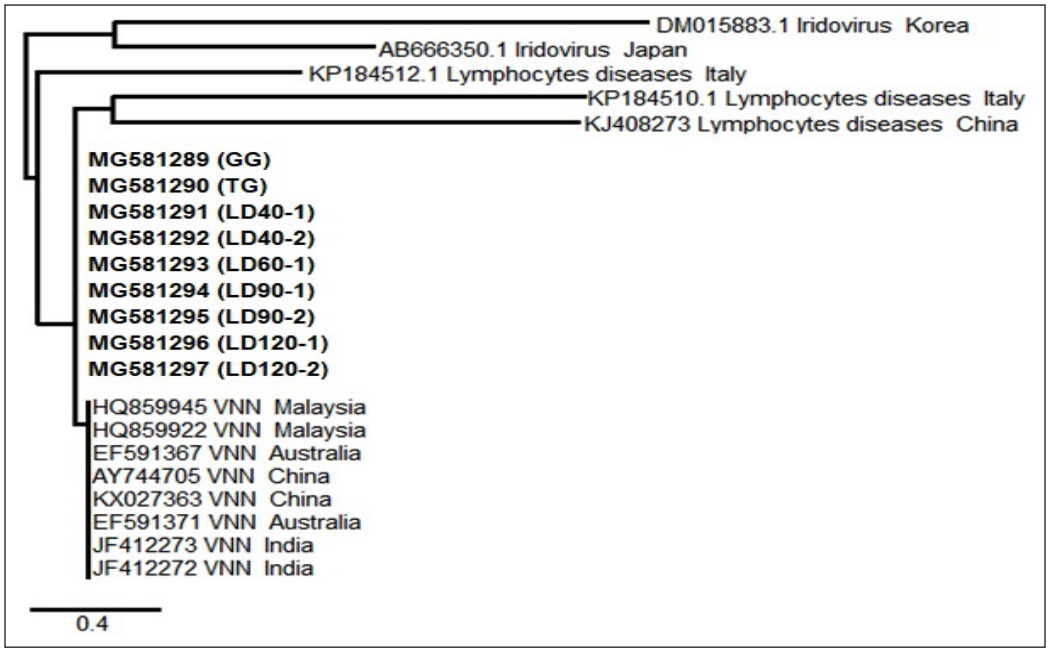

Figure 2. Phylogenetic tree showing the relationship between the NNV strains from this study with other NNV strains and fish viruses. NNV strains in this study were closely related between each other and distinctively grouped from other strains, including from east Malaysia (HQ859945 and HQ859922).GG: giant grouper; TG: tiger grouper; LD: Produced hybrid grouper fry whether at 40, 60, 90 and 120 days post-hatch

DISCUSSION

The prevalence of NNV among broodstocks in this hatchery was low. However, when at least one of the broodstocks was positive to NNV during breeding, all produced HG fry were NNV-positive at high prevalence. NNV is known as a disease that mainly affects the larvae and juveniles of cultured fish, resulting in high mortalities (Kokawa et al., 2008; Muroga 2001). However, under suitable conditions, sub-adults, market-size and adult fish (including broodstocks) can also be affected (Yanong 2016). Since this study was done under a non-stressful environment and no symptom or clinical sign of NNV infection was observed, the virus might shed from the broodstocks and the produced HG fry (Costa & Thompson, 2016). Moreover, the combination of RT and nested PCR methods used in this study were very sensitive and capable to identify the detected *Betanodavirus* as RGNNV genotype, which were usually isolated from warm-water fishes (Nishioka et al., 2010; OIE, 2017). In addition, without disease outbreak as observed in this study period, the high prevalence of NNV in HG fry is an important alert to the hatchery operators and farmers to consider.

This study revealed that iron and ammonia levels consistently showed significant correlations with the detection of NNV in broodstocks and GG+ × TG+ fry. Therefore, in order to reduce the risk of NNV infection, hatchery operators should closely monitor the two water quality

parameters, especially during the larval and fry periods. Moreover, water temperature has also been reported to be a risk factor in NNV infection in fish (Iwamoto et al., 2000; Yuasa et al., 2007). In this study, fresh fish, live, and commercial feed tested negative for NNV. Contrarily, previous studies in other countries showed that trash fishes are the main sources of betanodaviruses in cultured fish, and that they posed a serious risk for outbreaks of NNV in susceptible cultured fish (Doan et al., 2017; Gomez et al., 2010).

Phylogenetic tree analysis revealed possible vertical and horizontal transmissions of NNV in this hatchery. The virus might be transmitted horizontally among the broodstocks and fry. Similarly, vertical transmission occurred between broodstocks and fry, since infected broodstocks were most likely to transmit the virus to their respective fry. Furthermore, the NNV strains detected in this study were closely related with each other and distinctively grouped from other NNV strains, including from east Malaysia, suggesting the same virus strain is circulating within the hatchery system. Thus, the implementation of biosecurity measures is an important step towards controlling the disease.

CONCLUSION

The results of this study revealed the field scenario of the prevalence, risk factors, and transmission of NNV in a real hatchery environment. Hatchery operators are recommended to screen their broodstocks and produced fry before introduced into grow out farms in order to reduce the disease transmission and economic losses due to NNV. It is also expected that with the information obtained, a thorough biosecurity measure could be formulated and implemented to control NNV in the hatchery.

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APPENDIX

Appendix 1
*Water quality during the culture period of male GG (*Epinephelus lanceolatus*) broodstocks*

Month and Year	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
Mar 16	27.20±4.74	7.95±0.07	5.00±0.29	29.89±0.27	49.52±1.71	30.15±0.29	0.32±0.13	0.058±0.042	1.23±0.08	2.29±0.97	0.03±0.01
April 16	29.02±1.69	7.92±0.09	5.00±1.12	28.55±2.13	47.86±4.68	28.85±1.93	0.65±0.13	0.011±0.001	1.05±0.13	0.68±0.08	0.05±0.01
July 16	30.00±0.02	8.12±0.01	4.05±0.00	31.02±0.01	51.69±0.03	30.88±0.00	0.34±0.01	0.009±0.001	1.05±0.07	0.50±0.00	0.33±0.39
Aug 16	30.67±0.01	8.19±0.01	4.97±0.00	30.26±0.01	52.01±0.01	29.58±0.04	0.33±0.01	0.005±0.001	0.15±0.07	0.425±0.02	0.02±0.01
Sept 16	30.51±0.00	7.91±0.00	4.12±0.01	29.66±0.01	52.31±0.01	30.91±0.01	0.28±0.03	0.011±0.001	1.40±0.14	0.40±0.00	0.03±0.00
Oct 16	30.26±0.63	8.07±0.14	4.77±0.44	30.19±0.08	51.79±0.40	35.38±11.01	0.34±0.06	0.003±0.002	0.30±0.18	0.31±0.15	0.03±0.01
Nov 16	29.72±0.08	8.00±0.33	4.09±0.03	30.91±0.01	52.00±1.32	30.68±0.62	0.18±0.01	0.008±0.004	1.10±0.14	1.75±0.21	0.04±0.01
Dec 16	30.56±0.01	8.02±0.01	4.68±0.01	30.12±0.01	51.45±0.01	30.22±0.02	0.3±0.00	0.004±0.000	0.30±0.00	0.41±0.01	0.01±0.00
Jan 17	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.99±0.01	29.90±0.01	0.32±0.04	0.005±0.000	1.10±0.00	1.05±0.07	0.045±0.01
Feb 17	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.24±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
Mar 17	29.89±0.65	8.12±0.03	4.91±0.67	29.58±0.66	51.03±0.19	29.55±0.69	0.06±0.02	0.003±0.002	1.03±0.24	0.68±0.66	0.03±0.02
April 17	30.25±0.07	8.19±0.02	5.66±0.00	30.2±0.00	50.45±0.07	30.33±0.01	0.05±0.01	0.004±0.000	1.15±0.07	0.09±0.01	0.01±0.00

Appendix 2
*Water quality during the culture period of female TG (*Epinephelus fuscoguttatus*) broodstocks*

Month and Year	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
Mar 16	27.20±4.74	7.95±0.07	5.00±0.29	29.89±0.27	49.52±1.71	30.15±0.29	0.32±0.13	0.058±0.042	1.23±0.08	2.29±0.97	0.03±0.01
April 16	29.02±1.69	7.92±0.09	5.00±1.12	28.55±2.13	47.86±4.68	28.85±1.93	0.65±0.13	0.011±0.001	1.05±0.13	0.68±0.08	0.05±0.01
July 16	30.00±0.02	8.12±0.01	4.05±0.00	31.02±0.01	51.69±0.03	30.88±0.00	0.34±0.01	0.009±0.001	1.05±0.07	0.50±0.00	0.33±0.39
Aug 16	30.67±0.01	8.19±0.01	4.97±0.00	30.26±0.01	52.01±0.01	29.58±0.04	0.33±0.01	0.005±0.001	0.15±0.07	0.425±0.02	0.02±0.01
Sept 16	30.51±0.00	7.91±0.00	4.12±0.01	29.66±0.01	52.31±0.01	30.91±0.01	0.28±0.03	0.011±0.001	1.40±0.14	0.40±0.00	0.03±0.00
Oct 16	30.26±0.63	8.07±0.14	4.77±0.44	30.19±0.08	51.79±0.40	35.38±11.01	0.34±0.06	0.003±0.002	0.30±0.18	0.31±0.15	0.03±0.01
Nov 16	29.72±0.08	8.00±0.33	4.09±0.03	30.91±0.01	52.00±1.32	30.68±0.62	0.18±0.01	0.008±0.004	1.10±0.14	1.75±0.21	0.04±0.01
Dec 16	30.56±0.01	8.02±0.01	4.68±0.01	30.12±0.01	51.45±0.01	30.22±0.02	0.3±0.00	0.004±0.000	0.30±0.00	0.41±0.01	0.01±0.00
Jan 17	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.99±0.01	29.90±0.01	0.32±0.04	0.005±0.000	1.10±0.00	1.05±0.07	0.045±0.01
Feb 17	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.24±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
Mar 17	29.89±0.65	8.12±0.03	4.91±0.67	29.58±0.66	51.03±0.19	29.55±0.69	0.06±0.02	0.003±0.002	1.03±0.24	0.68±0.66	0.03±0.02
April 17	30.25±0.07	8.19±0.02	5.66±0.00	30.2±0.00	50.45±0.07	30.33±0.01	0.05±0.01	0.004±0.000	1.15±0.07	0.09±0.01	0.01±0.00

Appendix 3

Water quality during the culture period of GG+ × TG- fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	27.48±0.00	7.90±0.01	5.24±0.03	26.81±0.01	43.86±0.01	27.22±0.01	0.02±0.00	0.012±0.001	0.90±0.00	0.50±0.01	0.06±0.00
10	27.43±0.00	7.85±0.00	4.71±0.01	26.76±0.01	43.76±0.02	27.19±0.01	0.02±0.00	0.008±0.001	0.95±0.21	0.26±0.00	0.05±0.01
20	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.01±0.00	0.001±0.000	1.50±0.00	0.22±0.01	0.08±0.00
30	28.43±0.00	8.04±0.01	5.13±0.03	28.65±0.01	47.39±0.00	28.90±0.02	0.01±0.00	0.003±0.001	0.95±0.21	0.14±0.01	0.01±0.01
40	30.19±0.49	8.26±0.01	4.78±0.02	31.04±0.05	53.05±0.01	31.04±0.08	0.09±0.00	0.005±0.001	1.50±0.14	0.25±0.01	0.05±0.00
60	30.20±0.01	7.99±0.01	4.79±0.12	29.71±0.01	50.63±0.04	29.93±0.01	0.18±0.01	0.007±0.001	1.30±0.00	0.54±0.01	0.03±0.01
90	31.10±0.23	8.22±0.01	3.88±0.04	30.91±0.01	53.52±0.00	31.02±0.11	0.38±0.09	0.005±0.001	1.10±0.14	0.21±0.00	0.08±0.01
120	28.74±0.00	8.25±0.00	4.35±0.00	29.01±0.01	48.23±0.01	30.12±0.01	0.38±0.01	0.005±0.000	1.55±0.07	0.20±0.01	0.05±0.00

Appendix 4

Water quality during the culture period of GG+ × TG+ fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	27.55±0.00	7.84±0.01	5.97±0.11	26.70±0.06	43.81±0.09	27.18±0.04	0.05±0.01	0.010±0.001	1.00±0.14	0.46±0.01	0.05±0.01
10	27.57±0.01	7.86±0.01	3.72±0.05	26.78±0.00	43.90±0.00	27.24±0.00	0.04±0.00	0.005±0.000	0.95±0.07	0.42±0.01	0.04±0.01
20	28.70±0.01	8.23±0.00	4.66±0.01	28.68±0.00	47.59±0.02	28.91±0.02	0.06±0.01	0.002±0.001	1.10±0.00	0.15±0.03	0.02±0.01
30	28.74±0.01	8.24±0.01	4.37±0.01	28.98±0.01	48.24±0.01	29.24±0.00	0.02±0.00	0.006±0.001	1.65±0.21	0.20±0.01	0.05±0.00
40	30.31±0.00	8.00±0.02	4.83±0.11	29.72±0.00	50.65±0.00	29.98±0.05	0.06±0.01	0.005±0.001	1.25±0.07	0.65±0.01	0.02±0.00
60	30.01±0.01	7.74±0.01	4.81±0.01	30.03±0.04	50.57±0.71	30.24±0.01	0.26±0.04	0.011±0.001	1.25±0.07	0.15±0.01	0.05±0.02
90	30.48±0.00	8.00±0.02	4.02±0.01	30.40±0.02	51.92±0.02	30.52±0.03	0.55±0.02	0.005±0.000	1.10±0.14	0.13±0.01	0.05±0.00
120	30.00±0.00	7.34±0.01	4.03±0.01	31.63±0.05	44.56±0.00	29.89±0.01	0.24±0.01	0.005±0.001	0.95±0.07	0.09±0.00	0.04±0.01

Appendix 5

Water quality during the culture period of GG- × TG- fry

Days Post Hatch	Temperature (°C)	pH (1-14)	Dissolved Oxygen (mg/l)	Salinity (ppt)	Conductivity (uS/cm)	Dissolved Particles (mg/l)	Ammonia (mg/l)	Nitrite (mg/l)	Nitrate (mg/l)	Phosphate (mg/l)	Iron (mg/l)
5	28.71±0.01	8.36±0.04	5.17±0.00	28.64±0.01	47.66±0.02	28.91±0.00	0.03±0.01	0.001±0.000	1.50±0.00	0.39±0.01	0.08±0.00
10	29.95±0.01	7.37±0.06	4.37±0.09	31.42±0.04	52.98±0.01	29.90±0.01	0.01±0.00	0.005±0.000	1.10±0.00	0.25±0.02	0.05±0.01
20	29.36±0.00	7.24±0.01	5.24±0.04	30.85±0.00	52.94±0.01	28.92±0.00	0.05±0.00	0.003±0.000	1.15±0.07	0.11±0.01	0.01±0.00
30	31.06±0.01	7.89±0.01	4.89±0.02	30.77±0.01	51.15±0.00	28.90±0.02	0.02±0.00	0.001±0.000	1.05±0.07	0.31±0.01	0.03±0.01
40	30.25±0.01	7.58±0.01	5.26±0.00	30.11±0.01	50.57±0.07	29.72±0.00	0.04±0.00	0.005±0.001	1.45±0.07	0.35±0.01	0.03±0.02
60	30.21±0.01	7.43±0.01	4.79±0.01	30.27±0.01	46.66±0.00	26.70±0.06	0.02±0.01	0.002±0.001	1.20±0.00	0.17±0.01	0.01±0.00
90	31.45±0.01	7.25±0.00	4.58±0.02	30.85±0.01	50.10±0.00	29.24±0.00	0.02±0.00	0.004±0.000	1.00±0.00	0.32±0.01	0.04±0.01
120	31.13±0.03	7.44±0.00	4.93±0.02	31.02±0.00	48.45±0.01	30.02±0.00	0.01±0.00	0.001±0.000	1.30±0.14	0.19±0.00	0.01±0.00