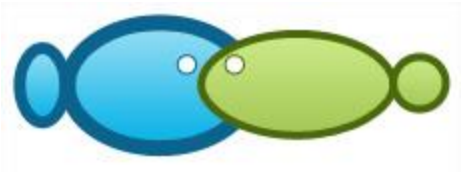

Occurrence of *Vibrio* spp. and viral nervous necrosis (VNN) in cultured hybrid grouper *Epinephelus fuscoguttatus* ♀ x *E. lanceolatus* ♂ and wild fish at Seribu Islands, Indonesia

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Abstract. This study aimed to identify the occurrence of *Vibrio* sp. and viral nervous necrosis (VNN) in hybrid grouper and wild fish in a floating-net cage system at Kelapa Dua Island, Seribu Islands, Jakarta. This study was performed from February 2021 to January 2022. The samples contained five cultured fish and five wild fish observed for 12 months with a purposive-sampling method. *Vibrio* spp. bacteria were isolated from the fish liver and kidney, while *the* VNN virus was isolated from the fish brain and eyes. The average total abundance level of *Vibrio* spp. bacteria (Log 10 CFU/ml) in cultured fish and wild fish was at the highest value in February, while the lowest abundance level in wild fish was found in March. Based on the identification results, bacteria found in the isolated organs were *Vibrio alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*. From the detection results, VNN was found in cultured fish in March and April at 80%, while in wild fish in March and November at 60%. Therefore, *Vibrio* spp. and VNN that attack both cultured and wild fish indicate cross-infection or a disease distribution from the cultured fish to wild fish, and vice versa.
Key Words: cage, disease, grouper.

Introduction. Indonesia is the main coral fish producer traded for consumption, specifically grouper (Khasanah et al 2020). The world's fish consumption per capita is estimated at 19.6 kg per year and will be predicted by 2030 at 22.5 tons per year (KKP 2019). FAO (2020) also predicts that the contribution of aquaculture to world fisheries by 2030 is expected to reach 58%. Prospects for aquaculture can grow and increase significantly with high economic value both in domestic and export markets in Asia, especially Hong Kong, China, Taiwan, and Singapore (Rochmad & Mukti 2020). However, a challenge, namely disease attack, emerged due to the low susceptibility of fish against pathogenic microorganisms, such as the viral nervous necrosis (VNN) virus and *Vibrio* spp. bacteria (Novriadi et al 2014; Assefa & Abunna 2018; Huang & Nitin 2019).

Seribu Islands are part of the Jakarta region located outside Jakarta Bay and has high potential of grouper fish culture development (Ghani et al 2015). This water area is ocean-geographically susceptible to pollution threats, as the location is directly connected to Jakarta Bay, downstream from 13 rivers that flow through densely populated and industrialized Jakarta City (Sachoemar 2008). In addition, pathogenic microorganisms and environmental degradation quality become the main inhibiting factors in aquaculture sustainable production.

Good fish health management can have a great impact on sustainable aquaculture production achievement with qualified broodstock, seeds, feed management, water quality management, culture waste management, biosecurity, traceability, and fish health management (Leaño 2019). To support sustainable aquaculture, good fish health

management at the cultivator level and optimal result maintenance at the end of the production cycle must be controlled.

The grouper culture technology at Seribu Islands can be conducted using the floating-net cage system. Commonly, there are three segmentations in grouper production, namely breeding (5-7 cm fingerling production), nursery (9-13 cm seed production), and growing out (consume size production). Nursery activity is part of rearing the fish fingerlings to reach eligible size for growing-out culture in the floating-net cage (Rochmad & Mukti 2020; KKP 2019). Based on various problems and descriptions above, this study aimed to identify the existence of *Vibrio* spp. bacteria, and virus nervous necrosis (VNN) virus in cultured fish (*Epinephelus fuscoguttatus* ♀ x *E. lanceolatus* ♂ hybrid) and wild fish at the floating-net cage area in Kelapa Dua Island, Seribu Islands, Jakarta.

Material and Method

Period and location. This study was performed from February 2021 to January 2022. Sampling was performed on a floating-net cage in Kelapa Dua Island, Seribu Islands, Jakarta (Figure 1).

Figure 1. Sampling location of hybrid grouper fish and wild fish in the floating-net cage area (Depicted from Google Earth and BIG 2021).

Fish sampling. Hybrid grouper (*Epinephelus fuscoguttatus* ♀ x *E. lanceolatus* ♂) sampling was performed by a purposive sampling method, based on the bacterial and VNN clinical signs in the floating-net cage. The clinical signs of bacterial disease were appetite loss, discolored skin, red and necrotic skin, and blisters on the body surface, which caused an open wound. Erythema has also occurred around the fin and mouth. The VNN virus clinical signs were abnormal swimming, benthic swimming, surface floating, swim-bladder swelling, and appetite loss. Infected grouper displays a nerve disruption related to nervous and retinal vacuolization (Novriadi et al 2015).

Five cultured fish (hybrid grouper) from the floating net cages and five wild fish (*Sphaeramia orbicularis*) from around the net cages were sampled every month for 12 months of observation and transported in a plastic bag filled with water and oxygen at a 30:70 ratio. Transportation was performed by sea transport and vehicle protected from direct sunlight. Moreover, sample analysis was performed in the Laboratory of Fish Health, Reproduction, and Genetics, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University.

Vibrio spp. isolation and identification. Isolation was performed using grouper liver and kidney at 1 g by crushing them in physiological solution (NaCl 0.85%), before serially diluting. The suspension from the 10^{-6} dilution was taken at 0.1 mL, spread twice on Thiosulphate Citrate Bile Sucrose (TCBS) agar, and incubated for 24 hours at 28°C. Different colonies were identified morphologically based on shapes and colors (Royan et al 2014). Isolates were re-isolated for purification and cultured in sea water complete

(SWC) slant agar media routinely. Based on biochemical characteristics, isolates were identified through biochemical tests, namely Gram staining, catalase, oxidase, oxidative/fermentative, and motility, followed by kit API 20 NE (Biomeureux ®). These test results were matched with bacterial characteristics based on Bergey's Manual of Determinative Bacteriology (Bergey & Holt 1994).

Polymerase chain reaction (PCR)

Sample preparation. Tissue samples from target organs of five hybrid grouper and five wild fish, namely eyes and brains, were analyzed based on the existence of Nodaviridae as a VNN causative agent in GENEzol™ (Geneaid, Taiwan) solution. The sample was then preserved in a polystyrene box to prevent contamination.

RNA extraction. Using GENEzol™ (Geneaid, Taiwan) solution, the 30-50 mg organ samples were lysed with 1 mL GENEzol™ reagent and incubated at room temperature for 5 minutes, before centrifugation at 12,000 rpm for 20 minutes at 4°C. The supernatant was taken and 200 µL of chloroform was added, then incubated at 4°C for 20 minutes and centrifuged at 12,000 rpm for 15 minutes at 4°C. A pellet as an RNA sample was taken and isopropanol was added at a 1:1 ratio, and incubated at 4°C. The supernatant was removed, and the pellet was then washed with ethanol 70% at 1 mL and centrifuged at 12,000 rpm for 15 minutes at 4°C. Furthermore, the supernatant was removed and air-dried. In the RNA sample 150 mL of TE (Tris-EDTA) was added for 50 mg of tissue (Manan & Fitriatin 2015).

Reverse transcriptase-polymerase chain reaction (RT-PCR). VNN detection with conventional PCR method procedure has been previously reported by Novriadi et al (2015). This analysis was performed with polymerase-chain-reaction (PCR) with specific primers, namely VNN 1, forward: 5'-ACGCAAAGGTGAGAAGAAA-3', reverse: 5'-GTCCCAGATGCCCCA-3', and VNN 2, forward: 5'-AACTGACAACGACCACACCTT-3', reverse: 5'-TGTGGAAAGGGAATCGTTG-3. Detailed temperature and time cycles for PCR VNN phase 1 amplification comprised: initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55.5°C for 45 seconds. PCR VNN phase 2 comprised: initial denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55.5°C for 15 seconds, extension at 72°C for 20 seconds, and 25 cycles of final extension at 72°C for 3 minutes. The PCR product obtained was 5 µL.

Nested-PCR. VNN primers to detect VNN and β-actin gene primers as internal sample control were used in this procedure. VNN amplification was performed with the nested-PCR method to avoid a false-negative condition. Each reaction was supported by non-template control (NTC) to avoid a false-positive condition. After being processed in a nested PCR, the VNN DNA band was formed at 313 bp. The primers used coat protein gene-specific primer from GVNN (grouper VNN).

Electrophoresis. The amplification results of conventional PCR were visualized through the gel electrophoresis method with 1.5% agarose gel and electrophoresis machine (Muppid-2 Plus/Submarine Type electrophoresis system advance) in 1X TAE-buffer, colored with ethidium bromide, observed in a UV-transilluminator, documented by gel camera.

Data analysis. Total *Vibrio* sp. abundance was tested with its significant variance (ANOVA) to testify the hypotheses, namely H0: no difference in parameter data for 12 months; H1: a difference was found in parameter data for 12 months. If the results show a Sig value < 0.05 (α = 5%), then H0 is rejected and H1 is accepted, while if the Sig value is ≥ 0.05 (α = 5%), then H0 is accepted and H1 is rejected. Tukey test was applied for further statistical analysis. Descriptively, the existence of VNN was calculated based on the prevalence formula, namely:

$$\text{Prevalence} = \frac{\text{Infected fish samples}}{\text{Total fish samples}} \times 100$$

Results

Clinical signs. Hybrid grouper fish in floating-net cages generally present clinical signs of *Vibrio* spp. and VNN, as shown in Table 1.

Table 1

Clinical signs of infected hybrid grouper with *Vibrio* spp. and *VNN* virus

Hybrid grouper fish	*Clinical signs*
Vibrio spp.	Behavior changes, such as slow movement, imbalance condition, and surface-swimming.
VNN virus	Abnormal swimming movement, several fish sinking at the net cage bottom and then floated to the water surface, darker body color, and appetite loss.

Morphological change in the grouper body and infection symptoms were suspected due to *Vibrio* spp. bacteria and VNN virus attack, based on pigmentation change in the fish body, followed by head and abdomen lesions, body and caudal fin hemorrhage (Figure 2).

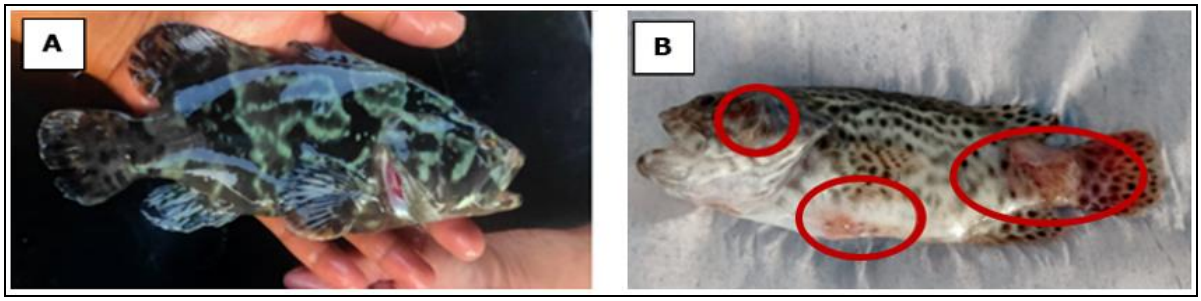

Figure 2. (A) Normal/healthy fish. Fish with (B) lesions on the head, abdomen, and outside body (hemorrhage), and caudal fin lesion.

Total Vibrio spp. abundance. The analysis of variance (ANOVA) on the average of total bacterial abundance (Log 10 CFU/mL) in hybrid grouper fish and wild fish for 12 months is presented in Figure 3.

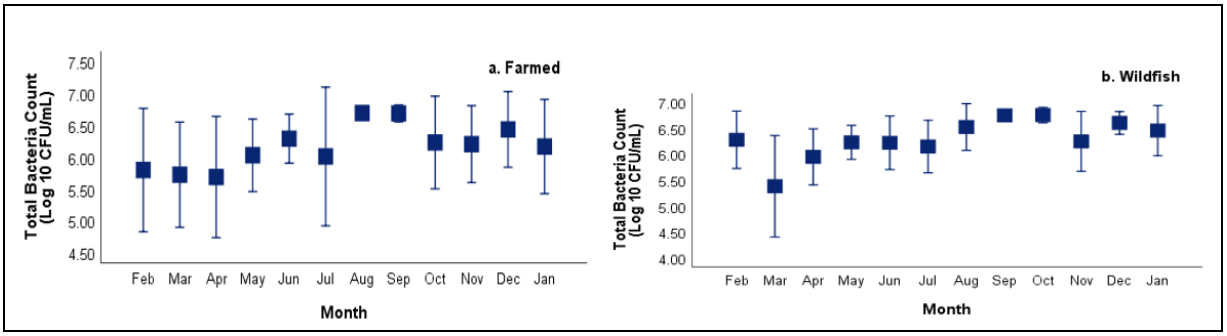

Figure 3. Total bacteria abundance during study (February 2021 – January 2022).

The ANOVA test results of total bacterial abundance in hybrid grouper fish obtained a Sig value of $p>0.05$, which indicates that the H0 is accepted and H1 is rejected, thus no significant difference was found in the total bacterial abundance in cultured fish (hybrid grouper fish) for 12 months. In Figure 3a, the highest value of total bacterial abundance

is present in August, while the lowest is found in April. Meanwhile, the ANOVA results indicate the total bacterial abundance in wild fish at a Sig value of ($p<0.05$), which indicates that the H0 is rejected and H1 is accepted. Furthermore, the Tukey test was performed to determine which month obtained a significant difference in total bacterial abundance in wild fish. In Figure 3b, the highest abundance level of bacteria in wild fish is presented in October, while the lowest is found in March. Meanwhile, a significantly different value of total bacterial abundance is obtained from October, September, December, August, January, February, and March.

Detailed ANOVA results of total *Vibrio* spp. (Log 10 CFU/mL) in hybrid grouper fish and wild fish for 12 months in the floating-net cage are shown in Figure 4.

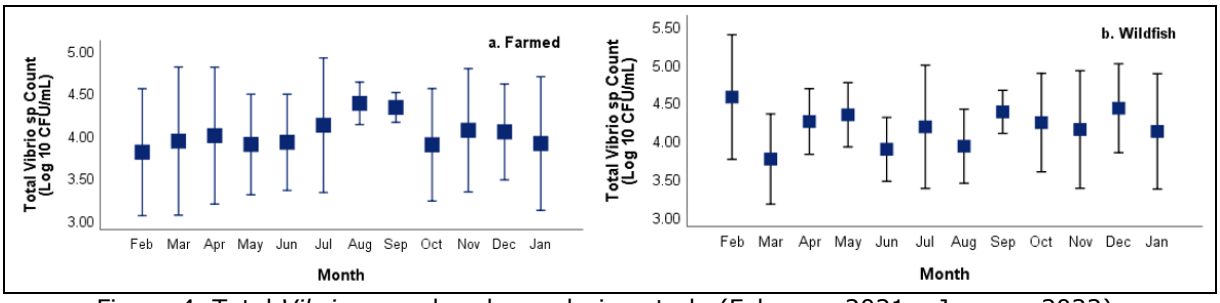

Figure 4. Total *Vibrio* spp. abundance during study (February 2021 – January 2022).

The ANOVA test results on total *Vibrio* spp. in cultured fish (hybrid grouper fish) and wild fish obtained a Sig value of $p>0.05$, which means that H0 is accepted and H1 is rejected, thus there is no significant difference. In Figure 4a, the greatest number of total bacteria is found in August, while the lowest is shown in February. In Figure 4b, the greatest number of total bacteria is found in February, while the lowest is shown in March.

Isolation and identification of *Vibrio* spp. on TCBS agar media were also identified using Kit API 20NE biochemical tests. The physiological and biochemical test results on *Vibrio* spp. isolates are presented in Table 2, while Table 3 shows the monthly identification results.

Table 2

Characteristics of *Vibrio* sp. isolated during study and API 20 NE kit identification

No	*Genus/Bacteria group*	*Isolate*			
		1	*2*	*3*	*4*
1	Cell shape	Rod	Rod	Rod	Bent-Rod
2	Gram	-	-	-	-
3	Catalase	+	+	+	+
4	Oxidase	+	+	+	+
5	O/F	F	F	F	F
Tests	*Active ingredients*	*Isolate*			
		1	*2*	*3*	*4*
KNO_3	potassium nitrate	+	+	+	+
TRP	L-tryptophan	+	+	+	-
GLU	D-glucose fermentation	+	+	+	+
ADH	L-arginine	-	-	-	+
URE	urea	-	-	-	+
ESC	gelatin	+	-	+	-
GEL	(ovine origin)	+	+	+	-
PNG	4-nitrophenyl-ßD-galactopyranoside	-	+	+	-
GLU	D-glucose oxidation	+	+	-	+

ARA	L-arabinose	-	+	-	+
MNE	D-mannose	-	+	-	+
MAN	D-mannitol	+	+	-	+
NAG	N-acetyl-glucosamine	-	+	-	+
MAL	D-maltose	-	+	-	-
GNT	potassium gluconate	+	-	-	-
CAP	capric acid	-	-	-	-
ADI	adipic acid	-	-	-	+
MLT	malic acid	+	+	+	-
CIT	trisodium citrate	-	+	-	-
PAC	phenylacetic acid	-	-	-	-
OX	(see oxidase test package insert)	+	+	+	+
	Code	7454644	7077345	7430044	5347124
	% ID	99.90%	99.20%	99.80%	99.90%
	Species	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*	*V. cholerae*

Note: O/F - fermentation-oxidation; KNO_3 - potassium nitrate; TRP – L-tryptophane; GLU - D-glucose; ADH – L-arginie; URE – urea; ESC – Gelatin; GEL – ovine origin; PNG – 4-nitrophenyl-ßD-galactopyranoside; ARA – L-arabinose; MNE – D-mannose; MAN – D-mannitol; NAG – N-acetyl-glucosamine; MAL – D-maltose; GNT – Potasium gluconate; CAP – capric acid; ADI – adipic acid; MLT – malic acid; CIT – trisodium citrate; PAC – phenylacetic acid; OX – see oxidase test package insert.

Table 3

Vibrio sp. isolated during study

Month	*Species*			
	V. alginolyticus	*V. parahaemolyticus*	*V. vulnificus*	*V. cholerae*
February	√	√	-	-
March	√	√	√	-
April	√	√	-	-
May	-	√	√	√
June	√	√		-
July	√	√	√	-
August	√	-	√	-
September	√	-	√	-
October	-	√	√	√
November	√	-	-	√
December	√	√	-	-
January	-	√	√	√

PCR (Polymerase Chain Reaction). Based on the VNN detection in fish samples, Table 4 presents the infection results as positive (+) and negative (-). Meanwhile, the analysis results of PCR from the electrophoresis method can be seen in Figure 5.

Table 4

PCR detection on the occurrence of VNN in hybrid grouper and wild fish

Month	Type of diseases	Conventional PCR examination results					
		Number of fish	Farmed (%)		Number of fish	Wild (%)	
			+	-		+	-
February	Viral Nervous Necrosis (VNN)	5	60 (3)	40 (2)	5	20 (1)	80 (4)
March		5	80 (4)	20 (1)	5	60 (3)	40 (2)
April		5	80 (4)	20 (1)	5	0	0
May		5	0	0	5	40 (2)	60 (3)
June		5	0	0	5	0	0
July		5	20 (1)	80 (4)	5	0	0
August		5	60 (3)	40 (2)	5	0	0
September		5	20 (1)	80 (4)	5	0	0
October		5	0	0	5	0	0
November		5	40 (2)	60 (3)	5	60 (3)	40 (2)
December		5	0	0	5	0	0
January		5	60 (3)	40 (2)	5	20 (1)	80 (4)

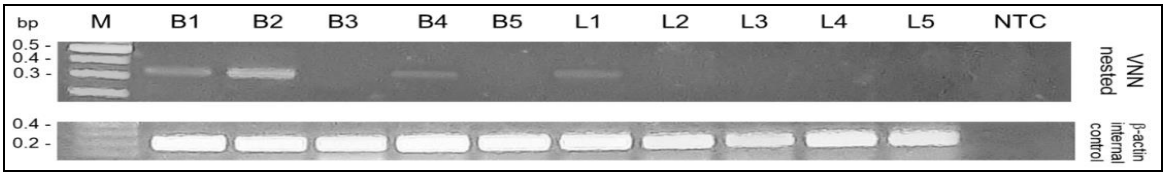

Figure 5. Nested-PCR results on the detection of VNN in fish samples Trip 1. M=DNA marker, B1-B5 = cultured fish/hybrid grouper, L1-L5 = Wild fish, NTC = no-template control.

The prevalence of VNN virus disease in cultured fish (hybrid grouper) and wild fish were observed in February 2021 – January 2022. The results of VNN virus detection from the floating-net cage and prevalence percentage each month are different, namely 60% of cultured fish and 20% of wild fish suffered from VNN in February; 80% of cultured fish and 60% of wild fish suffered from VNN in March; 80% of cultured fish and 0% of wild fish suffered from VNN in April; 0% of cultured fish and 40% of wild fish suffered from VNN in May; 0% of cultured and wild fish suffered from VNN in June; 20% of cultured fish and 0% of wild fish suffered from VNN in July; 60% of cultured fish and 0% of wild fish suffered from VNN in August; 20% of cultured fish and 0% of wild fish suffered from VNN in September; 0% of cultured and wild fish suffered from VNN in October; 40% of cultured fish and 60% of wild fish suffered from VNN in November; 0% of cultured and wild fish suffered from VNN in December; 60% of cultured fish and 20% of wild fish suffered from VNN in January.

Discussion. Clinical symptoms of the *Vibrio* spp. bacterial disease in grouper are observed from changes in behavior and morphology. Behavior changes contain slow movements, imbalance condition, and surface-swimming, while morphological changes contain pale body pigmentation, head and abdomen lesions, body hemorrhage, and caudal fin lesions (Figure 2). Similar clinical symptoms were reported (Dahlia et al 2019; Hastari et al 2014; Nitimulyo et al 2005), that hybrid grouper infected with *Vibrio* spp. bacteria presented several changes, namely disturbed balance, slow movements, fin rot, lesions in several parts of the body and ulcers.

Clinical symptoms of grouper viral disease caused by the VNN virus are abnormal swimming behavior, several fish sinking to the bottom of the net, then floating again to the water surface, darker body color, and appetite loss. This condition is influenced by stress factors, handling, and transportation systems, resulting in morphological changes

such as lesions on the head, abdomen, body, caudal fin, and gill hemorrhage. Similar research results were also reported (Hastari et al 2017; Shen et al 2017; Zhu et al 2018; Ben-Asher et al 2019; Mohamad et al 2019; Xiao et al 2019; Leaño 2019; Mahardika et al 2020; Nurlita et al 2020), that fish suffering from VNN has several behavioral and morphological changes, such as lesions and external organ hemorrhage.

Based on the analysis results of bacterial abundance, *Vibrio* spp. (Log 10 CFU/mL) in cultured fish and wild fish showed no significant difference in the average total bacterial abundance and total *Vibrio* spp. for 12 months ($p>0.05$). This was thought as *Vibrio* spp. bacteria were transferred from cultured fish to wild fish, and vice versa. Moreover, *Vibrio* spp. mass infection in other aquatic organisms can also be influenced by extreme weather changes, fish stress, and poor handling systems. Previous studies (Nitimulyo et al 2005; Novriadi et al 2014; Dahlia et al 2019; Huang & Nitin 2019; Mohamad et al 2019; Seniati et al 2019), reported that a high abundance of *Vibrio* spp. bacteria was caused by handling factors, transportation, environment, and climate change. In addition, the ANOVA and Tukey test results on total bacteria in wild fish obtained a significant difference ($p<0.05$) in October, September, December, August, January, February, and March. This study on the average total abundance of bacteria in wild fish suggests that the abundance of bacteria is caused by extreme weather changes and environmental changes, as similarly reported by Mahardika et al (2020) and Nitimulyo et al (2005).

Based on the plot in Figures 3 and 4, the average total bacteria and *Vibrio* spp. obtained the same pattern. This indicates the disease transfer from cultured fish to wild fish, which will have an impact on the sustainability of hybrid grouper aquaculture production. Similar conditions were also reported (Shen et al 2017; Dahlia et al 2019; Huang & Nitin 2019), that grouper fish can also be infected by *Vibrio* spp., as well as other aquatic organisms. The standard deviation shows that the abundance of *Vibrio* sp. bacteria varies, excepting the months of August and September. Meanwhile, the average total abundance of *Vibrio* spp. in wild fish obtained the highest level in February and the lowest level in March, whereas the standard deviation for *Vibrio* spp. is varied and uniform. The *Vibrio* sp. bacterium types based on the Kit API 20NE were identified as *Vibrio vulnificus, V. alginolyticus, V. parahaemolyticus,* and *V. cholerae* bacteria. A similar condition was also reported by Nitimulyo et al (2005). This condition will have an impact on the production of hybrid grouper culture.

In general, virus detection using the conventional PCR method indicates that cultured fish and wild fish in floating net cages are proven to be infected with the *VNN* virus, based on the emergence of the white band at 313 bp size for VNN (Figure 5). This condition follows other research (Novriadi et al 2015; Kurniawati et al 2019; Xiao et al 2019; Huang et al 2020; Nurlita et al 2020). The presence of the VNN virus in cultured fish reached 80% in March and April, while wild fish obtained a 60% prevalence level in March and November (Table 4). These results indicate that the pattern of the VNN virus from both cultured and wild fish is caused by various factors, such as transportation system, handling, fish size, rainfall, and extreme weather changes, resulting in fish stress, appetite loss, and decreased immunity level. If the VNN virus attack increase, fish will be vulnerable to death. The present study results were following the results of previous research (Cao et al 2018; Mahardika et al 2020; Novriadi et al 2015; Nurlita et al 2020), who mentioned that VNN is one of the important pathogens according to World Organisation for Animal Health (WOAH) (Fujita 2010), that has become an epidemic all over the world by killing grouper fish up to 100% in a short time. A trigger that causes this disease attack is an imbalance between carrying capacity and production quantity in the culture area, affecting fish, pathogens, and environment balance (Snieszko 1974). An effort that can be performed to avoid VNN transmission from the sick fish and virus distribution in the floating-net cage is safe and clean biosecurity application in the grouper fish culture environment.

Conclusions. Based on the analysis results of *Vibrio* spp. abundance and VNN virus detection, *Vibrio* spp. and VNN virus that infected both cultured hybrid fish and wild fish indicates a disease transfer from cultured to wild fish and vice versa.

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Conflict of interest. The authors declare that there is no conflict of interest.

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