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Bacteria associated with mass mortality of hybrid grouper *Epinephelus* sp. in East Java Province Indonesia

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Abstract
The purpose of this study was to determine the cause of the mass mortalities of hybrid grouper *Epinephelus* sp. Infection by various diseases causes a huge loses in Indonesian aquaculture, especially in larval stage, because infected fish live only 12 hours post infection. Analysis of cause for the mortality involves 3 species of bacteria that infect fish, *Vibrio alginolitycus, Vibrio harveyi* and *Sreptococcus iniae*. Blood glucose in stress fish elevated to 88-151 mg/dL (control) and 44-65 mg/dL (moribund fish). The phagocytic rate decreased to 32-33 (control) and 21-23 (moribund fish).

Keywords: *Epinephelus* sp, blood glucose, *Vibrio alginolitycus, Vibrio harveyi, Streptococcus iniae,* phagocytosis

1. Introduction
Hybrid grouper *Epinephelus* sp. is cross breeding between *Epinephelus lanceolatus* and *Epinephelus fuscogutatus* [1]. For breeding it is expected that the fish grow very fast and resistant to various diseases. Giant grouper *(Epinephelus lanceolatus),* also known as brindlebass, cod, or grouper as in Queensland, Australia, is the largest bony fish found in coral reefs. Fish was found in near of surface to a depth of 100 m (330 ft.) in reefs throughout the Indo-Pacific region, except Persian Gulf. These fish also live in the estuary reaches up to 2.7 m (8.9 ft) in length and 400 kg [2].
In Indonesia it is known that two types of Vibrio bacteria infect groupers, they are *Vibrio alginolyticus* and *Vibrio anguillarum* [3], while those infect shrimp are *Vibrio harveyi* and *Vibrio splendidus*, similar to those seen in the Philippines [4]. In Malaysia vibriosis is caused by bacteria *V. alginolyticus* and *V. parahaemolyticus* [5], while in Japan the *Vibrio sp*. reportedly attacked prawns *P. japonicas* [6]. In China vibriosis found in shrimp larvae is mainly caused by *V. alginolyticus* and *V. parahaemolyticus* [7]. The transportation of live fishes from breeding to farm is very stressful and may lead to some physiological changes such as elevation of blood glucose level [8]. Mass mortality may occur after the transportation. This research was conducted due to high mortality of grouper *Epinephelus fuscogutatus* and survival rate of only about 2%. This study aimed to find the cause of death in grouper hybrid.

2. Materials and Methods
2.1 Isolation and culture of bacteria
Samples were taken from dying fish, then washed with tap water to clean the mud. The kidney was taken and clean fish were then dissected into small part. The sterilized kidney were aseptically minced, vortexed with 0.8% saline solution and quartz sand, and then decimally diluted in 0.8% saline solution. Sterility check test was done to insure that the strains were isolated from fish.

2.2 Phenotyping characterization of the isolates
The isolates of bacteria were phenotypically characterized as described by McFadding [9] using the following tests: Gram staining, cytochrome oxidase, motility, oxidation and fermentation of glucose, ornithine and lysine decarboxylase, amylase, gelatinase, lipase production, etc.

2.3 Blood sugar
Blood was drawn from the caudal peduncle and stored in a test tube. Previously the test tube was poured with anticoagulant to prevent blood to clot. Blood sugar was measured by Glucosure Star (Apex Biotechology Corp., Taiwan). The blood that has been taken from the fish was then examined for sugar content and the result was read from the device in mg/dL.

2.4 Isolation of macrophage
Grouper were injected intraperitoneally with 0.1 ml glycogen to stimulate macrophage production. After 24 hours the fish were anesthetized with MS-222 and blood was taken from the base of the tail so that the macrophage was not contaminated with red blood. One milliliters of medium Leibovitz (L-15) containing 2% Fetal Bovine Serum (FBS) and 10 units/ml heparin was injected into the grouper intraperitoneally. Fish maw was sorted-sequence and then the macrophages were harvested by vacuuming with a syringe. Remaining macrophages were then washed with 0.5 ml more L-15. Macrophage cells were then centrifuged at 400xg for 10 minutes and dissolved in L-15. Viable cells (living cells) seen and counted with trypan blue staining, then the cell density was calculated 2-5x106 cells/ml wear haemocytometer.

2.5 Phagocytosis
Bacteria and macrophage (dying fish and control) were mixed and then incubated at 25 ˚C for 30 minutes with shaking at 25 rpm to create contact between macrophages and bacteria. Macrophages were removed and smeared on glass slides that had been coated with albumin and stained with May-Grunwald Giemsa, and then phagocytosis was calculated.

3. Results and Discussion
It is well known that two types of *Vibrio* bacteria infect groupers. Those bacteria are *V alginolyticus* and *V. anguillarum* [3], while those infecting shrimp are *Vibrio harvey*i and *Vibrio splendidus*. In Southeast Asian ccountries those bacteria were found in the Philippines [4]. In Malaysia vibriosis was reported by Anderson [5], while in Japan *Vibrio* sp. reportedly infected prawns *P. japonicas* [6]. In China vibriosis was found in shrimp larvae, mainly caused by *V. alginolyticus* and *V. parahaemolyticus* [7]. Infected fish cannot survive more than 12 hours and in the case of *C. altivellis* survived larvae are no more than 3 percent. This disease is a big problem in grouper farming in Indonesia and is still difficult to overcome by the farmer. All the results of the identification of Pond No.1 (Table 1) and Pond No 2 (Table 2), all the physical and chemical properties are presented in Table 3.

Table 1: Identified bacteria from fish pond no 1

Pond No 1

No of Fish	Bacteria	No of Fish	Bacteria
1	*V.alginolitycus*	11	*Sreptococcus iniae*
2	*V.alginolitycus*	12	*S. iniae*
3	*V. harveyi*	13	*V. alginolitycus*
4	*V. harveyi*	14	-
5	*V. harveyi*	15	*V. alginolitycus*
6	*S. iniae*	16	*S. iniae*
7	*V.alginolitycus*	17	*S. Iniae*
8	*V.alginolitycus*	18	*S. iniae*
9	*V. harveyi*	19	*V. alginolitycus*
10	*V. harveyi*	20	*V. alginolitycus*

Table 2: Identified bacteria from fishes Pond no 2

Pond No 2

No of Fish	Bacteria	No of Fish	Bacteria
1	*V. alginolitycus*	11	*V. harveyi*
2	-	12	*V. alginolitycus*
3	.*V.alginolitycus*	13	*V. harveyi*
4	*V.alginolitycus*	14	*S. iniae*
5	*S. iniae*	15	-
6	*S. iniae*	16	*S. iniae*
7	*V.alginolitycus*	17	*V. alginolitycus*
8	*V. harveyi*	18	*S. iniae*
9	*V. harveyi*	19	*S. iniae*
10	*V. harveyi*	20	*V. harveyi*

Three bacteria found infecting hybrid grouper were *V. alginolyticus, V. harveyi* and *S. iniae* (Table 3). This is the first report of the disease that occurred in hybrid grouper. On seeds transport, the death rate is even higher if it is not carefully handled due to the fluctuation of dissolved oxygen in tropical area. In some cases, we put ice to cold water to prevent acute stress of fish. All vibrio diseases are well known, except *S. iniae* so that it can be said that infection of grouper hybrid is found now in Indonesia and this is the first report [3].

Table 3: The characteristics of isolated bacteria

Characteristics		*V. alginolyticus*	*V. harveyi*	*S. iniae*
Morphology at TSA 2%	Color	Yellow	Yellow	Yellow
	Shape	Swarming	Swarming	Swarming
	Edge	Flat	Flat	Flat
	Elevation	Flat	Flat	Raised
Gram test		-	-	+
Morphology		Rod	Rod	Coccus
Biochemical test	Oxidase	+	+	-
	Katalase	+	+	-
	O/F	F	F	F
	TSIA – H_2S	+	+	-
	LIA	+	+	-
	Motile	+	+	-
	Indole	+	+	-
	Ornithine	+	+	-
	MR	-	-	+
	VP	+	+	-
	Simmon Citrate	-	-	+
	Novobiocin	+	+	+
	Gelatin	+	+	+
Glucose test	Glucose	+	+	+
	Maltose	-	-	+
	Sucrose	+	+	+
	Arbinose	-	-	-
	Manitol	-	+	+
	Inositol	-	-	-
	Swarming	+	-	
	Pigment		-	
	Arginine	_	-	
	Growth 4 ºC	-	-	
	Growth 30 ºC	+	+	
	Growth 35 ºC	+	+	
	Growth 40 ºC	+	+	
	Na + required	+	+	

Observation of water quality at the sampling time actually showed nothing unusual (Table 4). Because there is no contamination in pond in time of sampling, the water is suitable for fish.

Table 4: Water quality in the time of sampling

Time	No Pond	Temp ºC	pH	DO	Salinity	Ammonia
Morning	1	30	8,25	5,5	32	0,003
	2	28	8,11	5,3	32	0,031
Afternoon	1	28,1	8,23	5,5	31	0,009
	2	31	8,8	5,6	31	0,047
Evening	1	30	8,39	4,8	32	<0,001
	2	31	8,,4	5,7	32	<0,001

Physiological blood sugar reflects the state of the fish (Table 5). In control fish average blood sugar value was 124 mg/dL, but in dying fish the value was 55.6mg/dL, indicating that hypoglycemia occurred in those fish. The range of blood glucose level in non- transported common carp was recorded to be 19.0-49.6mg/dL and in post-transported was 20-126 ml/dL [9]. The use of blood sugar as an indicator of environmental stress [11] is essential to know what other factors of physiological stress may influence of concentration of sugar in blood.

Table 5: Blood glucose of moribund and control fish

Treatment of Fish	Number of Fish		
	1	2	3
Control	88	133	151
Moribund Fish	44	58	65

Low blood sugar may be associated with lower phagocytosis activity in macrophages (Table 6), whether the fish is in short of energy for activity needs further research. Blood sugar levels of dying fish are very low, this condition due to stress for long time. Physiologically infected fish rarely move, and laid the bottom of the tank due to lack of energy.

Table 6: Number of phagocytosis by macrophage

Treatment of Fish	Number of Fish		
	1	2	3
Control	32	34	33
Moribund Fish	21	33	23

4. Conclusion

We concluded that mass mortality of grouper is caused by *V alginolitycus, V harveyi,* and *S iniae.* The content of blood glucose in stress fish elevated to 88-151mg/dL control) and 44-65 mg/dL (moribund fish) and phagocytic rate decreased to 32-33 (control) and 21-23 (moribund fish). The viral nervous necrosis (VNN) was not found in this experiment.

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