

EFFECTIVITY OF INACTIVE GSDIV (GROUPEE SLEEPY DISEASE IRIDOVIRUS) VACCINE IN GROUPEE FISH (*Cromileptes altivelis* and *Epinephelus fuscoguttatus*) AGAINST GSDIV INFECTION

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ABSTRACT

Grouper sleepy disease iridovirus (GSDIV) has been known as viral agent causing mass mortality and significant economic losses in Indonesian aquaculture industry. The aim of this research was to know the effectivity of formalin-inactivated GSDIV vaccine to prevent GSDIV infection in humpback grouper (*Cromileptes altivelis*) and tiger grouper (*Epinephelus fuscoguttatus*). The vaccine was derived from GF cells infected-GSDIV which activated using formalin. Used vaccine was contained titer virus of $10^{2.80}$ - $10^{5.80}$ TCID₅₀/mL. Result of vaccination test indicated that a vaccinated grouper fish with formalin-inactive GSDIV vaccine should decreased mortality rate of 5%-85.4% in humpback grouper and tiger grouper that infected with GSDIV. Histopathologically, infected fish from vaccinated and control groups showed formation of enlarged cells as well as necrotized cells especially in spleen and kidney tissues. On the other hand, surviving fish from both vaccinated and control groups did not contained formation of enlarged cells in spleen, kidney, liver, and stomach.

KEYWORDS: GSDIV, cultured cells, CPE, inactive GSDIV vaccine, grouper

INTRODUCTION

Iridovirus belonging to the family *Iridoviridae* is one of viruses causing morbidity and mortality among grouper fish *Epinephelus* sp. Iridovirus infecting orange spotted grouper has been term as grouper sleepy disease iridovirus (GSDIV) base on clinical sign of sleepy behavior (Danayadol *et al.*, 1997). Base on similarity on the ATPase and the MCP gene (>99.6% and >97.2%), GSDIV as well as SBIV (sea bass iridovirus), RSIV (red sea bream iridovirus), ALIV (African lampeye iridovirus), and DGIV (dwarf gourami iridovirus) were proposed to be members of genus "Tropivirus" for tropical iridovirus as the third piscine iridovirus in the family *Iridoviridae* (Sudthongkong *et al.*, 2002). However, GSDIV was different with GIV (grouper iridovirus) derived from yellow grouper *Epinephelus awoara* that caused severe mortality in Taiwan (Murai *et al.*, 2002; Sudthongkong *et al.*, 2002). GIV has been

determined to be a member of the genus *Ranavirus*. Recently, member of "Tropivirus" and ISKNV (infectious spleen kidney necrosis virus) were proposed to be members of newly published genus in International Committee on Taxonomy of Viruses that was genus *Megalocytivirus* (Chinchar *et al.*, 2005).

In Indonesia, GSDIV has been reported to infect and caused mass mortality in several grouper fish culture such as orange spotted grouper *Epinephelus coioides* (Koesharyani *et al.*, 2001; Mahardika *et al.*, 2001), humpback grouper *Cromileptes altivelis* (Mahardika *et al.*, 2004a), tiger grouper *E. fuscoguttatus*, marbled grouper *E. polyphkadion*, coral trout grouper *Plectropormus indicus* (Mahardika *et al.*, 2004b; 2009), and sea bass *Lates calcariifer* (Mastuti *et al.*, 2010). Thus, GSDIV is the one problem in cultured grouper and sea bass

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in Indonesia which occurred every year.

Recently, a formalin-inactivated spleen-GSDIV have shown good protecting vaccinated humpback grouper against GSDIV (Mahardika *et al.*, 2003). However, it is problem on availability of virus in big scales, virus concentration in spleen and supernatant contents. Mahardika *et al.* (2004) reported that spleen-GSDIV supernatant contained substance that could prohibit infectivity of virus in fish. A commercialized formalin-inactivated GF-RSIV vaccine has been shown to be highly efficient in protecting vaccinated red sea bream and other cultured marine fishes against RSIV (Nakajima *et al.*, 1997; 2002). It was possible to use in grouper since it vaccine was shown to be effective decreasing mass mortality of vaccinated humpback grouper against GSDIV (Mahardika *et al.*, 2008). However, its vaccine was expensive when imported from Japan and might be a risk in contaminant. For these reasons, we tried to cultivate and propagate GSDIV in cultured GF (grunt fin) cell. Its virus was propagated in cultured GF cell resulting in $10^{5.4}$ TCID₅₀/mL of virus titer (Mahardika *et al.*, submitted data). This research was to know the effectivity of formalin-inactive GF-GSDIV vaccine to covered vaccinated humpback grouper and tiger grouper against GSDIV. This paper described the vaccine test in grouper with histopathological changed and detection of PCR amplification, without titer antibody test because it difficult to collected the blood from small fish (<15 cm).

MATERIALS AND METHODS

Experimental Fish

Experimental fish used in this study was healthy humpback grouper and tiger grouper with total length ranged of 8-10 cm. Those fish derived from Research Institute for Mariculture (RIM) Gondol and private hatcheries around Gondol. Several fish were analyzed PCR (polymerase chain reaction) to ensure they were free from VNN and GSDIV following the methods previously described by Nishizawa *et al.* (1997) and Kurita *et al.* (1998).

Vaccine Preparation

GF-GSDIV virus was prepared from spleens of the GSDIV-infected humpback grouper from RIM-Gondol, as same as our previous preparation of GF-GSDIV virus (Mahardika *et al.*, inpress). The procedures were as follows:

spleen-GSDIV inoculum was inoculated into 50% confluent of subsequent monolayer GF cell. Inoculated-GF cell was incubated at 25°C until cytopathic effect (CPE) occurred. When many of the enlarged cells as CPE apparent in all parts of cell-flask, CPE was harvested for stock of GSDIV. A small volume of GSDIV was used to calculate the virus titer containing in GF cell (see result).

Inactive virus GSDIV was done using 0.1% of formaldehyde and kept in room temperature for one day. Then, formalin-inactivated GSDIV vaccine was kept in 4°C until used.

Effectivity Test

Fourth experiment were conducted with vaccinated and unvaccinated (control) groups. The vaccinated fish were intramuscularly injected with 0.1 mL of the formalin-inactivated GSDIV vaccine. The unvaccinated fish were intramuscularly injected with the same amount of Eagle's minimum essential medium (MEM) supplemented with 2% fetal bovine serum.

First experiment: each of 15 humpback grouper were injected with formalin-inactive GSDIV vaccine derived from 6 dpi of harvested CPE (VD6H), formalin-inactive GSDIV vaccine derived from 2 dpi of harvested CPE (VD2H) and control (EMEM-2). Seven days post first vaccinated, vaccinated fish were injected with second vaccine (booster) with same vaccine and methods as well as control fish injected with second times of EMEM-2. The fish were held in separated bucket, and then these buckets were floated together into rearing water in 8 ton of concrete tank for one month.

Second experiments: 29 of tiger grouper were injected with formalin-inactive GSDIV vaccine (VD2H), and 30 fish were injected with EMEM-2. The fish were held in separated bucket and floated in rearing water as well as first experiment.

Third experiments: each of 50 humpback grouper were injected with formalin-inactive GSDIV vaccine derived from 5 dpi of harvested CPE (VD5H) and EMEM-2 as control. The fish were held in tanks (200L) at 28°C to 31°C with aeration and daily water exchange. Seven days after first vaccinated, all fish gave booster vaccine and EMEM-2 with same vaccine and methods (*double vaccine/DV* dan *control double vaccine/CDV*). At same time, the other two groups (50 fish per group) gave formalin-inactive GSDIV vaccine (VD5H) and EMEM-2

as control formalin-inactive GSDIV vaccine derived from 5 dpi of harvested CPE (VD5H) and EMEM-2 as control (*single vaccine/SV* dan *control single vaccine/CSV*). Each 15 to 20 fish per group were challenged intramuscularly with spleen-GSDIV inoculum after 7, 14, and 20 days post booster vaccine and observed for 15 days.

Fourth experiments: Each 30 and 29 of humpback grouper were injected with formalin-inactive GSDIV vaccine (VD5H: SV) and EMEM-2 as control. After 20 days post vaccination, all fish were challenged intramuscularly with spleen-GSDIV inoculum and observed for 19 days.

Histopathology Observation

Internal organs (spleen, kidney, liver, and stomach) derived from diseased and remain surviving fish at the end of observation were collected and fixed in 10% buffered formalin. Histopathological preparation was followed the methods previously described by Mahardika *et al.* (2004a). Briefly: All samples were dehydrated in serial concentration of alcohol, clearing with xylene and embedded in paraffin wax. The paraffin-blocks were cut 3-5 μm in microtome and stained using Mayer's hematoxylin-eosin.

PCR Amplification

PCR test was applied to confirm the moribund and fish just died were exactly infected with GSDIV. DNA-template derived from extract spleen and kidney were amplified using primer 1-F and 1-R. PCR amplification was done under condition which previously described by Kurita *et al.* (1998).

RESULT

Virus Titer

Four different viral titer of GF-GSDIV were found from four cultured-flask as follows: GF-GSDIV harvested at 6 dpi resulted on $10^{2.8}$ TCID₅₀/mL, GF-GSDIV harvested at 2 dpi was

$10^{4.3}$ TCID₅₀/mL, and two cultured flask of GF-GSDIV harvested at 5 dpi were $10^{3.55}$ and $10^{4.3}$ TCID₅₀/mL. These GF-GSDIV were proceed for formalin-inactive GSDIV vaccine.

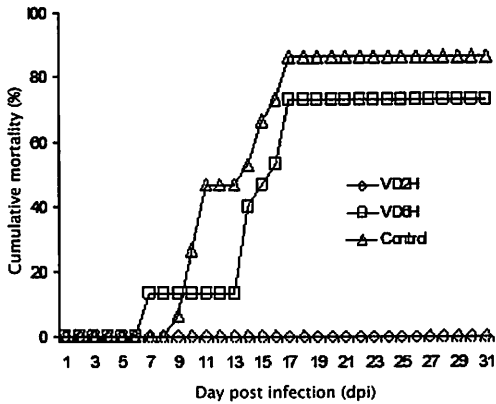
Effectivity of Formalin-Inactive GSDIV Vaccine

In the first vaccination trial, formalin-inactive GSDIV vaccine (VD6H) resulted in high mortality (73.33%) for 31 days observation from booster vaccination. Its mortality was similar than mortality in control group (86.67%). However, fish given formalin-inactive GSDIV vaccine (VD2H) still surviving until the end of experiment (0% mortality) (Figure 1A). In the second vaccination trial, mortality of vaccinated tiger grouper (VD2H) was lower (10.71%) than control group (96.15%) (Figure 1B). In the third vaccination trial, vaccinated (double/DV and single vaccine/SV) humpback grouper after 7 and 14 days post challenged GSDIV shown lower mortalities (60%-66.67% of DV and 73.33%-86.7% of SV) than control groups (CDV and CSV: 100%). However, fish mortalities were similar (81.25%-95%) between vaccinated and control groups after 20 days challenged with GSDIV (Figure 2A, B, and C). In the fourth vaccination trial, vaccinated humpback grouper gave lower mortality (63.33%) than control group (86.73%) (Figure 2D).

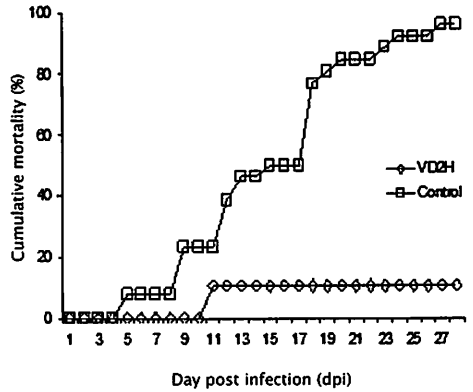
Infected fish shown lost appetite followed with resting in the bottom tank. In several hours, fish became sleepy on their one body side before dead. It took 4-5 days from initial lost appetite to dead. On the other hand, surviving fish did not shown any infected symptom and still active to capture the food.

Histopathology Observation

Dissected moribund (Figure 3A) and fish just after dead showed enlarged spleen and kidney. The size infected-spleen was 2 until 3 time bigger (splenomegally) than the normal spleen size (Figure 4B and 4C). Some fish showed hemorrhage of liver.

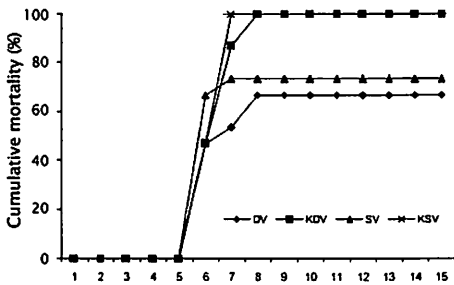


Graphic 1a

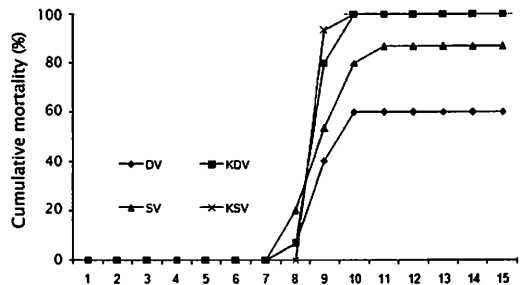


Graphic 1b

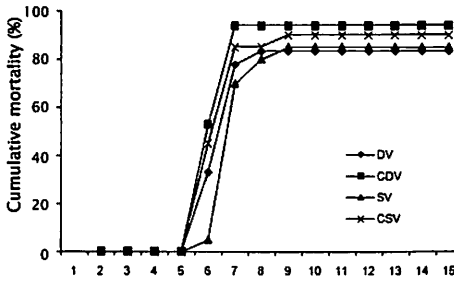
Figure 1. Daily mortality of grouper fish with natural GSDIV infection after vaccinated with inactive GF-GSDIV vaccine; A) Daily mortality of juvenile humpback grouper in the first vaccination trial; B) Daily mortality of juvenile tiger grouper in the second vaccination trial; VD6H: formalin-inactive GSDIV vaccine from harvested CPE on 6 dpi; VD2H: formalin-inactive GSDIV vaccine from harvested CPE on 2 dpi, control: fish injected with EMEM-2



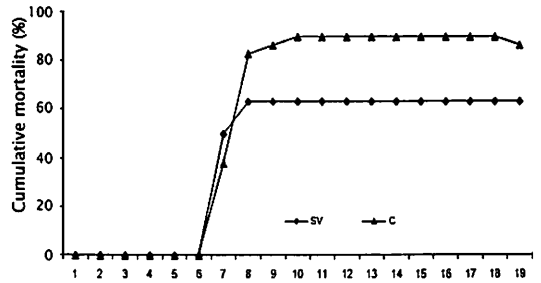
Graphic 3a



Graphic 3b



Graphic 3c



Graphic 3d

Figure 2. Daily mortality of juvenile humpback grouper challenged with virus GSDIV after vaccination in the third vaccination trial; A) Daily mortality of fish challenged with virus GSDIV after 7 days of vaccination; B) Daily mortality of fish challenged with virus GSDIV after 14 days of vaccination; C) Daily mortality of fish challenged with virus GSDIV after 20 days of vaccination; D) Daily mortality of juvenile humpback grouper in the fourth vaccination trial; DV: double vaccination, CDV: control DV, SV: single vaccination, CSV: control SV, control: fish injected with EMEM-2

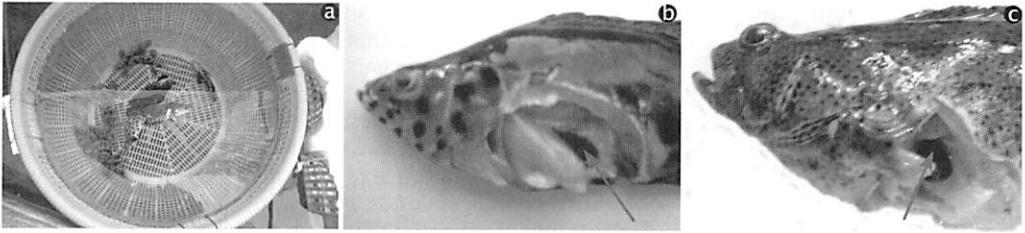


Figure 3. A) Control fish were moribund and dead after naturally infected with virus GSDIV; B) diseased humpback grouper fish shows enlarged of spleen (arrow); C) diseased tiger grouper fish shows splenomegaly (arrow)

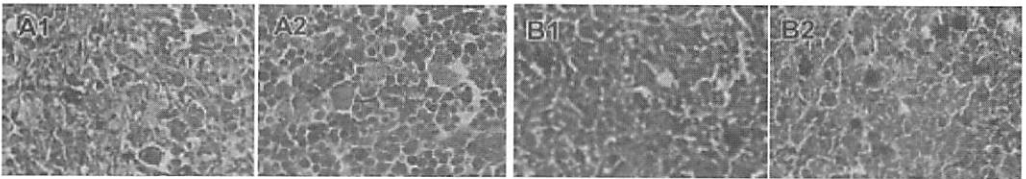


Figure 4A. Histopathological feature of vaccinated tiger grouper fish after challenged with virus GSDIV; A1) spleen, and A2) head kidney of diseased fish from control groups shows formation of enlarged cells (arrows) as well as necrotized cells; B1) spleen, and B2) trunk kidney of surviving fish from vaccinated groups shows normal splenocyte and hematopoietic cells (scale bar: 50 μ m)

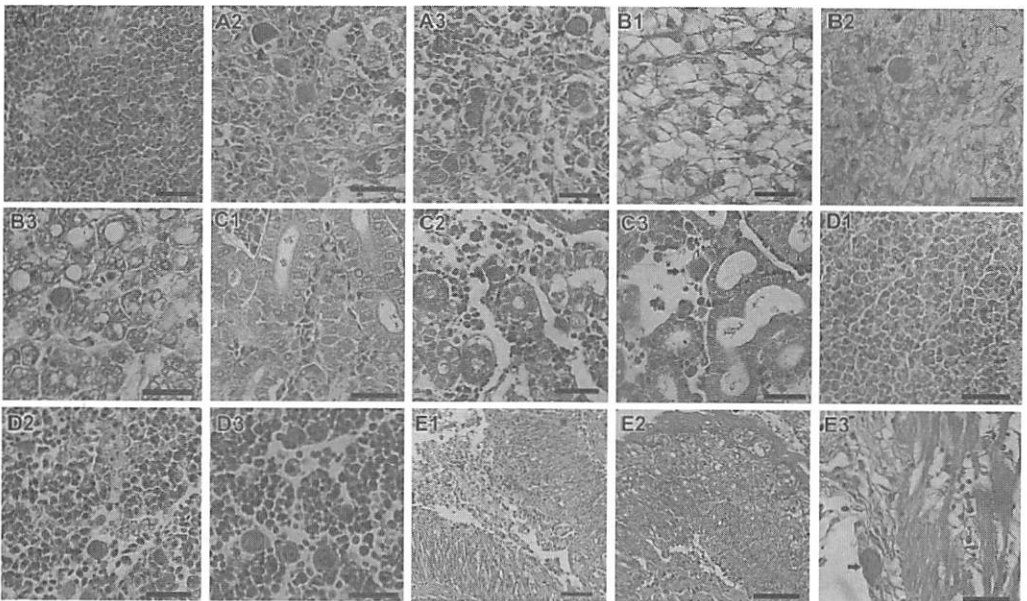


Figure 4B. Histopathological feature of vaccinated and unvaccinated (control) humpback grouper fish after GSDIV exposure. A1-D1]: surviving fish from double-vaccinated group (DV), fish showed normal organs without formation of enlarged cells in the spleen (A1), liver (B1), posterior kidney (C1), head kidney (D1), stomach (E1); A2-D2]: Moribund fish from single-vaccinated group (SV) showed formation of enlarged cells (arrows) as well as necrotic cells in the spleen (A2), liver (B2), posterior kidney (C2), head kidney (D2), stomach (E2); A3-D3]: fish just after die from control group shows formation of enlarged cells (arrows) and necrotic cells in the spleen (A3), liver (B3), posterior kidney (C3), head kidney (D3), stomach (E3) (scale bar: 50 μ m)

Histopathologically, all moribund and newly died fish showed formation of enlarged cells and necrotized cells in the spleen, head kidney, trunk kidney, and liver. Hemorrhage was also observed in the liver. However, a few numbers of enlarged cells were found in the stomach. Formation of enlarged cells and necrotized cells were not shown in the surviving fish from vaccinated and control group

(Figure 4A and B). Histopathological analyze indicated that many enlarged cells were found in the spleen, head, and trunk kidney. It was predominantly found in the spleen following by head and trunk kidney (Table 1 and 2). The size of enlarged cells was different appearance from those of normal cells. However, the size of enlarged cells were variety between each other with average diameter between 10.23-13.67 μm (Table 2).

Table 1. Percent of internal organs contained enlarged cells of diseased fish from third vaccinated trial

Treatments	Total of fish (pcs)	Spleen (%)	Liver (%)	Posterior kidney (%)	Head kidney (%)	Stomach (%)	
Challenge test step 1	SV	10	100.0	45.5	72.7	90.9	0.0
	DV	7	87.5	11.1	66.7	66.7	0.0
	CSV	11	100.0	64.3	100.0	100.0	0.0
	CDV	12	100.0	23.1	8.1	92.3	0.0
Challenge test step 2	SV	11	90.9	7.7	83.3	83.3	100.0
	DV	11	72.7	7.7	33.3	58.3	7.7
	CSV	10	100.0	20.0	100.0	100.0	7.1
	CDV	12	91.7	28.6	85.7	92.9	13.4
Challenge test step 3	SV	12	82.4	43.8	82.4	82.4	0.0
	DV	13	70.0	7.7	76.9	56.3	0.0
	CSV	17	100.0	33.3	94.4	100.0	5.6
	CDV	16	100.0	25.0	87.5	100.0	13.3

Table 2. Number per field view and diameter of enlarged cells founded in spleen, liver, head and posterior kidney, and stomach of diseased fish from third vaccinated treatments

Treatments	Number of enlarged cells per field-view (0.93 mm ²)				
	Spleen	Liver	Posterior kidney	Head kidney	Stomach
SV	7.33	3.00	1.67	15.67	-
CSV	13.67	2.33	4.33	14.67	0.33
DV	5.67	0.33	0.33	12.33	0.33
CDV	10.67	0.67	9.00	18.67	0.33
Treatments	Average of enlarged cells diameter (μm)				
	Spleen	Liver	Posterior kidney	Head kidney	Stomach
SV	12.32	12.12	10.23	10.41	-
CSV	13.48	13.04	11.93	13.14	13.67
DV	13.48	12.44	12.59	10.66	11.31
CDV	12.51	12.07	12.03	11.75	12.60

SV: single vaccine, CSV: control SV, DV: double vaccine, CDV: control DV, -: No enlarged cells

Viral-DNA Detection by PCR

GSDIV infection; A) juvenile humpback grouper in the first treatment; B) juvenile tiger grouper in the second treatments; M: marker 100 bp, P: positive control, N: negative control

PCR amplification indicated that dead humpback grouper (Figure 5A) and tiger grouper (Figure 5B) from vaccinated and control groups were positive infected with GSDIV. It indicated by DNA-band align position with positive control in agarose gel. On the other hand, surviving fish from vaccinated and control groups did not contain DNA GSDIV.

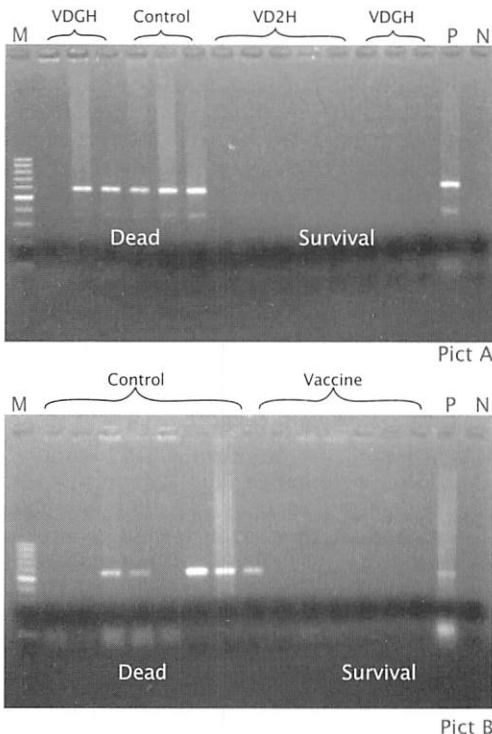


Figure 5. PCR result of fish vaccinated were dead and still survive after effected with natural

DISCUSSION

Commercial inactive-RSIV vaccine was reported to be successful to increased specific immunity indicating by enhanced expression of MHC class 1 and increased the level of serum neutralization antibody (Caipang *et al.*, 2006). Histopathologically, vaccinated fish did not showed formation of enlarged cells as well as necrotic cells in the hematophoitic

cells instead melanomacrophage center. Moreover, no virus particles were found in the melanomacrophage center and hematophoitic cells under electron microscopic observation (Mahardika *et al.*, 2008). In this research, formalin-inactive GSDIV vaccine could decreased mortality of juvenile humpback grouper and tiger grouper against natural and experimental GSDIV infection. This vaccine was effective when it titer virus more than 10^3 TCID₅₀/mL.

Light microscopy observation showed that the vaccinated fish did not contain enlarged cells and necrotic cells in the spleen, kidney, and stomach. On the other hand, enlarged, and necrotic cells were found in the internal-observed organs of control vaccinated groups. These enlarged cells were mainly found in the spleen and kidney with variety in size. Enlarged cells as well as necrotic cells are characteristic sign of *Megalocytivirus* disease (Inouye *et al.*, 1992; Jung *et al.*, 1997; Sudthongkong *et al.*, 2002; Mahardika *et al.*, 2004a; Chao *et al.*, 2004; Miyazaki, 2007). Enlarged cells were firstly found in the spleen tissue, subsequently spread trough circulatory blood system to head and trunk kidney, heart, liver, gill, and digestive tract. (Chao *et al.*, 2004, Mastuti *et al.*, 2010). The viruses released from these infected cells spread to other organs, possibly by infected phagocytes or simply through the circulatory system. A consequence of the presence of enlarged cells in the circulatory system, these cells are trapped in the capillaries, especially in the gills, resulting in insufficient gas exchange, and finally in the death of the fish (Chao *et al.*, 2004). There is might be possible occurred, however, histopathological observation showed that degradation of hematophoitic cells causing by necrotic and replaced-enlarged cells were involved to the dead fish.

In conclusion, formalin-inactive GSDIV vaccine was effective to prevent GSDIV infection in grouper fish. The GF cells infected-GSDIV was available to produce, however, the viral titer still fluctuative or not stable yet. In future, it is need the primary cultured cells from grouper which have high sensitivity to GSDIV virus.

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