Indonesian Aquaculture Journal, 11 (2), 2016, 87-95

Available online at: http://ejournal-balitbang.kkp.go.id/index.php/iaj

ADDITION OF ADJUVANTS IN RECOMBINANT SUBUNIT VACCINES FOR THE PREVENTION OF GROUPER SLEEPY DISEASE IRIDOVIRUS (GSDIV) INFECTION IN HUMPBACK GROUPER, *Cromileptes altivelis*

Ketut Mahardika#, Indah Mastuti, Ahmad Muzaki, and Sudewi

Institute for Mariculture Research and Development

ABSTRACT

Infection of grouper sleepy disease iridovirus (GSDIV) which is a member of *Megalocytivirus* causes mass mortalities in marine fish in Indonesia. This study was conducted to know the effectiveness of recombinant subunit vaccine of GSDIV with an addition of adjuvants against GSDIV infection. Inactive bacteria *Eschericia coli* containing recombinant MCP-GSDIV protein was added with montanide ISA adjuvant at a ratio of 3:7. The vaccine was administered to humpback grouper, *Cromileptes altivelis* by intramuscular and intraperitoneal injection at a dose of 0.1 mL/fish. Test of the vaccine in humpback grouper was performed in four replicates (four trials). Results of the vaccination showed that the recombinant protein vaccine added with the adjuvant increased immunity of humpback grouper, indicated by higher relative percent survival (RPS = 77.78%) compared to negative control (PBS) and 50% higher compared to protein control (pET Sumo CAT) at two weeks post vaccination. The RPS values of the recombinant protein vaccine were still higher (53.57%-72.73%) than those of the control vaccine and 25%-53.33% of the protein control in the 4th week post vaccination. GSDIV detection by PCR showed that MCP-GSDIV-DNA and pET Sumo CAT-DNA were not detected in the vaccinated fish after one, two, three, and four weeks post vaccination. The fish died in both of vaccinated and control groups after experimental challenge with GSDIV were found to be infected with GSDIV. It can be stated that recombinant subunit vaccine of GSDIV with the addition of montanide ISA adjuvant could be used to prevent and diminish mortalities of grouper against GSDIV infection.

KEYWORDS: MCP-GSDIV; *Megalocytivirus*; subunit vaccine; humpback grouper

INTRODUCTION

Economic losses caused by iridovirus infection in aquaculture were considerably high. The virus causes mass mortality in both marine and fresh water fish. In Indonesia, iridovirus was known to infect marine fish such as orange-spotted grouper (*Epinephelus coioides*) (Koesharyani *et al*., 2001), tiger grouper (*E. fuscoguttatus)*, camouflage grouper (*E. polyphekadion*), coral-trout grouper (*Plectropomus leopardus*), and cobia (*Rachycentron canadum*) (Mahardika *et al*., 2009). Fresh water fish, for instance Dwarf gurami (*Colisa lalia*), African lampeye (*Aplocheilichthys normani*) (Sudthongkong *et al*., 2002a), gourami (*Osphronemus goramy*), and *Osteochillus hasselti* (Koesharyani, *unpublished data*) had also been reported to be infected with iridovirus.

Correspondence: Institute for Mariculture Research and Development. Br. Gondol, Ds. Penyabangan, Kec. Gerokgak, Kab. Buleleng, P.O. Box 140, Singaraja 81101, Bali, Indonesian.
Phone: + (0362) 92278
E-mail: *kmahardika@yahoo.com*

Grouper sleepy disease iridovirus (GSDIV) is a member of the genus *Megalocytivirus*, belongs to the family *Iridoviridae*. GSDIV has been known to attack blood-forming organs (hematopoietic cells) including spleen and kidney. Infection of GSDIV causes anemia symptoms such as body weakened and lying on the bottom of the tank with a pale or black body color (Mahardika *et al*., 2009). In addition, according to histopathological study, GSDIV infection can be detected by the presence of inclusion body bearing cells (IBCs) and necrotic cells that are observed as enlarged cells under a light microscope. Morphologycally, iridovirus is hexagonal in shape, 150-200 nm in diameter, and multiplies in the cytoplasm of the infected cells (Miyazaki, 2007).

In the previous study, an attempt had been made to develop recombinant subunit vaccine using bacteria *E. coli* which containing of the Major Capsid Protein (MCP) gene of Indonesian isolate of fish *Megalocytivirus*. The recombinant subunit vaccine of GSDIV could increase immunity of humpback grouper after experimental challenge with GSDIV.

However, the effectiveness of the vaccine was relatively short (only two weeks post vaccination) (Mahardika & Mastuti, 2015). Therefore, it is required to add an adjuvant which is known to enhance immune responses and improve vaccine potency. Adjuvants assist with early immunity onset, long effector response (antibody titres; T cell activity) and diminishing requirement of boosters. The main function of adjuvants is the antigen presentation process, distinguishing them in Signal-1 (actual antigenic presentation) and Signal-2 (secondary signals) facilitators; both involved in the activation of lymphocytes T and B (Tafalla *et al*., 2013 in Roldan, 2014). Adjuvants can be classified by physical or chemical characteristics, but also by the immunomodulating activity they develop. Signal-1 adjuvants include oil emulsions and micro/nanoparticles. Oil emulsion adjuvants have the advantage of achieving a slow release that increases immunogenicity; the emulsion is a dispersion of a non-miscible liquid (dispersed phase = antigenic media) in a second liquid (continuous phase = oil). The first oil based adjuvant was Freund´s adjuvant based on heat killed Mycobacteria plus mineral oil and surfactant. Later came oil adjuvants that aimed to increase efficacy and delivery, such as Montanide™ (SEPPIC, France). These are mineral oil adjuvants, optimized to improve efficacy, stability and reduce side effects; they are based in mineral oil, non-mineral oil, and mixtures, with mannitol oleate as surfactant (Roldan, 2014). This study was conducted as an effort to increase immunity of grouper against GSDIV infection, and to enhance protection period of the subunit vaccine of GSDIV through the addition of Montanide™ ISA 763 A VG.

MATERIALS AND METHODS

Virus Stock

The fish *Megalocytivirus* used in the present study was isolated from the naturally infected humpback grouper (*Cromileptes altivelis*). The virus was obtained from the spleen and kidney of the infected fish. The spleen and kidney was homogenized in 9 x volume of EMEM-2 and was centrifuged at 3,000 rpm for 15 minutes. Then, the supernatant was filtered at 0.45 μm^2. The filtrate was then maintained at -80°C, and used for DNA template, test vaccine, and virus propagation in healthy fish.

Propagation of Bacteria as A Carrier of Recombinant MCP-GSDIV Protein

The bacteria cell (*E. schericia coli* strain BL-21) as a carrier of recombinant MCP-GSDIV protein vaccine was confirmed by PCR and protein sequencing (Mahardika & Mastuti, 2015). The bacteria cell were cultured at mass scale in luria bertani (LB) broth media containing 50 μg/mL kanamycin at 37°C in rotary shaker with speed of 225 rpm/minute for 24 h. Density of the harvested bacteria cell was measured by plated into LB agar.

Expression of Protein Using IPTG

Cultured *E. coli* strain BL-21 was then induced using 0.1 mM IPTG (Isopropyl-β-D-Thiogalactopyranoside) and incubated in 37°C in rotary shaker with speed of 225 rpm/minute for eight hour. A 50 mL of *E. coli* strain BL-21 culture that had been induced using IPTG was centrifuged at 6,000 rpm for ten minutes. After that, the pellet was lysed enzymatically using lysozyme, and mechanically by not only freezing and thawing method, but also sonication to obtain the protein. Lysing enzymatically was performed by incubating 100 μL of the pellet with 150 μL lysozyme 200 mM at 37°C for five minutes. The cells were then lysed by 30 cycles of freezing and thawing (freezing at -80°C and thawing at 42°C), and by sonication for 4 x 15 minutes. The lysed cells were centrifuged at 15,000 rpm (maximum speed) for one minute. A 10-15 μL of the pellet and 25 μL of the supernatant were used for vertical electrophoresis using SDS-page according to the method in our previous study (Mahardika & Mastuti, 2015).

Preparation of The Subunit Vaccine

Subunit vaccine is a vaccine produced from specific protein subunits of a virus and thus having less risk of adverse reactions than whole virus vaccines. In this study was used a subunit vaccine involves putting an MCP gene from the GSDIV into bacteria vector such *E. coli* to make a recombinant virus to serve as the important component of a recombinant vaccine (called a recombinant subunit vaccine).

A 5 mL of *E. coli* strain BL-21 culture that had been induced with IPTG, density of 1.25 x 10^7 CFU/mL, and had known to express protein by SDS-page was inactivated by adding formalin (40%) until final concentration of 0.03%. The inactivation process was done overnight at room temperature. The formalin was then cleaned by centrifugation at 6,000 rpm for five minutes to obtain pellet, and then the pellet was lysed by sonication (4x 15 minutes). Following that, the pellet was diluted in PBS in order to obtain density of 10^7 CFU/mL. At the 4th vaccination trial, the volume of the vaccine was reduced at two times by centrifugation; therefore, the concentration of the bacteria was doubled.

The Addition of Adjuvant

Montanide™ ISA 763 AVG (Seppic, France) is a common adjuvant to be used for vaccination in aquaculture. This product was selected based on the previous experience which was no mortality of fish after one week administration of the adjuvant. *E. coli* BL-21 was inactivated and then added with montanide ISA 763 AVG (Seppic) at a volume ratio of 3:7 according to the manual. Compound of the vaccine and the adjuvant was emulsified or homogenized using a syringe without rubber (Discofix-3, Braun-Jermany) in a clean bench. The homogenized vaccine was then stored at 4°C until use. As well as recombinant subunit vaccine of GSDIV, the recombinant subunit of pET Sumo CAT as protein control was also emulsified with montanide ISA 763 AVG with same volume ratio.

Test of The Recombinant Subunit Vaccine of GSDIV in Humpback Grouper

Test of the subunit GSDIV vaccine was performed in a total of 292 juvenile humpback grouper. The juvenile were 6-8 cm in size, and had been determined negative to VNN and GSDIV by PCR analysis. The juvenile were divided into three groups for experiment vaccination test. In group-I (vaccinated group), a total of 105 fish were administered with the emulsified of recombinant subunit vaccine of GSDIV and (Ia) or without (Ib) montanide ISA 763 AVG. In group-II (protein control group), a total of 84 fish were injected with emulsified of recombinant subunit of pET Sumo CAT and montanide ISA 763 AVG. In group-III (negative control), a total of 103 fish were injected with phosphate buffer saline (PBS). Vaccination tests were administrated by either intramuscular or intraperitonial injection at a dose of 0.1 mL/fish. The tests were done at a laboratory scale. The experiment was carried out in four trials. In the 1st trial, the fish were divided into three groups (group-Ia, II, and III) which each group containing 25 fish, while in the 2nd trial, it was divided into four groups (group-Ia, Ib, II, and III) which each group containing 10 fish. The 2nd trial was performed to know the effects of the vaccination with and without addition of montanide ISA 763 AVG. In the 3rd and the 4th trials, the test fish were divided into three groups (group-I, II, and III) which each group containing 30 fish. All fish for each treatment were stocked into a happa with a size of 1 m x 1 m x 0.75 m and divided into four holes which were floated in a 1,000 m^3 tank. The vaccine was administered once time according to the manual of adjuvant Seppic manufacture's distributor. Experimental challenge test with GSDIV virus was performed after 14 and/or 30 days post vaccination by intramuscular injection (IM) at a dose of 0.1 mL/fish for the 1st and 2nd trials, and by intraperitoneal injection (IP) at a dose of 0.1 mL/fish for the 3rd and 4th trials. Particularly for the 4th trial, the experiment was done in triplicates. Mortalities were recorded daily for 15 days after the challenge test. Dead fish were autopsied to determine the cause of death and to detect the presence of virus.

Effectiveness of the vaccine was determined by relative percent survival (RPS) value after the experimental challenge with GSDIV virus with lethal dose of 80%. Data resulted from the challenge test were presented in percent of mortality and RPS was calculated using the following formula:

$$RPS = \frac{(1 - \%\ \text{mortality of treatment})}{\%\ \text{mortality of control}} \times 100$$

In the 4th trial, data are expressed as mean ± SD. A statistical analysis was performed using one-way ANOVA for the RPS value. $P < 0.05$ was taken to indicate statistical significance.

Detection of GSDIV Virus by PCR Analysis

Detection of GSDIV virus by conventional PCR analysis was done for sample of blood, spleen, and liver after 1, 2, 3, and 4 weeks post vaccination, five fish each. The PCR detection was also performed for fish that were died after challenge test. The samples were extracted using ISOGEN according to the manual. PCR amplification was done using GoTaq® PCR Core System I kit (Promega M7660) and the primer used were MCP and pET Sumo (CAT) with a molecular weight target of 1,362 bp and 1,313 bp. Temperature of amplification was following the previous method (Mahardika & Mastuti, 2015). Results of the amplification were analyzed by electrophoresis at a voltage of 100 V and 1.5% of gel agarose using TAE buffer (2 Na EDTA 2 Na, Triss and Aquades) for 20-25 minutes. Staining was done using 0.5 mg/mL ethidium bromide in TAE buffer. After the electrophoresis, the gel was read using ultraviolet transiluminator.

Histopathological Observation

Histopathological observation was conducted to the test fish with clinical sign (moribund). The fish observed were 2-3 of the test fish at the last sampling for each treatment, and five fish at 1, 2, 3, and 4 weeks post vaccination each. Condition of the internal organs of the fish was observed after dissection. Then, the internal organs (spleen, liver, kidney, heart, stomach, and intestine) were fixed in 10% of

phosphate buffer formalin. Particularly for gills, fixation was followed by decalcification using 5% formic acid in 10% phosphate buffer formalin for 1-2 days and was neutralized using 5% Na_2SO_4 in aquades. The samples were processed using a method by Mahardika *et al*. (2009). All samples were then embedded in paraffin wax and were cut at a thick of 3–5 μm. Staining was done using haematoxylin-eosin (H&E).

RESULTS AND DISCUSSIONS

Expression of Protein

Recombinant plasmid was successfully expressed the MCP-GSDIV protein in bacteria cells of *E. coli* strain BL-21 induced by IPTG. The targeted MCP gene could be expressed at a concentration of 0.1 mM IPTG. Results of electrophoresis using SDS-page gel showed molecular weight of the MCP recombinant at 60 kDa (Figure 1).

Cloning of MCP gene from the virus isolate of GSDIV derived from humpback grouper had been successfully performed at Institute for Mariculture Research and Development (IMRAD) at Gondol, in 2012 (Mahardika & Mastuti, 2015). The MCP gene had been expressed completely and resulted in peptides with a number of amino acid 453. The sequence of the amino acids found in the present study was similar to the MCP of Red Sea Bream Iridovirus (MCP-RSIV: gi|29467056|BAC66968.1), and was agreed with the sequence of MCP *Megalocytivirus* gene isolated from South China Sea and South East Asian countries, which consists of 1,362 and 1,372 base pairs and encode

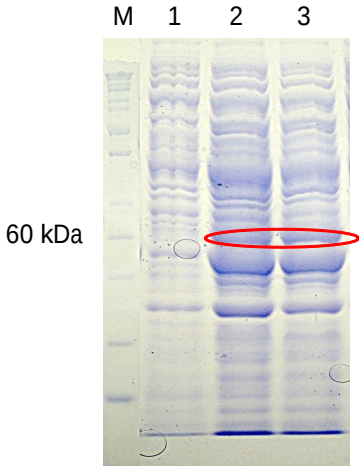

Figure 1. SDS-page gel shows target protein expression of the MCP gene resulting in 60 kDa (circle line). Line M: protein marker, line-1: *E. coli* strain BL-21, lines-2 & 3: *E. coli* strain BL-21 containing gen MCP-GSDIV

454 amino acids (Sudthongkong *et al*., 2002b). The MCP-RSIV had been reported to be used as recombinant protein vaccine to prevent RSIV virus infection (Shimmoto *et al*., 2010). The sequence of MCP-GSDIV isolated locally from Indonesia was similar to the sequence of MCP-RSIV which belongs to genus *Megalocytivirus*. Therefore, recombinant protein of GSDIV in this study could be used as recombinant vaccine against *Megalocytivirus* infection in Indonesia. Potency of the recombinant MCP-GSDIV gene as a vaccine will be discussed later.

In this study, the vaccine was constructed from *E. coli* strain BL-21 as host cells inactivated in 0.03% formalin and partially lysed by sonication. This procedure allows modulation of the non-specific immune system in fish due to protein content in the bacteria (Shimmoto *et al*., 2010). Proteomic analysis of peripheral blood mononuclear cells (PBMC) confirmed that *E. coli* lipopolysaccharide induced differentially over-expressed immune proteins such as complement component C-3 and lysozyme C-2 precursor (Hang *et al*., 2013).

Potency of The Protein Vaccine Against GSDIV Infection

Administration of vaccine by intramuscular injection (IM) in the first trial (Table 1) showed that recombinant subunit vaccine of GSDIV with addition of montanide ISA 763 AVG could increase the immunity of humpback grouper against GSDIV infection after 14 and 30 days post vaccination. First, at 14 days post vaccination, the increased in immunity of the vaccinated fish (GSDIV + ISA) was indicated by higher RPS (77.78%) compared to either of the fish administered CAT (control protein) + ISA (55.56%) or the non-vaccinated fish (injected with PBS). Second, at 30 days post vaccination, level of RPS of the fish vaccinated with recombinant protein of GSDIV + ISA was remained higher (57.14%) than that of the fish administered by CAT + ISA (14.28%) and the non-vaccinated fish.

In the 2nd vaccination (trial 2), intramuscular injection of the emulsified of montanide ISA 763 AVG and recombinant subunit vaccine of GSDIV showed higher RPS value (60% than those of the control fish group (PBS) at 30 days post vaccination. The high RPS value was also achieved by vaccination using recombinant subunit vaccine of GSDIV without montanide ISA 763 AVG (16.67%) compared to the control fish group (PBS) (Table 2).

Vaccination of emulsified of the montanide ISA 763 AVG with recombinant subunit vaccine of GSDIV protein through intraperitoneal injection (the 3rd trial) was also indicated higher RPS value (> 50%) than those

Table 1. RPS values of the vaccinated fish by intramuscular injection (IM)

Treatments	14 days post vaccination			30 days post vaccination		
	Number of fish	Mortality (%)	RPS (%)	Number of fish	Mortality (%)	RPS (%)
MCP + ISA	10	20	77.78	15	40	57.14
CAT (protein control) + ISA	10	40	55.56	15	80	14.28
PBS (negative control)	10	90		15	93.33	

of emulsified of the recombinant subunit of pET Sumo CAT and negative control fish groups (Table 3).

In the last trial (the 4th trial), mortality of the fish was relatively high. Therefore, average survival rate was considerably low (< 20%) (Tabel 4). Nevertheless, administration of inactive bacteria containing recombinant MCP-GSDIV protein at high concentration of the *E. coli* (10^{12} CFU/mL) and the addition of the adjuvant, resulted in higher survival rate (33.33 ± 3.33[a]) than those of the fish injected with protein control CAT ($P<0.05$). However, survival rate of the fish vaccinated with emulsified of the montanide ISA 763 AVG with recombinant subunit of GSDIV was not significantly different with the non-vaccinated fish ($P>0.05$) (Table 4).

Recombinant MCP-GSDIV protein vaccine has the potential to induce immune system of fish against GSDIV infection. This potency was indicated by higher RPS value obtained in the vaccinated fish group than that of the unvaccinated fish group (Mahardika & Mastuti, 2015). Similarly, RPS value of the fish vaccinated by recombinant MCP-GSDIV protein vaccine was higher compared to the fish administered by inactive *E. coli* containing recombinant pET Sumo CAT protein. Results of the MCP-GSDIV test vaccine at a laboratory scale exhibited relatively good protection effect at 14 days post vaccination (RPS = 55%), although it decreasing at 20-21 days post vaccination (RPS < 33%).

Protection period of the recombinant protein MCP-GSDIV vaccine was relatively short (< 20 days). Therefore, montanide ISA adjuvant was added into the vaccine. Results of the vaccine trials showed an increase in immunity, and longer period of protec-

Table 2. RPS values of the fish vaccinated with and without adjuvant in the second trial

Treatments (vaccine + adjuvant)	Number of fish	Days post vaccination		Treatments (vaccine without adjuvant)	Number of fish	Days post vaccination	
		30 days				30 days	
		Mortality (%)	RPS (%)			Mortality (%)	RPS (%)
MCP +ISA	10	20	60	MCP	10	30	16.67
PBS (negative control)	10	50		PBS (negative control)	10	40	

Table 3. RPS values of the vaccinated fish by intraperitoneal injection (IP)

Treatments	Number of fish	30 days post vaccination	
		Mortality (%)	RPS comparison to PBS (RPS comparison to CAT) (%)
Tank 1 (IP)			
MCP + ISA	15	25	53.57 (53.13)
CAT (protein control) + ISA	15	53.33	0.95
PBS (negative control)	13	53.85	
Tank 2 (IP)			
MCP + ISA	15	20	72.73 (53.33)
CAT (protein control) + ISA	14	42.86	41.56
PBS (negative control)	15	73.33	

Table 4. Average survival rate of the test fish after the experimental challenge with GSDIV virus for 15 days observation (Mean ± SD)

Treatments (Vaccine + montanide ISA 763 AVG (by IP))	Survival rate
MCP + ISA	33.33 ± 3.33[a]
CAT + ISA	30.0 ± 5.77[a]
PBS	33.33 ± 14.52[a]

tion was achieved. This achievement indicated by higher RPS value of the vaccinated fish, not only at 14 days post vaccination (> 70%) and (23%-73%) but also at 30 days post vaccination compared to the unvaccinated fish. RPS value of the fish group vaccinated with adjuvant was also higher than those of the fish vaccinated without adjuvant. Those results showed that the addition of montanide ISA adjuvant in the recombinant MCP-GSDIV protein vaccine could increase the effectiveness of the vaccine in stimulating immune system indicated by the increase in RPS value until one month post vaccination. In another study, the increase in specific immunity was also achieved when the RSIV vaccine was used to alleviate RSIV infection, signified by the increase in serum antibody and expression of MHC class 1 which are produced in response to infection (Caipang *et al*., 2006). An MHC class I protein is anchored into the cell membrane and extends upright into the extracellular area. At the top of MHC class-I is a small groove, which binds a specific peptide from a protein that has been digested within the cell. Tc cells have T cell receptors (TCRs) on their membranes that can bind to an MHC class-I that is presenting a antigen. These TCRs can determine whether this peptide is representative of normal cell function or an implication that the cell has been infected (Campbell, 2010).

Virus Detection by PCR

Vaccination using inactive *E. coli* containing recombinant MCP-GSDIV protein and pET Sumo CAT showed that there were either no MCP-GSDIV-DNA or pET Sumo CAT-DNA detected in the vaccinated fish at 1, 2, 3, and 4 weeks post vaccination (Figure 2). These results revealed that MCP-GSDIV-DNA or pET Sumo CAT-DNA was cleared and could not be detected in blood or target organs of GSDIV virus. Other possibility is that those DNA had interacted with the fish antibody and produce specific antibody that would memorize the DNA. Similar to the vaccinated fish, the PCR results of the unvaccinated fish did not show any band in the gel agarose. Then, after the challenge test, the survivors of all treatments were also negative to GSDIV infection confirmed by PCR. Contrastingly, the fish that were died after the challenge test were positively infected with GSDIV, indicated by the presence of a band in the gel agarose. The results are shown in Figure 2.

Histopathologycal Observation

Generally, vaccination by intramuscular injection showed no irritation in the injection site of the vaccinated fish. However, several fish suffered ulcers at the injection site. On the contrary, most of the fish vaccinated through intraperitoneal injection (> 80%) in the fourth trial exhibited pustule accumulation in the abdominal cavity at the injection site (Figure 3), whereas the internal organs such as liver, intestine, stomach, spleen, kidney, and the heart were appeared normal. Any pathological changes in the internal organs were also not found in the survivors after the challenge test. However, the fish that were died after the challenge test showed dilatation in the spleen, and several of the fish showed black color or pale in its hearts.

Histopathologically, clumps of pus in the abdominal-cavity appeared as abscess in the mesenterium tissues. The abscess was red in color by hematoxylin-eosin staining (Figure 4A), and rounded by fibrinogen wall. The central part of the fibrinogen wall showed cavity due to the pus run out of the wall when embedding process (Figure 4B). Whereas other internal organs were seen normal (Figure 4D). Abscess was not found in the fish injected with PBS (Figure 4C).

The newly dead fish of all treatments after the challenge test showed the formation of enlarged cells in the spleen and head kidney tissues (Figure 4E). Those cells spread evenly and some of the cells were contained in melanomacrophage center (MMC). The enlarged cells appear bluish and reddish with some variations in size. While in the heart tissue, the red blood cells were accumulated in the blood vessels (hyperemi), and outside of the blood vessel along

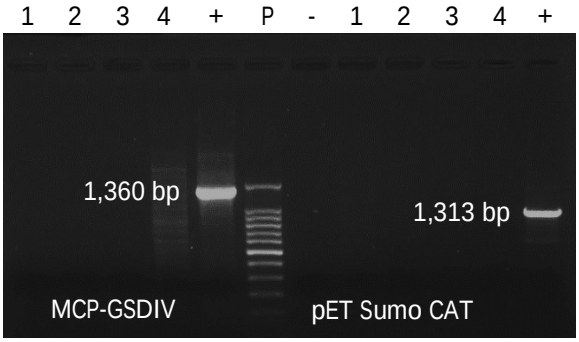

Figure 2. PCR results of the test fish at 1, 2, 3, and 4 weeks after administration of recombinant MCP-GSDIV protein vaccine, pETSumo CAT (protein control), and PBS (negative control or unvaccinated group). P : DNA Marker 100 bp, +: positive control, -: negative control

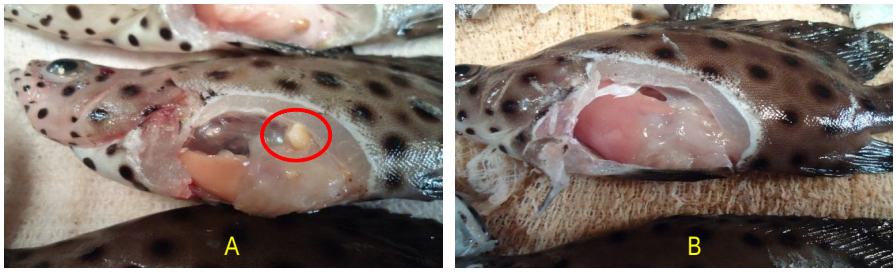

Figure 3. Pathological sign of the vaccinated fish in the 4th trial; A) fish injected with recombinant MCP-GSDIV vaccine showed pustule accumulation in the abdominal-cavity (circle line) at the injection site; B) unvaccinated fish showed normal abdominal-cavity without any pathological changed

haemotocyte tissue nearby the blood vessels (haemorrarghie). In addition, several enlarged cells were circulated in the blood vessels.

While in the survivors of the control fish were exhibited groups of enlarged cells in the spleen and head kidney tissues (Figure 4F). The enlarged cells together with other cells including melamine and hemosiderin, forming a group of cells defined as MMC.

Based on histopathology observation, the moribund and fish just died in both of vaccine and control groups revealed necrotic and formation of enlanged cells in the kidney and spleen resulting in spleenomegally. Dead fish were caused by anemia due to damage of haematopitic cells. On the other hand, necrotic, and enlarged cells were not observed in survival fish. Spleen tissue just contained melanomacrophage center (MMC) which normaly seen in the fish recovered from GSDIV-disease (Miyazaki, 2007). Fish in the trial 1 to 3 did not shown any injury or abcess at the injection site of vaccine. In the previous study, formation of enlarged cells and necrosis were not found in the hematopoietic tissue of the vaccinated fish. However, MMC was detected. Although the MMC was found, there was no virus particles observed in either the MMC or in the hematopoietic cells under an electron microscope observation (Mahardika *et al*., 2008).

In the present study, the concentration of *E. coli* was increased by decreasing the initial volume of the bacteria up to 100 times. Therefore, it was detected that the concentration of inactive *E. coli* reached up to 10^{12} CFU/mL. The use of high concentration of the bacteria with the addition of the adjuvant (in the 4th trial) causing accumulation of the vaccine and the adjuvant at the injection site in the abdominal cavity. This accumulation causing pustules that observed until the fourth week post vaccination. The emergence of the pustules was probably caused by the vaccine which was not absorbed completely into the organs or tis-

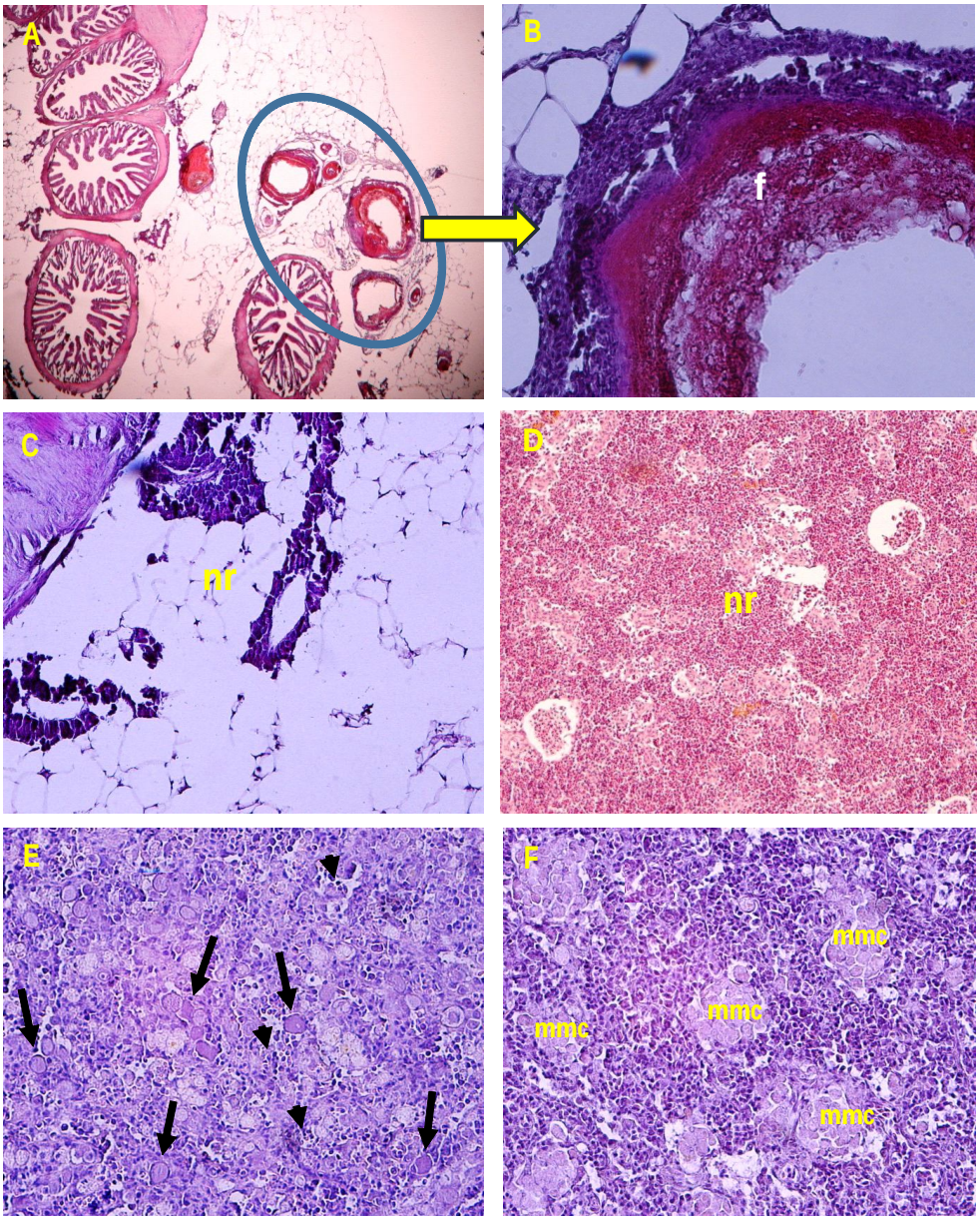

Figure 4. Histopatological sign of the test fish administered by recombinant protein vaccine added with adjuvant; A) fish administered by recombinant pET Sumo CAT in the 4th trial showed some abcess in the abdominal-cavity (circle line) (magnification of 40X), B) detail of the abcess in figure A (arrow) the abdominal-cavity was surrounded by fibrinogen (f) with bacterial invasion (magnification of 400X), C) mesenterium tissue of the control fish (PBS) was normal (nr) with peripheral blood vessels (magnification of 200X), D) spleen tissue of the fish administered by recombinant protein MCP-GSDIV vaccine was seen normal (nr) (magnification of 400X), E) spleen of fish just died of control fish group after the experimental challenge with GSDIV showed formation of enlarged cells (arrows) as well as necrotic cells (arrow heads), F) spleen of the survivors of the control fish group after the experimental challenge with GSDIV showed groups of enlarged cells defined as melanomacrophage center (mmc) (magnification of 400X)

sues of the fish, and could affect the health of the fish. Consequently, the survival rate of the vaccinated fish was low and statistically not different with those of the unvaccinated fish after the experimental challenge with GSDIV virus.

CONCLUSION

The recombinant subunit vaccine of GSDIV with the addition of montanide ISA 763 AVG adjuvant could be used to prevent and to decrease mortalities of grouper against GSDIV infection. The humpback grouper have a longer protection that indicating by the better Relative Percentage Survival (RPS) value.

ACKNOWLEDGEMENT

The authors thank to Sri Suratmi, Ketut M. Arya Sadewa, and Kadek Sulandra for the help during vaccination trials and the laboratory works. We gratefully acknowledge Research Prof. Dr. Haryanti, M.S. and Prof. Dr. I G.N. Kade Mahardika for the suggestion and guidance throughout the study. We would also like to thank the manufacturer's distributor of the Seppic Montanide adjuvant who's provided support for the experiment.

REFERENCES

Caipang, C.M.A., Takano, T., Hirono, I., & Aoki, T. (2006). Genetic vaccine protect red sea bream iridovirus, *Pagrus major*, upon challenge with Red Sea Bream Iridovirus (RSIV). *Fish and Shellfish Immunology*, 21, 130-138.

Campbell, M. (2010). The structure and function of MHC class I. Department of Biology, Davidson College, Davidson, NC 28036. http://bio.davidson.edu/Courses/Molbio/MolStudents/spring2010/Reilly/MHC.

Hang, B.T.B., Milla, S., Gillardin, V., Phuong, N.T., & Kestemont, P. (2013). In vivo effects of *Escherichia coli* lipopolysaccharide on regulation of immune response and protein expression in striped catfish (*Pangasianodon hypophthalmus*). *Fish & Shellfish Immunology*, 34, 339-347.

Koesharyani, I., Roza, D., Mahardika, K., Johnny, F., Zafran, & Yuasa, K. (2001). Iridovirus. *In* Sugama, K., Ikenoue, H., & Kawahara, S. (Eds.). Manual for fish disease diagnosis-II. Marine fish and crustacean diseases in Indonesia. Gondol Marine Research for Mariculture, Central Research Institute for Sea Exploration and Fisheries, Dep. of Marine Affair and Fisheries, and Japan International Cooperation Agency, p. 5-7 (in Indonesia).

Mahardika, K., Haryanti, Muzaki, A., & Miyazaki, T. (2008). Histopathological and ultrastructural features of enlarged cells of humpback grouper *Cromileptes altivelis* challenged with *Megalocytivirus* (Family *Iridoviridae*) after vaccination. *Diseases of Aquatic Organisms,* 79, 163-168.

Mahardika, K., Muzaki, A., & Suwirya, K. (2009). Pathogenecity of grouper sleepy disease iridovirus (GSDIV: *Megalocytivirus*, Family *Iridovirdae*) to coral trout grouper *Plectrophomus leopardus*. *Indonesian Aquaculture Journal*, 4(2), 121-130.

Mahardika, K., & Mastuti, I. (2015). The effect of crude recombinant viral protein vaccines against grouper sleepy disease iridovirus (GSDIV) on humpback grouper (*Cromileptes altivelis*). *Indonesian Aquaculture Journal*, 10(2), 163-172.

Miyazaki, T. (2007). Color atlas of fish histopathology. Shin-Suisan Shimbun-Sha, Tokyo. Japan, 2, 325-335.

Roldan, M.A.M. (2014). *Development of a vaccine against Francisella noatunensis subsp. orientalis in red nile tilapia (Oreochromis niloticus)*. Ph.D Thesis. Institute of Aquaculture University of Stirling FK9 4LA. United Kingdom, p. 1-38.

Shimmoto, H., Kawai, K., Ikawa, T., & Oshima, S-I. (2010). Protection of red sea bream *Pagrus major* against red sea bream iridovirus infection by vaccination with a recombinant viral protein. *Microbiol. Immunol.*, 54, 135-142.

Sudthongkong, C., Miyata, M., & Miyazaki, T. (2002a). Iridovirus disease in two ornamental tropical freshwater fishes: African lampeye and dwarf gourami. *Diseases of Aquatic Organisms,* 48, 163-173.

Sudthongkong, C., Miyata, M., & Miyazaki, T. (2002b). Viral DNA sequences of genes encoding the ATPase and the major capsid protein of tropical iridovirus isolates which are pathogenic to fishes in Japan, South China Sea and southeast Asian countries. *Archives Virology*, 147, 2089-2109.