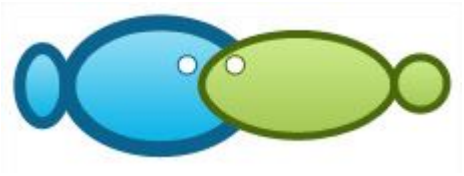

The hematological response of cantang hybrid grouper (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*) injected with extracellular product, intracellular component and whole cell vaccine of *Vibrio alginolyticus*

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Abstract. Vibriosis is a bacterium disease that is often found in grouper cultivation from the eggs, larvae, seeds, and broodstocks. One of the alternatives to overcome fish infection is vaccine administration. A material can be used as a vaccine if it has an immunogenic substance. Several vaccines are used in fish culture such as whole cell vaccine (WCV), extracellular products (ECP), intracellular components (ICC). The aim of this study was to determine the effectiveness of WCV, ECP, and ICC of *Vibrio alginolyticus* and to compare the various vaccines which had the best influence on the hematological response of the hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*). This study was conducted by design of Complete Randomized Design (CRD) with four treatments, i.e. treatment A (i.p injection with 0.1 mL phosphate buffer saline per fish as a control), B (i.p injection with 0.1 mL whole cell vaccine per fish), C (i.p injection with 0.1 mL extracellular products per fish) and D (i.p injection with 0.1 mL intracellular components per fish), each treatment repeated three times. A second vaccination (booster) was carried out a week after the first vaccination. Blood collection was carried out four times during the study on day 7, 14, 21 and 28 after the first vaccination. The results showed that WCV, ECP, ICC of *V. alginolyticus* produced total leukocytes/WBC ($33.83\pm0.96 \times 10^3$ μL^{-1}, $35.17\pm2.19 \times 10^3$ μL^{-1} and $27.33\pm1.37 \times 10^3$ μL^{-1} respectively) lower compared to the control fish ($41.60\pm6.25 \times 10^3$ μL^{-1}) on the 28th day. Group D also showed total erythrocytes/RBC ($1.46\pm0.27 \times 10^6$ μL^{-1}), hematocrit (20.13±0.40%), thrombocytes/PLT ($248.33\pm12.34 \times 10^3$ μL^{-1}) and blood glucose levels (39.00±9.85 mg dL^{-1}) better than treatments B, C, and A two weeks after the challenge test. Treatment D provides 80% of the highest survival rate two weeks post-challenge.

Key Words: hematology, bacterial vaccine, vaccination, cantang hybrid grouper, *Vibrio alginolyticus*.

Introduction. Outbreaks of fish disease have become a significant obstacle to fish farming. According to Istiqomah et al (2006) vibriosis is a bacterial disease that is often found in grouper cultivation from the eggs, larvae, seeds and broodstocks. *Vibrio* sp. is also a pathogen in marine and brackish fish (Reed & Floyd 1996). So this disease caused by *Vibrio* is a very serious problem.

One of the alternatives to overcome fish infection is vaccine administration. Vaccines are preventive strategies that are determined by the ability of antigens to stimulate the immune system of animal targets (Amrullah 2014). A material can be used as a vaccine if it has an immunogenic substance. Extracellular products are important virulent factors of fish pathogens and sufficiently immunogenic to provide protection to fish during challenging tests. Extracellular products are products of bacterial metabolism which have toxic substance that can stimulate antibody formation. Exotoxins released by

bacteria are secreted proteins (Nindarwi 2006). Proteins of extracellular products can activate the immune response of the host because they are removed from the cell and easily come in contact with the host (Amrullah 2014; Zhang et al 2014). Besides, there is an intracellular component part of a bacterial cell that can be used as an antigen. It is made from an intracellular fluid of bacteria that is able to activate lymphocytes so it can produce fish antibodies (Mulia & Purbomartono 2007). The whole cell vaccines are also widely used as an antigen because whole cell vaccines contain an endotoxin component as an antigen to stimulate antibody formation (Herlina et al 2003).

Blood figures can be used to determine the health condition of fish. The physiological deviation of fish causes changes in blood components. Changes in the figure and blood chemistry both quantitatively and qualitatively can determine their health condition (Yuwono 2001). The blood will have serious changes if it is infected with a disease (Amlacher 1970). The aim of this study was to determine the effectiveness of whole cell vaccines, extracellular products, and intracellular products of *Vibrio alginolyticus* and to compare the various vaccines which had the best influence on the hematological responses as an indication of hybrid grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) immunity.

Material and Method

Experimental fish. The experimental subject consisted of a hybrid grouper from cross-bred of female tiger grouper and male giant grouper (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) named 'cantang' with a mean body weight of 30 g per fish. The fish were obtained from a local fish hatchery in Gerokgak, Buleleng, Indonesia and must be in healthy condition. The fish were acclimatized in a concrete tank for two weeks before use. This study was conducted in July 2018.

Vibrio alginolyticus strain. The *Vibrio alginolyticus* bacterial strain that was used in this study was isolated from ill tiger grouper fish and infected with *V. alginolyticus*. The bacteria were a collection from Marine Aquaculture Research Center and Fisheries Extension, Gondol-Bali/Balai Besar Riset Budidaya Laut dan Penyuluhan Perikanan (BBRBLPP) Gondol-Bali.

Bacterial malignancy. Bacterial malignancy was carried out by infecting white snapper (*Lates calcarifer*) with *V. alginolyticus* with a dosage 0.1 mL per fish and maintained until the fish becomes sick and moribund then *V. alginolyticus* were isolated from the lesion and spleen of the fish.

Preparation of WCV. Whole cells vaccines (WCV) are made based on the method of Suprapto et al (1995) with some modifications. *V. alginolyticus* was cultured on Trypticase Soy Agar (TSA, Merck) with a cellophane plate for 24-48 hours, then it was killed with 3% formalin for 72 hours. The culture was centrifuged at 2,000 rpm for 15 min and the supernatant was removed. The pellets obtained are whole cells that work as antigens and they were washed three times using 0.01 M phosphate-buffered saline (PBS) and centrifuged for 10 minutes at 2,000 rpm. The vaccine was stored at -4^{o}C until used.

Preparation of ECP. Bacterial cells were harvested from cellophane plates with 2 mL of PBS pH 7.0. The cell suspension was centrifuged at 12,000 g for 20 minutes. The supernatant was sterilized with a 0.45-µm syringe filter. The supernatant was dialyzed with PBS at 4°C for a night then the supernatant was stored in a tube as an ECP vaccine and stored at -4^{o}C until used (Suprapto et al 1995).

Preparation of ICC. Bacterial pellets from cellophane plates were washed three times using PBS and centrifuged for 10 minutes at 2,000 rpm. The cell concentration was adjusted to 50 mg mL^{-1} on PBS. Cells were broken down by sonication for 20 min. Debris and ICC were separated by centrifugation at 12,000 g for 20 min and filtered with a

0.45-µm syringe filter. The filtrate was stored in a tube as an ICC vaccine and stored at -4°C after sterility check (Suprapto et al 1995). The ICC was suspended in 5-7 mL 0.01 M PBS pH 7.4 (Mulia & Purbomartono 2007).

Treatment and fish maintenance. In this study, 120 test fish were used with four treatments. Ten fish were injected with three replications in each treatment. Fish are injected with PBS as control or treatment A, whole cell vaccine (WCV) as treatment B, extracellular product (ECP) as treatment C and intracellular component (ICC) as treatment D. Test fish were injected intraperitoneally with a dose of 0.1 mL per fish for each treatment. Fish were anesthetized using 100 ppm Eugenol before the vaccination. Fish were kept in an 80 L fiber tank for each treatment and each replication with aeration and fed with commercial grouper feed (Megami GR3) *ad libitum* three times a day. Vaccination was carried out on the 1st day, then performed a booster (second vaccination) with the same dose and method on the 8th day.

Blood collection. Fish hematology observation was carried out to analyze hybrid grouper immune response vaccinated with WCV, ECP, and ICC of *V. alginolyticus*. A blood sample is collected from the caudal vein (Grant 2015). Fish were anesthetized using 100 ppm Eugenol then blood is collected at the base of the tail using 1 cc sterile plastic syringe. The amount of blood is 0.5 mL per fish. The syringe is coated with ethylenediaminetetraacetic acid (EDTA) and the blood should be placed into vacuette tubes containing EDTA (One Med®) and stored at 4°C to prevent blood clots.

Blood parameters. The types of blood were then subjected to white blood cell/leukocyte (WBC), hematocrit levels (HCT), red blood cell/erythrocyte (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), thrombocyte/platelet levels (PLT) using Hematology Blood Cell Counter (SFRI Medical Diagnostic Contender 20+ Hematology) and blood glucose level test using digital blood glucose test (Easy Touch®). The paper strip inserted into the blood glucose device until the blood image appears, then a blood sample is dropped on a strip of paper and the results will be visible on the screen after 10 seconds. Blood glucose levels are expressed in mg/dL. The amount of blood was 4 µL per sample.

Challenge test. Challenge tests are carried out two weeks after the first vaccination or a week after the second vaccination. The fish were starved 24 hours before the challenge test. Fish were anesthetized using 100 ppm Eugenol. Fish were challenged by injecting *V. alginolyticus* intraperitoneally with dosage 0.5 mL per fish and 10^9 CFU mL^{-1} bacterial concentration. Fish were re-maintained and the mortality was recorded up to 14 days of post-challenge. The measurement to calculate the survival rate (SR) according to Effendie (2002):

$$SR = \frac{Nt}{No} x\ 100\%$$

where: SR = survival rate (%);
Nt = a number of fish at the end of maintenance;
N0 = a number of fish at the beginning of maintenance.

Data analysis. Statistical analysis using analysis of variants (ANOVA) and determined the best treatment performed Duncan's Multiple Range Test (DMRT). Data analysis using SPSS Statistics 21 Program.

Results and Discussion. The observation of hematological parameters of cantang hybrid grouper (*E. fuscoguttatus* × *E. lanceolatus*) can be seen in Figure 1.

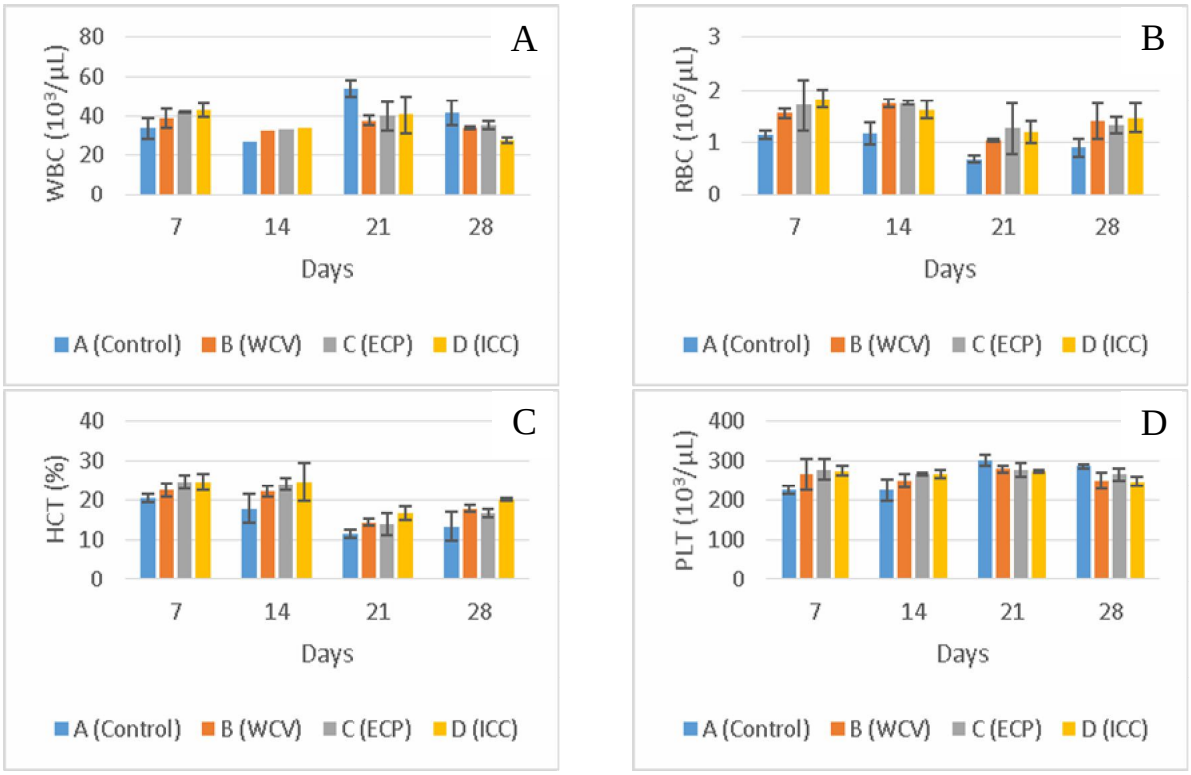

Figure 1. Hematological parameters of hybrid groupers in each treatment on day 7, 14, 21 and 28, A: white blood cells/leucocyte, B: red blood cells/erythrocyte, C: hematocrit, D: thrombocyte/platelets.

Total white blood cells. From Figure 1A above it can be seen that WBC/leukocyte of the fish injected with the three types of vaccines (WCV, ECP and ICC) shows a higher number on days 7 and 14 (38.93±4.88 x 10^3 μL^{-1} and 32.13±5.77 x 10^3 μL^{-1}, 41.90±0.60 x 10^3 μL^{-1} and 33.03±7.75 x 10^3 μL^{-1}, 42.63±3.48 x 10^3 μL^{-1} and 34.13±1.09 x 10^3 μL^{-1}, respectively) compared to the control group (33.57±5.19 x 10^3 μL^{-1} and 27.07±10.50 x 10^3 μL^{-1}). The statistical analysis showed significantly differences ($p < 0.05$) on day 7 and not significantly different between treatments on day 14.

One week after challenge test (day 21) group A showed a higher number of WBC (53.83±4.25 x 10^3 μL^{-1}) compared to the three types of vaccines group (B: 37.57±2.42 x 10^3 μL^{-1}, C: 40.00±7.35 x 10^3 μL^{-1}, and D: 40.53±9.23 x 10^3 μL^{-1}). The statistical analysis shows there are significant differences ($p < 0.05$) between treatment A and treatments B, C and D.

Total WBC decreased in all treatments on day 28 (two weeks after the challenge test), but treatment A showed highest total WBC (41.60±6.25 x 10^3 μL^{-1}) compared to the group injected with all three types of vaccine (B: 33.83±0.96 x 10^3 μL^{-1}, C: 35.17±2.19 x 10^3 μL^{-1}, and D: 27.33±1.37 x 10^3 μL^{-1}). Statistical analysis showed significant differences. Treatment D was significantly different ($p < 0.05$) with treatment B, C, and A. Treatment B and C was significantly different ($p < 0.05$) with treatment A.

Blood cells have a very important role in the immune system, especially leukocytes or white blood cells (Johnny et al 2005). WBC (leukocytes) are part of the non-specific immune system. Factors affecting the number of leukocytes are the condition and health of the fish (Chinabut et al 1991). WBC values were increased post-vaccination showing that the presence of the antigen into the body as the body's defense effort. Vaccines are considered as foreign substances into the body so that the fish will produce more WBC. The number of WBC also increased due to bacteria-infected fish after the challenge test. There are more leukocytes than normal conditions in pathogen-infected fish because one of the body's anticipations is to prevent the bacterial expansion by sending more blood to the infections area (Hardi et al 2011a).

WBC was decreased on day 28 which indicates the fish is in the healing process. WBC values in treatments B and C ($33.83 \pm 0.96 \times 10^3$ μL^{-1} and $35.17 \pm 2.19 \times 10^3$ μL^{-1} respectively) showed that WBC values decreased at day 28 post-challenge test. It shows that WCV and ECP vaccines can provide an immune response, but the value is not approaching the normal value of grouper WBC which indicates that even though fish are given a vaccine, the fish has not been able to fully suppress *V. alginolyticus*. WBC in treatment D was $27.33 \pm 1.37 \times 10^3$ μL^{-1}, this value was close to the normal value of tiger grouper WBC which was 2.72×10^4 cell mm^{-3} (Supriyono et al 2010). WBC in treatment A is still high on day 28, it is because there are wounds on the fish body so that fish are still trying to phagocytose bacteria by producing and sending more WBC to the infection area.

Total red blood cells. Total red blood cells (RBC) or erythrocyte in all three vaccine types (WCV, ECP and ICC) showed higher values on days 7 and 14 ($1.55 \pm 0.09 \times 10^6$ μL^{-1} and $1.74 \pm 0.07 \times 10^6$ μL^{-1}, $1.70 \pm 0.48 \times 10^6$ μL^{-1} and $1.75 \pm 0.05 \times 10^6$ μL^{-1}, $1.82 \pm 0.17 \times 10^6$ μL^{-1} and $1.61 \pm 0.17 \times 10^6$ μL^{-1}, respectively) compared to the control treatment ($1.13 \pm 0.08 \times 10^6$ μL^{-1} and $1.16 \pm 0.21 \times 10^6$ μL^{-1}). Total RBC in treatments C and D was significantly different ($p < 0.05$) with treatment A on day 7 and total RBC in treatment B, C, and D were significantly different ($p < 0.05$) with treatment A on day 14, but there is no significant difference between vaccine treatments.

Total RBC on day 21 were decreased then increased on day 28. The lowest value was obtained by treatment A ($0.67 \pm 0.06 \times 10^6$ μL^{-1}) on day 21. Statistical analysis showed that total RBC in treatment C and D was significantly different ($p < 0.05$) with treatment A on day 21 and total RBC in treatment B and D was significantly different ($p < 0.05$) to treatment A in day 28 with higher RBC produced by treatment D ($1.46 \pm 0.27 \times 10^6$ μL^{-1}). The decrease was due to the fish become infected after a challenge test (see Figure 1B). This value is below the range of normal values because according to Roberts (1978) normal erythrocyte range in teleost fish is between 1.05 and 3.00×10^6 cell mm^{-3}. Treatment A provides the lowest RBC value because the fish were not vaccinated. Fish in treatment A suffered a wound in the area of the post-challenge injection causing bleeding in the fish body. A total of RBC below the normal range due to bleeding causes anemia in fish. Pathogenic infections cause the fish to become stressed and followed by erythroblastosis which causes a decrease of RBC count so that the fish suffered severe anemia condition (Sabri et al 2009; Ebi et al 2018) and according to Fadhilah (2009) and Matofani et al (2013) bacteria that enter the body will occur phagocytosis process so that phagocytic cells will recognize and digest the particles of bacteria that need oxygen so that number of RBC was decreased. This is causing decreased RBC of hybrid grouper on day 21. Increased RBC on day 28 indicates that fish is in the healing process. Increased RBC is a fish body's defense mechanism against pathogenic infections, so the body will produce more erythrocytes to replace the lysis of erythrocytes due to infection (Dangeubun & Metungun 2017).

Dewantoro (2006) states that antigens are foreign materials that can stimulate specific immune responses in the form of antibodies circulating in the bloodstream. WCV contain endotoxin components as antigens to stimulate antibody formation, including lipopolysaccharide derived from cell walls of Gram-negative bacteria. Lipopolysaccharide is a major component of the outer of Gram-negative bacteria, the main junction of the structural integrity of the bacteria and protects membranes from various damages caused by chemicals (Johnny et al 2008). Lipopolysaccharide can induce the immunity needed for fish (Herlina et al 2003), while ECP is an exotoxin containing the polypeptide, a peptide containing more than two amino acids bound by peptide bonds. Polypeptides have lower immunogenic properties than lipopolysaccharide (Nindarwi 2006). Therefore, the hematological value of fish vaccinated with the WCV (treatment B) has a better value than fish vaccinated with ECP (treatment C). Soluble protein in the bacterium contains several kinds of enzymes that are useful as effective antigens (Austin & Austin 1989; Dewantoro 2006). ICC is made from bacterial intracellular fluid capable of activating lymphocytes that can produce antibodies (Mulia & Purbomartono 2007). There is a correlation between RBC and hemoglobin. The main function of RBC is to transport

hemoglobin which has the role of carrying oxygen throughout the body tissues. Low numbers of RBC caused the fish not being able to supply oxygen in large quantities despite sufficient availability of oxygen in the waters. Thus, the fish are coming in anoxia (lack of oxygen) (Fujaya 2008; Rahma et al 2015). Low erythrocyte levels indicate anemia, whereas high erythrocyte levels indicate that the fish is under stress (Wedemeyer & Yasutake 1977). Treatment A obtained erythrocyte values below the normal range on days 7 and 14 post-challenged tests that indicated fish in an anemic condition.

Hematocrit levels. Hematocrit levels of hybrid grouper on treatments B, C, and D showed higher values on days 7 and 14 compared to treatment A. The statistical analysis of hematocrit levels on day 7 showed treatments C and D were significantly different ($p < 0.05$) compared to treatment A. Treatment D was significantly different ($p < 0.05$) compared to treatment A on day 14. Hematocrit levels in four treatments on day 21 showed a decreased level and showed increased levels on day 28. Hematocrit levels of treatment A, B, C, and D on day 21 were 11.63±1.04%, 14.33±0.87%, 14.00±2.77%, and 16.80±1.77%, respectively. Statistical analysis showed that hematocrit levels of treatment D were significantly different ($p < 0.05$) with treatment A, but treatments B and C showed that were not significantly different from treatment A on day 21. Treatment B and D showed a significant difference ($p < 0.05$) for treatment A with higher hematocrit levels was produced by treatment D on day 28 (20.13±0.40%).

Treatment D resulted in a higher hematocrit value compared to other treatments but the value was still low on day 21. According to Bond (1979) and Setiawati et al (2017) hematocrit value in teleost fish ranges from 20 to 30% and in some marine fish species around 42%. A hematocrit value of less than 20% in teleost fish is a condition of anemia in fish (Clauss et al 2008; Ebi et al 2018). Hematocrit level of grouper fish was increased with the highest value resulting in treatment D (20.13±0.40%) on day 28. Treatment D obtained normal values, which indicates the fish has recovered. While hematocrit levels of treatments A, B, and C was 13.37±3.65%, 17.83±0.91%, and 16.63±1.11%, respectively (see Figure 1C). Hematocrit showed an increased level on day 28 compared to level on day 21 but the value was still below the normal value which indicates the fish in the healing process.

Hematocrit levels are one of the references that can be used as the health status of fish. Low hematocrit levels can indicate protein deficiencies in the diet, food and vitamin deficiencies, and as an indicator of infectious pathogens that cause fish refusing to eat (Blaxhall & Daisley 1973; Setiawati et al 2017). Low hematocrit levels are also caused by the low value of RBC causing fish to become anemic (Roberts 1978). According to Wedemeyer & Yasutake (1977) high hematocrit levels indicate fish under stressful conditions. The health status of the fish on day 21 or one week after the challenge test showed that disruption to fish physiological condition resulting from bacterial infection. Increased total WBC and decreased total RBC explain that fish are sick and injured as a result of infection, so the body anticipates producing more WBC as an immune response. Low hematocrit levels are also associated with low RBC causing the fish to be anemic.

Thrombocytes levels. In Figure 1D, it can be known if the value of hybrid grouper thrombocytes levels on day 7 shows a high number (225.67±10.07 x 10^3 μL^{-1}, 266.00±40.29 x 10^3 μL^{-1}, 278.33±25.93 x 10^3 μL^{-1}, and 275.67±12.10 x 10^3 μL^{-1}, respectively) and decreased on day 14 (225.67±28.5 x 10^3 μL^{-1}, 250.67±17.90 x 10^3 μL^{-1}, 267.00±3.60 x 10^3 μL^{-1}, and 267.67±11.68 x 10^3 μL^{-1}, respectively). Statistical analysis of thrombocyte levels in treatments C and D were significantly different ($p < 0.05$) compared to treatment A on day 7 and 14. Thrombocyte levels in the four treatments showed an increased level on day 21 and decreased levels on day 28. Thrombocyte levels in treatment A increased up to 302.67±15.28 x 10^3 μL^{-1} and decreased on day 28 287.00±5.57 x 10^3 μL^{-1}, but the value was higher when compared to thrombocyte levels of three vaccine treatments (B = 250.00±20.81 x 10^3 μL^{-1}, C = 267.00±15.72 x 10^3 μL^{-1}, and D = 248.33±12.34 x 10^3 μL^{-1}) on day 28. Statistical analysis of thrombocyte levels showed that treatments C and D were significantly different ($p < 0.05$) with treatment A

on day 21 but treatment B and D showed significant differences ($p < 0.05$) for treatment A on day 28.

Increased thrombocyte levels at day 21 showed if the fish was injured due to *V. alginolyticus* infection and thrombocyte levels began to decline due to fish in the healing phase on day 28. Thrombocytes play an important role in the process of coagulation and blood clotting (Grant 2015). According to Roberts (1978) thrombocytes are responsible for blood clotting and they are important in preventing the loss of tissue fluids as a result of wounds on the body surface. High thrombocyte values indicate if the fish is injured. Thrombocytes are actively helping clear the wound due to pathogens and cell debris by phagocytosis (Schmidt et al 2013).

Red blood cell indices

Mean corpuscular volume. Mean corpuscular volume (MCV) is the average volume of erythrocytes that is affected by the number of erythrocytes and hematocrit values (Lavabetha et al 2015). Figure 2A showed the value of MCV on days 7 to 28 indicated if treatment A provides the highest MCV value (180.86±14.61 fL, 155.87±34.22 fL, 176.33±31.26 fL, and 149.67±28.24 fL) when compared to treatment B, C, and D but there was no significant difference between these treatments. A high value on treatment A is caused by the fish in an inactive condition to swim due to *V. alginolyticus* infection causing the fish was starved, while the fish that have been vaccinated with WCV, ECP, and ICC are still actively swimming. These results are in accordance with the statement of Hrubec & Smith (2000) that fish with high oxygen demand are likely to have small erythrocyte size with low MCV and high erythrocyte counts. Rios et al (2005) and Grant (2015) also states that traíra fish (*Hoplias malabaricus*) in a starving condition will cause an increasing number of MCV. Increased MCV can be associated with erythrocyte swelling which causes macrocytic anemia or impaired water balance (osmotic stress) or macrocytic anemia in fish under stressful conditions (Tort & Torres 1988; Harikrishnan et al 2012).

Mean corpuscular hemoglobin. Based on Figure 2B it can be known if the value of mean corpuscular hemoglobin (MCH) in treatment A shows the highest MCH value on days 7, 21, and 28 (48.68±6.50 pg, 52.21±5.50 pg, and 49.16±16.95 pg, respectively) compared to fish group who were given all three types of vaccines (treatments B, C, and D). MCH value for each treatment showed no significant difference. Although MCH value in treatment A during maintenance shows a high value compared to other treatments, it is still in the range of normal values because according to Hrubec & Smith (2010) normal value of MCH in teleost is 30-100 pg. MCH is a hemoglobin content in each erythrocyte cell and can be used as an indicator in determining the health status of fish (Frandson 1992).

Mean corpuscular hemoglobin concentration. Mean corpuscular hemoglobin concentration (MCHC) is the mean concentration value of hemoglobin per unit erythrocyte volume. Figure 2C showed the value of MCHC in all treatments showed up and down values and there was no significant difference between treatments. MCHC value on treatments C and D approached normal range (30.06±6.12 g dL^{-1} and 30.79±0.24 g dL^{-1}, respectively) on day 28, while treatments A and B get MCHC value above normal range (33.52±12.59 g dL^{-1} and 31.51±3.83 g dL^{-1}, respectively). The normal MCHC reference value of teleost fish is 18-30 g dL^{-1} (Hrubec & Smith 2010). According to Frandson (1992) and Lavabetha et al (2015), the values of MCH and MCHC can be used in fish health examinations. Lower MCHC value than normal range is known as hypochromic anemia and a higher value than normal range is known as hyperchromic anemia.

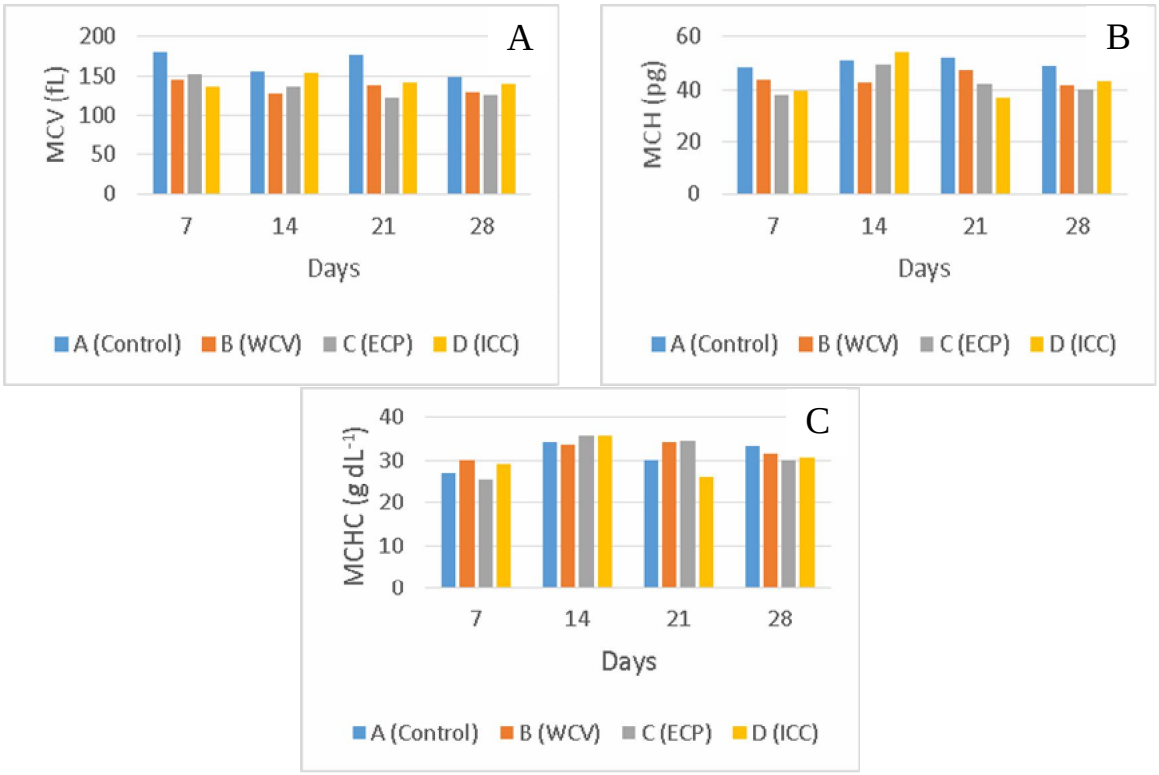

Figure 2. Red blood cell indices of hybrid groupers in each treatment on day 7, 14, 21 and 28, A: mean corpuscular volume (MCV), B: mean corpuscular hemoglobin (MCH), C: mean corpuscular hemoglobin concentration (MCHC).

Blood glucose levels. Based on Figure 3 it can be known that treatments B, C, and D on days 7 and 14 provide higher value compared to treatment A. Statistical analysis showed that blood glucose levels in treatment D were significantly different ($p < 0.05$) with treatment A on day 7. There was an increased blood glucose levels in all treatments on day 21 and had a decrease on day 28. Treatment D obtain blood glucose level approached the normal value (39.00±9.85 mg dL^{-1}) and treatments A, B, and C obtain higher values (64.33±14.57 mg dL^{-1}, 46.33±12.27 mg dL^{-1} and 46.00±10.39 mg dL^{-1}, respectively) on day 28. Statistical analysis showed that blood glucose levels in treatment C were significantly different ($p < 0.05$) with treatment A, but treatment C showed results that were not significantly different from treatments B and D on day 21. Treatment D showed significantly different ($p < 0.05$) to treatment A, but there was no significant difference between treatment D with treatments B and C on day 28. The normal value of blood glucose in groupers according to Martínez-Porchas et al (2009) is 28.8-34.2 mg dL^{-1}. Treatment D results were close to normal blood glucose levels on day 28.

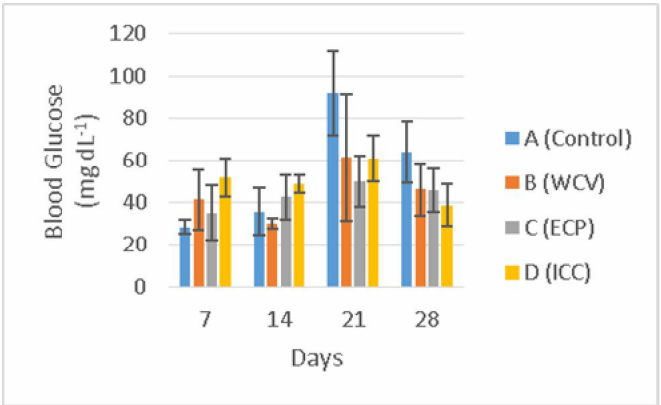

Figure 3. Blood glucose levels of hybrid groupers in each treatment on day 7, 14, 21, and 28.

Blood can be used to determine fish health conditions. Stress responses in animals can be seen from changes in hormone levels of cortisol, blood glucose, hemoglobin, and hematocrit (Torres et al 1986; Setiawati et al 2017). Blood glucose is one parameter that can be used as an indicator of stress in fish. Increased blood glucose on day 21 is related to stress conditions caused by *V. alginolyticus* infection in fish. Blood glucose can be an immunosuppressor in fish, because when glucose level in the blood is high, the kidneys work harder to maintain balance of the body so the function and work of the kidneys as organs that play a role in the immune system is impaired (Anderson 1990; Hardi et al 2011b). Kidneys are valuable organs with primary regulatory functions and central organs for immune-endocrine interactions (Press & Evensen 1999; Kum & Sekkin 2011). Thus the immune system of the fish will decrease so the pathogens more rapidly grow and develop in fish that cause fish to become sick.

Survival rate (SR). WCV, ECP, and ICC are some kinds of different antigens so the ability to provide protection to the fish is also different. From the results of the study it can be seen that the highest survival rate was obtained in treatment D (80.00±10.00%) and the lowest survival rate was obtained in treatment A (26.67±15.28%). Statistical analysis indicates the fish group vaccinated with ICC provides significant differences ($p < 0.05$) compared to non-vaccinated fish and vaccinated fish with ECP.

In Table 1, it can be seen that ICC bacterial vaccine can provide the highest survival rate of the grouper fish (80.00±10.00%). It shows that the ICC vaccines are capable of increasing the immune response so it can provide protection against *V. alginolyticus* infections. Non-vaccinated fish gives the lowest survival rate (26.67±15.28%) and it showed clinical symptoms that the skin was injured at the injection areas and some fish showed a perforated skin because the flesh was peeled off, then bleeding in the mouth and dorsal fin and excessive mucus production (Figure 4). While the internal symptoms caused are the liver shrinks and bleeding, enlarged spleen and kidney organs. This is similar with the clinical symptoms of 'cantik' hybrid grouper infected by *V. alginolyticus* reported by Roza (2017), which the fish body looks reddish, inflammation, necrosis and ulcer, swimming behaviors begin to interfere with swirling or dwell, refusing to eat and increased mucus production.

Table 1

Survival rate (SR) in each treatment at the end of the study

Treatment	*Survival rate (%)*
A	26.67±15.28[a]
B	76.67±11.55[bc]
C	53.33±15.28[b]
D	80.00±10.00[c]

[a,b,c] Means bearing different superscript letters in the same column showed significant differences ($p < 0.05$)

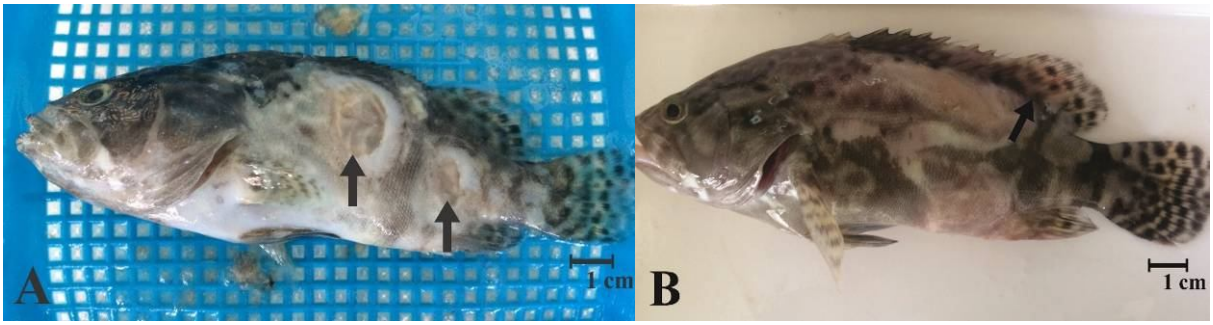

Figure 4. Clinical symptoms in 'cantang' hybrid grouper fish, A: perforated skin, B: bleeding in the dorsal fin.

Treatments B and C also provide a higher survival rate compared to treatment A (control), but survival rate of these two vaccines was lower than treatment D. From these data it can be seen that the ECP and WCV can also increase the immune response, but

when compared with the results of treatment D, it appears to be better. Providing booster on day 7 is an attempt to enhance the immune response of fish. According to Campbell et al (2004) and Dewantoro (2006) when the fish are re-exposed to the same antigen (booster) sometime later the immune response will be faster (2 to 7 days) is referred to as a secondary immune response. Bagni et al (2000) and Roza (2017) also states the booster can improve leukocyte function, defense against infectious diseases and does not cause fish to become stressed. The ability of the immune system to generate a secondary immune response is the basis of immunological memory. The initial exposure of an antigen will produce not only short-lived effector cells and attacking the antigen, but also long-lived T and B memory cell clones. Memory cells are prepared to proliferate and differentiate when it comes in contact with the same antigen (Dewantoro 2006). Previous studies conducted by Suprapto (2005) showed the administration of ICC vaccine mixture of *V. parahaemolyticus* and *V. alginolyticus* in *Penaeus monodon* shrimp which challenged with *V. parahaemolyticus* and *V. alginolyticus* give 70 and 75% survival rate (SR) respectively.

Conclusions. Based on the results of administration of whole-cell vaccines (WCV), extracellular products (ECP), and intracellular components (ICC) in hybrid groupers it can be concluded that treatment D (ICC vaccine) shows total leukocytes/WBC (27.33±1.37 x 10^3 μL^{-1}), total erythrocytes/RBC (1.46±0.27 x 10^6 μL^{-1}), hematocrit (20.13±0.40%), thrombocytes/PLT (248.33±12.34 x 10^3 μL^{-1}) and blood glucose levels (39.00±9.85 mg dL^{-1}) are better than treatment B, C and A on day 28. Treatment D provides 80% highest survival rate of hybrid grouper two weeks post-challenge.

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